Functionalization of second harmonic generation nanoparticles for theranostic applications

Présentée le 13 décembre 2019
à la Faculté des sciences de base
Groupe SCI-SB-SG
Programme doctoral en chimie et génie chimique

pour l'obtention du grade de Docteur ès Sciences

par

Jérémy VUILLEUMIER

Acceptée sur proposition du jury
Dr. R. Hovius, président du jury
Prof. S. Gerber, directrice de thèse
Prof. Ph. Renaud, rapporteur
Dr. Y. Chevolot, rapporteur
Dr A.-S. Chauvin, rapporteuse
Acknowledgement

During the time of the doctoral studies, I had the pleasure to meet and work with many wonderful people. And I would like to thank several people who supported me and who contributed to the success of this thesis.

First of all, I would like to gratefully thank Prof. Sandrine Gerber-Lemaire for giving me the opportunity to work in her research group and to work on this interesting interdisciplinary project. Her support, her patience and guidance helped me immensely throughout the course of the thesis.

I wish to express my gratitude to the jury members, Prof. Phillipe Renaud, Dr Yann Chevolot and Dr. Anne-Sophie Chauvin for taking some of their precious time to examine and review the manuscript of my thesis. I also thank Dr. Ruud Hovius for chairing the jury.

I sincerely thank all our partners in Geneva; Epithelix, Oncotheis and the Group of Biophotonic at the University of Geneva partners for the nice and profitable collaboration. I especially thank Dr. Christophe Mas who performed the biological assays and for his valuable exchange. I would like to thank warmly Dr. MER Luigi Bonacina, Gabriel Campargue and especially Geoffrey Gaulier for their time and patience, their expertise for the physical point of view and without whom this thesis could not have been realized.

I would like to thank all the personal of the ISIC’s Mass Spectroscopy service of EPFL: Dr. Laure Menin, Dr. Natalia Gasilova, Francisco Sepulveda and especially Daniel Ortiz who took the time to solve my experimental problems and who kept smiling even when I brought him hundreds of samples. I also appreciated the helpful and friendly teams of NMR service, Pascal Miéville and Aurélien Bornet, of the chemical stores, Anne-Lise, Gladys, Benjamin, Jacques and Marie.

I would like to thank warmly all the present and former members of the Group for Functionalized Biomaterials. Solène who welcomed me warmly in the lab and for her help at the beginning of the thesis; François “Franky the papy” for his jokes and his relax attitude; especially Raphaël for his insightful remarks that have advanced the project on the nanoparticles and his “expertise” (not always true) in a number of subjects, mainly for food; Laura for her kindness and her good mood; Luca and Céline for the amazing atmosphere they created in the lab.

I would like to thank all the master students and lab apprentices who cam in the lab: Adrian, Denis, Alice, Julie, Florence, Anthony, Anthony, Lucas “Mayoutz”, Yannick and David who shared our lab and for bringing a good working atmosphere.

Thank to the former and present members of the Zhu group with whom we shared daily lunches. Especially, Dylan, Mathias, Antho, Magic Raph, Nico, Alex, Dina, Bastien and Rémi. I also would like to thanks our lab neighbor, Christo for the nice discussions during the coffee breaks and for his interesting facts.
I am very grateful to Sophie who added to support me during these four years and who encouraged me in the most difficult moments.

Finally, I would like to thank my family for their support and encouraging during all my academic studies.
Abstract

Nowadays, cancer is one of the leading causes of death in the developed countries. The actual medical tools to detect cancer at early stage suffer to their low sensitivity. The design of multimodal nanodevices for in vivo imaging offers the perspective of cancer detection at a very early stage. The recent progress in the development of stimuli-responsive nanocarriers allows to achieve higher concentration in tumor site and reduces the side effects of active molecules. The combination of controlled release of therapeutics and imaging properties in a single nanocarrier has a great potential for theranostic applications. In this context, harmonic nanocarriers, which are composed by non-centrosymmetric materials, can be easily imaged by their second harmonic generation signal in multiphoton imaging platforms and functionalized with photosensitive systems for controlled-drug delivery.

The research project focused on the development of functionalized second-harmonic nanoparticles that exhibited imaging properties for theranostic application. The first part devoted to the development of photosensitive molecules based on coumarinyl and ortho-nitrobenzyl moieties and thereafter, several cargos molecules (tryptophan, organic dyes and anticancer drugs) where covalently conjugated to these tethers via carbamate or ester linkages. The coating of the harmonic nanoparticles, BiFeO$_3$ and LiNbO$_3$, via formation of silica shell increased their biocompatibility. The coated nanoparticles were functionalized with photo-responsive tethers via covalent linkage through bioorthogonal click reactions.

The ability of the phototrigger to induce the selective release of the caged compound was first evaluated under UV-light excitation. The amount of uncaged molecule was quantified by UHPLC-MS. Excitation of these functionalized harmonic nanoparticles in the near IR region generated second harmonic UV emission and subsequent selective release of the caged compound. This strategy was first evaluated with tryptophan as model cargo using femtosecond pulsed laser. NIR irradiation and functionalized nanoparticles exhibited good biocompatibility in vitro. The methodology was applied to the controlled delivery of anticancer drug (erlotinib and chlorambucil). The suitability of this methodology for the decoupled imaging of cancer cells and exposure to uncaged molecular cargos was investigated in vitro by tuning the excitation wavelength in multiphoton imaging setup.

In summary, harmonic nanoparticles were functionalized with several photosensitive tethers bearing molecular cargos that can be released in a controlled and selective manner upon UV- or NIR-light irradiation. In addition, the system exhibited high potency for in vivo and ex vivo cancer treatment.

Keywords: nanoparticles, imaging, theranostic, anticancer drug, surface functionalization, second-harmonic generation, controlled-release, light-triggered uncaging.
Résumé

De nos jours, le cancer est l’une des principales causes de décès dans les pays développés. Les outils médicaux actuels pour détecter le cancer à un stade précoce souffrent de leur faible sensibilité. La conception de nano-dispositifs multimodaux pour l’imagerie in vivo offre la perspective de détecter les cancers à un stade très précoce de développement. Les récents progrès dans le développement de nano-transporteurs réactifs aux stimuli permettent d’atteindre une concentration plus élevée dans les sites cancéreux et permet de réduire les effets secondaires des molécules actives. La combinaison de plusieurs propriétés tels que la libération contrôlée de médicaments et d’imagerie dans un seul nanodispositif a un grand potentiel pour les applications thérapeutiques. Dans ce contexte, les nanoparticules harmoniques, qui sont composées de matériaux non-centrosymétriques, peuvent être facilement imitées par leur signal de seconde harmonique dans des plateformes d’imagerie multi-photon et fonctionnalisées avec des systèmes photosensibles pour le relargage contrôlé de médicaments.

Le projet de recherche a porté sur le développement et la fonctionnalisation de nanoparticules générant du second harmonique pour l’imagerie ainsi que pour une application thérapeutique. La première partie est consacrée au développement de molécules photosensibles basées sur des dérivés de coumarine et d’ortho-nitrobenzyle et, par la suite, différents molécules cargos (tryptophane, colorants organiques et médicaments anticancéreux) y sont conjugués de façon covalente par des liaisons carboamate ou ester. L’enrobage des nanoparticules harmoniques, BiFeO₃ et LiNbO₃, par la formation d’une couche de silice augmente leur biocompatibilité. Les nanoparticules enrobées ont été fonctionnalisées avec les systèmes composés de molécules photosensibles liés à des molécules cargos par liaisons covalentes via des réactions clics bio-orthogonales.

La capacité du photo-déclencheur à provoquer la libération sélective du composé encapsulé a d’abord été évaluée sous une lampe UV. La quantité de molécules relâchées dans le milieu a été quantifiée par UPLC-MS. L’excitation de ces nanoparticules harmoniques fonctionnalisées dans l’infrarouge proche a généré de la lumière UV grâce aux propriétés des nanoparticules de générer du second harmonique et, cela a conduit à une libération sélective du composé encapsulé. Cette stratégie a d’abord été évaluée avec du tryptophane comme molécule modèle en utilisant un laser pulsé femtoseconde. L’exposition à la lumière NIR et les nanoparticules fonctionnalisées présentent une bonne biocompatibilité in vitro. La méthodologie a été appliquée au relargage contrôlé de médicaments anticancéreux (erlotinib et chlorambucil). Cette méthodologie pour l’imagerie découplée des cellules cancéreuses et au relargage des cargos moléculaires a été étudiée in vitro en ajustant la longueur d’onde d’excitation dans un instrument d’imagerie multi-photon.

En résumé, les nanoparticules harmoniques ont été fonctionnalisées avec plusieurs systèmes photosensibles possédant des liaisons pouvant être cassées lors de l’irradiation avec de la lumière UV ou NIR cela permet de libérer, de manière contrôlée et sélective, des molécules cargos. En outre, le système présentait un grand potentiel pour le traitement du cancer in vivo et ex vivo.
Mots-Clés: nanoparticules, imagerie, théranostique, médicaments anticancéreux, fonctionnalisation de surface, génération de seconde harmonique, relargage contrôlé, relargage déclenché par la lumière.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AgNPs</td>
<td>Silver nanoparticles</td>
</tr>
<tr>
<td>AIBN</td>
<td>Azobisisobutyronitrile</td>
</tr>
<tr>
<td>APTES</td>
<td>(3-Aminopropyl)triethoxysilane</td>
</tr>
<tr>
<td>aq.</td>
<td>Aqueous</td>
</tr>
<tr>
<td>Ar</td>
<td>Aromatic</td>
</tr>
<tr>
<td>AuNPs</td>
<td>Golds nanoparticles</td>
</tr>
<tr>
<td>BFO</td>
<td>Bismuth ferrite, BiFeO$_3$</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>calc.</td>
<td>Calculated</td>
</tr>
<tr>
<td>Cbl</td>
<td>Chlorambucil</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CM</td>
<td>Coumarin</td>
</tr>
<tr>
<td>CNTs</td>
<td>Carbon nanotubes</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CuAAC</td>
<td>Copper(I)-catalyzed azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>Cy3</td>
<td>Cyanine 3</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DIBO</td>
<td>4-Dibenzo-cyclooctyne</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N'-Diisopropylethylamine</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMF-DMA</td>
<td>Dimethylformamide-dimethylacetal</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DSC</td>
<td>N,N'-disuccinimidyl carbonate</td>
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<tr>
<td>EDCI</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>eq</td>
<td>Equivalent</td>
</tr>
<tr>
<td>erlo</td>
<td>Erlotinib</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization-mass spectrometry</td>
</tr>
<tr>
<td>Et$_3$N</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FAPi</td>
<td>Inhibitor of fibroblast activation protease-α</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HATU</td>
<td>(1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate)</td>
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<tr>
<td>HNPs</td>
<td>Harmonic nanoparticles</td>
</tr>
<tr>
<td>HOBt</td>
<td>Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HRMS</td>
<td>High Resolution Mass Spectrometry</td>
</tr>
<tr>
<td>IONPs</td>
<td>Iron oxide nanoparticles</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LNO</td>
<td>Lithium nobiate, LiNbO$_3$</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drugs resistance</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MES buffer</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MONB</td>
<td>metha-methoxy-ortho-nitrobenzyl</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSNs</td>
<td>Mesoporous silica nanoparticles</td>
</tr>
<tr>
<td>MW</td>
<td>Microwave</td>
</tr>
<tr>
<td>NaBH4</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAMDIATREAM</td>
<td>Nanotechnological toolkits for multi-modal disease diagnostics and treatments monitoring</td>
</tr>
<tr>
<td>NBS</td>
<td>N-Bromosuccinimide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NIR</td>
<td>Near-infrared</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small-cell lung carcinoma</td>
</tr>
<tr>
<td>ONB</td>
<td>ortho-Nitrobenzyl</td>
</tr>
<tr>
<td>PAA</td>
<td>Polycrylic acid</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Poly(amidoamine)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDT</td>
<td>Photodynamic therapy</td>
</tr>
<tr>
<td>PE</td>
<td>Petroleum ether</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylene-imine</td>
</tr>
<tr>
<td>PET</td>
<td>Positron-emission tomography</td>
</tr>
<tr>
<td>PGA</td>
<td>Polyglycolide</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PMHS</td>
<td>Polymethylhydrosiloxane</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts-per-million</td>
</tr>
<tr>
<td>PTT</td>
<td>Photothermal therapy</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>QDs</td>
<td>Quantum dots</td>
</tr>
<tr>
<td>r.t.</td>
<td>Room temperature</td>
</tr>
<tr>
<td>redox</td>
<td>Reduction-oxidation</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive-oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>sat.</td>
<td>Saturated</td>
</tr>
<tr>
<td>SHG</td>
<td>Second-harmonic generation</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SPAAC</td>
<td>Strain-promoted azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single-photon-emission computerized tomography</td>
</tr>
<tr>
<td>SPION</td>
<td>Superparamagnetic iron oxide nanoparticles</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>STEM</td>
<td>Scanning transmission electron microscopy</td>
</tr>
<tr>
<td>TBAB</td>
<td>Tetra-n-butylammonium bromide</td>
</tr>
<tr>
<td>TEG</td>
<td>Tetraethylene glycol</td>
</tr>
<tr>
<td>TEOS</td>
<td>Tetraethyl orthosilica</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>THG</td>
<td>Triple-harmonic generation</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>UCNPs</td>
<td>Up-conversion nanoparticles</td>
</tr>
<tr>
<td>UHPLC</td>
<td>Ultra High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>ZP</td>
<td>Zeta potential</td>
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1 Introduction

Nowadays, cancer is the second leading cause of death in the developed countries after cardiovascular diseases. According to the GLOBOCAN 2018 report from the World Health Organization, the incidence of cancer was estimated at 18.1 million of new cases and the number of deaths caused by cancer reached 9.6 million. Lung and breast cancers are the two most diagnosed (both 11.6%, Figure 1a), followed by colorectum and prostate cancers (10.2% and 7.1% respectively). However, lung cancer is by far the one that caused the highest number of deaths worldwide (18.4%, 1.8 million, Figure 1b). 24.2% (2.1 Million) of new detected cancer cases for the women population is breast cancer while prostate cancer affects 1.3 million (13.5% of incidence) of males. Nevertheless, prostate cancer is only the fifth cancer type in term of mortality for males that indicated good progress in prostate cancer treatment.

![Figure 1](image.png)

Figure 1: A) estimated number of new cancer cases in 2018 worldwide for both sexes; B) estimated number of deaths caused by cancers in 2018 worldwide for both sexes. Reprinted (adapted) from Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians. Copyright 2018.

According to the GLOBOCAN 2018 report, the number of new cases of cancer detected will rise up to 29.5 million in 2040 and, leading to 16.4 million of deaths. This increase can be associated with several factors such as world population growth, longer life expectancy and lifestyle-associated risks (alcohol consumption, cigarette smoking or obesity).

While significant progress were made over the last two decades for the diagnosis and treatment of cancer, several issues still need to be addressed, in particular the detection of cancer at early stage and the development of more selective therapies to reduce the impact of side effects. The design of multimodal nanodevices offers the perspective to overcome several of these limitations. The recent progress in the development of stimuli-responsive nanocarriers allowed to achieve higher drug local concentration and to reduce the side effects of active molecules. The combination of controlled release of therapeutics and imaging properties in a single nanocarrier has a great potential for theranostic (therapy and diagnostic) applications.
1.1 Diagnosis, imaging and treatment of cancer

1.1.1 Cancer diagnosis and imaging

Several techniques are currently used to diagnose and image cancer tumors. The non-invasive simplest medical exams are the analysis of blood, serum or urine to detect the presence of specific biomarkers released from cancer cells.\(^3\) Suspicion of tumor allows the use of several imaging techniques to localize the tumor in the patient body such as computed tomography (CT), magnetic resonance imaging (MRI), ultrasound (US), single-photon-emission computerized tomography (SPECT), positron-emission tomography (PET) or fluorescent and bioluminescent imaging.\(^4-9\) Furthermore, to confirm the suspicion of cancer, surgical biopsy is performed and the tissues are analyzed by histological techniques.\(^10\)

- **CT** is an imaging method based on the combination of X-ray images taken from different angles to give a three-dimensional image of organs and internal tissues. CT is currently used for detecting tumors in the head, lung and abdominal region.\(^11-13\)

- **MRI** is based on same principle than nuclear magnetic resonance (NMR). A radio frequency pulse is applied in the presence of a static magnetic field and an image is obtained as a result of the different relaxation time of hydrogen atoms in the body. MRI is used to diagnose soft tissue tumor and for neuroimaging. Injection of a contrast agent, based on paramagnetic complexes of gadolinium or iron oxide nanoparticles (IONPs), is usually required to obtain higher resolution images.\(^14,15\) The relaxation time of water protons can be enhanced in two ways by the contrast agents: reduction of T\(_1\) (spin-lattice relaxation time) or T\(_2\) (spin-spin relaxation time).

- **Medical US** makes use of high frequency sound waves, which have the ability to penetrate deeply inside the body. The reflection of the sound wave with the tissues is recorded and a two-dimensional image is built. Furthermore, this method allows real-time images of the body and can be used to detect breast, lung or prostate cancer.\(^16-18\) However, this technique give relative low-resolution images.

- **PET and SPECT** are techniques using a radionuclide source that is injected into the bloodstream of the patient. Gamma rays are detected in SPECT from \(^{99m}\text{Tc, }^{123}\text{I or }^{111}\text{In isotopes}.\(^19-21\) Positron-emitting isotopes, such as \(^{18}\text{F radiotracer, are used in PET.}\(^22\) These imaging techniques are able to detect bone marrow metastasis and provide valuable imaging of the metabolic state of cancer cells.\(^23,24\)

CT, US and MRI imaging techniques are categorized as non-invasive methods contrary to SPECT or PET technologies which require the injection of a radionuclide source. Furthermore, optical and fluorescent nanosensors as well as nuclear imaging give the possibility to obtain information at the molecular level. In contrary, US, MRI and CT techniques only provide macroscopic information (Figure 2). High resolution images are obtained with MRI and CT but the sensitivity of the contrast agent is low.\(^25\) In contrary, PET and SPECT techniques have high sensitivity and are able to monitor the accumulation, pharmacokinetics and biodistribution of the radionuclide. However, these imaging techniques offer limited spatial resolution.
Multimodal imaging consists in the combination of two or more imaging technologies to overcome the limitations of a single imaging technique. The combination of SPECT and CT imaging was the first multimodal instrument developed for clinical application. This hybrid SPECT-CT system allows having high signal coming from the SPECT tracer and high-resolution images thanks to CT. For example, prostate tumor and its metastasis could be detected with CT in combination with $^{111}$In radiotracer bearing antibody against prostate specific membrane antigen. Other hybrid techniques are routinely in tumor detection such as PET-CT or PET-MRI.

1.1.2 Cancer-chemotherapy

Three main therapeutic strategies are currently used to treat cancer: surgery, radiotherapy and chemotherapy. Surgery consists in the removal of cancer tissues and it is performed only if the tumor is found in a single area of the patient’s organs. Radiotherapy is a method using high doses of X-ray radiation to kill cancer cells through irreversible damage to their DNA. Surgery and radiotherapy are generally combined to increase the chance of remission. The history of chemotherapy started in the 1940’s with the discovery of nitrogen mustard as potent cytotoxic agents. Since this period, the number of anticancer drugs approved by the food and drug administration (FDA) gradually increased. They can be divided into several classes based on their mechanism of action (non-exhaustive list): alkylating agents which cause direct damages on deoxyribonucleic acid (DNA) such as cisplatin, anti-metabolites such as 5-fluorouracil (5-FU) that substitute natural DNA building blocks, topoisomerase inhibitors which block DNA replication such as daunorubicin and signal-transduction agents that block signalling pathways such as gefitinib. However, the poor selectivity of anticancer drugs causes side-damage to non-cancerous cells, which is one of the major limitations of current chemotherapies. Consequently, high doses of chemotherapeutics are required to reach cytotoxic effects and to reduce tumor growth. To overcome these limitations, nanocarriers have generated high expectation as selective drug delivery systems for cancer treatment. The following section presents anticancer therapeutics which are commonly used in chemotherapies and which hold the potential to be associated with nanocarriers.

Erlotinib was discover in 1997 and approved by the FDA in 2004 for the treatment of metastatic non-small cell lung cancer (NSCLC). Erlotinib is a small synthetic molecule composed by an aminoquinoline core linked to an alkyne-substituted aromatic ring. Erlotinib acts as a reversible inhibitor of epidermal growth factor receptor (EGFR) that is a trans-membrane protein tyrosine kinase overexpressed in NSCLC tumors. The interaction of EGFR with specific ligands in the extracellular matrix triggers homo-dimerization of the receptor which leads to activation of the tyrosine kinase domain (Figure 3). It was established that Erlotinib
interacts with the tyrosine residue of EGFR through the aminoquinoline moiety while alkyne-substituted aromatic ring binds to the ATP receptor, leading to disruption of the replication cycle.

**Figure 3:** Left part: signaling pathway via EGFR; right part: inhibited signal pathway thanks to erlotinib.

Furthermore, erlotinib in contrary to several anticancer therapeutics do not show severe side effects.\(^40\) Unfortunately, after several months of treatment with erlotinib, the mutation of EGFR is invariably observed, resulting in the inactivation of the drug. To overcome this limitation, new generations of EGFR inhibitors were developed such as osimerinib or poziotinib that bind covalently to the receptor.\(^41,42\) Modification on the side chain of erlotinib on the aminoquinoline and further functionalization with protein or bovine serum albumin (BSA) increased the efficacy of the inhibition (Figure 4).\(^43,44\) Additionally, the development of a molecular system based on erlotinib derivatives for dual-targeting allowed to increase the potency of erlotinib.\(^45\)

**Figure 4:** Structure and activity relationship of erlotinib for the inhibition of EGFR.

Doxorubicin (DOX) was isolated from *Streptomyces peucetius* bacteria and exhibited a wide spectrum of antitumor activity.\(^46\) Breast cancer, solid-tumors or non-Hodgkin lymphomas can be cured with DOX treatment.\(^47-49\) DOX is composed by a tetracyclic anthraquinone fluorophore (anthracycline) linked to an amino-sugar moiety (daunosamine) that increased its solubility in aqueous solution.\(^50\) When DOX is injected into the blood stream, a rapid uptake in cells is observed via passive diffusion.\(^51\) Several mechanisms of action were highlighted for DOX cytotoxic activity. First, DOX demonstrates a strong affinity with DNA double helix and ribonucleic acid (RNA) via intercalation between the strands thanks to the anthracycline moiety (Figure 5).\(^52\) Furthermore, topoisomerase II enzymes that are involved in regulation of DNA supercoiling are inhibited and as a result, replication of DNA cannot occur. The intercalation process and the inhibition of topoisomerase II lead to double-DNA stranded breaking that induces cell apoptosis. Several other targets are identified for DOX binding such as RNA and DNA polymerase, proteasome and mitochondrial DNA.\(^50,53,54\) Moreover, free radicals can be generated by DOX due to its ability to act as electron acceptor via cytochrome
reductase catalysis in the presence of NAD$^+$. Those free radicals cause oxidative stress leading to DNA degradation or cleavage. However, the poor selectivity for cancer cells and its high toxicity limited the application of DOX in chemotherapy. Acute damages were observed in liver, brain, kidney and heart. Furthermore, the activity of the immune system is reduced during DOX chemotherapy due to the killing of immune cells. The generation of reactive oxygen species (ROS) can also affect cardiomyocytes in the heart, which can lead to heart failure.

Figure 5: Mechanism of action of DOX.

To overcome these limitations, liposomal encapsulation of DOX was developed and approved by the FDA in 1995. This formulation that is composed by a lipid double layer coated with polyethylene glycol (PEG) for the encapsulation of DOX and showed better pharmacokinetic properties such as longer circulation time, better accumulation into cancer cells and longer half-life of DOX.

Chlorambucil (Cbl) is a small molecular anticancer drug deriving from nitrogen mustard and was first synthesized by Everett and co-workers in 1953. This molecule is used to treat different types of cancer such as chronic lymphatic leukemia (CLL), breast and ovarian carcinomas. Cbl acts as an alkylating agent that is able to bind to DNA via the N7 nitrogen on the DNA base guanine, resulting in disruption of cell replication (Figure 6). In more details, the bis(2-chloroethyl)amine moiety undergoes an intramolecular cyclization leading to the formation of a highly reactive and unstable aziridinium cation. This cation is attacked by guanidine through $S_N2$ reaction forming an alkylated adduct. Thereafter, this reaction can occur a second time to form a cross-link between the two DNA strands.
However, Cbl has limited clinical applications due to its non-specific cytotoxicity that causes a wide panel of side effects such as bone marrow suppression, anemia, nausea or neurotoxicity. Development of prodrugs based on Cbl have been reported to reduce side effects and to achieve more selective delivery into tumor sites. Conjugation of serum transferrin protein to Cbl resulted in higher selectivity thanks to the interaction of the protein with transferring receptors on cancer cell membrane. Alternatively, conjugation of Cbl to saccharides or to peptides reduced the side effects of the native drug and increased the selectivity towards cancer cells. Cbl linked to cyclic peptide Arg-Gly-Asp (RGD) sequence was selective to cancer cells that overexpressed integrins receptors on their surface and higher cytotoxicity was observed compared to the drug alone. Moreover, combination therapy with Cbl and the antibody rituximab increased the efficiency of Cbl for the treatment of CLL cancers in phase II clinical trial.

1.2 Nanoparticles for cancer imaging and therapy

The following section presents an overview of the nanomaterials, which have been developed for cancer imaging and treatment, with particular emphasis on inorganic nanoparticles. Since the 1990s, the emergence of new multimodal nanodevices for in vivo imaging applications offers the perspective of cancer detection at a very early stage to improve the chance for preventing tumor growth and metastasis, and to favor complete recovery for the patients. A variety of organic or inorganic nanomaterials were developed and showed promising applications in oncology, including therapeutics, magnetic hyperthermia or imaging thanks to their physical and chemical properties. Nanoparticles have high surface area and surface to volume ratio that allow high loading of therapeutic agents and / or imaging probes. In addition, their surface can be easily modified with a variety of functional groups such thiol, carboxylic acid, silane or amine and further functionalized with small molecules.

Most nanoparticles present dimensions ranging from 5 to 200 nm, which are thus in the same range as biological entities such as proteins or viruses. Therefore, they can interact with molecular components at the cell surface or within intra-cellular compartments. However, if the nanoparticles exceed 200 nm dimensions, they can be cleared by the reticuloendothelial system (RES), whereas very small particles (below 5 nm) are cleared by the kidneys. Nanocarriers are designed to discriminate between cancer and healthy cells due to the specific behavior of neoplastic cells which show uncontrolled growth and more acidic pH of
their extracellular matrix compared with non-cancer cells. Two major approaches were reported to target cancerous sites: passive and active targeting. Nanomaterials using passive targeting are preferably accumulated into cancerous cells due to the enhanced permeability and retention (EPR) effect (Figure 7). EPR effect is due to the fast growth of abnormal and non-structured blood vessels, in form and architecture, at tumor sites. This abnormal structure leads to leaky vascular system and dysfunctional lymphatic draining, thus favoring passive accumulation of nanomaterials. In addition, the circulation time of nanoparticles in the blood stream is largely influenced by their surface charge. Positively charged nanoparticles tend to be easily internalized and accumulated in tumor tissues. However, high positive charge density favors non-specific interactions with cells. In addition, hydrophobic nanoparticles or nanoparticles bearing hydrophobic groups tend to form aggregate which are rapidly cleared by RES.

![Figure 7: Passive accumulation of nanomaterials into cancer cells via EPR effect.](image)

Cancer cells are generally overexpressing different surface receptors. Nanomaterials functionalized with targeting ligands including proteins, small molecules, peptides and antibodies are expected to specifically address cancer cells and tumor tissues through active recognition of these overexpressed biomarkers (Figure 8). Interaction with cell surface receptors induces enhanced cell internalization through endocytosis. Furthermore, the tumor microenvironment was recognized as a valuable target that can be addressed by molecular ligands targeting the extra-cellular matrix, cancer associated fibroblasts or tumor associated vasculature. Folate receptors are overexpressed in numerous cancer cells and several nanocarriers bearing folic acid, which is one of the most reported targeting ligands, are used to actively target tumor sites. Addition of folic acid on the nanocarrier overcomes the drawbacks of the poor selectivity of the nanocarriers towards tumor sites. And inversely, the nanocarrier increases the solubility of folic acid in aqueous media. Several cancer cells show an overexpression of αvβ3 integrin, which plays a crucial role in cell survival, differentiation, proliferation and migration. This receptor is a target for nanoparticles bearing RGD peptide mimetics. Active targeting is not only applied to the specific recognition of cancer cells and tissues, but was also reported to overcome multi-drug resistance (MDR). Nanocarriers decorated with targeting ligands and loaded with prodrugs or encapsulated drugs are accumulated into cancer cells and the payload is generally further released through the intervention of an internal or external stimuli (more details in section 1.4).
Imaging and/or therapeutic nanoparticles can be mainly divided into two categories: organic nanoparticles and inorganic nanoparticles. Organic nanomaterials are generally composed of one or double lipid layers (micelles and liposome), by highly organized polymers such as dendrimers or polymeric nanogels (Figure 9). Carbon nanotubes (CNTs), quantum dots (QDs), noble metals nanoparticles (gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs), meso-porous silica nanoparticles (MSNs), up-conversion nanoparticles (UCNPs) and super-paramagnetic iron oxide (SPIONs) are the most commonly used inorganic nanomaterials for cancer imaging and treatment.

Liposomes demonstrated good biocompatibility with stable and high drug loading efficiency. Double phospholipids bi-layers are able to encapsulate both hydrophobic and hydrophilic payloads. In addition, the outside membrane of liposomes can be easily modified or functionalized with targeting ligands to mimic the cell membrane. DOX, which is a hydrophobic drug, encapsulated within PEGylated-liposomes was the first FDA-approved nano-drug for cancer treatment. Micelles are able to encapsulate hydrophobic payloads in their core due to their amphiphilic properties. Micelles loaded with two anticancer drugs were reported to overcome MDR and to improve the selective delivery to cancer cells. Polymeric nanogels are formed either through electrostatic interactions or through chemical cross-linking, resulting in a three-dimensional network, presenting highly porous structures and high solvent content. Dendrimers are hyper-branched and well chemically defined polymers exhibiting good biocompatibility and solubility in physiological media. The inner part of dendrimers is generally loaded with hydrophobic payloads while their hydrophilic
surface can be covalently conjugated to cancer cell targeting ligands.\textsuperscript{101} DOX covalently bounded on polyamidoamine (PAMAM) dendrimer via amide linkage was released upon acidic cleavage into epidermoid carcinoma cancer cells.\textsuperscript{102}

Despite a wide range of applications, organic-based nanocarriers suffer from several drawbacks. Liposomes and micelles showed premature release of the encapsulated compounds, insufficient drug loading or reduced circulation time in the blood stream.\textsuperscript{103} Dendrimers are rapidly cleared by the immune system and are less internalized inside cancer cells.\textsuperscript{104} Nanocarriers based on polymers suffer from poor reproducibility of their chemical composition between batches and uncontrolled release of caged compounds. Inorganic nanomaterials are able to overcome several of these limitations and in addition, most of the inorganic materials show intrinsic imaging properties.

### 1.2.1 Inorganic nanoparticles

The recent progress in nanotechnologies has generated high expectation that inorganic nanomaterials could provide unprecedented contrast agents in imaging set-ups.\textsuperscript{105} Moreover, multimodal nanodevices are developed using the combination of their inherent imaging properties with the addition of a second imaging probe or in combination with anticancer drug. Different imaging techniques can be combined to achieve nano-hybrid systems such as PET-MRI, SPECT-CT, CT-MRI or PET-CT.\textsuperscript{106–109} For instance, PET-MRI nanodevices combine simultaneously the paramagnetic properties of IONPs and the information coming from the $^{64}$Cu radiotracer.\textsuperscript{110} Moreover, inorganic nanoparticles have proved their potential as multifunctional platforms for sustained and controlled drug delivery for cancer diagnosis and therapeutic applications in vivo (Figure 10).\textsuperscript{111} Numerous inorganic nanomaterials showed excellent imaging properties and can be easily post-functionalized with a variety of payloads to overcome MDR and to achieve controlled delivery at specific sites. Furthermore, targeting ligands added to the nanocarriers allows the specific targeting of cancer cells and tumor tissues.

**Figure 10:** Schematic representation of multifunctional nanodevices for multimodal imaging applications and therapeutic payload delivery.
Following their approval by the FDA for their use in basic medical care applications, MSNs were extensively reported in the literature as drug nanocarriers.\textsuperscript{112} MSNs exhibit many favorable properties, including excellent biocompatibility, high stability, tunable pore sizes and non-premature release of caged compounds.\textsuperscript{113} MSNs can be degraded under biological conditions and excreted by the kidney. However, one of the limitations of MSNs \textit{in vivo} is their limited circulation half-lives due to hemolysis of red blood cells.\textsuperscript{104} MSNs are generally synthesized by sol-gel, template directed method or via microwave assisted technique.\textsuperscript{114} Furthermore, the surface of MSNs can be easily tuned by introduction of amino or carboxylic groups, which can be post-functionalized with targeting ligands. Conjugation of MSNs with folic acid allowed to increase their local concentration into cancer cells in comparison with bare MSNs.\textsuperscript{115} Alternatively, mesoporous silica can be applied as a coating shell around inorganic nanomaterials such as QDs, UCNPs or noble metal nanoparticles in order to improve their biocompatibility.\textsuperscript{116} While mesoporous silica does not bring any imaging capability, electrostatic or covalent conjugation to imaging agents can be easily achieved. For instance, a silylated gadolinium complex was directly introduced via ligand exchange on the surface of MSNs and showed significant T\textsubscript{1}-weighted enhancement \textit{in vivo}.\textsuperscript{117} Noteworthy, hydrophobic anticancer drugs can be easily encapsulated into the porous structure of MSNs and can be released via several stimuli such as pH change. This strategy improved drug accumulation at tumor site, minimized the side effects of the free drugs and overcame MDR. In particular, a combination of rose Bengal and DOX loaded on MSNs was reported for chemo-photodynamic combination therapy.\textsuperscript{118}

CNTs are composed by carbon atoms assembled into a two-dimensional hexagonal lattice bent to form a hollow cylinder. CNTs exhibit high thermal conductivity, good mechanical stiffness and tensile strength.\textsuperscript{119} For biomedical applications, CNTs have generally average 10 nm in diameter and 1 to 100 mm length.\textsuperscript{120} CNTs are generally synthesized by carbon arc-discharge, laser-ablation and chemical vapor deposition techniques.\textsuperscript{121} Apart from their relevance for the biomedical field, CNTs are used for several applications such as water desalination, hydrogen storage and as semi-conductors.\textsuperscript{122–124} CNTs are able to adsorb or conjugate with a large panel of active molecules, which make them excellent nanocarriers for drug delivery.\textsuperscript{125} Nevertheless, impurities coming from the synthesis and the hydrophobicity of CNTs are major limitations for their use \textit{in vivo}.\textsuperscript{126} While CNTs do not exhibit intrinsic imaging capabilities, their ability to absorb light in the near-infrared (NIR) region followed by conversion into heat was used for hyper-thermic ablation of cancer cells.\textsuperscript{127} For example, De La Zerda and co-workers developed a nanosystem based on single-walled CNTs for cancer detection in living mice using photo-acoustic imaging.\textsuperscript{128} The ease to chemically modify CNTs through hydrophobic or covalent conjugation makes them good candidates for theranostic applications. Multi-walled CNTs decorated with a combination of folic acid (acting as targeting ligand), Alexa-fluor (fluorescent probe), technetium-99m (radiotracer for SPECT imaging) and methotrexate (anticancer drug) were used to image and treat mice bearing paclitaxel-resistant 4T1 murine breast tumor.\textsuperscript{129}

IONPs are composed by magnetite (Fe\textsubscript{3}O\textsubscript{4}) and several methods are currently used to synthesize these nanoparticles such as chemical synthesis, co-precipitation or thermal decomposition techniques.\textsuperscript{130} IONPs below 150 nm size exhibit magnetic properties, present high surface to volume ratio, reduced \textit{in vitro} an \textit{in vivo} toxicity as well as biodegradable properties.\textsuperscript{131} Below 20 nm, these nanoparticles display a unique phenomenon called superparamagnetism. In the absence of magnetic field, the overall magnetization is equal to zero. However, when strong external magnetic field is applied, the SPIONs developed a strong internal magnetization. This effect affects the relaxivity of the \textsuperscript{1}H atoms of water molecules present in the tissues, which makes SPIIONs excellent MRI contrast agents.\textsuperscript{132} In addition, SPIIONs can be guided with an external magnet to accumulate inside tumor tissues. Several formulations based on SPIIONs were approved
Introduction

by the FDA for medical imaging and therapeutic applications. One can mention Feridex IV®, for liver and spleen imaging, Lumiren® for bowel imaging and Ferumoxytol® for iron replacement therapy.\textsuperscript{133-135} Surface coating of SPIONs with organic polymers or via formation of a silica shell was applied to decrease their aggregation in physiological medium and to reduce surface oxidation that can altering their magnetic properties.\textsuperscript{136} Dextran coated SPIONs functionalized with multiple antibodies were reported to target mammary carcinoma cells lines and to increase MRI contrast \textit{in vitro}.\textsuperscript{137} Moreover, when an alternative external magnetic field is applied, SPIONs generate heat that can be used for thermal ablation of cancer cells. Silica coated SPIONs were injected into patients having prostate cancer and after application of local magnetic field, temperature increase was observed locally in the prostate leading to thermal-ablation of cancer cells without touching the healthy cells.\textsuperscript{138} In addition, loading of SPIONs with therapeutic payloads offers the possibility for controlled release. For instance, coated SPIONs with a double layer of polydopamine and human serum albumin were disclosed by Quan and co-workers. for the targeted delivery of DOX in 4T1 breast tumor bearing mice, resulting in the significant decrease of solid tumor size.\textsuperscript{139}

AuNPs are generally obtained from the reduction of a commercial gold source to produce tunable size and shape of the resulting nanomaterials. While bare AuNPs injected in the blood stream undergo rapid clearance by the RES due to the formation of a protein corona,\textsuperscript{140} coating with polymers such as PEG generally result in extended circulation time.\textsuperscript{141} Upon laser irradiation, the electrons of AuNPs are able to enter into resonance and the incoming light is scattered, this phenomena is called surface plasmon resonance (SPR). SPR peaks are depended of the size and the shape of the nanomaterial and the plasmonic resonance can be shifted from visible to NIR region.\textsuperscript{142} The surface of AuNPs is easily functionalized with polymers containing amine, thiol or phosphine groups.\textsuperscript{143} Enhancement of the contrast between blood and the surrounding tissues can be achieved thanks to PEGylated AuNPs for photoacoustic tomography imaging.\textsuperscript{144} However, AuNPs are mostly used for photothermal therapy (PTT). Local heat produced by the nanoparticles was able to disrupt cell membranes and causing protein denaturation.\textsuperscript{145} AuNPs coated with PEG-g-PGA (polyglycolide) copolymer were functionalized with folic acid which was acting as targeting ligand and with cisplatin anticancer drug.\textsuperscript{146} After injection into mice bearing breast tumor, the accumulation of the nanocarrier into tumor site was imaged by bioluminescence. Thereafter, upon NIR-light irradiation, local heat was generated leading to the necrosis of tumor tissues.

QDs are inorganic semi-conductor fluorophores containing elements from groups II-VI or III-V such as CdSe, CdTe, CdS, ZnSe or InP. They are generally coated with a second layer of inorganic semi-conductors such as ZnS or CdS, which have a wider bandgap for the enhancement of their imaging properties.\textsuperscript{147} QDs, which are mainly produced by electron beam lithography, are characterized by the following physical properties: small size (1 to 10 nm), high photostability, sharp emission spectra, high extinction coefficient and quantum yield.\textsuperscript{148} Furthermore, the emission wavelengths and absorption spectra can be tuned from the NIR to the ultra-violet (UV) region by changing the size of the nanomaterial.\textsuperscript{149} However, the major concern about QDs is the cytotoxicity resulting from the heavy metal ions, which limits their use to \textit{in vitro} applications. Coating with hydrophilic ligands was reported to increase the biocompatibility of QDs. For instance, QDs coated with long PEG chains displayed longer circulation time in the blood stream in comparison with QDs functionalized with short PEG chains, that accumulated mostly in the kidney.\textsuperscript{150} Thanks to their unique optical properties, QDs are used as fluorescent probes for biomedical applications. QDs bearing monoclonal anti-HER2 antibody were injected in mice bearing breast cancer.\textsuperscript{151} The localization of the QDs were monitored in real-time using a high-speed confocal microscope.
UCNPs are nanoparticles less than 100 nm composed by inorganic crystals based on NaYF$_4$, NaGdF$_4$ or YAlO$_3$ doped with trivalent lanthanide ions such as Yb$^{3+}$, Er$^{3+}$, Ho$^{3+}$ or Tm$^{3+}$ that act as optically active centers.$^{152}$ Three different methods are generally reported for the synthesis of UCNPs with precise control of their morphology: hydro-solvo-thermal, thermolysis and co-precipitation methods, leading to nanoparticles below 100 nm size range.$^{153}$ Upon continuous-wave excitation, UCNPs absorb two or more low energy photons (generally in the NIR region) to reach long lifetime real excited states.$^{154}$ Thereafter, the energy is released into sharp and well defined emission peaks in the visible and UV region. Core-shell structures presenting a second layer of UCNPs crystals are generally required to enhance the luminescence efficiency.$^{155}$ A large variety of bioimaging applications were reported for UCNPs due to their high brightness and resistance to photobleaching as well as the deep penetration of NIR-light irradiation.$^{156}$ Surface coating of UCNPs through silanization or polymer coating was developed to modulate their hydrophobicity and to reduce the toxicity of lanthanide ions.$^{157}$ Furthermore, these nanoparticles can be used as multimodal imaging agents via functionalization with probes for MRI, CT, SPECT or PET.$^{158-160}$ Encapsulation of DOX within mesoporous silica-coated UCNPS was also disclosed for targeted release triggered by pH variation.$^{161}$

While a large variety of inorganic nanoparticles were successfully applied to targeted cancer bioimaging and controlled drug release, in vitro and in vivo, several limitations still hamper their rapid translation to clinical procedures.

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Table 1: Advantages and drawbacks of inorganic nanoparticles for theranostic applications.
Harmonic nanoparticles for imaging applications

Since the two last decades, harmonic nanoparticles (HNPs) have emerged as a new class of imaging probes in nanomedicine.\(^{181-183}\) These materials are composed by non-centrosymmetric metal oxide nanocrystals and present several favorable properties for bioimaging applications. In particular, HNPs show excellent non-linear response to excitation in the region from UV to mid-IR.\(^{184,185}\) Two photons coinciding in time and space are combined by non-centrosymmetric materials but their frequencies are not added up to an existing transition of the system (virtual state). A new photon with twice the frequency and half the wavelength of the initial excitation is then generated (Figure 11). This process is called second harmonic generation (SHG). In addition, some HNPs show the ability for triple harmonic generation (THG) or fourth harmonic generation.\(^{186}\)

**Figure 11**: Second harmonic generation from HNPs.

HNPs can be easily imaged by their SHG signal in multiphoton imaging platforms.\(^{187}\) These nanomaterials exhibit several advantages compared to organic probes such as long-term photo-stability under pulsed laser irradiation due to low energy deposition on the HNPs surface, sharp and narrow emission peak, as well as the absence of blinking or bleaching processes.\(^{188-191}\) The nonlinear optical response of HNPs being independent from the incoming wavelength, the excitation wavelength can be easily tuned to match the optical properties of the sample. The range used for SHG application is generally selected between 700 to 1200 nm, offering deep penetration capabilities and reduced photodamages to biological samples. However, the application of harmonic generation for bioimaging requires the use of a pulsed laser system such as Ti:Sapphire based laser.

Different metal oxide based HNPs, including bismuth ferrite (BiFeO\(_3\), BFO), barium titanate (BaTiO\(_3\)), zinc oxide (ZnO), iron iodate (Fe(IO\(_3\))\(_3\)), silicon carbide (SiC), potassium niobate (K\(_2\)NbO\(_6\)) or lithium niobate (Li\(_2\)NbO\(_6\), LNO) have demonstrated good SHG properties.\(^{192-196}\) Evaluation of the cytotoxicity of these HNPs against a panel of human cells indicated that only ZnO HNPs showed detrimental effects to all cell lines.\(^{192,197}\) The other HNPs revealed low cytotoxicity after 72 h of incubation (20-30% decrease in cell viability) and moderate hemolytic effect on human red blood cells (5-7%). However, after surface coating with PEG derivatives, BFO HNPs reached excellent in vitro cell compatibility and reduced hemolytic effect (Figure 12).\(^{198}\) PEGylated BFO HNPs decorated with targeting ligands, RGD peptidomimetics and inhibitor of prolyl oligopeptidases, were able to target and to inhibit the grow of tumor cells in vitro.\(^{199}\) Furthermore, these BFO HNPs were used for stem cells tracking in tissue depth by using their second and third harmonic signals.\(^{200}\) BFO HNPs were successfully used to track pulmonary macrophages in mice with allergic airway inflammation.\(^{201}\) Other recent applications of HNPs include the labelling of liver cancer cells with folic acid-decorated SiC HNPs and photodynamic therapy (PDT) protocol based on the combination of BaTiO\(_3\) HNPs with rose Bengal.\(^{203}\)
1.3 Functionalization of inorganic nanoparticles

1.3.1 Coating of nanoparticles

In vivo applications of nanocarriers and imaging nanoparticles have to face several biological barriers. In the blood stream, nanovectors interact non-specifically with plasma proteins that can trigger an adverse response from the immune system.\(^{131}\) Adsorption of proteins that act as ligands on the surface of the nanoparticles leads to their recognition by phagocytic cells. As a result, nanocarriers, which cannot be digested by phagocytes, have the tendency to accumulate into the spleen and liver. Furthermore, due to their surface hydrophobicity, inorganic nanoparticles generally suffer from rapid aggregation in physiological medium and blood stream.\(^{204}\) To overcome these limitations, surface derivatization with polymers and biomolecules or silica surface coating are necessary to improve the interactions of inorganic nanoparticles with biological media. In addition, surface modification can increase circulation time, colloidal stability and reduce the clearance of nanomaterials (Figure 13).\(^{205}\)

The majority of the inorganic nanoparticles are generally coated with PEG derivatives. However, other types of natural and synthetic polymers are also used to modify the surface of nanosystems, including polyvinyl alcohol (PVA), polyacrylic acid (PAA), polylactic acid (PLA), polyethylene-imine (PEI) or carbohydrates such as chitosan or dextran (Figure 14).\(^{205–211}\) Commercially available polymers are easily tailored. Due to their good biocompatibility, polymers are widely used to coat inorganic nanomaterials. Two main methods are currently applied to functionalize inorganic nanoparticles: i) formation of polymeric shell via electrostatic interaction; ii) adsorption of the polymer through ligand exchange.\(^{212}\)
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In 1990, the FDA approved the clinical application of PEG as excipient for pharmaceutical formulations. PEG is a linear biocompatible synthetic polyether available in a large range of sizes (e.g. 1 to 20 kDa). Furthermore, the termination groups can be easily modified for the introduction of several functionalities such as azido, amino, carboxylic, maleimide, thiol or silano reactive groups. Consequently, PEGylation of nanoparticles induces “stealth” properties due to the low toxicity and immunogenicity of PEG towards recognition by the RES. For instance, PEGs containing thiol ends are generally used to functionalize AuNPs via coordination interactions. In addition, PEGylated nanoparticles can be easily post-functionalized with targeting ligands, anticancer drugs, antibodies, peptides and RNA.

Surface silanization of inorganic nanoparticles is a common alternative to polymeric coating. Silica is known to be stable in aqueous media, biocompatible and optically transparent, thus avoiding loss of optical efficiency when applied to the coating of optically active nanoparticles. Silanization generally involves the formation of a primary silica shell through reaction with tetraethyl silicate (TEOS), followed by post-conjugation with organoalkoxysilane derivatives that allowed the introduction a variety of functional groups such as amino, thiol or carboxylic reactive ends. In addition, the structure is generally porous, which is used to encapsulate therapeutic payloads.

Two major methods are employed to introduce protein coating on nanoparticles: either via adsorption or via chemical conjugation. Among the most used proteins for inorganic nanoparticles coating, one can mention albumin, streptavidin and avidin which can be conjugated by electrostatic interactions or covalent coupling.

1.3.2 Conjugation on the nanoparticles surface

To covalently functionalize the coated surface of inorganic nanoparticles, different methods were developed: reactions leading to covalent linkage such as click azide-alkyne cycloaddition, amidation and esterification reactions using coupling agents, Diels-Alder, Staudinger ligation, native chemical ligation and Michael addition; or via formation of non-covalent but strong binding such as biotin-avidin interaction, DNA or RNA strand hybridization.

Frequently the coated nanoparticles exhibited carboxylic acid or amino groups at the surface, which can be easily functionalized with payloads bearing amino or carboxylate functionalities through amidation thanks to coupling agents. In example, mesoporous silica coated UCNPs exhibited amino groups at the surface and were functionalized with photoresponsive pyrenemethyl ligand via amidation reaction using N-hydroxysuccinimidy (NHS) and 1-ethyl-3-dimethyaminopropyl carbodiimide (EDCI). Staudinger ligation

![Figure 14: Examples of polymers usually used for the coating of inorganic nanoparticles.](image)
Introduction

 consist of a chemical reaction between a phosphine-containing molecule and a molecule bearing an azide functional group leading to the creation of amide linkage in high yield. AuNPs coated with PEG polymer bearing phosphine derivative reacted through Staudinger ligation with azide-labelled CRGDK peptide. The most popular click reaction is the Huisgen 1,3 dipolar cycloaddition using copper(I) as catalyst between alkyne and azide functionalities leading to the formation of 1,2,3-triazoles. However, copper(I)-mediated azide-alkyne cycloaddition (CuAAC) required highly toxic copper(I) that limited its application in vivo. PEGs bearing both fluorescence probe and polyarginine were introduced on silica coated \( \text{Ho}_2\text{O}_3 \) nanoparticles by CuAAC. To avoid the use cytotoxic Cu(I) catalyst, strained molecules were developed to achieve strain-promoted alkyne-azole cycloadditions (SPAAC). Moreover, SPAAC do not require the addition of any catalyst. Biomolecule, \( \text{Fe}_2\text{-transferrin} \), bearing strained-cyclooctyne molecule was covalently conjugated via SPAAC on polymer-coated core-shell QDs. Furthermore, avidin-coated nanoparticles react with biotinylated compounds or vice versa to form a strong non-covalent complex. Tekle and co-workers developed a nanosystem based on avidin-coated QDs functionalized with biotinylated ricin to image the accumulation of nanocarrier inside cancer cells.

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<th>Reaction type</th>
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<td>RNA hybridization</td>
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Table 2: Selected examples of reactions for the functionalization of inorganic nanoparticles.
1.4 Triggered release from functionalized nanoparticles

This section gives an overview of stimuli-sensitive nanocarriers in the context of drug delivery, with particular emphasis on photosensitive molecules and NIR-light responsive nanomaterials.

Since the two last decades, the development of stimuli-responsive nanodevices has generated high expectation for the controlled and selective release of therapeutic payloads to overcome MDR and to reduce side effects. A stimuli-responsive system is based on a cargo (i.e. small molecule, peptide, protein, antibody, nucleic acid) which is temporarily inactivated and caged at the surface or within the structure of nanoparticles. Upon exposure to external or internal stimulus, the cargo is uncaged from the nanocarrier to retrieve its activity. The nanosystems should satisfy some requirements such as zero premature release, high loading capacity and high accumulation into the targeted site. Furthermore, those stimuli nanocarriers have to respond specifically to the stimulus.

These stimuli are divided in two main categories: external and endogenous stimuli (Figure 15). External stimuli are the application of an external magnetic field, electric field, ultrasound, temperature gradient, X-rays and light. Endogenous conditions such as pH change, reduction-oxidation (redox) conditions or ion strength are able to trigger the release of caged compounds. Moreover, caged therapeutics can be released through bio-chemical process such as enzymatic cleavage.

![Figure 15: External and endogenous stimuli for payloads delivery from nanoparticles.](image)

One of the most common triggers for the release of cargo molecules from nanocarriers in vitro or in vivo is the pH change. The strategy is to design a nanocarrier in which a hydrophobic cargo molecule is encapsulated or which contain acetal or hydrazone bonds that can be hydrolyzed under acidic pH. Chen and co-workers designed a multifunctional nano-platform for theranostic application combining immunotherapy, PTT and pH-responsive chemotherapy for the treatment of malignant liver tumor. Nanosystems responsive to redox conditions are usually responding to glutathione (L-γ-glutamyl-L-cysteine-L-glycine, GSH) that are overexpressed in numerous cancerous cells. GSH reduced disulfide and thioether
bonds leading to the release of the caged payload. DOX encapsulated into MSNs was released after the cleavage of disulfide bond between MSNs and cyclodextrin by GSH. Magnetic nanosystems accumulate at the tumor site when external magnetic field is applied via magnetic guidance. Moreover, upon application of an alternative magnetic field, local heat (up to 46°C) is generated leading to thermal ablation of tumors. Curcumin as anticancer drug was loaded into polymer coated IONPs, and after application of an alternative magnetic field, cell death was induced by combination of thermal ablation and chemotherapy. US waves are able to cause hyperthermia by cavitation. Luo and co-workers designed a nano-bubble stabilized by lipids and loaded with paclitaxel. When mice bearing breast tumor were exposed to US irradiation, the bubble collapsed, heat was generated and the drug was released.

1.4.1 Photosensitive systems

Photo-responsive nanocarriers have been developed to achieve on-demand release of caged compounds thanks to light. Upon applying a specific wavelength, payloads are released specifically in localized areas thanks to the spatiotemporal control and non-invasiveness of the light source. Moreover, the intensity and the power of the light source can be fine tuned to reduce side effects. Light sensitive nanosystems are good candidates for therapeutic and diagnostic carriers for in vivo applications. Photosensitive systems are divided into four groups: nanocarriers based on inorganic materials able to induce PTT; systems that are able to generate ROS through photosensitization process; photo-isomerization of small molecules that induces changes of the initial properties; and systems based on photocleavable groups that are cleaved upon light irradiation at a specific wavelength (Figure 16).

Photosensitizers generate ROS upon light irradiation leading to trigger apoptosis of cancer cells through PDT. Porphyrin, which is a photosensitizer for PDT, was covalently conjugated on MSNs surface. After accumulation into cancerous cells, UV-light irradiation was applied leading to the generation of ROS and causing cells death. Light is able to trigger photo-isomerization of small molecule such as azobenzenes, spirospyrans or diarylethenes inducing changes in their physical and optical properties. Pyrene organic dye was encapsulated into micelles composed by hydrophobic spiropyran. Upon exposition to UV-light, spiropyran isomerized into hydrophilic merocyanine leading to the disruption of the micelles structure and subsequently, the dye was released in lung cancer cells. Some nanomaterials, such as CNTs and noble metal (i.e. AuNPs) nanoparticles, are able to convert light into heat. This process is known under the denomination of PTT. Overheating localized in a specific region promoted damages on biological tissues triggering cell death. Gold nanorods bearing DOX and siRNA were exposed to light. The heat generated by the nanoparticles conducted to thermal ablation of cancerous cells and simultaneously, released of anticancer drug.
Varieties of small molecules are able to absorb light and in excited state, photoreaction occurs leading to the cleavage of chemical bonds.\textsuperscript{272,273} Since the three last decades, strategies based on photosensitive molecules acting as photo-protecting groups or as phototrigger to release cargo payloads have emerged. In more details, active therapeutic is temporary inactivated through conjugation to a phototrigger and after light exposition, the bioactivity of the caged compound is restored.\textsuperscript{264} The photocleavable ligands that are able to induce the photo-uncaging of caged molecules are based on aromatic rings, which absorbed generally light in the UV region. The most common photosensitive molecules are derivatives from ortho-nitrobenzyl (ONB), coumarin (CM), quinolone, nitroindoline, p-hydroxyphenacyl or xanthene (Figure 17).\textsuperscript{274–279} For \textit{in vivo} applications, these photoactivable molecules should have some requirements such as fast and clean photoreaction, excellent quantum yield, high thermal stability, good biocompatibility and low toxicity (for both photocleavable ligand and photo-product).\textsuperscript{280} In addition, the photolysis product should have low absorption at the excitation wavelength to avoid competitive absorption with the native product. Furthermore, these photolabiles groups are easily tuned by addition of several substituents.\textsuperscript{281} This can help to increase the water solubility or to tune the absorption properties. Caged compounds are generally introduced on the phototriggers via nucleophilic substitution thanks to amino-, hydroxyl-, carboxylate-, thiol- or phosphate groups.\textsuperscript{282–286} A large variety of caged payloads are conjugated to the photocleavable linkers such as active chemical drugs, amino acids, RNA and DNA, nucleotides, peptides, proteins or fatty acids derivatives.\textsuperscript{287–293}

![Figure 16: Light induced photo-isomerization, photocleavage, photosensitization and PTT.](image)

![Figure 17: Selected examples of photosensitive ligands for controlled drug release upon light irradiation.](image)
ONB photosensitive derivatives are the most commonly employed as photo-protecting group and as phototriggers for drug delivery systems. They are highly flexible towards modification on the aromatic scaffold, such as introduction of methoxy group in para position of nitro substituent or extension of the aromatic core to form napthalen derivatives. Those modifications tune the absorbance properties of the molecule, for example, from 254 (UV-B) to 365 nm (UV-A). However, ONB phototriggers generate, upon UV-light illumination, toxic and strongly absorbent photo-degradation products, which cause potentially serious side effects. CM is also the central core of several photocleavable molecules absorbing in the UV-region. As for ONB linkers, the absorbance of coumarinyl ligand is tuned in the UV region by addition of substituents (from 320 to 475 nm). Molecular cargos are covalently conjugated to the methyl group on the C-4 position and further modifications on the C-7 or / and C-6 positions are usually made through introduction of methoxy or amino groups. However, photolysis process of coumarinyl phototriggers require the presence of a nucleophile, typically protic solvents, to achieve good photolysis process.

In the literature, controlled photochemical release was applied to the delivery of therapeutic payloads from phototigger-drug complexes for several biomedical applications. Serotonin, which is a neurotransmitter, was conjugated to ONB phototigger and upon UV-light irradiation, selective delivery into neuronal cells was demonstrated (Figure 18). Hydrophobic therapeutic agent, progesterone, coupled to coumarinyl ligand exhibited fast photolysis process leading to the improvement of sperm mobility. Santos and co-workers designed a photo-caged peptide for psoriasis treatment. Upon UV-light irradiation, the peptide was uncaged and retrieved its efficacy against psoriasis. Efficient photo-releasable system based on paclitaxel conjugated to CM was developed by Noguchi and co-workers. Upon UV-light excitation, paclitaxel was released from water-soluble coumarinyl prodrug through cleavage of carbamate linkage.

Figure 18: Selected examples of phototrigger-drug molecular systems for controlled drug release applications.

AuNPs coated with amphiphilic tetraethylene glycol (TEG) were functionalized with photosensitive system based on ONB bearing 5-FU (Figure 19). UV-light irradiation induced the uncaging of 5-FU and conducted to the reduction of the cell viability of breast cancer cells. Furthermore, Jin and co-workers synthesized a micelle-based nanocarrier bearing coumarinyl phototigger. 5-FU was covalently introduced on coumarinyl moiety via photo-irradiation using UV-A (366 nm) light source. Then, 5-FU was released through UV-B (254 nm) irradiation. In a similar way, micelle bearing folic acid (acting as targeting ligand) and photo-caged camptothecin (anticancer drug) was released upon UV irradiation into A549 cells. PAMAM dendrimer nanocarriers were functionalized with ONB phototigger bearing anticancer drug (methotrexate or DOX) and folic acid. Upon 15 min of continuous irradiation with UV-light source, up to 70% of caged payload was released in KB cancer cells. In addition, folic acid enhanced the internalization of the nanocarrier into cancer cells.
cells. MSNs loaded with caged Cbl accumulated into KB cancer cells. The activity of Cbl was restored through UV-light irradiation. In addition, upon two-photon excitation in the NIR region, coumarinyl moiety was able to absorb NIR-light via two-photon absorption process inducing the release of Cbl.

![Figure 19: Selected example of UV-vis triggered release of caged anticancer drug from nanoparticles.](image)

### 1.4.2 NIR-infrared light responsive nanomaterials

UV- and/or visible light used to trigger the release of caged compounds suffer of several drawbacks that limit the *in vitro* and *in vivo* applications. The major limitation is the low tissue penetration depth, less than 1 cm, due to endogenous scattering coming from tissue chromophores such as hemoglobin, melanin or amino acids. In addition UV-light induces photochemical reactions or heating effects that causes DNA damage, protein denaturation or lipid peroxidation. NIR-light can overcome some of these limitations. For examples, NIR-light penetrates up to 5 cm tissue depth and exhibits less cytotoxic effect. However, the therapeutical window is limited approximately from 650 to 1700 nm due to, as mentioned previously, the scattering of biological tissue and by water molecules. Furthermore, NIR window is generally divided into two sub-groups: NIR-I (650-900 nm) and NIR-II (1000 to 1700 nm). Several parameters such as power density, beam diameter, wavelength, exposure time and irradiation mode determined the efficacy of NIR-light excitation for biomedical applications. Irradiation mode is a key parameter for triggering the release. Continuous wave laser or pulsed laser source is chosen depending the type of nanomaterial. For example, pulsed laser is used to excite fluorophore via a two photons absorption process or to generate SHG or THG from HNPs (Figure 20). On the other hand, continuous wave irradiation is able to excite UCNPs for the generation of up-converted luminescence.
Several NIR responsive nanocarriers are developed for theranostic applications. These nanosystems are combining imaging properties and therapy such as controlled drug release, PDT or PTT on the same plateform. In addition, some molecular chromophores are also able to absorb in the NIR region thanks to the fine tuning of their optical properties for *in vitro* imaging. In addition, noble metals such as AuNPs convert NIR-light into heat. UCNPs are able to transform NIR-light into up-converted luminescence, which make them good candidates as imaging probe and as on-demand drug release nanoplatforms.

Upon NIR-light irradiation using a pulsed laser, several molecular chromophores are able to absorb simultaneously two low energy photons and then, convert them into UV or visible light.\(^{319}\) The efficiency of the two-photon absorption process of organic molecules is strongly depending of the two-photon cross-section. Chitose and co-workers demonstrated the ability of modified coumarinyl phototrigger to induce the release of benzoic acid as model cargo through two-photon excitation process.\(^{320}\) CM phototrigger bearing Cbl was covalently conjugated to MSNs. Upon NIR-light irradiation, 40% of the caged Cbl was released after 2 h of irradiation.\(^{308}\) Core-shell UCNPs coated with inorganic material such as carbon or gold shell make them good candidates as NIR-triggered PTT agents. Gold nanoclusters were encapsulated into mesoporous silica-coated UCNPs.\(^{321}\) The accumulation of the nanocarriers into mice bearing liver tumor was followed in real-time by photoacoustic imaging techniques. Thereafter, upon irradiation at 808 nm, heat was locally generated leading to cancer cell death. Highly functionalized UCNPs for multimodal imaging and multiple therapies are currently developed. Liu and co-workers designed a nanocarrier based on mesoporous silica coated UCNPs.\(^{322}\) The core acted as imaging probe for X-ray, CT and up-converted luminescence imaging in mice bearing cervical carcinoma tumor. In addition, caged DOX was released in tumor site due to the acidic tumor microenvironment and upon NIR-light irradiation, PDT was induced by polyoxometalate nanoclusters.

Up-converted UV-vis luminescence generated by UCNPs is able to induce the cleavage of photosensitive linkage between the phototrigger and the anticancer drug. This methodology leads to a precise and controlled release of therapeutic payload into tumor sites. In 2010, Carling and co-workers demonstrated the ability of the up-converted luminescence to trigger the uncaging of cargo model molecule from the UCNPs surface (Figure 21).\(^{323}\) When the UCNPs were irradiated with NIR, up-converted luminescence emission peaks were generated (at 290 nm, 350 nm and 450 nm) and UV-light was able to trigger the photorelease of acetic acid model molecule from benzoin photosensitive ligand. PAMAM dendrimer bearing folic acid and ONB-DOX photosensitive system was covalently conjugated to UCNPs surface.\(^{324}\) Upon NIR-light irradiation, UCNPs generated emission bands in the UV region leading to the cleavage of the ONB-DOX linkage. Up to 90% of the caged DOX was released within 60 min of irradiation in human cervix carcinoma cells. Yang and co-workers designed in nanocarrier based on silica-coated UCNPs for siRNA delivery.\(^{224}\) The coated UCNPs were functionalized with ONB photosensitive ligand bearing a cationic moiety, which was able to adsorbed siRNA. Upon 980 nm light irradiation, the UCNPs emitted UV-light and the photo responsive ONB moiety uncaged
the cationic moiety causing the release of siRNA into HeLa cells. Moreover, photosensitive molecule, 4,5-dimethoxy-2-nitroacetophenone, was covalently conjugated to the phosphate backbone of siRNA.\textsuperscript{325} This modification temporarily inactivated the siRNA. Thereafter, the complex was loaded into mesoporous silica coated UCNPs. Upon NIR-light illumination, up-converted emission at 350 nm induced the cleavage of the photosensitive protecting group leading to the release the siRNA into skin melanoma cancer cells. UCNPs coated with phosphorylethanolamine were post-functionalized with 5-FU conjugated to ONB phototrigger.\textsuperscript{326} Upon NIR-light irradiation, UCNPs generated emission bands in the UV region leading to the cleavage of ONB-5-FU linkage. Up to 80% of the caged 5-FU was released after 14 min of irradiation with NIR-light. Zhao and co-workers designed a nanosystem based on yolk-shell structure.\textsuperscript{268} The UCNPs were encapsulated into mesoporous silica hollow spheres. Thereafter, photosensitive system composed of Cbl and coumarinyl bearing long hydrophobic alkane chains was loaded into the hollow cavity through physical adsorption. Upon NIR-light irradiation, the coumarinyl moiety absorbed the UV-light emitted by the UCNPs leading to the release of Cbl. Up to 80% of the treated mice, bearing murine sarcoma tumor, survived after 35 days of treatment. Photo-responsive luciferin was covalently conjugated on silica-coated UCNPs and upon 980 nm excitation, the generated UV-light triggered the release of luciferin into mice bearing breast cancer.\textsuperscript{327} Afterwards, lucerifase was injected and the bioluminescence was detected in the tumor site that indicated the successful photorelease of the caged molecule \textit{in vivo}. Dai and co-workers developed a theranostic nanocarrier based on UCNPs coated with a PEG-PEI copolymer and post-functionalized with cisplatin prodrug.\textsuperscript{328} After injection of the nanocarrier into mice bearing murine hepatocarcinoma tumor, the biodistribution was followed in real-time by CT and MRI imaging techniques. Thereafter, the UCNPs converted the NIR-light into UV light and the drug retrieved its activity through photocleavage and the reduction of the platinum Pt(iv) into Pt(ii).
Introduction

UCNPs exhibit high potency for multimodal imaging and drug release upon NIR irradiation. However, they suffer from some drawbacks: i) the wavelengths used for the generation of up-converted luminescence are defined (i.e. 808 and 980 nm); ii) there is no possibility to decouple imaging and photo-activation protocols. HNPs can overcome those limitations. There is no wavelength constrains (excepted the wavelengths range that can be achieve by the pulsed laser) and HNPs offer the possibility to image at a wavelength where the phototrigger do not absorb light. Then, by changing the wavelength, the SHG emission from the harmonic core can induced the selective release of the caged compound.

1.5 Presentation of the project

Over the last 8 years, our group contributed to the design, preparation and evaluation of new nanodevices based on HNPs to detect and image tumors at their earliest stage of growth, in the frame of the European project NAMDIATREAM (nanotechnological toolkits for multimodal disease diagnostics and treatment monitoring). Particular focus was put on the most common cancers that are breast, lung and prostate cancer. From this study, BFO HNPs were identified as highly promising bioimaging probes due to their high harmonic efficiency. After coating and post-functionalization with targeting ligands, BFO HNPs were successfully applied to the labelling and tracking of human cancer cells and human muscle stem cells thanks to the simultaneous acquisition of the second and third harmonic signals from the inorganic core. In addition, the generation of deep ultraviolet radiation allowing direct interaction with cancer cells nuclear DNA was demonstrated with BFO HNPs in the absence of photosensitizing molecules.
The present project focused on the development of HNP-based systems for theranostic applications, combining the imaging properties of the inorganic core with light-sensitive chemical functionalization for both targeting and controlled photorelease of molecular cargos relevant for anticancer chemotherapy. The ultimate goal of such approach would be to combine on the same nanoplatform targeting ligands addressing cancer cells biomarkers, caged chemotherapeutics and complementary probes for multimodal imaging. In this study, we concentrated on the demonstration that functionalized HNPs hold the potential for decoupled optical imaging and therapeutic intervention by simple tuning of the incoming irradiation. In more details, we envisaged the conjugation of coated BFO or LNO HNPs to phototrigger-drug complexes. Bare HNPs were available through a collaboration with the Laboratoire des Systèmes et Matériaux pour la Mecatronique (SYMME, Prof. Ronan Le Dantec) at the University Savoie Mont-Blanc (Annecy-le-Vieux, France).

In this approach, we intended to make use of the nonlinear response of HNPs to convert (N)IR- or visible light into UV-light for the release of therapeutic cargos caged with photosensitive spacers at the surface of HNPs. Photorelease experiments and imaging sessions were performed in collaboration with the Department of Applied Physics at the University of Geneva (MER Dr Luigi Bonacina, Prof. Jean-Pierre Wolf).

The first phase of the project was devoted to the development of the phototrigger-drug conjugates. Two classes of photocleavable linkers, based on coumarinyl (blue color) and o-nitrobenzyl (pink color) cores, were synthesized for further caging of molecular cargos and conjugation with coated HNPs (Figure 22). Proof-of-concept was established with tryptophan (Trp) as model molecular cargo and could be extended to several anticancer drugs, including doxorubicin, chlorambucil and erlotinib analogues. These systems were expected to be cleaved upon irradiation with UV-light resulting from the harmonic emission of HNPs, thus inducing the release of the cargo molecule (dash bonds indicate the broken bonds).

![Figure 22: Cargo molecule conjugated to coumarinyl (blue) and o-nitrobenzyl (pink) phototriggers.](image)

Then, silica coated BFO and LNO HNPs were functionalized with the caged molecular cargos using copper free click reaction. Following laser-pulsed NIR irradiation, the uncaging and controlled release of the different payloads was quantified by UHPLC-MS analysis (Scheme 1).

![Scheme 1: NIR irradiation leads to generation of SHG signal in the UV region that excited the photosensitive linker leading to the release of the cargo molecule.](image)
In parallel, Raphaël de Matos (PhD student in our group) was developing targeting ligands based on erlotinib analogues and new generation of FAP inhibitors. In addition, the synthesis of MRI probe was developed based on gadolinium chelates for multimodal imaging (combination of MRI and fluorescence imaging techniques). Combination of all these components within the same HNP-based system is still under investigation (Figure 23).

**Figure 23:** HNPs for multimodal application: inorganic core and imaging probe as imaging modalities; coating to increase the biocompatibility and colloidal stability; targeting ligand to selectively target cancer cells; and photo-responsive system for controlled drug delivery upon NIR-light irradiation.
2 Results and discussion

2.1 Development of photosensitive tethers for caging molecular cargos

In this section, the first efforts were devoted to the synthesis of photocleavable linkers based on CM and ONB cores. Thereafter, a model cargo molecule, Trp, was conjugated to the linkers via the formation of a carbamate linkage. Finally, the on-demand release ability of the photo-responsive tethers was tested using UV-light.

2.1.1 Synthesis of photosensitive tethers

Two types of photosensitive tethers were investigated, based on nitrobenzyl and coumarinyl cores. The photocaging linkers based on coumarinyl derivatives containing an alkyne moiety for click azide-alkyne cycloaddition or an ester moiety for post-functionalization were synthesized. These linkers were bearing a hydroxymethyl group at C-4 position for conjugation to molecular cargo and, in addition, this bond was cleaved upon irradiation in the UV-region. The second class of linkers was composed of nitrobenzyl core that was containing different substitution patterns on the aromatic ring for tuning the absorption properties. All the ONB phototriggers were bearing carboxylate moieties, which would be further hydrolyzed for further conjugation to molecules bearing reactive ends. Nitro group in alpha position of hydroxymethyl group was required for the uncaging of the cargo molecule (see mechanism in section 2.1.3).

The coumarinyl ligands would be synthesized starting from 3-aminophenol (Scheme 2). The coumarinyl core would be accessed through a Pechmann condensation. Thereafter, functionalization of the aniline moiety was investigated through sequential alkylation reactions and Riley oxidation using selenium dioxide followed by reduction to provide the hydroxymethyl residue.

![Scheme 2: Retrosynthetic pathway for the synthesis of coumarinyl (blue), o-nitrobenzyl (pink) and m-o-nitrobenzyl (green) photocleavable ligands.](image-url)
ONB photosensitive systems would be accessed from 4-methyl-3-nitrophenol through alkylation reaction and the hydroxymethyl residue would be obtained via radical bromination followed by substitution reaction. Preparation of a third class of linkers was envisaged from commercially available vanillin. Regioselective nitration of the aromatic ring, followed by substitution reaction on the phenol group and reduction of the aldehyde will be performed.

**Synthesis of ortho-nitrobenzyl photocleavable linkers**

ONB linkers were synthesized in three steps following procedure adapted from the protocol developed by Baker and co-workers.\(^{230}\) The synthesis started from 4-methyl-3-nitrophenol that was alkylated on the phenol moiety to afford intermediates \(1-3\) in high yields (Scheme 3). A sequence of radical bromination on the methyl substituent, followed by substitution reaction in the presence of water and \(\text{AgNO}_3\) to trap the free bromide ions led to the formation of ONB linkers \(7-9\) in moderate yields (30 to 56% yield over two steps). Compound 5 was obtained in higher yield when DCM was replaced by trifluorotoluene for the radical bromination to achieve higher yield (90% vs 66% yield).

**Synthesis of meta-methoxy-ortho-nitrobenzyl photocleavable linkers**

Preparation of MONB linkers was achieved starting from commercially available vanillin. Two synthetic pathways were explored to obtain advanced intermediates \(15-17\) (Scheme 4). In pathway A, the hydroxyl group of vanillin was protected as a benzyl ether in 93% yield. Thereafter, regioselective nitration on the phenol led to compound 11 in 86% yield. Acidic cleavage of the benzyl ether with trifluoroacetic acid (TFA) followed by alkylation in the presence of tert-butyl bromoacetate delivered the advanced intermediate 15 in high yield.

In pathway B, vanillin was alkylated with tert-butyl-2-bromoacetate to form 13 in high yield. Unfortunately, regioselective nitration on the phenol ring led simultaneously to the cleavage of the tert-butyl ester. Different ester groups (methyl-, ethyl- and tert-butyl ester) were re-installed in moderate to high yields (49% to quantitative yields) via acid catalyzed esterification (for 16 and 17) or via coupling reaction using \(N,N'\)-dicyclohexylcarbodiimide (DCC) (for 13).

Pathway A provided advanced intermediate 15 in 40% yield over four steps against 22% yield over three steps for pathway B.
Results and discussion

Scheme 4: Synthesis of the MONB linkers 18-20: Pathway A: i) benzyl chloride, K$_2$CO$_3$, acetone, reflux, 16 h; ii) 70% HNO$_3$, r.t., 1 h; iii) TFA, r.t., 72 h; iv) tert-butyl bromoacetate, K$_2$CO$_3$, DMF, r.t., 16 h; Pathway B: v) tert-butyl bromoacetate, K$_2$CO$_3$, DMF, r.t., 16 h; vi) 70% HNO$_3$, 0°C, 45 min; vii) For 15: tert-butanol, DCC, DMAP, r.t., 5 h.; viii) For 16 and 17: conc. H$_2$SO$_4$ cat., solvent, reflux, 12 h; ix) NaBH$_4$, THF / MeOH (1:1), 0°C, 30 min.

The last step of the synthesis was the reduction of the aldehyde into alcohol in the presence of sodium borohydride (NaBH$_4$) to afford MONB linkers 18-20 in 53% to 99% yields. Interestingly, when the reaction time of the reduction with NaBH$_4$ was extended, reduction of ester into alcohol was also observed.

Synthesis of coumarinyl photocleavable linkers

Two families of coumarinyl linkers containing different reactive groups were synthesized. A common intermediate 24 was prepared in five steps (Scheme 5). The amino group of 3-aminophenol was first selectively protected as ethyl carbamate in moderate yield due to the formation of the corresponding ammonium chloride salt. The coumarinyl core 22 was then formed in high yield in one-pot reaction via acid-catalyzed Pechmann condensation followed by cleavage in situ of the ethyl carbamate. Tosylation followed by nucleophilic substitution with bromoethane afforded intermediate 24 in high yield (92% over two steps).

Scheme 5: Synthesis of the coumarinyl key intermediate 24: i) ethyl chloroformate, Et$_2$O, r.t., 2 h; ii) ethyl acetoacetate, H$_2$SO$_4$, EtOH, r.t., 4 h; then iii) H$_2$SO$_4$, acetic acid, reflux, 2 h; iv) TsCl, pyridine, DCM, r.t., 24 h; v) bromoethane, K$_2$CO$_3$, TBAB, NaI, ACN, reflux, 6 h.
A coumarinyl linker bearing alkyne reactive end was first synthesized following a procedure adapted from the report of Lin and co-workers (Scheme 6). Introduction of the primary alcohol was achieved by Riley oxidation of the methyl group followed by reduction of the carbonyl intermediate with NaBH₄ to afford the intermediate 25 in 63% yield. Thereafter, the tosyl group was removed under acidic conditions followed by substitution with 3-bromoprop-1-yne to introduce the alkyne moiety. Unfortunately, this last step delivered 27 in low yield. Several attempts were performed to improve the yield including changing the solvent, the base or to perform the reaction at higher temperature under microwave irradiation but did not meet with success.

![Scheme 6: Synthesis of 27](image)

The second family of coumarinyl linkers was designed to contain a carboxylate functionality. The synthesis started from intermediate 24 by removal of tosyl group in presence of sulfuric acid to afford 28 in high yield (Scheme 7). Afterwards, the secondary amine was alkylated with ethyl bromoacetate or tert-butyl bromoacetate to give respectively 29 and 30 in 90% and 75% yields. Following the previous pathway, Riley oxidation followed by reduction with NaBH₄ afforded the photocleavable linkers 31 and 32 in moderate yields. Thereafter, 31 was activated using 4-nitrophenyl chloroformate to deliver 34 moderate yield. In addition, tert-butyl ester group from compound 32 was removed in presence of TFA to form 33 in 85% yield.
Scheme 7: Synthesis of coumarinyl derivatives 31 and 32: i) H$_2$SO$_4$, 0°C, 1 h; ii) for 29: ethyl bromoacetate, K$_2$CO$_3$, TBAB, NaI, ACN, reflux, 46 h; for 30: tert-butyl bromoacetate, K$_2$CO$_3$, TBAB, NaI, ACN, reflux, 5 days; iii) SeO$_2$; p-xylene, reflux 24 h; then NaBH$_4$, MeOH, r.t., 3 h; iv) For 32: TFA, r.t., 2 h; v) For 31: 4-nitrophenyl chloroformate, DIPEA, DCM, r.t., 16 h.

2.1.2 Functionalization of photosensitive tethers with Trp as model molecular cargo

The cargo molecule was conjugated to the photocleavable tethers on the hydroxyl group. In the literature, several covalent photosensitive linkages are reported including carbamate-, ester-, amino- or phosphate bonds. Carbamate was chosen due to its good stability in physiological media compared to ester bond that is cleaved by esterase. Trp was chosen as model cargo molecule and was introduced via a two-step procedure: first, the primary alcohol was activated with 4-nitrophenyl chloroformate; second, the leaving group, 4-nitrophenol, was replaced by Trp through substitution reaction (Scheme 8).
Functionalization of ortho-nitrobenzyl photocleavable linkers with Trp

Ortho-nitrobenzyl-tryptophan (ONB-Trp) photosensitive systems 35-37 were synthesized in a one-pot procedure using optimized conditions (Scheme 9). The photocleavable ligands were first activated in presence of 4-nitrophenyl chloroformate under dark conditions followed by addition of tert-butyl L-tryptophanate. Several conditions to selectively saponify the ester group on the nitrobenzyl moiety were tested. Unfortunately, selective cleavage of ethyl- or tert-butyl ester from 37 and, respectively, 35 led only to degradation or double deprotection. Methyl ester saponification from 36, in the presence of LiOH, afforded the desired product 38 in 25% yield over three steps.

![Scheme 9](image)

Scheme 9: Synthesis of the o-nitrobenzyl-Trp linker 38: i) 4-nitrophenyl chloroformate, Et$_3$N, DCM, r.t., 16 h, dark conditions; Trp-OtBu, Et$_3$N, DCM, r.t., 24 h, dark conditions; vi) LiOH, MeOH / H$_2$O (5:1), r.t., 16 h, dark conditions.

Functionalization of meta-methoxy-ortho-nitrobenzyl photocleavable linkers with Trp

Similar strategy was applied for the synthesis of meta-methoxy-ortho-nitrobenzyl-tryptophan (MONB-Trp) 42. Different organic bases and solvents were screened to optimize the activation step and subsequent formation of the carbamate linkage to tert-butyl L-tryptophanate. Using optimized conditions, photosystems 39-41 were obtained in moderate to quantitative yields (Scheme 10). Thereafter, selective removal of methyl- or ethyl ester from 40 and 41 under basic conditions afforded 42 in high yields (84% to 91%).

![Scheme 10](image)

Scheme 10: Synthesis of the MONB-Trp linker 42: i) 4-nitrophenyl chloroformate, DIPEA, DCM, r.t., 16 h, dark conditions; then, Trp-OtBu, DIPEA, DCM, r.t, 24 h, dark conditions; ii) LiOH, MeOH / H$_2$O (5:1) r.t., 4 h, dark conditions.
Functionalization of coumarinyl photocleavable linker with Trp

The coumarin-tryptophan (CM-Trp) linker 44 was synthesized using similar procedure described above (Scheme 11). In a one-pot protocol, the hydroxyl group of 27 was activated with 4-nitrophenyl chloroformate followed by reaction with tert-butyl L-tryptophanate to afford 43 in 69% yield. tert-Butyl ester was cleaved in presence of TFA to provide 44 in moderate yield. Thus, amino-TEG-azide spacer 47 was introduced on 44 via copper click reaction followed by coupling with activated strained-dibenzo-cyclooctyne (DIBO) 48 to deliver 46 in low yield.

![Scheme 11: Synthesis of CM-Trp photosensitive linker 46](image)

2.1.3 Photorelease assays from molecular photosensitive tethers

Photorelease tethers were first exposed to UV-light to test their ability to induce the release of cargo molecule (Trp) via an one-photon absorption process. The photo-responsive systems were placed into a multi-well plate or into NMR tubes and were irradiated with 312 or 366 nm light using a Sylvania UV lamp (8 W power) or Spectroline EF-16F (15 W power) as light sources.

One-photon release of Trp from ortho-nitrobenzyl phototrigger

The photochemical process that occurs upon photo-irradiation and induces the release of the caged compound is different between coumarinyl and nitrobenzyl phototriggers. According to the literature, the proposed mechanism for photorelease from nitrobenzyl derivative is depicted in Scheme 12. Upon irradiation in the UV region, de-aromatization followed by proton transfer to the nitro group affords the aci-nitro intermediate ii. Afterwards, 1,5-cyclization delivers cyclic intermediate iii, which is the rate determining step in the photolytic process. Finally, the hemiacetal is opened, and the cargo molecule is released via
extrusion of a molecule of carbon dioxide. The photolysis process can be followed by several techniques, including $^1$H-NMR spectroscopy and absorbance measurements.

Scheme 12: Proposed mechanism for the release of cargo molecule from o-nitrobenzyl photosensitive ligand. Adapted from ref 305.

The absorption maxima of phototriggers 7 and 20 were measured to further tune the irradiation wavelength. Using a BioTek Synergy 2 multi-mode reader instrument, the absorption maxima of ONB 7 were measured at 275 and 330 nm, which was consistent with the literature for this type of phototrigger (Figure 24). MONB photosensitive linker 20 showed bathochromic displacement of absorption maximal compared to ONB 7 due to the methoxy group on the aromatic scaffold. The maxima in absorption for 38 and 40 photoconjugates were in both cases around 285 nm that corresponded to the overlapping between the absorbance of ONB photocleavable ligand and Trp.

Figure 24: Extinction spectra of ONB 7 (black), MONB 20 (red), ONB-Trp 38 (green) and MONB-Trp 40 (blue) between 250 and 500 nm.

To test the ability of the nitrobenzyl photosensitive linkers to release a cargo molecule, compounds 7 and 20 were exposed to 312 nm wavelength using a Spectroline EF-16F (15W) UV lamp as light source. At this wavelength, the linkers are able to absorb light and to induce the release of a water molecule via the formation of a cyclic intermediate (Scheme 13). Due to the formation of several photo-products ix and x, the progress of the photorelease reaction was followed by absorbance and $^1$H-NMR spectroscopy. At different
times points $^1$H-NMR spectra were recorded and the absorbance spectra were measured. Release profiles were obtained by area integration of characteristic peaks of the starting material and photo-products, and compared with the area integration of solvent peak (DMSO, 2.5 ppm).

Scheme 13: Release of water molecule from ortho-nitrobenzyl photocleavable linkers upon UV-light irradiation.

The photorelease profile of MONB derivatives was explored using MONB 20 as model. After 50 min of irradiation, 50% of the starting material was consumed (integration area of the aromatic peak at 7.64 ppm, Figure 25b, blue color curve). Moreover, appearance of a peak at 12.0 ppm corresponding to the formation of photo-product bearing aldehyde functional group and a peak at 5.91 ppm attributed to the hydrate form of the aldehyde was consistent with the literature ($^1$H NMR spectra in section 4.5.2, Figure 74). Significant changes in absorption spectra confirmed the generation of photo-products, thus the release of a water molecule (Figure 25a).

Figure 25: A) Extinction spectra at different time point of MONB 20 between 200 and 500 nm; B) photolysis of MONB 20 (blue) and photo-product formation (red) profiles by exposure to UV-light (312 nm); C) extinction spectra at different time point of ONB 7 between 200 and 500 nm; D) photolysis of ONB 7 (blue) and photo-product formation (red) profiles by exposure to UV-light (312 nm).
ONB photosensitive ligand 7 was irradiated at 312 nm. After 120 min of irradiation, 80% of the starting material was converted into the photo-products (area integration of aromatic peak of ONB 7 at 7.52 ppm, Figure 25d, blue color curve). In addition, the appearance of $^1$H NMR peaks at 11.0 ppm and 6.01 ppm was attributed to the formation of photo-products ($^1$H NMR spectra in section 4.5.2, Figure 85). Moreover, significant changes in absorption spectra indicated the successful release of a water molecule from 7 (Figure 25c). Other wavelengths were applied (254 and 366 nm) but could not induce efficient release of a water molecule.

Based on the results, the experiment was repeated using nitrobenzyl-based phototriggers bearing Trp. The photosensitive systems were irradiated at 312 nm and the progress of the photolytic reaction was monitored by $^1$H-NMR and absorbance spectroscopy (Scheme 14).

![Scheme 14: Release of caged Trp from ortho-nitrobenzyl photosensitive tethers.](image)

Half of caged Trp was released from MONB-Trp 40 within 60 min of irradiation (determined by area integration of aromatic proton peak at 7.39 ppm, Figure 26b, blue color curve). Furthermore, integration of aromatic peak at 6.98 ppm of Trp showed no variation along time, which indicated the good photo-stability of Trp upon UV-light irradiation (Figure 26b, red color curve). Significant changes in absorbance spectra were also observed confirming the photocleavage of MONB-Trp 40 upon UV-light irradiation (Figure 26a).
Upon UV-irradiation of ONB-Trp 38 at 312 nm, only 75% of rp was released after 3.5 h (area integration of peak area at 4.52 ppm, Figure 26d, blue color curve). Furthermore, drastic changes in absorption spectra were observed confirming the successful release of Trp from ONB-Trp 38 (Figure 26c).

**One-photon release of Trp from coumarinyl phototrigger**

As for ONB-derived phototriggers, the ability of coumarinyl linkers to induce the release of caged molecule was assessed under direct UV-light irradiation. According to the literature, the coumarinyl core viii absorbs UV-light leading to the formation of the excited state xiv (Scheme 15).336 Thereafter, the C-O bond (in red color) undergoes heterolysis cleavage to generate two ions. Sub-product xvii is obtained through recombination between the ion pairs with the loss of carbon dioxide molecule which corresponds to the less favored pathway according to Schade and co-workers.337 The fastest pathway is the separation of tight ion pairs xviii and xix by the solvent leading to separate ions. The positive charged coumarinyl core reacts with the solvent (in this case water) to afford compound xx. Carbon dioxide is released from intermediate xix leading to the formation of neutral Trp molecule vii. If the photorelease experiment is performed in aprotic solvent, carbon dioxide is first released and the two ion pairs are combined to re-form the starting molecule with amine bond instead of carbamate linkage. As consequence, the cargo molecule is not released.
Results and discussion

Scheme 15: Proposed mechanism for the release of Trp molecule from coumarinyl photosensitive ligand. Adapted from ref 336,337.

The extinction spectra of CM-Trp derivatives were measured in phosphate-buffered saline (PBS) solution between 200 nm to 500 nm. Maximum absorption of CM-Trp 42 was observed at 375 nm (Figure 27, red color curve). This value is consistent with the literature for amino-coumarin dye. Conjugation to DIBO led to an additional absorbance band at 300 nm (Figure 27, blue color curve).

Figure 27: Normalized extinction spectra of CM-Trp 42 (red) and CM-Trp 45 (blue) between 250 and 500 nm.

Thereafter, the ability of CM-Trp 45 to release Trp via an one-photon absorption process was assessed at 366 nm (Sylvania UV lamp, 8 W power). CM-Trp 45 was irradiated for 6 h and the released Trp was quantified by UHPLC-MS (measured mass 205.0938, mass error 16 ppm). After 2 h of irradiation, 76% of the Trp was uncaged and no additional release was observed within the additional 4 h of experiment (Figure 28).
In summary, synthesis of ONB phototriggers was achieved in 3 steps and afterwards, \textit{tert}-butyl \textit{L}-tryptophanate was introduced via formation of carbamate linkage. However, selective deprotection of ethyl ester on the nitrobenzyl moiety was not succeeded excepted for 39. MONB photo-responsive linkers were obtained through two-pathways. Irradiation at 312 nm conducted to the release of the caged Trp from ONB- and MONB-Trp photosensitive systems.

Photocleavable linkers based on a coumarinyl core bearing several functional groups (carboxylate and alkyne moieties) were synthesized in a 9 steps reaction sequence. Addition of Trp was achieved in a one-pot reaction procedure. When photosensitive tether 45 was exposed to 366 nm UV-light, Trp was released. For the further experiments, coumarinyl photocleavable linkers were used for the conjugation to HNPs. The main reasons were the brighter fluorescence and absorbance at a longer wavelength of coumarinyl tethers compared to ONB linkers (375 nm vs 340 nm respectively).

### 2.2 Development of BFO HNPs based stimuli sensitive nanocarriers

This section has led to the publication: Two-photon-triggered photorelease of caged compounds from multifunctional harmonic nanoparticles. \textit{ACS Appl. Mater. Interfaces}, \textbf{2019}, \textit{11}, 30, 27443-27452. First different methods for coating the BFO HNPs are presented. Then, the coumarinyl phototriggers bearing organic dye or Trp were introduced on the BFO HNPs through biorthogonal reactions. Finally, proof of concept of the release of caged compounds from the surface of BFO HNPs induced by the SHG emitted under NIR irradiation was demonstrated.

#### 2.2.1 Coating of BFO HNPs

Protocol for surface coating of metal oxide using hetero-bifunctionalized PEGs was previously developed in the group.\textsuperscript{339} However due to several issues such as presence of unreacted PEGs in the supernatant and low amount of phototrigger conjugated on the surface, alternative coating procedures were explored: first, inorganic coating leading to the formation of a silica shell around the harmonic core (Figure 29). Thereafter, coating using a biomolecule avidin protein was performed.
Formation of a silica shell around the nanoparticles increases the biocompatibility of various inorganic nanomaterials. However, several parameters need to be controlled such as shell size and porosity. In this project, BFO HNPs were coated with a silica shell bearing two reactive functionalities, amino and azido groups. Finally, BFO HNPs were coated with the biomolecule avidin via first formation of silica shell bearing carboxylic acid functional groups and thereafter, avidin was covalently conjugated to BFO HNPs through amidation reaction using coupling agents.

Figure 29: Coating of BFO HNPs with biomolecule (avidin protein) and through inorganic surface passivation (formation of silica shell).

APTES-coated HNPs

Following the demonstration that surface passivation with silica shell could reduce the cytotoxicity of inorganic nanoparticles, in vitro and in vivo, we explored the coating of BFO HNPs with (3-aminopropyl)triethoxysilane (APTES) derivatives. First, small APTES derivative bearing azido moiety was synthesized. Substitution of 4-bromobutanoate in the presence of sodium azide, followed by saponification of ethyl ester afforded 50 in 73% yield (Scheme 16). Activation of carboxylate with NHS afforded compound 51 in 72% yield. Conjugation of 51 to APTES led to APTES-N₃ 52 in high yield which was used for surface modification of BFO HNPs.

Scheme 16: Synthesis of APTES-N₃ 52: i) NaN₃, MeOH / H₂O, reflux, 7 h; ii) KOH, MeOH / H₂O, 0°C to r.t., 6 h; iii) DCC, NHS, DCM, r.t., 4 h; iv) APTES, DCM, r.t., 5 h.

BFO HNPs were produced by a precipitation route developed by Tytus and co-workers (SYMME, Annecy). Briefly, bismuth nitrate pentahydrate and iron nitrate nonahydrate were dissolved in a solution of nitric acid. This solution was added to a stirred solution of sodium hydroxide leading to the formation of a precipitate. The solid was crystallized at high temperature to produce the nanocrystals. The synthetic
procedure to form of silica shell around BFO HNPs was based on a protocol developed for the coating of UCNPs. BFO HNPs were suspended in a mixture of ethanol and cyclohexane (1:1), followed by addition of TEOS, APTES and APTES-N₃ 52 (2:1:1) (Scheme 17). Aqueous ammonia was added, and the suspension was ultra-sonicated for 16 h at 40°C. After removal of unreacted organic molecules, the APTES-N₃-coated HNPs (BFO-APTES-N₃) were characterized by their hydrodynamic diameter and surface charge.

Scheme 17: Surface passivation of BFO HNPs with APTES derivatives.

Drastic change in zeta potential value (-27.8 ± 0.7 mV to 20.2 ± 0.4 mV) was measured, that indicated the addition of positive charges on the BFO HNPs surface. Large increase of mean hydrodynamic diameter (from 331.7 ± 53.3 nm to 464.3 ± 120.2 nm) was consistent with formation of a silica shell around the BFO HNPs. Moreover, the silica shell around the BFO HNPs was evidenced by STEM images (Figure 30). The average silica coating layer determined by STEM observation and was about 80-90 nm thick, which was coherent with dynamic light scattering (DLS) measurement.

Figure 30: Representative STEM images of BFO-APTES-N₃ HNPs: A) high-angle annular dark-field image; B) Si EDX map; C) O EDX map; D) Bi EDX map; E) Fe EDX map.
Avidin-coated BFO HNPs

First, BFO HNPs were covered with a silica shell using TEOS and APTES-CO$_2$H 53 following the protocol described for BFO-APTES-N$_3$ (Scheme 18). APTES-CO$_2$H coating of BFO HNPs was evidenced by the following measurements: i) the zeta potential value decreased from -27.8 ± 0.7 mV to -34.6 ± 0.9 mV due to the presence of carboxylate groups; ii) the mean hydrodynamic diameter shifted from 331.7 ± 53.3 nm to 114.1 ± 9.6 nm.

Then, surface carboxylic acids were activated with sulfo-NHS to covalently conjugate avidin on the BFO HNPs surface. Finally, the unreacted carboxylic groups were treated with ethylene diamine in order to add positive charges on the BFO HNPs surface to increase their colloidal stability. This step led to the decrease of average hydrodynamic diameter (114.1 ± 9.6 nm to 77.0 ± 9.1 nm) and diminution of the surface potential (-34.6 ± 0.9 mV to -52.8 ±3.9 mV). That was due to the better colloidal stability of BFO-Avidin HNPs compared to bare and BFO-APTES-CO$_2$H HNPs. In addition, STEM images showed the presence of silica and oxygen atoms on the surface of BFO-Avidin HNPs (Section 4.4.3, Figure 63). Unfortunately, the Bi and Fe atoms could not be localized due to the thick layer of silica around the BFO HNPs. All data confirmed the successful functionalization of the BFO HNPs with avidin.

2.2.2 Real time release of Cy3 organic dye from BFO-Avidin HNPs

In this section, coumarinyl photo-responsive linker 27 was functionalized with a Cy3-derived organic dye to follow in real-time the release of caged molecule from the surface of BFO HNPs. The dye should fulfill a number of requirements such as no or low absorbance overlapping between the dye and the phototrigger, high photo-stability and strong fluorescence intensity. For these reasons, cyanine3 dye (Cy3) was chosen. Cyanine dyes are composed by two indolenine rings linked via a polymethyne chain acting as both electron acceptors and donors. These dyes exhibit bright fluorescence in the visible region (570 to 700 nm), high extinction coefficients and good quantum yields. The absorbance wavelength can be easily tuned by elongating the polymethylene chain or by addition of a second benzyl ring. Furthermore, sulfonated groups can be conjugated on the indolenine scaffold to increase the solubility of cyanine dye in aqueous solutions. However, they undergo photobleaching upon long irradiation. Cyanine dyes are currently used for several applications such as fluorescent imaging probe, organic solar cells or in histological staining. In addition, the FDA approved indocyanine green in 1958 for medical diagnosis application. Cy3 derivative was first
conjugated to coumarinyl photosensitive linker, followed by addition of biotinylated moiety for reaction with avidin-coated BFO HNPs (Scheme 19).

![Scheme 19: Retrosynthetic pathway for the synthesis of photosensitive tether bearing Cy3 organic dye.](image)

Coumarinyl-containing derivative 27 was first activated with 4-nitrophenyl chloroformate in 78% yield, followed by conjugation with Cy3 derivative 57 through a carbamate linkage (Scheme 20). Thereafter, 55 reacted with biotinylated TEG 58 via CuAAC to produce 56 in low yield.

![Scheme 20: Synthesis of CM-Cy3 56: i) 4-nitrophenyl chloroformate, DIPEA, DCM, r.t., 16 h; ii) 57, DIPEA, DMF, r.t., 16 h; iii) biotin-TEG-N₃ 58, CuSO₄, sodium ascorbate, H₂O / THF (1:1), r.t., 24 h.](image)

The extinction spectrum of CM-Cy3 56, measured in ACN, showed two distinct absorption bands around 400 nm and 530 nm, which were attributed to CM and Cy3 respectively (Figure 31). In addition, no overlapping between the absorption of CM and Cy3 moieties was observed. These conditions should thus allow to decouple imaging and monitoring of molecular cargo release upon NIR excitation.
Results and discussion

Avidin, which is composed by four homo-units, is able to form one of the strongest non-covalent complex with biotinylated compounds with an association constant of $10^{15}$ M$^{-1}$. Moreover, avidin is stable under extreme conditions such as pH and denaturants. BFO-Avidin HNPs were incubated with the photosensitive system 56 to afford BFO-Avidin-CM-Cy3 which were characterized by DLS and zeta potential measurements (Scheme 21). Slight increase in size (from $77.0 \pm 9.1$ nm to $80.9 \pm 14.1$ nm) was measured. While, significant change in zeta potential (from $-52.8 \pm 3.9$ mV to $-43.7 \pm 1.3$ mV) was observed, indicating the addition of positive charges coming from Cy3 dye.

BFO-Avidin-CM-Cy3 were exposed to NIR pulsed laser irradiation setup coupled to a multiphoton inverted microscope system. An image of BFO-Avidin-CM-Cy3 was first recored before photolysis using a 950 nm wavelength. At this wavelength, only the Cy3-derived dye 57 absorbed light and not the coumarinyl phototrigger (Figure 32a, black color curve). Afterwards, the wavelength was shifted to 750 nm and the generated SHG signal by the BFO HNPs in the UV region (at 375 nm) started to excite the coumarinyl phototrigger. This process induced the release of the caged Cy3-derived dye 57. Moreover, the uncaging process was followed in real-time by measuring the decrease of the fluorescence intensity from Cy3-derived dye 57 present on the BFO HNPs surface. After 5 min of irradiation, the wavelength was shifted back to 950 nm and a new image was recorded. Disappearance of the fluorescence emission signal belonging to Cy3-derived dye 57 was observed (Figure 32a, red color curve). The uncaging experiment was tested at different wavelengths (880 nm and 950 nm) and, as expected, slower released rates were obtained when longer wavelengths were used (Figure 32h, red and green color curves). That was attributed to the decrease absorption efficiency of CM at longer wavelengths (see absorption spectra, Figure 31). In addition, when lower power was applied, slower release rate of Cy3-derived dye 57 was measured (Figure 32h, blue color curve).

Figure 31: Normalized extinction spectrum of Biotin-CM-Cy3 56 between 250 and 500 nm.

Scheme 21: Incubation of CM-Cy3 56 with BFO-Avidin HNPs.
Figure 32: A) Emission spectrum of BFO-Avidin-CM-Cy3 upon excitation at 950 nm before (black) and after (red) photolysis at 750 nm, obtained from selected regions of multiphoton multispectral images; multiphoton multispectral microscopy of BFO-Avidin-CM-Cy3 HNPs at 950 nm, before photolysis: B) SHG channel images of BFO HNPs, C) merged channel images, D) sum of intensity of all channels in the 530-650 nm spectral region (fluorescence of Cy3 dye); and after photolysis: E) SHG channel images of BFO HNPs, F) merged channel images, G) sum of intensity of all channels in the 530-650 nm spectral region; H) rate release profiles of Cy3 dye 57 from BFO-Avidin-CM-Cy3 HNPs at different wavelengths and power densities; I) Cy3 dye 57 release profiles following irradiation at 750 nm of BFO-Avidin-CM-Cy3 (red), SiO2-Avidin-CM-Cy3 (green) and SiO2-Avidin-biotin-Cy3 (blue).

To confirm that the uncaging process was induced by the SHG emission from the harmonic core, bare silica nanoparticles were coated with avidin and functionalized with CM-Cy3 photosensitive system 56 as negative control system (Scheme 22). In addition, BFO-Avidin HNPs and silica-avidin nanoparticles were conjugated with biotin-Cy3 59, which do not contain any photocleavable linker to ensure that the release was effectively induced by the SHG emission from the harmonic core. All these systems were exposed to 810 nm NIR-light using 20% of power density and the uncaging rate was followed in real-time by measuring the maximal fluorescence intensity of Cy3-derived dye 57 (Figure 32i). Unfortunately, similar release curves were obtained in all cases meaning that only photobleaching of Cy3-derived 57 or degradation of the avidin coating were measured. As a conclusion, Cy3 organic dye was not suitable for following the release of caged compound in real-time.
Results and discussion

2.2.3 Photo-controlled release from BFO-APTES-N$_3$ HNPs with Trp as model molecular cargo

Due to photobleaching of the organic dye upon NIR-light irradiation, coumarinyl photosystem bearing Trp was used as model for controlled release of caged molecular cargo. In this section, the ability of the SHG induced by the BFO HNPs to uncage the Trp was explored. In a first part, CM-Trp linker was covalently conjugated on the BFO-APTES-N$_3$ HNPs surface by copper free click reaction. Thereafter, the release of Trp using UV-light was demonstrated, followed by NIR triggered uncaging process.

To conjugate the photosensitive system on the BFO HNPs surface, spontaneous [3+2] cycloaddition between the strained 4-dibenzocyclooctyne moiety present on the coumarinyl photocleavable linker and azido groups on the BFO-APTES-N$_3$ HNPs surface was investigated (Scheme 23). A solution of CM-Trp was added to a suspension of BFO-APTES-N$_3$ HNPs in a mixture of ethanol / DMF (1:1). Then, the suspension of BFO HNPs was ultra-sonicated for 16 h at 40°C under dark conditions. Thereafter, the unreacted ligands were removed by successive cycles of centrifugation (10 min, 4 700 rpm) and washing with DMF and EtOH.

Scheme 22: Functionalization of BFO-Avidin HNPs with Biotin-Cy3; functionalization of SiO$_2$ NPs with CM-Cy3 and Biotin-Cy3.

Scheme 23: Functionalization of BFO-APTES-N$_3$ HNPs with CM-Trp photosensitive tether via copper free click reaction.
After functionalization with CM-Trp 46, the mean hydrodynamic size did not change (464.3 ± 120.2 nm to 440.9 ± 76.1 nm). However, the zeta potential value shifted from 20.2 ± 0.4 mV to -3.0 ± 0.4 mV. These data were consistent with the evolution of the charge surface composition (amino group were replaced by carboxylate moieties from Trp). The initial amount of photosensitive tether 46 present on the surface of the BFO HNPs was determined by measuring the absorbance of BFO-APTES-CM-Trp HNPs and compared to calibration curves of CM-Trp 46 between 0 to 1000 μM (section 4.5.1, Figure 69). The maximal amount of CM-Trp 46 was evaluated at 3.29 μM per mg of BFO HNPs (Figure 33).

Figure 33: Extinction spectra of CM-Trp 46 (500 μM, red), BFO (0.5 mg/ml, black), BFO-APTES-N₃ (0.5 mg/ml, blue) and BFO-APTES-CM-Trp (2 mg/ml, green) between 200 to 700 nm. Reprinted (adapted) with permission from Two-photon-triggered photorelease of caged compounds from multifunctional harmonic nanoparticles. ACS Appl. Mater. Interfaces. Copyright 2019.

Multiphoton multispectral imaging was performed on a Nikon multiphoton inverted microscope (A1R-MP) coupled with tunable Ti:Sapphire laser source (100 fs, 80 MHz, 700–1000 nm). Co-localization was observed between the bright structure (larges structures corresponded to particles aggregate) in the images in Figure 34a and 34b, which corresponded, respectively, to SHG channel image of BFO HNPs and to the coumarinyl moiety (image obtained by summing the intensity of all channels in the spectral range). To confirm this attribution, multiphoton multispectral microscopy images of non-functionalized BFO HNPs were performed under the same excitation and detection conditions. As expected, only SHG emission at 405 nm was detected (Figure 34c-d). In Figure 34e, typical emission spectrum of BFO-APTES-CM-Trp was reported. Narrow peak at short wavelengths corresponding to the SHG emission from the BFO HNPs and broad luminescence signal attributed to coumarinyl moiety were detected. All these results confirmed the successful surface functionalization of BFO HNPs with 46.
Results and discussion

Figure 34: Multiphoton multispectral microscopy of BFO-APTES-CM-Trp HNPs: (A) SHG channel image and (B) sum of intensity of all channels in the 450-560 nm spectral range for BFO-APTES-CM-Trp HNPs (large structures correspond to particles aggregates); multiphoton multispectral microscopy of BFO HNPs: (C) SHG channel image and (D) sum of intensity of all channels in the 450-560 nm spectral range for BFO HNPs; E) emission spectrum of BFO-APTES-CM-Trp HNPs upon excitation at 800 nm, obtained from selected regions of multiphoton multispectral images. Reprinted (adapted) with permission from Two-photon-triggered photorelease of caged compounds from multifunctional harmonic nanoparticles. ACS Appl. Mater. Interfaces. Copyright 2019.

Photorelease at one and two photon from BFO-APTES-CM-Trp HNPs

The photosensitive nanocarriers were first exposed to UV-light to test the ability of coumarinyl photocleavable linker to induce the release of Trp from the BFO HNPs surface via one-photon absorption process (Scheme 24). The CM-Trp ligand 46 and BFO-APTES-CM-Trp HNPs were placed into a multi-wells plate and irradiated at 366 nm using a Sylvania UV lamp (8 W power) as light source. The released Trp in the supernatant was quantified by UHPLC-MS. Thereafter, the ability of SHG emission from the BFO HNPs to cause the cleavage of CM-based tether and subsequently inducing the release of caged Trp was demonstrated upon femtosecond pulsed NIR irradiation at 790 nm. In more details, the laser setup was composed of a Ti:Sapphire laser system (Asterlla Coherent) with a 5 W average output power and the laser output corresponded to 6.5 mm (FWHM) with a 430 GW / cm² peak intensity at the sample. The samples were placed into a multi-wells plate and were irradiated at 790 nm for a given time and the Trp released in the supernatant was quantified by UHPLC-MS. HCC827 cells were exposed to laser pulsed irradiation and/or to BFO HNPs in order to ensure that these conditions were compatible for in vitro experiment.
The ability of coumarinyl photosensitive tether to induce the release of caged Trp was tested using a UV lamp (366 nm, 8 W). A 4 μM solution of photosensitive system 46 in PBS was first exposed for 6 h to UV-light and the products were analyzed and quantified by UHPLC-MS (measured mass 304.0871, mass error 50 ppm). The uncaged compound was confirmed to be Trp (co-elution with Trp standard at 1.5 min and accurate measured mass error < 10 ppm). After 2 h of irradiation, 76% of the caged Trp was released and then, reached a plateau without significant release within the remaining 4 h of experiment (Figure 35, red color curve). Afterwards, the ability of coumarinyl tether to induce the uncaging of Trp from the surface of BFO HNPs was assessed by direct UV-light irradiation in PBS medium. The release profile showed a slower release rate compared to free photosensitive conjugate 46 (65% of uncaged Trp after 2 h vs 76% for 46, Figure 35 blue color curve). After 6 h of irradiation, more than 85% of caged Trp was released from the surface of BFO HNPs. Moreover, BFO-APTES-CM-Trp HNPs kept in the dark and at 37°C did not showed any trace of released Trp in the supernatant (Figure 35, green color curve). For quantitative comparison, the rate of the photocleavage mechanism was determined by fitting the progress curves with bi-exponential function. The initial rate ($k_0$) was determined at 5 min using normalized parameters. As expected, faster rate was obtained for the free ligand 46 compared to the ligand covalently bounded to BFO HNPs surface (16 nM/min vs 9 nM/min respectively), which was consistent with the decreased efficiency of photorelease mechanism in suspension compared in solution. This led to the conclusion that uncaging process occurred only in the presence of UV-light and the system was stable at physiological temperature.
Results and discussion

Figure 35: UV-light-triggered release of Trp from BFO-APTES-CM-Trp HNPs: Trp release profiles following irradiation at 366 nm of BFO-APTES-CM-Trp HNPs (blue), CM-Trp 46 (red) and BFO-APTES-CM-Trp HNPs (dark conditions, green). Reprinted (adapted) with permission from Two-photon-triggered photorelease of caged compounds from multifunctional harmonic nanoparticles. *ACS Appl. Mater. Interfaces.* Copyright 2019.

Based on the results obtained upon direct irradiation using UV-light, the ability of SHG emission from the BFO harmonic core to induce the uncaging of Trp was assessed using an NIR pulsed Ti:Sapphire laser setup as excitation source. In view to potential *in vitro* and *in vivo* applications, the cytotoxic effect of NIR triggered photo-activation protocol was first evaluated on lung adenocarcinoma HCC827 cells. The cells were exposed to laser pulsed irradiation at 790 nm. Cytotoxicity effect caused by the NIR exposition was determined in function of the irradiation time by dosing the lactate dehydrogenase (LDH) released in the cell media. High cell viability, more than 80%, was observed after 15 minutes of continuous irradiation on HCC827 cells at 790 nm (Figure 36a). Thereafter, BFO-APTES-CM-Trp HNPs (10 μg/mL) were incubated with HCC827 cells at 37°C for 16 h. BFO-APTES-CM-Trp HNPs did not affected the cell viability in comparison with the control (more than 90% of cell viability in both cases, Figure 36b). When the HCC827 cells were incubated with BFO-APTES-CM-Trp HNPs at 37°C for 16 h, followed by 15 minutes of continuous irradiation at 790 nm, the cell viability remained high (more than 80%). Consequently, the cell exposure to NIR-light did not induced high cytotoxicity and allowed to use this setup for control and selective drug release in physiological medium without causing severe side damages.

Figure 36: A) Effect of NIR irradiation time on the viability of HCC827 cells and the cytotoxicity was determined by LDH released in the cell culture supernatant; B) effect of the NIR triggered photoactivation process on the viability of HCC827 cells: HCC827 cells were exposed for 16 h to BFO-APTES-CM-Trp HNPs, for to 15 min laser pulsed irradiation at 790 nm or for 16 h to BFO-APTES-CM-Trp HNPs followed by 15 min laser pulsed irradiation at 790 nm. Cytotoxicity was evaluated by LDH released in the cell culture supernatant. Data were compared using Student’s t-test. Significance is expressed as: ns, p > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001. Reprinted (adapted) with permission from Two-photon-triggered photorelease of caged compounds from multifunctional harmonic nanoparticles. *ACS Appl. Mater. Interfaces.* Copyright 2019.
Based on the results obtained by irradiation with UV-light, the experiment was repeated using NIR pulsed laser irradiation light source. First, a suspension of BFO-APTES-CM-Trp HNPs in PBS was exposed at 790 nm for 30 min. After 15 min of irradiation, approximately 60% of Trp was released in the supernatant (quantification by UHPLC-MS). Afterwards, slower uncaging rate was measured (70% of Trp released after 30 min, Figure 37, blue color curve). Control experiments were performed to confirm that the release mechanism resulted from photocleavage of CM-Trp carbamate linkage induced by the SHG emitted from the BFO HNPs. First, BFO-APTES-N3 were functionalized with DIBO-Trp 60, which do not contain any photocleavable tether. As expected, no released Trp was detected from BFO-APTES-DIBO-Trp by UHPLC-MS within 60 min of irradiation (Scheme 25 and Figure 37, green color curve). Due to the ability of the coumarinyl photosensitive ligand to absorb NIR-light via two-photon absorption process and subsequently inducing the release of the caged compounds, a 40 μM solution of CM-Trp 46 was exposed to 790 nm light laser excitation. After 10 min of irradiation, only 10% of Trp was uncaged from the ligand 46 and no additional release was observed after 30 min of exposure to 790 nm laser excitation (Figure 37, green color curve).

Scheme 25: Functionalization of BFO-APTES-N3 HNPs via SPAAC using ligands bearing Trp with (BFO-APTES-CM-Trp) or without (BFO-APTES-DIBO-Trp) a photosensitive caging group.

For quantitative comparison of the uncaging rate, the photolysis progress was determined by fitting the release curves. The initial rate constant ($k_0$) was measured at 5 min using normalized parameters deriving from mono-exponential fitted curves. As expected, the initial kinetic rate of Trp uncaging from BFO-APTES-CM-Trp HNPs was almost ten times higher than the CM-Trp ligand 46 in solution (36.5 nM/min and 4.5 nM/min respectively). All these data confirmed the successful released of Trp from the surface of functionalized BFO HNPs resulting from the cleavage of carbamate linkage triggered by the SHG emission generated by the harmonic core.
To ensure that the non-linear properties of BFO HNPs were not affected by the NIR irradiation, the SHG signal was measured before (left image, Figure 38) and after (right image) NIR exposition. The images showed no modifications of the average SHG response from the BFO HNPs upon irradiation at 790 nm (430 GW/cm²) for 10 min.

In summary, CM-Trp photocleavable linker 46 was conjugated to the BFO-APTES-N3 HNPs by SPAAC. First, the ability of coumarinyl phototrigger to release caged Trp from the BFO HNPs surface was demonstrated using UV-light. The SHG emission from BFO HNPs was able to trigger the uncaging of Trp under NIR-light irradiation. These results constitute the basis for the development of protocols based on HNPs for decoupled imaging and photo-activation of caged molecular cargos.
2.3 Application of the methodology to the controlled release of anticancer drugs

Part of this section is based on the manuscript "Photocontrolled release of the anticancer drug chlorambucil with caged harmonic nanoparticles", in preparation. The methodology presented in the section 2.2 was applied to the release of anticancer drugs (erlotinib analogue, DOX and Cbl) from LNO-APTES-N3 HNPs. Anticancer drugs were first conjugated to coumarinyl phototriggers via either carbamate or an ester linkages. Afterwards, the photo-responsive tethers were covalently conjugated to the LNO HNPs surface through copper free click reaction. Finally, the ability of phototigger to induce the release of anticancer drug was tested using, first, UV-light then NIR-light excitation sources.

In this section, another type of HNP were used, LNO HNPs, which exhibited better colloidal stability (monitored over 72 h) and more robust and reproducible synthetic protocol compared to BFO HNPs. However, the SHG efficiency of LNO HNPs is lower compared to BFO HNPs. 351

2.3.1 Functionalization of coumarinyl ligand with anticancer drugs

Anticancer drugs, erlotinib analogue, DOX and Cbl, were introduced on coumarinyl linkers via either carbamate or ester linkage. DOX was conjugated on the coumarinyl phototigger via its amino group, while the carboxylate moiety of Cbl was used (Figure 39). Erlotinib analogue bearing amino group was synthesized prior reaction with coumarinyl tether.

As presented in the section 1.1.2, DOX is a highly active anticancer drug that intercalates into the double DNA strands. However, this drug causes several side effects and if the molecule is covalently bounded to nanoparticles, its cytotoxicity can be reduced. DOX was directly linked to the coumarinyl phototigger 33 without further modifications thanks to the amino group present on the amino-sugar moiety.

The synthesis started by amidation reaction between coumarinyl ligand 33 and DIBO-NH$_2$ 63 in presence of HOBt and EDCI to form 61 in moderate yield (Scheme 26). In an one-pot synthesis, the hydroxyl group of 61 was first activated with 4-nitrophenyl chloroformate followed by reaction with DOX hydrochloride to form the carbamate linkage between the DOX and the coumarinyl linker. CM-DOX photocleavable linker 62 was obtained in low yield (18% yield).
Erlotinib is an anticancer drug that inhibited reversibly and selectively the ATP binding pocket of EGFR. As presented in section 1.1.2, the position C6 and C7 are pointing outside of the pocket. One of these positions was modified to introduce amino moiety for the conjugation to coumarinyl phototriggers via formation of carbamate linkage. In addition, Boobalan and co-workers tested the cell viability of several erlotinib analogues bearing iodo-, mesyl-, amino- or acetate groups at the C-6 position. No significant cytotoxicity was observed compared to commercially available erlotinib (excepted for iodo-compound). As a consequence, introduction of amino group at the C-7 position should not induced higher toxicity than erlotinib.

Erlotinib analogue 74 was synthesized using an adapted procedure developed in the group (Scheme 27). Starting from vanillin, nitrile 64 was obtained in high yield through condensation between vanillin and hydroxylamine followed by in situ dehydration of the intermediate. The hydroxyl group was first alkylated with 2-chloroethanol followed by protection with acetyl chloride to afford 66 in high overall yield. Regioselective nitration on the aromatic ring using fuming nitric acid led to the formation of the intermediate 67 in 74% yield. Thereafter, the nitro group was reduced into amino moiety using polymethylhydroxysiloxane (PMHS) and catalytic amount of Pd(OAc)$_2$, followed by condensation with DMF-DMA to afford 69 in 77% yield (over two steps). Erlotinib analogue 71 was obtained through Dimroth rearrangement under acidic condition followed by removal of acetyl group. Then, the hydroxyl moiety reacted first with mesyl chloride followed by substitution reaction with sodium iodide to provide 73 in moderate yield. Afterwards, the intermediate 73 reacted with a solution of ammonia in methanol to afford erlotinib analogue bearing amino moiety 74 in quantitative yield.
Scheme 27: Synthesis of erlotinib analogue 74: i) NH₂OH·HCl, AcOH, reflux, 90 min; ii) 2-chloroethanol, K₂CO₃, DMF, 150°C, 8 h; iii) Acetyl chloride, pyridine, 0°C, 2 h; iv) HNO₃, 0°C to r.t., 35 min; v) Pd(OAc)₂, PMHS, KF, THF, r.t., 30 min; vi) DMF-DMA, ACOH, toluene, reflux, 2 h; vii) 3-ethynylaniline, ACOH, 130°C, 2 h; viii) K₂CO₃, MeOH, r.t., 20 min; ix) Ms-Cl, DMAP, pyridine, 0°C to r.t., 5 h; x) NaI, acetone, 80°C, 16 h; xi) 7N ammonia in MeOH, 85°C, 16 h.

Subsequently, erlotinib analogue 74 reacted with activated coumarinyl linker 34 to form 75 in 63% yield (Scheme 28). Thereafter, the ethyl ester on the coumarinyl moiety was saponified using lithium hydroxide to give the intermediate 76 in moderate yield. Finally, 76 reacted with DIBO-NH₂ 63 to afford CM-erlo 77 in 40% yield.

Scheme 28: Synthesis of CM-erlo 77: i) DIPEA, DMF, r.t., 16 h; ii) LiOH, MeOH / H₂O (5:1), r.t., 6 h; iii) 63, HOBT, DMAP, EDCI, DIPEA, DMF, r.t., 24 h.
Cbl was one of the first discovered anticancer drugs. An alkylating agent that covalently binds to DNA strand through guanidine nucleotide leading to cell death. Di-chloro-ethyl moiety is the active part of Cbl and cannot be modified without loss of its activity. Therefore, the carboxylic acid end was used to introduce Cbl on the coumarinyl photo-responsive tether 61 via esterification reaction using EDCI and HOBt as coupling agents (Scheme 29). Finally, CM-Cbl 78 was obtained in 58% yield.

Scheme 29: Synthesis of CM-Cbl 78: i) Cbl, HOBt, EDCI, DIPEA, DCM, r.t., 16 h.

In summary, three anticancer drugs (DOX, erlotinib analogue and Cbl) were covalently conjugated on coumarinyl phototrigger (Figure 40). Afterwards they would be introduced on the LNO-APTES-N₃ surface through SPAAC.

Figure 40: Coumarinyl-anticancer drug photosensitive tethers

2.3.2 Functionalization of LNO-APTES-N₃ HNPs with CM-anticancer drug photosensitive tethers

LNO HNPs were coated using a mixture of TEOS, APTES and APTES-N₃ 52 following the procedure detailed in part 2.2.1 (Scheme 30). The surface potential shifted from -39.0 ± 1.2 mV to 25.5 ± 0.7 mV indicated the large addition of positive charges on the LNO HNPs surface. Furthermore, increase of the mean hydrodynamic diameter of LNO-APTES-N₃ HNPs (from 61.2 ± 9.4 nm to 165.3 ± 33.8 nm) was measured. Representative STEM images confirmed the formation of silica shell around LNO HNPs. The LNO-APTES-N₃ HNPs were functionalized with Cy3 dye (DIBO-Cy3 79) via SPAAC. Multiphoton multispectral microscopy images showed a co-localization between from SHG signal of LNO harmonic core and the fluorescence signal from Cy3 dye (Section 4.4.2, Figure 53).
Scheme 30: Coating of LNO HNPs with APTES derivatives.

Figure 41: Representative STEM images of LNO-APTES-N₃ HNPs: A) high-angle annular dark-field image; B) Si EDX map; C) O EDX map; D) Nb EDX map; E) C EDX map.

The LNO-APTES-N₃ were functionalized with CM-drug using the procedure described previously in the section 2.1.2. CM-DOX 62 was first conjugated on LNO-APTES-N₃ HNPs (Scheme 31). As expected, no significant variation in the charge surface (from 25.5 ± 0.7 mV to 22.5 ± 0.4 mV) was measured, as the photo-responsive tether 62 was not adding any positive or negative charges. The mean hydrodynamic size of nanocarrier do not changed (from 165.3 ± 33.8 nm to 164.1 ± 30.5 nm). However, the white colored LNO HNPs turned purple colored after functionalization that indicated the addition of ligand 62 on the LNO HNPs surface. Furthermore, images obtained by multiphoton multispectral microscopy confirmed the addition of CM-DOX ligand 62 on the LNO HNPs via co-localization between the SHG signal from LNO harmonic core and both fluorescence signals from coumarinyl and DOX moieties (Section 4.4.2, Figure 57).
Scheme 31: Functionalization of LNO-APTES-N₃ HNPs with 62, 77 and 78 photosensitive tethers.

The initial amount of DOX present on the LNO HNPs surface was quantified by absorbance measurement and was determined at 4.27 μM of DOX per mg of LNO HNPs (Figure 42). Interestingly, the maximum in absorbance shifted from 492 nm to 540 nm.

Figure 42: Extinction spectra of CM-DOX 62 (red) and of LNO-APTES-CM-DOX (black) in PBS between 250 to 700 nm.

Subsequently, LNO-APTES-N₃ HNPs were functionalized with CM-erlo photosensitive tether 77. Increase in mean hydrodynamic size of the LNO HNPs (from 165.3 ± 33.8 nm to 200.6 ± 33.2 nm) and no change in zeta potential (from 25.5 ± 0.7 mV to 23.7 ± 0.6 mV) were measured. Furthermore, multiphoton multispectral microscopy images confirmed the presence of photo-responsive system 77 on the LNO HNPs via colocalization between the SHG signal from the LNO HNPs and the fluorescence signal from coumarinyl moiety (Section 4.4.2, Figure 55). The amount of erlotinib analogue 74 present on the surface of LNO HNPs was determined by measuring the absorbance of supernatant after coating and compared to standard curves (section 4.5.1, Figure 70). The initial amount of erlotinib analogue 74 on the HNPs was evaluated at 4.98 μM of 74 per mg of LNO HNPs (Figure 45).
Results and discussion

Figure 43: Extinction spectra of CM-erlo 77 (blue) and the supernatant (red) after copper free click reaction between CM-erlo 77 and LNO-APTES-N₃ HNPs in DMF between 250 and 500 nm.

LNO-APTES-N₃ HNPs were functionalized with CM-Cbl photo-responsive system 78 via SPAAC. The functionalized nanoparticles were characterized by their mean hydrodynamic diameter and the zeta potential: the zeta potential slightly decreased (from 25.5 ± 0.7 mV to 20.4 ± 0.2) and the mean hydrodynamic diameter increased from 165.3 ± 33.8 nm to 297.4 ± 60.9 due to the addition of hydrophobic Cbl anticancer drug. Moreover, co-localization between the SHG signal of LNO HNPs and the fluorescence emission spectrum of CM was obtained (Section 4.4.2, Figure 59). The amount of Cbl present of the LNO HNPs surface was determined by measuring the absorbance between 200 to 700 nm and compared to standard curves (section 4.5.1, Figure 72). The initial amount of Cbl present on the HNPs was evaluated at 3.64 μM of Cbl per mg of HNPs (Figure 44).

Figure 44: Extinction spectra of CM-Cbl 78 (blue) and LNO-APTES-CM-Cbl HNPs (red) in PBS between 250 and 600 nm.

2.3.3 Photorelease assays at one and two photon from LNO-APTES-CM-drug HNPs

As for previously reported with BFO-APTES-CM-Trp HNPs, a first set of experiments was performed to characterize the photocleavage and subsequent release of caged anticancer drug from the LNO HNPs surface using a UV lamp (366 nm, 8 W). Thereafter, the ability of the SHG emission from LNO HNPs to induce the uncaging of the anticancer drug was explored using a Ti:Sapphire pulsed laser setup as excitation source.

First, LNO-APTES-CM-DOX HNPs were exposed to UV-light (366 nm) using a Sylvania lamp (8 W) as light source and the amount of released DOX in the supernatant was quantified by UHPLC-MS. Unfortunately, only traces of free DOX were detected (47.5 nM), which corresponded to 1% of DOX uncaged. The experiment
was repeated via irradiation of free CM-DOX ligand 62, and as for the functionalized LNO HNPs, only traces of DOX were detected. Nonetheless, the amount of intact ligand 62 decreased with time indicated either the product was degraded or DOX was released under several degradation products. To ensure this hypothesis, photo-, pH- and solvent stability assays were performed and as a result, degradation products of DOX in aqueous solvent were detected by UHPLC-MS. Moreover, fragmentation pathway under acidic and photolytic conditions, measured by HRMS, was in accordance with the literature (Scheme 32).\textsuperscript{352} The sugar moiety was first hydrolyzed; afterwards hydroxyl group was eliminated to form new aromatic ring and, finally, the intermediate lost of a water molecule or C\textsubscript{2}H\textsubscript{2}O\textsubscript{2}.

\begin{scheme}
\centering
\includegraphics[width=\textwidth]{scheme.png}
\end{scheme}

\textbf{Scheme 32:} Fragmentation pathway of DOX and fragments identified by HRMS in accordance with ref \textsuperscript{352}.

Alternative methods for quantification were explored. LNO-APTES-CM-DOX HNPs were exposed to UV-light and the fluorescence intensity of supernatant was measured (excitation wavelength at 485 nm, emission at 590 and 645 nm). However, no change in fluorescence was detected. Dcona and co-workers developed a nanosystem based on UCNPs, where ONB-DOX photosensitive tether was loaded through electrostatic interactions.\textsuperscript{282} They observed a red-shift in the absorption spectrum and it was attributed to the formation of coordination complex through coordination of the anthraquinone moiety of DOX and Y\textsuperscript{3+} ions present on the UCNPs surface. To avoid the formation of this complex, they first formed a DOX-dimer complex via coordination with Mn\textsuperscript{2+} ions prior reaction with the UCNPs. And finally, they were able to release DOX upon NIR-light irradiation. As previously mention (see Figure 45), red-shift was also observed in absorbance spectrum after functionalization with ligand 62 meaning that DOX formed potentially strong complex with Nb\textsuperscript{2+} ions present on the LNO HNPs surface. Consequently, CM-DOX ligand 62 was first incubated with manganese chloride prior reaction with the LNO-APTES-N\textsubscript{3} HNPs. However, after UV irradiation, no change
in fluorescence spectroscopy was observed. Moreover, another hypothesis is the following: the free DOX stayed trapped inside the silica shell around the LNO HNPs through electrostatic interactions.

A suspension of LNO-APTES-CM-erlo HNPs in PBS medium was first exposed to UV-light (366 nm, 8 W) for 90 min and the supernatant was quantified by UHPLC-MS (measured mass 335.1502, mass error 50 ppm). The release of erlotinib analogue 74 cumulated to 60% within 20 min of irradiation and then reached a plateau with almost no significant additional release (Figure 45a). The rate of the photocleavage mechanism was determined by fitting the progress curve with bi-exponential function. The initial rate ($k_0$) was determined at 5 min using normalized parameters. Faster rate was obtained for uncaging of erlotinib analogue 74 from LNO-APTES-CM-erlo compared to the release of Trp from BFO-APTES-CM-Trp (358.9 nM / min and 9 nM / min respectively). That was attributed to the smaller mean diameter of functionalized LNO HNPs compared to bigger mean sized BFO HNPs (Section 2.2.3, Figure 354).

![Figure 45: A) UV-light-triggered release of erlotinib analogue 74 from LNO-APTES-CM-erlo HNPs: erlotinib analogue 74 release profile following irradiation of LNO-APTES-CM-erlo at 366 nm; B) NIR-light-triggered release of erlotinib analogue 74 from LNO-APTES-CM-erlo HNPs: erlotinib analogue 74 release profile following irradiation of LNO-APTES-CM-erlo at 790 nm.](image)

Thereafter, the ability of the SHG signal emission from LNO-APTES-CM-erlo HNPs to trigger the release of caged erlotinib analogue 74 was studied using NIR pulsed Ti:Sapphire laser system as excitation source. When LNO-APTES-CM-erlo were exposed to 790 nm, up to 80% of caged erlotinib analogue 74 was released within 30 min of irradiation (Figure 45b). The initial rate constant was measured at 5 min using normalized parameters coming from mono-exponential fitted curves. The release rate of erlotinib analogue 74 from LNO-APTES-CM-erlo HNPs was approximately four times faster compared to BFO-APTES-CM-Trp (136.9 nM / min and 36.5 nM / min respectively).

Afterwards, the LNO-APTES-CM-erlo HNPs were incubated with HCC827 cells for 16 h at 37°C at different concentrations in HNPs. Thereafter, cell samples incubated with LNO-APTES-CM-erlo HNPs were exposed to NIR pulsed laser irradiation for 15 min. The amount of erlotinib analogue 74 released in the supernatant was quantified by UHPLC-MS and reliable values were obtained in respect of the concentration in LNO HNPs (Figure 46). However, viability assays were performed and gave not any cytotoxicity values even at high doses of commercial erlotinib (1 μM). Mutation on the EGFR receptor occurred quite frequently leading to MDR and that can explain the low or absence of toxicity of erlotinib in vitro even at high doses.
Figure 46: HCC827 cells were incubated for 16 h with LNO-APTES-CM-erlo at different concentrations and exposed to laser irradiation at 790 nm for 15 min. The amount of erlotinib analogue 74 released in the supernatant was quantified by UHPLC-MS.

The ability of the coumarinyl photosensitive tether to induce the release of caged Cbl was tested using a UV lamp (366 nm, 8 W). A 2 μM solution of CM-Cbl photosensitive tether 78 in PBS medium was first exposed for 3 h to UV-light and the products were analyzed and quantified by UHPLC-MS (measured mass 304.0871, mass error 50 ppm). After 20 minutes of irradiation, more than 50% of caged Cbl was released in the supernatant. A plateau was reached after 60 minutes of irradiation and no more Cbl was uncaged (70% of released molecules, Figure 47a, blue color curve). Afterwards, the ability of coumarinyl tether to trigger the release Cbl from the LNO HNPs surface was assessed. Similar release profile than ligand 78 in solution was attained for LNO-APTES-CM-Cbl. 50% of caged Cbl was released within 20 min of irradiation and a small fraction of Cbl was released within the remaining 160 min of experiment (Figure 47b, red color curve). Moreover, when the LNO-APTES-CM-Cbl HNPs were kept in the dark and heated at 37°C, no significant release of Cbl in the supernatant was measured ( > 5% of release, Figure 47a, green color curve). For quantitative comparison of the photolysis rates, the progress curves were fitted and normalized using bi-exponential function. Interestingly, the initial kinetic rate (k0) was faster for uncaging of Cbl from LNO-APTES-CM-Cbl than for the release of erlotinib 74 from LNO HNPs (305.0 nM/min vs 208.8 nM/min respectively). Moreover, the initial release rate constant was in the same order than for the release of erlotinib analogue 74 from LNO HNPs (305.0 nM/min vs 358.9 nm/min respectively).

Figure 47: A) UV-light-triggered release of Cbl from LNO HNPs: Cbl release profiles following irradiation of LNO-APTES-CM-Cbl (red), of CM-Cbl 78 (blue) and LNO-APTES-CM-Cbl kept in dark (green); B) NIR-light-triggered release of Cbl from functionalized LNO HNPs: Cbl release profiles following irradiation of LNO-APTES-CM-Cbl (red), of CM-Cbl 78 (blue), of control LNO-APTES-DIBO-Cbl (black) and LNO-APTES-CM-Cbl kept in dark (green).
Results and discussion

Based on the results obtained by direct irradiation with UV-light, the experiment was repeated using femtosecond pulsed laser system as excitation source. First, the CM-Cbl ligand 78 in PBS medium (2 μM) was exposed to 790 nm light and after 30 min of irradiation, 30% of Cbl was uncaged (Figure 47b, blue color curve). This released was attributed to the two-photon absorption ability of coumarinyl linker. Then, the ability of the SHG emission from the harmonic core to trigger the release of Cbl from the HNPs surface was performed. After 30 min of irradiation at 790 nm, the release of Cbl from LNO-APTES-CM-Cbl reached almost 80% (Figure 47b, red color curve). Furthermore, control experiment was assessed using LNO HNPs functionalized with DIBO-Cbl ligand 80, which do not contain any photosensitive linkage (Scheme 33). As expected, upon NIR irradiation, negligible amount of Cbl was detected by UHPLC-MS (Figure 47b, black color curve).

\[
\text{Scheme 33: Functionalization of LNO-APTES HNPs via SPAAC using ligands bearing Cbl with (LNO-APTES-CM-Cbl) or without (LNO-APTES-Cbl) a photosensitive caging group.}
\]

The initial kinetic rate constant \( k_0 \) was determined at 5 min using normalized parameters from fitted mono-exponential curves of the release profiles. The initial rate constant of Cbl release was, as expected, twice faster for the LNO-APTES-CM-Cbl HNPs compared to free ligand 78 (218.8 nM/min vs 105.3 nM/min respectively). All these data demonstrated the successful released of Cbl anticancer drug from the LNO HNPs surface thanks to the SHG signal emitted from the harmonic core.

In summary, coumarinyl phototrigger bearing anticancer drugs (erlotinib analogue, DOX and Cbl) were conjugated on the LNO-APTES-N\(_3\) HNPs by copper free click reaction. Erlotinib analogue was released from LNO HNPs at one and two-photon. In addition, promising in vitro application of this methodology was demonstrated. Unfortunately, DOX was not able to be release from the LNO HNPs. However, no examples of uncaging of DOX from coumarinyl linkers were reported in the literature. Nonetheless, the SHG emission from LNO HNPs was able to trigger the release of Cbl anticancer drug under NIR-light irradiation.
3 Conclusion and perspectives

Significant progress were achieved during the last decades to improve the cancer detection at its earliest stage of development. The design of stimuli-responsive nanocarriers has raised much attention to achieve higher local concentration of therapeutics and mitigate the appearance of drug resistance. The combination of imaging properties and controlled photorelease of active molecules within the same nanoconjugate has a great potential for theranostic applications. This thesis was devoted to the development of nanosystems based on HNPs for on-demand drug delivery. In particular, functionalized nanocarriers based on HNPs for NIR-light triggered release of molecular cargos induced by the second harmonic emission of HNPs were developed for in vitro applications.

At first, synthetic routes were investigated for the development of two phototriggers based on coumarinyl and ortho-nitrobenzyl cores. Coumarinyl linkers bearing different functionalities on the 4-amino moiety for direct functionalization on the coated HNPs via CuAAC or further derivatization with DIBO were synthesized in 9 steps. The o-nitrobenzyl linkers 7-9 were obtained in three steps and in good overall yields (26% to 47% yields). The two pathways for the synthesis of the p-methoxy-o-nitrobenzyl linker 15 were designed. Thereafter, robust protocols for the conjugation of cargo molecules on the phototriggers were developed. Trp was first conjugated to the phototriggers via the formation of carbamate linkage in a one-pot reaction. Derivatization of CM-Trp photo-responsive systems with DIBO moiety were developed for conjugation at the surface of BFO-APTES-N3 BFO HNPs via copper-free azide-alkyne [3+2] cycloaddition.

Robust protocols for the coating of HNPs were developed to ensure biocompatibility of the inorganic materials in vitro. Coating protocols leading to the formation of silica shell on the HNPs surface were developed and exhibited several advantages: ease to remove unreacted reactants, high amount of photocleavable systems were loaded on the surface and good biocompatibility. However, the silica coated HNPs tended to form aggregates. This versatile strategy allowed introducing a variety of functional groups on the HNPs surface (i.e. amino, azido and carboxylic acid functionalities). Moreover, avidin biomolecule was covalently conjugated to the BFO HNPs surface using coupling agents. Then, the photo-responsive systems were conjugated to the coated HNPs. Based on the previous work in the group, robust protocols were developed in particular copper free [3+2] cycloaddition. Photosensitive tethers were introduced on the HNPs surface via click reaction between alkyne moieties present on the linkers and the azido groups present on the HNPs. This strategy ensured the formation of a covalent linkage between the HNPs and the ligand. In addition, the formation of a triazole moiety offered high stability toward a large range of external stimuli (pH variation and enzymatic cleavage) and thus preserved the nanocarrier system from premature undesired release. The effectiveness of the functionalization and the complete removal of unreacted ligands supported by the co-localization between the SHG signal from the HNPs and the fluorescence of the coumarinyl moiety in a multiphoton imaging platform.

Real-time monitoring of the release of caged compounds was performed using fluorescent organic dye. However, due to the photobleaching of these dyes under NIR-light excitation, monitoring of the photorelease was further studied by UHPLC-MS quantification of the supernatant. For the first time, uncaging of cargo molecules from phototriggers covalently bounded on the HNPs surface induced by the SHG emission upon NIR irradiation was established. The irradiation protocol using NIR-light excitation did not induce cytotoxic
Conclusion and perspectives

Effect on HCC827 cells. First, the ability of BFO-APTES-CM-Trp HNPs to induce the photorelease from the HNPs surface was showed using UV-light. The Trp release profiles from BFO-APTES-CM-Trp HNPs irradiated at 790 nm demonstrated the efficiency of the second harmonic generation to induce the photolytic cleavage of CM-Trp linkage. Complementary experiments involving Trp-conjugated BFO HNPs but lacking the CM-based tether and CM-Trp confirmed the NIR-light triggered cleavage of CM-Trp carbamate linkage was effectively induced by the SHG emission (Scheme 34).

Afterwards, the methodology was extended to anticancer drugs. First, erlotinib analogue, DOX and Cbl were conjugated to the coumarinyl-phototrigger either through carbamate or ester linkages. The release of caged DOX in aqueous conditions was not successful due to several reasons: possible electrostatic binding between the aromatic moiety of DOX and the HNPs, photo-degradation or instability in aqueous solution. Analogue of erlotinib and Cbl were successfully released from HNPs surface upon NIR irradiation within 30 min of irradiation. Moreover, this methodology exhibited great potency for in vitro applications. Successful quantification of erlotinib released after NIR irradiation in the cell media was demonstrated. Studies are in progress to determine the cytotoxicity effect of the released anticancer drug in cancer cells lines.

These promising results will be the basis to improve the performance of functionalized HNPs as cancer theranostic probes and further improvements are required to develop in vivo applications of these nanocarriers, including:

- Derivatization of ONB and MONB phototriggers with DIBO to further conjugation on the coated HNPs through copper free click reaction and with anticancer drug such as chlorambucil, doxorubicin or erlotinib analogue as cargo molecules. Afterwards, the functionalized ONB ligands could be introduced on the coated HNPs and finally the NIR-triggered photo-activation protocol could be applied for the uncaging of anticancer drugs. Furthermore, conjugation of cargo molecules through thio-acetal photocleavable linkage could achieved simultaneous dual-release of payloads from the same phototrigger. This strategy could increase the number of caged compounds on the functionalized HNPs. Moreover, the THG generated from the harmonic core of HNPs was up to now used only to track stem cells in vitro. It is envisaged to use the THG produced by the HNPs to trigger
the uncaging of anticancer drug from the surface of functionalized HNPs. In addition, the HNPs could be functionalized simultaneously with different phototriggers such as ONB, MONB and CM. This strategy allowed sequential delivery of payload by tuning the wavelength thanks to the different absorption properties of phototriggers. In addition, real-time release of the caged compound could be assessed by using lanthanide chelates that are more stable towards photobleaching compared to organic dyes.

- Improvements of synthesis and coating protocols will be necessary to further increasing the biocompatibility and colloidal stability of HNPs. In particular, the synthesis protocol of BFO HNPs to obtain a better reproducibility between batches. For instance, ball mill apparatus could be used to provide BFO HNPs with a narrow size distribution comprised between 10 and 100 nm. This size range is optimal for the accumulation of nanomaterials into cancer cells. Evaluation of the biocompatibility of silica-coated HNPs should be tested in vitro on healthy and cancer cell lines. In addition, the porosity of the silica shell could be tested by nitrogen absorption / desorption technique. If the pores are large enough, hydrophobic payloads could be encapsulated into the pores and released through protonation in the tumor microenvironment. Moreover, the stability of the avidin coating under NIR irradiation conditions should be assessed to ensure that the protein is not denatured.

- Combination of targeting ligands, imaging probe and photo-responsive system on the same nanoplatform could provide highly functionalized nanocarriers for theranostic application. The design of targeting ligands such as FAP inhibitor could allow to target specially cancer cells and increasing the HNPs concentration into tumor site. In addition, development of imaging probes allows multimodal detection not only by multiphoton imaging but also by MRI. For MRI application, the development of gadolinium chelating probe showed promising results as T₁ contrast agent without affecting the capacity of HNPs to produce SHG. Replacing gadolinium ions by other lanthanides could allow imaging the cancer cells by fluorescence. Finally, dual therapy could be achieved by combination of the controlled release of anticancer drug from phototrigger and PDT by functionalization of coated HNPs with photosensitizers such as rose Bengal.

- Prior application of the NIR-activation protocol in vivo, several improvements are required. In example, the ability of functionalized HNPs to cross the mucus layer in the lungs. This assessment could be achieved by incubation the functionalized HNPs with 3D model of human functional lung epithelium (MucilAir™-HF, EPITHELIX) based on the co-culture of bronchial cells and lung fibroblasts. Moreover, the colloidal stability and the toxicity of the functionalized HNPs could be evaluated on a ship mimicking the blood stream and which is containing tissues from several organs.

In conclusion, the SHG produced by the harmonic core was, for the first time, used to trigger the release of caged compounds from the surface of HNPs in a control and selective manner. In addition, the independence of the SHG process on the laser excitation wavelengths allow decoupling imaging and photo-activation protocols for theranostic applications.
4 Experimental part

4.1 General Methods

Reagent-grade solvents (Fluka, Riedel-de-Haën) and chemicals (Aldrich, Acros, Fluka, Sigma, Maybridge, TCI Chemicals, Apollo and Fluorochem) were reagent-grade, used without further purification. All the reactions were performed in flame dried glassware under an inert atmosphere of nitrogen. Evaporation and concentration in vacuo were conducted by Rotavapor from Büchi. All products were dried under high vacuum (10^{-2} Bar) before analytic characterization.

Thin Layer Chromatography (TLC): Pre-coated aluminium plates SiO$_2$ 60 F$_{254}$ from Merck. The compounds were visualized by 254 nm light or stained with solutions of KMnO$_4$, panchaldi reagent [(NH$_4$)$_6$MoO$_4$, Ce(SO$_4$)$_2$, H$_2$SO$_4$, H$_2$O], ninhidrine or iodine vapors

Flash Column Chromatography (FC): SiO$_2$ 60 (230-400 mesh, particle size 40-63 μm) from Fluka. All solvents used have been distilled under reduced pressure.

NMR spectra $^1$H- and $^{13}$C-NMR spectra: NMR spectra were recorded on Bruker Avance III-400, Bruker Avance-400 or Bruker DRX-400 spectrometers (Bruker, Billerica, MA, USA) at room temperature (r.t.), unless otherwise stated. All spectra were recorded at room temperature, unless otherwise stated. Chemical shifts are reported downfield from tetramethylsilane. All $^1$H signals are reported in ppm with the internal chloroform signal at 7.26 ppm, the internal DMSO signal at 2.50 ppm, as internal references. All $^{13}$C-NMR signals are reported in ppm with the internal chloroform signal at 77.00 ppm, the internal DMSO signal at 39.5 ppm, as internal references. The chemical values are given in ppm. The resonance multiplicity is described as s (singulet), d (doublet), t (triplet), q (quartet), quin (quintet), m (multiplet). Broad signals are indicated as br. coupling constant $J$ are given in Hz. In general, spectra are performed in chloroform-d, if another solvent was used, it is indicated.

Accurate Mass: The qualitative accurate masses were measured by ESI-TOF using the Xevo G2-S QTOF (Waters) and nanoESI-FT-MS using the Elite™ Hybrid Ion Trap-Orbitrap (ThermoFisher) Mass Spectrometer. Quantitative MS analyses were performed on the 6530 Accurate-Mass Q-TOF LC/MS mass spectrometer coupled to the 1290 Infinity UHPLC system (Agilent Technologies, USA). The separation was achieved using an ACQUITY UPLC® BEH C18 1.7µm column, 2.1 mm x 50 mm (Waters) heated at 30°C using water and acetonitrile as mobile phases.

IR: All infrared spectra were recorded on JASCO FT-IR-4100 spectrometer outfitted with a PIKE technology MIRacle™ ATR accessory as neat films compressed onto a Zinc Selenide window. Spectra are described as cm$^{-1}$ (w = weak, m = medium, s = strong, sh = shoulder).

Dynamic Light Scattering (DLS) and Zeta potential: measurements made with Malvern NanoZ instrument.

Sonicator: VWR USC 900 TH cleaner sonicator; Branson 1800 Ultraonic Cleaner sonicator

Centrifugator: HERAEUS Biofuge 13 centrifuge; Beckman Coulter Allegra X-30 centrifuge

Microwave: Anton Paar Monowave 400 Microwave Synthesis Reactor
4.2 Synthesis of photocleavable linkers

4.2.1 Synthesis of o-nitrobenzyl-linkers

**Scheme 35: Synthesis of the o-nitrobenzyl linkers 7-9:** i) tert-butyl bromoacetate, K$_2$CO$_3$, THF, r.t., 16 h; ii) For 1 and 3: NBS, AIBN, DCM, reflux, 48 h; iii) For 2: NBS, AIBN, trifluorotoluene, reflux, 24 h iv) AgNO$_3$, H$_2$O, acetone, r.t., 16 h.

**tert-Butyl 2-(4-methyl-3-nitrophenoxy)acetate (1)**

tert-Butylbromoacetate (1.2 mL, 7.9 mmol, 1.2 eq) was added to a solution of 4-methyl-3-nitrophenol (1.0 g, 6.6 mmol, 1.0 eq) and potassium carbonate (1.8 g, 13.2 mmol, 2.0 eq) in THF (45 mL). The reaction mixture was stirred at r.t. for 16 h and the solvent was removed under reduced pressure. The residue was dissolved in H$_2$O (100 mL) and extracted with ethyl acetate (3 X 150 mL). The organic layers were combined, dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was purified via flash chromatography eluting with a mixture of EtOAc / PE (1:9). **tert-Butyl 2-(4-methyl-3-nitrophenoxy)acetate (1)** (1.75 g, 6.6 mmol, quant.) was obtained as a yellow oil.

$^1$H NMR (400 MHz, Chloroform-$d$): δ 7.51 (d, $J = 2.7$ Hz, 1H, Ar-$H$), 7.28 (s, 1H, Ar-$H$), 7.12 (dd, $J = 8.5$, 2.8 Hz, 1H, Ar-$H$), 4.58 (s, 2H, CH$_2$), 2.56 (s, 3H, CH$_3$), 1.52 (s, 9H, 3 X CH$_3$ tert-butyl).

The analytical data were in accordance with previously reported data.$^{330}$
Experimental part

** tert-Butyl 2-(4-(hydroxymethyl)-3-nitrophenoxy)acetate (7) **

To a solution of 4 (10.6 g) in acetone (250 mL) was added dropwise a solution silver nitrate (4.56 g, 29.25 mmol, 1.1 eq.) in H₂O (250 mL). The reaction mixture was stirred at r.t. for 16 h. The reaction mixture was filtered through a pad of Celite® eluting with ethyl acetate and the organic layer was washed with brine (3 X 250 mL). The organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. The crude mixture was purified by column chromatography eluting with a mixture of PE / EtOAc (8:2) to afford tert-butyl 2-(4-(hydroxymethyl)-3-nitrophenoxy)acetate (7) (3.46 g, 12.2 mmol, 38% yield over 2 steps) as a yellow solid.
**Experimental part**

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 7.62 (d, $J = 8.6$ Hz, 1H, Ar-H), 7.59 (d, $J = 2.7$ Hz, 1H, Ar-H), 7.22 (dd, $J = 8.6$, 2.7 Hz, 1H, Ar-H), 4.88 (d, $J = 2.5$ Hz, 1H, OH-CH$_2$-Ar), 4.59 (s, 2H, CH$_2$), 2.53 (s, 1H, OH), 1.50 (s, 9H, 3 X CH$_3$ tert-butyl).

The analytical data were in accordance with previously reported data.$^{330}$

$^1$H NMR spectrum for 7:

**Ethyl 2-(4-methyl-3-nitrophenoxy)acetate (3)**

Ethylbromoacetate (4.35 mL, 39.2 mmol, 1.2 eq) was added to a solution of 4-methyl-3-nitrophenol (5.0 g, 32.5 mmol, 1.0 eq) and potassium carbonate (9.0 g, 63.5 mmol, 2.0 eq) in THF (250 mL) and the reaction mixture was stirred at r.t. for 16 h. The solvent was removed under reduced pressure. The residue was dissolved in H$_2$O (100 mL) and extracted with ethyl acetate (3 X 150 mL). The organic layers were combined, dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was purified via column chromatography eluting with a mixture of EtOAc / PE (1:9). Ethyl 2-(4-methyl-3-nitrophenoxy)acetate (3) (6.77 g, 28.3 mmol, 87% yield) was obtained as a yellow solid.
**Experimental part**

**H NMR** (400 MHz, Chloroform-\(d\)): \(\delta 7.48 (d, J = 2.6 \text{ Hz}, 1H, \text{Ar-H}), 7.23 (d, J = 8.5 \text{ Hz}, 1H, \text{Ar-H}), 7.08 (dd, J = 8.5, 2.6 \text{ Hz}, 1H, \text{Ar-H}), 4.64 (s, 2H, RO-CH\(2\)-CO\(2\)Et), 4.26 (q, J = 7.2 \text{ Hz}, 2H, CH\(2\) ester), 2.50 (s, 3H, Ar-CH\(3\)), 1.28 (t, J = 7.2 \text{ Hz}, 3H, CH\(3\) ester).

**C NMR** (101 MHz, Chloroform-\(d\)): \(\delta 168.2 (\text{CO}), 156.3 (\text{C Ar}), 149.3 (\text{C Ar}), 133.7 (\text{CH Ar}), 126.8 (\text{C Ar}), 120.5 (\text{CH Ar}), 110.3 (\text{CH Ar}), 65.7 (\text{CH}_2), 61.7 (\text{CH}_2), 19.8 (\text{CH}_3), 14.2 (\text{CH}_3).

**IR** (\(\nu_{\text{max}}, \text{ cm}^{-1}\)): 2980 (s), 1754 (s), 1619 (s), 1524 (s), 1439 (s), 1343 (s), 1196 (s), 1089 (s), 1015 (s), 810 (s).

**HRMS (APCI/QTOF) m/z**: [M + H]\(^+\) Calcd for C\(_{11}\)H\(_{14}\)NO\(_5\)\(^+\) 240.0866; Found 240.0869.

**H NMR and C spectra for 3:**
Experimental part

**Ethyl 2-(4-bromomethyl-3-nitrophenoxy)acetate (6)**

Ethyl 2-(4-methyl-3-nitrophenoxy)acetate (3) (1.00 g, 4.2 mmol, 1.0 eq.), AIBN (0.14 g, 0.82 mmol, 0.2 eq.) and n-bromosuccinimide (0.74 g, 6.30 mmol, 1.5 eq.) were dissolved in trifluorotoluene (30 mL) and the reaction mixture was refluxed under argon atmosphere for 5 h. The reaction mixture was cooled down to r.t. and the solvent was removed under reduced pressure. The crude mixture was purified by column chromatography eluting with a mixture of PE / EtOAc (9:1 to 8:2). Ethyl 2-(4-bromomethyl-3-nitrophenoxy)acetate (6) (0.94 g, 2.96 mmol, 71% yield) was obtained as a yellow oil.

\[\text{H NMR (400 MHz, Chloroform-}\delta\text{): } \delta\text{ 7.55 (d, }J = 2.7 \text{ Hz, 1H, Ar-H), 7.47 (d, }J = 8.6 \text{ Hz, 1H, Ar-H), 7.14 (dd, }J = 8.6, 2.7 \text{ Hz, 1H, Ar-H), 4.78 (s, 2H, RO-CH}_{2}\text{-CO}_{2}\text{Et), 4.69 (s, 2H, Ar-CH}_{2}\text{), 4.28 (q, }J = 7.1 \text{ Hz, 2H, CH}_{2}\text{ ester), 1.30 (t, }J = 7.1 \text{ Hz, 3H, CH}_{3}\text{ ester).}\]

\[\text{C NMR (101 MHz, Chloroform-}\delta\text{): } \delta\text{ 167.8 (C }\text{O), 158.3 (C }\text{Ar), 148.6 (C }\text{Ar), 133.9 (CH }\text{Ar), 126.0 (C }\text{Ar), 120.5 (C }\text{Ar), 111.4 (CH }\text{Ar), 65.6 (CH}_{2}\text{), 61.9 (CH}_{2}\text{), 29.0 (CH}_{2}\text{), 14.3 (CH}_{3}\text{).}\]

IR (\(\nu_{\text{max}}\) cm\(^{-1}\)): 2950 (s), 1762 (s), 1624 (s), 1525 (s), 1432 (s), 1349 (s), 1206 (s), 1091 (s), 821 (s).

HRMS (ESI/QTOF) m/z: [M]\(^+\) Calcd for C\(_{11}\)H\(_{12}\)NO\(_5\)\(^+\) 238.0710; Found 238.0712.
$^1$H NMR and $^{13}$C spectra for 6:
Experimental part

Ethyl 2-\((4\text{-hydroxymethyl}-3\text{-nitrophenoxy})\)acetate (9)

To a solution of 6 (0.94 g, 2.96 mmol, 1.0 eq) in acetone (30 mL) was added dropwise a solution silver nitrate (0.60 g, 3.56 mmol, 1.2 eq) in \(\text{H}_2\text{O}\) (30 mL). The reaction mixture was stirred at r.t. for 16 hr. The reaction mixture was filtered through a pad of Celite® eluting with ethyl acetate (60 mL) and the organic layer was washed with brine (2 X 45 mL). The organic layer was dried over \(\text{MgSO}_4\), filtered and concentrated under reduced pressure and the crude was purified by column chromatography eluting with a mixture of PE / EtOAc (8:2). Ethyl 2-\((4\text{-hydroxymethyl}-3\text{-nitrophenoxy})\)acetate (9) (0.31 g, 1.23 mmol, 42% yield) was obtained as a yellow solid.

\(^1\text{H NMR}\) (400 MHz, Chloroform-\(d\)): \(\delta\) 7.68 (d, \(J = 2.6\) Hz, 1H, Ar-H), 7.52 (d, \(J = 8.6\) Hz, 1H, Ar-H), 7.23 (dd, \(J = 8.6, 2.7\) Hz, 1H, Ar-H), 5.80 (s, 2H, RO-\(\text{CH}_2\)-CO\(\text{Et}\)), 4.71 (s, 2H, Ar-\(\text{CH}_2\)), 4.29 (q, \(J = 7.2\) Hz, 2H, \(\text{CH}_2\) ester), 1.31 (t, \(J = 7.2\) Hz, 3H, \(\text{CH}_3\) ester).

\(^{13}\text{C NMR}\) (101 MHz, Chloroform-\(d\)): \(\delta\) 167.8 (CO), 158.7 (C \(\text{Ar}\)), 148.5 (C \(\text{Ar}\)), 131.2 (C \(\text{Ar}\)), 121.8 (C \(\text{Ar}\)), 120.8 (CH \(\text{Ar}\)), 111.6 (CH \(\text{Ar}\)), 70.5 (CH\(_2\)), 65.7 (CH\(_2\)), 62.0 (CH\(_2\)), 14.3 (CH\(_3\)).

\(\text{IR (}\nu_{\text{max}}, \text{ cm}^{-1})\): 2986 (w), 2937 (w), 1754 (m), 1638 (s), 1536 (s), 1441 (w), 1349 (w), 1281 (s), 1206 (s), 1088 (w), 858 (m), 821 (w).

\(\text{HRMS (ESI) m/z: \text{calcd for C}_{11}\text{H}_{11}\text{N}_2\text{O}_5^+ [M]^+ 238.0716; found 238.0721.}\)
$^1$H NMR and $^{13}$C spectra for 9:
Methyl 2-(4-methyl-3-nitrophenoxy)acetate (2)

Methylbromoacetate (2.3 mL, 23.5 mmol, 1.2 eq) was added to a solution of 4-methyl-3-nitrophenol (3.0 g, 19.6 mmol, 1.0 eq) and potassium carbonate (5.6 g, 39.2 mmol, 2.0 eq) in THF (200 mL) and the reaction mixture was stirred at r.t. for 16 h. The solvent was removed under reduced pressure. The residue was dissolved in H₂O (100 mL) and extracted with ethyl acetate (3 X 150 mL). The organic layers were combined, dried over MgSO₄, filtered and concentrated under reduced pressure. The residue was purified via column chromatography eluting with a mixture of EtOAc / PE (1:9 to 2:8). Methyl 2-(4-methyl-3-nitrophenoxy)acetate (2) (4.5 g, 17.6 mmol, 90% yield) was obtained as a yellow solid.

¹H NMR (400 MHz, Chloroform-d): δ 7.52 (d, J = 2.8 Hz, 1H, Ar-H), 7.28 (s, 1H, Ar-H), 7.11 (dd, J = 8.5, 2.8 Hz, 1H, Ar-H), 4.69 (s, 2H, RO-CH₂-CO₂Me), 3.83 (s, 3H, CH₃ ester), 2.55 (s, 3H, Ar-CH₃).

¹³C NMR (101 MHz, Chloroform-d): δ 168.7 (CO), 149.5 (C Ar), 156.3 (C Ar), 133.9 (CH Ar), 127.0 (C Ar), 120.6 (CH Ar), 110.3 (CH Ar), 110.3 (CH Ar), 65.6 (CH₂), 52.6 (CH₂), 19.9 (CH₃).

IR (νmax, cm⁻¹): 2960 (s), 1751 (s), 1621 (s), 1526 (s), 1439 (s), 1344 (s), 1307 (s), 1210 (s), 1092 (s), 1055 (s), 808 (s).

HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for C₁₀H₁₂NO₅+: 226.0710; Found 226.0707.
$^1$H NMR and $^{13}$C spectra for 2:
**Experimental part**

**Methyl 2-(4-(bromomethyl)-3-nitrophenoxy)acetate (5)**

![Structural formula](image)

Methyl 2-(4-methyl-3-nitrophenoxy)acetate (2) (1.00 g, 4.4 mmol, 1.0 eq.), AIBN (0.126 g, 0.76 mmol, 0.2 eq.) and n-bromosuccinimide (1.05 g, 5.9 mmol, 1.5 eq) were dissolved in dry DCM (20 mL) and the reaction mixture was refluxed under argon atmosphere for 24 h. The reaction mixture was cooled down to r.t. and the solvent was removed under reduced pressure. The crude mixture was purified by column chromatography eluting with a mixture of PE / EtOAc (9:1 to 8:2). Methyl 2-(4-(bromomethyl)-3-nitrophenoxy)acetate (5) (1.21 g, 3.9 mmol, 90% yield) was obtained as a yellow solid.

$^1$H NMR (400 MHz, Chloroform-d): δ 7.65 (d, $J = 2.6$ Hz, 1H, Ar-H), 7.50 (d, $J = 8.6$ Hz, 1H, Ar-H), 7.21 (dd, $J = 8.6$, 2.6 Hz, 1H, Ar-H), 5.77 (s, 2H, Ar-CH$_2$), 4.72 (s, 2H, RO-CH$_2$-CO$_2$Me), 3.81 (s, 3H, CH$_3$ ester).

$^{13}$C NMR (101 MHz, Chloroform-d): δ 168.2 (C=O), 158.6 (C$_{Ar}$), 148.5 (C$_{Ar}$), 131.4 (CH$_{Ar}$), 121.6 (C$_{Ar}$), 120.5 (CH$_{Ar}$), 111.6 (CH$_{Ar}$), 70.5 (CH$_2$), 65.4 (CH$_2$), 52.6 (CH$_3$).

IR ($\nu_{max}$ cm$^{-1}$): 2960 (s), 1754 (s), 1634 (s), 1537 (s), 1434 (s), 1343 (s), 1274 (s), 1214 (s), 1092 (s), 906 (s), 843 (s), 729 (s).

HRMS (APCI/QTOF) m/z: [M]$^+$ Calcd for C$_{10}$H$_{10}$NO$_5$ $^+$ 224.0553; Found 224.0556.

$^1$H NMR and $^{13}$C spectra for 5:
Experimental part

Methyl 2-(4-(hydroxymethyl)-3-nitrophenoxy)acetate (8)

To a solution of 5 (1.06 g, 4.2 mmol, 1.0 eq) in acetone (40 mL) was added dropwise a solution silver nitrate (0.73 g, 4.3 mmol, 1.1 eq) in H2O (40 mL). The reaction mixture was stirred at r.t. for 12 hr. The reaction mixture was filtered through a pad of Celite® eluting with ethyl acetate and the organic layer was washed with brine (3 X 100 mL). The organic layer was dried over MgSO4, filtered and concentrated under reduced pressure. The residue was purified via column chromatography eluting with a mixture of EtOAc / PE 1:9 to 2:8) to afford methyl 2-(4-(hydroxymethyl)-3-nitrophenoxy)acetate (8) (0.55 g, 2.3 mmol, 62% yield) as a yellow solid.

1H NMR (400 MHz, Chloroform-d): δ 7.67 (dd, J = 2.7, 0.9 Hz, 1H, Ar-H), 7.52 (d, J = 8.5 Hz, 1H, Ar-H), 7.23 (dd, J = 8.5, 2.6 Hz, 1H, Ar-H), 4.88 (s, 2H, Ar-CH2), 4.74 (d, J = 1.0 Hz, 3H, RO-CH2-CO2Me), 3.83 (s, 3H, CH3 ester).

13C NMR (101 MHz, Chloroform-d): δ 168.6 (CO), 157.5 (C Ar), 148.4 (C Ar), 131.7 (CH Ar), 130.1 (C Ar), 120.9 (CH Ar), 110.8 (CH Ar), 110.8 (CH Ar), 65.5 (CH2), 62.3 (CH2), 52.7 (CH3).

IR (νmax, cm⁻¹): 3549 (w), 2920 (w), 2851 (w), 1751 (m), 1623 (w), 1529 (s), 1439 (w), 1348 (w), 1214 (s), 1088 (m), 1039 (w), 819 (w).

$^1$H NMR and $^{13}$C spectra for 8:
4.2.2 Synthesis of \(m\)-methoxy-o-nitrobenzyl linkers

![Scheme 36: Synthesis of the MONB linkers 18-20](image)

Pathway A

4-Formyl-2-methoxyphenyl benzoate (10)

Benzyl bromide (4.3 mL, 36 mmol, 1.1 eq) was added to a solution of vanillin (5.0 g, 33 mmol, 1.0 eq.) and \(K_2CO_3\) (4.5 g, 36 mmol, 1.0 eq.) in acetone (50 mL). The reaction mixture was refluxed for 16 h. \(H_2O\) (150 mL) and EtOAc (200 mL) were added and the aqueous layer was extracted with EtOAc (2 X 200 mL). The combined organic layers were dried over MgSO\(_4\), filtered and concentrated under reduced pressure. 4-formyl-2-methoxyphenyl benzoate (10) (7.9 g, 31 mmol, 93% yield) was obtained as yellow oil.

\(^1H\) NMR (400 MHz, Chloroform-\(d\)): \(\delta\) 9.84 (s, 1H, CHO), 7.48 – 7.28 (m, 7H, Ar-H), 6.99 (d, \(J = 8.2\) Hz, 1H, Ar-H), 5.25 (s, 2H, RO-\(CH_2\)-Ph), 3.95 (s, 3H, O-\(CH_3\)).

The analytical data were in accordance with previously reported data.
Experimental part

\(^1\)H NMR spectrum for 10:

4-Formyl-2-methoxy-5-nitrophenyl benzoate (11)

4-Formyl-2-methoxyphenyl benzoate (10) (1.00 g, 4.1 mmol, 1.0 eq) was dissolved in 70% nitric acid (6 mL) at 0°C. The reaction mixture was stirred at 0°C for 2 h. The solution was poured into a mixture of ice / H\(_2\)O (30 mL), neutralized with sat. NaHCO\(_3\) solution (100 mL) and extracted with EtOAc (3 X 150 mL). The combined organic layers were dried over MgSO\(_4\), filtered and concentrated under reduced pressure. The crude product was recrystallized from EtOAc. 4-formyl-2-methoxy-5-nitrophenyl benzoate (11) (1.02 g, 3.5 mmol, 86% yield) was obtained as yellow needles.

\(^1\)H NMR (400 MHz, Chloroform-\(d\)): \(\delta\) 10.44 (d, \(J = 3.3\) Hz, 1H, CHO), 7.67 (d, \(J = 3.2\) Hz, 1H, Ar-H) 7.50 – 7.34 (m, 6H, 6 X Ar-H), 5.27 (d, \(J = 3.2\) Hz, 2H, RO-CH\(_2\)-Ph), 4.02 (d, \(J = 3.4\) Hz, O-CH\(_3\)).

The analytical data were in accordance with previously reported data.\(^\text{35a}\)
Experimental part

$^1$H NMR spectrum for 11:

4-Hydroxy-5-methoxy-2-nitrobenzaldehyde 12

4-Formyl-2-methoxy-5-nitrophenyl benzoate (11) (1.6 g, 5.6 mmol, 1.0 eq) was dissolved in TFA (10 mL) and the reaction mixture was stirred at r.t. for 3 days. The solvent was removed under reduced pressure and the crude was partitioned between DCM (50 mL) and H$_2$O (50 mL). The aqueous layer was extracted with DCM (2 X 50 mL) and the combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography eluting with a mixture of EtOAc / PE (1:1 to 7:3). 4-hydroxy-5-methoxy-2-nitrobenzaldehyde (12) (0.85 g, 4.2 mmol, 78% yield) was obtained as a yellow solid

$^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.11 (s, 1H, OH), 10.16 (s, 1H, CHO), 7.51 (s, 1H, Ar-H), 7.36 (s, 1H, Ar-H), 3.94 (s, 3H, O-CH$_3$).

The analytical data were in accordance with previously reported data.\(^{353}\)
Experimental part


\[ ^1H \text{NMR spectrum for } 12: \]

\[
\begin{align*}
\text{\textit{\textbf{tert-Butyl 2-(4-formyl-2-methoxy-5-nitrophenoxyl)acetate (15)}}} \\
\text{tert-Butyl bromoacetate (0.7 mL, 4.8, 1.1 eq) was added to a solution of 4-hydroxy-5-methoxy-2-nitrobenzaldehyde (12) (0.85 g, 4.2 mmol, 1.0 eq) and K}_2\text{CO}_3 (0.89 g, 6.5 mmol, 1.1 eq) in DMF (10 mL). The reaction mixture was stirred at r.t. for 16 h. The solvent was removed under reduced pressure and the residue was partitioned between EtOAc (100 mL) and H}_2\text{O (100 mL). The aqueous layer was extracted with EtOAc (2 X 100 mL) and the combined organic layers were dried over MgSO}_4, filtered and concentrated under reduced pressure. tert-Butyl 2-(4-formyl-2-methoxy-5-nitrophenoxyl)acetate (15) (1.26 g, 4.0 mmol, 93% yield) was obtained as a yellow solid.} \\
\text{\textit{\textbf{\textsuperscript{1}H NMR (400 MHz, Chloroform-\textit{d})}}:} \delta 10.46 (\text{s}, 1H, \text{CHO}), 7.51 (\text{s}, 1H, \text{Ar-H}), 7.44 (\text{s}, 1H, \text{Ar-H}), 4.73 (\text{s}, 2H, \text{ROCH}_2\text{CO}_2\text{tBu}), 4.03 (\text{s}, 3H, \text{OCH}_3), 1.51 (\text{s}, 9H, 3 \times \text{CH}_3 \text{tert-butyl}). \\
\text{\textit{\textbf{\textsuperscript{13}C NMR (101 MHz, DMSO-\textit{d}_6):} } \delta 188.6 (\text{CHO}), 166.9 (\text{CO}), 152.7 (\text{C} \text{Ar}), 150.1 (\text{C} \text{Ar}), 143.1 (\text{C} \text{Ar}), 125.4 (\text{C} \text{Ar}), 110.5 (\text{CH} \text{Ar}), 108.9 (\text{CH} \text{Ar}), 82.0 (\text{C} \text{Ar}), 65.8 (\text{CH}_2), 56.6 (\text{CH}_3), 27.7 (3 \times \text{CH}_3). \\
\text{\textit{\textbf{IR (\nu_max \text{ cm}^{-1})}}:} 2915 (\text{s}), 2858 (\text{s}), 1746 (\text{s}), 1692 (\text{s}), 1576 (\text{s}), 1522 (\text{s}), 1462 (\text{s}), 1392 (\text{s}), 1368 (\text{s}), 1325 (\text{s}), 1293 (\text{s}), 1215 (\text{s}), 1155 (\text{s}), 1073 (\text{s}), 873 (\text{s}).}
\end{align*}
\]

86
HRMS (APCI/QTOF) m/z: [M + H]+ Calcd for C_{14}H_{18}NO_{7}+ 312.1078; Found 312.1078.

^1^H NMR and ^13^C spectra for 15:
Experimental part

Pathway B

**tert-Butyl 2-(4-formyl-2-methoxyphenoxy)acetate (13)**

To a solution of vanillin (0.50 g, 3.3 mmol, 1.0 eq) and K$_2$CO$_3$ (0.91 g, 6.6 mmol, 2.0 eq) in DMF (5 mL) was added tert-butyl bromoacetate (0.42 mL, 3.6 mL, 1.1 eq). The reaction mixture was stirred at r.t. for 16 h. The solvent was evaporated under reduced pressure and the residue was partitioned between EtOAc (50 mL) and H$_2$O (50 mL). The aqueous layer was extracted with EtOAc (2 X 50 mL) and the combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure. **tert-Butyl 2-(4-formyl-2-methoxyphenoxy)acetate (13)** (0.81 g, 3.0 mmol, 92% yield) was obtained as a slightly yellow solid.

$^1$H NMR (400 MHz, Chloroform-d): δ 9.86 (s, 1H, CHO), 7.42 (d, $J = 10.2$ Hz, 2H, 2 X Ar-H), 6.85 (d, $J = 8.0$ Hz, 1H, Ar-H), 4.68 (s, 2H, RO-CH$_2$-CO$_2$Bu), 3.95 (s, 3H, O-CH$_3$), 1.47 (s, 9H, 3 X CH$_3$ tert-butyl).

The analytical data were in accordance with previously reported data.$^{354}$

$^1$H NMR spectrum for 13:
Experimental part

2-(4-Formyl-2-methoxy-5-nitrophenoxo)acetic acid (14)

\[ \text{Me} \quad \text{O} \quad \text{O}_2\text{N} \quad \text{O} \quad \text{CO}_2\text{H} \]

tert-Butyl 2-(4-formyl-2-methoxyphenoxy)acetate (13) (7.09 g, 26.5 mmol, 1.0 eq) was dissolved in 70% nitric acid (50 mL) at 0°C. The reaction mixture was allowed to warm up to r.t. and was stirred for 45 min. The solution was poured into a mixture of ice / H\(_2\)O and neutralized with sat. NaHCO\(_3\). The aqueous layer was extracted with EtOAc (3 x 250 mL) and the combined organic layers were dried over MgSO\(_4\), filtered and concentrated under reduced pressure. 2-(4-formyl-2-methoxy-5-nitrophenoxy)acetic acid (14) (3.24 g, 12.7 mmol, 48% yield) was obtained as a yellow solid.

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 13.27 (s, 1H, CO\(_2\)H), 10.19 (s, 1H, CHO), 7.66 (s, 1H, Ar-H), 7.39 (s, 1H, Ar-H), 4.98 (s, 2H, RO-\(\text{CH}_2\)-CO\(_2\)H), 3.97 (s, 3H, O-\(\text{CH}_3\)).

The analytical data were in accordance with previously reported data.\(^{354}\)

\(^1\)H NMR spectrum for 14:
**Experimental part**

**tert-Butyl 2-(4-formyl-2-methoxy-5-nitrophenoxo)acetate (15)**

![Chemical structure of tert-Butyl 2-(4-formyl-2-methoxy-5-nitrophenoxo)acetate (15)](attachment)

2-(4-Formyl-2-methoxy-5-nitrophenoxo)acetic acid (14) (50 mg, 0.19 mmol, 1.0 eq) and DMAP (12 mg, 0.09 mmol, 0.5 eq) were dissolved in mixture of dry DCM (5 mL) and tert-butanol (23 µL, 0.24 mmol, 1.2 eq.) under argon atmosphere. A solution of DCC (48 mg, 0.24 mmol, 1.2 eq) in DCM (1 mL) was added dropwise and the reaction mixture was stirred at r.t. for 5 h. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography eluting with a mixture of PE / EtOAc (9:1 to 6:4). **tert-Butyl 2-(4-formyl-2-methoxy-5-nitrophenoxo)acetate (15)** (30 mg, 0.09 mmol, 49% yield) was obtained as a yellow solid.

**H NMR** (400 MHz, Chloroform-d): δ 10.46 (s, 1H, CHO), 7.51 (s, 1H, Ar-H), 7.44 (s, 1H, Ar-H), 4.73 (s, 2H, RO-C$_2$H$_4$CO$_2$tBu), 4.03 (s, 3H, O-CH$_3$), 1.51 (s, 9H, 3 X C$_3$H$_7$ tert-butyl).

**13C NMR** (101 MHz, Chloroform-d): δ 167.0 (C=O), 154.4 (C$_{Ar}$), 146.2 (C$_{Ar}$), 139.5 (C$_{Ar}$), 133.5 (C$_{Ar}$), 111.6 (CH$_{Ar}$), 110.2 (CH$_{Ar}$), 83.3 (Cq), 66.5 (CH$_{2}$), 62.9 (CH$_{2}$), 56.7 (CH$_{3}$), 28.2 (3 X CH$_{3}$ tert-butyl).

**IR (ν$_{max}$, cm$^{-1}$):** 3543 (w), 2980 (w), 2933 (w), 1747 (m), 1582 (w), 1518 (s), 1370 (w), 1328 (m), 1281 (m), 1212 (w), 1158 (s), 1083 (m).

**HRMS (ESI) m/z:** calcd for C$_{14}$H$_{19}$NNaO$_7$ [M+Na]$^+$ 336.1054; found 336.1056.

---

**Sodium borohydride (0.06 g, 1.6 mmol, 1.0 eq) was added to a mixture of tert-butyl 2-(4-formyl-2-methoxy-5-nitrophenoxo)acetate (15) (0.50 g, 1.6 mmol, 1.0 eq) in a mixture of THF / MeOH (1:1, 10 mL) at 0°C. The reaction mixture was stirred at 0°C for 30 min.** H$_2$O (5 mL) and sat. NH$_4$Cl (5 mL) was added to quench the reaction and the solution was stirred 30 min. The crude product was extracted with EtOAc (3 X 50 mL) and the combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure. **tert-Butyl 2-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxo)acetate (18)** (0.49 g, 1.6 mmol, 99% yield) was obtained as a slightly yellow cristal.

**H NMR** (400 MHz, Chloroform-d): δ 7.63 (s, 1H, Ar-H), 7.22 (s, 1H, Ar-H), 4.97 (s, 2H, Ar-C$_2$H$_4$), 4.65 (s, 2H, RO-CH$_2$CO$_2$tBu), 4.03 (s, 3H, O-CH$_3$), 1.51 (s, 9H, 3 X CH$_3$ tert-butyl).

**13C NMR** (101 MHz, Chloroform-d): δ 167.0 (C=O), 154.4 (C$_{Ar}$), 146.2 (C$_{Ar}$), 139.5 (C$_{Ar}$), 133.5 (C$_{Ar}$), 111.6 (CH$_{Ar}$), 110.2 (CH$_{Ar}$), 83.3 (Cq), 66.5 (CH$_{2}$), 62.9 (CH$_{2}$), 56.7 (CH$_{3}$), 28.2 (3 X CH$_{3}$).

**IR (ν$_{max}$, cm$^{-1}$):** 3543 (w), 2980 (w), 2933 (w), 1747 (m), 1582 (w), 1518 (s), 1370 (w), 1328 (m), 1281 (m), 1212 (w), 1158 (s), 1083 (m).

**HRMS (ESI) m/z:** calcd for C$_{14}$H$_{19}$NNaO$_7$ [M+Na]$^+$ 336.1054; found 336.1056.
$^1$H NMR and $^{13}$C spectra for 18:
General procedure A:

2-(4-Formyl-2-methoxy-5-nitrophenoxo)acetic acid (0.90 g, 3.53 mmol, 1.0 eq) was dissolved in a mixture of solvent / conc. sulfuric acid (10:1) and the reaction mixture was refluxed for 12 h. The solvent was removed under reduced pressure and the residue was neutralized with sat. NaHCO₃. The precipitate was filtered, dissolved in EtOAc, dried over MgSO₄, filtered and the solvent was removed under reduced pressure.

Methyl 2-(4-formyl-2-methoxy-5-nitrophenoxo)acetate (16)

Following the general procedure A, 2-(4-formyl-2-methoxy-5-nitrophenoxo)acetic acid (14) was dissolved in MeOH to afford methyl 2-(4-formyl-2-methoxy-5-nitrophenoxo)acetate (16) (0.83 g, 3.1 mmol, 78% yield) as a yellow solid.

¹H NMR (400 MHz, Chloroform-d): δ 10.44 (s, 1H, CHO), 7.53 (s, 1H, Ar-H), 7.43 (s, 1H, Ar-H), 4.84 (s, 2H, RO-CH₂-CO₂Me), 4.03 (s, 3H, O-CH₃), 3.83 (s, 3H, CH₃ester).

¹³C NMR (101 MHz, Chloroform-d): δ 187.7 (C=O), 167.8 (C), 153.8 (C₆), 150.4 (C₆), 143.4 (C₆), 126.8 (C₆), 110.7 (CH₃), 109.2 (CH₃), 66.1 (CH₃), 57.0 (CH₃), 52.8 (CH₃).

IR (νmax cm⁻¹): 2960 (s), 1747 (s), 1685 (s), 1574 (s), 1501 (s), 1281 (s), 1205 (s), 1073 (s), 728 (s).

HRMS (APCI/QTOF) m/z: [M + H]+ Calcd for C₁₁H₁₂NO₇ 270.0608; Found 270.0608.
$^1$H NMR and $^{13}$C spectra for 16:
Ethyl 2-(4-formyl-2-methoxy-5-nitrophenoxo)acetate (17)

Following the general procedure A, 2-(4-formyl-2-methoxy-5-nitrophenoxo)acetic acid (14) was dissolved in EtOH to afford ethyl 2-(4-formyl-2-methoxy-5-nitrophenoxo)acetate (17) (0.99 g, 3.5 mmol, quant.) as a yellow solid.

\(^1\)H NMR (400 MHz, Chloroform-\(d\)): \(\delta\) 10.40 (s, 1H, CHO), 7.51 (s, 1H, Ar-H), 7.40 (s, 1H, Ar-H), 4.81 (s, 2H, RO-CH\(_2\)-CO\(_2\)Et), 4.27 (q, \(J = 7.2\) Hz, 2H, CH\(_2\)ester), 4.01 (s, 3H, O-CH\(_3\)), 1.29 (t, \(J = 7.3\) Hz, 3H, CH\(_3\)ester).

\(^1\)C NMR (101 MHz, Chloroform-\(d\)): \(\delta\) 187.7 (C\(_{HO}\)), 167.4 (C\(_{O}\)), 153.8 (C\(_{Ar}\)), 150.4 (C\(_{Ar}\)), 143.4 (C\(_{Ar}\)), 126.7 (C\(_{Ar}\)), 110.7 (CH\(_{Ar}\)), 109.3 (CH\(_{Ar}\)), 66.2 (CH\(_3\)), 62.1 (CH\(_3\)), 56.9 (CH\(_3\)), 14.2 (CH\(_3\)).

IR (\(\nu_{max}\), cm\(^{-1}\)): 2947 (s), 1747 (s), 1688 (s), 1577 (s), 1515 (s), 1330 (s), 1289 (s), 1196 (s), 1076 (s), 1017 (s), 875 (s), 782 (s), 729 (s), 649 (s).

HRMS (APCI/QTOF) m/z: [M + H]\(^+\) Calcd for C\(_{12}\)H\(_{14}\)NO\(_7\)\(^+\) 284.0765; Found 284.0766.

\(^1\)H NMR and \(^1\)C spectra for 17:
Experimental part

Sodium borohydride (0.12 g, 3.1 mmol, 1.0 eq) was added to a mixture of methyl 2-(4-formyl-2-methoxy-5-nitrophenoxyl)acetate (16) (0.83 g, 3.1 mmol, 1.0 eq) in a mixture of THF / MeOH (1:1, 10 mL) at 0°C. The reaction mixture was stirred at 0°C for 30 min. H₂O (10 mL) and sat. NH₄Cl (10 mL) was added to quench the reaction and the solution was stirred 30 min. The crude product was extracted with EtOAc (3 X 100 mL) and the combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. Methyl 2-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxyl)acetate (19) (0.53 g, 1.9 mmol, 64% yield) was obtained as a slightly yellow crystal.

\[ \text{Methyl 2-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxyl)acetate (19)} \]

\[
\text{\textsuperscript{1}H NMR (400 MHz, Chloroform-\text{d}): } \delta 7.65 (s, 1H, Ar-H), 7.24 (s, 1H, Ar-H), 4.97 (s, 2H, Ar-CH₂), 4.77 (s, 2H, RO-CH₂-CO₂Me), 4.01 (s, 3H, O-C₃H₃), 3.92 (s, 3H, O-C₃H₃ ester), 2.63 (s, 1H, OH)
\]

\[
\text{\textsuperscript{13}C NMR (101 MHz, Chloroform-\text{d}): } \delta 168.5 (C=O), 154.5 (C₆), 146.0 (C₅), 139.5 (C₆), 133.9 (C₆), 111.6 (CH₆), 110.6 (CH₆), 66.2 (CH₂), 62.9 (CH₂), 56.7 (CH₃), 52.7 (CH₃).
\]

IR (\(v_{\text{max}}\) cm\(^{-1}\)): 3528 (w), 2954 (w), 1755 (m), 1518 (s), 1438 (w), 1329 (w), 1279 (m), 1208 (m), 1084 (m), 878 (w).

HRMS (ESI) m/z: calcd for C₁₁H₁₃NNaO₇ \([\text{M+Na}]^+\) 294.0584; found 294.0584.
$^1$H NMR and $^{13}$C spectra for 19:
Experimental part

Ethyl 2-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxy)acetate (20)

Sodium borohydride (0.13 g, 3.5 mmol, 1.0 eq) was added to a mixture of ethyl 2-(4-formyl-2-methoxy-5-nitrophenoxy)acetate (17) (0.99 g, 3.5 mmol, 1.0 eq) in a mixture of THF / MeOH (1:1, 10 mL) at 0°C. The reaction mixture was stirred at 0°C for 30 min. H2O (10 mL) and sat. NH4Cl (10 mL) was added to quench the reaction and the solution was stirred 30 min. The crude product was extracted with EtOAc (3 X 100 mL) and the combined organic layers were dried over MgSO4, filtered and concentrated under reduced pressure. Ethyl 2-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxy)acetate (20) (0.54 g, 1.9 mmol, 53% yield) was obtained as a slightly yellow crystal.

1H NMR (400 MHz, Chloroform-d): δ 7.66 (s, 1H, Ar-H), 7.23 (s, 1H, Ar-H), 4.98 (d, J = 6.4 Hz, 2H, Ar-CH2), 4.75 (s, 2H, RO-CH2-CO2Et), 4.29 (q, J = 7.1 Hz, 2H, CH3 ester), 4.02 (s, 3H, O-C6H3), 2.58 (t, J = 6.5 Hz, 1H, OH), 1.31 (t, J = 7.1 Hz, 3H, CH3 ester).

13C NMR (101 MHz, Chloroform-d): δ 168.0 (CO), 154.5 (C Ar), 146.1 (C Ar), 139.5 (C Ar), 133.8 (C Ar), 111.6 (CH Ar), 110.6 (CH Ar), 66.3 (CH2), 62.9 (CH2), 61.9 (CH2), 56.7 (CH3), 14.3 (CH3).

IR (νmax, cm⁻¹): 3481 (w), 2941 (w), 1753 (m), 1518 (s), 1329 (w), 1279 (s), 1202 (m), 1084 (m), 1026 (w), 878 (w), 796 (w).

HRMS (ESI) m/z: calcd for C12H13NNaO7 [M+Na]⁺ 308.0741; found 308.0739.
$^1$H NMR and $^{13}$C spectra for 20:
4.2.3 **Synthesis of the coumarinyl-linkers**

**Synthesis of the coumarinyl precursor 24:**

Scheme 37: Synthesis of the coumarinyl key intermediate 24: i) ethyl chloroformate, Et₂O, r.t., 2 h; ii) ethyl acetoacetate, H₂SO₄, EtOH, r.t., 4 h; Then iii) H₂SO₄, acetic acid, reflux, 2 h; iv) TsCl, pyridine, DCM, r.t., 24 h; v) bromoethane, K₂CO₃, TBAB, NaI, ACN, reflux, 6 h.

**Ethyl (3-hydroxyphenyl)carbamate (21)**

Ethyl chloroformate (9.0 mL, 92 mmol, 1.0 eq) was added to a stirred suspension of aminophenol (10.0 g, 92 mmol, 1.0 eq) in diethyl ether (400 mL). The reaction mixture was stirred at r.t. for 2 h. The solid was removed by filtration. The filtrate was concentrated under reduced pressure to give ethyl (3-hydroxyphenyl)carbamate (21) (8.63 g, 47.6 mmol, 52% yield) as a white solid.

\[ ^1H \text{NMR (400 MHz, Chloroform-}d\text{)}: 5.723 (s, 1H, NH), 7.14 (t, J = 8.1 Hz, 1H, Ar-H), 6.71 (dd, J = 7.9, 2.1 Hz, 1H, Ar-H), 6.63 – 6.52 (m, 2H, 2x Ar-H), 5.45 (s, 1H, OH), 4.22 (q, J = 7.2 Hz, 2H, CH₂ ester), 1.31 (t, J = 7.1 Hz, 3H, CH₃ ester). \]

The analytical data were in accordance with previously reported data.\(^{355}\)
Experimental part

$^1$H NMR spectrum for 21:

7-Amino-4-methyl-$2H$-chromen-2-one (22)

Ethyl (3-hydroxyphenyl)carbamate (21) (24.3 g, 134.1 mmol, 1.0 eq) was suspended in 70% ethanolic $\text{H}_2\text{SO}_4$ solution (150 mL) at 0°C. Ethyl acetoacetate (17.4 mL, 134.1 mmol, 1.0 eq) was added slowly and the reaction mixture was stirred at r.t. for 4 h. Completion of the reaction was monitored by TLC. Acetic acid (70 mL) was added and the reaction mixture was refluxed for 2 h. The reaction mixture was poured into ice (approx. 400 mL) and NaOH was added until pH = 9. The solid was filtered and 7-amino-4-methyl-$2H$-chromen-2-one (22) (21.2 g, 121.1 mmol, 90% yield) was obtained as a yellow solid.

$^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.40 (d, $J = 8.7$ Hz, 1H, coumarin-H), 6.56 (dd, $J = 8.7$, 2.1 Hz, 1H, coumarin-H), 6.40 (d, $J = 2.1$ Hz, 1H, coumarin-H), 6.11 (s, 2H, NH$_2$), 5.90 (s, 1H, coumarin-H), 2.30 (s, 3H, CH$_3$coumarin).

The analytical data were in accordance with previously reported data.$^{355}$
A solution of tosyl chloride (23.0 g, 123.2 mmol, 1.2 eq) in pyridine (150 mL) was added slowly to a solution of 7-amino-4-methyl-2H-chromen-2-one (22) (17.9 g, 102.7 mmol, 1.0 eq) in DCM (200 mL). The reaction mixture was stirred at r.t. for 24 h. DCM and pyridine was removed under reduced pressure. The crude mixture was dissolved in DCM (300 mL) and washed with 20% citrate solution (3 X 250 mL) and brine (1 X 250 mL). The organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. 4-Methyl-N-(4-methyl-2-oxo-2H-chromen-7-yl)benzenesulfonamide (23) (31.1 g, 94.5 mmol, 92% yield) was obtained as a yellow solid.

$^1$H NMR (400 MHz, DMSO-d₆): δ 10.93 (s, 1H, NH), 7.74 (d, $J = 8.0$ Hz, 2H, 2x tosyl-H), 7.64 (d, $J = 8.6$ Hz, 1H, coumarin-H), 7.38 (d, $J = 8.1$ Hz, 2H, 2x tosyl-H), 7.09 (dd, $J = 8.7, 2.2$ Hz, 1H, coumarin-H), 7.03 (d, $J = 2.0$ Hz, 1H, coumarin-H), 6.24 (s, 1H, coumarin-H), 2.33 (s, 6H, CH₃ coumarin and CH₃ tosyl).

The analytical data were in accordance with previously reported data. 355
Experimental part

$^1$H NMR spectrum for 23:

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 7.59 (d, $J = 8.5$ Hz, 1H, coumarin-$H$), 7.48 (d, $J = 8.1$ Hz, 2H, 2x tosyl-$H$), 7.29 – 7.25 (m, 2H, 2x tosyl-$H$), 7.23 (dd, $J = 8.4$, 2.1 Hz, 1H, coumarin-$H$), 6.89 (d, $J = 2.3$ Hz, 1H, coumarin-$H$), 5.30 (s, 1H, coumarin-$H$), 3.64 (q, $J = 7.1$ Hz, 2H, $R_2$N-CH$_2$-CH$_3$), 2.45 (s, 3H, $CH_3$ coumarin), 2.44 (s, 3H, $CH_3$ tosyl), 1.10 (t, $J = 7.1$ Hz, 3H, $R_2$N-CH$_2$-CH$_3$)

The analytical data were in accordance with previously reported data.$^{331}$

$N$-Ethyl-4-methyl-$N$-(4-methyl-2-oxo-$2H$-chromen-7-yl)benzenesulfonamide (24)

4-Methyl-$N$-(4-methyl-2-oxo-$2H$-chromen-7-yl)benzenesulfonamide (23) (31.1 g, 94.5 mmol, 1.0 eq), $K_2$CO$_3$ (19.6 g, 141.7 mmol, 1.5 eq), NaI (1.42 g, 9.45 mmol, 0.1 eq) and tetrabutylammonium bromide (3.05 g, 9.45 mmol, 0.1 eq) were dissolved in acetonitrile (350 mL). Bromoethane (14 mL, 188.9 mmol, 2 eq) was added and the mixture was refluxed for 6 h. The reaction mixture was cooled down to r.t., filtered and the solvent was removed under reduced pressure. The residue was dissolved in DCM (300 mL) and washed with $H_2O$ (3 X 250 mL). The organic layer was dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was recrystallized from ethanol to afford $N$-ethyl-4-methyl-$N$-(4-methyl-2-oxo-$2H$-chromen-7-yl)benzenesulfonamide (24) (34.8 g, 141.7 mmol, quant.) as a brown solid.
Synthesis of alkyne functionalized coumarinyl linker:

Scheme 38: Synthesis of 27: i) SeO₂; p-xylene, reflux 24 h; then NaBH₄, MeOH, r.t., 3 h; ii) H₂SO₄, 0°C, 1 h; iii) 3-bromoprop-1-yn, K₂CO₃, TBAB, NaI, acetone, reflux, 16 h; iv) 4-nitrophenyl chloroformate, DIPEA, DCM, r.t., 16 h.

N-Ethyl-N-(4-(hydroxymethyl)-2-oxo-2H-chromen-7-yl)-4-methylbenzenesulfonamide (25)

N-Ethyl-4-methyl-N-(4-methyl-2-oxo-2H-chromen-7-yl)benzenesulfonamide (24) (0.51 g, 1.41 mmol, 1.0 eq) and SeO₂ (0.31 g, 2.81, 2.0 eq) were dissolved in p-xylene (12 mL). The reaction mixture was refluxed with vigorous stirring under argon atmosphere for 24 h. The reaction mixture was cooled down to r.t., filtered
through paper filter and concentrated under reduced pressure. The crude was dissolved in methanol (12 mL) and NaBH₄ (0.11 g, 2.81 mmol, 2.0 eq) was added. The reaction mixture was stirred at r.t. for 3 h and the suspension was neutralized with 1M HCl and H₂O. The mixture was extracted with DCM (3 X 50 mL) and the combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. The crude mixture was purified via column chromatography eluting with a mixture of EtOAc / PE (1:1). N-Ethyl-N-(4-(hydroxymethyl)-2-oxo-2H-chromen-7-yl)-4-methylbenzenesulfonamide (25) (0.33 g, 0.89 mmol, 63% yield) was obtained as a yellow solid.

¹H NMR (400 MHz, Chloroform-d): δ 7.48 (m, 3H, 2x tosyl-H and coumarin-H), 7.28 (s, 1H, coumarin-H), 7.23 (dd, J = 8.6, 2.1 Hz, 2H, 2x tosyl-H), 6.90 (d, J = 2.1 Hz, 1H, coumarin-H), 6.63 (s, 1H), coumarin-H, 4.92 (d, J = 5.0 Hz, 2H, CH₂-coumarin), 3.63 (q, J = 7.2 Hz, 2H, R₂N-CH₂-CH₃), 2.44 (s, 3H, CH₃-tosyl), 2.05 (s, 1H, OH), 1.09 (t, J = 7.2 Hz, 3H, R₂N-CH₂-CH₃).

The analytical data were in accordance with previously reported data.³³¹

¹H NMR spectrum for 25:
N-Ethyl-N-(4-(hydroxymethyl)-2-oxo-2H-chromen-7-yl)-4-methylbenzenesulfonamide (25) (8.33 g, 22.3 mol, 1.0 eq) was added to conc. sulfuric acid (60 mL) and the reaction mixture was stirred at 0°C for 1 h. The reaction mixture was poured into H₂O (60 mL) and neutralized with sat. NaHCO₃ (300 mL) and solid NaHCO₃. The aqueous layer was extracted with EtOAc (5 X 300 mL). The combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. The crude mixture was purified via column chromatography eluting with a mixture of EtOAc / PE (1:1) to afford 7-(ethylamino)-4-(hydroxymethyl)-2H-chromen-2-one (26) (4.21 g, 19.2 mmol, 86% yield) as a yellow solid.

¹H NMR (400 MHz, DMSO-d₆): δ 7.35 (d, J = 8.8 Hz, 1H, coumarin- H), 6.60 (t, J = 5.4 Hz, 1H, NH), 6.56 (dd, J = 8.7, 2.3 Hz, 1H, coumarin-H), 6.39 (d, J = 2.3 Hz, 1H, coumarin-H), 6.05 (d, J = 1.4 Hz, 1H, coumarin-H), 5.48 (t, J = 5.6 Hz, 1H, OH), 4.64 (dd, J = 5.6, 1.5 Hz, 2H, CH₂coumarin), 3.11 (qd, J = 7.1, 5.0 Hz, 2H, RNH-CH₂-CH₃), 1.17 (t, J = 7.0 Hz, 3H, RNH-CH₂-CH₃).

The analytical data were in accordance with previously reported data.↵331

¹H NMR spectrum for 26:
Experimental part

7-(Ethyl(prop-2-yn-1-yl)amino)-4-(hydroxymethyl)-2H-chromen-2-one (27)

7-(Ethylamino)-4-(hydroxymethyl)-2H-chromen-2-one (26) (2.00 g, 9.12 mmol, 1.0 eq), K$_2$CO$_3$ (2.52 g, 18.2 mmol, 2.0 eq), NaI (0.14 g, 0.91 mmol, 0.1 eq) and tetrabutylammonium bromide (0.30 g, 0.91 mmol, 0.1 eq) were dissolved in acetone (200 mL). 3-bromoprop-1-yn (2.0 mL, 18.2 mmol, 2.0 eq) was added and the reaction mixture was refluxed for 16 h. The reaction mixture was cooled down to r.t., filtered and the solvent was removed under reduced pressure. The crude mixture was purified via column chromatography eluting with a mixture of EtOAc / PE (1:1) and 7-(ethyl(prop-2-yn-1-yl)amino)-4-(hydroxymethyl)-2H-chromen-2-one (27) (0.46 g, 1.79 mmol, 23% yield) was obtained as an orange solid.

$^1$H NMR (400 MHz, Chloroform-d): δ 7.37 (d, $J = 8.6$ Hz, 1H, coumarin-H), 6.72 – 6.66 (m, 2H, 2x coumarin-H), 6.33 (d, $J = 1.4$ Hz, 1H, coumarin-H), 4.86 (d, $J = 5.4$ Hz, 2H, CH$_2$coumarin), 4.08 (d, $J = 2.4$ Hz, 2H, R$_2$N-CH$_2$-C≡CH), 3.53 (q, $J = 7.2$ Hz, 2H, R$_2$N-CH$_2$-CH$_3$), 2.25 (t, $J = 2.4$ Hz, R$_2$N-CH$_2$-C≡CH), 1.86 (t, $J = 7.1$ Hz, OH), 1.27 (t, $J = 7.2$ Hz, 3H, R$_2$N-CH$_2$-CH$_3$).

The analytical data were in accordance with previously reported data.
Experimental part

(7-(Ethyl(prop-2-yn-1-yl)amino)-2-oxo-2H-chromen-4-yl)methyl (4-nitrophenyl) carbonate 54

\[
\text{\textbf{[Image of chemical structure]}}
\]

7-(Ethylamino)-4-(hydroxymethyl)-2H-chromen-2-one (27) (20.0 mg, 77.7 μmol, 1.0 eq) and 4-nitrophenyl chloroformate (18.8 mg, 93.2 μmol, 1.2 eq) were dissolved in dry DCM (1 mL) under argon atmosphere. DIPEA (52 μL, 0.23 mmol, 4.0 eq) was added and the reaction mixture was stirred at r.t. for 16 h under dark conditions. The solvent was removed under reduced pressure and the crude was purified by column chromatography eluting with a mixture of PE / EtOAc (8:2 to 6:4). (7-(Ethyl(prop-2-yn-1-yl)amino)-2-oxo-2H-chromen-4-yl)methyl (4-nitrophenyl) carbonate (54) (25.7 mg, 60.8 μmol, 78% yield) was obtained as yellow foam.

\[\text{\textsuperscript{1}H NMR} (400 MHz, Chloroform-\text{d}): \delta 8.34 – 8.29 (m, 2H, 2 X Ar-H), 7.46 – 7.40 (m, 2H, 2 X Ar-H), 7.38 (d, \textit{J} = 8.9 Hz, 1H, coumarin-H), 6.75 (dd, \textit{J} = 8.9, 2.6 Hz, 1H, coumarin-H), 6.71 (d, \textit{J} = 2.6 Hz, 1H, coumarin-H), 6.30 (d, \textit{J} = 1.3 Hz, 1H, coumarin-H), 5.42 (d, \textit{J} = 1.3 Hz, 2H, CH\text{\textsubscript{2}}coumarin), 4.10 (d, \textit{J} = 2.5 Hz, 2H, R\textsubscript{2}N-CH\text{\textsubscript{2}}C≡C), 3.55 (q, \textit{J} = 7.2 Hz, 2H, R\textsubscript{2}N-CH\text{\textsubscript{2}}-CH\textsubscript{3}), 2.25 (t, \textit{J} = 2.4 Hz, 1H, R\textsubscript{2}N-CH\text{\textsubscript{2}}C≡C), 1.28 (t, \textit{J} = 7.1 Hz, 3H, R\textsubscript{2}N-CH\text{\textsubscript{2}}-CH\textsubscript{3}).\]

\[\text{\textsuperscript{13}C NMR} (101 MHz, Chloroform-\text{d}): \delta 161.4 (C\text{\textsubscript{coumarin}}), 158.5 (CO), 156.1 (C\text{\textsubscript{Ar}}), 155.3 (C\text{\textsubscript{coumarin}}), 152.3 (C\text{\textsubscript{coumarin}}), 147.7 (C\text{\textsubscript{Ar}}), 145.8 (C\text{\textsubscript{coumarin}}), 125.6 (2 X CH\textsubscript{2}Ar), 124.4 (CH\text{\textsubscript{coumarin}}), 121.9 (2 X CH\textsubscript{Ar}), 110.0 (C\text{\textsubscript{coumarin}}), 110.0 (C\text{\textsubscript{coumarin}}), 108.5 (CH\text{\textsubscript{coumarin}}), 99.9 (CH\text{\textsubscript{coumarin}}), 79.0 (Calkyne), 72.6 (CH\text{\textsubscript{alkyne}}), 65.8 (CH\text{\textsubscript{2}}), 46.3 (CH\text{\textsubscript{2}}), 40.0 (CH\text{\textsubscript{2}}), 12.4 (CH\text{\textsubscript{3}}).\]

\[\text{IR} (\textit{ν}_{\text{max}}, \text{cm}^{-1}): 3366 (m), 1715 (m), 1612 (m), 1593 (s), 1491 (s), 1446 (m), 1337 (s), 1295 (s), 1169 (m), 1114 (s), 845 (s), 753 (m), 692 (w).\]

\[\text{HRMS (ESI/QTOF) m/z: \text{[M + Na]}^+ \text{Calcd for C}_{22}\text{H}_{18}\text{N}_{2}\text{NaO}_{7}^+ 445.1006; Found 445.1004.}\]
$^1$H NMR and $^{13}$C spectra for 54:
Synthesis of ester functionalized coumarinyl linkers:

Scheme 39: Synthesis of CM derivatives 31 and 32: i) H$_2$SO$_4$, 0°C, 1 h; ii) for 29: ethyl bromoacetate, K$_2$CO$_3$, TBAB, NaI, ACN, reflux, 46 h; for 30: tert-butyl bromoacetate, K$_2$CO$_3$, TBAB, NaI, ACN, reflux, 5 days; iii) SeO$_2$: p-xylene, reflux 24 h; then NaBH$_4$, MeOH, r.t., 3 h; iv) For 32: TFA, r.t., 2 h; v) 4-nitrophenyl chloroformate, DIPEA, DCM, r.t., 16 h.

7-(Ethylamino)-4-methyl-2H-chromen-2-one (28)

4-Methyl-N-(4-methyl-2-oxo-2H-chromen-7-yl)benzenesulfonamide (24) (1.1 g, 3.1 mmol, 1.0 eq) was added to conc. sulfuric acid (5 mL) and the reaction mixture was stirred at 0°C for 1 h. The reaction mixture was poured into H$_2$O (20 mL) and neutralized with sat. NaHCO$_3$ (150 mL). The aqueous layer was extracted with DCM (3 X 200 mL). The combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure. The crude mixture was purified via column chromatography eluting with a mixture of EtOAc / PE (6:4) to afford 7-(ethylamino)-4-methyl-2H-chromen-2-one (28) (0.6 g, 2.9 mmol, 97% yield) as a yellow solid.

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 7.35 (d, $J = 8.6$ Hz, 1H, coumarin-$H$), 6.49 (dd, $J = 8.6$, 2.4 Hz, 1H, coumarin-$H$), 6.44 (d, $J = 2.3$ Hz, 1H, coumarin-$H$), 5.98 (d, $J = 1.5$ Hz, 1H, coumarin-$H$), 4.11 (s, 1H, N-$H$), 3.22 (qd, $J = 7.1$, 5.2 Hz, 2H, CH$_2$ ester), 2.34 (d, $J = 1.2$ Hz, 3H, CH$_3$ coumarin), 1.29 (t, $J = 7.2$ Hz, 3H, CH$_3$ ester).

The analytical data were in accordance with previously reported data.
Experimental part

$^1$H NMR spectrum for 28:

Ethyl N-ethyl-N-(4-methyl-2-oxo-2H-chromen-7-yl)glycinate 29

7-(Ethylamino)-4-methyl-2H-chromen-2-one (28) (1.0 g, 4.9 mmol, 1.0 eq), K$_2$CO$_3$ (2.0 g, 14.7 mmol, 3.0 eq), NaI (0.15 g, 0.98 mmol, 0.2 eq) and tetrabutylammonium bromide (0.32 g, 0.92 mmol, 0.2 eq) were dissolved in acetonitrile (50 mL). Ethyl bromoacetate (5.5 mL, 49.0 mmol, 10.0 eq) was added and the mixture was refluxed for 48 h. The reaction mixture was cooled down to r.t., filtered and the solvent was removed under reduced pressure. The residue was dissolved in EtOAc (100 mL) and washed with H$_2$O (3 X 100 mL). The organic layer was dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was purified via column chromatography eluting with a mixture of EtOAc / PE (6:4) to afford ethyl N-ethyl-N-(4-methyl-2-oxo-2H-chromen-7-yl)glycinate (29) (1.29 g, 4.5 mmol, 90% yield) as a yellow solid.

$^1$H NMR (400 MHz, Chloroform-d): δ 7.40 (d, $J$ = 8.9 Hz, 1H, coumarin-H), 6.56 (dd, $J$ = 8.9, 2.6 Hz, 1H, coumarin-H), 6.48 (d, $J$ = 2.6 Hz, 1H, coumarin-H), 6.48 (d, $J$ = 2.6 Hz, 1H, coumarin-H), 5.99 (d, $J$ = 1.5 Hz, 1H, coumarin-H), 4.22 (q, $J$ = 7.1 Hz, 2H, CH$_2$ ester), 4.07 (s, 2H, RN-CH$_2$-CO$_2$Et), 3.52 (q, $J$ = 7.1 Hz, 2H, R$_2$N-CH$_2$-CH$_3$), 2.34 (d, $J$ = 1.1 Hz, 3H, CH$_3$ coumarin), 1.27 (dt, $J$ = 12.5, 7.2 Hz, 6H, R$_2$N-CH$_2$-CH$_3$ and CH$_3$ ester).

$^{13}$C NMR (101 MHz, Chloroform-d): δ 170.2 (CO), 162.1 (C coumarin), 155.9 (C coumarin), 152.9 (C coumarin), 150.9 (C coumarin), 125.7 (CH coumarin), 110.5 (C coumarin), 109.9 (CH coumarin), 108.8 (CH coumarin), 98.7 (CH coumarin), 61.6 (CH$_3$), 52.3 (CH$_3$), 46.8 (CH$_2$), 18.6 (CH$_3$), 14.4 (CH$_3$), 12.4 (CH$_3$).
Experimental part

IR (νmax cm⁻¹): 2966 (w), 1726 (s), 1618 (s), 1536 (w), 1409 (m), 1194 (m), 1080 (m), 770 (m), 713 (s).

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: [M + H]^+ Calcd for C_{16}H_{20}NO_4 + 290.1387; Found 290.1385.

1H NMR and 13C spectra for 29:
Experimental part

Ethyl N-ethyl-N-(4-(hydroxymethyl)-2-oxo-2H-chromen-7-yl)glycinate 31

Ethyl N-ethyl-N-(4-methyl-2-oxo-2H-chromen-7-yl)glycinate (29) (1.29 g, 4.46 mmol, 1.0 eq) and SeO₂ (0.99 g, 8.92, 2.0 eq) were dissolved in p-xylene (40 mL). The reaction mixture was refluxed with vigorous stirring under argon atmosphere for 24 h. The reaction mixture was cooled down to r.t., filtered through paper filter and concentrated under reduced pressure. The crude was dissolved in methanol (30 mL) and NaBH₄ (0.34 g, 8.92 mmol, 2.0 eq) was added. The reaction mixture was stirred at r.t. for 3 h and the suspension was neutralized with 1M HCl (10 mL) and H₂O (100 mL). The mixture was extracted with DCM (3 X 150 mL) and the combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. The crude mixture was purified via column chromatography eluting with a mixture of EtOAc / PE (3:7 to 6:4). Ethyl N-ethyl-N-(4-(hydroxymethyl)-2-oxo-2H-chromen-7-yl)glycinate (31) (0.75 g, 2.46 mmol, 55% yield) was obtained as a yellow solid.

¹H NMR (400 MHz, Chloroform-d): δ 7.32 (d, J = 8.9 Hz, 1H, coumarin-H), 6.53 (dd, J = 8.9, 2.6 Hz, 1H, coumarin-H), 6.49 (d, J = 2.6 Hz, 1H, coumarin-H), 6.30 (s, 1H, coumarin-H), 4.81 (d, J = 6.1 Hz, 2H, CH₂coumarin), 4.22 (q, J = 7.1Hz, 2H, CH₂ester), 4.07 (s, 2H, R₂N-CH₂-CO₂Et), 3.51 (q, J = 7.1 Hz, 2H, RΝH-CH₂-C₂H₅), 2.07 (t, J = 6.1 Hz, 1H, ROH), 1.28 (t, J = 7.1 Hz 3H, CH₃ester), 1.25 (t, J = 7.1 Hz 3H, R₂N-CH₂-C₂H₅).

¹³C NMR (101 MHz, Chloroform-d): δ 170.1 (CO), 162.1 (C coumarin), 155.9 (C coumarin), 154.2 (C coumarin), 150.7 (C coumarin), 124.4 (CH coumarin), 108.8 (CH coumarin), 107.5 (C coumarin), 106.6 (CH coumarin), 98.7 (CH coumarin), 61.5 (CH₂), 61.0 (CH₃), 52.1 (CH₂), 46.7 (CH₂), 14.2 (CH₃), 12.3 (CH₃).

IR (νmax, cm⁻¹): 1713 (m), 1612 (s), 1517 (m), 1441 (w), 1403 (w), 1188 (m), 1093 (m), 808 (m), 663 (s).

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: [M + H]+ Calcd for C₁₆H₂₀NO₅ 306.1336; Found 306.1332.
$^1$H NMR and $^{13}$C spectra for 31:
Experimental part

Ethyl $\text{N-ethyl-N-}(4-(((4\text{-nitrophenoxy})\text{carbonyl})\text{oxy})\text{methyl})-2\text{-oxo-2H-chromen-7-yl})\text{glycinate (34)}$

Ethyl $\text{N-ethyl-N-}(4-((\text{hydroxymethyl})-2\text{-oxo-2H-chromen-7-yl})\text{glycinate (31})$ (0.10 g, 0.33 mmol, 1.0 eq) and 4-nitrophenyl chloroformate (99 mg, 0.49 mmol, 1.5 eq) were dissolved in dry DCM (2.5 mL) under argon atmosphere and dark conditions. DIPEA (0.20 mL, 1.32 mmol, 4.0 eq) was added and the reaction mixture was stirred at r.t. for 24 h. The solvent was removed under reduced pressure and the crude was purified via column chromatography eluting with a mixture of EtOAc / PE (7:3 to 5:5). Ethyl $\text{N-ethyl-N-}(4-(((4\text{-nitrophenoxy})\text{carbonyl})\text{oxy})\text{methyl})-2\text{-oxo-2H-chromen-7-yl})\text{glycinate (34)}$ (78.7 mg, 0.17 mmol, 51% yield) was obtained as a yellow oil.

$^1\text{H NMR}$ (400 MHz, Chloroform-$d$): $\delta$ 8.30 (d, $J = 9.1$ Hz, 2H, Ar-H), 7.42 (d, $J = 9.1$ Hz, 2H, Ar-H), 7.35 (d, $J = 9.0$, 1H, coumarin-H), 6.59 (dd, $J = 9.0$, 2.6 Hz, 1H, coumarin-H), 6.53 (d, $J = 2.6$ Hz, 1H, coumarin-H), 6.28 (s, 1H, coumarin-H), 5.40 (s, 2H, $\text{CH}_2\text{coumarin}$), 4.23 (q, $J = 7.1$ Hz, 2H, $\text{CH}_2\text{ester}$), 4.09 (s, 2H, $\text{R}_2\text{N-CH}_2\text{CO}_2\text{Et}$), 3.54 (q, $J = 7.1$ Hz, 2H, $\text{R}_2\text{N-CH}_2\text{CH}_3$), 1.29 (t, $J = 7.1$ Hz, 3H, $\text{C}_2\text{H}_3\text{ester}$), 1.27 (t, $J = 7.2$ Hz, 3H, $\text{R}_2\text{N-CH}_2\text{CH}_3$).

$^{13}\text{C NMR}$ (101 MHz, Chloroform-$d$): $\delta$ 170.0 (CO), 161.4 ($\text{C coumarin}$), 156.2 ($\text{C coumarin}$), 155.3 ($\text{C Ar}$), 152.3 ($\text{C coumarin}$), 151.3 ($\text{C coumarin}$), 147.7 ($\text{C Ar}$), 125.6 ($\text{CH Ar}$), 124.5 ($\text{CH coumarin}$), 121.9 ($\text{CH Ar}$), 109.2 ($\text{CH coumarin}$), 108.3 ($\text{CH coumarin}$), 107.0 ($\text{C coumarin}$), 99.0 ($\text{CH coumarin}$), 65.8 ($\text{CH}_2$), 61.7 ($\text{CH}_2$), 52.3 ($\text{CH}_2$), 46.9 ($\text{CH}_2$), 14.4 ($\text{CH}_3$), 12.4 ($\text{CH}_3$).

IR ($\nu_{\text{max}}$, cm$^{-1}$): 2961 (w), 1729 (s), 1610 (s), 1522 (w), 1410 (m), 1278 (w), 1184 (m), 1166 (m), 1065 (w), 859 (w).

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: [M + Na]$^+$ Calcd for C$_{23}$H$_{22}$N$_2$NaO$_9$ 493.1218; Found 493.1207.
$^1$H NMR and $^{13}$C spectra for 34:
**Experimental part**

**tert-Butyl N-ethyl-N-(4-methyl-2-oxo-2H-chromen-7-yl)glycinate (30)**

7-(Ethylamino)-4-methyl-2H-chromen-2-one (28) (3.8 g, 18.5 mmol, 1.0 eq), K$_2$CO$_3$ (11.9 g, 36.9 mmol, 2.0 eq), NaI (0.55 g, 3.69 mmol, 0.2 eq) and tetrabutylammonium bromide (1.1 g, 3.69 mmol, 0.2 eq) were dissolved in acetonitrile (150 mL). tert-Butyl bromoacetate (25 mL, 184.5 mmol, 10 eq) was added and the mixture was refluxed for 5 days. The reaction mixture was cooled down to r.t., filtered and the solvent was removed under reduced pressure. The residue was dissolved in DCM (500 mL) and washed with H$_2$O (3 X 500 mL). The organic layer was dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was purified via column chromatography eluting with a mixture of EtOAc / PE (3:7 to 6:4) to afford tert-butyl N-ethyl-N-(4-methyl-2-oxo-2H-chromen-7-yl)glycinate (30) (4.4 g, 13.8 mmol, 75%) as a yellow solid.

$^1$H NMR (400 MHz, Chloroform-d): $\delta$ 7.40 (d, $J = 8.9$ Hz, 1H, coumarin-H), 6.56 (dd, $J = 8.9, 2.6$ Hz, 1H, coumarin-H), 6.48 (d, $J = 2.6$ Hz, 1H, coumarin-H), 6.00 (d, $J = 1.2$ Hz, 1H, coumarin-H), 3.97 (s, 2H, R$_2$N-CH$_2$-CO$_2$Bu), 3.51 (q, $J = 7.2$ Hz, 2H, R$_2$N-CH$_2$-CH$_3$), 2.35 (d, $J = 1.1$ Hz, 3H, CH$_3$coumarin), 1.46 (s, 9H, 3 X CH$_3$tert-butyl), 1.24 (dd, $J = 7.2, 4.5$ Hz, 3H, R$_2$N-CH$_2$-CH$_3$).

The analytical data were in accordance with previously reported data.

$^1$H NMR spectrum for 30:
tert-Butyl N-ethyl-N-(4-(hydroxymethyl)-2-oxo-2H-chromen-7-yl)glycinate (32)

tert-Butyl N-ethyl-N-(4-methyl-2-oxo-2H-chromen-7-yl)glycinate (30) (0.57 g, 1.8 mmol, 1.0 eq) and SeO₂ (0.40 g, 3.6, 2.0 eq) were dissolved in p-xylene (45 mL). The reaction mixture was refluxed with vigorous stirring under argon atmosphere for 24 h. The reaction mixture was cooled down to r.t., filtered through paper filter and concentrated under reduced pressure. The crude was dissolved in methanol (50 mL) and NaBH₄ (0.14 g, 3.6 mmol, 2.0 eq) was added. The reaction mixture was stirred at r.t. for 3 h and the suspension was neutralized with 1M HCl (20 mL) and H₂O (120 mL). The mixture was extracted with DCM (3 X 300 mL) and the combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. The crude mixture was purified via column chromatography eluting with a mixture of EtOAc / PE (3:7 to 6:4). tert-Butyl N-ethyl-N-(4-(hydroxymethyl)-2-oxo-2H-chromen-7-yl)glycinate (32) (0.36 g, 1.1 mmol, 60% yield) was obtained as a brown solid.

³H NMR (400 MHz, Chloroform-d): δ 7.31 (d, J = 8.9 Hz, 1H, coumarin-H), 6.52 (dd, J = 8.9, 2.6 Hz, 1H, coumarin-H), 6.48 (d, J = 2.6 Hz, 1H, coumarin-H), 6.32 – 6.23 (m, 1H, coumarin-H), 4.81 (d, J = 1.4 Hz, 2H, CH₂ coumarin), 3.96 (s, 2H, R₂N-CH₂-CO₂tBu), 3.50 (q, J = 7.2 Hz, 2H, R₂N-CH₂-CH₃), 1.92 (s, 1H, OH), 1.46 (s, 9H, 3 X CH₃ tert-buty), 1.24 (t, J = 7.1 Hz, 3H, R₂N-CH₂-CH₃).

The analytical data were in accordance with previously reported data.³⁰₈
**Experimental part**

$^1$H NMR spectrum for 32:

![NMR Spectrum](image)

$N$-Ethyl-$N$-(4-(hydroxymethyl)-2-oxo-2H-chromen-7-yl)glycine (33)

To a solution of tert-butyl $N$-ethyl-$N$-(4-(hydroxymethyl)-2-oxo-2H-chromen-7-yl)glycinate (32) (40.0 mg, 0.12 mmol, 1.0 eq) in DCM (3 mL) was added dropwise TFA (3 mL) and the reaction mixture was stirred at r.t. for 2 h. The solvents were removed under reduced pressure and the crude was purified by column chromatography eluting with a mixture of DCM / MeOH (9:1). $N$-Ethyl-$N$-(4-(hydroxymethyl)-2-oxo-2H-chromen-7-yl)glycine (33) (28.2 mg, 0.10 mmol, 85% yield) was obtained as a brown amorphous solid.

$^1$H NMR (400 MHz, Methanol-$d_4$): δ 7.38 (d, $J$ = 9.0 Hz, 1H, coumarin-H), 6.62 (dd, $J$ = 9.0, 2.5 Hz, 1H, coumarin-H), 6.45 (d, $J$ = 2.5 Hz, 1H, coumarin-H), 6.25 – 6.16 (m, 1H, coumarin-H), 4.73 (d, $J$ = 1.4 Hz, 2H, CH$_2$coumarin), 4.09 (s, 2H, R$_2$N-CH$_2$-CO$_2$H), 3.50 (q, $J$ = 7.1 Hz, 2H, R$_2$N-CH$_2$-CH$_3$), 3.32 (s, 1H, OH), 1.20 (t, $J$ = 7.1 Hz, 3H, R$_2$N-CH$_2$-CH$_3$).

The analytical data were in accordance with previously reported data.$^{308}$
Experimental part

$^1$H NMR spectrum for 33:

4.3 Photosensitive tethers

4.3.1 Synthesis of small spacers and DIBO

Synthesis of tetraethylene glycol spacers

Scheme 40: Synthesis of 47 and 85: i) TsCl, Et$_3$N, DMAP, DCM, r.t., 16 h; ii) NaN$_3$, DMF, 90°C, 16 h; iii) PPh$_3$, 5% HCl, toluene, 0°C to r.t., 6 h; iv) Pd/C, H$_2$, EtOAc, MeOH, r.t., 4 h.

Tetraethylene glycol di(p-toluenesulfonate) 81

Tetraethylene glycol (8.9 mL, 51.4 mmol, 1.0 eq) was dissolved in DCM (100 mL) at 0°C under argon atmosphere. Ts-Cl (29.4 g, 154.6 mmol, 3.0 eq), Et$_3$N (22.0 mL, 154.6 mmol, 3.0 eq) and DMAP (0.3 g, 2.3
mmol, 5 mol%) were added and the reaction mixture was stirred at r.t. for 16 h. The mixture was washed with H$_2$O (3 X 100 mL) and the organic layer was dried over MgSO$_4$, filtered and concentrated under reduced pressure. The crude mixture was purified via column chromatography eluting with a mixture of EtOAc / PE (1:1). (81) (22.4 g, 44.6 mmol, 87% yield) was obtained as yellow oil.

The analytical data were in accordance with previously reported data.$^{350}$

$^1$H NMR (400 MHz, Chloroform-d): $\delta$ 7.78 (d, $J = 8.2$ Hz, 4H, 4 X Ar-H), 7.33 (d, $J = 8.0$ Hz, 4H, 4 X Ar-H), 4.23 – 4.08 (m, 4H, 2 X CH$_2$-OTs), 3.71 – 3.63 (m, 4H, 2 X CH$_2$-CH$_2$-OTs), 3.55 (s, 8H, 4X CH$_2$-O-CH$_2$), 2.43 (s, 6H, 2 x Ar-CH$_3$).

$^1$H NMR spectrum for 81:

Tetraethylene glycol di(azide) 82

Sodium azide (6.9 g, 107.0 mmol, 2.4 eq) was added to a solution of 81 (22.4 g, 44.6 mmol, 1.0 eq) in DMF (100 mL). The reaction mixture was stirred at 90°C for 16 h. The solvent was removed under reduced pressure and the crude was purified via column chromatography eluting with a mixture of EtOAc / PE (3:7). 82 (10.5 g, 42.9 mmol, 96% yield) was obtained as a yellow oil.

The analytical data were in accordance with previously reported data.$^{350}$
Experimental part

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 3.67 (m, 12H, 6 $\times$ CH$_2$-O-CH$_2$), 3.38 (t, $J = 5.0$ Hz, 4H, 2 $\times$ CH$_2$-N$_3$).

$^1$H NMR spectrum for 82:

1-Amino, 11-azido-3,6,9-trioxaundecane hydrochloride (47)

![NMR spectrum image]

To a solution of 82 (10.5 g, 42.9 mmol, 1.0 eq) dissolved in toluene (50 mL) was added a 5% HCl solution (50 mL). The reaction mixture was cooled down to 0°C and PPh$_3$ (10.7 g, 40.8 mmol, 0.95 eq) was added. The reaction mixture was allowed to warm up to r.t. and was stirred for 6 h. The organic layer was separated and the aqueous layer was concentrated under reduced pressure, followed by co-evaporation with toluene. 47 (10.2 g, 40.2 mmol, 94% yield) was obtained as a white amorphous solid.

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 8.25 (s, 2H, NH$_2$), 3.84 (t, $J = 4.9$ Hz, 2H, CH$_2$-CH$_2$-N$_3$), 3.78 – 3.64 (m, 10H, 5 $\times$ CH$_2$-O-CH$_2$), 3.46 (t, $J = 5.0$ Hz, 2H, CH$_2$-N$_3$), 3.25 (q, $J = 5.4$ Hz, 2H, CH$_2$-NH$_2$).

The analytical data were in accordance with previously reported data.$^{350}$
Experimental part

$^1$H NMR spectrum for 47:

1-Amino-2-[2-{2-(2-aminoethoxy)ethoxy}ethoxy]ethane 83

2-{2-[2-{2-Azidoethoxy}ethoxy]ethoxy}ethanamine hydrochloride (47) (0.5 g, 1.96 mmol, 1.0 eq) and 10% Pd/C (0.1 mol%) were dissolved in a mixture of EtOAc / MeOH (3:1, 13 mL). The reaction mixture was stirred at r.t. under hydrogen atmosphere. Completion of the reaction was monitored by TLC. The mixture was filtrated on a pad of Celite® and the filtrate was concentrated under reduced pressure. 83 (0.45 g, 1.96 mmol, quant.) was obtained as a yellow oil.

$^1$H NMR (400 MHz, Chloroform-d): δ 5.83 – 5.60 (m, 4H, $2 \times NH_2$) 3.78 (t, $J = 5.02$ Hz, 4H, $2 \times CH_2-CH_2-NH_2$), 3.73 – 3.62 (m, 10H, $5 \times CH_2-O-CH_2$), 3.09 (t, $J = 4.9$ Hz, 4H, $2 \times CH_2-NH_2$).

The analytical data were in accordance with previously reported data.\(^\text{350}\)
**Experimental part**

$^1$H NMR spectrum for 83:

### Synthesis of biotinylated spacers

![Synthesis of biotinylated spacers](image)

Scheme 41: Synthesis of biotinylated spacer 85: EDCI, NHS, DMF, r.t., 16 h; ii) 47, Et$_3$N, DMF, r.t., 48 h; iii) 10% Pd / C, EtOAc, H$_2$, r.t., 3 h.

### 2,5-Dioxopyrrolidin-1-yl 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (84)

Biotin (2.0 g, 8.2 mmol, 1.0 eq), EDCI (2.4 g, 12.3 mmol, 1.5 eq) and NHS (1.9 g, 16.4 mmol, 2.0 eq) were dissolved in DMF (65 mL). The reaction mixture was stirred at r.t. for 16 h. The mixture was poured into a mixture of ice / H$_2$O (200 mL) and the precipitate was collected by filtration. The solid was co-evaporated with toluene (3 X 10 mL) to afford biotin-OSu 84 (2.4 g, 6.9 mmol, 85% yield) as a white solid.
**Experimental part**

\[^1^H\text{NMR}\ (400 \text{ MHz, DMSO-}d_6): \delta 6.41 \text{ (s, 1H, NH)}, 6.35 \text{ (s, 1H, NH)}, 4.30 \text{ (dd, } J = 7.7, 5.1 \text{ Hz, 1H, NH-CH-CH}_2), 4.19 - 4.10 \text{ (m, 1H, NH-CH-CH)}, 3.14 - 3.03 \text{ (m, 1H, CH-S)}, 2.85 \text{ (d, } J = 5.1 \text{ Hz, 1H, H-CH-S)}, 2.81 \text{ (s, 4H, CH}_2\text{OSuc)}, 2.67 \text{ (t, } J = 7.4 \text{ Hz, 2H, CH}_2\text{-CO}_2\text{R)}, 2.58 \text{ (d, } J = 12.3 \text{ Hz, 1H, H-CH-S)}, 1.65 \text{ (p, } J = 7.3 \text{ Hz, 2H, CH}_2\text{-CH}_2\text{-CO}_2\text{R)}.

The analytical data were in accordance with previously reported data.\(^{350}\)

\[^1^H\text{NMR spectrum for 84:}\]

Azido-tetraethylene glycol-biotin 58

A solution of 47 (0.28 g, 1.1 mmol, 1.1 eq) and Et\(_3\)N (0.35 mL, 2.5 mmol, 2.5 eq) in DMF (5 mL) was added to a solution of biotin-OSu 84 (0.34 g, 1.0 mmol, 1.0 eq) in DMF (5 mL). The reaction mixture was stirred at r.t. for 48 h. The solvent was removed under reduced pressure and the crude was extracted with EtOAc (20 mL) and washed with sat. NH\(_4\)Cl (20 mL). The aqueous layer was extracted with EtOAc (3 X 20 mL) and the combined organic layers dried over MgSO\(_4\), filtered and concentrated under reduced pressure. The crude was purified via column chromatography eluting with a mixture of DCM / MeOH (6:1). Azido-tetraethylene glycol-biotin 60 (0.34 g, 0.77 mmol, 70% yield) was obtained as a yellow oil.

\[^1^H\text{NMR (400 MHz, Chloroform-d): } \delta 6.58 \text{ (t, } J = 5.6 \text{ Hz, 1H, NH} \text{amide}), 6.06 \text{ (s, 1H, NH)}, 5.17 \text{ (s, 1H, NH), 4.51 (dd, } J = 7.9, 4.9 \text{ Hz, 1H, NH-CH-CH}_2), 4.32 \text{ (dd, } J = 8.0, 4.7 \text{ Hz, 1H, NH-CH-CH}), 3.70 - 3.60 \text{ (m, 11H, CH}_2\text{-NHCOR and 4 X CH}_2\text{-O-CH}_2), 3.56 \text{ (t, } J = 5.0 \text{ Hz, 2H, CH}_2\text{-CH}_2\text{-N}_3), 3.48 - 3.42 \text{ (m, 2H, RO-CH}_2\text{-CH}_2\text{-NHCOR), 3.40 (t, } J =
5.0 Hz, 2H, $\text{CH}_2\text{-N}_3$), 3.15 (td, $J = 7.2$, 4.5 Hz, 1H, $\text{CH}$-$\text{S}$), 2.91 (dd, $J = 13.0$, 5.2 Hz, 1H, $\text{H-CH-S}$), 2.80 – 2.68 (m, 1H, $\text{H-CH-S}$), 2.23 (td, $J = 7.2$, 2.5 Hz, 2H, $\text{CH}_2\text{-CONHR}$), 1.71 (d, $J = 13.7$ Hz, 4H, $\text{CH}_2\text{(CH}_2)_2\text{-CONHR}$ and $\text{CH}_2\text{(CH}_2)_2\text{-CONHR}$), 1.45 (p, $J = 7.6$ Hz, 2H, $\text{CH}_2\text{-CH}_2\text{-CONHR}$).

The analytical data were in accordance with previously reported data.\textsuperscript{350}

$^1\text{H NMR}$ spectrum for 58:

Azido-tetraethylene glycol-biotin 85

58 (0.45 g, 1.0 mmol, 1.0 eq) was dissolved in EtOAc (4 mL) and 10% Pd / C (0.1 mol%) was added. The reaction mixture was stirred at r.t. for 3 h under H$_2$ atmosphere. The mixture was filtered over a pad of Celite\textsuperscript{®} and the solvent was removed under reduced pressure. 85 (0.25 g, 0.59 mmol, 61% yield) was obtained as a yellow oil.

$^1\text{H NMR}$ (400 MHz, Chloroform-$d$): $\delta$ 7.83 (s, 1H, $\text{NH}_{\text{amide}}$), 6.99 (s, 1H, NH), 5.23 (s, 1H NH), 4.50 (q, $J = 7.0$ Hz, 1H, $\text{NH-CH-CH}_2$), 4.43 – 4.28 (m, 1H, $\text{NH-CH-CH}_2$), 3.82 – 3.53 (m, 1H, $\text{RO-CH}_2\text{-CH}_2\text{-NHCOR}$, $\text{CH}_2\text{-CH}_2\text{-NH}_2$ and 4 X $\text{CH}_2\text{-O-CH}_2$), 3.46 – 3.39 (m, 1H, $\text{CH}_2\text{-NH}_2$), 3.19 – 3.05 (m, 1H, $\text{CH}$-$\text{S}$), 2.92 (dt, $J = 12.7$, 4.4 Hz, 1H, $\text{H-CH}$-$\text{S}$), 2.73 (dd, $J = 12.8$, 7.9 Hz, 1H, $\text{H-CH-S}$), 2.61 (s, 2H, NH$_2$), 2.23 (q, $J = 7.3$ Hz, 1H, $\text{CH}_2\text{-CONHR}$), 1.86 - 1.60 (m, 4H, $\text{CH}_2\text{(CH}_2)_2\text{-CONHR}$ and $\text{CH}_2\text{(CH}_2)_2\text{-CONHR}$), 1.46 (q, $J = 8.7$, 7.8 Hz, 2H, $\text{CH}_2\text{-CH}_2\text{-CONHR}$).

The analytical data were in accordance with previously reported data.\textsuperscript{350}
**Synthesis of amino functionalized cyclooctyne**

2-Phenylacetaldehyde (9.7 mL, 83.2 mmol, 1.0 eq) was dissolved in dry DCM (45 mL) and the solution was cooled down to 0°C. Trimethylsilyl iodide (12.1 mL, 84.9 mmol, 1.02 eq) was added dropwise and the reaction mixture was stirred at 5°C for 7 days under argon atmosphere. A sat. aqueous solution of Na₂S₂O₃ (30 mL) and DCM (20 mL) were added and the reaction mixture was stirred until the iodine color vanished. The aqueous layer was extracted with DCM (2 X 25 mL) and the combined organic layers were dried over

**Scheme 42: Synthesis of DIBO derivatives 48 and 63:**

i) Me₃Si, DCM, 5°C, 5 days; ii) BuLi, THF, r.t., 4 h; iii) Br₂, CHCl₃, r.t., 2 h; iv) LDA, THF, r.t., 1 h; v) 4-nitrophenyl chloroformate, pyridine, DCM, r.t., 16 h; vi) ethylenediamine, Et₃N, DCM, r.t., 2 h.

5,6,11,12-Tetrahydro-5,11-epoxydibenzo[α,e][8]annulene (86)
MgSO₄, filtered and concentrated under reduced pressure. The crude mixture was purified by column chromatography eluting with a mixture of PE / EtOAc (50:1 to 1:1) to afford 86 (3.6 g, 16.2 mmol, 40% yield) as a light brown solid.

³¹H NMR (400 MHz, Chloroform-d): δ 7.17 – 7.04 (m, 6H, 6 X Ar-H), 6.98 (d, J = 7.6 Hz, 2H, 2 X Ar-H), 5.30 (d, J = 6.0 Hz, 2H, 2 X CH), 3.56 (dd, J = 16.2, 6.1 Hz, 2H, 2 X HC-H), 2.78 (d, J = 16.2 Hz, 2H, 2 X HC-H).

The analytical data were in accordance with previously reported data.³⁵⁶

³¹H NMR spectrum for 86:

(Z)-5,6-Dihydrodibenzo[a,e][8]annulen-5-ol (87)

A 2.1 M solution of n-BuLi (14.9 mL, 32.4 mmol, 2.0 eq.) in pentane was added dropwise to a solution of 5,6,11,12-tetrahydro-5,11-epoxydibenzo[a,e][8]annulene (86) (3.6 g, 16.2 mmol, 1.0 eq) in dry THF (180 mL). The reaction mixture was stirred at r.t. for 4 h under argon atmosphere. The mixture was quenched by addition of H₂O (15 mL) and THF was removed under reduced pressure. The aqueous layer was extracted with DCM (3 X 25 mL) and the combined organic layers were washed with brine (1 X 40 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. The crude mixture was purified by column chromatography eluting with a mixture of PE / EtOAc (5:1). 87 (3.0 g, 13.5 mmol, 83% yield) was obtained as a white solid.
Experimental part

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 7.49 (d, $J = 7.0$ Hz, 1H, Ar-H), 7.28 (d, $J = 4.1$ Hz, 1H, Ar-H), 7.25 – 7.10 (m, 6H, 6 X Ar-H), 6.93 – 6.79 (m, 2H, RHC=CHR), 5.31 (dt, $J = 10.3$, 5.1 Hz, 1H, CH), 3.49 (dd, $J = 13.8$, 6.1 Hz, 1H, HC-H), 3.36 (dd, $J = 13.8$, 10.0 Hz, 1H, HC-H), 1.88 (d, $J = 4.6$ Hz, 1H, OH).

The analytical data were in accordance with previously reported data.\(^{357}\)

$^1$H NMR spectrum for 87:

![NMR Spectrum for 87](image)

11,12-Dibromo-5,6,11,12-tetrahydrodibenzo[a,e][8]annulen-5-ol (88)

Bromine was added dropwise (0.23 mL, 4.5 mmol, 1.0 eq) to a solution of (Z)-5,6-dihydrodibenzo[a,e][8]annulen-5-ol (87) (1.00 g, 4.5 mmol, 1.0 eq) in CHCl$_3$ (13.5 mL). The reaction mixture was stirred at r.t. for 2 h. The mixture was quenched by addition of sat. solution of Na$_2$S$_2$O$_3$ in H$_2$O (30 mL). The aqueous layer was extracted with CHCl$_3$ (3 X 30 mL) and the combined organic layers were washed with brine (1 X 30 mL), dried over MgSO$_4$, filtered and concentrated under reduced pressure. The crude mixture was purified by column chromatography eluting with a mixture of PE / DCM (2:1). 88 (0.74 g, 2.0 mmol, 45% yield) was obtained as a yellow oil.

$^1$H NMR (400 MHz, Chloroform-$d$, major diastereoisomer): $\delta$ 7.60 (dd, $J = 13.2$, 7.2 Hz, 1H, Ar-H), 7.39 (d, $J = 7.2$ Hz, 1H, Ar-H), 7.24 – 6.99 (m, 5H, 5 X Ar-H), 6.87 (d, $J = 7.4$ Hz, 1H, Ar-H), 5.82 (s, 1H, R$_2$BrC-H), 5.46 (d, $J$
Experimental part

\[ \text{3.1 Hz, 1H, R}_2\text{BrC-H}, 5.29 (d, J = 6.3 \text{ Hz}, 1H, CH), 3.59 (dd, J = 16.7, 6.7 \text{ Hz}, 1H, HC-H), 2.85 (d, J = 16.4 \text{ Hz}, 1H, HC-H). \]

The analytical data were in accordance with previously reported data.\(^{358}\)

\(^1\)H NMR spectrum for 88:

![NMR spectrum](image)

DIBO 89

Freshly prepared 0.8 M solution of LDA (10.2 mL, 8.1 mmol, 5.8 eq) in dry THF was added dropwise at 0°C to a solution of 11,12-dibromo-5,6,11,12-tetrahydrodibenzo[α,e][8]annulen-5-ol (88) (0.52 g, 1.4 mmol, 1.0 eq) in dry THF (17 mL). The reaction mixture was allowed to warm up to r.t. and was stirred for 1 h under argon atmosphere. The mixture was quenched by addition of \(\text{H}_2\text{O} \) (10 mL) and THF was evaporated under reduced pressure. The aqueous layer was extracted with DCM (3 X 10 mL) and the combined organic layers were washed with \(\text{H}_2\text{O} \) (1 X 10 mL), brine (1 X 10 mL), dried over MgSO\(_4\), filtered and concentrated under reduced pressure. The crude mixture was purified by column chromatography eluting with a mixture of PE / DCM (1:3) 89 (0.23 g, 1.1 mmol, 75% yield) was obtained as a white solid.

\(^1\)H NMR (400 MHz, Chloroform-\(d\)): \(\delta\) 7.75 (d, \(J = 7.8 \text{ Hz}, 1\text{H}, \text{Ar-H})\), 7.48 – 7.27 (m, 7\text{H}, 7 \times \text{Ar-H}), 4.64 (d, \(J = 5.8 \text{ Hz}, 1\text{H}, \text{CH})\), 3.11 (dd, \(J = 14.7, 2.2 \text{ Hz}, 1\text{H}, \text{HC-H})\), 2.94 (dd, \(J = 14.7, 3.8 \text{ Hz}, 1\text{H}, \text{HC-H})\), 2.19 (s, 1\text{H}, \text{OH}).
The analytical data were in accordance with previously reported data.\textsuperscript{358}

\textsuperscript{1}H NMR spectrum for 89:

![NMR spectrum image]

11,12-Didehydro-5,6-dihydrodibenzo[a,e][8]annulen-5-yl 4-nitrophenyl carbonate (48)

DIBO 89 (0.50 g, 2.3 mmol, 1.0 eq) and 4-nitrophenyl chloroformate (0.92 g, 4.6 mmol, 2.0 eq) were dissolved in DCM (70 mL). Pyridine was added (0.9 mL, 11.4 mmol, 5.0 eq) was added and the reaction mixture was stirred at r.t. for 16 h. The mixture was washed with brine (2 X 10 mL) and the organic layer was dried over MgSO\textsubscript{4}, filtered and concentrated under reduced pressure. The crude mixture was purified by column chromatography eluting with a mixture of PE / EtOAc (5:1). 48 (0.83 g, 2.1 mmol, 94\% yield) was obtained as a white solid.

\textsuperscript{1}H NMR (400 MHz, Chloroform-\textit{d}): \textit{d} 8.28 (d, \textit{J} = 9.1 Hz, 2H, 2 X Ar-H), 7.62 (dq, \textit{J} = 7.8, 1.0 Hz, 1H, DIBO-H), 7.47 – 7.29 (m, 9H, 7 X DIBO-H and 2 X Ar-H), 5.59 (t, \textit{J} = 3.0 Hz, 1H, CH\textsubscript{DIBO}), 3.34 (dd, \textit{J} = 15.4, 2.2 Hz, 1H, H-CH\textsubscript{DIBO}), 3.05 (dd, \textit{J} = 15.3, 4.0 Hz, 1H, H-CH\textsubscript{DIBO}).

The analytical data were in accordance with previously reported data.\textsuperscript{358}
**Experimental part**

1H NMR spectrum for 48:

A solution of 48 (0.40 g, 1.04 mmol, 1.0 eq) in dry DCM (5 mL) was added dropwise to a solution of ethylenediamine (0.35 mL, 5.19 mmol, 5.0 eq) and Et3N (0.43 mL, 3.11 mmol, 3.0 eq) in dry DCM (25 mL). The reaction mixture was stirred at r.t. for 2 h under argon atmosphere and the solvent was removed under reduced pressure. The crude mixture was purified by column chromatography eluting with a mixture of DCM / MeOH (5:1). 63 (0.29 g, 0.96 mmol, 92% yield) was obtained as a slightly yellow oil.

DIBO-NH₂ 63

A solution of 48 (0.40 g, 1.04 mmol, 1.0 eq) in dry DCM (5 mL) was added dropwise to a solution of ethylenediamine (0.35 mL, 5.19 mmol, 5.0 eq) and Et3N (0.43 mL, 3.11 mmol, 3.0 eq) in dry DCM (25 mL). The reaction mixture was stirred at r.t. for 2 h under argon atmosphere and the solvent was removed under reduced pressure. The crude mixture was purified by column chromatography eluting with a mixture of DCM / MeOH (5:1). 63 (0.29 g, 0.96 mmol, 92% yield) was obtained as a slightly yellow oil.

1H NMR (400 MHz, Chloroform-d): δ 7.51 (d, J = 7.7 Hz, 1H, Ar-H), 7.41 – 7.24 (m, 7H, 7 × Ar-H), 5.50 (d, J = 3.3 Hz, 1H, CH), 5.38 (s, 1H, NH), 3.26 (q, J = 6.0 Hz, 2H, RNH-CH₂-CH₂-NH₂), 3.17 (dd, J = 15.1, 2.2 Hz, 1H, HC-H), 2.99 – 2.90 (m, 1H, HC-H), 2.90 – 2.83 (m, 2H, CH₂-NH₂).

The analytical data were in accordance with previously reported data.³⁵⁹
1H NMR spectrum for 63:

4.3.2 Synthesis of phototrigger-Trp conjugates

Synthesis of o-nitrobenzyl-Trp linkers

Scheme 43: Synthesis of o-nitrobenzyl-trp linker 38: i) 4-nitrophenyl chloroformate, Et3N, DCM, r.t., 16 h, dark conditions; Then, Trp-OtBu, Et3N, DCM, r.t., 24 h, dark conditions; vi) LiOH, MeOH / H2O (5 : 1), r.t., 16 h, dark conditions.
**Experimental part**

**tert-Butyl (((4-(2-(tert-butoxy)-2-oxoethoxy)-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (35)**

**tert-Butyl 2-(4-(hydroxymethyl)-3-nitrobenzoxy)acetate (7) (50 mg, 0.18 mmol, 1.0 eq) and 4-nitrophenyl chloroformate (54 mg, 0.27 mmol, 1.5 eq.) were dissolved in dry DCM (2.5 mL). Et$_3$N (49 µL, 0.35 mmol, 2.0 eq.) was added and the reaction mixture was stirred at r.t. for 16 h under argon atmosphere and dark conditions. Then, a solution of tert-butyl L-tryptophanate (58 mg, 0.19 mmol, 1.1 eq), Et$_3$N (49 µL, 0.35 mmol, 2.0 eq.) in dry DCM (1 mL) were added and the reaction mixture was stirred for 24 h under argon atmosphere. The mixture was diluted with DCM (40 mL) and washed with H$_2$O (3 X 50 mL). The organic layer was dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was purified via column chromatography eluting with a mixture of EtOAc / PE (3:7 to 7:3). **tert-Butyl (((4-(2-(tert-butoxy)-2-oxoethoxy)-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (35) (72 mg, 0.13 mmol, 72% yield)** was obtained as a yellow foam.

**1H NMR** (400 MHz, Chloroform-d): δ 8.11 (s, 1H, N$_{\text{H indole}}$), 7.61 – 7.54 (m, 2H, indole- H and Ar- H$_{\text{nitrobenzyl}}$), 7.40 (d, J = 8.7 Hz, 1H, Ar- H$_{\text{nitrobenzyl}}$), 7.36 (d, J = 8.1 Hz, 1H, indole- H), 7.22 – 7.17 (m, 1H, indole- H), 7.16 – 7.09 (m, 2H, indole- H and Ar- H$_{\text{nitrobenzyl}}$), 7.00 (d, J = 2.2 Hz, 1H, indole- H), 5.49 – 5.35 (m, 3H, CH$_2$ nitrobenzyl and NH amide), 4.65 – 4.59 (m, 1H, CH$_2$Tryp), 4.58 (s, 2H, RO-CH$_2$-CO$_2$-tBu), 3.32 (dd, J = 14.8, 5.6 Hz, 1H, CH$_2$Tryp), 3.23 (dd, J = 14.9, 6.1 Hz, 1H, CH$_2$Tryp), 1.50 (s, 9H, 3 X CH$_3$ tert-buty ester), 1.40 (s, 9H, 3 X CH$_3$ tert-buty Trp).

**13C NMR** (101 MHz, Chloroform-d): δ 171.1 (C O), 167.2 (CO), 157.7 (C Ar), 155.4 (CO), 148.0 (C Ar), 136.2 (C indole), 130.5 (CH Ar), 127.9 (C indole), 126.1 (C Ar), 122.8 (CH indole), 122.3 (CH indole), 120.8 (CH Ar), 119.7 (C indole), 111.3 (C indole), 111.3 (C indole), 110.4 (C Ar), 110.3 (C indole), 83.3 (Cq), 82.4 (Cq), 66.0 (CH$_2$), 63.2 (CH$_2$), 55.1 (CH), 28.2 (3 X CH$_3$), 28.1 (CH$_2$ and 3 X CH$_3$).

**IR** ($\nu_{\text{max}}$, cm$^{-1}$): 3730 (w), 3396 (w), 2979 (w), 1721 (s), 1533 (s), 1345 (w), 1228 (m), 1156 (s), 1082 (w), 1055 (w), 743 (w).

**HRMS (ESI) m/z**: calcd for C$_{29}$H$_{35}$N$_3$NaO$_9$" [M+Na]$^+$ 592.2266; found 592.2269.
$^1$H NMR and $^{13}$C spectra for 35:
Experimental part

(S)-2-((1-((tert-Butoxy)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)carbamoyl)oxy)methyl)-3-nitrophenoxy)acetic acid (38)

Methyl 2-(4-(hydroxymethyl)-3-nitrophenoxy)acetate (8) (0.55 g, 2.3 mmol, 1.0 eq) and 4-nitrophenyl chloroformate (0.55 g, 2.75 mmol, 1.2 eq.) were dissolved in dry DCM (10 mL). Et$_3$N (0.64 mL, 4.56 mmol, 2.0 eq.) was added and the reaction mixture was stirred at r.t. for 16 h under argon atmosphere and dark conditions. Then, a solution of tert-butyl L-tryptophanate (0.75 g, 2.51 mmol, 1.1 eq) and Et$_3$N (0.64 mL, 4.56 mmol, 2.0 eq.) in dry DCM (1 mL) were added and the reaction mixture was stirred for 24 h under argon atmosphere. The mixture was diluted with DCM (100 mL) and washed with H$_2$O (3 X 100 mL). The organic layer was dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was purified via column chromatography eluting with a mixture of EtOAc / PE (3:7 to 7:3). tert-Butyl (((4-(2-ethoxy-2-oxoethoxy)-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (0.33 g) (36) was obtained as an impure mixture.

The crude tert-butyl (((4-(2-ethoxy-2-oxoethoxy)-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (0.33 g) was dissolved in a mixture of LiOH (0.27 g, 11.4 mmol, 5.0 eq) in MeOH / H$_2$O (5:1) and the reaction mixture was stirred for 16 h under dark conditions. The solvent was removed under reduced pressure and the mixture was diluted with DCM (30 mL) and 1M sulfuric acid (5 mL). The aqueous layer was extracted with EtOAc (3 X 50 mL) and the combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was purified via column chromatography eluting with a mixture of DCM / MeOH (8:2). (S)-2-((1-((tert-Butoxy)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)carbamoyl)oxy)methyl)-3-nitrophenoxy)acetic acid (38) (0.29 g, 0.56 mmol, 25% yield over 3 steps) was obtained as a brown solid.

$^1$H NMR (400 MHz, DMSO-d$_6$): δ 11.00 (d, J = 2.5 Hz, 1H, NH$_{indole}$), 7.78 (d, J = 8.1 Hz, 1H, NH$_{amide}$), 7.50 (d, J = 7.8 Hz, 1H, indole-H), 7.49 (d, J = 2.6 Hz, 1H, Ar-H$_{nitrobenzyl}$), 7.41 (d, J = 8.7 Hz, 1H, Ar-H$_{nitrobenzyl}$), 7.36 (d, J = 8.1 Hz, 1H, indole-H), 7.17 (dd, J = 8.6, 2.7 Hz, 1H, indole-H), 7.09 – 7.03 (m, 2H, indole-H and Ar-H$_{nitrobenzyl}$), 6.98 (t, J = 7.4 Hz, 1H, indole-H), 5.20 (s, 2H, CH$_2$$_{nitrobenzyl}$), 4.30 (s, 2H, RO-CH$_2$-CO$_2$H), 4.17 (ddd, J = 9.4, 8.0, 5.3 Hz, 1H, CH$_{Trp}$), 3.11 (dd, J = 14.6, 5.3 Hz, 1H, CH$_2$$_{Trp}$), 2.97 (dd, J = 14.5, 9.5 Hz, 1H, CH$_2$$_{Trp}$), 1.32 (s, 9H, 3 X CH$_3$ tert-butyl).

$^{13}$C NMR (101 MHz, DMSO-d$_6$): δ 171.2 (CO), 169.8 (CO), 158.9 (C$_{Ar}$), 155.6 (CO), 147.8 (C$_{Ar}$), 136.1 (C$_{indole}$), 130.2 (CH$_{Ar}$), 127.1 (C$_{indole}$), 123.7 (C$_{Ar}$), 123.3 (CH$_{indole}$), 120.9 (CH$_{indole}$), 120.4 (CH$_{Ar}$), 118.3 (CH$_{indole}$), 118.0 (CH$_{indole}$), 111.5 (CH$_{Ar}$), 110.3 (CH$_{indole}$), 109.7 (C$_{indole}$), 81.0 (C$_{O}$), 69.8 (CH$_2$), 68.0 (CH$_2$), 61.9 (CH$_2$), 55.5 (CH), 28.5 (3 X CH$_3$), 27.6 (CH$_2$).

IR (ν$_{max}$, cm$^{-1}$): 3750 (w), 2967 (w), 1710 (m), 1622 (w), 1531 (s), 1428 (w), 1346 (m), 1231 (m), 1162 (m), 1054 (s), 821 (w), 743 (w).

HRMS (ESI) m/z: calcld for C$_{25}$H$_{27}$N$_3$NaO$_9$ [M+Na]$^+$ 536.1640; found 536.1640.
$^1$H NMR and $^{13}$C spectra for 38:
Experimental part

Synthesis of \(m\)-methoxy-o-nitrobenzyl-Trp linkers

![Scheme 44: Synthesis of MONB-Trp linker 42](image)

**Scheme 44:** Synthesis of MONB-Trp linker 42: i) 4-nitrophenyl chloroformate, DIPEA, DCM, r.t., 16 h, dark conditions; Then, Trp-OtBu, Et3N, DCM, r.t, 24 h, dark conditions; ii) LiOH, MeOH / H2O (5:1) r.t., 4 h, dark conditions.

**tert-Butyl (((4-(2-(tert-butoxy)-2-oxoethoxy)-5-methoxy-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (39)**

To a solution of tert-butyl 2-(4-(hydroxymethyl)-2-methoxy-5-nitrophenox)acetate (18) (11 mg, 35.1 \(\mu\)mol, 1.0 eq) and 4-nitrophenyl chloroformate (10 mg, 52.7 \(\mu\)mol, 1.5 eq.) in dry DCM (1 mL) was added DIPEA (22 \(\mu\)L, 70.2 mmol, 2.0 eq). The reaction mixture was stirred at r.t. for 16 h under argon atmosphere and dark conditions. Then, a solution of tert-butyl L-tryptophanate (10 mg, 38.6 mmol, 1.1 eq) and Et3N (18 \(\mu\)L, 140 mmol, 4.0 eq) in dry DCM (1 mL) was added and the reaction mixture was stirred at r.t. for 24 h under argon atmosphere. The solvent was removed under reduced pressure and the crude was purified by column chromatography eluting with a mixture of PE / EtOAc (8:2 to 7:3). tert-Butyl (((4-(2-(tert-butoxy)-2-oxoethoxy)-5-methoxy-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (39) (22 mg, 35.1 \(\mu\)mol, quant. yield over 2 steps) was obtained as a yellow foam.

**\(^1\)H NMR** (400 MHz, Chloroform-d): \(\delta\) 8.17 (s, 1H, NH indole), 7.61 (s, 1H, Ar-H nitrobenzyl), 7.58 (d, \(J = 8.0\) Hz, 1H, indole-H), 7.37 (dd, \(J = 16.4, 8.4\) Hz, 1H indole-H), 7.17 (ddd, \(J = 8.2, 7.0, 1.2\) Hz, 1H, indole-H), 7.09 (ddd, \(J = 8.0, 7.0, 1.0\) Hz, 1H, indole-H), 7.00 (dd, \(J = 2.3\) Hz, 1H, indole-H), 6.92 (s, 1H, Ar-H nitrobenzyl), 5.49 – 5.35 (m, 3H, \(CH_2\) nitrobenzyl and NH amide), 4.61 (dd, \(J = 8.4, 5.8\) Hz, 1H, \(CH_2\) Trp), 4.58 (s, 2H, RO-\(CH_2\)-CO\(_2\)tBu), 3.79 (s, 3H, OMe), 3.32 (dd, \(J = 14.8, 5.5\) Hz, 1H, \(CH_2\) Trp), 3.23 (dd, \(J = 14.9, 6.1\) Hz, 1H, \(CH_2\) Trp), 1.50 (s, 9H, 3 \(CH_3\) tert-butyl ester), 1.40 (s, 9H, 3 \(CH_3\) tert-butyl Trp).

**\(^{13}\)C NMR** (101 MHz, Chloroform-d): \(\delta\) 171.3 (CO), 167.2 (CO), 155.5 (CO), 154.0 (C\(_A\)), 146.0 (C\(_A\)), 139.0 (C\(_A\)), 136.1 (C\(_A\)), 129.5 (C\(_A\)), 127.7 (C\(_A\)), 122.8 (CH\(_A\) indole), 122.3 (CH\(_A\) indole), 119.7 (CH\(_A\) indole), 118.8 (CH\(_A\) indole), 111.4 (CH\(_A\) indole), 110.2 (CH\(_A\)), 110.1 (C\(_A\) indole), 109.8 (CH\(_A\)), 83.4 (C\(_A\)), 82.5 (C\(_A\)), 66.2 (CH\(_A\)), 63.7 (CH\(_A\)), 56.5 (CH\(_A\)), 55.0 (CH\(_A\)), 28.1 (3 \(CH_3\) tert-butyl ester), 28.1 (3 \(CH_3\) tert-butyl Trp), 28.0 (CH\(_A\)).
**Experimental part**

**IR ($\nu_{\text{max}}$ cm$^{-1}$):** 3397 (w), 2981 (w), 2943 (w), 1731 (s), 1587 (w), 1520 (s), 1335 (m), 1285 (m), 1156 (s), 1084 (m), 848 (w), 747 (w).

**HRMS (ESI) m/z:** calcd for C$_{30}$H$_{37}$N$_3$NaO$_{10}$ $^+$ [M+Na]$^+$ 622.2371; found 622.2376.

$^1$H NMR and $^{13}$C spectra for 39:
Experimental part

tert-Butyl (((5-methoxy-4-(2-methoxy-2-oxoethoxy)-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (40)

To a solution of methyl 2-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxy)acetate (19) (0.53 g, 1.9 mmol, 1.0 eq) and 4-nitrophenyl chloroformate (0.59 g, 2.9 mmol, 1.5 eq.) in dry DCM (5 mL) was added DIPEA (1.4 mL, 7.84 mmol, 4.0 eq). The reaction mixture was stirred at r.t. for 16 h under argon atmosphere and dark conditions. A solution of tert-butyl L-tryptophanate (0.64 g, 2.16 mmol, 1.1 eq) and Et3N (1.1 mL, 7.8 mmol, 4.0 eq) in dry DCM (5 mL) was added and the reaction mixture was stirred at r.t. for 24 h under argon atmosphere. The solvent was removed under reduced pressure and the crude was purified by column chromatography eluting with a mixture of PE / EtOAc (8:2 to 7:3). tert-Butyl (((5-methoxy-4-(2-methoxy-2-oxoethoxy)-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (40) (0.36 g, 0.64 mmol, 33% yield over 2 steps) was obtained as a yellow foam.

^1H NMR (400 MHz, Chloroform-d): δ 8.14 (s, 1H, NH indole), 7.64 (s, 1H, Ar-H nitrobenzyl), 7.59 (d, J = 8.0 Hz, 1H, indole-H), 7.36 (d, J = 8.1 Hz, 1H, indole-H), 7.22 – 7.15 (m, 1H, indole-H), 7.10 (td, J = 7.4, 6.9, 1.0 Hz, 1H, indole-H), 7.01 (d, J = 2.2 Hz, 1H, indole-H), 6.95 (s, 1H, Ar-H nitrobenzyl), 5.50 (d, J = 3.2 Hz, 2H, CH_2 nitrobenzyl), 5.41 (d, J = 8.5 Hz, 1H, NH amide), 4.76 (s, 2H, RO-CH_2-CO_2Me), 4.64 (dt, J = 8.3, 5.8 Hz, 1H, CH_Tp), 3.82 (s, 6H, CH_3
ester and O-CH₃), 3.34 (dd, \( J = 15.0 \), 5.4 Hz, 1H, CH₂Trp), 3.23 (dd, \( J = 15.0 \), 6.3 Hz, 1H, CH₂Trp), 1.42 (s, 9H, 3 X CH₃ tert-butyl).

\(^{13}\text{C} \text{NMR} \) (101 MHz, Chloroform-\( d_2 \)): 171.3 (CO), 168.5 (CO), 155.4 (CO), 154.3 (C Ar), 146.0 (C Ar), 139.2 (C Ar), 136.2 (C indole), 130.1 (C Ar), 127.9 (C indole), 122.7 (CH indole), 122.4 (CH indole), 119.8 (CH indole), 118.9 (CH indole), 111.3 (CH indole), 110.5 (CH Ar), 110.4 (C indole), 110.4 (CH Ar), 82.4 (Cq), 66.1 (CH₂), 63.7 (CH₂), 56.6 (CH₂), 55.1 (CH), 52.7 (CH₃), 28.1 (3 X CH₃), 28.0 (CH₂).

\( \text{IR (} \nu_{\text{max}}, \text{ cm}^{-1} \): 2956 (w), 2919 (w), 2851 (w), 1731 (w), 1523 (w), 1267 (w), 1200 (w), 1086 (w), 738 (s), 706 (w).

\( \text{HRMS (ESI) m/z: calcd for C}_{27}\text{H}_{31}\text{N}_{3}\text{NaO}_{10}^{+} [M+Na]^+ 586.1904; found 586.1909.}

\(^{1}\text{H} \text{NMR and } ^{13}\text{C} \text{ spectra for 40:}

![NMR and C spectra for 40]
Experimental part

**tert-Butyl (((4-(2-ethoxy-2-oxoethoxy)-5-methoxy-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (41)**

To a solution of ethyl 2-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxy)acetate (20) (0.55 g, 1.9 mmol, 1.0 eq) and 4-nitrophenyl chloroformate (0.57 g, 2.8 mmol, 1.5 eq.) in dry DCM (5 mL) was added DIPEA (1.3 mL, 7.8 mmol, 4.0 eq). The reaction mixture was stirred at r.t. for 16 h under argon atmosphere and dark conditions. Then, a solution of tert-butyl L-tryptophanate (0.61 g, 2.1 mmol, 1.1 eq) and Et$_3$N (1.1 mL, 7.5 mmol, 4.0 eq) in dry DCM (5 mL) was added and the reaction mixture was stirred at r.t. for 24 h under argon atmosphere. The solvent was removed under reduced pressure and the crude was purified by column chromatography eluting with a mixture of PE / EtOAc (8:2 to 7:3). **tert-Butyl (((4-(2-ethoxy-2-oxoethoxy)-5-methoxy-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (41)** (0.59 g, 1.0 mmol, 55% yield over 2 steps) was obtained as a yellow foam.

$^1$H NMR (400 MHz, Chloroform-d): $\delta$ 8.14 (s, 1H, NH$_{\text{indole}}$), 7.65 (s, 1H, Ar$_{-}$H$_{\text{nitrobenzyl}}$), 7.59 (d, $J = 8.0$ Hz, 1H, indole-H$_3$), 7.36 (d, $J = 8.1$ Hz, 1H, indole-H$_4$), 7.19 (ddd, $J = 8.2$, 7.0, 1.2 Hz, 1H, indole-H$_5$), 7.13 – 7.07 (m, 1H, indole-H$_6$), 7.01 (d, $J = 2.3$ Hz, 1H, indole-H$_7$), 6.94 (s, 1H, Ar$_{-}$H$_{\text{nitrobenzyl}}$), 5.50 (d, $J = 2.2$ Hz, 2H, CH$_2$$_{\text{nitrobenzyl}}$), 5.40 (d, $J = 8.5$ Hz, 1H, NH$_{\text{amide}}$), 4.74 (s, 2H, RO-CH$_2$-CO$_2$Me), 4.64 (dt, $J = 8.4$, 5.9 Hz, 1H, CH$_2$$_{\text{Trp}}$), 4.29 (qd, $J = 7.2$, 1H, RO-CH$_2$-CO$_2$Et).
Experimental part

1.5 Hz, 2H, \( CH_2 \text{ester} \), 3.81 (s, 3H, O-CH\(_3\)), 3.34 (dd, \( J = 15.0, 5.4 \) Hz, 1H, \( CH_2 \text{Trp} \)), 3.25 (dd, \( J = 15.0, 5.4 \) Hz, 1H, \( CH_2 \text{Trp} \)), 1.41 (s, 9H, \( CH_3 \text{tert-butyl} \)), 1.31 (td, \( J = 7.1, 1.6 \) Hz, 3H, \( CH_3 \text{ester} \)).

\(^{13}\text{C NMR} \) (101 MHz, Chloroform-\( d \)): \( \delta \) 171.2 (CO), 168.1 (CO), 155.4 (CO), 154.3 (C\( \text{Ar} \)), 146.1 (C\( \text{Ar} \)), 139.2 (C\( \text{Ar} \)), 136.2 (C\( \text{indole} \)), 130.0 (C\( \text{Ar} \)), 127.9 (C\( \text{indole} \)), 122.7 (CH\( \text{indole} \)), 122.4 (CH\( \text{indole} \)), 119.8 (CH\( \text{indole} \)), 118.9 (CH\( \text{indole} \)), 111.3 (CH\( \text{indole} \)), 110.5 (CH\( \text{Ar} \)), 110.5 (C\( \text{indole} \)), 110.4 (C\( \text{Ar} \)), 82.4 (C\( q \)), 66.3 (CH\( 2 \)), 63.7 (CH\( 2 \)), 61.9 (CH\( 2 \)), 56.6 (CH\( 3 \)), 55.1 (CH), 28.1 (3 X CH\( 3 \)), 28.1 (CH\( 2 \)), 14.3 (CH\( 3 \)).

IR (\( \nu_{\text{max}}, \text{cm}^{-1} \)):
3730 (w), 2958 (w), 2918 (w), 2851 (w), 1733 (w), 1265 (w), 736 (s), 706 (m).

HRMS (ESI) m/z: calcd for \( C_{28}H_{33}N_3\text{NaO}_{10} \) \([M+\text{Na}]^+\) 594.2060; found 594.2065.

\(^1\text{H NMR and }^{13}\text{C spectra for 41}\):
**Experimental part**

(5)-2-{4-(((1-(tert-Butoxy)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)carbamoyl)oxy)methyl)-2-methoxy-5-nitrophenoxy)acetic acid (42)

General procedure:

tert-Butyl ((5-R-4-(2-methoxy-2-oxoethoxy)-2-nitrobenzyl)oxy)carbonyl-s-tryptophanate (69.9 µmol, 1.0 eq) was dissolved in a solution of LiOH (16.5 mg, 0.69 mmol, 10.0 eq) in a mixture of MeOH / H₂O (5:1, 6 mL) at 0°C. The reaction mixture was allowed to warm up to r.t. and the mixture was stirred for 4 h under dark conditions. The mixture was diluted with H₂O (100 mL) and acidified with 1M HCl (5 mL). The aqueous layer was extracted with EtOAc (3 X 150 mL) and the combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure and the crude was purified by column chromatography eluting with a mixture of DCM / MeOH (8:2) to afford (S)-2-{4-(((1-(tert-butoxy)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)carbamoyl)oxy)methyl}-2-methoxy-5-nitrophenoxy)acetic acid (42) as a brown solid.

Following the general procedure, using tert-butyl ((5-methoxy-4-(2-methoxy-2-oxoethoxy)-2-nitrobenzyl)oxy)carbonyl-L-tryptophanate (40) (39 mg, 69.9 µmol, 1.0 eq), (S)-2-{4-(((1-(tert-butoxy)-3-(1H-
Experimental part

indol-3-yl)-1-oxopropan-2-yl(carbamoyl)oxy)methyl)-2-methoxy-5-nitrophenoxy)acetic acid (42) (32 mg, 58.9 µmol, 84% yield) was obtained as a brown solid.

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 10.93 (d, \(J = 2.4\) Hz, 1H, \(\text{NH}_{\text{indole}}\)), 7.94 (d, \(J = 8.0\) Hz, 1H, \(\text{NH}_{\text{amide}}\)), 7.51 (m, 2H, indole-\(H\) and Ar-\(H_{\text{nitrobenzyl}}\)), 7.38 – 7.29 (m, 1H, indole-\(H\)), 7.13 (d, \(J = 2.4\) Hz, 1H, indole-\(H\)), 7.11 (s, 1H, Ar-\(H_{\text{nitrobenzyl}}\)), 7.06 (ddd, \(J = 8.1, 1.2\) Hz, 1H, indole-\(H\)), 6.98 (ddd, \(J = 8.0, 7.0, 1.1\) Hz, 1H, indole-\(H\)), 5.38 – 5.21 (m, 2H, CH\(_2_{\text{nitrobenzyl}}\)), 4.44 (s, 2H, RO-C\(_2\)H), 4.22 (ddd, \(J = 9.2, 7.9, 5.5\) Hz, 1H, CH\(_{\text{Trp}}\)), 3.82 (s, 3H, CH\(_3\) ester), 3.13 (dd, \(J = 14.7, 5.6\) Hz, 1H, CH\(_2_{\text{Trp}}\)), 3.00 (dd, \(J = 14.6, 9.3\) Hz, 1H, CH\(_2_{\text{Trp}}\)), 1.31 (s, 8H, 3 X C\(_3\)H\(_3\) tert-butyl).

\(^{13}\)C NMR (101 MHz, DMSO-\(d_6\)): \(\delta\) 171.4 (C\(_O\)), 169.9 (C\(_O\)), 155.6 (C\(_O\)), 153.4 (C\(_\text{Ar}\)), 147.0 (C\(_\text{Ar}\)), 138.9 (C\(_\text{Ar}\)), 136.2 (C\(_{\text{indole}}\)), 127.5 (C\(_\text{Ar}\)), 127.0 (C\(_{\text{indole}}\)), 123.7 (C\(_{\text{indole}}\)), 121.0 (C\(_{\text{indole}}\)), 118.4 (C\(_{\text{indole}}\)), 118.1 (C\(_{\text{indole}}\)), 111.5 (C\(_{\text{indole}}\)), 110.3 (C\(_\text{Ar}\)), 109.7 (C\(_{\text{indole}}\)), 109.2 (C\(_\text{Ar}\)), 80.6 (C\(_q\)), 67.3 (CH\(_2\)), 62.5 (CH\(_2\)), 56.1 (CH\(_3\)), 55.5 (CH), 27.6 (3 X CH\(_3\)), 27.1 (CH\(_2\)).

IR (\(\nu_{\text{max}}, \text{cm}^{-1}\)): 3670 (w), 2983 (s), 2899 (m), 1717 (w), 1521 (w), 1399 (w), 1245 (w), 1072 (s).

HRMS (ESI) m/z: calcd for C\(_{26}\)H\(_{30}\)N\(_3\)O\(_{10}\)\([\text{M+H}]^+\) 544.1926; found 544.1928.

\(^1\)H NMR and \(^{13}\)C spectra for 42:
Following the general procedure, using tert-butyl ((4-(2-ethoxy-2-oxoethoxy)-5-methoxy-2-nitrobenzyl)oxy)carbonyl)-L-tryptophanate (41) (40 mg, 69.9 µmol, 1.0 eq), (S)-2-(4-(((1-(tert-butoxy)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)carbamoyl)oxy)methyl)-2-methoxy-5-nitrophenoxy)acetic acid (42) (35 mg, 63.5 µmol, 91% yield) was obtained as a brown solid.

$^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 10.93 (d, $J = 2.4$ Hz, 1H, NH$_{indole}$), 7.94 (d, $J = 8.0$ Hz, 1H, NH$_{amide}$), 7.51 (m, 2H, indole-$H$ and Ar-$H_{nitrobenzyl}$), 7.38 – 7.29 (m, 1H), 7.13 (d, $J = 2.4$ Hz, 1H, indole-$H$), 7.11 (s, 1H, Ar-$H_{nitrobenzyl}$), 7.06 (ddd, $J = 8.1$, 6.9, 1.2 Hz, 1H, indole-$H$), 6.98 (ddd, $J = 8.0$, 7.0, 1.1 Hz, 1H, indole-$H$), 5.38 – 5.21 (m, 2H, CH$_2$$_{nitrobenzyl}$), 4.44 (s, 2H, RO-CH$_2$-CO$_2$H), 4.22 (ddd, $J = 9.2$, 7.9, 5.5 Hz, 1H, CH$_{Trp}$), 3.82 (s, 3H, CH$_2$$_{ester}$), 3.13 (dd, $J = 14.7$, 5.6 Hz, 1H, CH$_2$$_{Trp}$), 3.00 (dd, $J = 14.6$, 9.3 Hz, 1H, CH$_2$$_{Trp}$), 1.31 (s, 9H, 3 X CH$_3$ tert-butyl).
Experimental part

Synthesis of coumarinyl-Trp linkers

Scheme 45: Synthesis of coumarinyl-Trp linker 46: i) 4-nitrophenyl-chloroformate, DIPEA, DCM, r.t., 16 h, dark conditions; Trp-OTBu HCl, DIPEA, DCM, r.t., 24 h, dark conditions; ii) TFA, DCM, r.t., 6 h, dark conditions; iii) CuSO₄, sodium ascorbate, THF, H₂O, r.t., 24 h, dark conditions; iv) 48, DIPEA, DMF, r.t., 24 h, dark conditions; v) Trp, DIPEA, DMF, r.t., 24 h.

**tert-Butyl (((7-(ethyl(prop-2-yn-1-yl)amino)-2-oxo-2H-chromen-4-yl)methoxy)carbonyl)-L-tryptophanate (43)**

7-(Ethylamino)-4-(hydroxymethyl)-2H-chromen-2-one (27) (0.13 g, 0.51 mmol, 1.0 eq) and 4-nitrophenyl chloroformate (0.12 g, 0.62 mmol, 1.2 eq) were dissolved in dry DCM (10 mL). DIPEA (0.28 mL, 1.64 mmol, 4.0 eq) was added and the reaction mixture was stirred at r.t. for 16 h under argon atmosphere and dark conditions. Then, a solution of tert-butyl L-tryptophanate (0.17 g, 0.56 mmol, 1.1 eq) and DIPEA (0.17 mL, 1.02 mmol, 2.0 eq) in dry DCM (2 mL) was added to the reaction mixture and stirred at r.t. for 24 h under argon atmosphere. The solvent was removed under reduced pressure and the crude was purified by column chromatography eluting with a mixture of PE / EtOAc (7:3 to 3:7). **tert-Butyl (((7-(ethyl(prop-2-yn-1-yl)amino)-2-oxo-2H-chromen-4-yl)methoxy)carbonyl)-L-tryptophanate (43)** (0.19 g, 0.35 mmol, 69% yield) was obtained as a yellow foam.
Experimental part

$^1$H NMR (400 MHz, Chloroform-d): $\delta$ 8.35 (s, 1H, NH indole), 7.60 (d, $J = 8.0$ Hz, 1H, indole-H), 7.37 (d, $J = 8.1$ Hz, 1H, indole-H), 7.29 (d, $J = 8.6$ Hz, 1H, coumarin-H), 7.22 – 7.15 (m, 1H, indole-H), 7.12 (dt, $J = 7.8$, 1.4 Hz, 1H, indole-H), 7.05 (d, $J = 2.3$ Hz, 1H, indole-H), 6.68 (d, $J = 8.2$ Hz, 2H, 2 X coumarin-H), 6.06 (t, $J = 1.4$ Hz, 1H, coumarin-H), 5.45 (d, $J = 8.3$ Hz, 1H, NH amide), 5.19 (ddd, $J = 77.8$, 15.5, 1.4 Hz, 2H, CH$_2$coumarin), 4.68 – 4.58 (m, 1H, CH$_2$Trp), 4.07 (d, $J = 2.4$ Hz, 2H, R$_2$N-CH$_2$-C≡CH), 3.52 (q, $J = 7.1$ Hz, 2H, R$_2$N-CH$_2$-CH$_3$), 3.37 (dd, $J = 14.8$, 5.4 Hz, 1H, C$_2$H$_2$Trp), 2.24 (t, $J = 2.4$ Hz, 1H, R$_2$N-CH$_2$-C≡CH), 1.43 (s, 9H, 3 X CH$_3$ tert-butyl), 1.29 – 1.23 (m, 3H, R$_2$N-CH$_2$-CH$_3$).

$^{13}$C NMR (101 MHz, Chloroform-d): $\delta$ 171.0 (CO), 162.0 (C Ar-coumarin), 155.9 (C Ar-coumarin), 154.9 (CO), 150.5 (C Ar-coumarin), 150.5 (C Ar-coumarin), 136.3 (C indole), 127.7 (C indole), 124.5 (CH Ar-coumarin), 123.1 (CH indole), 122.4 (CH indole), 119.7 (CH indole), 118.8 (CH indole), 111.5 (CH indole), 110.2 (C indole), 109.9 (CH Ar-coumarin), 107.5 (C Ar-coumarin), 107.2 (CH Ar-coumarin), 99.7 (CH Ar-coumarin), 82.5 (Cq), 79.2 (C alkynelc), 72.5 (CH alkynelc), 61.8 (CH$_2$), 55.1 (CH Trp), 46.2 (CH$_2$), 39.9 (CH$_3$), 28.2 (CH$_2$Trp), 28.1 (3 X CH$_3$), 12.4 (CH$_3$).

IR ($\nu_{\text{max}}$, cm$^{-1}$): 3359 (w), 3304 (w), 2977 (w), 2931 (w), 1713 (s), 1609 (s), 1524 (w), 1420 (w), 1337 (w), 1227 (w), 1156 (m), 1084 (w), 912 (w), 847 (w), 741 (w).

HRMS (ESI) m/z: calcd for C$_{31}$H$_{33}$N$_3$NaO$_6$ $^+$ [M+Na]$^+$ 566.2262; found 566.2265.

$^1$H NMR and $^{13}$C spectra for 43:
**Experimental part**

TFA (2.5 mL) was added to a solution of 43 (0.27 g, 0.49 mmol, 1.0 eq.) in DCM (10 mL) and the reaction mixture was stirred at r.t. for 6 h under dark conditions. The solvents were removed under reduced pressure and the crude mixture was purified by column chromatography eluting with a mixture of DCM / MeOH (9:1). (((7-(Ethyl(prop-2-yn-1-yl)amino)-2-oxo-2H-chromen-4-yl)methoxy)carbonyl)-L-tryptophan (44) (0.16 g, 0.34 mmol, 68% yield) was obtained as yellow foam.

**1H NMR** (400 MHz, DMSO-d$_6$): $\delta$ 10.85 (d, $J = 2.3$ Hz, 1H, NH$_{\text{indole}}$), 7.62 (bs, 1H, NH$_{\text{amide}}$), 7.54 (d, $J = 8.0$ Hz, 1H, indole-$H$), 7.46 (d, $J = 9.0$ Hz, 1H, coumarin-$H$), 7.32 (d, $J = 8.0$ Hz, 1H, indole-$H$), 7.14 (d, $J = 2.3$ Hz, 1H, indole-$H$), 7.07 (t, $J = 7.5$ Hz, 1H, indole-$H$), 6.96 (t, $J = 7.3$ Hz, 1H, indole-$H$), 6.78 (dd, $J = 9.1$, 2.6 Hz, 1H, coumarin-$H$), 6.68 (d, $J = 2.5$ Hz, 1H, coumarin-$H$), 6.06 (d, $J = 1.5$ Hz, 1H, coumarin-$H$), 5.20 (q, $J = 16.0$ Hz, 2H, CH$_2$coumarin), 4.23 (d, $J = 2.5$ Hz, 2H, R$_2$N-CH$_2$-C≡CCH), 4.15 (td, $J = 8.2$, 4.5 Hz, 1H, CH$_{\text{TPr}}$), 3.52 (d, $J = 8.7$ Hz, 1H, CH$_2$TPr), 3.19 (t, $J = 2.3$ Hz, 1H, R$_2$N-CH$_2$-C≡CCH), 3.02 (dd, $J = 14.5$, 8.7 Hz, 1H, CH$_2$TPr), 1.15 (t, $J = 7.0$ Hz, 3H, R$_2$N-CH$_2$-CH$_2$H$_3$).

**13C NMR** (101 MHz, DMSO-d$_6$): $\delta$ 160.7 (C Ar-coumarin), 155.4 (C Ar-coumarin), 155.3 (C Ar-coumarin), 151.9 (CO), 150.4 (C Ar-coumarin), 136.2 (C indole), 127.4 (C indole), 125.3 (CH Ar-coumarin), 123.7 (CH indole), 121.0 (CH indole), 118.5 (CH indole), 118.3 (CH indole), 111.5 (CH indole), 111.1 (C indole), 109.9 (CH Ar-coumarin), 106.5 (C Ar-coumarin), 105.7 (CH Ar-
Experimental part

coumarin), 98.6 (CH<sub>Ar</sub>-coumarin), 80.5 (CH<sub>alkyne</sub>), 74.5 (CH<sub>alkyne</sub>), 61.0 (CH<sub>2</sub>coumarin), 55.8 (CH<sub>Trp</sub>), 45.4 (CH<sub>2</sub>coumarin), 39.3 (CH<sub>2</sub>coumarin), 27.3 (CH<sub>2</sub>Trp), 27.3 (CH<sub>3</sub>coumarin).*

IR (ν<sub>max</sub>, cm<sup>-1</sup>): 3907 (w), 3379 (w), 2928 (w), 2861 (w), 1688 (s), 1607 (s), 1526 (w), 1424 (w), 1343 (w), 1209 (m), 1143 (m), 1086 (w), 748 (w).

HRMS (ESI) m/z: calcd for C<sub>27</sub>H<sub>26</sub>N<sub>3</sub>O<sub>6</sub> [M+H]<sup>+</sup> 488.1816; found 488.1819.

* One carbon is not resolved (CO<sub>2</sub>H).

<sup>1</sup>H NMR and <sup>13</sup>C spectra for 44:
Experimental part

(((7-(((1-((2-((2-Aminoethoxy)ethoxy)ethoxy)ethyl)-1H,1,2,3-triazol-4-yl)methyl)(ethyl)amino)-2-oxo-2H-chromen-4-yl)methoxy)carbonyl)-L-tryptophan hydrochloride (45)

(((7-(Ethyl(prop-2-yn-1-yl)amino)-2-oxo-2H-chromen-4-yl)methoxy)carbonyl)-L-tryptophan (44) (65.4 mg, 0.13 mmol, 1.0 eq) was added to a solution of azido-PEG3-amine hydrochloride (47) (37.5 mg, 0.15 mmol, 1.1 eq) dissolved in a mixture of THF / H2O (5 ml, 1:1). CuSO4 (10.4 mg, 0.07 mmol, 0.5 eq) and sodium ascorbate (26.2 mg, 0.13 mmol, 1.0 eq) were added and the reaction mixture was stirred at r.t. for 24 h under argon atmosphere and dark conditions. The solvents were removed under reduced pressure and the crude product was purified by preparative chromatography eluting with a mixture of ACN / H2O (8:2). The pure product (45) (60.7 mg, 0.8 mmol, 61% yield) was obtained as a brown salt.

1H NMR (400 MHz, Methanol-d4): δ 7.79 (s, 1H, triazole-H), 7.64 (d, J = 7.8 Hz, 1H, indole-H), 7.41 (d, J = 9.0 Hz, 1H, coumarin-H), 7.31 (d, J = 8.0 Hz, 1H, indole-H), 7.13 (s, 1H, indole-H), 7.05 (t, J = 7.5 Hz, 1H, indole-H), 6.97 (t, J = 7.4 Hz, 1H, coumarin-H), 6.76 (dd, J = 9.0, 2.5 Hz, 1H, coumarin-H), 6.61 (d, J = 2.5 Hz, 1H, coumarin-H), 6.10 (s, 1H, coumarin-H), 5.16 (dd, J = 4.0 Hz, 2H, CH2-coumarin), 4.71 (s, 2H, R2N-CH2-triazole), 4.51 (t, J = 4.9 Hz, 2H, R-CH2-CH2-triazole), 4.34 (dd, J = 7.6, 4.5 Hz, 1H, CH2-Trp), 3.78 (t, J = 4.9 Hz, 2H, R-CH2-CH2-triazole), 3.62 (dd, J = 14.3, 7.2, 2H, R2N-CH2-CH3), 3.48 (t, J = 5.1 Hz, 2H, R-CH2-CH2-NH2), 3.42 (t, J = 4.5 Hz, 1H, CH2-Trp),

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Experimental part

3.32 (m, 8H, CH$_2$-O-CH$_2$), 3.14 (dd, J = 14.5, 7.6 Hz, 1H, CH$_2$Trp), 2.97 (t, J = 5.1 Hz, 2H, R-CH$_2$-CH$_2$-NH$_2$), 1.25 (t, J = 7.0 Hz, 3H, R$_2$N-CH$_2$-CH$_3$).

$^{13}$C NMR (101 MHz, Methanol-d$_4$): δ 164.0 (CO Ar-coumarin), 157.2 (2 X C Ar-coumarin), 153.6 (CO carbamate), 152.3 (C Ar-coumarin), 129.3 (C triazole), 127.7 (C Ar-indole), 126.3 (CH Ar-coumarin), 124.9 (CH triazole), 124.5 (CH indole), 122.1 (CH indole), 119.6 (2 X CH indole), 112.2 (CH indole), 111.0 (CH Ar-coumarin), 108.1 (C Ar-coumarin), 107.2 (CH Ar-coumarin), 99.3 (CH Ar-coumarin), 71.3 (CH$_2$ TEG), 71.3 (CH$_2$ TEG), 71.2 (CH$_2$ TEG), 71.0 (CH$_2$ TEG), 70.3 (CH$_2$ TEG), 67.8 (CH$_2$ TEG), 62.8 (CH$_2$ coumarin), 58.7 (CH$_2$ Trp), 51.4 (CH$_2$ TEG), 46.9 (CH$_2$ coumarin), 46.6 (CH$_2$ coumarin), 40.5 (CH$_2$ TEG), 29.3 (CH$_2$ Trp), 12.7 (CH$_3$ coumarin).*

IR (ν$_{\text{max}}$, cm$^{-1}$): 732 (m), 688 (m), 707 (m), 758 (m), 2017 (w), 891 (w), 1530 (w).

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: [M + H]$^+$ Calcd for C$_{35}$H$_{44}$N$_7$O$_9$ 706.3195; Found 706.3180.

*Three carbons are not resolved (CO$_2$H, C Ar-coumarin and C indole).

$^1$H NMR and $^{13}$C spectra for 45:
DIPEA (9.4 μL, 53.8 μmol, 2.0 eq) was added to a stirred solution of 45 (20.0 mg, 26.9 μmol, 1.0 eq) dissolved in dry DMF (1 mL) under argon atmosphere and dark conditions. 11,12-didehydro-5,6-dihydrodibenzo[α,e]cycloocten-5-yl carbonic acid 4-nitrophenyl ester (48) (11.4 mg, 29.6 μmol, 1.1 eq) was added and the reaction mixture was stirred at r.t. for 24 h. The solvent was removed under reduced pressure and the crude product was purified by preparative chromatography eluting with a mixture of DCM / MeOH (8.5:1.5). The pure product 46 (8.7 mg, 9.1 μmol, 34% yield) was obtained as a yellow solid.

$^1$H NMR (400 MHz, Methanol-$d_4$): δ 7.83 (s, 1H, triazole-H), 7.62 (d, J = 7.8 Hz, 1H, indole-H), 7.54 (d, J = 7.6 Hz, 1H, coumarin-H), 7.37 – 7.24 (m, 9H, indole-H and 8 $CH_2$ cyclooctyne), 7.12 (s, 1H, indole-H), 7.05 (t, J = 7.5 Hz, 1H, indole-H), 6.97 (t, J = 7.5 Hz, 1H, indole-H), 6.73 (dd, J = 9.0, 2.6 Hz, 1H, coumarin-H), 6.60 (s, 1H, coumarin-H), 6.07 (s, 1H, coumarin-H), 5.42 – 5.30 (m, 1H, $CH_2$ cyclooctyne), 5.25 – 5.05 (m, 2H, $CH_2$ coumarine), 4.65 (s, 2H, R$_2$N-CH$_2$-triazole), 4.48 (t, J = 5.1 Hz, 2H, R-CH$_2$-CH$_2$-triazole), 4.45 (dd, J = 7.9, 4.4 Hz, 1H, $CH_{Trp}$), 3.80
- 3.77 (m, 2H, R-CH$_2$-CH$_2$-triazole), 3.66 (s, 2H, O-CH$_2$-CH$_2$-NHRamide), 3.56 (q, $J = 6.9$ Hz, 2H, R$_2$N-CH$_2$-CH$_3$), 3.50 – 3.42 (m, 8H, CH$_2$-O-CH$_2$), 3.40 – 3.35 (m, 1H, CH$_2$-Trp), 3.29 – 3.22 (m, 2H, O-CH$_2$-CH$_2$-NHRamide), 3.21 – 3.10 (m, 1H, CH$_2$-Trp), 2.76 (dd, $J = 15.1$, 4.3 Hz, 1H, HC-H cycloctyne), 2.06 (dd, $J = 15.5$, 8.6 Hz, 2H, HC-H cycloctyne), 1.20 (t, $J = 7.1$ Hz, 3H, R$_2$N-CH$_2$-CH$_3$).

$^{13}$C NMR (101 MHz, Methanol-d$_4$): δ 173.3 (CO$_2$H), 157.1 (C Ar-coumarin), 153.6 (CO carbamate), 153.5 (CO carbamate), 152.4 (C Ar-DIBO), 152.4 (C Ar-coumarin), 145.8 (C Ar-DIBO), 137.9 (C Ar-indole), 131.1 (C triazole), 129.3 (CH Ar-DIBO), 129.3 (CH Ar-DIBO), 129.2 (CH Ar-DIBO), 128.3 (CH Ar-DIBO), 128.2 (CH Ar-DIBO), 127.2 (CH Ar-DIBO), 126.9 (CH Ar-DIBO), 126.2 (CH Ar-DIBO), 125.0 (CH Ar-coumarin), 124.9 (CH triazole), 124.4 (CH Ar-indole), 122.4 (C Ar-DIBO), 122.2 (CH Ar-indole), 119.7 (CH Ar-indole), 119.5 (CH Ar-indole), 112.2 (CH Ar-indole), 111.8 (C Ar-DIBO), 111.0 (C Ar-coumarin), 111.0 (C Ar-coumarin), 110.0 (C Ar-coumarin), 106.9 (C Ar-coumarin), 99.3 (CH-ar-coumarin), 78.0 (CH-DIBO), 71.5 (CH$_2$ TEG), 71.4 (CH$_2$ TEG), 71.4 (CH$_2$ TEG), 71.2 (CH$_2$ TEG), 70.9 (CH$_2$ TEG), 70.3 (CH$_2$ TEG), 62.8 (CH$_2$ coumarin), 58.0 (CH$_2$ Trp), 51.4 (CH$_2$ TEG), 46.6 (CH$_2$ coumarin), 46.4 (CH$_2$ coumarin), 41.8 (CH$_2$ TEG), 28.1 (CH$_2$ Trp), 26.7 (CH$_2$ DIBO), 12.6 (CH$_3$ coumarin).

IR (ν$_{max}$ cm$^{-1}$): 3017 (m), 2162 (s), 1979 (s), 1890 (s), 1587 (s), 1340 (s), 1163 (s), 1023 (m), 758 (s).

HRMS (ESI/QTOF) m/z: [M + Na]$^+$ Calcd for C$_{52}$H$_{53}$N$_4$NaO$_{11}$: 974.3695; Found 974.3701.

* Five carbons are not resolved (2 X Cq alkyne, 2 X C Ar-coumarin, C Ar-indole).

$^1$H NMR and $^{13}$C spectra for 46:
DIBO-Trp 60

Trp (11.7 mg, 57.1 μmol, 1.1 eq) and DIBO 48 (20.0 mg, 51.9 μmol, 1.0 eq) were dissolved in dry DMF (1 mL). DIPEA (18 μL, 103.8 μmol, 2.0 eq) was added and the reaction mixture was stirred at r.t. for 24 h under argon atmosphere. The solvent was removed under reduced pressure and the residue was purified by preparative TLC eluting with a mixture of DCM / MeOH (9:1) to afford DIBO-Trp 60 as a light yellow oil (22.0 mg, 48.8 μmol, 94%).

\(^1\)H NMR (400 MHz, Methanol-\(d_4\)): \(\delta\) 7.62 (q, \(J = 8.4\) Hz, 2H, \(\text{CH}_{\text{Ar-cyclooctyne}}\)), 7.40 – 7.29 (m, 2H, \(\text{CH}_{\text{Ar-cyclooctyne}}\)), 7.32 – 6.88 (m, 9H, 4 X \(\text{CH}_{\text{Ar-cyclooctyne}}\) and 5 X \(\text{CH}_{\text{indole-H}}\)), 5.30 (d, \(J = 13.9\) Hz, 1H, \(\text{CH}_{\text{Ar-cyclooctyne}}\)), 4.46 (td, \(J = 9.6, 9.1, 4.4\) Hz, 1H, \(\text{CH}_{\text{Trp}}\)), 3.45 – 3.33 (m, 1H, \(\text{H}_{\text{C-H}}\text{Trp})\), 3.16 (dddd, \(J = 14.3, 12.3, 9.8, 4.6\) Hz, 1H, \(\text{H}_{\text{C-H}}\text{Trp})\), 2.90 (dd, \(J = 15.0, 2.1\) Hz, 1H, \(\text{H}_{\text{C-H}}\text{cyclooctyne})\), 2.72 (ddd, \(J = 23.6, 14.9, 3.9\) Hz, 1H, \(\text{H}_{\text{C-H}}\text{cyclooctyne})\).

\(^{13}\)C NMR (101 MHz, Methanol-\(d_4\)): \(\delta\) 175.3 (\(\text{C}_2\text{O}2\text{H})\), 152.0 (\(\text{CO})\), 150.9 (\(\text{C}_{\text{Ar-DIBO}}\)), 136.7 (\(\text{C}_{\text{Ar-DIBO}}\)), 133.3 (\(\text{C}_{\text{Ar-indole}}\)), 129.6 (\(\text{CH}_{\text{Ar-DIBO}}\)), 127.9 (\(\text{CH}_{\text{Ar-DIBO}}\)), 127.6 (\(\text{CH}_{\text{Ar-DIBO}}\)), 126.8 (\(\text{CH}_{\text{Ar-DIBO}}\)), 126.6 (\(\text{CH}_{\text{Ar-DIBO}}\)), 125.6 (\(\text{CH}_{\text{Ar-DIBO}}\)), 125.2 (\(\text{CH}_{\text{Ar-DIBO}}\)), 124.0 (\(\text{CH}_{\text{Ar-indole}}\)), 123.5 (\(\text{CH}_{\text{Ar-indole}}\)), 123.2 (\(\text{CH}_{\text{Ar-indole}}\)), 121.0 (\(\text{CH}_{\text{Ar-indole}}\)), 119.6 (\(\text{C}_{\text{Ar-DIBO}}\)), 118.5 (\(\text{CH}_{\text{Ar-indole}}\)), 118.0 (\(\text{CH}_{\text{Ar-indole}}\)), 112.3 (\(\text{C}_{\text{Ar-DIBO}}\)), 110.9 (\(\text{C}_{\text{Ar-indole}}\)), 110.9 (\(\text{CH}_{\text{Ar-DIBO}}\)), 110.1 (\(\text{C}_{\text{Alkyne}}\)), 109.4 (\(\text{C}_{\text{Alkyne}}\)), 76.8 (\(\text{CH}_{\text{DIBO}}\)), 55.6 (\(\text{CH}_{\text{Trp}}\)), 46.0 (\(\text{CH}_{2\text{DIBO}}\)), 27.7 (\(\text{CH}_{2\text{Trp}}\)).

IR (\(v_{\text{max}}, \text{cm}^{-1}\)): 3396 (w), 2023 (w), 1713 (m), 1403 (w), 1213 (w), 1042 (w), 656 (w), 758 (s).
HRMS (ESI-QTOF) m/z: [M-H] - Calcd for C_{28}H_{21}N_{2}O_{4} - 449.1501; Found m/z 449.1500.

\(^1\)H NMR and \(^{13}\)C spectra for 60:
4.3.3 Synthesis of coumarinyl-Cy3 dye conjugates

Synthesis of coumarinyl-Cy3 linkers

Synthesis of Cy3 dye

Scheme 46: Synthesis of Cy3 dye 57: i) MeI, ACN, reflux, 24 h; ii) 6-bromohexanoic acid, 80°C, MW, 20 min; iii) N,N'-diphenylformamidine, acetic anhydride, 120°C, 30 min; 90, pyridine, r.t., 16 h; iv) DSC, DCM, r.t., 2 h; v) ethylenediamine, DIPEA, DMF, r.t., 30 min.

1,2,3,3-Tetramethyl-3H-indol-1-ium Iodide (90)

2,3,3-Trimethylindolenine (0.16 mL, 1.0 mmol, 1.0 eq) and MeI (0.13 mL, 2.0 mmol, 2.0 eq) were dissolved in dry ACN (0.5 mL) and the reaction mixture was reflux for 24 h under argon atmosphere. The mixture was cooled down to r.t. and filtered. The precipitate was washed with Et₂O to afford 1,2,3,3-tetramethyl-3H-indol-1-ium iodide (90) (0.26 g, 0.9 mmol, 87% yield) as a white solid.

$^1$H NMR (400 MHz, DMSO-d$_6$): δ 7.93 – 7.85 (m, 1H, Ar-H), 7.83 – 7.73 (m, 1H, Ar-H), 7.68 – 7.46 (m, 2H, 2 X Ar-H), 3.94 (s, 3H, CH$_3$), 2.73 (s, 3H, CH$_3$), 1.50 (s, 6H, 2 X CH$_3$).

The analytical data were in accordance with previously reported data.$^{360}$
Experimental part

$^1$H NMR spectrum for 90:

1-(5-Carboxypentyl)-2,3,3-trimethyl-3H-indol-1ium bromide (91)

2,3,3-Trimethylindolenine (1.9 mL, 12.0 mmol, 1.2 eq) and 6-bromohexanoic acid (1.95 g, 10.0 mmol, 1.0 eq) were putted into a microwave vial. The reaction mixture was stirred at 80°C for 20 min in a microwave apparatus. The mixture was cooled down to r.t., filtered and the solid was washed with ACN. 1-(5-Carboxypentyl)-2,3,3-trimethyl-3H-indol-1ium bromide (91) (3.18 g, 8.9 mmol, 89% yield) was obtained as a purple solid.

$^1$H NMR (400 MHz, DMSO-d$_6$): δ 11.99 (s, 1H, CO$_2$H), 8.00 – 7.93 (m, 1H, Ar-H), 7.89 – 7.80 (m, 1H, Ar-H), 7.66 – 7.59 (m, 2H, 2 X Ar-H), 4.45 (t, J = 7.7 Hz, 2H, R-CH$_2$-(CH$_2$)$_4$-CO$_2$H), 2.83 (s, 3H, CH$_3$), 2.23 (t, J = 7.2 Hz, 2H, R-CH$_2$-CO$_2$H), 1.84 (p, J = 7.9 Hz, 2H, R-CH$_2$-(CH$_2$)$_3$-CO$_2$H), 1.59 – 1.55 (m, 2H, R-CH$_2$-CH$_2$-CO$_2$H), 1.53 (s, 6H, 2 X CH$_3$), 1.47 – 1.38 (m, 2H, R-CH$_2$-(CH$_2$)$_3$-CO$_2$H).

The analytical data were in accordance with previously reported data.\textsuperscript{361}
Experimental part

$^1$H NMR spectrum for 91:

1-(5-Carboxypentyl)-3,3-dimethyl-2-((E)-3-((E)-1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium chloride (92)

1-(5-Carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (91) (1.06 g, 3.0 mmol, 1.0 eq) and N,N-diphenylformamidine (0.71 g, 3.6 mmol, 1.2 eq) were dissolved in acetic anhydride (6.0 mL) and the reaction mixture was stirred at 120°C for 30 min. The mixture was cooled down to r.t. and a solution of 1,2,3,3-tetramethyl-3H-indol-1-ium iodide (90) (0.90 g, 3.0 mmol, 1.0 eq) in pyridine (6.0 mL) was added. The reaction mixture was stirred at r.t. for 16 h. The solvents were removed under reduced pressure and the crude mixture was purified via column chromatography eluting with a mixture of DCM / MeOH (1:0 to 20:1). (92) (1.25 g, 2.5 mmol, 84% yield) was obtained as a purple solid.

$^1$H NMR (400 MHz, DMSO-$d_6$): δ 12.03 (s, 1H, CO$_2$H), 8.34 (t, J = 13.4 Hz, 1H, R-CH=CH-CH=R), 7.64 (d, J = 7.5 Hz, 2H, 2 X Ar-H), 7.45 (dd, J = 8.2, 4.5 Hz, 4H, 4 X Ar-H), 7.35 – 7.25 (m, 2H, 2 X Ar-H), 6.48 (dd, J = 13.4, 5.1 Hz, 2H, R-CH=CH-CH=R), 4.11 (t, J = 7.5 Hz, 2H, R-CH$_2$-(CH$_2$)$_4$-CO$_2$H), 3.66 (s, 3H, CH$_3$), 2.22 (t, J = 7.2 Hz, 2H, R-
CH$_2$-CO$_2$H), 1.69 (s, 14H, R-CH$_2$-(CH$_2$)$_4$-CO$_2$H and 4 X CH$_3$), 1.56 (q, $J$ = 7.9, 7.3 Hz, 2H, R-CH$_2$-CH$_2$-CO$_2$H), 1.42 (t, $J$ = 7.7 Hz, 2H, R-CH$_2$-(CH$_2$)$_2$-CO$_2$H).

The analytical data were in accordance with previously reported data.$^{362}$

$^1$H NMR spectrum for 92:

1-(6-((2,5-Dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-2-((E)-3-((E)-1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium chloride (93)

92 (0.86 g, 1.8 mmol, 1.0 eq) and DIPEA (0.6 mL, 3.5 mmol, 2.0 eq) were dissolved in dry DCM (20) and DSC (0.49 g, 1.9 mmol, 1.1 eq) was added in one portion. The reaction mixture was stirred at r.t. for 2 h under argon atmosphere. The mixture was diluted with DCM (20) and washed with H$_2$O (80 mL), 1M HCl (80 mL) and brine (80 mL). The organic layer was dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was triturated with Et$_2$O to afford 93 (0.88 g, 1.5 mmol, 85% yield) as a purple solid.
\( ^1H \) NMR (400 MHz, DMSO-\( d_6 \)): \( \delta 8.36 \) (t, \( J = 13.5 \) Hz, 1H, R-CH=CH=CH=R), 7.65 (d, \( J = 7.6 \) Hz, 2H, 2 X Ar-H), 7.46 (t, \( J = 6.8 \) Hz, 4H, 4 X Ar-H), 7.35 – 7.25 (m, 2H, 2 X Ar-H), 6.48 (dd, \( J = 13.5, 8.8 \) Hz, 2H, R-CH=CH=CH=R), 4.12 (d, \( J = 8.1 \) Hz, 2H, R-CH\(_2\)(CH\(_2\))\(_3\)CO\(_2\)Suc), 3.67 (s, 3H, CH\(_3\)), 2.82 (s, 4H, CH\(_2\)Suc), 2.72 (t, \( J = 7.3 \) Hz, 2H, R-CH\(_2\)-CO\(_2\)Suc). 1.84 – 1.63 (m, 16H, R-CH\(_2\)(CH\(_2\))\(_3\)CO\(_2\)Suc, R-CH\(_2\)-CH\(_2\)-CO\(_2\)Suc and 4 X CH\(_3\)), 1.63 – 1.44 (m, 2H, R-CH\(_2\)(CH\(_2\))-CO\(_2\)Suc).

The analytical data were in accordance with previously reported data.\(^{362}\)

\( ^1H \) NMR spectrum for 93:

1-(6-((2-Aminoethyl)amino)-6-oxohexyl)-3,3-dimethyl-2-((\(E\))-3-((\(E\))-1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium chloride (57)

A solution of 93 (50.0 mg, 85.0 \( \mu \)mol, 1.0 eq) in dry DMF (5 mL) was added dropwise to a stirred solution of ethylenediamine (8.5 \( \mu \)L, 0.13 mmol, 1.5 eq) and DIPEA (28.8 \( \mu \)L, 0.17 mmol, 2.0 eq) in dry DMF (5 mL). The reaction mixture was stirred at r.t. for 30 min under argon atmosphere. The solvent was removed under reduced pressure and the crude was purified by preparative TLC eluting with a mixture of ACN / H\(_2\)O (8:2). 57 (45.5 mg, 85.0 \( \mu \)mol, quant.) was obtained as a purple solid.
Experimental part

IR ($\nu_{\text{max}}$ cm$^{-1}$): 653 (w), 705 (w), 765 (w), 817 (w), 881 (w), 2024 (w), 2152 (w), 3732 (w).

HRMS (ESI/QTOF) m/z: [M]$^+$ Calcd for C$_{32}$H$_{43}$N$_4$O$^+$ 499.3431; Found 499.3441.

Synthesis of Biotin-CM-Cy3 58

Scheme 47: Synthesis of biotin-CM-Cy3 56: i) 57, DIPEA, DMF, r.t., 16 h; ii) biotin-TEG-N$_3$ 58, CuSO$_4$, sodium ascorbate, H$_2$O / THF (1:1), r.t., 24 h; iii) 85, DIPEA, DMF, r.t., 24 h; iv) 63, DIPEA, DMF, r.t., 24 h.
**Experimental part**

**Coumarin-Cy3 55**

![Diagram of Coumarin-Cy3 55]

Cy3-NH₂ 57 (18.7 mg, 44.3 μmol, 1.1 eq) and 7-{(ethyl(prop-2-yn-1-yl)amino)-2-oxo-2H-chromen-4-yl)methyl (4-nitrophenyl) carbonate (56) (21.5 mg, 40.0 μmol, 1.0 eq) were dissolved in dry DMF (2 mL). DIPEA (28 μL, 0.16 mmol, 4.0 eq) was added and the reaction mixture was stirred at r.t., under argon atmosphere and dark conditions for 16 h. The solvent was removed under reduced pressure and the crude product was purified by prep TLC eluting with a mixture of DCM / MeOH (9:1). 55 (24.2, 29.6 μmol, 73% yield) was obtained as a purple solid.

**1H NMR** (400 MHz, Methanol-d₄): δ 8.36 (t, J = 13.4 Hz, 1H, R-CH=CH-CH=R), 7.45 – 7.37 (m, 2H, 2 X Ar-H (cy)), 7.37 – 7.28 (m, 3H, 2 X Ar-H (cy3) and coumarin-H), 7.24 – 7.12 (m, 4H, 4 X Ar-H (cy3)), 6.66 (dd, J = 9.0, 2.6 Hz, 1H, coumarin-H), 6.48 (d, J = 2.5 Hz, 1H, coumarin-H), 6.30 (dd, J = 13.5, 10.3 Hz, 2H, 2 X R-CH=CH-CH=R), 5.99 (d, J = 1.4 Hz, 1H, coumarin-H), 5.15 (d, J = 1.4 Hz, 2H, CH₂-coumarin), 4.00 (d, J = 2.4 Hz, 2H, R₂N-CH₂-C≡CH), 3.94 (t, J = 7.7 Hz, 2H, R-CH₂-(CH₂)₄-CONHR), 3.55 (s, 3H, CH₃-cy), 3.39 (q, J = 7.1 Hz, 2H, R₂N-CH₂-CH₃), 3.19 (s, 4H, R̵NHOC-CH₂-CH₂-NHOR), 2.51 (t, J = 2.4 Hz, 1H, R₂N-CH₂-C≡CH), 2.13 (t, J = 7.1 Hz, 2H, R-CH₂-CONHR), 1.66 (s, 4H, R-CH₂-(CH₂)₃-CONHR and R-CH₂-CH₂-CONHR), 1.63 (s, 6H, 2 X CH₂-cy), 1.61 (s, 6H, 2 X CH₂-cy), 1.39 (q, J = 7.9, 6.9 Hz, 2H, R-CH₂-(CH₂)₂-CONHR), 1.09 (t, J = 7.1 Hz, 3H, R₂N-CH₂-CH₃).

**13C NMR** (101 MHz, Methanol-d₄): δ 175.1 (C₂-cy), 174.9 (C₃-cy), 174.4 (CO-camid), 162.5 (CO Ar-coumarin), 156.7 (CO carbamate), 155.6 (C Ar-coumarin), 152.5 (C Ar-coumarin), 150.9 (CH₂-cy), 150.6 (C Ar-coumarin), 142.6 (C Ar-cy), 141.8 (C Ar-cy3), 140.7 (C Ar-cy3), 140.7 (C Ar-cy), 128.6 (CH Ar-cy), 128.5 (CH Ar-cy), 125.3 (CH Ar-cy), 124.5 (CH Ar-coumarin), 122.1 (CH Ar-cy), 121.9 (CH Ar-cy), 111.0 (CH Ar-cy), 110.8 (CH Ar-cy), 109.9 (CH Ar-coumarin), 106.7 (C Ar-coumarin), 105.1 (CH Ar-coumarin), 102.5 (CH₃-cy), 102.2 (CH₃-cy), 98.4 (CH Ar-coumarin), 79.0 (C₂-cy), 72.1 (CH₂-cy), 61.3 (CH₂-coumarin), 49.2 (C₃-cy), 49.2 (C₃-cy), 45.4 (CH₂-coumarin), 43.7 (CH₂-cy), 40.2 (CH₂-cy), 39.1 (CH₂-cy), 38.9 (CH₂-coumarin), 35.4 (CH₂-cy), 30.4 (CH₃-cy), 27.0 (2 X CH₃-cy), 26.8 (2 X CH₃-cy and CH₂-cy), 25.7 (CH₂-cy), 25.01 (CH₂-cy), 11.2 (CH₃-coumarin).

**IR** (νmax cm⁻¹): 2352 (w), 2162 (m), 1080 (w), 1023 (w), 935 (w), 865 (w), 827 (w), 758 (s), 669 (m), 712 (m), 692 (m).

**HRMS (ESI/QTOF) m/z**: [M]+ Calcd for C₄₈H₅₆N₄O₇⁺ 782.4276; Found 782.4291.
$^1$H NMR and $^{13}$C spectra for 55:
CM-Cy3 55 (21.2 mg, 25.9 μmol, 1.0 eq) and Biotin-TEG-N₃ 58 (13.8 mg, 31.1 μmol, 1.2 eq) were dissolved in a mixture of H₂O / THF (1:1, 4 mL). Sodium ascorbate (5.1, 25.9 μmol, 1.0 eq) and CuSO₄ (2.0, 12.9 μmol, 0.5 eq) were added and the reaction mixture was stirred at r.t. for 24 h under dark conditions. The solvents were removed under reduced pressure and the crude was purified via preparative TLC eluting with a mixture of DCM / MeOH (9:1). 56 (6.9 mg, 5.4 μmol, 21% yield) was obtained as a red solid.

³H NMR (400 MHz, Methanol-d₄): δ 8.38 (t, J = 13.5 Hz, 1H, R-CH=CH-CH=R), 7.82 (s, 1H, CH triazole), 7.42 (dd, J = 7.7, 2.5 Hz, 2H, 2 X Ar-H (Cy3)), 7.37 – 7.28 (m, 3H, 2 X Ar-H (Cy3) and coumarin-H)), 7.26 – 7.13 (m, 4H, 4 X Ar-H (Cy3)), 6.69 (dd, J = 9.0, 2.5 Hz, 1H, coumarin-H)), 6.49 (d, J = 2.5 Hz, 1H, coumarin-H)), 6.32 (dd, J = 13.4, 10.1 Hz, 2H, 2 X R-CH=CH-CH=R), 5.96 (d, J = 1.3 Hz, 1H, coumarin-H)), 5.13 (d, J = 1.4 Hz, 2H, CH₂ coumarin), 4.53 (s, 2H, R₂N-CH₂-C triazole), 4.43 (t, J = 4.9 Hz, 2H, triazole-CH₂-CH₂-OR TEG), 3.47 (dd, J = 8.1, 4.9 Hz, 1H, NH-CH-biotin), 4.21 – 4.14 (m, 1H, NH-CH-biotin), 3.96 (t, J = 7.6 Hz, 2H, R-CH₂-(CH₂)₂-CO-CONH-Cy3), 3.74 (t, J = 5.0 Hz, 2H, triazole-CH₂-CH₂-OR TEG), 3.61 – 3.57 (m, 9H, 2 X RO-CH₂-CH₂-OR TEG, RO-CH₂-CH₂-NH TEG and CH₂Cy3), 3.47 – 3.40 (m, 2H, R₂N-CH₂-CH₂), 3.40 – 3.34 (m, 4H, 2 X RO-CH₂-CH₂-OR TEG), 3.30 – 3.24 (m, 2H, RO-CH₂-CH₂-NH TEG), 3.22 – 3.17 (m, 4H, RNHOC-CH₂-CH₂-NHCOR), 3.08 (tt, J = 9.6, 4.6 Hz, 1H, NH-CH-CH-biotin), 2.86 – 2.79 (m, 1H, NH-CH-CH₂ biotin), 2.64 – 2.59 (m, 1H, NH-CH-CH₂ biotin), 2.16 – 2.05 (m, 4H, CH₂-CONH-biotin and R-CH₂-CO-CONH-Cy3), 1.67 (s, 2H, CH₂-(CH₂)₂-CO-CONH-biotin and CH₂-(CH₂)₂-CO-CONH-biotin), 1.64 (s, 10H, 2 X CH₃-Cy3, R-CH₂-(CH₂)₂-CO-CONH-Cy3 and R-CH₂-CH₂-CO-CONH-Cy3), 1.62 (s, 6H, 2 X CH₃-Cy3), 1.55 – 1.45 (m, 4H, CH₂-CH₂-CONH-biotin), 1.39 – 1.29 (m, 2H, R₂N-CH₂-CH₂).

¹³C NMR (101 MHz, Methanol-d₄): δ 175.1 (Cq-Cy3), 174.8 (Cq Cy3), 174.7 (CO camid), 174.6 (CO camide), 162.5 (CO Ar-coumarin), 156.6 (CO carbamate), 155.7 (C Ar-coumarin), 152.5 (C Ar-coumarin), 151.1 (CH Cy3), 150.6 (C Ar-coumarin), 142.7 (C Ar-Cy3), 141.8 (C Ar-Cy3), 140.8 (C Ar-Cy3), 140.7 (C Ar-Cy3), 128.6 (CH Ar-Cy3), 128.5 (CH Ar-Cy3), 125.3 (CH Ar-Cy3), 124.7 (CH Ar-coumarin), 123.7 (CH triazole), 122.1 (CH Ar-Cy3), 122.0 (CH Ar-Cy3), 111.0 (CH Ar-Cy3), 110.9 (CH Ar-Cy3), 109.6 (CH Ar-coumarin), 106.4 (C Ar-coumarin), 104.8 (C Ar-coumarin), 102.5 (CH Cy3), 102.2 (CH Cy3), 97.9 (CH Ar-coumarin), 70.2 (CH₂ TEG), 70.1 (CH₂ TEG), 70.0 (CH₂ TEG), 69.8 (CH₂ TEG), 69.2 (CH₂ TEG), 69.0 (CH₂ TEG), 62.0 (CH biotin), 61.3 (CH₂ coumarin), 60.2 (CH biotin), 55.6 (CH biotin), 50.4 (CH₂ TEG), 50.1 (CH₂ TEG), 49.2 (Cq Cy3), 49.2 (Cq Cy3), 45.2 (CH₂ coumarin), 44.9 (CH₂ coumarin), 43.7 (CH₂ Cy3), 39.7 (CH₂ biotin), 39.0 (CH₂ Cy3), 38.9 (CH₂ Cy3), 35.4 (CH₂ Cy3), 35.3 (CH₂ biotin), 29.4 (CH₂ Cy3), 28.4 (CH₂ biotin), 28.1 (CH₂ Cy3), 27.0 (2 X CH₃ Cy3), 26.8 (2 X CH₃ Cy3), 25.8 (CH₂ biotin), 25.4 (CH₂ Cy3), 25.1 (CH₂ biotin), 11.2 (CH₃ coumarin).*
**Experimental part**

**IR** ($\nu_{\text{max}} \text{ cm}^{-1}$): 2915 (w), 1707 (m), 1604 (w), 1554 (m), 1416 (m), 1155 (w), 1119 (m), 757 (m), 683 (m), 926 (w).

**HRMS (ESI/QTOF)** $m/z$: [M]$^+$ Calcd for C$_{66}$H$_{88}$N$_{11}$O$_{10}$S$^+$ 1226.6431; Found 1226.6451.

*One carbon is not resolved (Cq triazole).*

$^1$H NMR and $^{13}$C spectra for 56:
Biotin-Cy3 59

Biotin-TEG-NH$_2$ 85 (12.9 mg, 30.8 μmol, 1.0 eq) and 93 (20.0 mg, 33.9 μmol, 1.1 eq) were dissolved in dry DMF (1 mL). DIPEA (10.7 μL, 61.6 μmol, 2.0 eq) was added and the reaction mixture was stirred at r.t. for 24 h under argon atmosphere and dark conditions. The solvent was removed under reduced pressure and the crude was purified by preparative TLC eluting with a mixture of DCM / MeOH (9:1). 59 (9.3 mg, 10.8 μmol, 35% yield) was obtained as a purple solid.

IR ($\nu_{\text{max}}$, cm$^{-1}$): 3115 (w), 1712 (m), 1559 (w), 1454 (m), 1406 (m), 1255 (w), 1122 (m), 926 (w), 857 (m), 653 (m).

HRMS (ESI/QTOF) m/z: [M]$^+$ Calcd for C$_{48}$H$_{69}$N$_6$O$_6$S$^+$ 857.4999; Found 857.0004.
DIBO-Cy3 79

DIBO 63 (18.8 mg, 61.6 μmol, 1.0 eq) and 93 (40.0 mg, 67.8 μmol, 1.1 eq) were dissolved in dry DMF (1 mL). DIPEA (21.4 μL, 0.12 mmol, 2.0 eq) was added and the reaction mixture was stirred at r.t. for 24 h under argon atmosphere and dark conditions. The solvent was removed under reduced pressure and the crude was purified by preparative TLC eluting with a mixture of DCM / MeOH (18:1). 79 (27.9 mg, 35.7 μmol, 58% yield) was obtained as a purple solid.

IR (ν_{max}, cm^{-1}): 3335 (w), 1862 (m), 1542 (w), 1420 (m), 1401 (m), 1185 (w), 1128 (m), 978 (w), 929 (s), 827 (m), 669 (m).

HRMS (ESI/QTOF) m/z: [M]^+ Calcd for C_{49}H_{53}N_{4}O_{3}^+ 745.4118; Found 745.4115.

4.3.4 Synthesis of coumarinyl-drug conjugates

Synthesis of erlotinib 74

Scheme 48: Synthesis of erlotinib derivative 74: i) NH₂OH · HCl, AcOH, reflux, 90 min; ii) 2-chloroethanol, K₂CO₃, DMF, 150°C, 8 h; iii) Acetyl chloride, pyridine, 0°C, 2 h; iv) HNO₃, 0°C to r.t., 35 min; v) Pd(OAc)₂, PMHS, KF, THF, r.t., 30 min; vi) DMF-DMA, ACOH, toluene, reflux, 2 h; vii) 3-ethynylaniline, ACOH, 130°C, 2 h; viii) K₂CO₃, MeOH, r.t., 20 min; ix) Ms-Cl, DMAP, pyridine, 0°C to r.t., 5 h; x) Nal, acetone, 80°C, 16 h; xi) 7N ammonia in MeOH, 85°C, 16 h.
To a solution of vanillin (10.0 g, 65.7 mmol, 1.0 eq) in AcOH (80 mL) was added hydroxylamine hydrochloride (6.9 g, 98.6 mmol, 1.5 eq). The reaction mixture was refluxed for 1.5 h. The mixture was cooled down to r.t., diluted with Et₂O (100 mL). The organic layer was washed with H₂O (2 X 100 mL), brine (1 X 100 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. The crude product was purified via column chromatography eluting with a mixture of PE / EtOAc (1:1). 4-Hydroxy-3-methoxybenzonitrile (64) (7.7 g, 51.4 mmol, 78% yield) was obtained as a pale yellow solid.

¹H NMR (400 MHz, Chloroform-d): δ 7.23 (dd, J = 8.3, 1.8 Hz, 1H, Ar-H), 7.08 (d, J = 1.8 Hz, 1H, Ar-H), 6.96 (d, J = 8.2 Hz, 1H, Ar-H), 6.08 (s, 1H, OH), 3.93 (s, 3H, CH₃).

The analytical data were in accordance with previously reported data.³⁶³

¹H NMR spectrum for 64:
Experimental part

4-(2-Hydroxyethoxy)-3-methoxybenzonitrile (65)

4-Hydroxy-3-methoxybenzonitrile (64) (7.7 g, 51.4 mmol, 1.0 eq), 2-chloroethanol (3.8 mL, 56.6 mmol, 1.1 eq) and K₂CO₃ (21.3 g, 154.2 mmol, 3.0 eq) were dissolved in DMF (100 mL). The reaction mixture was stirred at 150°C for 8 h. The mixture was cooled down to r.t. and diluted with sat. NH₄Cl (300 mL) and extracted with DCM (3 X 300 mL). The combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. The crude product was purified via column chromatography eluting with a mixture of PE / EtOAc (1:1). 4-(2-Hydroxyethoxy)-3-methoxybenzonitrile (65) (8.9 g, 45.9 mmol, 89% yield) was obtained as a white solid.

¹HNMR (400 MHz, Chloroform-d): δ 7.28 (d, J = 1.8 Hz, 1H, Ar-H), 7.10 (d, J = 1.9 Hz, 1H, Ar-H), 6.93 (d, J = 8.3 Hz, 1H, Ar-H), 4.20 – 4.12 (m, 2H, CH₂-OAr), 4.07 – 3.99 (m, 2H, CH₂-OH), 3.89 (s, 3H, CH₃), 2.25 (t, J = 6.3 Hz, 1H, OH).

The analytical data were in accordance with previously reported data.³⁶⁴
Experimental part

2-(4-Cyano-2-methoxyphenoxy)ethyl acetate (66)

Acetyl chloride (4.9 mL, 68.9 mmol, 1.5 eq) and pyridine (5.6 mL, 68.9 mmol, 1.5 eq) were added dropwise to a solution of 4-(2-hydroxyethoxy)-3-methoxybenzonitrile (65) (8.9 g, 45.9 mmol, 1.0 eq) in THF (70 mL) at 0°C. The reaction mixture was allowed to warm up to r.t. and was stirred for 2 h. The mixture was diluted with DCM (300 mL) and washed with sat. copper sulfate solution (2 X 300 mL) and H₂O (1 X 300 mL). The organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. 2-(4-Cyano-2-methoxyphenoxy)ethyl acetate (66) (10.5 g, 44.5 mmol, 97% yield) was obtained as a white solid.

¹H NMR (400 MHz, Chloroform-d): δ 7.26 (s, 1H, Ar-H), 7.10 (d, J = 1.9 Hz, 1H, Ar-H), 6.92 (d, J = 8.3 Hz, 1H, Ar-H), 4.51 – 4.39 (m, 2H, CH₂-OAc), 4.27 (t, J = 4.9 Hz, 2H, CH₂-OAr), 3.89 (s, 3H, CH₃-OAr), 2.10 (s, 3H, CH₃-Ac).

The analytical data were in accordance with previously reported data.

¹H NMR spectrum for 66:
2-(4-Cyano-2-methoxy-5-nitrophenoxy)ethyl acetate (67)

2-(4-Cyano-2-methoxyphenoxy)ethyl acetate (66) (0.5 g, 2.1 mmol, 1.0 eq) was added slowly to fuming HNO₃ (0.7 mL) at 0°C. The reaction mixture was allowed to warm up to r.t. and was stirred for 35 min. The mixture was poured into a mixture of ice / H₂O. The precipitate was filtered and recrystallized from EtOAc and 2-(4-cyano-2-methoxy-5-nitrophenoxy)ethyl acetate (67) (0.4 g, 1.6 mmol, 74% yield) was obtained as a white solid.

³¹H NMR (400 MHz, Chloroform-d): δ 7.83 (s, 1H, Ar-H), 7.22 (s, 1H, Ar-H), 5.13 (s, 2H), 4.50 (dd, J = 5.5, 3.8 Hz, 2H, CH₂-OAc), 4.40 – 4.28 (m, 2H, CH₂-OAr), 4.01 (s, 3H, CH₃-OAr), 2.10 (s, 3H, CH₃Ac).

The analytical data were in accordance with previously reported data.

³¹H NMR spectrum for 67:
Experimental part

2-(5-Amino-4-cyano-2-methoxyphenoxy)ethyl acetate (68)

Pd(OAc)$_2$ (0.23 g, 1.0 mmol, 5 mol%) was added to a solution of 2-(4-cyano-2-methoxy-5-nitrophenoxy)ethyl acetate (67) (5.61 g, 40.0 mmol, 1.0 eq) in dry and degassed THF (100 mL), followed by a 1M KF solution in H$_2$O (40 mL, 80.0 mmol, 2.0 eq). PHMS (4.8 mL, 80.0 mmol, 4.0 eq) was slowly added and the reaction mixture was stirred at r.t. for 30 min. The solution was diluted with Et$_2$O (200 mL) and H$_2$O (160 mL). The aqueous layer was extracted with Et$_2$O (3 X 200 mL). The combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure. The crude product was purified via column chromatography eluting with a mixture of EtOAc. 2-(5-Amino-4-cyano-2-methoxyphenoxy)ethyl acetate (68) (10.02 g, 40.0 mmol, quant.) was obtained as brown solid.

$^1$H NMR (400 MHz, Chloroform-d): δ 6.82 (s, 1H, Ar-H), 6.27 (s, 1H, Ar-H), 4.45 (t, $J = 4.9$ Hz, 3H, CH$_2$-OAc), 4.20 (t, $J = 4.9$ Hz, 3H, CH$_2$-OAr), 3.79 (s, 3H, CH$_3$-OAr), 2.10 (s, 3H, CH$_3$Ac).

The analytical data were in accordance with previously reported data.

$^1$H NMR spectrum for 68:
Experimental part

\((E)\)-2-(4-Cyano-5-(((dimethylamino)methylene)amino)-2-methoxyphenoxy)ethyl acetate (69)

To a solution of 2-(5-amino-4-cyano-2-methoxyphenoxy)ethyl acetate (68) (10.02 g, 20.0 mmol, 1.0 eq) in toluene (120 mL) was added DMF-DMA (10.6 mL, 80.0 mmol, 2.0 eq) and AcOH (0.7 mL, 12.0 mmol, 0.3 eq). The reaction mixture was refluxed for 2 h. The mixture was cooled down to r.t. and concentrated under reduced pressure. The resulting oil was triturated with heptane and sonicated. The precipitate was filtered and washed with heptane. \((E)\)-2-(4-Cyano-5-(((dimethylamino)methylene)amino)-2-methoxyphenoxy)ethyl acetate (69) (9.45 g, 30.9 mmol, 77% yield) was obtained as yellow solid.

\(^1\)H NMR (400 MHz, Chloroform-\(d\)): \(\delta\) 7.58 (d, \(J = 2.8\) Hz, 1H, \(CH=\text{N-Ar}\)), 6.95 (d, \(J = 2.5\) Hz, 1H, Ar-H), 6.54 (s, 1H, Ar-H), 4.50 – 4.40 (m, 2H, \(CH_2\)-OAc), 4.24 (p, \(J = 3.2\) Hz, 2H, \(CH_2\)-OAr), 3.83 (d, \(J = 2.5\) Hz, 3H, \(CH_3\)-OAr), 3.08 (s, 6H, 2 X RN-\(CH_3\)), 2.09 (d, \(J = 2.6\) Hz, 3H, \(CH_3\)Ac).

The analytical data were in accordance with previously reported data. 350

\(^1\)H NMR spectrum for 69:
2-((4-((3-Ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethyl acetate (70)

3-Ethynylaniline (4.2 mL, 37.1 mmol, 1.2 eq) was added to a solution of (E)-2-(4-cyano-5-(((dimethylamino)methylene)amino)-2-methoxyphenoxy)ethyl acetate (69) (9.45 g, 30.9 mmol, 1.0 eq) in AcOH (80 mL). The reaction mixture was stirred at 130°C for 2 h. The mixture was cooled down to r.t. and concentrated under reduced pressure. The residue was recrystallized from toluene to afford 2-((4-((3-ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethyl acetate (70) (6.58 g, 17.4 mmol, 56% yield) as yellow solid.

$^1$H NMR (400 MHz, Chloroform-d): $\delta$ 8.68 (s, 1H, Ar-H (2)), 7.84 (s, 1H, Ar-H (2')), 7.77 (d, $J = 8.2$ Hz, 1H, Ar-H (6')), 7.37 (t, $J = 7.9$ Hz, 1H, Ar-H (5')), 7.30 – 7.26 (m, 2H, Ar-H (8) and Ar-H (4')), 7.12 (s, 1H, NH), 7.01 (s, 1H, Ar-H (5)), 4.59 – 4.47 (m, 2H, CH$_2$-OAc), 4.38 (t, $J = 4.8$ Hz, 2H, CH$_2$-OAr), 4.03 (s, 3H, CH$_3$-OAr), 3.11 (s, 1H, CH≡C-Ar), 2.12 (s, 3H, CH$_3$-Ac).

The analytical data were in accordance with previously reported data.$^{350}$

$^1$H NMR spectrum for 70:
Experimental part

2-((4-((3-Ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethan-1-ol (71)

K₂CO₃ (12.0 g, 87.1 mmol, 5.0 eq) was added to a solution of 2-((4-((3-ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethyl acetate (70) (6.58 g, 17.4 mmol, 1.0 eq) was dissolved in MeOH (400 mL). The reaction mixture was stirred at r.t. for 20 min and diluted with H₂O (100 mL). MeOH was evaporated under reduced pressure, the precipitate was filtered and washed with H₂O. 2-((4-((3-Ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethan-1-ol (71) (5.7 g, 16.9 mmol, 98% yield) was obtained as a yellow solid.

¹H NMR (400 MHz, DMSO-d₆): δ 9.47 (s, 1H, NH), 8.44 (s, 1H, Ar-H (2)), 7.94 (d, J = 2.2 Hz, 1H, Ar-H (2')), 7.85 (dd, J = 8.1, 2.1 Hz, 1H, Ar-H (6')), 7.79 (s, 1H, Ar-H (8)), 7.36 (t, J = 7.9 Hz, 1H, Ar-H (5')), 7.16 (d, J = 6.6 Hz, 2H, Ar-H (4') and Ar-H (5)), 4.92 (s, 1H, OH), 4.14 (s, 1H, CH≡C-Ar), 4.11 (t, J = 4.9 Hz, 2H, CH₂-OAr), 3.92 (s, 3H, CH₂-OAr), 3.76 (t, J = 4.9 Hz, 2H, CH₂-OH).

The analytical data were in accordance with previously reported data.³⁵⁰

¹H NMR spectrum for 71:
Experimental part

2- ((4-((3-Ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethyl methanesulfonate (72)

Mesyl chloride (0.12 mL, 1.49 mmol, 5.0 eq) was added dropwise to a stirred solution 2-((4-((3-ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethan-1-ol (71) (100.0 mg, 0.30 mmol, 1.0 eq) and DMAP (18.2 mg, 0.15 mmol, 0.5 eq) in dry pyridine (5 mL) at 0°C. The reaction mixture was allowed to warm up to r.t. and stirred for 5 h under argon atmosphere. The completion of the reaction was monitored by ESI-MS. The reaction mixture was quenched by addition of sat. NaHCO₃ solution (15 mL) and extracted with EtOAc (5 X 30 mL). The combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure to give 2-((4-((3-ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethyl methanesulfonate (72) (114.8 mg, 0.28 mmol, 93% yield) as a yellow solid.

1H NMR (400 MHz, DMSO-d₆): δ 9.53 (s, 1H, NH), 8.51 (s, 1H, CH₆(2)), 8.00 (d, J = 5.0 Hz, 1H, CH₆(2')), 7.91 (d, J = 8.1 Hz, 1H, CH₆(6')), 7.87 (s, 1H, CH₆(8)), 7.41 (t, J = 7.9 Hz, 1H, CH₆(5')), 7.26 (s, 1H, CH₆(5)), 7.21 (d, J = 7.7 Hz, 1H, CH₆(4')), 4.64 (dt, J = 4.2, 2.2 Hz, 2H, CH₂-OMs), 4.45 (dd, J = 5.4, 2.8 Hz, 2H, CH₂-OAr), 4.20 (s, 1H, H-C≡C-Ar), 3.98 (s, 3H, CH₃-OAr), 3.29 (s, 3H, CH₃mesyl).

13C NMR (101 MHz, DMSO-d₆): δ 156.1 (C Ar), 152.8 (C Ar), 152.8 (C Ar), 148.8 (C Ar), 146.9 (C Ar), 139.8 (C Ar), 128.9 (CH Ar), 126.4 (CH Ar), 124.8 (CH Ar), 122.6 (CH Ar), 121.7 (C Ar), 109.2 (C Ar), 108.2 (CH Ar), 102.2 (CH Ar), 83.5 (C alkyn), 80.6 (CH alkyn), 68.6 (CH₂), 66.6 (CH₂), 56.3 (CH₃), 36.9 (CH₃).

IR (νmax, cm⁻¹): 2921 (s), 2851 (s), 1613 (s), 1505 (s), 1458 (s), 1427 (s), 1330 (s), 1249 (s), 1168 (s), 978 (s), 926 (s), 809 (s).

HRMS (ESI/QTOF) m/z: [M + H]⁺ Calcd for C₂₀H₂₀N₃O₅S⁺ 414.1118; Found 414.1112.
$^1$H NMR and $^{13}$C spectra for 72:
Experimental part

\[ N-(3-\text{Ethynylphenyl})-7-(2-\text{iiodoethoxy})-6-\text{methoxyquinazolin}-4-\text{amine} \ (73) \]

\[ \text{2-}((4-((3-\text{Ethynylphenyl})\text{amino})-6-\text{methoxyquinazolin-7-yl} \text{oxy})\text{ethyl methanesulfonate} \ (72) \ \text{and NaI} \ (0.45 \ \text{g}, \ 2.53 \ \text{mmol}, \ 10.0 \ \text{eq}) \ \text{were dissolved in acetone} \ (5 \ \text{mL}) \ \text{in a sealed tube and the reaction mixture was stirred at} \ 80^\circ \text{C for} \ 16 \ \text{h}. \ \text{The mixture was filtered, the solvent was removed under reduced pressure and the crude was purified via column chromatography eluting with EtOAc}. \ \text{N-}((3-\text{Ethynylphenyl})-7-(2-\text{iiodoethoxy})-6-\text{methoxyquinazolin-4-amine} \ (73) \ (92.8 \ \text{mg}, \ 0.21 \ \text{mmol}, \ 68\% \ \text{yield}) \ \text{was obtained as a yellow solid.} \]

\[ \text{\textsuperscript{1}H NMR} \ (400 \ \text{MHz, Chloroform-}d): \ \delta \ 8.60 \ (s, \ 1H, CH_{Ar}(2)), \ 7.86 \ (t, \ J = 1.8 \ Hz, \ 1H, CH_{Ar}(2^{''})), \ 7.77 \ (dt, \ J = 8.2, 1.5 \ Hz, \ 1H, CH_{Ar}(6^{''})), \ 7.35 \ (t, \ J = 7.9 \ Hz, \ 1H, CH_{Ar}(5^{''})), \ 7.31 - 7.27 \ (m, \ 1H, CH_{Ar}(4^{''})), \ 7.24 \ (s, \ 1H, CH_{Ar}(8)), \ 7.17 \ (s, \ 1H, CH_{Ar}(5)), \ 4.40 \ (t, \ J = 7.1 \ Hz, \ 2H, CH_{2}-OAr), \ 4.04 \ (s, \ 3H, CH_{3}-OAr), \ 3.55 - 3.46 \ (m, \ 2H, CH_{2}-I), \ 3.10 \ (s, \ 1H, HC≡C-Ar). \]

\[ \text{\textsuperscript{13}C NMR} \ (101 \ \text{MHz, Chloroform-}d): \ \delta \ 168.5 \ (C_{Ar}), \ 156.3 \ (C_{Ar}), \ 153.6 \ (CH_{Ar}), \ 150.0 \ (C_{Ar}), \ 143.7 \ (C_{Ar}), \ 138.4 \ (C_{Ar}), \ 129.2 \ (CH_{Ar}), \ 128.4 \ (CH_{Ar}), \ 125.5 \ (CH_{Ar}), \ 123.1 \ (CH_{Ar}), \ 122.8 \ (C_{Ar}), \ 108.1 \ (CH_{Ar}), \ 100.6 \ (CH_{Ar}), \ 83.3 \ (C_{alkyne}), \ 77.8 \ (CH_{alkyne}), \ 69.6 \ (CH_{2}), \ 57.0 \ (CH_{2}), -0.5 \ (CH_{2}). \]

\[ \text{IR} \ (\nu_{\text{max}} \ \text{cm}^{-1}): \ 3428 \ (m), \ 2390 \ (s), \ 1631 \ (m), \ 1504 \ (m), \ 1435 \ (m), \ 1245 \ (s), \ 1220 \ (m), \ 979 \ (m), \ 878 \ (m), \ 815 \ (s). \]

\[ \text{HRMS (ESI/QTOF) m/z: [M + H]}^+ \ \text{Calcd for C}_{19}\text{H}_{17}\text{IN}_{3}\text{O}_{2}^+ \ 446.0360; \ \text{Found} \ 446.0360. \]

\* One carbon is not resolved (C_{Ar}).
**Experimental part**

$^1$H NMR and $^{13}$C spectra for 73:
Experimental part

7-(2-Aminoethoxy)-N-(3-ethynylphenyl)-6-methoxyquinazolin-4-amine (74)

N-(3-Ethynylphenyl)-7-(2-iodoethoxy)-6-methoxyquinazolin-4-amine (73) (92.8 mg, 0.21 mmol, 1.0 eq) was dissolved in a 7N ammonia in methanol solution (3 mL) in a sealed tube. The reaction mixture was stirred at 85°C for 16 h. The solvent was removed under reduced pressure to afford 7-(2-aminoethoxy)-N-(3-ethynylphenyl)-6-methoxyquinazolin-4-amine (74) (70.2 mg, 0.21 mmol, quant.) as a yellow solid.

\( ^1 \text{H NMR} \) (400 MHz, Methanol-\( \text{d}_4 \)): \( \delta \) 8.51 (s, 1H, \( \text{CH}_\text{Ar}(2) \)), 7.94 (t, \( J = 1.9 \text{ Hz} \), 1H, \( \text{CH}_\text{Ar}(2') \)), 7.90 (s, 1H, \( \text{CH}_\text{Ar}(5) \)), 7.80 (dd, \( J = 8.2, 2.3 \text{ Hz} \), 1H, \( \text{CH}_\text{Ar}(6') \)), 7.41 (t, \( J = 7.9 \text{ Hz} \), 1H, \( \text{CH}_\text{Ar}(5') \)), 7.31 (dt, \( J = 7.6, 1.3 \text{ Hz} \), 1H, \( \text{CH}_\text{Ar}(4') \)), 7.26 (s, 1H, \( \text{CH}_\text{Ar}(8) \)), 4.49 – 4.40 (m, 2H, \( \text{CH}_2\text{-OAr} \)), 4.12 (s, 3H, \( \text{CH}_3\text{-OAr} \)), 3.55 (s, 1H, \( \text{H}_\text{C≡C-Ar} \)), 3.53 – 3.47 (m, 2H, \( \text{CH}_2\text{-NH}_2 \)).

\( ^{13} \text{C NMR} \) (101 MHz, Methanol-\( \text{d}_4 \)): \( \delta \) 160.1 (\( \text{C}_\text{Ar} \)), 155.9 (\( \text{C}_\text{Ar} \)), 153.4 (\( \text{C}_\text{Ar} \)), 152.6 (\( \text{CH}_\text{Ar} \)), 149.6 (\( \text{C}_\text{Ar} \)), 141.0 (\( \text{C}_\text{Ar} \)), 128.6 (\( \text{CH}_\text{Ar} \)), 127.7 (\( \text{CH}_\text{Ar} \)), 125.9 (\( \text{CH}_\text{Ar} \)), 123.1 (\( \text{CH}_\text{Ar} \)), 122.9 (\( \text{CH}_\text{Ar} \)), 121.2 (\( \text{C}_\text{Ar} \)), 107.1 (\( \text{CH}_\text{Ar} \)), 101.9 (\( \text{CH}_\text{Ar} \)), 82.8 (\( \text{C}_\text{alkyne} \)), 77.4 (\( \text{CH}_\text{alkyne} \)), 65.2 (\( \text{CH}_2 \)), 55.7 (\( \text{CH}_3 \)), 38.7 (\( \text{CH}_3 \)).

IR (\( \nu_{\text{max}}, \text{cm}^{-1} \)): 3453 (s), 2923 (s), 1624 (s), 1526 (s), 1509 (s), 1453 (s), 1431 (s), 1280 (s), 1244 (s), 1155 (s), 993 (s).

HRMS (ESI/QTOF) m/z: \([\text{M} + \text{H}]^+\) Calcd for \( \text{C}_{19}\text{H}_{19}\text{N}_4\text{O}_2^+ \) 335.1503; Found 335.1511.

\( ^1 \text{H NMR} \) and \( ^{13} \text{C} \) spectra for 74:

\( ^1 \text{H NMR} \) and \( ^{13} \text{C} \) spectra for 74:
DIBO functionalized CM-erlotinib derivative:

Scheme 49: Synthesis of 77: i) DIPEA, DMF, r.t., 16 h; ii) LiOH, MeOH / H₂O (5:1), r.t., 6 h; iii) 63, HOBt, DMAP, EDCI, DIPEA, DMF, r.t., 24 h.
Experimental part

Ethyl \( N \)-ethyl-\( N \)-\{4-\{(2-\{(3-ethynylphenyl)amino\}-6-methoxyquinazolin-7-yl\}oxy\}ethyl\}carbamoyl)oxy)methyl\}-2-oxo-2\( H \)-chromen-7-yl\}glycinate (75)

![Chemical Structure](image)

\[ \text{34} \text{ (100.0 mg, 0.21 mmol, 1.0 eq) and erlotinib-NH}_2 \text{ 74 (71.2 mg, 0.21 mmol, 1.0 eq) were dissolved in dry DMF (10 mL). DIPEA (0.15 mL, 0.85 mmol, 4.0 eq) was added and the reaction mixture was stirred at r.t. for 16 h under argon atmosphere and dark conditions. The solvent was removed under reduced pressure and the crude was purified via column chromatography eluting with a mixture of PE / EtOAc (5:5 to 0:1) to afford 75 (139.1 mg, 0.21 mmol, 99% yield) as a yellow solid.} \]

\( ^1 \text{H NMR (400 MHz, DMSO-}d_6) \): \( \delta \) 9.60 (s, 1H, \( \text{NH} \text{erlotinib} \)), 8.49 (s, 1H, \( \text{CH}_2(2) \)), 8.00 (d, \( J = 1.9 \text{ Hz} \), 1H, \( \text{CH}Ar(2') \)), 7.95 – 7.87 (m, 2H, \( \text{CH}Ar(5) \) and \( \text{CH}Ar(6') \)), 7.81 (t, \( J = 5.6 \text{ Hz} \), 1H, \( \text{NH carbamate} \)), 7.51 – 7.46 (m, 1H, coumarin-H), 7.40 (t, \( J = 7.8 \text{ Hz} \), 1H, \( \text{CH}Ar(5') \)), 7.20 (dd, \( J = 16.9, 9.5 \text{ Hz} \), 2H, \( \text{CH}Ar(4') \) and \( \text{CH}Ar(8') \)), 6.64 (dd, \( J = 9.1, 2.6 \text{ Hz} \), 1H, coumarin-H), 6.52 (d, \( J = 2.5 \text{ Hz} \), 1H, coumarin-H), 6.05 (s, 1H, coumarin-H), 5.25 (s, 2H, \( \text{CH}_2 \text{coumarin} \)), 4.28 (s, 2H, \( \text{R}_2\text{N-CH}_2\text{-CO}_2\text{Et} \)), 4.22 (t, \( J = 5.5 \text{ Hz} \), 2H, \( \text{RCO}_2\text{NH-CH}_2\text{-CH}_2\text{-OAr} \)), 4.19 (s, 1H, \( \text{H} \text{C}\text{e}\text{C-Ar} \)), 4.12 (q, \( J = 7.2 \text{ Hz} \), 2H, \( \text{CH}_2 \text{ester} \)), 3.97 (s, 3H, \( \text{CH}_3 \text{-OAr} \)), 3.50 (d, \( J = 5.0 \text{ Hz} \), 4H, RRN-\( \text{CH}_2 \text{-CH}_3 \) and \( \text{RCO}_2\text{NH-CH}_2\text{-CH}_2\text{-OAr} \)), 1.28 – 1.17 (m, 3H, \( \text{R}_2\text{N-CH}_2\text{-CH}_2 \)), 1.11 (t, \( J = 7.0 \text{ Hz} \), 3H, \( \text{CH}_2 \text{ester} \)).

\( ^{13} \text{C NMR (101 MHz, DMSO-}d_6) \): \( \delta \) 170.5 (\( \text{CO}_2\text{Et} \)), 161.1 (\( \text{CO coumarin} \)), 156.6 (\( \text{C erlo} \)), 155.8 (\( \text{C coumarin} \)), 153.8 (\( \text{C erlo} \)), 153.2 (\( \text{CH erlo} \)), 152.2 (\( \text{CO carbamate} \)), 151.4 (\( \text{C coumarin} \)), 149.5 (\( \text{C erlo} \)), 147.4 (\( \text{C erlo} \)), 140.3 (\( \text{C erlo} \)), 129.3 (\( \text{CH erlo} \)), 126.8 (\( \text{CH erlo} \)), 125.7 (\( \text{CH coumarin} \)), 125.2 (\( \text{CH erlo} \)), 123.1 (\( \text{CH erlo} \)), 122.2, 109.5 (\( \text{CH coumarin} \)), 108.5 (\( \text{CH erlo} \)), 106.7 (\( \text{CH coumarin} \)), 106.5 (\( \text{C coumarin} \)), 105.3 (\( \text{C erlo} \)), 102.7 (\( \text{CH erlo} \)), 98.0 (\( \text{CH coumarin} \)), 84.0 (\( \text{C alkyn} \)), 81.0 (\( \text{CH alkyn} \)), 67.6 (\( \text{CH erlo} \)), 61.5 (\( \text{CH}_2 \text{coumarin} \)), 61.1 (\( \text{CH}_2 \text{coumarin} \)), 56.8 (\( \text{CH}_3 \text{erlo} \)), 51.9 (\( \text{CH}_2 \text{coumarin} \)), 46.2 (\( \text{CH}_2 \text{coumarin} \)), 40.2 (\( \text{CH}_2 \text{erlo} \)), 14.6 (\( \text{CH}_3 \text{coumarin} \)), 12.5 (\( \text{CH}_3 \text{coumarin} \)).

\( IR (\nu_{max}, \text{cm}^{-1}) \): 3346 (s), 1960 (w), 1618 (m), 1701 (m), 1156 (w), 834 (m), 770 (m), 713 (m).

\( HRMS (ESI/QTOF) m/z \): [M + H]\(^+\) Calcd for \( \text{C}_{38}\text{H}_{36}\text{N}_{5}\text{O}_{8} \) 666.2558; Found 666.2566.

*Two carbons are not resolved (\( \text{C erlo} \) and \( \text{C coumarin} \)).
$^1$H NMR and $^{13}$C spectra for 75:
**Experimental part**

\(N\)-Ethyl-\(N\)-(4-(((2-((4-(3-ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethyl)carbamoyl)oxy)methyl)-2-oxo-2H-chromen-7-yl)glycine 76

75 (139.1 mg, 0.21 mmol, 1.0 eq) was dissolved in a mixture of LiOH (100 mg, 4.2 mmol, 20.0 eq) in MeOH / H\(_2\)O / DMF (8 mL, 5:1:1) and the reaction mixture was stirred at r.t. for 6 h under dark conditions. The solvent was removed and the reaction mixture was diluted with H\(_2\)O (50 mL) and 1M hydrochloric acid (8 mL). The aqueous layer was extracted with EtOAc (3 X 60 mL) and the combined organic layers were dried over MgSO\(_4\), filtered and concentrated under reduced pressure. The residue was purified via column chromatography eluting with a mixture of DCM / MeOH (9:1). 76 (118.7 mg, 0.19 mmol, 89\% yield) was obtained as a slightly yellow solid.

IR (\(\nu_{\text{max}}, \text{cm}^{-1}\)): 3296 (s), 2943 (s), 2860 (s), 2321 (s), 1694 (s), 1614 (s), 1530 (s), 1429 (s), 1243 (s), 1154 (s), 990 (s), 802 (s).

HRMS (ESI/QTOF) m/z: [M + H]\(^+\) Calcd for C\(_{34}\)H\(_{32}\)N\(_5\)O\(_8\) 638.2245; Found 638.2237.

CM-erlo 77

76 (25.0 mg, 39.2 \(\mu\)mol, 1.0 eq), DIBO-NH\(_2\) 63 (13.2 mg, 43.1 \(\mu\)mol, 1.1 eq), DMAP (2.4 mg, 19.6 \(\mu\)mol, 0.5 eq), HOBt (21.2 mg, 0.16 mmol, 4.0 eq), and EDCI (30.1 mg, 0.16 mmol, 4.0 eq) were dissolved in dry DMF (2.5 mL). DIPEA (2.7 \(\mu\)L, 0.16 mmol, 4.0 eq) was added and the reaction mixture was stirred at r.t. for 24 h under argon atmosphere and dark conditions. The solvent was removed under reduced pressure and the crude was purified by preparative TLC eluting with a mixture of DCM / MeOH (18:1). 77 (14.7 mg, 15.8 \(\mu\)mol, 40\% yield) was obtained as yellowish solid.

\(^1\)H NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) 9.54 (s, 1H, NH erlotinib), 9.45 (d, \(J = 7.9\) Hz, 1H, NH amide), 8.50 (d, \(J = 5.4\) Hz, 1H, CH\(_{\alpha\beta}(2)\)), 7.99 (d, \(J = 12.5\) Hz, 1H, CH\(_{\alpha\beta}(2')\)), 7.77 – 7.86 (m, 2H, CH\(_{\alpha\beta}(5)\) and CH\(_{\alpha\beta}(6')\)), 7.81 (d, \(J = 8.4\) Hz, 1H, CH \(\text{Ar-cyclooctyne}\)), 7.60 (dt, \(J = 11.5, 5.7\) Hz, 1H, NH carbamate), 7.56 – 7.49 (m, 1H, coumarin-H and CH \(\text{Ar-cyclooctyne}\)), 7.47 – 7.27 (m, 7H, CH\(_{\alpha\beta}(5')\) and 6 X CH \(\text{Ar-cyclooctyne}\)), 7.26 – 7.18 (m, 2H, CH\(_{\alpha\beta}(4')\) and CH\(_{\alpha\beta}(8')\)), 6.56 (dd, \(J = 21.0, 8.6\) Hz, 1H, coumarin-H), 6.51 – 6.40 (m, 1H, coumarin-H), 6.04 (d, \(J = 3.1\) Hz, 1H, coumarin-H), 5.27 (m, 2H, NH carbamate and CH \(\text{DIBO}\)), 5.23 (s, 2H, CH\(_2\) coumarin), 4.39 (t, \(J = 5.6\) Hz, 1H, RCO\(_2\)NH-CH\(_2\)-CH\(_2\)-OAr), 4.23 (t, \(J = 5.9\) Hz, 1H, RCO\(_2\)NH-CH\(_2\)-CH\(_2\)-OAr), 4.20 (d, \(J = 5.1\) Hz, 1H, CH alkene), 3.97 (s, 3H, CH\(_3\)-O), 3.96 – 3.84 (m, 4H, R\(_2\)N-CH\(_2\)-CONHR and RCO\(_2\)NH-CH\(_2\)-CH\(_2\)-OAr), 3.58 – 3.47 (m, 2H, R\(_2\)N-CH\(_2\)-CH\(_3\)), 3.17 (q, \(J = 6.3, 5.6\) Hz, 3H, RCONH-)
Experimental part

CH₂-CH₂-NHCO₂R and H-CH_DIBO, 3.06 (q, J = 7.6, 6.8 Hz, 2H, RCONH-CH₂-CH₂-NHCO₂R), 2.75 (dd, J = 15.0, 4.4 Hz, 1H, H-CH_DIBO), 1.06 (t, J = 7.0 Hz, 1H, R₂N-CH₂-CH₃).

¹³C NMR (101 MHz, DMSO-d₆): δ 171.2 (CO amide), 169.4 (CO amide), 159.8 (CO coumarin), 156.6 (C erlo), 155.8 (C coumarin), 155.8 (C erlo), 155.8, 153.2 C erlo, 152.1 (CH erlo), 151.3 (C coumarin), 149.5 (C DIBO), 142.8 (C erlo), 146.1 (C erlo), 140.3 (C erlo), 129.3 (CH erlo), 128.9 (CH DIBO), 127.8 (CH DIBO), 127.8 (CH DIBO), 126.5 (CH erlo), 126.2 (CH erlo), 125.2 (CH erlo), 124.3 (CH coumarin), 123.9 (CH DIBO), 123.4 (CH DIBO), 123.0 (CH erlo), 122.2 (CH DIBO), 120.8 (C DIBO), 119.0 (C DIBO), 113.0 (CH coumarin), 109.5 (CH coumarin), 108.5 (CH erlo), 107.1 (C coumarin), 105.6 (CH coumarin), 102.6 (CH erlo), 98.0 (CH coumarin), 92.0 (C alkyl DIBO), 84.0 (C alkyl erlo), 81.0 (CH alkene), 75.9 (CH DIBO), 68.8 (CH₂ erlo), 61.9 (CH₂ coumarin), 56.6 (CH₃ erlo), 53.5 (CH₂ coumarin), 46.1 (CH₂ coumarin), 45.9 (CH₂ DIBO), 40.9 (CH₂ erlo), 40.7 (CH₂ DIBO), 39.1 (CH₂ DIBO), 12.1 (CH₃ coumarin).*

IR (νmax cm⁻¹): 2955 (s), 2918 (s), 1704 (s), 1657 (s), 1619 (s), 1575 (s), 1531 (s), 1423 (s), 1258 (s), 1084 (s), 1019 (s), 793 (s), 770 (s).

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: [M + H]⁺ Calcd for C₅₃H₄₈N₇O₉ 926.3508; Found 926.3539.

*Five carbons are not resolved (Cq alkyl DIBO, CO carbamate, C erlo, C coumarin and C DIBO).

¹H NMR and ¹³C spectra for 77:
Synthesis of CM-DOX 62:

Scheme 50: Synthesis of CM-DOX 62: i) 63, HOBt, EDCI, DIPEA, DMF, r.t., 16 h; ii) 4-nitrophenyl chloroformate, DIPEA, DMF, r.t., 6 h; then DOX HCl, DIPEA, r.t., 16 h.
Experimental part

DIBO-CM 61

![Chemical Structure](image)

33 (0.24 g, 0.87 mmol, 1.0 eq), DIBO-NH2 63 (0.28 g, 0.91 mmol, 1.05 eq), HOBT (0.24 g, 1.74 mmol, 2.0 eq) and EDCI (0.33, 1.74 mmol, 2.0 eq) were dissolved in dry DMF (22 mL). DIPEA (0.3 mL, 1.74 mmol, 2.0 eq) was added and the reaction mixture was stirred at r.t. for 16 h under argon atmosphere. The solvent was removed under reduced pressure and the crude mixture was purified by column chromatography eluting with a mixture of DCM / MeOH (50:1 to 25:1). 61 (0.29 g, 0.51 mmol, 59% yield) was obtained as a yellow solid.

$^1$H NMR (400 MHz, Chloroform-d): δ 7.45 (d, $J = 7.6$ Hz, 1H, Ar-H$_{DIBO}$), 7.38 – 7.18 (m, 7H, 7 X Ar-H$_{DIBO}$), 6.23 (dd, $J = 9.0$, 2.5 Hz, 1H, coumarin-H), 6.05 (s, 1H, coumarin-H), 5.57 (t, $J = 5.6$ Hz, 1H, N-H), 5.38 (t, $J = 2.8$ Hz, 1H, CH$_{DIBO}$), 4.58 – 4.37 (m, 2H, CH$_2$coumarin), 4.37 (s, 1H, OH), 2.97 (s, 1H, O-H), 2.86 (dd, $J = 15.1$, 3.8 Hz, 1H, HC-H$_{DIBO}$), 0.99 (t, $J = 7.0$ Hz, 3H, N$_2$-CH$_2$-CH$_3$).

$^{13}$C NMR (101 MHz, Chloroform-d): δ 170.5 (C=Oamide), 162.4 (C=Ocoumarin), 156.6 (C=Ocarbamate), 155.4 (C=Ocoumarin), 154.9 (C=Ocoumarin), 152.0 (C=Ar-DIBO), 151.1 (C=Ar-DIBO), 150.5 (C=Ar-DIBO), 148.3 (CH$_{Ar-DIBO}$), 128.8 (CH$_{Ar-DIBO}$), 127.4 (CH$_{Ar-DIBO}$), 127.3 (CH$_{Ar-DIBO}$), 126.4 (CH$_{Ar-DIBO}$), 126.1 (CH$_{Ar-DIBO}$), 124.5 (CH$_{coumarin}$), 123.8 (CH$_{Ar-DIBO}$), 123.8 (CH$_{Ar-DIBO}$), 123.8 (CH$_{Ar-DIBO}$), 121.4 (C=Ar-DIBO), 121.4 (C=Ar-DIBO), 109.3 (CH$_{coumarin}$), 107.8 (C=Ar-DIBO), 106.5 (CH$_{coumarin}$), 99.0 (C=Ar-DIBO), 92.1 (Cq_alkyne), 92.1 (Cq_alkyne), 77.3 (CH$_{DIBO}$), 60.5 (CH$_2$coumarin), 54.6 (CH$_2$coumarin), 46.2 (CH$_2$DIBO), 46.0 (CH$_2$coumarin), 41.2 (CH$_2$), 40.2 (CH$_2$), 11.4 (CH$_3$coumarin).

IR ($\nu_{\text{max}}$, cm$^{-1}$): 2928 (m), 1715 (s), 1609 (m), 1531 (m), 1510 (m), 1450 (m), 1412 (m), 1294 (s), 1225 (s), 1163 (s), 1111 (s), 1042 (m), 830 (m).

HRMS (ESI/QTOF) m/z: [M + H]$^+$ Calcd for C$_{33}$H$_{32}$N$_3$O$_6$: 566.2286; Found 566.2298.
$^1$H NMR and $^{13}$C spectra for 61:
Experimental part

CM-DOX 62

61 (38.1 mg, 67.3 μmol, 1.0 eq) and 4-nitrophenyl chloroformate (20.3 mg, 0.10 mmol, 1.5 eq) were dissolved in dry DMF (2 mL) and DIPEA (47 μL, 0.26 mmol, 4.0 eq) was added. The reaction mixture was stirred at r.t. for 6 h under argon atmosphere and dark conditions. Then DOX HCl (42.9 mg, 74.0 μmol, 1.1 eq) and DIPEA (24 μL, 0.13 mmol, 2.0 eq) were added and the reaction mixture was stirred at r.t. for 16 h. The solvent was removed under reduced pressure and the crude was purified by preparative TLC eluting with a mixture of DCM / MeOH (18:1). 62 (13.9 mg, 12.2 μmol, 18% yield) as a red solid.

1H NMR (400 MHz, DMSO-d6): δ 13.23 (d, J = 14.8 Hz, 1H, OH), 8.02 (s, 1H, NH carbamate), 7.88 (s, 2H, CHα(5) and CHα(4)), 7.66 – 7.57 (m, 2H, CHα(3) and NH amide), 7.51 (d, J = 8.0 Hz, 1H, CH DIBO), 7.45 – 7.30 (m, 8H, coumarin-H and 7 X CH DIBO), 7.24 (d, J = 8.1 Hz, 1H, NH carbamate), 6.54 (d, J = 9.3 Hz, 1H, coumarin-H), 6.46 (s, 1H, coumarin-H), 6.05 (s, 1H, coumarin-H), 5.46 (s, 1H, OH), 5.30 (s, 1H, OH), 5.25 (s, 2H, CH(1') and CH DIBO), 5.13 (s, 2H, CH2 coumarin), 4.94 (s, 1H, CH(14)), 4.85 (d, J = 5.8 Hz, 1H, OH), 4.81 (d, J = 5.9 Hz, 1H, OH), 4.58 (d, J = 6.1 Hz, 2H, HO-CH2-C=OR), 4.20 – 4.14 (m, 1H, CH(5')), 3.97 (d, J = 4.1 Hz, 3H, OCH3 DOX), 3.92 (s, 2H, R2N-CH2-CO(CH2)3), 3.75 (s, 1H, CH(3')), 3.47 (s, 1H, CH(4')), 3.40 (d, J = 7.3 Hz, 2H, R2N-CH2-CH3), 3.15 (d, J = 13.2 Hz, 3H, RCONH-CH2-CH2-NHCOR and HC-H DIBO), 3.03 (d, J = 14.5 Hz, 2H, RCO2NH-CH2-CH2-NHCOR), 2.96 - 2.89 (m, 2H, CH2(11)), 2.72 – 2.65 (m, 1H, HC-H DIBO), 2.21 (d, J = 12.2 Hz, 1H, HC-H(13)), 2.14 – 2.05 (m, 1H, HC-H(13)), 1.99 – 1.85 (m, 2H, CH2(2')), 1.14 (d, J = 6.4 Hz, 3H, C(5')-CH3), 1.04 (t, J = 6.9 Hz, 3H, R3N-CH2-CH3).

13C NMR (101 MHz, DMSO-d6): δ 214.2 (CO), 187.0 (CO), 186.9 (CO amide), 161.2 (C Ar DOX), 160.4 (CO coumarin), 158.2 (C Ar DOX), 156.6 (C coumarin), 155.8 (C coumarin), 155.1 (C Ar DIBO), 154.9 (C Ar DIBO), 151.5 (C coumarin), 151.3 (CO carbamate), 137.1 (CH Ar DOX), 136.0 (C Ar DOX), 135.1 (C Ar DOX), 134.5 (C Ar DOX), 130.6 (CH Ar DOX), 128.9 (CH Ar DOX), 128.8 (CH Ar DOX), 127.8 (CH Ar DOX), 127.7 (CH Ar DIBO), 126.5 (CH Ar DIBO), 126.2 (CH Ar DIBO), 126.2 (CH coumarin), 124.6 (CH Ar DIBO), 124.3 (C Ar DIBO), 120.7 (C Ar DIBO), 120.2 (CH Ar DOX), 119.4 (C Ar DOX), 119.4 (C Ar DOX), 111.2 (C Ar DOX), 111.1 (C Ar DOX), 110.3 (C coumarin) 106.4 (C coumarin), 105.4 (CH coumarin), 100.9 (CH DOX), 98.0 (CH coumarin), 92.1 (Cq alkyne), 92.0 (Cq alkyne), 75.6 (Cq DOX), 75.4 (CH DIBO), 70.5 (CH DOX), 68.4 (CH DOX), 67.1 (CH DOX), 64.1 (CH2 DOX), 61.2 (CH2 coumarin), 57.0 (CH3 DOX), 53.4 (CH2 coumarin), 47.8 (CH DOX), 46.0 (CH2 coumarin), 45.9 (CH2 DIBO), 40.2 (CH2 DIBO), 39.2 (CH2 DIBO), 37.1 (CH2 DOX), 32.5 (CH2 DOX), 29.5 (CH2 DOX), 17.5 (CH3 DOX), 12.1 (CH3 coumarin).*

IR (vmax, cm⁻¹): 3371 (s), 2936 (s), 2658 (s), 1690 (s), 1613 (s), 1436 (s), 1393 (s), 1256 (s), 1179 (s), 1082 (s), 1015 (s), 793 (s).

HRMS (ESI/QTOF) m/z: [M + Na]⁺ Calcd for C61H58Na2NaO18⁺ 1157.3638; Found 1157.3634.

*Two carbons are not resolved (C Ar DOX and C carbamate).
$^1$H NMR and $^{13}$C spectra for 62:
Experimental part

Synthesis of CM-Cbl 79:

**Scheme 51: Synthesis of CM-Cbl 79 and DIBO-Cbl 80:** i) Cbl, HOBt, EDCI, DIPEA, DCM, r.t., 16 h; ii) Cbl, HOBt, EDCI, DIPEA, DCM, r.t., 16 h.

**CM-Cbl 78**

![Chemical structure of CM-Cbl 78](image)

**1H NMR** (400 MHz, Chloroform-d): δ 7.68 (d, J = 24.9 Hz, 1H, NH), 7.44 – 7.27 (m, 8H, 8 X Ar-H (DIBO)), 7.14 (d, J = 8.9 Hz, 1H, coumarin-H), 7.09 – 7.02 (m, 2H, 2 X Ar-H (Cbl)), 6.64 – 6.57 (m, 2H, 2 X Ar-H (Cbl)), 6.37 (d, J = 2.5 Hz, 1H, coumarin-H), 6.31 (dd, J = 9.0, 2.6 Hz, 1H, coumarin-H), 6.10 (s, 1H, coumarin-H), 5.48 (t, J = 5.8 Hz, 1H, NH), 5.29 (d, J = 3.4 Hz, 1H, CH (DIBO)), 5.09 (s, 2H, CH2 (coumarin)), 3.76 (d, J = 6.5 Hz, 2H, R2N-CH2-CONHR), 3.68 – 3.54 (m, 4H, 2 X C1-CH2-CH2-CHR2), 3.46 – 3.33 (m, 2H, ROCN-CH2-CH2-NHCO2R), 3.28 – 3.03 (m, 5H, ROCN-CH2-CH2-NHCO2R), R2N-CH2-CH3 and HC-H (DIBO), 2.85 (dd, J = 15.1, 4.0 Hz, 1H, HC-H (DIBO)), 2.58 (t, J = 7.5 Hz, 2H, ROOC-(CH2)2-CH3), 2.43 (t, J = 7.5 Hz, 2H, ROOC-(CH2)2-CH3), 1.96 (p, J = 7.5 Hz, 2H, ROOC-(CH2)2-CH3), 0.96 (t, J = 7.0 Hz, 3H, R3N-CH2-CH3).

**13C NMR** (101 MHz, Chloroform-d): δ 172.6 (CO (ester)), 170.0 (CO (amide)), 161.4 (CO (coumarin)), 156.6 (CO (carbamate)), 155.5 (C (coumarin)), 151.7 (C (Ar-DIBO)), 150.9 (C (Ar-DIBO)), 150.5 (C (coumarin)), 149.3 (C (coumarin)), 144.3 (2 X C (Cbl)), 130.1 (CH (Ar-DIBO)), 130.0 (2 X C (Cbl)), 129.7 (2 X CH (Cbl)), 128.0 (CH (Ar-DIBO)), 128.0 (CH (Ar-DIBO)), 127.2 (C (Ar-DIBO)), 127.1 (C (Ar-DIBO)), 126.3 (C (Ar-DIBO)), 126.0 (CH (Ar-DIBO)), 124.4 (C (coumarin)), 123.7 (C (Ar-DIBO)), 123.5 (C (Ar-DIBO)), 121.2 (C (Ar-DIBO)), 112.2 (2 X C (Cbl)), 109.3 (C (coumarin)), 107.6 (C (coumarin)), 107.6 (C (coumarin)), 99.1 (C (coumarin)), 77.2 (CH (DIBO)), 60.9
(CH₂ coumarin), 54.5 (CH₂ coumarin), 53.6 (2 X CH₂ Cbl), 46.0 (CH₂ coumarin), 45.8 (CH₂ DIBO), 41.0 (CH₂ DIBO), 40.5 (2 X CH₂ Cbl), 33.9 (CH₂ Cbl), 33.3 (CH₂ Cbl), 26.5 (CH₂ Cbl), 11.2 (CH₃ coumarin).*

IR (νmax, cm⁻¹): 3324 (s), 1656 (s), 1620 (s), 1487 (m), 1448 (m), 1319 (m), 1293 (m), 1245 (m), 1097 (w), 1017 (m), 807 (m), 698 (m).

HRMS (ESI/QTOF) m/z: [M + H]^+ Calcd for C₄₇H₄₉Cl₂N₄O₇+ 851.2973; Found 851.2959.

*Two carbons are not resolved (2 X Cq DIBO)

³¹H NMR and ¹³C spectra for 78:
Experimental part

DIBO-Cbl 80

DIBO 89 (20.0 mg, 90.8 μmol, 1.0 eq), Cbl (33.2 mg, 0.11 mmol, 1.2 eq), HOBt (24.5 mg, 0.18 mmol, 2.0 eq) and EDCI (34.8 mg, 0.18 mmol, 2.0 eq) were dissolved in dry DCM (2 ml). DIPEA (31.75 μL, 0.18 mmol, 2.0 eq) was added and the reaction mixture was stirred at r.t for 16 h under argon atmosphere and dark conditions. The solvent was removed and the crude mixture was purified by preparative TLC eluting with a mixture of DCM / PE (3:1). DIBO-Cbl 80 (8.9 mg, 17.6 μmol, 19%) was obtained as a slightly yellow oil.

$^1$H-NMR (400 MHz, Chloroform-\(d\)): δ 7.49 (d, \(J = 7.7\) Hz, 1H, CH\(_{Ar-DIBO}\)), 7.42 – 7.28 (m, 7H, 7 X CH\(_{Ar-DIBO}\)), 7.12 (d, \(J = 8.4\) Hz, 2H, 2 X CH\(_{Cbl}\)), 6.71 (d, \(J = 8.3\) Hz, 2H, 2 X CH\(_{Cbl}\)), 5.56 (s, 1H, CH\(_{DIBO}\)), 3.76 – 3.67 (m, 4H, 2 X R\(_2\)N-CH\(_2\)-CH\(_2\)-Cl), 3.63 (ddd, \(J = 8.3, 6.6, 1.8\) Hz, 4H, 2 X R\(_2\)N-CH\(_2\)-CH\(_2\)-Cl), 3.11 (d, \(J = 15.1\) Hz, 2H, H-CH\(_{DIBO}\)), 2.94 (dd, \(J = 15.1, 4.0\) Hz, 1H, H-CH\(_{DIBO}\)), 2.63 (t, \(J = 7.5\) Hz, 2H, Ar-CH\(_2\)-(CH\(_2\))\(_2\)-CO\(_2\)R), 2.54 (t, \(J = 7.5\) Hz, 2H, Ar-(CH\(_2\))\(_2\)-CH\(_2\)-CO\(_2\)R), 2.02 (p, \(J = 7.4\) Hz, 2H, Ar-CH\(_2\)-CH\(_2\)-CH\(_2\)-CO\(_2\)R).

$^{13}$C NMR (101 MHz, Chloroform-\(d\)): 172.0 (CO\(_{ester}\)), 151.1 (C\(_{Ar-DIBO}\)), 150.8 (C\(_{Ar-DIBO}\)), 140.9 (C\(_{Ar-Cbl}\)), 131.4 (C\(_{Ar-Cbl}\)), 130.1 (2 X CH\(_{Cbl}\)), 129.9 (CH\(_{Ar-DIBO}\)), 128.1 (CH\(_{Ar-DIBO}\)), 127.9 (CH\(_{Ar-DIBO}\)), 127.3 (C\(_{Ar-DIBO}\)), 127.2 (CH\(_{Ar-DIBO}\)), 126.4 (CH\(_{Ar-DIBO}\)), 126.0 (CH\(_{Ar-DIBO}\)), 123.8 (CH\(_{Ar-DIBO}\)), 123.7 (C\(_{Ar-DIBO}\)), 115.9 (2 X CH\(_{Cbl}\)), 90.5 (C\(_{q}\)), 88.0 (C\(_{q}\)), 76.3 (CH\(_{DIBO}\)), 55.0 (CH\(_{2-Cbl}\)), 55.0 (CH\(_{2-Cbl}\)), 46.5 (CH\(_{2-DIBO}\)), 39.4 (CH\(_{2-Cbl}\)), 39.3 (CH\(_{2-Cbl}\)), 34.2 (CH\(_{2-Cbl}\)), 33.8 (CH\(_{2-Cbl}\)), 26.7 (CH\(_{2}\)).
**Experimental part**

IR ($\nu_{\text{max}}$ cm$^{-1}$): 2953 (s), 2919 (s), 2850 (s), 1733 (s), 1614 (s), 1517 (s), 1449 (s), 1352 (m), 1247 (m), 1179 (m), 1143 (m), 1015 (w), 803 (m), 755 (m).

**HRMS (ESI/QTOF) m/z:** [M + H]$^+$ Calcd for C$_{30}$H$_{30}$Cl$_2$NO$_2$ 506.1648; Found 506.1648.

$^3$H NMR and $^{13}$C spectra for 80:
4.4 Coating and functionalization of HNPs

4.4.1 Coating of HNPs with APTES derivatives and functionalization with CM-cargo molecules

**Synthesis of azizo-modified APTES-N$_3$**

![Scheme 52: Synthesis of 52](image)

**Ethyl 4-azidobutanoate (49)**

Ethyl 4-bromobutanoate (2.2 mL, 15.4 mmol, 1.0 eq) and NaN$_3$ (2.0 g, 30.8 mmol, 2.0 eq) were dissolved in a mixture of MeOH / H$_2$O (4:1, 30 mL) and the reaction mixture was refluxed for 7 h. MeOH was removed under reduced pressure and the residue was diluted with H$_2$O (100 mL). The aqueous layer was extracted with DCM (3 X 100 mL). The combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure. Ethyl 4-azidobutanoate (49) (2.55 g, 15.4 mmol, quant.) was obtained as a colorless oil.

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 4.14 (q, $J = 7.1$ Hz, 2H, CH$_2$-ester), 3.35 (t, $J = 6.7$ Hz, 2H, EtCO$_2$-CH$_2$-R), 2.40 (t, $J = 7.2$ Hz, 2H, R-CH$_2$-N$_3$), 1.91 (p, $J = 7.0$ Hz, 2H, R-CH$_2$-CH$_2$-N$_3$), 1.26 (t, $J = 7.1$ Hz, 3H, CH$_3$ ester).

The analytical data were in accordance with previously reported data.
Experimental part

$^1$H NMR spectrum for 49:

4-Azidobutanoic acid (50)

KOH (4.32 g, 77.0 mmol, 5.0 eq) was added to a solution of ethyl 4-azidobutanoate (49) (2.55 g, 15.4 mmol, 1.0 eq) in a mixture of MeOH / H$_2$O (55:45, 60 mL) at 0°C. The reaction mixture was allowed to warm up to r.t. and was stirred for 6 h. MeOH was removed under reduced pressure and the residue was diluted with H$_2$O (50 mL). The aqueous layer was extracted with DCM (2 X 100 mL), then acidified to pH=1 with HCl 1M and extracted with Et$_2$O (5 X 100 mL). The combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure. 4-Azidobutanoic acid (50) (1.52 g, 11.8 mmol, 73% yield) was obtained as a colorless oil.

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 5.72 (s, 1H, CO$_2$H), 3.37 (t, $J = 6.7$ Hz, 2H, HCO$_2$-CH$_2$-R), 2.46 (t, $J = 7.2$ Hz, 2H, R-CH$_2$-N$_3$), 1.91 (p, $J = 7.0$ Hz, 2H, R-CH$_2$-CH$_2$-N$_3$).

The analytical data were in accordance with previously reported data.\textsuperscript{365}
Experimental part

$^1$H NMR spectrum for 50:

2,5-Dioxopyrrolidin-1-yl 4-azidobutanoate (S1)

DCC (2.92 g, 14.1 mmol, 1.2 eq) was added to a solution of 4-azidobutanoic acid (50) (1.52 g, 11.8 mmol, 1.0 eq) and NHS (1.63 g, 14.1 mmol, 1.2 eq) in dry DCM (65 mL). The reaction mixture was stirred at r.t. for 4 h. Saturated KCl solution (50 mL) was added and the organic layer was separated, then washed with H$_2$O (50 mL), dried over MgSO$_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography eluting with a mixture of DCM / MeOH (99:1). 2,5-Dioxopyrrolidin-1-yl 4-azidobutanoate (S1) (1.93 g, 8.5 mmol, 72% yield) was obtained as a white solid.

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 3.45 (t, $J = 6.6$ Hz, 2H, Suc-CO$_2$-CH$_2$-R), 2.89 – 2.80 (m, 4H, 2 X CH$_2$suc), 2.73 (t, $J = 7.2$ Hz, 2H, R-CH$_2$-N$_3$), 2.01 (p, $J = 6.9$ Hz, 2H, R-CH$_2$-CH$_2$-N$_3$).

The analytical data were in accordance with previously reported data.$^{365}$
Experimental part

$^1$H NMR spectrum for 51:

$$\text{NH}_2$$ ($\delta$ 5.79 (s, 1H, NHamide), 3.82 (q, $J = 7.0$ Hz, 4H, 2 X CH$_2$OSi), 3.72 (q, $J = 7.0$ Hz, 2H, CH$_2$CONHR), 3.26 (td, $J = 6.9, 5.8$ Hz, 2H, CH$_2$-NHCOR), 2.25 (t, $J = 7.2$ Hz, 2H, CH$_3$-N$_3$), 1.93 (p, $J = 6.9$ Hz, 2H, CH$_2$-CH$_2$-N$_3$), 1.68 – 1.59 (m, 2H, CH$_2$-CH$_2$Si), 1.23 (td, $J = 7.0, 5.1$ Hz, 9H, 3 X CH$_2$-CH$_2$-OSi), 0.68 – 0.61 (m, 2H, CH$_2$-Si).

$^{13}$C NMR (101 MHz, Chloroform-d): $\delta$ 171.7 (CO), 58.6 (2 X CH$_2$), 58.6 (CH$_2$), 51.0 (CH$_3$), 42.0 (CH$_2$), 33.4 (CH$_2$), 25.0 (CH$_3$), 23.0 (CH$_2$), 18.6 (CH$_3$), 18.4 (2 X CH$_2$), 7.9 (CH$_3$).

IR ($\nu_{\text{max}}$ cm$^{-1}$): 3327 (w), 2947 (m), 2087 (s), 1644 (s), 1561 (m), 1283 (w), 1201 (w), 1087 (s), 764 (m).

HRMS (ESI/QTOF) m/z: [M+Na]$^+$ Calcd for C$_{13}$H$_{28}$N$_4$NaO$_4$Si$^+$ 355.1772; Found 355.1776.

4-Azido-N-(3-(triethoxysilyl)propyl)butanamide (52)

2,5-Dioxopyrrolidin-1-yl 4-azidobutanoate (51) (0.10 g, 0.44 mmol, 1.0 eq) was dissolved in dry DCM (5 mL) under argon atmosphere. APTES (0.10 mL, 0.44 mmol, 1.0 eq) and Et$_3$N (0.12 mL, 0.88 mmol, 2.0 eq) were added and the reaction mixture was stirred at r.t. for 5 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography eluting with a mixture of PE / EtOAc (6:4 to 4:6). 4-Azido-N-(3-(triethoxysilyl)propyl)butanamide (52) (0.14 g, 0.42 mmol, 95% yield) was obtained as a colorless oil.

$^1$H NMR (400 MHz, Chloroform-d): $\delta$ 5.79 (s, 1H, NHamide), 3.82 (q, $J = 7.0$ Hz, 4H, 2 X CH$_2$OSi), 3.72 (q, $J = 7.0$ Hz, 2H, CH$_2$-OSi), 3.35 (t, $J = 6.6$ Hz, 2H, CH$_2$-CONHR), 3.26 (td, $J = 6.9, 5.8$ Hz, 2H, CH$_2$-NHCOR), 2.25 (t, $J = 7.2$ Hz, 2H, CH$_3$-N$_3$), 1.93 (p, $J = 6.9$ Hz, 2H, CH$_2$-CH$_2$-N$_3$), 1.68 – 1.59 (m, 2H, CH$_2$-CH$_2$-Si), 1.23 (td, $J = 7.0, 5.1$ Hz, 9H, 3 X CH$_2$-CH$_2$-OSi), 0.68 – 0.61 (m, 2H, CH$_2$-Si).
Experimental part

$^1$H NMR and $^{13}$C spectra for 52:
Experimental part

Coating with APTES derivatives and functionalization with CM-Cargo of HNPs

Scheme 53: Functionalization of HNPs with CM-Cargo: i) APTES, 52, NH₄OH 25% aq, cyclohexane, EtOH, US, 40°C, 16 h; ii) CM-Cargo, DMF, US, 40°C, 16 h, dark conditions.

General procedure of coating of the HNPs with APTES derivatives

Scheme 54: General procedure for the formation of silica shell around the HNPs.

Cyclohexane (2 mL) was added to a suspension of HNPs in EtOH (2 mg, 2 mL) and the suspension was ultra-sonicated for 30 min. TEOS (2.0 μL, 10 μmol, 2.0 eq), APTES (1.2 μL, 5 μmol, 1.0 eq) and a solution of 4-azido-N-(3-(triethoxysilyl)propyl)butanamide (52) (1.7 mg, 5 μmol, 1.0 eq) in EtOH (100 μL) were added to the suspension of HNPs and ultra-sonicated for 30 min. NH₄OH aq (0.1 mL, 25%) was added and the suspension was ultra-sonicated at 40°C for 12 h under argon atmosphere. The suspension was centrifuged (10 min, 4700 rpm) and the supernatant was removed. The residue was washed with EtOH (5 X, 1 mL) and the solid residue was suspended in EtOH (1 mL).

BFO-APTES-N₃ HNPs

Following the general procedure, BFO HNPs were coated with TEOS, APTES and 4-azido-N-(3-(triethoxysilyl)propyl)butanamide (52). An aliquot of the BFO-APTES-N₃ HNPs suspension (10 μL) was diluted with H₂O (1 mL) and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

Table 3: Values of dynamic light scattering by number and zeta potential measurements for BFO-APTES-N₃ HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFO</td>
<td>331.7 ± 53.3</td>
<td>-27.8 ± 0.7</td>
</tr>
<tr>
<td>BFO-APTES-N₃</td>
<td>464.3 ± 120.2</td>
<td>20.2 ± 0.4</td>
</tr>
</tbody>
</table>
Experimental part

Figure 48: A) Dynamic light scattering by number of BFO-APTES-N₃ HNPs; B) Zeta potential of BFO-APTES-N₃ HNPs.

**LNO-APTES-N₃ HNPs**

Following the general procedure, LNO HNPs were coated with TEOS, APTES and 4-azido-N-(3-(triethoxysilyl)propyl)butanamide (52). An aliquot of the LNO-APTES-N₃ HNPs suspension (10 μL) was diluted with H₂O (1 mL) and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

**Table 4:** Values of dynamic light scattering by number and zeta potential measurements for LNO-APTES-N₃ HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO</td>
<td>61.2 ± 9.4</td>
<td>-39.0 ± 1.2</td>
</tr>
<tr>
<td>LNO-APTES-N₃</td>
<td>165.3 ± 33.8</td>
<td>25.5 ± 0.7</td>
</tr>
</tbody>
</table>

Figure 49: A) Dynamic light scattering by number of LNO-APTES-N₃ HNPs; B) Zeta potential of LNO-APTES-N₃ HNPs.
General procedure for functionalization of APTES-N₃ HNPs with CM-Cargo

**Scheme 55:** General procedure for functionalization of APTES-N₃ HNPs with CM-Cargo.

APTES-N₃ HNPs (2 mg) were suspended in a mixture of EtOH: DMF (1:1, 2 mL) and a solution of CM-Cargo was added. The suspension was ultra-sonicated at 40°C for 16 h under dark conditions. The suspension was centrifuged (10 min, 4 700 rpm). The solid residue was sequentially washed and centrifuged with DMF (1 X, 1 mL) and EtOH (3 X, 1 mL). Finally, the resulting HNPs-APTES-CM-Cargo HNPs were suspended in EtOH (1 mL) for further experiments.

**Functionalization of BFO-APTES-N₃ HNPs with CM-Trp 46**

Following the general procedure of functionalization, a 2 mM solution of CM-Trp 46 (50 μL, 95.2 μg, 0.1 μmol) in DMF was added to a suspension of BFO-APTES-N₃ HNPs. An aliquot of the BFO-APTES-CM-Trp HNPs suspension (10 μL) was diluted with H₂O (1 mL) and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

**Table 5:** Values of dynamic light scattering by number and zeta potential measurements for BFO-APTES-CM-Trp HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFO-APTES-N₃</td>
<td>464.3 ± 120.2</td>
<td>20.2 ± 0.4</td>
</tr>
<tr>
<td>BFO-APTES-CM-Trp</td>
<td>440.9 ± 76.1</td>
<td>9.8 ± 1.4</td>
</tr>
</tbody>
</table>
Experimental part

Figure 50: A) Dynamic light scattering by number of BFO-APTES-CM-Trp HNPs; B) Zeta potential of BFO-APTES-CM-Trp HNPs.

Functionalization of BFO-APTES-N$_3$ HNPs with DIBO-Trp 60

Following the general procedure of functionalization, a 2 mM solution of DIBO-Trp 60 (25 μL, 11.0 μg, 24.5 nmol) in DMF was added to a suspension of BFO-APTES-N$_3$ HNPs. An aliquot of the BFO-APTES-DIBO-Trp HNPs suspension (10 μL) was diluted with H$_2$O (1 mL) and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

Table 6: Values of dynamic light scattering by number and zeta potential measurements for BFO-APTES-DIBO-Trp HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFO-APTES-N$_3$</td>
<td>464.3 ± 120.2</td>
<td>20.2 ± 0.4</td>
</tr>
<tr>
<td>BFO-APTES-DIBO-Trp</td>
<td>127.0 ± 22.6</td>
<td>25.8 ± 0.6</td>
</tr>
</tbody>
</table>

Figure 51: A) Dynamic light scattering by number of BFO-APTES-DIBO-Trp HNPs; B) Zeta potential of BFO-APTES-DIBO-Trp HNPs.
Functionalization of LNO-APTES-N\textsubscript{3} HNPs with DIBO-Cy3 79

Following the general procedure of functionalization, 5 mM solution of DIBO-Cy3 79 (25 µL, 78.1 µg, 0.1 µmol) in DMF was added to a suspension of LNO-APTES-N\textsubscript{3} HNPs. An aliquot of the LNO-APTES-DIBO-Cy3 HNPs suspension (20 µL) was diluted with H\textsubscript{2}O (1 mL and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

Table 7: Values of dynamic light scattering by number and zeta potential measurements for LNO-APTES-DIBO-Cy3 HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO-APTES-N\textsubscript{3}</td>
<td>165.3 ± 33.8</td>
<td>25.5 ± 0.7</td>
</tr>
<tr>
<td>LNO-APTES-DIBO-Cy3</td>
<td>328.9 ± 141.8</td>
<td>24.4 ± 1.0</td>
</tr>
</tbody>
</table>

Figure 52: A) Dynamic light scattering by number of LNO-APTES-DIBO-Cy3 HNPs; B) Zeta potential of LNO-APTES-DIBO-Cy3 HNPs.
Experimental part

Figure 53: Multiphoton multispectral microscopy images of LNO-APTES-DIBO-Cy3 HNPs: A) SHG channel image of LNO HNPs; B) sum of intensity of all channels in the 460-650 nm spectral range (fluorescence of Cy3 dye); C) merged image; D) emission spectrum of LNO-APTES-DIBO-Cy3 HNPs upon excitation at 800 nm, obtained from selected regions of multiphoton multispectral images.

Functionalization of LNO-APTES-N₃ HNPs with CM-erlo 77

Following the general procedure of functionalization, 5 mM solution of CM-erlo 77 (25 μL, 78.1 μg, 0.1 μmol) in DMF was added to a suspension of LNO-APTES-N₃ HNPs. An aliquot of the LNO-APTES-CM-erlo HNPs suspension (20 μL) was diluted with H₂O (1 mL and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

Table 8: Values of dynamic light scattering by number and zeta potential measurements for LNO-APTES-CM-erlo HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO-APTES-N₃</td>
<td>165.3 ± 33.8</td>
<td>25.5 ± 0.7</td>
</tr>
<tr>
<td>LNO-APTES-CM-erlo</td>
<td>200.6 ± 33.2</td>
<td>23.7 ± 0.6</td>
</tr>
</tbody>
</table>
Experimental part

Figure 54: A) Dynamic light scattering by number of LNO-APTES-CM-erlo HNPs; B) Zeta potential of LNO-APTES-CM-erlo HNPs.

Figure 55: Multiphoton multispectral microscopy images of LNO-APTES-CM-erlo HNPs: A) SHG channel image of LNO HNPs; B) sum of intensity of all channels in the 420-560 nm spectral range (fluorescence of CM); C) merged channel; D) emission spectrum of LNO-APTES-CM-erlo HNPs upon excitation at 800 nm, obtained from selected regions of multiphoton multispectral images.
Functionalization of LNO-APTES-N₂ HNPs with CM-DOX 62

Following the general procedure of functionalization, 8 mM solution of CM-DOX 62 (12.5 μL, 113.5 μg, 0.1 μmol) in DMSO was added to a suspension of LNO-APTES-N₂ HNPs. An aliquot of the LNO-APTES-CM-DOX HNPs suspension (20 μL) was diluted with H₂O (1 mL and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

**Table 9:** Values of dynamic light scattering by number and zeta potential measurements for LNO-APTES-CM-DOX HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO-APTES-N₂</td>
<td>165.3 ± 33.8</td>
<td>25.5 ± 0.7</td>
</tr>
<tr>
<td>LNO-APTES-CM-DOX</td>
<td>164.1 ± 30.5</td>
<td>22.5 ± 0.4</td>
</tr>
</tbody>
</table>

![Figure 56: A) Dynamic light scattering by number of LNO-APTES-CM-DOX HNPs; B) Zeta potential of LNO-APTES-CM-DOX HNPs.](image)
Experimental part

Figure 57: Multiphoton multispectral microscopy images of LNO-APTES-CM-DOX HNPs: A) SHG channel image of LNO HNPs; B) sum of intensity of all channels in the 430-500 nm spectral range (fluorescence of CM); C) sum of intensity of all channels in the 530-600 nm spectral range (fluorescence of DOX); D) merged channel; E) emission spectrum of LNO-APTES-CM-DOX HNPs upon excitation at 810 nm, obtained from selected regions of multiphoton multispectral images.

Functionalization of LNO-APTES-N₃ HNPs with CM-Cbl 78

Following the general procedure of functionalization, 24.9 mM solution of CM-Cbl 78 (4 μL, 81.8 μg, 0.1 μmol) in DMF was added to a suspension of LNO-APTES-N₃ HNPs. An aliquot of the LNO-APTES-CM-Cbl HNPs suspension (20 μL) was diluted with H₂O (1 mL) and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

Table 10: Values of dynamic light scattering by number and zeta potential measurements for LNO-APTES-CM-Cbl HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO-APTES-N₃</td>
<td>165.3 ± 33.8</td>
<td>25.5 ± 0.7</td>
</tr>
<tr>
<td>LNO-APTES-CM-Cbl</td>
<td>297.4 ± 60.9</td>
<td>20.4 ± 0.2</td>
</tr>
</tbody>
</table>
Experimental part

Figure 58: A) Dynamic light scattering by number of LNO-APTES-CM-Cbl HNPs; B) Zeta potential of LNO-APTES-CM-Cbl HNPs.

Figure 59: Multiphoton multispectral microscopy images of LNO-APTES-CM-Cbl HNPs: A) SHG channel image of LNO HNPs; B) sum of intensity of all channels in the 420-560 nm spectral range (fluorescence of CM); C) merged channel; D) emission spectrum of LNO-APTES-CM-Cbl HNPs upon excitation at 800 nm, obtained from selected regions of multiphoton multispectral images.

Functionalization of LNO-APTES-N\textsubscript{3} HNPs with DIBO-Cbl 79

Following the general procedure of functionalization, 18.4 mM solution of DIBO-Cbl 79 (5.4 μL) in DMF was added to a suspension of LNO-APTES-N\textsubscript{3} HNPs. An aliquot of the LNO-APTES-DIBO-Cbl HNPs suspension (20
μL) was diluted with H₂O (1 mL) and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

Table 11: Values of dynamic light scattering by number and zeta potential measurements for LNO-APTES-DIBO-Cbl HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO-APTES-N₃</td>
<td>165.3 ± 33.8</td>
<td>25.5 ± 0.7</td>
</tr>
<tr>
<td>LNO-APTES-DIBO-Cbl</td>
<td>206.6 ± 37.8</td>
<td>19.2 ± 0.4</td>
</tr>
</tbody>
</table>

Figure 60: A) Dynamic light scattering by number of LNO-APTES-DIBO-Cbl HNPs; B) Zeta potential of LNO-APTES-DIBO-Cbl HNPs.

4.4.2 Coating of BFO HNPs with Avidin and functionalization with CM-Cy3 molecules

Synthesis of carboxylic modified APTES-CO₂H 53 and coating of BFO HNPs

![Chemical structure of 53](image)

Scheme 56: Coating of BFO HNPs with avidin and functionalization with biotin-(CM)-Cargo linkers: i) succinic anhydride, dioxane, r.t., 1 h; ii) BFO HNPs, 53, TEOS, NH₄OH 25% aq, EtOH, cyclohexane, US, 40°C, 16 h; iii) 1) Sulfo-NHS ester, EDCI, MES Buffer, US, 30 min, 2) Avidin, PBS, 2 h, 3) ethylene diamine, PBS, 2 h; iv) Biotin-(CM)-Cargo, PBS, 2 h, dark conditions.
Experimental part

4-Oxo-4-((3-(triethoxysilyl)propyl)amino)butanoic acid (53)

Succinic anhydride (0.59 g, 4.99 mmol, 1.1 eq) was dissolved in dioxane (50 mL) at 60°C. The solution was cooled down to r.t. and APTES (1.1 mL, 4.54 mmol, 1.0 eq) was added dropwise. The reaction mixture was stirred at r.t. for 1 h. The solution was filtered and the solvent was removed under reduced pressure. 4-Oxo-4-((3-(triethoxysilyl)propyl)amino)butanoic acid (53) (1.44 g, 4.48 mmol, 90% yield) was obtained as a colorless oil.

$^1$H NMR (400 MHz, Chloroform-$d$): δ 6.25 (s, 1H, NH amide), 3.82 (q, $J = 7.0$ Hz, 6H, 3X CH$_2$-OSi), 3.27 (q, $J = 6.5$ Hz, 2H, CH$_2$-NHCOR), 2.69 (dd, $J = 7.7$, 5.1 Hz, 2H, CH$_2$-CO$_2$H), 2.51 (dd, $J = 7.7$, 5.2 Hz, 2H, CH$_2$-CONHR), 1.64 (p, $J = 6.9$ Hz, 2H CH$_2$-CH$_2$-Si), 1.23 (t, $J = 7.0$ Hz, 9H, 3X CH$_3$-CH$_2$-OSi), 0.64 (t, $J = 8.0$ Hz, 2H, CH$_2$-Si).

The analytical data were in accordance with previously reported data.$^{366}$

$^1$H NMR spectrum for 53:
BFO-APTES-CO₂H HNPs

Cyclohexane (2 mL) was added to a suspension of BFO HNPs in EtOH (2 mg, 2 mL) and the suspension was ultra-sonicated for 30 min. TEOS (4 μL, 14.3 mmol, 4.0 eq) and a solution of 4-oxo-4-((3-(triethoxysilyl)propyl)amino)butanoic acid (53) (1.4 mg, 3.6 μmol, 1.0 eq) in EtOH (100 μL) were added to the suspension of BFO HNPs and ultra-sonicated for 30 min. NH₄OH aq (0.1 mL) was added and the suspension was ultra-sonicated at 40°C for 16 h under argon atmosphere. The crude was divided into eppendorfs and centrifuged (10 min, 13 000 rpm). The residue was washed with EtOH (4 X). The BFO-APTES-CO₂H HNPs were re-suspended in EtOH (2 mL).

An aliquot of the BFO-APTES-CO₂H HNPs suspension (20 μL) was diluted with H₂O (1 mL) and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

Table 12: Values of dynamic light scattering by number and zeta potential measurements for BFO-APTES-CO₂H HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFO</td>
<td>331.7 ± 53.3</td>
<td>-27.8 ± 0.7</td>
</tr>
<tr>
<td>BFO-APTES-CO₂H</td>
<td>114.1 ± 9.6</td>
<td>-34.6 ± 0.9</td>
</tr>
</tbody>
</table>

Figure 61: A) Dynamic light scattering by number of BFO-APTES-CO₂H HNPs; B) Zeta potential of BFO-APTES-CO₂H HNPs.

General procedure for the coating of NPs with Avidin

A suspension of NPs were centrifuged (10 min, 13 000 rpm), the solvent was removed and re-suspended in 0.1 M MES buffer (0.5 mL). A solution of 40 mM sulfo-NHS ester (0.25 mL) in 0.1 mM MES buffer and a solution of 16 mM EDCI (0.25 mL) in 0.1 MES buffer were added and the mixture was shaken for 30 min. The suspension was centrifuged (10 min, 13 000 rpm), the solvent was removed, washed with PBS (1 X 1 mL) and re-suspended in PBS (1 mL). A solution of Avidin (1 mg/ mL, 100 μL) in PBS was added and the suspension was shaken for 2 h. Ethylene diamine (4 μL) was added to the suspension and shaken for 2 h. The NPs were centrifuged (10 min, 13 000 rpm), washed with PBS (1 X) and re-suspended in quenching solution (40 mM.
Experimental part

TRIS-HCl with 0.05 % BSA, 1 mL). The suspension was shaken for 1 h, centrifuged (10 min, 13 000 rpm). The solvent was removed, the solid residue was washed with PBS (3 X 1 mL) and re-suspended in PBS (0.5 mL).

BFO-Avidin HNPs

Following the general procedure, a suspension of BFO-APTES-CO$_2$H HNPs (0.5 mg) in EtOH (0.5 mL) was coated with avidin. An aliquot of the BFO-Avidin HNPs suspension (20 μL) was diluted with H$_2$O (1 mL) and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

Table 13: Values of dynamic light scattering by number and zeta potential measurements for BFO-Avidin HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFO-APTES-CO$_2$H</td>
<td>114.1 ± 9.6</td>
<td>-34.6 ± 0.9</td>
</tr>
<tr>
<td>BFO-Avidin</td>
<td>77.0 ± 9.1</td>
<td>-52.8 ± 3.9</td>
</tr>
</tbody>
</table>

Figure 62: A) Dynamic light scattering by number of BFO-Avidin HNPs; B) Zeta potential of BFO-Avidin HNPs.
Experimental part

**Figure 63**: Representative STEM images of BFO-Avidin HNPs: A) high-angle annular dark-field image; B) Si EDX map; C) O EDX map; D) Bi EDX map; E) Fe EDX map; F) C EDX map.

**SiO$_2$-Avidin NPs**

Following the general procedure, a suspension of SiO$_2$ NPs (0.5 mg) in EtOH (0.2 mL) was coated with avidin. An aliquot of the SiO$_2$-Avidin NPs suspension (20 μL) was diluted with H$_2$O (1 mL) and ultra-sonicated for 30 min. The supernatant was analyzed with a Malvern NanoZ.

**Table 14**: Values of dynamic light scattering by number and zeta potential measurements for SiO$_2$-Avidin NPs.

<table>
<thead>
<tr>
<th>NPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO$_2$</td>
<td>76.4 ± 12.3</td>
<td>-24.5 ± 0.8</td>
</tr>
<tr>
<td>SiO$_2$-Avidin</td>
<td>52.2 ± 9.0</td>
<td>-31.9 ± 1.3</td>
</tr>
</tbody>
</table>

**Figure 64**: A) Dynamic light scattering by number of SiO$_2$-Avidin NPs; B) Zeta potential of SiO$_2$-Avidin NPs.
General procedure for functionalization of NPs-Avidin with Biotin-(CM)-Cargo

Scheme 57: General procedure for functionalization of Avidin-coated NPs with biotinylated-cargo molecules.

A solution of Biotin-(CM)-Cargo was added to a suspension of NPs-Avidin (1 mg) in PBS (1 mL). The suspension was shaken for 2 h, divided into eppendorfs, centrifuged (10 min, 13 000 rpm) and the supernatant was discarded. The NPs were washed with PBS (4 X 1 mL) and re-suspended in PBS (1 mL).

An aliquot of the Biotin-functionalized NPs suspension (20 μL) was diluted with H2O (1 mL) and shaken for 30 min. The supernatant was analyzed with a Malvern NanoZ.

Functionalization of BFO-Avidin HNPs with CM-Cy3 56

Following the general procedure, the BFO-Avidin HNPs were functionalized with a 0.78 mM solution of CM-Cy3 ligand 56 (36 μL, 45.5 μg, 36.0 nmol) in PBS to afford BFO-Avidin-CM-Cy3 HNPs.

Table 15: Values of dynamic light scattering by number and zeta potential measurements for BFO-Avidin-CM-Cy3 HNPs.

<table>
<thead>
<tr>
<th>HNPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFO-Avidin</td>
<td>77.0 ± 9.1</td>
<td>-52.8 ±3.9</td>
</tr>
<tr>
<td>BFO-Avidin-CM-Cy3</td>
<td>80.9 ± 14.1</td>
<td>-43.7 ± 1.3</td>
</tr>
</tbody>
</table>

Figure 65: A) Dynamic light scattering by number of BFO-Avidin-CM-Cy3 HNPs; B) Zeta potential of BFO-Avidin-CM-Cy3 HNPs.
Functionalization of BFO-Avidin HNPs with Biotin-Cy3

Following the general procedure, the BFO-Avidin HNPs were functionalized with a 1 mM solution of Biotin-Cy3 ligand in PBS to afford BFO-Avidin-Biotin-Cy3 HNPs.

Table 16: Values of dynamic light scattering by number and zeta potential measurements for BFO-Avidin-Biotin-Cy3 HNPs.

<table>
<thead>
<tr>
<th>NPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFO-Avidin</td>
<td>77.0 ± 9.1</td>
<td>-52.8 ± 3.9</td>
</tr>
<tr>
<td>BFO-Avidin-Biotin-Cy3</td>
<td>89.5 ± 13.3</td>
<td>-37.2 ± 3.8</td>
</tr>
</tbody>
</table>

Figure 66: A) Dynamic light scattering by number of BFO-Avidin-Biotin-Cy3 HNPs; B) Zeta potential of BFO-Avidin-Biotin-Cy3 HNPs.

Functionalization of SiO₂-Avidin HNPs with CM-Cy3

Following the general procedure, the SiO₂-Avidin HNPs were functionalized with a 0.78 mM solution of CM-Cy3 ligand (36 μL, 45.5 μg, 36.0 nmol) in PBS to afford SiO₂-Avidin-CM-Cy3 HNPs.

Table 17: Values of dynamic light scattering by number and zeta potential measurements for SiO₂-Avidin-CM-Cy3 HNPs.

<table>
<thead>
<tr>
<th>NPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO₂-Avidin</td>
<td>52.2 ± 9.0</td>
<td>-31.9 ± 1.3</td>
</tr>
<tr>
<td>SiO₂-Avidin-CM-Cy3</td>
<td>69.7 ± 16.5</td>
<td>32.1 ± 0.9</td>
</tr>
</tbody>
</table>
Experimental part

Figure 67: A) Dynamic light scattering by number of SiO$_2$-Avidin-CM-Cy3 HNPs; B) Zeta potential of SiO$_2$-Avidin-CM-Cy3 HNPs.

Functionalization of SiO$_2$-Avidin HNPs with Biotin-Cy3 59

Following the general procedure, the SiO$_2$-Avidin HNPs were functionalized with a 1 mM solution of Biotin-Cy3 ligand 59 in PBS to afford SiO$_2$-Avidin-Biotin-Cy3 HNPs.

Table 18: Values of dynamic light scattering by number and zeta potential measurements for SiO$_2$-Avidin-Biotin-Cy3 HNPs.

<table>
<thead>
<tr>
<th>NPs</th>
<th>Size (d, nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO$_2$-Avidin</td>
<td>52.2 ± 9.0</td>
<td>-31.9 ± 1.3</td>
</tr>
<tr>
<td>SiO$_2$-Avidin-Biotin-Cy3</td>
<td>89.5 ± 13.3</td>
<td>-32.1 ± 0.8</td>
</tr>
</tbody>
</table>

Figure 68: A) Dynamic light scattering by number of SiO$_2$-Avidin-Biotin-Cy3 HNPs; B) Zeta potential of SiO$_2$-Avidin-Biotin-Cy3 HNPs.
4.5 Photorelease assays at one and two photon

4.5.1 Absorbance measurements

The absorbance measurements were measured with BioTek Synergy 2 multi-mode reader instrument between 200 to 700 nm with increment of one nm in a 96-well plate (Corning® UV-Transparent microplate). The absorbance of ligands was measured in solvent and in a concentration range between 0 to 1000 μM. The absorbance of HNPs was measured in PBS or EtOH and in a concentration range between 0.1 to 2 mg/mL.

Qualibration curves for quantification of Trp on BFO-APTES-CM-Trp HNPs

The UV-visible absorption of BFO-APTES-CM-Trp HNPs was measured and compared to standard curves between 0 and 1000 μM for CM-Trp 46. The total amount of CM-Trp 46 present was determined to 3.29 μM per mg of BFO HNPs.

Figure 69: A) Extinction spectra of CM-Trp 46 in PBS at different concentrations; B) extinction spectra of BFO-APTES-CM-Trp HNPs in PBS at different concentrations; C) absorbance and corrected absorbance in function of the concentration of CM-Trp 46; D) corrected absorbance in function of the concentration of BFO-APTES-CM-Trp HNPs.
Qualibration curves for quantification of erlotinib on LNO-APTES-CM-erlo HNPs

The UV-visible absorption of the supernatant of LNO-APTES-CM-erlo HNPs was measured and compared to standard curves between 0 and 250 μM for CM-erlo 77 in PBS. The total amount of CM-erlo 77 present was determined to 4.98 μM per mg of LNO-APTES-CM-erlo HNPs.

![Extinction spectra of CM-erlo 77 in PBS at different concentrations](image1)

![Extinction spectrum in DMF of the supernatant (green) after functionalization of LNO-APTES-CM-erlo HNPs](image2)

![Absorbance and corrected absorbance in function of the concentration of CM-erlo 77](image3)

**Figure 70:** A) Extinction spectra of CM-erlo 77 in PBS at different concentrations; B) extinction spectrum in DMF of the supernatant (green) after functionalization of LNO-APTES-CM-erlo HNPs; C) absorbance and corrected absorbance in function of the concentration of CM-erlo 77.

Qualibration curves for quantification of DOX on LNO-APTES-CM-DOX HNPs

The UV-visible absorption of LNO-APTES-CM-DOX HNPs was measured and compared to standard curves between 0 and 1000 μM for CM-DOX 62 in DMF. The total amount of CM-DOX 62 present was determined to 4.27 μM per mg of LNO HNPs.
Figure 71: A) Extinction spectra of CM-DOX 62 in DMF at different concentrations; B) extinction spectra in DMF of LNO-APTES-CM-DOX (red) and the supernatant (blue) after functionalization; C) absorbance and corrected absorbance in function of the concentration.

Qualibration curves for quantification of Cbl on LNO-APTES-CM-Cbl HNPs

The UV-visible absorption of LNO-APTES-CM-Cbl HNPs was measured and compared to standard curves between 0 and 50 μM for CM-Cbl 78 in DMF. The total amount of CM-Cbl 78 present was determined to 3.64 μM per mg of LNO-APTES-CM-Cbl HNPs.
Experimental part

Figure 72: A) Extinction spectra of CM-Cbl 78 in DMF at different concentrations; B) extinction spectra of LNO-APTES-CM-Cbl HNPs in DMF at different concentrations; C) absorbance and corrected absorbance in function of the concentration of CM-Cbl 78.

4.5.2 Photorelease experimental procedures at one and two photon

General procedure A for one-photon release assay

One-photon photolysis experiment was performed using Spectroline EF-16F (312 nm, 15 W) as light source. A solution of ligands in solvent was putted into an NMR tube and the solution was irradiated at 312 nm. Between a certain time intervals NMR spectra were recorded and the total amount of starting material was calculated by integration of peaks of interest and comparing with internal standard (solvent peak).

General procedure B for one-photon release assay

One-photon photolysis experiment was performed using Sylvania UV-light tube (366 nm, 8 W) as light source. A suspension of HNPs or solutions of ligands in PBS was putted into an 8-well plate (Lab-Tek II Chambered Coverglass) and the suspension / solution was irradiated with UV-A (366 nm) light. Between a certain time intervals, aliquots of the suspension / solution were taken out, diluted with solvent to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of released cargo molecule was quantified by UHPLC-MS.
Procedure for the release of H$_2$O from ONB 7

Following the general procedure A, a 26.5 mM solution ONB 7 in deuterated DMSO was irradiated at 312 nm. NMR spectra were recorded after 0, 1, 2, 5, 10, 20, 30, 40, 50, 60 and 90 min and the amount of intact molecule was calculated by integration of peak area.

![Figure 73: Stack $^1$H NMR spectra of ONB 7 at 0 (red), 10 min (green), 50 min (blue) and 90 min (purple).](image-url)
Experimental part

Procedure for the release of H$_2$O from MONB 17

Following the general procedure A, a 24.3 mM solution MONB 17 in deuterated DMSO was irradiated at 312 nm. NMR spectra were recorded after 0, 2, 5, 10, 20, 30, 45, 60, 90, 120, 150, 180 and 210 min and the amount of intact molecule was calculated by integration of peak area.

Figure 74: Stack $^1$H NMR spectra of MONB 17 at 0 (red), 30 min (green), 150 min (blue) and 210 min (purple).
Experimental part

Procedure for the release of Trp from ONB-Trp 38

Following the general procedure A, a 23.3 mM solution ONB-Trp 38 in deuterated DMSO was irradiated at 312 nm. NMR spectra were recorded after 0, 1, 2, 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 150, 180 and 210 min and the amount of intact molecule was calculated by integration of peak area.

Figure 75: Stack $^1$H NMR spectra of ONB-Trp 38 at 0 (red), 30 min (green), 150 min (blue) and 210 min (purple).
**Procedure for the release of Trp from MONB-Trp 40**

Following the general procedure A, a 26.0 mM solution MONB-Trp 40 in deuterated DMSO was irradiated at 312 nm. NMR spectra were recorded after 0, 10, 20, 30, 45, 60, 90, 120, 150, 180 and 210 min and the amount of intact molecule was calculated by integration of peak area.

**Figure 76:** Stack $^1$H NMR spectra of MONB-Trp 40 at 0 (red), 30 min (green), 150 min (blue) and 210 min (purple).
Experimental part

Procedure for the release of Trp from BFO-APTES-CM-Trp HNPs kept in dark

A suspension of BFO-APTES-CM-Trp in PBS (1 mg/mL, 1.0 mL) was kept in dark at 37°C. Aliquots (135 μL, 0, 30, 60, 120 and 360 min) of the suspension were taken out, diluted with PBS to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free Trp was quantified by UHPLC-MS.

**Table 19:** Ratio and percentage of Trp released of BFO-APTES-CM-Trp NPs (loading 2 mg/mL) in PBS medium kept in the dark. Aliquots of the suspension were withdrawn at the indicated time points, diluted 1:1 in PBS and centrifuged (20 min, 13’000 rpm). Quantification of Trp in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Ratio Trp / HNPs (μM/ mg)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>4</td>
<td>120</td>
<td>0.00 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>360</td>
<td>0.09 ± 0.05</td>
<td>2.7 ± 1.5</td>
</tr>
</tbody>
</table>

Procedure for the release of Trp from CM-Trp 46

Following the general procedure B, a solution of CM-Trp 46 in PBS (4 μM, 1.0 mL) was irradiated with UV-A (366 nm) light. Aliquots (135 μL; 0, 5, 15, 30, 120, 240 and 360 min) of the solution were taken out, diluted with PBS to 270 μL, divided into MS vial (triplicate) and the amount of free Trp was quantified by UHPLC-MS.

**Table 20:** Ratio and percentage of Trp released upon irradiation of compound 46 (initial concentration 4 μM) at 366 nm (UV lamp) in PBS medium. Aliquots of the solution were withdrawn at the indicated time points and diluted 1:1 with PBS. Quantification of Trp was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Trp (μM)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>1.50 ± 0.04</td>
<td>37.5 ± 2.0</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>1.84 ± 0.02</td>
<td>46.0 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>2.58 ± 0.06</td>
<td>64.5 ± 3.0</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>3.04 ± 0.08</td>
<td>76.0 ± 4.0</td>
</tr>
<tr>
<td>6</td>
<td>240</td>
<td>3.08 ± 0.06</td>
<td>77.0 ± 3.0</td>
</tr>
<tr>
<td>7</td>
<td>360</td>
<td>3.12 ± 0.18</td>
<td>78.0 ± 9.0</td>
</tr>
</tbody>
</table>
**Procedure for the release of Trp from BFO-APTES-CM-Trp HNPs**

Following the general procedure B, a suspension of BFO-APTES-CM-Trp in PBS (1 mg/mL, 1.0 mL) was irradiated with UV-A (366 nm) light. Aliquots (135 μL, 0, 2, 5, 15, 30, 60, 120, 240 and 360 min) of the suspension were taken out, diluted with PBS to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free Trp was quantified by UHPLC-MS.

**Table 21:** Ratio and percentage of Trp released upon irradiation of BFO-APTES-CM-Trp NPs (loading 2 mg/mL) at 366 nm (UV lamp) in PBS medium. Aliquots of the suspension were withdrawn at the indicated time points, diluted 1:1 in PBS and centrifuged (20 min, 13'000 rpm). Quantification of Trp in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Ratio Trp / HNPs (μM/ mg)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0.24 ± 0.02</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>0.45 ± 0.04</td>
<td>13.7 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>0.70 ± 0.03</td>
<td>21.3 ± 0.9</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>1.37 ± 0.03</td>
<td>41.7 ± 0.9</td>
</tr>
<tr>
<td>7</td>
<td>120</td>
<td>2.13 ± 0.03</td>
<td>64.8 ± 0.9</td>
</tr>
<tr>
<td>8</td>
<td>240</td>
<td>2.66 ± 0.05</td>
<td>80.9 ± 1.5</td>
</tr>
<tr>
<td>9</td>
<td>360</td>
<td>2.82 ± 0.06</td>
<td>85.8 ± 1.8</td>
</tr>
</tbody>
</table>

**Procedure for the release of erlotinib from LNO-APTES-CM-erlo HNPs**

Following the general procedure B, a suspension of LNO-APTES-CM-erlotinib in PBS (2 mg/mL, 0.5 mL) was irradiated with UV-A (366 nm) light. Aliquots (33.75 μL, 0, 1, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120 and 180 min) of the suspension were taken out, diluted with PBS to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free erlotinib was quantified by UHPLC-MS.
Experimental part

Table 22: Ratio and percentage of erlotinib analogue 74 released upon irradiation of LNO-APTES-CM-erlo (loading 2 mg/mL) at 366 nm (UV lamp) in PBS medium. Aliquots of the suspension were withdrawn at the indicated time points, diluted 1:8 in PBS and centrifuged (20 min, 13'000 rpm). Quantification of erlotinib analogue 74 in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Ratio 74 / HNPs (nM/ mg)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>116.7 ± 7.8</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1073.7 ± 47.5</td>
<td>21.6 ± 1.0</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1241.7 ± 44.3</td>
<td>24.9 ± 0.9</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1804.1 ± 147.3</td>
<td>36.2 ± 3.0</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>2455.9 ± 77.1</td>
<td>49.3 ± 1.5</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>2866.4 ± 117.0</td>
<td>57.6 ± 2.3</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>3315.7 ± 183.2</td>
<td>66.6 ± 3.7</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>3433.0 ± 92.3</td>
<td>68.9 ± 1.9</td>
</tr>
<tr>
<td>9</td>
<td>45</td>
<td>3887.9 ± 175.3</td>
<td>78.1 ± 3.5</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>3804.8 ± 155.9</td>
<td>76.4 ± 3.1</td>
</tr>
<tr>
<td>11</td>
<td>90</td>
<td>3772.0 ± 283.6</td>
<td>75.7 ± 5.7</td>
</tr>
</tbody>
</table>

Procedure for the release of DOX from CM-DOX 62

Following the general procedure B, a 4 μM solution of CM-DOX 62 in PBS was irradiated with UV-A (366 nm) light. Aliquots (33.75 μL, 0, 1, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120 and 180 min) of the solution were taken out, diluted with PBS to 270 μL, divided into MS vial (triplicate) and the amount of free DOX was quantified by UHPLC-MS.

Procedure for the release of DOX from LNO-APTES-CM-DOX HNPs

Following the general procedure B, a suspension of LNO-APTES-CM-DOX in PBS (2 mg/mL, 0.5 mL) was irradiated with UV-A (366 nm) light. Aliquots (33.75 μL, 0, 1, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120 and 180 min) of the suspension were taken out, diluted with PBS to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free DOX was quantified by UHPLC-MS.

Procedure for the release of Cbl from CM-Cbl 78

Following the general procedure B, a 2 μM solution of CM-Cbl 78 in a mixture of PBS / ACN (1:1, 0.8 mL) was irradiated with UV-A (366 nm) light. Aliquots (33.75 μL, 2, 5, 10, 20, 30, 45, 60, 90, 120 and 180 min) of the solution were taken out, diluted with ACN to 270 μL, divided into MS vial (triplicate) and the amount of free Cbl was quantified by UHPLC-MS.
Table 23: Ratio and percentage of Cbl released upon irradiation of CM-Cbl 78 (2 μM) at 366 nm (UV lamp) in PBS / ACN medium. Aliquots of the solution were withdrawn at the indicated time points and diluted 1:8 in ACN. Quantification of Cbl in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Cbl (μM)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>232.9 ± 22.5</td>
<td>11.6 ± 1.1</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>364.2 ± 48.2</td>
<td>18.2 ± 2.4</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>729.8 ± 74.7</td>
<td>36.5 ± 3.7</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>1053.2 ± 82.2</td>
<td>52.7 ± 4.1</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>1144.9 ± 169.0</td>
<td>57.2 ± 8.4</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>1248.1 ± 107.8</td>
<td>62.4 ± 5.4</td>
</tr>
<tr>
<td>7</td>
<td>60</td>
<td>1518.3 ± 130.8</td>
<td>75.9 ± 6.5</td>
</tr>
<tr>
<td>8</td>
<td>90</td>
<td>1527.3 ± 195.5</td>
<td>76.4 ± 9.8</td>
</tr>
<tr>
<td>9</td>
<td>120</td>
<td>1428.2 ± 197.0</td>
<td>71.4 ± 9.9</td>
</tr>
<tr>
<td>10</td>
<td>180</td>
<td>1453.1 ± 200.9</td>
<td>72.7 ± 10.0</td>
</tr>
</tbody>
</table>

Procedure for the release of Cbl from LNO-APTES-CM-Cbl HNPs kept in dark

A suspension of LNO-APTES-CM-Cbl in a mixture of PBS / ACN (1:1, 0.8 ml, 2 mg/ml) was kept in dark at 37°C. Aliquots (33.75 μL, 0, 15, 30, 60 and 180 min) of the suspension were taken out, diluted with ACN to 270 μL and were centrifuged (20 min, 13'000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free Cbl was quantified by UHPLC-MS.

Table 24: Ratio and percentage of released Cbl upon irradiation of LNO-APTES-CM-Cbl (loading 2 mg/mL) in a mixture of PBS / ACN (1:1) kept in the dark at 37°C. Aliquots of the suspension were withdrawn at the indicated time points, diluted 1:8 in ACN and centrifuged (20 min, 13'000 rpm). Quantification of Cbl in the supernatant was performed by UHPLC-MS on triplicates

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Ratio Cbl / HNPs (nM/ mg)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>103.9 ± 8.8</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>120.6 ± 16.4</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>143.6 ± 3.8</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>131.6 ± 2.5</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>180</td>
<td>178.9 ± 29.2</td>
<td>4.9 ± 0.8</td>
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</table>

Procedure for the release of Cbl from LNO-APTES-CM-Cbl HNPs

Following the general procedure B, a suspension of LNO-APTES-CM-Cbl in a mixture of PBS / ACN (1:1, 0.8 ml, 2 mg/ml) was irradiated with UV-A (366 nm) light. Aliquots (33.75 μL, 0, 2, 5, 10, 15, 20, 30, 60, 90, 120 and 180 min) of the suspension were taken out, diluted with ACN to 270 μL and were centrifuged (20 min, 13'000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free Cbl was quantified by UHPLC-MS.
Experimental part

Table 25: Ratio and percentage of Cbl released upon irradiation of LNO-APTES-Cbl HNPs (loading 2 mg/mL) at 366 nm (UV lamp) in PBS / ACN medium. Aliquots of the solution were withdrawn at the indicated time points, diluted 1:8 in ACN and centrifuged (20 min, 13’000 rpm). Quantification of Cbl in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Ratio Cbl / HNPs (nM/ mg)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>103.9 ± 8.8</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>288.8 ± 26.0</td>
<td>7.9 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>425.4 ± 37.8</td>
<td>11.7 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1208.5 ± 48.0</td>
<td>33.2 ± 1.3</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>1733.5 ± 49.2</td>
<td>47.6 ± 1.4</td>
</tr>
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<td>6</td>
<td>15</td>
<td>1925.9 ± 73.7</td>
<td>52.9 ± 2.0</td>
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<td>20</td>
<td>2145.6 ± 121.5</td>
<td>58.9 ± 3.3</td>
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<td>8</td>
<td>30</td>
<td>2217.0 ± 155.6</td>
<td>60.8 ± 4.3</td>
</tr>
<tr>
<td>9</td>
<td>60</td>
<td>2576.5 ± 38.0</td>
<td>70.7 ± 1.0</td>
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<td>10</td>
<td>90</td>
<td>2621.5 ± 97.4</td>
<td>71.9 ± 2.7</td>
</tr>
<tr>
<td>11</td>
<td>120</td>
<td>2545.5 ± 108.2</td>
<td>69.9 ± 3.0</td>
</tr>
<tr>
<td>12</td>
<td>180</td>
<td>2417.3 ± 38.9</td>
<td>66.3 ± 1.1</td>
</tr>
</tbody>
</table>

General procedure for two-photon release assay

The setup was based on an amplified Ti:Sapphire laser system (Astrella, Coherent) with a 5 W average output at 1 KHz repetition rate. The system delivers laser pulses centered at 790 nm with 35 nm bandwidth corresponding to 27 fs transform limited pulse duration. The actual duration measured by a SHG Frequency Resolved Optical Gating (SHG-FROG) device (PulseCheck, APE Berlin) yields a value of 35 fs. The beam diameter measured using a beam profiler (Newport) placed at the laser output corresponds to 6.5 mm (FWHM). The beam was directed to the bottom surface of the multi-wells plate containing the sample solution through a 45-degree dielectric mirror, without focusing. The peak intensity at the sample corresponds to 430 GW/cm².
A suspension of HNPs or solutions of ligands in PBS was putted into an 8-well plate (Lab-Tek II Chambered Coverglass) and the suspension / solution was irradiated at 790nm. Between a certain time intervals, aliquots of the suspension / solution were taken out, diluted with solvent to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed and the amount of release cargo molecule was quantified by UHPLC-MS.

**Procedure for the release of Trp from CM-Trp 46**

A 40 μM solution of CM-Trp 46 in PBS (1.0 mL) was irradiated at 790nm. Aliquots (135 μL; 0, 2, 5, 10, 20, 30 and 60 min) of the suspension were taken out and diluted with PBS to 270 μL. The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free Trp was quantified by UHPLC-MS.

**Table 26**: Ratio and percentage of Trp released upon irradiation of compound 46 (initial concentration 40 μM) at 790 nm (Ti:sapphire pulsed laser) in PBS medium. Aliquots of the solution were withdrawn at the indicated time points and diluted 1:1 in PBS. Quantification of Trp was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Trp (μM)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1.28 ± 0.00</td>
<td>3.2 ± 0.00</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>3.02 ± 0.03</td>
<td>7.6 ± 0.20</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>3.70 ± 0.07</td>
<td>9.3 ± 0.40</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>4.54 ± 0.04</td>
<td>11.4 ± 0.20</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>4.76 ± 0.08</td>
<td>11.9 ± 0.40</td>
</tr>
<tr>
<td>7</td>
<td>60</td>
<td>5.08 ± 0.03</td>
<td>12.7 ± 0.20</td>
</tr>
</tbody>
</table>
Procedure for the release of Trp from BFO-APTES-CM-Trp HNPs

A suspension of BFO-APTES-CM-Trp in PBS (1 mg/mL, 1.0 mL) was irradiated at 790nm. Aliquots (135 μL; 0, 1, 2, 5, 10, 15, 20 and 30 min) of the suspension were taken out, diluted with PBS to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free Trp was quantified by UHPLC-MS.

Table 27: Ratio and percentage of Trp released upon irradiation of BFO-APTES-CM-Trp NPs (loading 2 mg/mL) at 790 nm (Ti:sapphire pulsed laser) in PBS medium. Aliquots of the suspension were withdrawn at the indicated time points, diluted 1:1 in PBS and centrifuged (20 min, 13’000 rpm). Quantification of Trp in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Ratio Trp / HNPs (μM/ mg)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.03 ± 0.00</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.03 ± 0.00</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.54 ± 0.04</td>
<td>16.4 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>1.00 ± 0.10</td>
<td>30.4 ± 3.0</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>1.88 ± 0.09</td>
<td>57.1 ± 2.7</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>2.10 ± 0.17</td>
<td>63.8 ± 5.2</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>2.34 ± 0.09</td>
<td>71.1 ± 2.7</td>
</tr>
</tbody>
</table>

Procedure for the release of Trp from BFO-APTES-DIBO-Trp HNPs

A suspension of BFO-APTES-DIBO-Trp in PBS (1 mg/mL, 1.0 mL) was irradiated at 790nm. Aliquots (135 μL; 0, 2, 5, 10, 20, 30 and 60 min) of the suspension were taken out, diluted with PBS to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free Trp was quantified by UHPLC-MS.

Table 28: Ratio and percentage of Trp released upon irradiation of BFO-APTES-DIBO-Trp NPs (loading 2 mg/mL) at 790 nm (Ti:sapphire pulsed laser) in PBS medium. Aliquots of the suspension were withdrawn at the indicated time points, diluted 1:1 in PBS and centrifuged (20 min, 13’000 rpm). Quantification of Trp in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Ratio Trp / HNPs (μM/ mg)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>7</td>
<td>60</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>
**Experimental part**

**Procedure for the release of erlotinib from LNO-APTES-CM-erlo HNPs**

A suspension of LNO-APTES-CM-erlotinib in PBS (2 mg/mL, 0.8 mL) was irradiated at 790 nm. Aliquots (67.5 μL, 0, 2, 5, 10, 15, 20 and 30 min) of the suspension were taken out, diluted with PBS to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free erlotinib was quantified by UHPLC-MS.

**Table 29:** Ratio and percentage of erlotinib analogue 74 released upon irradiation of LNO-APTES-CM-erlo (loading 2 mg/mL) at 790 nm (Ti:sapphire pulsed laser) in PBS medium. Aliquots of the suspension were withdrawn at the indicated time points, diluted 1:4 in PBS and centrifuged (20 min, 13 000 rpm). Quantification of erlotinib analogue 74 in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Ratio 74 / HNPs (nM/ mg)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>58.4 ± 3.9</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>547.4 ± 20.3</td>
<td>32.7 ± 1.2</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>695.8 ± 23.0</td>
<td>41.5 ± 1.4</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>810.1 ± 26.5</td>
<td>48.4 ± 1.6</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>936.5 ± 23.4</td>
<td>55.9 ± 1.4</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>1133.4 ± 29.3</td>
<td>67.7 ± 1.7</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>1134.4 ± 27.9</td>
<td>67.7 ± 1.7</td>
</tr>
</tbody>
</table>

**Procedure for the release of DOX from LNO-APTES-CM-DOX HNPs**

A suspension of LNO-APTES-CM-DOX in PBS (2 mg/mL, 0.8 mL) was irradiated at 790 nm. Aliquots (67.5 μL, 0, 2, 5, 10, 15, 20 and 30 min) of the suspension were taken out, diluted with PBS to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free DOX was quantified by UHPLC-MS.

**Procedure for the release of Cbl from CM-Cbl 78**

A 2 μM solution of CM-Cbl 78 in a mixture of PBS / ACN (1:1, 0.8 mL) was irradiated at 790 nm. Aliquots (67.5 μL, 2, 5, 10, 15, 20, 25 and 30 min) of the suspension were taken out and diluted with ACN to 270 μL. The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free Cbl was quantified by UHPLC-MS.
Table 30: Ratio and percentage of Cbl released upon irradiation of CM-Cbl 78 (2 μM) at 790 nm (Ti:sapphire pulsed laser) in PBS / ACN medium. Aliquots of the solution were withdrawn at the indicated time points and diluted 1:4 in ACN. Quantification of Cbl in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Cbl (nM)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>142.8 ± 13.6</td>
<td>7.1 ± 0.7</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>192.2 ± 4.2</td>
<td>9.6 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>434.8 ± 9.0</td>
<td>21.7 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>434.5 ± 19.4</td>
<td>21.7 ± 0.9</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>527.3 ± 50.0</td>
<td>26.4 ± 2.5</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>557.4 ± 5.3</td>
<td>27.8 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>656.8 ± 11.2</td>
<td>32.8 ± 0.6</td>
</tr>
</tbody>
</table>

Procedure for the release of Cbl from LNO-APTES-CM-Cbl HNPs

A suspension of LNO-APTES-CM-Cbl in mixture of PBS / ACN (1:1, 0.8 ml, 1 mg/ml) was irradiated at 790nm. Aliquots (67.5 μL, 0, 2, 5, 10, 15, 20, 25 and 30 min) of the suspension were taken out, diluted with ACN to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free Cbl was quantified by UHPLC-MS.

Table 31: Ratio and percentage of Cbl released upon irradiation of LNO-APTES-CM-Cbl HNPs (loading 1 mg/mL) at 790 nm (Ti:sapphire pulsed laser) in PBS / ACN medium. Aliquots of the solution were withdrawn at the indicated time points, diluted 1:4 in ACN and centrifuged (20 min, 13'000 rpm). Quantification of Cbl in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Ratio Cbl / HNPs (nM/ mg)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>103.9 ± 8.8</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>421.1 ± 19.4</td>
<td>11.6 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>879.6 ± 38.1</td>
<td>24.1 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>1353.5 ± 22.8</td>
<td>37.1 ± 0.6</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>1946.8 ± 47.5</td>
<td>53.4 ± 1.3</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>2350.6 ± 458.8</td>
<td>64.5 ± 4.4</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>2824.2 ± 54.5</td>
<td>77.5 ± 1.5</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>2816.7 ± 58.4</td>
<td>77.3 ± 1.6</td>
</tr>
</tbody>
</table>

Procedure for the release of Cbl from LNO-APTES-DIBO-Cbl HNPs

A suspension of LNO-APTES-DIBO-Cbl in mixture of PBS / ACN (1:1, 0.8 ml, 1 mg/ml) was irradiated at 790nm. Aliquots (67.5 μL, 0, 2, 5, 10, 15, 20, 25 and 30 min) of the suspension were taken out, diluted with ACN to 270 μL and were centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free Cbl was quantified by UHPLC-MS.
Table 32: Ratio and percentage of Cbl released upon irradiation of LNO-APTES-DIBO-Cbl HNPs (loading 1 mg/mL) at 790 nm (Ti:sapphire pulsed laser) in PBS/ACN medium. Aliquots of the solution were withdrawn at the indicated time points, diluted 1:4 in ACN and centrifuged (20 min, 13’000 rpm). Quantification of Cbl in the supernatant was performed by UHPLC-MS on triplicates.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (min)</th>
<th>Ratio Cbl / HNPs (nM/ mg)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>92.6 ± 7.9</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>123.12 ± 8.0</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>98.56 ± 1.3</td>
<td>2.7 ± 0.0</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>101.68 ± 6.3</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>119.4 ± 5.8</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>121.28 ± 3.5</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>99.4 ± 5.3</td>
<td>2.7 ± 0.1</td>
</tr>
</tbody>
</table>

Procedure for the release in presence of cells

Cells incubated with or without HNPs were irradiated for 15 min at 790 nm using the laser setup conditions. For cells incubated with LNO-APTES-CM-erlo and an aliquot (100 μL) of the supernatant was taken out, diluted with a solution of MeOH containing 0.1% of formic acid (200 μL) and was centrifuged (20 min, 13 000 rpm). The clear supernatant was removed, divided into MS vial (triplicate) and the amount of free drug was quantified by UHPLC-MS.

Kinetic calculation of photorelease

The amount of cargo molecule released upon UV-A irradiation or NIR excitation was plotted as a function of irradiation time and fitted with mono- or bi-exponential functions according to equations (1) and (2), respectively. Equations (1) and (2) were differentiated with respect of time (t) to get the initial release rate constant at a given time point (equations (3) and (4)).

\[
[Trp] = A(1 - \exp(-k * t)) \\
[Trp] = A_1(1 - \exp(-k_1 * t)) + A_2(1 - \exp(-k_2 * t)) \\
\frac{d}{dt}[Trp]_t = A * k(\exp(-k * t)) \\
\frac{d}{dt}[Trp]_t = A_1 * k_1(\exp(-k_1 * t)) + A_2 * k_2(\exp(-k_2 * t))
\]

A: pre-exponential factor
k: rate constant
t: time
Experimental part

UV-induced photolysis experiments were performed with a Sylvania UV-light tube (366 nm, 8W). The initial release rate ($k_0$), calculated at 5 min and normalized from fitted parameters.

Figure 78: Progress of the release of the caged molecule upon irradiation at 366 nm / 8W; the curves were plotted with a biexponential fit. A) release of Trp upon irradiation of CM-Trp 46; B) release of Trp upon irradiation of BFO-APTES-CM-Trp; C) release of erlotinib analogue 74 upon irradiation of LNO-APTES-CM-erlo; D) release of Cbl upon irradiation of CM-Cbl 78; E) release of Cbl upon irradiation of LNO-APTES-CM-Cbl.
NIR-induced photolysis experiments were performed with a Ti:Sapphire laser system (790 nm). The initial release rate ($k_0$), calculated at 5 min and normalized from fitted parameters.

**Figure 79**: Progress of the release of caged molecule upon irradiation with Ti:sapphire pulsed laser system at 790 nm; the curves were plotted with a monoeponential fit: A) release of Trp upon irradiation of CM-Trp 46; B) release of Trp upon irradiation of BFO-APTES-CM-Trp; C) release of erlotinib analogue 74 upon irradiation of LNO-APTES-CM-erlo; D) release of Cbl upon irradiation of CM-Cbl 78; E) release of Cbl upon irradiation of LNO-APTES-CM-Cbl.
4.5.3 Quantification of released molecular cargo by UHPLC-MS

Quantification of Trp:

MS experiments were runned on a 6530 Accurate Mass Q-TOF LCMS mass spectrometer coupled to the 1290 Infinity UHPLC system (Agilent Technologies, USA). Analyses were performed on an ACQUITY UPLC® BEH C18 1.7µm column, 2.1 mm x 50 mm (Waters) heated at 30°C. The mobile phase was maintained at a flow rate of 0.4 mL/min and contained 0.1% (v/v) formic acid water solution (A), and 0.1% (v/v) formic acid acetonitrile solution (B). Over a 6 minutes total run, the gradient was: 0-0.5 min, 1-5% B; 0.5-3 min, 5-95% B; 3-3.5 min, 95-1% B; 3.5-6 min, 1% B to re-equilibrate the system in initial conditions. The sample manager system temperature was fixed at 15°C and the injection volume was 5 µL. The ESI source was set in positive ionization mode ionization using the Dual AJS Jet stream ESI Assembly. The QTOF instrument was operated in the 4 GHz High Resolution mode in profile mode. The Instrument was calibrated in positive full scan mode using ESI-L+ solution (Agilent Technologies, USA). The TOF mass spectra were acquired over the range of m/z 100-1000 at an acquisition rate of 3 spectra/s. ESI AJS settings were as follows RF drying gas flow, 8 L/min; drying gas temperature, 300 °C; nebulizer pressure, 35 psi; capillary voltage, 3500 V; nozzle voltage, 1000 V; fragmentor voltage, 175 V; skimmer voltage, 65 V; octopole 1 RF voltage, 750 V; Sheath gas temperature, 350 °C; Sheath gas low; 11 L/min . Data were processed using the MassHunter Workstation (Agilent Technologies, USA).

Stock solutions of Trp were prepared at 1.0 mM in PBS. Working standards were prepared in PBS at concentrations of 10 µM, 1 µM, 0.5 µM and 0.1 µM by using serial dilutions of stock solutions. Extracted ions chromatograms (XIC) were based on a retention time (RT) of 1.5 min with a window of ±0.5 min using a mass-extraction-window (MEW) of ±50 ppm centered on the m/z theor 205.0971. The average peak area of three replicate injections at each concentration was used for each data point. Calibration curve were fitted with a polynomial order 2 equation, with R2 > 0.99 for all days. The %CV of the lowest calibration point (100 nM) was lower than 3% within the same batch of samples and <5% between all experiments.
Experimental part

Figure 80: (a) Typical XIC (205.0971 MEW of ±50 ppm) of a Trp standard at 10 µM eluted after 1.5 min; (b) Typical MS of Trp standard; (c) Typical calibration curve for Trp.

Quantification of erlotinib and DOX

MS analysis were analyzing using the 6530 Accurate Mass Q-TOF LCMS mass spectrometer coupled to the 1290 Infinity UHPLC system (Agilent Technologies, USA). Analysis were performed on an ACQUITY UPLC® BEH C18 1.7µm column, 2.1 mm x 50 mm (Waters) heated at 30°C. The mobile phase was maintained at a flow rate of 0.4 mL/min and contained 0.1% (v/v) formic acid water solution (A), and 0.1% (v/v) formic acid acetonitrile solution (B). Over a 4 minutes total run, the gradient was: 0-0.5 min, 1-5% B; 0.5-2 min, 5-95% B; 2-2.1 min, 95-1% B; 2.1-4 min, 1% B to re-equilibrate the system in initial conditions. The sample manager system temperature was fixed at 15°C and the injection volume was 5 µL. The ESI source was set in positive ionization mode ionization using the Dual AJS Jet stream ESI Assembly. The QTOF instrument was operated in the 4 GHz High Resolution mode in profile mode. The Instrument was calibrated in positive full scan mode using ESI-L+ solution (Agilent Technologies, USA). The TOF mass spectra were acquired over the range of m/z 100-600 at an acquisition rate of 3 spectra/s. ESI AJS settings were as follows RF drying gas flow, 8 L/min; drying gas temperature, 300 °C; nebulizer pressure, 35 psi; capillary voltage, 3500 V; nozzle voltage, 1000 V; fragmentor voltage, 175 V; skimmer voltage, 65 V; octopole 1 RF voltage, 750 V; Sheath gas temperature, 350 °C; Sheath gas low; 11 L/min. Data were processed using the MassHunter Workstation (Agilent Technologies, USA). Extracted ions chromatograms (XIC) were calculated with a window of ±0.5 min using a
mass-extraction-window (MEW) of ±50 ppm. The average peak area of three replicate injections at each concentration was used for each data point.

Stock solutions of DOX were prepared at 1 mM in DMSO. Working standards were prepared in MeOH at concentrations of 1000 nM, 500 nM, 100 nM, 50 nM and 10 nM by using serial dilutions of the stock solution. Extracted ions chromatograms (XIC) were based on a retention time (RT) of 2.23 min with a window of ±0.5 min using a mass-extraction-window (MEW) of ±50 ppm centered on the \( m/z \) theor 544.1819. The average peak area of three replicate injections at each concentration was used for each data point. Calibration curve were fitted with a polynomial order 2 equation, with \( R^2 > 0.99 \) for all days.

Stock solutions of erlotinib analogue 74 were prepared at 1 mM in DMSO. Working standards were prepared in PBS at concentrations of 5000 nM, 1000 nM, 500 nM, 100 nM, 50 nM and 10 nM by using serial dilutions of the stock solution. Extracted ions chromatograms (XIC) were based on a retention time (RT) of 2.51 min with a window of ±0.5 min using a mass-extraction-window (MEW) of ±50 ppm centered on the \( m/z \) theor 335.1502. The average peak area of three replicate injections at each concentration was used for each data point. Calibration curve were fitted with a polynomial order 2 equation, with \( R^2 > 0.99 \) for all days.

Figure 81: Left image: (a) Typical XIC (544.1819 MEW of ±50 ppm) of a DOX standard at 1 µM eluted after 2.23 min; (b) Typical MS of DOX standard; (c) Typical calibration curve for DOX. Right image: (a) Typical XIC (335.1502 MEW of ±50 ppm) of an erlotinib standard at 1 µM eluted after 2.51 min; (b) Typical MS of erlotinib analogue 74 standard; (c) Typical calibration curve for erlotinib analogue 74.
Experimental part

Quantification of Cbl

Qualitative and quantitative analyses were conducted on a Xevo G2-S QTOF mass spectrometer coupled to the Acquity UPLC Class Binary Solvent manager and BTN sample manager (Waters, Corporation, Milford, MA). Mass spectrometer detection was operated in positive ionization using the ZSpray™ dual-orthogonal multimode ESI/APCI/ESCI® source. The TOF mass spectra were acquired in the sensitive mode over the range of m/z 50-700 at an acquisition rate of 0.036 sec/spectra. The instrument was calibrated using a solution of sodium formate (0.01 mg/L in isopropanol/H₂O 90:10). A mass accuracy better than 5 ppm was achieved using a Leucine Enkephalin solution as lock-mass (200 pg/µL in ACN/H₂O (50:50)) infused continuously using the LockSpray source. Source settings were as follows: cone, 25V; capillary, 3 kV, source temperature, 150°C; desolvation temperature, 500°C, cone gas, 10 L/h, desolvation gas, 500 L/h. Data were processed using MassLynx™ 4.1 software and QuanLynx application for quantification. The separation was achieved using an ACQUITY UPLC® BEH C18 1.7µm column, 2.1 mm x 50 mm (Waters) heated at 30°C. Mobile phase consisted of 0.1% formic acid in water as eluent A and 0.1% formic acid in acetonitrile as eluent B. After Injection volume of 5 μL, the separation was carried out at 0.4 mL/min over a 2.5 min total run time using the following program: 0-1 min, 1-99% B; 1-1.5 min, 95% B; 1.5-1.6 min, 95-1% B; 1.6-3 min, 1% B to reequilibrated the system in initial conditions. Stock solutions of Cbl were prepared at 1 mM in DMSO. Working standards were prepared in MeOH at concentrations of 1000 nM, 500 nM, 100 nM, 50 nM and 10 nM by using serial dilutions of the stock solution. Extracted ions chromatograms (XIC) were based on a retention time (RT) of 1.42 min with a window of ±0.5 min using a mass-extraction-window (MEW) of ±50 ppm centered on the m/z theor 304.0871. The average peak area of three replicate injections at each concentration was used for each data point. Calibration curve were fitted with a polynomial order 2 equation, with R² > 0.99 for all days.
**Experimental part**

**4.5.4 In vitro experiments**

**General procedure for incubation of HNPs with cells**

A suspension of HNPs was ultra-sonicated for 15 min. An aliquot of the NPs suspension was collected and centrifuged (13 000 rpm, 10 min). The supernatant was discarded and the NPs were resuspended in cellular media to obtain the desired concentration. The suspension was sonicated for 30 min and incubated with cells in an 8-well plate (Lab-Tek II Chambered Coverglass, triplicate per conditions) at 37°C for 16 h.

**Incubation of BFO-APTES-CM-Trp HNPs in HCC827 cells**

Stock solution of BFO-APTES-CM-Trp (2 mg / mL in ethanol) was diluted to a final concentration of 10 μg / mL in cellular media and incubated with HCC827 cells.

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**Figure 82:** (a) Typical XIC (304.0871 MEW of ±50 ppm) of a Cbl standard at 1 μM eluted after 1.42 min; (b) Typical MS of Cbl standard; (c) Typical calibration curve for Cbl.
Incubation of LNO-APTES-CM-erlo HNPs in HCC827 cells

Stock solution of LNO-APTES-CM-erlo (2 mg / mL in ethanol) was diluted to 100, 200 and 400 μg / mL in cellular media and incubated with HCC827 cells.

Evaluation of cytotoxicity by dosing LDH release:

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that is rapidly released into the culture medium upon rupture of the plasma membrane (Decker, 1988). Irradiation and HNPs induced cytotoxicity was evaluated by measuring LDH activity from cell culture supernatants. For each conditions, 100 µl of culture supernatants were collected 48 hours after exposure and incubated with the reaction mixture from the LDH colorimetric detection kit, following manufacturer’s instructions (Roche, Cat.No.11644793001). LDH released was then quantified by measuring the absorbance of each sample at 490 nm with a spectrophotometric microplate reader (Victor2, Perkin Elmer). Experiments were conducted in triplicates. Mean ± SD were calculated. Comparison between cells exposed to control (no HNPs and no irradiation) or to laser irradiation (790 nm) and Functionalized HNPs (with or without laser irradiation at 790 nm) were done using a Student’s t-test: *p<0.05; **p<0.01; NS: p>0.05.
5 References


References


References


References


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Passemand, S. Functionization of Nanoparticles for Targeted Cancer Imaging and Diagnosis, EPFL TH N°6248.


6 Curriculum vitae

Jérémy Vuilleumier
28.05.1991
Avenue de Gilamont 21D
1800 Vevey, Switzerland
Mobile: +41787567731
E-mail: jeremy.vuilleumier@epfl.ch

Education

PhD study in organic chemistry and polymers functionalization
École Polytechnique Fédérale de Lausanne (EPFL), Switzerland
2015-2019

Master in molecular and biological chemistry
École Polytechnique Fédérale de Lausanne (EPFL), Switzerland
2013-2015

Bachelor in chemistry
University of Fribourg, Switzerland
2010-2013

Matura (High School Diploma), Option Biology and Chemistry
Gymnase français de Bienne, Biel / Bienne
2007-2010

Professional experience

PhD Student in the Group for Functionalized Biomaterials
August 2015-November 2019
(EPFL; Prof. Gerber-Lemaire Sandrine)
- Functionalization of inorganic nanoparticles with organic molecules
- Design and synthesis of bioactive molecules
- Collaboration with biologists and physicists
- Selective release of bioactive molecule thanks to NIR light
- Supervision of Masters Projects and practical laboratory assistant for undergraduate students
(Winner of the awards for best teaching assistant in 2018)

Master Thesis: Laboratory of Catalysis and Organic Synthesis
September 2014-February 2015
(EPFL, Prof. Waser Jerôme)
- Optimization of synthesis via [4+2] cycloaddition with aminocyclobutanes for the synthesis of nucleoside analogues

Semester project: Laboratory of Catalysis and Organic Synthesis
February 2014-June 2014
(EPFL, Prof. Waser Jerôme)
- Synthesis of nucleoside analogues via [3+2] cycloaddition with aminocyclopropanes

Skills

Synthesis: - molecules, design of synthetic pathway, polymer synthesis, functionalization of nanoparticles, photochemistry

Analytical: - NMR, LC-MS, IR, DLS (dynamic light scattering), ZP (zeta potential)
Microscopy: - TEM, fluorescence microscopy
Software: - ChemDraw, MestReNova, Topspin, SciFinder, Reaxys, ImageJ, microsoft office
Language:
  French: Mother tongue  
  English: Professional proficiency  
  German: Basic

Conferences

Oral communications:
Swiss Summer School 2016 in Chemical Biology, Villars, Switzerland, August 21-25, 2016. Functionalization of second harmonic generation nanoparticles for theranostic applications.

Poster presentations:
SCS (swiss chemical society) Fall Meeting, Zurich, Switzerland, September 6, 2019.
SCS (swiss chemical society) Fall Meeting, Lausanne, Switzerland, September 7, 2018.
SCS (swiss chemical society) Fall Meeting, Bern, Switzerland, August 21-22, 2017.
SCS (swiss chemical society) Fall Meeting, Zurich, Switzerland, September 15, 2016.
Swiss Summer School 2016 in Chemical Biology, Villars, Switzerland, August 21-25, 2016.

List of publications


