Surface functionalization of metal oxide harmonic nanoparticles for targeted cancer imaging

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par

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Abstract

Cancer is a major health burden worldwide and the leading cause of death in industrialized countries. Early detection is a primary factor of survival and remission rate. Classical imaging methods such as magnetic resonance imaging, ultrasound imaging and microscopy lack in sensitivity for early detection of precancerous lesions. Recent and rapid progresses in nanotechnologies have paved the way for the development of new tools for diagnosis and targeted treatment of cancer. Taking advantage of their surface properties for post-conjugation coupled to their optical characteristics, nanoparticles could provide new level of sensitivity for the detection and imaging of tumors. The investigation of functionalization of nanoparticles with small molecules for the targeting of cancer biomarkers was thus an important aim of this thesis. Secondly, the synthesis of a new multimodal probe coupling magnetic resonance imaging and multiphoton microscopy was also investigated.

This work focuses on the development and application of synthetic pathways for small molecules targeting neoplastic cells and tumor microenvironment. Erlotinib and an inhibitor of fibroblast activation protein α were selected for their specific interactions with the epithelial growth factor receptor, present on the surface of lung cancer cells, and cancer-associated fibroblasts respectively. Those molecules where first modified with a biotin residue to assess their biocompatibility and their affinity for the targeted biomarker. The targeting ligands were further equipped with a cyclooctyne moiety for coupling with coated nanoparticles through bioorthogonal reaction.

Nanoparticles based on non-centrosymmetric crystals were selected for their optical properties. Polymeric or inorganic coating, based on poly(ethylene glycol) derivatives and (3-aminopropyl)triethoxysilane respectively, were applied to decorate the nanomaterials with azide moieties. The resulting azides were used to perform copper-free click reaction with the modified targeting ligands. The conjugated nanoparticles where then assessed for their selective association to the targeted biomarker. Nanoparticles decorated with an inhibitor of fibroblast activation protein α revealed target-specific association to the enzyme. In vitro investigation of the association of Erlotinib-tagged nanoparticles to epithelial growth factor receptor positive cells is ongoing.

Finally, a gadolinium complex was modified with an alkyne functional group for copper-catalyzed $[3 + 2]$-cycloaddition to the surface of coated nanoparticles. The functionalized nanoparticles were further assessed for their efficiency as magnetic resonance imaging contrast agent. Results revealed that conjugation of the gadolinium complex to the nanomaterial afforded a potent T$_1$ contrast agent without affecting its optical properties.

To summarize, several nanosystems were prepared through bioorthogonal reactions. The first part of this thesis presents the synthesis of two small molecules and subsequent
coupling to coated nanoparticles for targeted tumor imaging. The second part of this work, discusses the preparation of a new multimodal device for magnetic resonance imaging coupled to multiphoton microscopy. Those nanoprobes may provide new sensitivity levels for the study and the early detection of cancers.

**Keywords:** Imaging, cancer targeting, small molecule ligands, nanoparticles, functionalization, nonlinear optics, multimodality.

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**Résumé**

Le cancer est un problème majeur de santé publique mondiale et la première cause de mortalité dans les pays industrialisés. Le point de détection est un facteur primordial à la survie et à la rémission des patients. Les méthodes d’imageries classiques telles que l’imagerie par résonance magnétique, l’imagerie par ultrason ou la microscopie manquent de sensibilité pour la détection précoce de lésions précancéreuses. La récente et rapide avancée dans les nanotechnologies a ouvert la voie à l’étude des nanoparticules pour les installations cliniques, le diagnostic et le traitement ciblé du cancer. Grâce à leurs propriétés de surface éligibles pour la post-conjugaison de ligands de ciblage, couplées à leurs caractéristiques optiques, les nanoparticules pourraient permettre d’accéder à de nouveaux niveaux de sensibilité dans la détection et l’imagerie des tumeurs. La fonctionnalisation de nanoparticules avec des petites molécules pour le ciblage de biomarqueur de cancers a donc été exploré dans ce projet.

Ce travail s’est concentré sur le développement et l’application de voies de synthèses pour de petites molécules ciblant des cellules néoplasiques et le microenvironnement tumoral. L’Erlotinib et un inhibiteur de la protéine activatrice de fibroblastes α ont été sélectionnées pour leurs interactions spécifiques avec le récepteur du facteur de croissance épithéliale, présent à la surface de cellules cancéreuses pulmonaires, et les fibroblastes associés aux cancers, respectivement. Ces molécules ont tout d’abord été modifiées avec un résidu de biotine, afin de déterminer leur biocompatibilité et leur affinité pour le biomarqueur correspondant. Les ligands de ciblage ont par la suite été équipés d’un fragment comprenant une cyclooctyne pour le couplage avec des nanoparticules enrobées au travers de réactions bioorthogonales. Dans un deuxième temps, la synthèse d’une nouvelle sonde multimodale pour le couplage d’imagerie par résonance magnétique et de microscopie multiphotonique, a été explorée.

Des nanoparticules basées sur des cristaux non-centrosymétriques ont été sélectionnées pour leurs propriétés optiques. Un enrobage polymérique ou inorganique, à base de dérivées de poly(éthylène glycol) et de (3-aminopropyl)triéthoxysilane respectivement, ont été appliqués afin de décorer les nanomatériaux avec des fonctions azoture. Ces dernières ont été utilisées afin d’effectuer des réactions click sans cuivre avec les ligands de ciblage modifiés. Les
nanoparticules conjuguées ont ensuite été évaluées pour leur association sélective au biomarqueur ciblé. Les nanoparticules décorées avec un inhibiteur de la protéine activatrice de fibroblastes α ont révélé la capacité à se lier sélectivement à l’enzyme correspondante. Des mesures sont actuellement en cours pour déterminer l’association de nanoparticules marquées avec de l’Erlotinib aux cellules exprimant le récepteur du facteur de croissance épithéliale.

Finalement, la synthèse d’une nouvelle sonde multimodale, couplant l’imagerie par résonance magnétique à la microscopie multiphotonique, a été explorée. Un complexe de gadolinium a été modifié avec un groupe alcyne afin de réagir à la surface des nanoparticules enrobées au travers d’une cycloaddition [3 + 2] catalysées par du cuivre. L’efficacité des nanoparticules fonctionnalisées comme agent de contraste pour l’imagerie par résonance magnétique a ensuite été mesurée. Les résultats ont révélé que la conjugaison de complexes de gadolinium sur le nanomatériau a fourni un puissant agent de contraste T₁ sans affecter ses propriétés optiques.

En résumé, plusieurs nanosystèmes ont été préparés au travers de réactions bioorthogonales. Deux petites molécules ont été synthétisées et couplées aux nanoparticules pour l’imagerie ciblée de tumeurs. De plus, un nouvel outil multimodal pour l’imagerie par résonance magnétique couplée à la microscopie multiphotonique a été préparé. Ces nano-sondes pourraient apporter un nouveau niveau de sensibilité pour l’étude et la détection précoce de cancers.

**Mots clefs** : Imagerie, ciblage du cancer, ligands à base de petites molécules, nanoparticules, fonctionnalisation, optique non linéaire, multimodalité.
Abbreviations

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<thead>
<tr>
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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AcOH</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>APTES</td>
<td>(3-Aminopropyl)triethoxysilane</td>
</tr>
<tr>
<td>AuNC</td>
<td>Gold nanocluster</td>
</tr>
<tr>
<td>AuNP</td>
<td>Gold nanoparticle</td>
</tr>
<tr>
<td>BARAC</td>
<td>Biarylazacyclooctyne</td>
</tr>
<tr>
<td>BFO</td>
<td>BiFeO$_3$</td>
</tr>
<tr>
<td>Boc</td>
<td>$\text{tert-Butyloxycarbonyl}$</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer-associated fibroblast</td>
</tr>
<tr>
<td>CBF</td>
<td>Cilia beat frequency</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
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<tr>
<td>DCC</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCE</td>
<td>Dichloroethane</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DIBAC</td>
<td>Dibenzoazacyclooctyne</td>
</tr>
<tr>
<td>DIBO</td>
<td>Dibenzylcyclooctyne</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>$N,N$-Dimethylformamide dimethyl acetal</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPP4</td>
<td>Dipeptidyl peptidase 4</td>
</tr>
<tr>
<td>EDCI</td>
<td>$N$-(3-Dimethylaminopropyl)-$N'$-ethylcarbodiimide hydrochloride</td>
</tr>
<tr>
<td>EDS</td>
<td>Energy-dispersive X-ray spectroscopy</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>Equiv.</td>
<td>Equivalent</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization-mass spectrometry</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FAP$\alpha$</td>
<td>Fibroblast activation protein $\alpha$</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>FCC</td>
<td>Flash column chromatography</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FDG</td>
<td>$^1$F-labelled fluoro-deoxy-d-glucose</td>
</tr>
<tr>
<td>FET</td>
<td>Fluoroethyltyrosine</td>
</tr>
<tr>
<td>FLT</td>
<td>Fluorothymidine</td>
</tr>
<tr>
<td>FMISO</td>
<td>Fluoromisonidazole</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>GCO</td>
<td>Global Cancer Observatory</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HER1</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>HER2</td>
<td>Epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HG</td>
<td>Harmonic generation</td>
</tr>
<tr>
<td>HNP</td>
<td>Harmonic nanoparticle</td>
</tr>
<tr>
<td>HOBt</td>
<td>1-Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>Human recombinant</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Half maximal inhibitory concentrations</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>K$_i$</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>LDA</td>
<td>Lithium diisopropylamide</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LNO</td>
<td>LiNbO$_3$</td>
</tr>
<tr>
<td>LSPR</td>
<td>Localized surface plasmon resonance</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NaAsc</td>
<td>Sodium ascorbate</td>
</tr>
<tr>
<td>NC</td>
<td>Nanocluster</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>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small-cell lung carcinoma</td>
</tr>
<tr>
<td>PAT</td>
<td>Photoacoustic tomography</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Petroleum ether</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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</table>
PET  Positron-emission tomography  
Ph   Phenyl  
PMHS Polymethylhydrosiloxane  
PREP Prolyl oligopeptidase  
PSMA Prostate-specific membrane antigen  
Py   Pyridine  
QD   Quantum dots  
RGD  Arginine-Glycine-Aspartic acid  
rpm Rotation per minute  
rt   Room temperature  
SHG  Second harmonic generation  
SI   Selectivity index  
SPECT Single-photon-emission computerized tomography  
SPION Superparamagnetic iron oxide  
TACN 1,4,7-Triazacyclononane  
TAM Tumor associated macrophage  
TEER Trans-epithelial electrical resistance  
TEM Transmission electron microscopy  
TEOS Tetraethyl orthosilicate  
TFA Trifluoroacetic acid  
TFAA Trifluoroacetic anhydride  
TfR Transferrin receptor  
THF Tetrahydrofuran  
THG Third harmonic generation  
TK Tyrosine kinase  
TLC Thin layer chromatography  
Tris Tris(hydroxymethyl)aminomethane  
Ts   Tosyl  
TsCl Tosyl chloride  
UCNP Upconversion nanoparticles  
UPLC Ultra-Performance Liquid Chromatography  
US Ultrasound  
UV Ultraviolet  
XPS X-ray photoelectron spectroscopy
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1 Introduction

1.1 Context

Cancer has become one of the major public health problems worldwide and the leading cause of death in industrialized countries.\textsuperscript{1,2} In 2018, the International Agency for Research on Cancer estimated that 18 million new cases appeared and more than 9 million people died of cancer.\textsuperscript{2} Those numbers are expected to increase due to population growth and aging but also to current lifestyle (overweight and solar exposure).\textsuperscript{3,4}

The Global Cancer Observatory (GCO) established that lung cancers were the most diagnosed and lethal type of cancer with an estimation of 2 million new cases and 1.7 million deaths in 2018 (Figure 1). Breast cancer is the second most commonly diagnosed cancer with an estimation of 2 million new cases in 2018, even though it only affects female patients. It also happens to be the leading cause of cancer-related death for female patients. Finally, for both sexes, colorectal cancer was the third most diagnosed cancer and the second most lethal cancer with an estimation of 1.8 million incidence cases and 880 thousand deaths.
Introduction

Survival and remission rates are highly correlated with detection point. In many cases, it is established that the development of malignant lesions requires substantial time. Therefore, there is an urgent need to develop sensitive and accurate methods able to detect precancerous lesions in their early stage of emergence. In addition to early diagnosis, therapeutic modalities featuring improved selectivity toward cancer cells and tumor tissues are strongly needed to reduce side-effects due to off-target activities.

1.2 Cancer diagnosis and imaging methods

Nowadays multiple techniques are used for cancer diagnosis. Simple medical examination or laboratory tests can be easily performed to detect specific cancer biomarkers in body fluids such as blood or urine. Those analyses usually represent the first step in diagnosis procedure. Complementary non-invasive imaging techniques can be used to visualize and localize tumors in the body, namely, computational tomography (CT), ultrasound (US) and
magnetic resonance imaging (MRI). Some invasive imaging techniques are also used for the same purpose such as single-photon-emission computerized tomography (SPECT), positron-emission tomography (PET) and fluorescence and bioluminescence imaging.\textsuperscript{8,9}

CT combines multiple X-ray images to generate a three-dimensional representation of the organs and internal tissues. CT is used, among others, to detect colorectal and lung cancers.

US imaging is based on the reflection of sound waves on tissues to afford an image. This technique uses high frequency sound waves that can penetrate deeply into the body.

In MRI imaging, a static magnetic field emits a radiofrequency pulse, which excites nuclear spins of tissue atoms. The difference in relaxation time of the hydrogens atoms present in the tissues allows the acquisition of a two-dimensional image.

SPECT and PET are nuclear imaging techniques that require intravenous injections of radionuclide sources. Gamma rays and positron-emitting isotopes are detected in SPECT and PET, respectively.

Optical fluorescence and bioluminescence imaging use genetically modified cells expressing fluorescent proteins or fluorescent-tagged molecules. The images are then obtained by optical microscopy.

Those techniques provide different levels of sensitivity (Figure 2). CT, US and MRI are used for macroscopic imaging while nuclear and optical nanosensors provide molecular-level imaging.

<table>
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<th>Anatomy</th>
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<td>CT</td>
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<td>US</td>
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<td>MRI</td>
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<td>SPECT/PET</td>
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<td>Optical</td>
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<td>Nanosensor</td>
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Figure 2: Overview of the sensitivity of different imaging techniques.

All these techniques have their advantages and drawbacks: CT acquisition time is short and affords high spatial resolution images but is limited by the radiation exposure of the subject; MRI has deep penetration in tissues and affords high spatial resolution images with great soft tissue contrast but has very low sensitivity leading to long acquisition time and need for contrast agents; PET and SPECT have excellent sensitivity and high depth penetration but are limited by the numbers of scan that a patient can have due to the use of ionizing radiations. In order to overcome those disadvantages and increase sensitivity, hybrid systems are commonly used. This approach is called multimodality.\textsuperscript{10} For instance, PET and CT can be used together to gain diagnostic accuracy.\textsuperscript{11} In this case the imaging is often done by
monitoring the accumulation of $^{18}F$-labelled fluoro-deoxy-d-glucose (FDG) in tumor tissues and is so called FDG-PET/CT. PET/MRI is another example of hybrid techniques.\textsuperscript{12} Due to interactions between usual PET detector and the MRI magnetic field, avalanche photodiodes are often used instead. Imaging is also done by monitoring the accumulation of $^{18}F$-labelled radio tracers like FDG, fluoromisonidazole (FMISO), fluorothymidine (FLT) or fluoroethyltyrosine (FET).\textsuperscript{13} PET/MRI shows good performances in comparison with PET/CT and even grants more precise definition on primary tumors.\textsuperscript{14}

For the last decades, great attention was given to nanoparticles (NPs) as imaging probes.\textsuperscript{15} The ability to produce NPs of tunable size combined to the versatility of their surface properties for post-functionalization have turned nanoprobes into the perfect candidates for multimodal imaging.\textsuperscript{16} Moreover, targeting ligands could also be combined with multimodal imaging NPs to afford high selectivity and allow precise detection of cancers.\textsuperscript{17} Furthermore, therapeutic agents could also be added on nanoprobes to allow the formation of theranostics tools (Figure 3).\textsuperscript{18}

![Figure 3: Schematic representation of multimodal theranostic nanoprobe.](image)

### 1.3 Nanoparticles in medicine

For the last twenty years, NPs have become major actors in the medicinal research field. The advances in producing different types of NPs, with tunable size and multiple shapes, allowed nanomedicine to develop.\textsuperscript{19} Taking advantage of their optical, electronic, magnetic, chemical and structural properties, a variety of organic or inorganic nanomaterials were developed and showed promising applications in drug delivery, diagnosis and specific targeting.\textsuperscript{20}

The unique physiochemical properties of NPs allow them to overcome pharmacological, biological and physical barriers of the body.\textsuperscript{21} Controlling the size and shape of NPs have been
widely studied. Indeed, it was pointed out that modification of the size and shape of NPs affected their internalization by cells through endocytosis. Moreover, the nanoscale of these particles plays a great role in cell permeability. This is especially true for cancer cells due to the enhanced permeability and retention effect (EPR) which allows specific accumulation of NPs in tumor cells (Figure 4). Moreover, the surface properties of NPs allow specific coating in order to increase their biocompatibility. Polyethylene glycol (PEG) was often used for this purpose as it highly increases the circulatory half-life of the NPs. For those reasons, the number of publications related to NP-based systems for disease diagnosis, controlled drug release and treatment monitoring has constantly increased over the last two decades.

Figure 4: Schematic representation of the EPR effect.

Most of NPs intended for biomedical applications are classified either as organic or inorganic NPs (Figure 5). Organic NPs are usually based on dendrimers, lipids, polymers and proteins. Their main advantage lies in their high biocompatibility. In addition, organic NPs do not need specific surface treatment. Several organic nanodevices have been approved by the FDA since the 1990s. In 1995, Doxil – a liposome formulation of the chemotherapeutic drug doxorubicin – was the first NP-based drug approved by the FDA. More recently, Abraxane, which is an albumin-bound form of the antineoplastic agent paclitaxel, was successively approved by the FDA for the treatment of metastatic breast cancer, advanced non-small cell lung cancer and late-stage pancreatic cancer.

Inorganic NPs are mainly composed of metals, metal oxides, quantum dots, silica and rare-earth elements. Bare inorganic NPs generally present unfavorable properties in physiological conditions (aggregation, hemolytic effect). However, their biocompatibility can be largely improved by surface coating with polymers (PEG, chitosan, dextran) or silica shells. Reactive functionalities displayed on the coating layer can be used for post-functionalization with targeting ligands, therapeutic payloads and imaging labels. A major
advantage of inorganic NPs, in comparison with organic ones, is their long lifetime. Accordingly, intensive research has been performed on this family of NPs for biomedical imaging applications. Several NP-based imaging probes were approved by the FDA or are under clinical trials, such as Feraheme® and Resovist® (superparamagnetic iron oxide NPs (SPION) used as contrast agent for MRI) or Corneel dots® (silica base NPs labeled with near infrared (NIR) fluorescent dye used for diagnosis of cancers).

![Diagram of organic and inorganic NPs for medicine](image-url)

**Figure 5:** Examples of different organic and inorganic NPs for medicine. “Reprinted (adapted) with permission from Nanotechnology in the Diagnosis and Treatment of Lung Cancer. Pharmacology & Therapeutics. Copyright (2019). Elsevier.”

### 1.3.1 Inorganic nanoparticles for imaging applications

Inorganic NPs are highly present in nanomedicine. The large versatility of their composition, structure and size, allows for a variety of applications, including drug delivery, photothermal and photodynamic therapies, as well as *in vitro* and *in vivo* imaging protocols. This section will focus on the main categories of inorganic NPs used as imaging probes.

#### 1.3.1.1 Superparamagnetic iron oxide nanoparticles

SPIONs are well-known and common NPs. Those magnetic NPs are made of maghemite ($\gamma$Fe$_2$O$_3$). Among the various protocols proposed for their synthesis, co-precipitation is by far the simplest technique to generate SPIONs. A mixture of Fe(II) and Fe(III) is reacted with hydroxide ions to form magnetite (Fe$_3$O$_4$) which precipitates at basic pH. Magnetite is then oxidized to maghemite in the presence of air, acid or base. The size of the NPs can be tuned by modulation of pH and Fe(II)/Fe(II) concentration ratio or by addition of chelating agents and polymers.
Due to their small size, SPIONs exhibit superparamagnetic properties. This implies that thermal energy is able to change randomly the orientation of the NPs and thus their magnetization. Therefore, the magnetic moment of a SPIONs system will be zero. When the system is in presence of an electromagnetic field, the magnetic moments align affording a total magnetic moment much stronger than in paramagnetic materials (Figure 6). Those physical properties depend on the size and shape of the SPIONs.

Taking advantage of their superparamagnetic properties, SPIONs can create small local magnetic fields that shorten the relaxation times (T₁ and T₂) of surrounding protons. This effect is much sought after in MRI because it increases contrast. T₁ and T₂ are called longitudinal and transversal relaxation times and play different roles in the interactions between protons. SPIONs are mainly used as T₂-agents.

In order to increase colloidal stability and biocompatibility, SPIONs were often coated with carboxylate or phosphate moieties, silica or polymers (dextran, PEG, etc.). Moreover, coating agents could be used for further conjugation with, for instance, targeting ligands. Nowadays, SPIONs are commonly used as MRI contrast agents as some of them were FDA approved, but they could also serve in other biological applications. Indeed, SPIONs were investigated for drug delivery or as therapeutic agents (hyperthermia).

1.3.1.2 Quantum dots

Quantum dots (QDs) are semi-conductor nanocrystals composed of II-VI or III-V elements. They are synthesized by deposition of surface-capping layers on a crystal core. The size of the crystals can be controlled by growing them at high temperature, which changes their optical properties (Figure 7).
Absorption of light by QDs allows excitation of their electrons, which reach higher energy levels. The electrons undergo vibrational relaxation followed by photon emission, while returning to their ground state. In this one-photon excited fluorescence pathway, the photons are emitted at a lower frequency than the excitation (Figure 8). The emission wavelength can be tuned by changing the size and the composition of the nanocrystals.\textsuperscript{58,59} The optical properties of QDs confer them great advantages with respect to usual dyes. The absorption spectrum of QDs are broad allowing excitation of multiple QDs with different emissions at the same time. This property can be very interesting in biological studies.\textsuperscript{60} Moreover, thanks to the sharp emission spectra of QDs, overlapping of emission is avoided. Furthermore, those nanocrystals show very good photostability over repeated absorption/emission cycles, unlike fluorescent dyes.\textsuperscript{61}

QDs are mainly insoluble compounds. In order to be soluble in water, QDs were often coated with hydrophilic molecules, like cyclodextrin,\textsuperscript{62} or hydrophilic polymers.\textsuperscript{63} The coating agents also allowed post-functionalization of ligands as proteins.\textsuperscript{64}

The major drawbacks of QDs are their high cytotoxicity and non-clearance from the body\textsuperscript{65}. This is mainly due to surface oxidation leading to release of cadmium in cells. Coating was proved to lower the toxicity but not to eliminate it. Those issues led to the use of QDs
mostly for *in vitro* imaging. Han *et al.* reported the use of zinc/cadmium/selenium based QDs for multicolor optical coding of biomolecules. Encapsulation of those NPs, having different sizes, in polymer microbeads allowed multicolour fluorescent image (Figure 9). The polymers were then tagged with DNA to perform DNA hybridization studies. The results proved the efficiency of their systems to bind selectively to the targeted DNA.

![Figure 9](image)

**Figure 9:** Different sized QDs encapsulated in tagged polymer microbeads for multicolor optical coding of biomolecules. “Reprinted (adapted) with permission from Quantum-Dot-Tagged Microbeads for Multiplexed Optical Coding of Biomolecules. Nature Biotechnology. Copyright (2011) Springer Nature.”

Recently, zinc/cadmium/selenium based QDs were reported as coupled imaging and drug delivery systems, for breast cancer cells. The NPs were coated with a polymer containing hyaluronic acid (a targeting ligand for breast cancer cells) and a tagged protein drug (CC-Cy5.5) was loaded on its surface. The QDs accumulated into cancer cells and the drug was specifically released into them through interactions with the microenvironment of the tumor (Figure 10).

![Figure 10](image)

**Figure 10:** Real-time imaging of CC-Cy5.5 release in breast cancer cells. “Reprinted (adapted) with permission from Multifunctional Hyaluronic Acid-Mediated Quantum Dots for Targeted Intracellular Protein Delivery and Real-Time Fluorescence Imaging. Carbohydrate polymers. Copyright (2019). Elsevier.”
1.3.1.3 Gold nanoparticles and nanoclusters

Gold NPs (AuNPs) are polycrystalline nanostructures with spherical shapes.\textsuperscript{67} Those nanocrystals are prepared by reduction of HAuCl\textsubscript{4} into Au(0) which precipitates and forms NPs.\textsuperscript{68} Gold nanoclusters (AuNCs) are ultra-small NPs, smaller than 2 nm.\textsuperscript{69} AuNCs are composed of a core of gold atoms surrounded by coordinating ligands.\textsuperscript{70} AuNCs can be separated in two categories: non-fluorescent and fluorescent. Non fluorescent AuNCs make use of phosphine\textsuperscript{71} or thiolate\textsuperscript{72} protecting ligands. Fluorescent AuNCs contain both Au(0) and Au(I) atoms. The presence of Au(I) allows those NCs to perform photoluminescence. They are formed using phosphor, sulfur or nitrogen containing ligands linked to Au(I) on the surface of the nanocrystals. Some groups presented the synthesis of AuNCs using thiolated-PEG,\textsuperscript{73} glutathione,\textsuperscript{74} small amines molecules,\textsuperscript{75,76} proteins\textsuperscript{77} or DNA as ligand.\textsuperscript{78}

AuNPs and AuNCs have very different optical properties. AuNPs are able to do localized surface plasmon resonance (LSPR).\textsuperscript{67} This physical phenomena happens when a small particle with a spherical shape is irradiated by light, inducing the free electrons of Au to oscillate collectively.\textsuperscript{79} LSPR is composed of two interactions: absorption, where the energy is converted into heat and scattering where the light is redirected to multiple directions. Together they form the extinction coefficient. The light scattered by AuNPs is much higher in intensity in comparison with organic dyes.\textsuperscript{80} The LSPR response can be tuned by changing the size, the shape and the morphology of the NPs.\textsuperscript{81}

Due to their small size, AuNCs do not perform LSPR, but can do photoluminescence.\textsuperscript{82} Similarly to QDs, AuNCs undergo one-photon excited fluorescence. The emission wavelength can be tuned by changing the size (number of gold atoms)\textsuperscript{83,84} and the ligands of the clusters.\textsuperscript{85}

Due to their low cytotoxicity,\textsuperscript{86} their large variety and their physical properties, intensive research has been made on AuNPs and NCs. Those nanostructures were shown to be applicable to sensing, imaging, therapeutic protocols and drug delivery both in vitro and in vivo.\textsuperscript{87-90} Wang et al. presented previously the use of nanoshells as contrast agents for photoacoustic tomography (PAT).\textsuperscript{91} Those nanoshells were composed of a silica core and a gold shell coated with PEG. In vivo PAT images of a rat brain were acquired after the injection of the nanoshells (Figure 11). High special resolution images could be acquired after 20 min and the NPs were rapidly cleared from the blood (significant decrease of the blood absorption after 6 hours).
AuNPs coupled with anti epidermal growth factor receptor (EGFR) antibody were also reported for imaging of cancer cells.\textsuperscript{92} Anti-EGFR monoclonal anti body were non-covalently attached to the surface of AuNPs. The obtained nanosystem proved to bind EGFR positive cancer cells at the membrane surface, while bovine serum albumin conjugated AuNPs (control) did not interact with the cells. Moreover, laser scanning confocal microscopy of cervical biopsies confirmed the specificity of anti-EGFR/AuNPs for tumor cells (Figure 12). Such system could be used as contrast agents for cancer diagnosis.

Figure 11: In vivo PAT imaging of a rat brain (A) before injection of nanoshells, (B) 20 min after injection of nanoshells and (C) 6 h after injection of nanoshells. “Reprinted (adapted) with permission from Photoacoustic Tomography of a Nanoshell Contrast Agent in the in Vivo Rat Brain. Nano Letters. Copyright (2004). American Chemical Society.”

Figure 12: Ex vivo laser scanning confocal reflectance of precancerous (A) and normal (C) cervical tissues. Ex vivo confocal fluorescence of precancerous (B) and normal (D) cervical tissues. “Reprinted (adapted) with permission from Real-Time Vital Optical Imaging of Precancer Using Anti-Epidermal Growth Factor Receptor Antibodies Conjugated to Gold Nanoparticles. Cancer Research. Copyright (2003). American Association for Cancer Research.”
1.3.1.4 Upconversion nanoparticles

Upconversion NPS (UCNPs) are inorganic materials doped with rare-earth elements (lanthanides). They are core-shell NPs formed by a host matrix such as fluorides, oxides and heavy halides. Among the variety of synthetic methods to generate UCNPs, one can cite:

- seed-mediated heat-up, where the shell layer grows around an existing core;
- Ostwald ripening strategy, which consist in growing larger NPs from less energetically stable smaller sacrificial NPs. Smaller NPs are less stable, due to their high surface-to-volume ratio;
- layer-by-layer assembly, which is based on seed-mediated heat-up techniques but with slow addition of shell precursor.

UCNPs have unique optical properties resulting from the presence of the doping lanthanides ions. These elements are able to absorb two or more photons of low energy and emit a photon with higher energy (Figure 13). Thereby, UCNPs can be excited with NIR light, which penetrates deeply into tissue, and emits visible or ultra violet (UV) light. The emission wavelength of UCNPs can be tuned by varying the composition of the nanocrystals. Moreover, their sharp emission pick coupled with high resistance to photobleaching and long fluorescence lifetime makes them important actors in the nanoprobe family.

In addition to their optical properties, UCNPs have a good biocompatibility. Due to their low cytotoxicity, rare metal-earth nanocrystals are present in many fields of biomedicine. Nowadays, UCNPs are widely investigated for cell imaging and drug delivery. Recently, Tian et al. reported the synthesis of silica coated UCNPs for in vivo colorectal tumor imaging. The coated UCNPs were functionalized with PEG, peptide or a colorectal cancer targeting ligand (UEA-I). In vitro and in vivo studies showed that the UCNPs did not exhibit cytotoxicity and were able to accumulate in tumor cells. In vivo images of mice bearing small tumor xenografts, injected with the ligand functionalized UCNPs, allowed specific tagging of the malignant cells (Figure 14). Thus, this nanosystem could be used for deep-tissue tumor imaging.
Figure 14: In vivo imaging of mice bearing small tumor xenografts treated with UCNP-UEA-I after 8 h. “Reprinted (adapted) with permission from Construction of Lanthanide-Doped Upconversion Nanoparticle-Ulex Europaeus Agglutinin-I Bioconjugates with Brightness Red Emission for Ultrasensitive in Vivo Imaging of Colorectal Tumor. Biomaterials. Copyright (2019) Elsevier.”

UCNPs were also reported for photodynamic therapy coupled to chemotherapy.\textsuperscript{104} The rare-earth doped UCNPs were first coated with poly(acrylic acid) to allow successive post-functionalization with a platinum prodrug, a photo sensitizer (rose Bengal) and PEG. The nanosystem was able to release the drug and to produce $^{1}O_2$ under NIR irradiation. Those coupled techniques allowed to increase the cell death of chemotherapeutic resistant cancer cells. Those NPs could become an alternative therapeutic agent for cis-platinum resistant tumors.

Moreover, reports showed that UCNPs could also be coupled with other NPs to make multimodal and theranostic systems.\textsuperscript{105,106}

\subsection*{1.3.1.5 Harmonic nanoparticles}

Harmonic NPs (HNPs) are based on non-centrosymmetric inorganic nanocrystals (Figure 15).\textsuperscript{107} Different synthetic pathways were developed depending on their chemical compositions. Soft-chemical routes\textsuperscript{108} and hydrothermal conditions\textsuperscript{109} can be used for the production of BaTiO$_3$ and ZnO. Niobate based HNPs can be prepared by hydrothermal\textsuperscript{110} or solvothermal\textsuperscript{111} treatment. Preparation of iron iodate Fe(IO$_3$)$_3$ was reported by co-precipitation.\textsuperscript{112} Bismuth ferrite (BiFeO$_3$) can be prepared by combustion,\textsuperscript{113} hydrothermal treatment\textsuperscript{114} and wet chemical method.\textsuperscript{115}
Nonlinear optic effects occur when an intense light source (laser) irradiates a material.\textsuperscript{116} The laser excitation is able to modify the optical properties of the material and thus its response. Multiple nonlinear effects exist and among them, second and third harmonic generations (HGs) are very interesting for bioimaging.

Second harmonic generation (SHG) is one of the first reported nonlinear effects.\textsuperscript{117} SHG is a scattering quantum phenomenon in which two photons of frequency $\omega$ are converted into a photon of frequency $2\omega$. When two photons of high intensity, coinciding in time and space, irradiate a non-centrosymmetric material, they are combined by the material which emits one photon with twice the frequency. This concept is described by Figure 16 (B) where the continuous line represents the group state of the material and the dashed lines represent virtual levels. This phenomenon can take place at any wavelength. Third harmonic generation (THG) is a similar phenomenon where three photons of frequency $\omega$ are converted into a photon of frequency $3\omega$. It also differs because both centro- and non-centrosymmetric material can generate THG. HGs display several advantages for microscopy such as increased spatial resolution, high penetration in tissues (NIR can be used as incident light), no photobleaching, sharp and tunable emission.\textsuperscript{107}
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Due to their non-centrosymmetric core, HNPs can perform HGs which make them very interesting targets for bioimaging. Studies performed in our group showed the low cytotoxicity of HNPs, which is a major asset for their use in biological media.\textsuperscript{118} Nevertheless, a slight hemolytic effect of those NPs was detected but could be countered by coating the HNPs, for instance with PEG.\textsuperscript{28,118} Lately, HNPs were reported as labelling agent for cell culture,\textsuperscript{28,119} in \textit{vivo} and \textit{ex vivo} imaging,\textsuperscript{120,121} drug delivery,\textsuperscript{122} and theranostic approaches.\textsuperscript{123}

Table 1 summarizes the major advantages of each inorganic NPs for imaging and biomedical applications.

<table>
<thead>
<tr>
<th>Types of NPs</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
</table>
| **SPIONs**   | • Paramagnetic by nature  
               • Highly controlled synthesis  
               | • Toxicity  
               • Low colloidal stability  
| **QDs**      | • High brightness  
               • High photostability  
               • Multicolor imaging  
               | • Toxicity  
               • Limited imaging resolution  
| **AuNPs**    | • Long observation time  
               • Biocompatible  
               • NIR light absorption  
               | • Limited penetration depth  
               • Strong illumination of the sample required  
| **UCNPs**    | • Narrow emission peak  
               • High photostability  
               • Upconversion emission  
               | • Broad size distribution  
               • Challenging synthesis  
| **HNPs**     | • Tunable wavelength of excitation and emission  
               • Non-linear HG  
               • High photostability  
               • High spatial resolution  
               • Deep imaging penetration  
               | • Require ultrafast laser system  

Table 1: Advantages and drawbacks of inorganic NPs for bioimaging.\textsuperscript{124}

The multiple advantages and few drawbacks of HNPs make them interesting materials for bioimaging. In this project, we will focus on the functionalization of HNPs for targeted imaging applications.
1.3.2 Multimodal imaging with inorganic nanoparticles

All the imaging techniques have their advantages and their drawbacks. As we discussed before, it is possible to avoid those drawbacks by combining different techniques. In this section, we will present some examples of inorganic nanosystems for multimodal imaging.

Taking advantage of their paramagnetic properties and their reactive surface, SPIONs were extensively investigated for multimodal imaging. SPIONs can for instance be functionalized with organic fluorophores. This kind of NPs can then be followed by MRI and optical scanners, fluorescent microscopy, confocal microscopy, etc. SPIONs can also be coated with other metals to obtain core-shell NPs with different features. For instance, coating with gold allows detection of NPs by MRI and colorimetric sensing. Lanthanides coatings were also reported to afford luminescent properties to SPIONs. Radiolabeled iron oxides were also synthesized for PET/MRI imaging.

AuNCs were investigated as multimodal imaging agents. By including radioactive gold in the composition of the NCs, AuCNs could be imaged by fluorescence and SPECT. Florescence was also coupled to CT imaging by functionalizing AuNCs with iodine. AuNCs was also used as MRI contrast agent by conjugation with GdO3 NPs using BSA as template, or by functionalizing the NCs with a Gd chelating agent. Furthermore, due to their physical properties, AuNCs could be used for CT imaging. Thus, gold-gadolinium based NCs were synthesized for triple imaging by NIR fluorescence, MRI and CT.

Part of the lanthanide family, Gd(III) is the most used extracellular MRI contrast agent due to its strong paramagnetic properties. Due to the toxicity of free Gd, this ion is often used with a chelating organic molecule as a T1-agent. Gd(III) can also serve as host for the formation of UCNPs and be used as MRI contrast agent. Gd(III) host was used to form UCNPs with paramagnetic properties. Those gadolinium host can be co-doped with, for instance, Yb and Er to obtain NPs with up-conversion florescent and MRI imaging properties. Gd(III) can also be co-doped on NaYF4 in the presence of other lanthanides to obtain similar properties. UCNPs can also be used as T2 contrast agent by coating SPIONs with lanthanides, as reported earlier. Incorporation of radioactive fluoride (18F) in UCNPs was also reported to allow PET imaging. Finally NaYF4 co-doped Gd, Yb, Er labeled with 18F were synthesized to do triple imaging by up-conversion luminescence, MRI and PET.

As MRI is an important feature for multimodality and Gd(III) is often used for this purpose, we will briefly present different chelating agents which are suitable for further conjugation to imaging NPs. Small molecular chelates, either macrocyclic or linear, are generally based on polyazacycloalkane cores substituted by carboxylate-containing fragments (Scheme 1). DOTAREM® and MAGNEVIST® are commercially available T1 contrast agents that exhibit similar 𝑟1 relaxivity between 3.5 and 3.8 mM⁻¹s⁻¹. Those chelating agents are
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commonly used by injection into the patient bloodstream and is rapidly cleared from the body through renal route.

Several modifications of the chelating agents can be done in order to increase their relaxivities. As the rotational motion of the complexe plays an important role in the relaxivity, optimization of this parameter could be done to enhance the efficiency of contrast agents. It was shown that slowing the rotational motion of the complexe did increase relaxivity. This could be done by using molecules with higher molecular weight. Thus, macrocyclic Gd(III) chelates, based on proteins, polymers and dendrimers, were reported to be more efficient as contrast agents. Furthermore, those complexes showed higher thermodynamic and kinetic stability. For instance, measurements on polylysine Gd-DTPA revealed a $r_1$ relaxivity 2.5 times higher than its corresponding small molecule. Several examples showed conjugation of Gd(III) on the surface of NPs through chelating agents. Macromolecular chelates are protein-, polymer- and dendrimer-based and are often used as such.

Scheme 1: Examples of Gd(III) chelating agents. “Reprinted (adapted) with permission from Gadolinium‐based contrast agents for magnetic resonance cancer imaging. WIREs Nanomedicine and Nanobiotechnology. Copyright (2012) John Wiley and Sons.”
Increasing the hydration number of the gadolinium ion could also be a solution to obtain a better relaxivity.\textsuperscript{149} This could be performed by using, for instance, heptadentate ligands instead of octadentate ones. Unfortunately, this approach often reduces the thermodynamic stability of the complex and increases the risk of Gd(III) release in the body. Nevertheless, some examples of stable Gd(III) heptadentate chelating agents were previously reported.\textsuperscript{149,150}

The rotation of the water molecules hydrating the Gd(III) ion also plays a role in the efficiency of the contrast agent.\textsuperscript{151} Minimization of this so-called internal motion increases the relaxivity. This could be performed by additions of moieties containing H-bond acceptors which would interact with the water molecule, slowing its rotation.\textsuperscript{152}

Water exchange rate is also an important factor for contrast agents efficiency.\textsuperscript{145} The water exchange has to be fast in order to propagate the relaxation effect efficiently. Increasing the exchange rate can be done by enhancing the steric hindrance around the water binding site.\textsuperscript{153} Nevertheless, those modifications showed to reduce the stability of the complexes and might induce toxicity.\textsuperscript{154}

Finally, relaxivity of Gd(III) complexes can be modulated by their coupling to the surface of NPs.\textsuperscript{155} High quantity of Gd(III) complexes can be present on the surface of the NPs allowing the increase of the contrast agent efficiency. Furthermore, the slow rotational motion of the NPs allows the enhancement of the relaxivity of the complexes. For instance, formation of Gd-nanotubes was reported to increase by 40 times the $r_1$ value of a Gd(III) complex.\textsuperscript{156}

So far, there are only a few precedents in the use of HNPs for contrast enhancement in MRI.\textsuperscript{157} However, the conjugation of Gd(III) chelated at the surface of HNPs could provide powerful imaging probes using both multiphotonics and MRI to achieve high resolution and contrast as well as deep penetration in tissues. Part of the present project focused on the design and synthesis of nanomaterials composed by a core of HNPs conjugated to Gd(III) chelates to produce dual MRI-multiphotonics.

### 1.4 Coating and functionalization of nanoparticles

As previously discussed, coating of inorganic NPs is generally used to improve their colloidal stability and their biocompatibility. Coating agents displaying reactive functionalities also play a key role for post-functionalization strategies. This section presents common coating agents for metal oxide NPs and some possible reactions for post-functionalization of coated NPs.

#### 1.4.1 Coating of metal oxide nanoparticles

Coating of metal oxide NPs is very important for colloidal stabilization in aqueous media and preventing aggregation.\textsuperscript{41} Different coating agents for metal oxide NPs were reported. Monomers, inorganic materials and polymers are commonly used to enhance their
stability in aqueous media and their biocompatibility. Polymers and silica are often preferred due to their versatility and synthetic accessibility.

Silica coating for metal oxide NPs was well described in the literature. Inert silica layer can be formed at the surface of NPs improving their stability in aqueous media and granting biocompatibility.\textsuperscript{158} By using functionalized silica, it is also possible to obtain functional groups on the surface of the coating agent, allowing simple post functionalization. Those groups can be for instance amines, carboxyls, thiols or azides.\textsuperscript{122,159}

Different types of polymers were reported as suitable coating agents for metal oxides NPs. They can be natural as dextran,\textsuperscript{160} alginate and chitosan or synthetic as polyvinyl alcohol or PEG. Scheme 2 presents the structure of those different polymers.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{polymers.png}
\caption{Examples of coating polymers.}
\end{figure}

PEG is widely used as coating agent for several types of NPS. It was shown that PEG stabilizes NPs in aqueous media, reduces toxicity, increases their circulation time in blood and promotes accumulation in tumor cells.\textsuperscript{161} Furthermore, chemical modification of PEG is easily accessible. Our group previously reported a synthesis of heterobifunctional PEG derivatives for coating metal oxide NPs and post-functionalization with small molecules for targeted cell imaging.\textsuperscript{162}

1.4.2 Post-functionalization of nanoparticle surface

Once the NPs are coated with material containing reactive functions, coupling of interesting moieties on the surface is straightforward. Multiple reactions at the surface of NPs were thereby reported. For instance, amide bond formation through activated acid or coupling agent mediated were often used towards this goal.\textsuperscript{163–165} Cross-linkers were also reported for bioconjugation on the surface of NPs. Those molecules contain two electrophilic sites at opposite ends of a spacer. Most common reactive functions are succinimidyl esters and maleimides (Scheme 3) for reactions with amines and thiols, respectively.\textsuperscript{166} Those reactive species were also coupled in order to react with both amines and thiols.\textsuperscript{167} Unfortunately, those methods often lack in selectivity and stability.
Bioorthogonal and chemoselective reactions were thus developed to create suitable linkage between NPs and biomolecules. Reactions as Diels-Alder cyclization,\textsuperscript{168} hydrazone and oxime ligation,\textsuperscript{169} native ligation\textsuperscript{170} and Staudinger-Bertozzi ligation\textsuperscript{171} were previously used for post-functionalization on NPs.

Among them, [3 + 2] cycloaddition is very appreciated for biological applications.\textsuperscript{172,173} Special attention was given to “click” chemistry\textsuperscript{174} due to its efficiency and simplicity. This reaction implies the use of azide and alkyne moieties in the presence of catalytic amount of Cu(I) to form a triazole. To avoid the cytotoxicity of copper, this reaction can also be performed without catalyst by using strained cyclic alkyne (Scheme 4).\textsuperscript{175}

Bioorthogonal and chemoselective reactions for bioconjugations to NPs are summarized in Figure 17.
Our group reported surface post-functionalization of NPs by both catalyzed and strain-promoted [3 + 2] cycloadditions.162,176

1.5 Targeted cancer imaging

As previously discussed, NPs hold the intrinsic capacity to accumulate in cancer cells and tumors due to the EPR effect. Nevertheless, passive targeting is generally not efficient to fully avoid off-target activities. The conjugation of targeting ligands at their surface allowed substantial improvement to guide NPs specifically to malignant cells and tumor tissues.177 Different types of targeting ligand can be used for cancer biomarkers as small molecules, peptides, antibodies and more.178 Antibodies have high binding affinities and high target selectivity. However, they are known to induce immunogenicity. Furthermore, their production is complex and expensive. By contrast, peptides do not induce immune response and can be produced at lower cost. Nevertheless, peptides usually exhibit lower target affinities with increased non-specific binding. Furthermore, peptides can be degraded by proteolytic cleavage. Small molecules are widely used as targeting ligands for cancer biomarkers due to their infinite diversity. Their small size also allows high loading ratios at the NPs surface, thus increasing the targeting capacities. In this section, we will present different biomarkers and their ligands for targeting neoplastic cells and the microenvironment of tumors.
1.5.1 Neoplastic cells biomarkers

The most straightforward method for targeting tumors is to address biomarkers overexpressed by the tumor cells. Those receptors are located at the surface of neoplastic cells and play a great role in their development.\textsuperscript{179,180}

\(\alpha_v\beta_3\) integrin is often used as a biomarker due to its overexpression in cancer cells and its low presence in normal tissues. They are very often addressed with peptides containing an Arginine-Glycine-Aspartic acid (RGD peptides) moiety due to their high affinity for this receptor.\textsuperscript{181}

EGFR and Epidermal growth factor receptor 2 (HER2) play an important role in cell growth and invasiveness\textsuperscript{182,183}. EGFR is present in many types of tumors, including breast and colorectal cancers, and a non-small-cell lung carcinoma (NSCLC),\textsuperscript{182} while HER2 is mainly overexpressed in breast cancers.\textsuperscript{180} These receptors can be addressed by small molecular drugs targeting their tyrosine kinase activity.\textsuperscript{183,184}

Folic acid receptors were also widely investigated as targets due to their overexpression in several types of cancers.\textsuperscript{179} Furthermore, its corresponding ligand, folic acid, is cheap and easily accessed.

Other overexpressed proteins were also reported as valuable neoplastic cells biomarkers, such as transferrin receptor (TfR) or prostate-specific membrane antigen (PSMA).

Table 2 summarizes the most relevant cancer cell biomarkers and their ligands.
Biomarkers | Roles in tumors | Types of cancer | Ligands
--- | --- | --- | ---
\(\alpha_3\beta_3\) integrin | Angiogenesis\textsuperscript{185} | Breast, melanoma, glioblastoma\textsuperscript{179} | RGD peptides
EGFR | Induce cell aggressive growth and invasiveness | Breast, neck, NSCLC, ovarian, colon | Erlotinib, Gefetinib
HER2 | Cell growth | Breast | Trastuzumab, Lapatinib
TfR | Iron uptake for tumor growth\textsuperscript{179} | Liver, breast, pancreatic\textsuperscript{179} | Transferrin\textsuperscript{186,187}
PSMA | Expressed in aggressive prostate tumors\textsuperscript{188} | Prostate\textsuperscript{180} | Docetaxel\textsuperscript{189}, Anti-PSMA antibody\textsuperscript{190}
Folate receptors | Tumor growth\textsuperscript{191} | Ovarian, epidermal, colon, adenocarcinoma | Folic acid

Table 2: Relevant neoplastic cells biomarkers and their ligands.

Due to its overexpression in cancers of high incidence, EGFR is one of the biomarkers that we selected for addressing imaging nanoparticles to cancer cells. The following section details the properties of epidermal growth factor receptors and the main therapeutic molecules which were developed for their targeting.

1.5.1.1 Epidermal growth factor receptors and their ligands

The epidermal growth factor receptor family is composed of four members, EGFR (or HER1), HER2, HER3 and HER4.\textsuperscript{192} These receptors are transmembrane proteins presenting a tyrosine kinase (TK) enzymatic activity. EGFR was identified for its important role in cell growth, differentiation and survival.\textsuperscript{193} Overexpression of this protein in solid tumors was reported to induce uncontrolled proliferation, invasiveness and chemoresistance.

EGFR is a glycoprotein composed of an extracellular domain with a binding site, a transmembrane part and an enzymatic tyrosine kinase (TK) intracellular domain.\textsuperscript{182} Binding of specific ligands to the extracellular site induces dimerization of the receptor. The receptor is then internalized promoting the phosphorylation of the TK domain. This last step triggers a signal transduction cascade, leading to cell proliferation (Figure 18 A). Inhibition of EGFR was reported to reduce the proliferation of tumors. This can be done on both external and intracellular domains by monoclonal antibodies and small molecular inhibitors respectively.\textsuperscript{194} Antibodies prevent the activation of the TK domain by blocking the interaction of EGFR.
ligand to the binding site. Small molecular inhibitors of the TK domain compete with adenosine triphosphate in the TK binding pocket and thus prevent phosphorylation (Figure 18 B).

Figure 18: EGFR signaling pathway. (A) Normal activation induction downstream signaling. (B) Inhibition of the TK domain by a small molecule.

Erlotinib and Gefenib are two $N$-phenylquinazolin-4-amine derivatives presenting potent TK domain inhibition. Both molecules were approved by the FDA for the treatment of NSCLC. In certain cases, Erlotinib is also prescribed to pancreatic cancer patients.

Scheme 5: Erlotinib and Gefenib structure and affinity for EGFR. Value taken from

1.5.2 Targeting the tumor microenvironment

Addressing malignant tumor cells has been widely studied for decades. Nevertheless, it has been proven to be ineffective for the treatment of several types of cancers. For the past ten years, research focusing on the tumor microenvironment revealed its importance in the development of cancers. More precisely, the microenvironment of a given tumor was described to play a crucial role in its progression, metastasis and response to chemotherapeutic interventions. In 2018, Sverdlov described the tumor-stroma symbiotic crosstalk as a new hallmark for cancers, emphasizing the significance of disrupting the tumor-stroma interactions in anticancer strategies. In particular, targeting the component of the tumor microenvironment rather than neoplastic cells could provide alternative therapeutic intervention for cancer treatment.
The microenvironment of tumors is composed of cells species (Figure 19) with specific roles. Among them, tumor associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) could be interesting targets for disrupting the tumor-stroma crosstalk.

![Simplified representation of the tumor microenvironment.](image)

TAMs are involved in tumor survival, growth and metastasis. They represent 30% of the immune cells and can constitute 50% of the tumor microenvironment in invasive cancers. Multiple studies were published on targeting and depletion of TAMs.

CAFs are predominant stromal cells in most carcinomas. They are mesenchymal cells that play a primary role in the regulation of tumor fibrosis, immunosuppression, angiogenesis and metastasis. Their major role is the secretion of stroma modeling factors such as collagen, cytokines and chemokines. It was reported that CAFs also interact with healthy macrophages to promote the formation of TAMs, inducing invasiveness and metastasis. Due to the importance in the development of cancers, multiple studies were made in order to understand and suppress their interactions with cancer cells.

Fibroblast activation protein α (FAPα) is a transmembrane protein overexpressed at the surface of CAFs. Studies pointed out that inhibition of FAPα could reduce tumor cell growth. This protein could thus be an interesting biomarker for cancer microenvironment targeting.

### 1.5.2.1 Fibroblast activation protein α and its ligands

FAPα is a transmembrane glycoprotein part of the prolyl oligopeptidase family. This enzyme is able to cleave proteins and peptides after a proline residue. The structure of FAPα is very similar to two other family members, dipeptidyl peptidase 4 (DPP4) and prolyl
Introduction

oligopeptidase (PREP). Different studies showed that this family of proteins plays a key role in several diseases, including diabetes, arthritis and cancer.214,215

Studies showed that FAP\(\alpha\) plays an important part in tumor invasion, metastasis and angiogenesis.216,217 Inhibition of this protein was shown to reduce tumor growth and metastasis.206,218 As FAP\(\alpha\) is overexpressed in 90\% of CAFs and is not found in normal, healthy adult tissues, except in granulation tissue of healing wounds, it is a highly valuable biomarker for targeted imaging or therapy.219 Unfortunately, DPP4 and PREP, which are closely related to FAP\(\alpha\), are widely present in the body.220 Thus, it is important to develop very specific ligands.

Scheme 6 presents different small molecules proposed as FAP\(\alpha\) inhibitors.221–223

Scheme 6: Different FAP\(\alpha\) inhibitors and their selectivity.

In all cases, modified prolines were used as targeting moiety. This is due to the fact that FAP\(\alpha\) prefers cleaving after proline residues. Boronic acid and nitrile groups play an important role in the affinity for this protein. Indeed, those functional groups allow covalent binding to the serine active site of FAP\(\alpha\).221 Moreover, presence of a glycine or alanine residue also enhanced the selectivity of the inhibitor for FAP\(\alpha\) with respect to PREP and DPP IV. Presence of an aromatic group further increased the affinity and selectivity for FAP\(\alpha\). Based on docking studies, cation-π-interactions of a FAP\(\alpha\) side chain, with the aromatic group, was hypothesised.223 In 2013, Jansen and al. reported the most selective inhibitor with the best affinity for FAP\(\alpha\) (Scheme 6 D). They also showed that modification at the methoxy position is preferable to avoid interaction with the binding pocket. This molecule could be used for further functionalization on NPs for targeted imaging of tumors.
2 Presentation of the project

This PhD thesis was part of a collaborative European project called NANOFIMT (Functionalized HNPs for ultra-sensitive imaging and theranostic). In particular harmonic properties could be used for both the tracking of malignant cells/tissues and the light-activated controlled release of molecular payloads conjugated to the surface of HNPs. NANOFIMT takes advantage of the expertise of different academic groups and SMES in the areas of controlled synthesis of NPs (TIBIO sagl, Switzerland and Materials and Mechatronics Laboratory (SYMME), University of Savoie Mont Blanc, France), organic synthesis and surface functionalization (Group for Functionalized Biomaterials (GBF), École Polytechnique Fédérale de Lausanne, Suisse), multiphoton imaging (Group of Applied Physics (GAP), University of Geneva, Switzerland) and biology (EPITHELIX Sàrl, France).

The present work focused on the synthesis of ligands for targeting cancer biomarkers. Those small molecules were then functionalized at the surface of coated HNPs. Biological evaluations of the targeting ligands were done in collaboration with EPITHELIX Sàrl (France). TIBIO Sagl (Switzerland) and SYMME (France) provided HNPs. Imaging of functionalized HNPs in in vitro cellular and tissue models were performed in partnership with EPITHELIX Sàrl and GAP (Switzerland).

HNPs based on BiFeO₃ (BFO) and LiNbO₃ (LNO) nanocrystals were selected due to their high capacity to produce multiharmonic signals and their colloidal stability. These HNPs were further coated with modified PEG derivatives, previously developed in our group, or with (3-aminopropyl)triethoxysilane (APTES) derivatives to increase their biocompatibility and stability and allow post functionalization. The resulting coating layer displayed amino and azido groups to modulate the surface charge of the nanomaterials and allow post-functionalization through bioorthogonal azide to alkyne [3 + 2]-cycloaddition (Figure 20).
As previously exposed, both neoplastic cells and tumor microenvironment represent valuable targets for cancer detection, cancer progression monitoring and therapeutic intervention. Our efforts were thus devoted to the development of functionalizing ligands based on Erlotinib for EGFR targeting and N-acylGlyPro derivatives for FAPα targeting. As depicted in Scheme 7, these molecules display functional motifs (highlighted in red) which should be modified without disrupting their affinity and specificity for the corresponding biomarkers. Synthetic pathways were developed to introduce reactive groups on the targeting core for post-conjugation to the surface of coated HNPs. Use of such targeting ligands on HNPs has no precedent and could allow significant improvements in cancer study and detection through new levels of sensitivity.

We also investigated the synthesis of dual MRI-multiphotonic nanoprobes by addition of Gd(III)-chelates at the surface of HNPs (Scheme 8). The chelating agent based on Hæbpatcn was selected for its high affinity for Gd³⁺. Functionalization with an alkyne moiety was envisaged for copper mediated azide to alkyne cycloaddition at the HNPs surface. This kind of multimodal device was not often investigated and could allow imaging cancer at early stage with high resolution and contrast combined to deep penetration in tissues.
Scheme 8: Schematic representation of dual MRI-multiphonic nanoprobes based on HNPs.
3 Results and Discussion

3.1 Synthesis and biological evaluation of cancer targeting ligands

In the following sections, we will present the synthesis and evaluation of targeting ligands addressing cancer cells and tumor microenvironment. We will discuss their synthetic pathways, their derivatizations for post-conjugation to HNPs and the evaluation of their targeting ability. Figure 21 presents the general approach for the preparation of HNPs conjugated to cancer targeting ligands.

![General approach for the preparation of cancer targeted NPs.](image)

The spacer will be composed of a PEG$_3$ fragment to modulate the polarity of the targeting ligand coupled to a DIBO moiety for $[3 + 2]$ cycloaddition at the surface of coated HNPs, or a biotin residue for biological evaluations (Figure 22).

![DIBO and Biotin derivatives structures.](image)

Figure 21: General approach for the preparation of cancer targeted NPs.

Figure 22: DIBO and Biotin derivatives structures.


3.1.1 Synthesis and biological evaluation of a FAPα inhibitor

This section is based on the manuscript entitled: “Inhibitor-Conjugated Harmonic Nanoparticles Targeting Fibroblast Activation Protein”, RSC Adv. 2019, 9, 31659-31669.

As we presented previously, FAPα has a very similar structure with respect to DPPIV and PREP. Most reported FAPα inhibitors to date are not entirely specific. Jansen et al.223 reported lately that (4-quinolinoyl)-glycyl-2-cyanopyrrolidine scaffold allowed highly potent inhibition of FAPα and sharp selectivity with respect to the inhibition of DPPs and PREP. Thus, we considered functionalizing the quinoline ring’s 6-position to introduce a suitable spacer between the FAPα targeting unit and the HNPs surface. Based on previous studies,176 PEG3 was selected to modulate the polarity of the targeting ligand, which is a prerequisite to promote further efficient click reaction at the surface of HNPs in EtOH:H2O medium. An acrylamide moiety was foreseen in order to perform cross-coupling to the 6-position of the quinoline ring. Due to the sensitivity of the strained cyclooctyne moiety, a biotin label was selected to perform the evaluation of the inhibitory activity of the targeting ligand. In addition, this moiety is known to favor solubility in aqueous medium. Scheme 9 presents the synthesis of PEG3 linkers 3 and 5 for conjugation with DIBO and for biological evaluations, respectively.

Scheme 9: Synthesis of PEG3 linkers 3 and 5. Reagents and conditions: i- Propargylamine, Et3N, DCM, rt, 1.5 h; ii- 6, CuSO4, NaAsc, THF/H2O (4:1), rt, 16 h; iii- 2, CuSO4, NaAsc, THF/H2O (4:1), rt, 16 h.

Starting from activated acrylic acid 1, precursor 2 was prepared by amide bond formation in the presence of propargylamine. Amide 2 was then coupled to PEG3mono-azide (6) by copper mediated azide alkyne cycloaddition to obtain spacer 3 in good yield. In a similar way, copper catalyzed click reaction was performed on Biotin-PEG3-N3 (4) in the presence of amide 2 to obtain biotin-containing linker 5 in 73% yield.
Those linkers were then coupled to the FAPα inhibitor core described by Jansen et al. (Scheme 10).

Scheme 10: Jansen et al. reported FAPα inhibitors.

Synthesis of FAPα inhibitors 13, Biotin-PEG$_7$-FAPi (16) and DIBO-PEG$_7$-FAPi (15) are depicted in Scheme 11.

Scheme 11: Synthesis of FAPα inhibitors. Reagents and conditions: i- Sodium pyruvate, NaOH 2.5 M, reflux, 4 h; ii- water, microwave, 200 °C, 10 min; iii- (COCl)$_2$, DMF 1 h, then NH$_3$ 25%, rt, 2 h; iv- KOH, DMF, rt, 1 h; then 12, DMF, rt, 1.5 h; v- 4-Nitrophenyl chloroformate, Py, DCM, rt, 16 h; vi- 17, Et$_3$N, DMF, rt, 5 h; vii- 5, Pd(OAc)$_2$ cat., PPh$_3$, Et$_3$N, DMF, 110 °C, 7 h; viii- 3, Pd(OAc)$_2$ cat., PPh$_3$, Et$_3$N, DMF, 110 °C, 7 h. “Reprinted (adapted) with permission from Inhibitor-conjugated harmonic nanoparticles targeting fibroblast activation protein. RSC Advances. Copyright (2019). Royal Society of Chemistry.”

The inhibitor core was synthesized from 5-bromoisatin (9) by a Pfitzinger reaction$^{224}$ in the presence of sodium pyruvate to afford the dicarboxylic intermediate 10 in 97% yield. Selective decarboxylation$^{225}$ was then accessed by microwave irradiation in high yield. Subsequent amide formation delivered 6-bromoquinoline-4-carboxamide (11). Nucleophilic substitution in the presence of cyano-proline derivative 12 (synthesis previously described by
our group\textsuperscript{176} afforded intermediate \textbf{13}. This product constituted the core of the FAP\textalpha\ inhibitor. Pd-catalyzed Heck reaction\textsuperscript{226} in the presence of linker \textbf{3} was then performed in moderate yield. Activation of the alcohol moiety with 4-nitrophenyl chloroformate allowed conjugation with DIBO derivative \textbf{14} (synthesis described in the experimental section) to yield \textbf{DIBO-PEG$_3$-FAPi} (15) (68\% yield, 2 steps), ready to be conjugated with coated HNPs. Pd-catalyzed Heck reaction was also performed on compound \textbf{13} in the presence of linker \textbf{5} to afford \textbf{Biotin-PEG$_3$-FAPi} (16) in moderate yield.

We then assessed the ability of the designed ligand, to target selectively FAP\textalpha\ by testing the inhibitory activity of compound \textbf{13} and \textbf{Biotin-PEG$_3$-FAPi} on human recombinant (hr) FAP\textalpha, DPP IV and PREP (Table 3). The enzymes were incubated with increasing concentrations (5, 10, 20, 50 and 100 nM) of compounds \textbf{13} and \textbf{Biotin-PEG$_3$-FAPi}, for 30 min at 37 °C. Then, the residual enzymatic activity was measured after exposure to 50 $\mu$M of the appropriate substrates (Z-Gly-Pro-AMC for hrFAP\textalpha\ and hrPREP, H-Gly-Pro-AMC for hrDPP IV). The half maximal inhibitory concentrations (IC$_{50}$) were graphically determined and the inhibition constants (Ki) were calculated\textsuperscript{227}.

Table 3: Kinetic characteristics for the inhibition of human recombinant prolyl-oligopeptidases. “Reprinted (adapted) with permission from Inhibitor-conjugated harmonic nanoparticles targeting fibroblast activation protein. RSC Advances. Copyright (2019). Royal Society of Chemistry.”

<table>
<thead>
<tr>
<th>Compound</th>
<th>hrPREP IC$_{50}$ (µM)</th>
<th>hrDPP IV IC$_{50}$ (µM)</th>
<th>hrFAP\textalpha IC$_{50}$ (µM)</th>
<th>Ki (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.84 ± 0.07</td>
<td>&gt; 100</td>
<td>0.014 ± 0.001</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>6.1 ± 0.4</td>
<td>&gt; 100</td>
<td>0.0092 ± 0.0005</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>0.03 ± 0.005</td>
<td>12 ± 1.8</td>
</tr>
<tr>
<td>Biotin-PEG$_3$-FAPi</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>0.0092 ± 0.0006</td>
<td>3.8 ± 0.23</td>
</tr>
</tbody>
</table>

Compound \textbf{5} exhibited good inhibition of FAP\textalpha\ and high selectivity toward the two close members of the prolyl-oligopeptidases family. Addition of a hydrophilic spacer greatly increased the inhibitory effect of this molecule allowing \textbf{Biotin-PEG$_3$-FAPi} to reach nanomolar IC$_{50}$ and Ki values. This could be explain by a better solubility of the inhibitor in water allowing a higher concentration of the product in the protein media affording more molecules to interact with the target\textsuperscript{228}. Moreover, sharp selectivity with respect to PREP and DPP IV was observed (selectivity index (SI)$_{FAP\alpha/DPP IV}$ and SI$_{FAP\alpha/PREP}$ higher than 10000). \textbf{Biotin-PEG$_3$-FAPi} affinity revealed to be as powerful as Jansen \textit{et al.} methoxy compound but its selectivity, with respect to PREP, was highly increased. This could be due to the higher
solubility of Biotin-PEG$_3$-FAPi thanks to its PEG$_3$ spacer. Those results emphasized the possibility of using Biotin-PEG$_3$-FAPi and DIBO-PEG$_3$-FAPi as potential FAP$\alpha$ targeting ligands.

Biocompatibility of the targeting ligand was assessed on a model of human functional lung epithelium (MucilAir$^{TM}$-HF) based on the co-culture of bronchial cells and lung fibroblasts (Figure 23).$^{229}$ MucilAir$^{TM}$ is composed of basal, ciliated and mucus cells to fully reproduce ciliated epithelia.$^{230}$ This 3D model holds the property to mimic in vivo mechanisms making it more relevant than cell cultures for long-term studies.$^{231,232}$

Repeated doses of Biotin-PEG$_3$-FAPi were applied to this 3D tissue model over 16 days. Measurements of the lactate dehydrogenase (LDH) release in the culture supernatant allowed quantification of the cytotoxicity (Figure 24 A). Cell viability was not affected by repeated exposure to low concentration (10 $\mu$M) of the inhibitor. Toxicity of the compound could only be observed after 8 days at high doses (100 $\mu$M). Comparable results were obtained by analyzing the trans-epithelial electrical resistance (TEER) and the cilia beat frequency (CBF) (Figure 24 B and Figure 24 C). Measurements also revealed that damage to the epithelium was only observed at high doses (100 $\mu$M) (CBF lost after day 8). These results proved the biocompatibility of our compound at appropriate concentrations for labelling and imaging applications.
Results and Discussion

Figure 24: Evaluation of the cytocompatibility of the targeting ligand on the human lung tissue model MucilAir™-HF. Tissue was exposed to repeated doses (10 μM or 100 μM) of Biotin-PEG3-FAPi (day 0, 2, 4, 7, 9, 11 and 14) and measurements were made at different time points (day 2, 4, 6, 8, 10, 12, 14 and 16). (A) Cytotoxicity was evaluated by dosing LDH in the culture supernatant. (B) Trans-epithelial electrical resistance (TEER) measurement. (C) Cilia beat frequency (CBF) measurement. Triton X-100 was used as positive control; DMSO (1%) was used as negative control. “Reprinted (adapted) with permission from Inhibitor-conjugated harmonic nanoparticles targeting fibroblast activation protein. RSC Advances. Copyright (2019). Royal Society of Chemistry.”

In summary, we prepared two FAPα inhibitors, Biotin-PEG3-FAPi and DIBO-PEG3-FAPi, containing a tetraethylene glycol spacer to increase their hydrophilicity and avoid loss of affinity for the enzyme. Their central cores were prepared from 5-bromoisatin and L-proline through a convergent pathway and was produced in a high yield. Compound 13 was also included in the evaluation of the inhibitory activity toward FAPα, DPPIV and PREP. Biotin-PEG3-FAPi proved to be a nanomolar FAPα inhibitor with high selectivity with respect to two closely-related members of the family. Conjugation of DIBO-PEG3-FAPi to the surface of HNPs will be discussed in the next chapter. The resulting systems will aim at targeting the fibroblastic element of the tumor stroma.

3.1.2 Synthesis of Erlotinib derivatives

In order to target more specifically lung and breast cancer cells, we decided to investigate the derivatization of Erlotinib for further conjugation of the surface of imaging NPs. As discussed previously, Erlotinib is a FDA approved drug for treatment of NSCLC. It is part of the 4-anilinoquinazolines family and inhibits the intracellular TK domain of EGFR. Previous studies conducted in our group233,234 showed that modifying the ether chains of Erlotinib did not disrupt its capacity to inhibit the phosphorylation of EGFR on human based-derived MDA-MB-231 and lung-derived NCI-H520 cancer cells positive for the expression of the receptor. Based on this work, we decided to modify Erlotinib with either a biotin or a DIBO group
connected to the inhibitor core through a PEG₃ spacer. Scheme 12 presents the synthesis PEG₃ linkers 19 and 22 equipped with a terminal tosylate group for the derivatization of Erlotinib.

Scheme 12: Synthesis of PEG₃ linkers 19 and 22. Reagents and conditions: i- TsCl, NaOH, THF, rt, 1 h; ii- PEG₃-NH₂, Et₃N, DMF, rt, 3 h; iii- 50 % NaOH in water, TsCl, DMF, rt, 24 h.

Activation of PEG₃-Biotin 18 (synthesis described in the experimental section) to obtain linker 19 was performed in the presence of tosyl chloride and sodium hydroxide in high yield. DIBO derivative 20 (synthesis described in the experimental section) was coupled to PEG₃-NH₂ (23) to afford carbamate 21 in quantitative yield. Finally, tosylation of the terminal alcohol was performed to obtain linker 22 in good yield.

Those two tosylated linkers were then coupled to the Erlotinib derivatives developed in our group. The synthesis of Biotin-PEG₃-Erlotinib and DIBO-PEG₃-Erlotinib are described in Scheme 13.
Results and Discussion

Scheme 13: Synthesis of Erlotinib derivatives. Reagents and conditions: i- NH₂OH·HCl, AcOH, reflux, 3.5 h; ii- 2-chloroethanol, K₂CO₃, DMF, 150 °C, 20 h; iii- Acetyl chloride, Py, rt, 3 h; iv- HNO₃, rt, 35 min; v- Pd(OAc)₂ cat., PMHS, KF, THF, rt, 1.5 h; vi- DMF-DMA, AcOH, toluene, reflux, 2 h; vii- 3-ethynylaniline, AcOH, reflux, 2 h; viii- K₂CO₃, MeOH, rt, 30 min; ix- NaH, DMF, 0 °C, 1 h, then 19, rt, 72 h; x- NaH, DMF, 0 °C, 1 h, then 22, rt, 24 h.

Condensation of vanillin 24 in the presence of hydroxylamine followed by in situ dehydration afforded nitrile 25 in 81% yield. Nucleophilic substitution with 2-chloroethanol followed by protection of the alcohol with an acetyl group provided ester 26 in moderate yield over two steps. Regioselective nitration was achieved by using fuming nitric acid to obtain intermediate 27 in 81% yield. The nitro group was reduced in the presence of polymethylhydrosiloxane and catalytic amount of palladium acetate to deliver amine 28 in
quantitative yield. Imine 29 was formed in high yield by double elimination of ethanol using DMF-DMA in acidic conditions. The protected erlotinib derivative 30 was obtained by Dimroth rearrangement\(^{235}\) in the presence of 3-ethynylaniline in 94 % yield. Deprotection of the alcohol under basic conditions followed by nucleophilic substitution on spacer 19 afforded **Biotin-PEG\(_3\)-Erlotinib** (31) which could be used for *in vitro* affinity assays. Aliquots could be further processed by PREP-HPLC to increase sample purity for biological evaluation but resulted in low yield of highly pure derivative. Similar reactions were performed on derivative 30 to obtain **DIBO-PEG\(_3\)-Erlotinib** (32).

In summary, we prepared two erlotinib derivatives, **Biotin-PEG\(_3\)-Erlotinib** and **DIBO-PEG\(_3\)-Erlotinib**, containing a tetraethylene glycol spacer to increase their hydrophilicity and avoid disrupting their ability to inhibit the phosphorylation of EGFR. Their central core was prepared from vanillin and the key step involved a Dimroth rearrangement. *In vitro* assays on EGFR positive human cells derived from breast and lung cancers (MDA-MB-231 and NCI-H520) revealed the inhibition of the Tyr\(^{845}\) residue of EGFR, present in the EGFR TK domain, by **Biotin-PEG\(_3\)-Erlotinib**. These data, which were not produced in the frame of my PhD thesis, indicated the ability of this ligand to target the EGFR TK domain. Conjugation of **DIBO-PEG\(_3\)-Erlotinib** to the surface of HNPs will be discussed in the next chapter.

### 3.2 Preparation of cancer targeted HNPs for bioimaging

In this chapter, we will present the functionalization of coated HNPs with the targeting ligands previously described and equipped with a strained cyclooctyne moiety for copper-free click reaction. We will then detail the characterization of those nanomaterials followed by their assessment as bioimaging nanoprobe. BiFeO\(_3\) (BFO) and LiNbO\(_3\) (LNO) NPs (Figure 25) were selected for their high second harmonic efficiency previously demonstrated by Hyper-Rayleigh Scattering measurements.\(^{118,236}\) Those HNPs were produced at SYMME (University of Savoie Mont Blanc, France) by wet chemical route\(^{236}\) and solvothermal approach\(^{237}\) for BFO and LNO respectively.
Two types of coating will be performed on those HNPs. A polymeric layer based on PEG derivatives or a denser layer based on (3-Aminopropyl)triethoxysilane (APTES) will be applied through ligand exchange.

3.2.1 Conjugation of FAPα inhibitors to BFO HNPs

This section is based on the manuscript entitled: “Inhibitor-Conjugated Harmonic Nanoparticles Targeting Fibroblast Activation Protein”, *RSC Adv.* 2019, 9, 31659-31669.

In order to perform bioorthogonal reactions on the surface of the HNPs, BFO NPs were coated with PEG derivatives (Scheme 14). As previously established, a 1:1 mixture of linear PEG derivatives containing amino and azido groups was used (synthesis described in the experimental section). Table 4 presents the size (mean hydrodynamic diameter) and surface charge (zeta potential) of bare and coated HNPs, evaluated by dynamic light scattering.
### Results and Discussion

<table>
<thead>
<tr>
<th>NPs</th>
<th>Mean hydrodynamic diameter [nm]</th>
<th>Zeta Potential [mV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFO</td>
<td>192 ± 28</td>
<td>-28.1 ± 0.48</td>
</tr>
<tr>
<td>BFO-PEG</td>
<td>92 ± 11</td>
<td>-13.4 ± 1.1</td>
</tr>
</tbody>
</table>

Table 4: Size and zeta potential of uncoated BFO and PEGylated BFO HNPs by DLS. Measurements were performed with a concentration of 20 μg/mL of HNPs in water.

Upon PEGylation, the mean hydrodynamic diameter decreased. This is consistent with the improvement of the dispersion in aqueous medium thus breaking BFO NPs aggregation. Due to the presence of amino groups on the coating polymer, the zeta potential value increased.

PEGylated NPs were then ultrasonicated in the presence of **DIBO-PEG₃-FAPi** (16 h, 40 °C) to allow copper-free click reaction and formation of a 1,2,3-triazole moiety (Figure 26 A). The resulting NPs were washed with EtOAc in order to discard unreacted ligand. The suspension was further centrifuged and the supernatant was removed. This procedure was repeated several times to obtain **BFO-PEG-FAPi** functionalized HNPs. While iron oxide NPs are generally separated from unreacted species by magnetic attraction, BFO NPs do not exhibit magnetic properties and were thus processed by cycles of centrifugation/re-suspension. The size and surface charge of the NPs were obtained by dynamic light scattering (DLS). Their mean hydrodynamic diameter was 50 ± 15 nm (Figure 26 B) and their zeta potential averaged -17.7 ± 1.6 mV. Decrease in size is certainly due to the good solubility of **DIBO-PEG₃-FAPi** in enhancing the colloidal stability of **BFO-PEG-FAPi** NPs, avoiding formation of aggregates. The slight surface charge change could be attributed to the washing of remaining PEG derivatives still present in the **BFO-PEG** NPs suspension. Bianco *et al.* reported that size and surface charge of NPs highly influence their circulation lifetime in biological media, their accumulations in tumor cells and their clearance from the system.²³⁸ Results showed that NPs with an average size of 100 nm and a neutral or slightly negative surface charge exhibited longer circulation half-lives in blood, less clearance by the organs and good accumulation in tumors. The size and surface charge of **BFO-PEG-FAPi** was, thus, in accordance with the principles of NPs’ design for biomedical applications, affording long-lasting systems in physiological environment.
Results and Discussion

Figure 26: (A) Conjugation of **DIBO-PEG-FAPi** to PEG coated BFO HNPs. (B) Mean hydrodynamic diameter of coated and functionalized HNPs. (C) Zeta potential of coated and functionalized HNPs. “Reprinted (adapted) with permission from Inhibitor-conjugated harmonic nanoparticles targeting fibroblast activation protein. RSC Advances. Copyright (2019). Royal Society of Chemistry.”

In order to further characterize **BFO-PEG-FAPi** NPs, transmission electron microscopy (TEM) images were acquired. Energy-dispersive X-ray spectroscopy (EDS) analysis (Figure 27 A) revealed the presence of silica on the surface of the NPs. Furthermore, TEM imaging (Figure 27 B) revealed a thin PEG layer of 8 nm. Nevertheless, presence of PEG residues in solution could not be avoided despite the multiple washes.
Results and Discussion

**Figure 27:** (A) EDS images of **BFO-PEG-FAPi** NPs. Bismuth in red, iron in yellow and silica in green; (B) TEM imaging of **BFO-PEG-FAPi** NPs. The coating layer is highlighted by the green mark.

**BFO-PEG-FAPi** NPs were then assessed for their association to hrFAPα in comparison with **BFO-PEG** NPs by reverse ELISA type assay. The functionalized or coated NPs were incubated for 2 h at 37 °C in the presence of hrFAPα and an excess of Human Fibroblast Activation Protein alpha /FAP AlexaFluor®594-conjugated Antibody. The suspension was then centrifuged to allow aggregation of the NPs and harvest of the supernatant. This mixture was further analyzed by fluorescence spectrophotometry (λ<sub>ex</sub>/λ<sub>em</sub> = 590/645 nm) (Figure 28). A solution of anti-FAPα and hrFAPα, at the same concentrations than the one used for incubation with NPs, was used as maximal intensity reference. Incubation of **BFO-PEG-FAPi** NPs with hrFAPα significantly decreased the fluorescence intensity of the antibody. This loss of fluorescence could have been optimised by lowering the concentration of enzyme and antibody, which would have allowed a better contrast with respect to the controls. In the presence of **BFO-PEG** NPs, a slight decrease in fluorescence intensity was observed, in comparison with the reference. This was certainly due to non-specific interactions of PEG with the enzyme. Those results showed that the functionalized NPs associated with FAPα via a target-specific interaction.
Results and Discussion

Figure 28: (A) FAPα dimer structure. (B) Association of BFO-PEG-FAPi NPs to FAPα through target-specific interactions. Results were analyzed using a Student’s t-test. Fluorescence intensity of BFO-PEG-FAPi NPs incubated with FAPα and anti-FAPα was significantly lower than BFO-PEG NPs incubated with FAPα and anti-FAPα and the fluorescence intensity of the controls (all comparisons *** p<0.001). “Reprinted (adapted) with permission from Inhibitor-conjugated harmonic nanoparticles targeting fibroblast activation protein. RSC Advances. Copyright (2019). Royal Society of Chemistry.”

Further analyses of the association of BFO-PEG-FAPi NPs to FAPα were performed by multiphoton microscopy. After similar incubation of hrFAPα and tagged antibody with BFO-PEG NPs or BFO-PEG-FAPi NPs, the suspensions were imaged. Using a pulsed laser at 840 nm, SHG from the NPs and fluorescent emission from the antibody were captured. Four different region of each sample were used in order to calculate the average ratio of colocalized SHG (420 nm) and fluorescence (580-650 nm) signals to the total emission of SHG channel. A manual threshold was performed on the photons emitted by AlexaFluor®594 that overlapped the NPs signals. Those summed areas were then divided by all SHG signals present on the image, to normalize the colocalized signals. Those measurements were performed on four regions of the sample to have homogeneous data over it (Figure 29).
Results and Discussion

Figure 29: Association of BFO-PEG NPs (A) and BFO-PEG-FAPi (B) NPs to hrFAPα multiphoton multispectral microscopy. SHG in blue, AlexaFluor®594-conjugated Antibody in red and merged channels in purple. “Reprinted (adapted) with permission from Inhibitor-conjugated harmonic nanoparticles targeting fibroblast activation protein. RSC Advances. Copyright (2019). Royal Society of Chemistry.”

Those data revealed a much higher association of BFO-PEG-FAPi NPs to hrFAPα than in the presence of BFO-PEG NPs, confirming the targeting ability of our system for FAPα. Quantification by multiphoton microscopy is presented in Figure 30.

In summary, copper-free [3 + 2] azide to alkyne cycloaddition was successfully applied to the conjugation of FAPα targeting ligand at the surface of coated imaging NPs. Analysis by
Results and Discussion

DLS and TEM highlighted that both the size (50-100 nm) and surface charge ($\approx -15$ mV) of 
**BFO-PEG-FAPi** NPs should provide long-lasting circulation time and prevent high rate of 
nonspecific uptake by cells. Further biological evaluation by reverse ELISA type assay and 
multiphoton imaging demonstrated the association of **BFO-PEG-FAPi** NPs to the targeted 
enzyme. Taken together, these data pave the way for the application of **BFO-PEG-FAPi** 
NPs in imaging applications targeting the fibroblastic element of the tumor stroma rather than 
malignant cancer cells. In particular, the ability of BFO NPs to be imaged in tissue depth by 
multiharmonic detection, combined with FAP$\alpha$ targeting could provide novel tools to study 
the role of FAP$\alpha$ activity in modulating tumor behavior.

3.2.2 Conjugation of Erlotinib derivatives to HNPs

3.2.2.1 Coating and post-functionalization using heterobifunctional PEG 
oligomers

Following the successful procedure established for the conjugation of FAP$\alpha$ targeting 
ligand to HNPs, both LNO and BFO NPs were first coated with heterobifunctional PEG 
derivatives and further reacted with **DIBO-PEG$_3$-Erlotinib** (Figure 31). Results are 
presented in Table 5.
Results and Discussion

Figure 31: (A) Conjugation of DIBO-PEG-Erlotinib to PEG coated LNO or BFO HNPs. (B) Mean hydrodynamic diameter of coated and functionalized HNPs. (C) Zeta potential of coated and functionalized HNPs.
Results and Discussion

<table>
<thead>
<tr>
<th>NPs</th>
<th>Mean hydrodynamic diameter [nm]</th>
<th>Zeta Potential [mV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFO</td>
<td>192 ± 28</td>
<td>-28.1 ± 0.48</td>
</tr>
<tr>
<td>BFO-PEG</td>
<td>92 ± 11</td>
<td>-13.4 ± 1.1</td>
</tr>
<tr>
<td>BFO-PEG-Erlotinib</td>
<td>52 ± 12</td>
<td>-21.3 ± 1.4</td>
</tr>
<tr>
<td>LNO</td>
<td>61.9 ± 4.4</td>
<td>-39.03 ± 0.99</td>
</tr>
<tr>
<td>LNO-PEG</td>
<td>99 ± 23</td>
<td>-25.3 ± 0.56</td>
</tr>
<tr>
<td>LNO-PEG-Erlotinib</td>
<td>102 ± 18</td>
<td>-22.63 ± 0.43</td>
</tr>
</tbody>
</table>

Table 5: Size and zeta potential of uncoated BFO and LNO, PEG-coated BFO and LNO NPs and Erlotinib functionalized BFO and LNO NPs by DLS.

Functionalized BFO NPs exhibited smaller sizes than LNO NPs. Nevertheless, LNO-PEG and LNO-PEG-Erlotinib NPs displayed better colloidal stability in comparison with their BFO homologues, which had tendency to aggregate and sediment. Both final functionalized nanosystems had similar surface charge value around -20 mV.

3.2.2.2 Coating and post-functionalization using APTES and derivatives

Meanwhile, research conducted by Jérémy Vuilleumier, PhD student in our group, revealed that coating of HNPs with short alkylsiloxanes resulted in a compact silica shell around the inorganic core offering higher density of surface reactive functionalities for further conjugation to molecular payloads. In addition, the higher colloidal stability of LNO NPs compared with BFO NPs led us to investigate the functionalization of LNO NPs using short alkylsiloxane derivatives as coating agents (Scheme 15).

APTES was reacted with activated 4-azidobutanoic acid (33) (synthesis described in the experimental section) to produce APTES-N₃ in 95 % yield.
Results and Discussion

Scheme 15: Coating of LNO NPs with short alkyloxyne derivatives

LNO NPs were then incubated with a 1:1 mixture of APTES and APTES-N₃, in the presence of tetraethyl orthosilicate (TEOS) and aqueous ammonia to promote surface activation. The resulting NPs presenting amino and azido surface functionalities displayed on the coating silica layer were analyzed by DLS (Table 6).

<table>
<thead>
<tr>
<th>NPs</th>
<th>Mean hydrodynamic diameter [nm]</th>
<th>Zeta Potential [mV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO</td>
<td>61.9 ± 4.4</td>
<td>-39.03 ± 0.99</td>
</tr>
<tr>
<td>LNO-APTES</td>
<td>149 ± 42</td>
<td>22.07 ± 0.094</td>
</tr>
</tbody>
</table>

Table 6: Size and zeta potential of uncoated LNO and silica coated LNO by DLS.

Due to residual aggregation, the average size reached almost 150 nm. The high density of amine and azido groups present at the NP surface resulted in highly positive zeta potential value (22.07 ± 0.094), which could be the reason for the formation of small aggregates.

As previously established, LNO-APTES NPs were ultrasonicated in the presence of DIBO-PEG₃-Erlotinib (16 h, 40 °C) to allow copper-free click reaction through strained promoted azide to alkyne cycloaddition (Figure 32 A). The resulting NPs were washed with EtOAc in order to discard unreacted ligand. The suspension was further centrifuged and the supernatant was removed. This procedure was repeated several times to obtain stable suspension of LNO-APTES-Erlotinib HNPs. Their size and surface charge were measured by DLS. Their mean hydrodynamic diameter was 178 ± 50 nm (Figure 32 B) and their zeta potential averaged 22.6 ± 1.2 mV. LNO-APTES-Erlotinib NPs displayed high suspension stability. The tendency of those NPs to aggregate might be attributed to their highly positive surface charge.
3.2.2.3 *In vitro* evaluation of targeting properties

Functionalized LNO and BFO NPs were assessed for their ability to target EGFR positive cancer cells. In a first attempt, **LNO-PEG-Erlotinib** and **BFO-PEG-Erlotinib** were incubated with co-cultures of hepatocellular carcinoma (HCC, positive for the expression of EGFR) and lung adenocarcinoma A549 cells (EGFR negative). However, the tendency of HCC to form clusters was highly detrimental to the internalization of functionalized NPs. In addition, the balance between EGFR expressing and non-expressing cells was difficult to control.

We thus turned our attention to the evaluation of the association of Erlotinib-containing HNPs to distinct cell lines. **LNO-APTES-Erlotinib** NPs were assessed for their selective internalization into adenosquamous carcinoma NCI-H596 cells (EGFR positive). Those cells were incubated with a suspension of **LNO-APTES-Erlotinib** NPs in cellular media (10 µg/mL) at 37 °C for 24 h. The cells were further washed with PBS in order to...
Results and Discussion

discard the non-associated NPs. The treated cells were subsequently stained with Nile Red (0.1 μg/mL in PBS) and fixed with paraformaldehyde. The fixed cells were further analyzed by multiphoton microscopy. Using a pulsed laser at 810 nm, SHG from the NPs and fluorescence from Nile Red (staining cells membrane) were captured (Figure 33). The NPs were internalized into the adenosquamous carcinoma cells in low quantity. Nevertheless, morphological changes of the cells tend to show that the NPs did interact with the cells and may have caused cellular death (round cells). Moreover, colocalization of the SHG signal and the Nile Red fluorescence could be observed at the periphery of the cells (Figure 33 A). Those results suggest that NPs were expelled out of the cells post-internalization. Further experiments in presence of Erlotinib conjugated NPs, containing non-hydrolysable and hydrolysable linkers, should be performed in order to confirm these results. Furthermore, shorter exposure time could avoid expulsion of the NPs from the cells and allow better accumulation of the probes into the neoplastic cells.
In summary, we prepared three nanosystems based on HNPs for targeting of EGFR positive cells. Two different coatings were performed in order to functionalize the NPs with DIBO-PEG$_2$-Erlotinib. The obtained nanodevices BFO-PEG-Erlotinib and LNO-PEG-Erlotinib were tested on co-culture of EGFR positive/negative cancer cells to assess their selectivity. Presence of Erlotinib on the surface of the NPs decreased non-specific uptake. LNO-APTES-Erlotinib NPs were prepared in a similar way to the other two nanoprobe. Those new core-shell nanodevices were incubated in the presence of NCI-H596 cells (EGFR positive) showing morphological changes, which might imply cellular death. In the future, association of LNO-APTES-Erlotinib to EGFR could be evaluated, for instance, by a procedure similar to the one previously used for BFO-PEG-FAPi. Further experiments will be also
conducted on LNO-APTES-Erlotinib NPs to have a better understanding of their interactions with EGFR positive cells.

3.3 Multimodal harmonic nanoparticles based on functionalized HNPs

The versatility of the post-conjugation strategy developed so far prompted us to investigate the functionalization of coated LNO NPs with Gd(III)-containing chelates. The approach was based on the reported chelating agent H₃ebpatcn which was selectively modified to introduce an alkyne moiety for click reaction at the surface of coated HNPs.

3.3.1 Synthesis of H₃ebpatcn chelate derivative

H₃ebpatcn (Figure 34) is a tripodal propionate ligand which was reported by Nonat et al.²³⁹ as powerful Gd (III) chelate for MRI contrast agent. We considered that slight modification of this compound could result in an appropriate functionality for bioorthogonal reaction at the surface of coated HNPs, without affecting the properties of the chelate. The resulting nanodevice could serve as bimodal probe for dual imaging by MRI and multiphoton microscopy.

Our initial plan was based on the modification of the propionic acid side chain of H₃ebpatcn to introduce an alkynyl moiety for click reaction at the surface of coated LNO NPs. However, no attempts to introduce such functionalization on the side chain was met with success. We thus explored the addition of a butanoyl side chain, as depicted in structure A. Based on previous reports for the functionalization of TACN, we first produced intermediate 38 from dipicolinic acid (34) (Scheme 16).

The synthetic pathway of intermediate 38 was based on several publications.²³⁹-²⁴¹ Dipicolinic acid (34) was esterified in good yield. Mono reduction of diester 35 to alcohol 21 was performed in the presence of NaBH₄. Subsequent Appel reaction²⁴² afforded brominated compound 37 in 69 % yield. Controlled nucleophilic substitution of TACN allowed the selective functionalization of 2 positions of the triazacycle, affording intermediate 38 in 63 % yield.
Results and Discussion

![Chemical structure](attachment:structure.png)

Scheme 16: Synthesis of disubstituted TACN derivative. i- H$_2$SO$_4$ conc. EtOH, reflux, 16 h; ii- NaBH$_4$, EtOH, reflux, 16 h; iii- CBr$_4$, PPh$_3$, DCM, rt, 3 h; iv- TACN - 3 HCl, DIPEA, ACN, reflux, 16 h.

Synthesis of the butanoyl side chain (Scheme 17) started from pentynoic acid (39) which was first activated as N-hydroxysuccinimide ester (40) and further coupled to α-amino-γ-butyrolactone in high yield. Ring opening and esterification were performed under acidic conditions in MeOH to afford the corresponding unstable alcohol that was directly subjected to Swern oxidation to obtain aldehyde (42) in 43% yield over 2 steps. Reductive amination was performed under mild conditions, in the presence of disubstituted TACN, to afford the protected chelating agent (43). Consecutive saponification followed by reaction with GdCl$_3$, as source of Gd(III) ions, delivered the conjugate (44) in quantitative yield. The identity and stability of this chelated was confirmed by ESI MS analysis.

![Chemical structure](attachment:structure.png)

Scheme 17: Synthesis of gadolinium chelate. Reagents and conditions: i- NHS, DCC, THF, rt, 2 h; ii- (S)-(−)-α-Amino-γ-butyrolactone hydrobromide, Et$_3$N, DCM, rt, 2 h; iii- H$_2$SO$_4$, MeOH, 24 h, rt; iv- (COCl)$_2$, DMSO, Et$_3$N, DCM 0 °C, 2 h; v- 22, NaBH$_3$CN, DCE/ACN, rt, 16 h; vi- KOH, THF, rt 4 h; then GdCl$_3$, H$_2$O, rt, 1 h.

In summary, we synthesized a new chelating agent for Gd(III) containing an alkyne functionality for post-functionalization on the surface of coated NPs. The chelating core was based on TACN and picolinic acid. The triple bound was introduced through a reductive
amination with a derivative of pentynoic acid and homo-serine. The obtained chelate proved to be stable and soluble in aqueous solution.

### 3.3.2 Preparation of LNO-gadolinium(III) conjugates for multimodal imaging

APTES derivatives were selected as coating reagents. LNO-APTES were ultrasonicated in the presence of chelate 44, copper sulfate and sodium ascorbate (16 h, 40 °C) to promote click reaction through azide to alkyne cycloaddition (Figure 35 A). In order to discard unreacted chelate and traces of copper catalyst, LNO-APTES-Gd NPs were processed through 4 cycles of centrifugation (10 min, 4700 rpm)/resuspension in water. In a first attempt, LNO-APTES-Gd were washed several times with 5 % cyclam aqueous solution to capture traces of copper catalyst used for the click reaction. However, this procedure led to partial decomplexation of Gd(III) (see XPS analysis below). Purified NPs were stored in distilled water for further analysis and imaging experiments. The size and surface charge of the NPs were obtained by DLS. Upon functionalization with Gd(III) complex 44, the mean averaged diameter remained in the same range (153 ± 11 nm) (Figure 35 B), but showed a better colloidal stability than coated LNO NPs. The surface charge decreased from 22.07 ± 0.094 to -8.59 ± 0.33 mV (Figure 35 C), which is consistent with the presence of carboxylates groups resulting from a fraction of non-chelated ligands.
In order to confirm the presence of Gd(III)-chelate at the surface of our nanosystem, X-ray photoelectron spectroscopy (XPS) was performed. The results are presented in Figure 36 A.
Results and Discussion

Gd3d and Cu2p were quantified to assess both the conjugation rate and the efficacy of the washing procedure to remove copper catalyst traces. The presence of gadolinium was confirmed on the surface of the functionalized NPs by the Gd3d peak at 1187.7 eV. For Cu2p, a peak at 933.5 eV would be expected. As depicted in Figure 36 B, the presence of Cu2p seems to be excluded or the concentration is below the detection limit of the measurement. Both washing procedures resulted in an efficient removal of copper catalyst from the suspended LNO-APTES-Gd NPs. However, treatment with 5 % cyclam aqueous solution led to a clear loss in Gd(III) content at the surface of the NPs (Figure 36 A, red curve), probably due to chelation exchange with cyclam. This hypothesis was supported by the surface charge of LNO-APTES-Gd NPs, which dropped to -16.0 ± 0.33 mV in case of washing cycles with cyclam, due to the presence of a larger number of non-chelated carboxylate groups.

To assess the ability of these Gd(III)-NP conjugate to be used as contrast agent, measurements of the relaxation rate R_1 and R_2 were performed on suspension of LNO, LNP-APTES and LNO-APTES-Gd NPs in water. The analyses were performed at 37 °C at 30 and 60 MHz fields. Results are presented in Figure 37.
Results and Discussion

As expected, LNO and LNO-APTES NPs did not exhibit change in the water proton relaxation rate. Under the same conditions, LNO-APTES-Gd NPs showed significant increase of the relaxation rates $R_1$ and $R_2$ of water protons. Those results were very encouraging and further analyses were performed on these NPs. To be able to compare our system to commercially available contrast agents, inductively coupled plasma mass spectrometry (ICP-MS) was performed to determine the concentration of gadolinium on the surface of the NPs.

The relaxivity per Gd(III)-complex (in mM$^{-1}$ s$^{-1}$) of the relaxing agent was calculated using the equation 1.244

$$r_i = \frac{1/T_i - 1/T_{id}}{C}$$  \hspace{1cm} (1)

With $i=1$ the longitudinal relaxation and $i=2$ the transverse relaxation of water in presence of the paramagnetic relaxing agent, $T_{id}$ the longitudinal or transverse relaxation of pure deionized water protons (diamagnetic relaxation) and $C$ the concentration of paramagnetic species in mM. Results are presented in Table 7.
Results and Discussion

<table>
<thead>
<tr>
<th></th>
<th>( r_1 ) (mM(^{-1})s(^{-1})) at 30MHz</th>
<th>( r_2 ) (mM(^{-1})s(^{-1})) at 30MHz</th>
<th>( r_1 ) (mM(^{-1})s(^{-1})) at 60MHz</th>
<th>( r_2 ) (mM(^{-1})s(^{-1})) at 60MHz</th>
<th>MW (g/mol)</th>
</tr>
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<tr>
<td>LNO-APTES-Gd</td>
<td>5.17</td>
<td>7.06</td>
<td>6.35</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>44</td>
<td>-</td>
<td>-</td>
<td>6.92</td>
<td>7.74</td>
<td>734.86</td>
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<td>Magnevist®</td>
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<tr>
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<tr>
<td>Primovist</td>
<td>-</td>
<td>-</td>
<td>4.7</td>
<td>5.1</td>
<td>725.72</td>
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<tr>
<td>Ferumoxides</td>
<td>-</td>
<td>-</td>
<td>10.1</td>
<td>120</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 7: Relaxivities of LNO-APTES-Gd, compound 44 and commercial contrast agents at 30 and 60 MHz

Both longitudinal and transversal relaxivities proved to be comparable to commercial contrast agents with similar molecular weight.\(^{245,246}\) As expected, \( r_1 \) gave better values which implies that LNO-APTES-Gd NPs could be used as positive contrast agent for MRI. Despite the large increase of the \( r_2 \) value, in comparison with the free Gd(III) complexe 44, its transversal relaxivity is far from recently developed SPIONs as Ferumoxides.\(^{247}\) Nevertheless, this difference tend to prove that the gadolinium complexe is well functionalized on the surface of the HNPs.

To verify that addition of Gd(III)-chelate 44 at the surface of HNPs did not affect their capacity to produce SHG, multiphoton imaging was performed at 810 nm (Figure 38). Intense SHG signal was detected, indicating the capability of those functionalized NPs to act as both MRI and multiphotonic probes.

![Figure 38: SHG signal of LNO-APTES-Gd deposited on a substrate when excited at 810 nm.](image)

In summary, Gd(III) chelate 44 was conjugated to the surface of silica coated LNO NPs by copper catalyzed \([3 + 2]\) cycloaddition resulting in homogeneous covering. Both XPS analyses and ICP-MS measurements confirmed the efficiency of the functionalization method. In addition, the protocol was repeated several times to ensure that Gd(III) loading was reproducible. Relaxivity of the system was measured and revealed that LNO-APTES-Gd
NPs could be used as efficient $T_1$ contrast agents. Furthermore, multiphoton microscopy showed that functionalization of the coated NPs did not alter their ability to produce SHG. Those results proved that LNO-APTES-Gd are promising nanopores for MRI coupled multiphoton spectroscopy dual imaging.

These data will be presented in the following article “Gd$^{3+}$-Functionalized Harmonic Nanoparticles for Multiphoton and Magnetic Resonance Multimodal Imaging.”, in preparation.
Conclusion and outlooks

4 Conclusion and outlooks

Because cancer is a major burden worldwide, early detection of malignant lesions is essential to increase survival and remission rate. Despite significant advances in cancer detection, tracking precancerous signs is still challenging. NPs have physical properties allowing them to overcome many of the limitations associated with traditional imaging probes, including photobleaching or wide emission peaks. The rapid progress in nanotechnologies have allowed NPs to become major actors in biomedical applications. Due to their versatility in size and composition, combined with surface accessibility to functionalization, inorganic NPs revealed to be promising materials for the development of sensitive and specific cancer imaging and therapeutic agents. During this thesis, efforts focused on the development of imaging probes based on multi-harmonic emission. The selected HNPs were coated with PEG or silica to improve their biocompatibility and to allow their conjugation to small molecules as ligands for targeting lung and breast cancer cells as well as the microenvironment of carcinomas. The ability of these nanodevices for selective association to cancer biomarkers was assessed through in vitro evaluation and multiphoton imaging protocols. In addition, dual MRI-multiphotonics nanoprobes were developed.

Two targeting ligands addressing EGFR and FAPα, respectively, and presenting a strained cyclooctyne residue for further conjugation to the surface of coated HNPs through copper free click reaction, were synthesized. Nanomolar inhibition of FAPα (evaluated on human recombinant enzyme) and sharp selectivity with respect to the close related oligopeptidases DPP IV and PREP was achieved with Biotin-PEG₃-FAPi. In addition, this ligand showed promising biocompatibility on human 3D tissue model reconstituting the complexity of lung epithelium. The Erlotinib derivative Biotin-PEG₃-Erlotinib induced the inhibition of EGFR phosphorylation in several cancer cell lines.

BFO and LNO HNPs were selected for post-functionalization with targeting ligands due to their high SHG efficiency and their low cytotoxicity. Coating with either heterobifunctional PEG derivatives or APTES derivatives resulted in surface modification and introduction of reactive azide functionalities for further conjugation to the targeting ligands. Strain-promoted azide to alkyne [3 + 2]-cycloaddition delivered BFO-PEG-FAPi NPs, targeting the fibroblastic element of the tumor stroma, and BFO-PEG-Erlotinib NPs, targeting the cells overexpression of EGFR. The affinity of BFO-PEG-FAPi NPs for FAPα was assessed by reverse Elisa Type assay. Both fluorescence spectroscopy and multiphoton imaging supported their association with the enzyme through targeted-specific interactions. BFO-PEG-Erlotinib NPs demonstrated reduction of non-specific cell uptake. Further experiments are currently in progress to confirm their selective association to EGFR positive cancer cells.
Finally, a gadolinium chelating agent was conjugated to silica coated LNO NPs through copper catalyzed [3 + 2] cycloaddition. T₁ and T₂ relaxation times were further measured on the resulting LNO-APTES-Gd NPs. Calculations revealed that the relaxivity of this nanosystem was comparable to commercially available T₁ contrast agents. Multiphoton imaging demonstrated that the addition of gadolinium chelates to the surface of HNPs did not affect the capacity of LNO to produce SHG. Further analyses are presently performed, on phantom gels containing these nanoprobes, to attest of the complex stability after multiphoton irradiation. Such system constitutes one of the first examples of dual MRI-multiphotonics nanoparticles based on functionalized HNPs.

Figure 39: Schematic summary of the project
Conclusion and outlooks

In the future, several modifications on those systems could improve the efficiency and the compatibility of HNPs for cancer diagnosis and targeted imaging. Among them:

- Coating improvements of HNPs could allow the increase of their colloidal stability and the modification of the amount of targeting ligand at their surface. For instance, using avidin could improve biocompatibility and would allow conjugation of biotin tagged targeting ligands through biotin-avidin interaction, which is considered as one of the strongest non-covalent interactions.

- Coupling of different molecules to the surface of HNPs could allow the formation of targeted theranostic or multimodal devices. Indeed, conjugation of both a targeting ligand and a drug attached through a photocleavable linker would enable real-time imaging of drug release. Similarly, multimodal-targeted imaging could be achieved by conjugating both a targeting ligand and gadolinium complexes. Ultimately, combining those three bioconjugates could lead to targeted drug delivery coupled to multimodal imaging.

- Minimization of the invasiveness of multiphoton imaging in vivo is the major limitation associated with the development of HNPs for in vivo bioimaging applications. Despite the deep penetration of NIR light into tissues, used as incident ray, the SHG signal would be difficult to image without surgical intervention. The development of fiber-optic fluorescence imaging could be a solution to this issue. Indeed, the recent progress in miniaturization of lasers and detectors could allow in vivo multiphoton imaging with minimal chirurgical intervention.

In conclusion, development of novel nanosystems conjugated to small molecules for targeted imaging of tumors based on HNPs, could provide new modalities for early detections and imaging of cancers. Moreover, use of functionalized HNPs targeting the tumor stroma could provide new insights in the role of cancer-associated fibroblasts. The ability of those NPs to produce SH and TH signals is an incredible asset for deep tissue imaging, with high spatial resolution and efficient segregation from endogenous harmonic emissions. Finally, the flexibility regarding the incident excitation wavelength could allow the generation of higher energy photons for UV-triggered release of molecular cargos or direct UV-induced DNA photodamage.
5 Experimental section

5.1 General information

Reagents and solvents were purchased from commercial sources (Aldrich, Acros, Merck, Fluka and VWR international) and preserved under argon. More sensitive compounds were stored in a desiccator or glove-box if required. Reagents were used without further purification unless otherwise noted.

All reactions were performed under argon (or nitrogen) unless otherwise noted. When needed, glassware was dried 12 h in an oven (T > 100 °C) or under vacuum with a heat gun (T > 200 °C).

When solvents are indicated as dry they were either purchased as such, distilled prior to use or were dried by a passage through a column of anhydrous alumina or copper using a Puresolv MD 5 from Innovative Technology Inc., based on the Grubb’s design.

Flash column chromatographies were performed using Silicycle P60 silica: 230-400 mesh (40-63 μm) silica.

Reactions were monitored using Merck Kieselgel 60F254 aluminium or glass backed plates. TLC’s were visualized by UV fluorescence (254 nm) then one of the following reagent: KMnO₄, molybdate, ninhydrine, Pancaldi, p-anisaldehyde, vanillin.

NMR spectra were recorded on a Bruker Avance III-400, Bruker Avance-400 or Bruker DRX-400 spectrometer at room temperature, ¹H frequency is at 400.13 MHz, ¹³C frequency is at 100.62 MHz. Chemical shifts (δ) were reported in parts per million (ppm) relative to residual solvent peaks rounded to the nearest 0.01 ppm for proton and 0.1 ppm for carbon (ref : CHCl₃ [¹H: 7.26 ppm, ¹³C: 77.2 ppm], MeOH [¹H: 3.31 ppm, ¹³C: 49.0 ppm], ACN [¹H: 1.94 ppm, ¹³C: 1.3 ppm], DMSO [¹H: 2.50 ppm, ¹³C: 39.5 ppm]). Coupling constants (J) were reported in Hz to the nearest 0.1 Hz. Peak multiplicity was indicated as follows s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad). Attribution of peaks was done using the multiplicities and integrals of the peaks. When needed, COSY, HSQC and HMBC experiments were used to confirm the attribution.

Relaxation measurements were performed at the Nuclear Magnetic Resonance service (NMR, EPFL, Lausanne, Switzerland) on two permanent magnets Bruker Minispec spectrometers with proton frequencies set at 30 MHz (0.71 T) and 60 MHz (1.41 T).

IR spectra were recorded in a Jasco FT/IR-4100 spectrometer outfitted with a PIKE technology MIRacleTM ATR accessory as neat films compressed onto a Zinc Selenide window.
Experimental section

The spectra are reported in cm$^{-1}$. Abbreviations used are: w (weak), m (medium), s (strong) and br (broad).

Mass spectra were obtained by using a Waters ACQUITY H-class UPLC/MS ACQ-SQD by electron ionisation (EI positive and negative) or a Finnigan TSQ7000 by electrospray ionization (ESI+). The accurate masses were measured by the mass spectrometry service of the EPFL by ESI-TOF using a QTOF Ultima from Waters. ICP-MS analyses were performed on a NexIon 350D from Perkin Elmer.

Measurements of the dynamic light scattering and zeta potential were obtained using a Malvern NanoZ instrument (Malvern Instruments, Malvern, UK).

Centrifugations were performed on HERAEUS Biofuge 13 centrifugator or Beckman Coulter Allegra X-30R Centrifuge.

Scanning transmission electron microscopy (STEM) was performed at the Interdisciplinary Centre for Electron Microscopy (CIME, EPFL, Lausanne, Switzerland) on a FEI Titan Themis 60-300 microscope.

X-ray photoelectron spectroscopy (XPS) was performed at the X-Ray Diffraction facility surface analytics (XRD, EPFL, Lausanne, Switzerland) on a Phi VersaProbe II (Physical Electronics Inc., MN, USA) using the monochromated Kα X-ray line of an aluminium anode (1486.6eV) operated at 50W. XPS spectra were referenced at 284.8eV with the C-C bound of the C1s line. XPS spectra were acquired with a step energy of 0.2eV/step at the pass energy of 46.95eV. CasaXPS was used for data processing.

For all general procedures the order of addition of reagents has to be respected.
5.2 Synthesis of coating agents

5.2.1 Synthesis of PEG 2000 derivatives

Scheme 18: Synthesis of coating PEG 2000 derivative. i- TsCl, Et3N, DMAP, DCM, rt 48 h; ii- NaN3, DMF, 48 h 90 °C; iii- PPh3, HCl aq., EtOH, rt, 72 h; iv- Succinic anhydride, Et3N, DCM, rt, 48 h; v- APTES, EDCI, DCM, rt, 48 h; vi- H2, Pd/C, DCM/MeOH, rt, 24 h.162

To a solution of PEG 2000 (1 equiv, 12.5 mmol, 25 g) in DCM (125 mL) TsCl (7 equiv, 87.5 mmol, 16.68 g), Et3N (7 equiv, 87.5 mmol, 12.2 mL) and DMAP (5 mol %, 0.63 mmol, 76 mg) were added and the reaction mixture was stirred at rt for 48 h. The reaction mixture was washed with sat. NH4Cl (100 mL) and the organic layer was dried over MgSO4, filtered and concentrated under reduce pressure. The crude product was purified by FCC (DCM/ MeOH 20:1) to afford 45 as a brown oil (8.01 mmol, 18.5 g, 64 %). The analytical data were in accordance with previously reported data.162

1H NMR (400 MHz, Chloroform-d) δ 7.79 (d, J = 8.1 Hz, 3H), 7.34 (d, J = 8.0 Hz, 4H), 4.15 (t, J = 4.9 Hz, 4H), 3.58 (s, 180H), 2.45 (s, 6H).
Experimental section

$^1$H NMR spectrum of 45

To a solution of 45 (1 equiv, 12.95 mmol, 29.9 g) in DMF (80 mL) NaN₃ (5 equiv, 64.75 mmol, 4.21 g) was added and the reaction mixture was stirred at 90 °C for 48 h. The mixture was concentrated *in vacuo* and the residue was redissolved in (DCM 100 mL), washed with sat. solution of NaHCO₃ in water (2 X 100 mL), dried over MgSO₄, filtered and concentrated *in vacuo* to afford 46 as a yellow oil (7.93 mmol, 21.03 g, 61 %). The analytical data were in accordance with previously reported data.\(^{162}\)
Experimental section

$^1$H NMR spectrum of 46

![NMR spectrum of 46](image)

To a solution of 46 (1 equiv, 7.93 mmol, 21.03 g) in toluene/EtOH (1:1, 64 mL) 5 % solution of HCl in water (158 mL) was added. The mixture was cooled down to 0 °C and PPh$_3$ (0.95 equiv, 7.53 mmol, 1.97 g) was added portion wise. The mixture was warmed up to rt and stirred for 72 h. The mixture was concentrated in vacuo and the crude product was purified by FCC (DCM/MeOH 10:1) to afford 47 as a brown oil (4.93 mmol, 10.15 g, 62 %). The analytical data were in accordance with previously reported data.$^{162}$

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 7.92 (s, 2H), 3.93 (t, $J = 4.9$ Hz, 2H), 3.84 – 3.41 (m, 180H), 3.17 (q, $J = 5.3$ Hz, 2H).
Experimental section

1H NMR spectrum of 47

To a solution of 47 (1 equiv, 3.06 mmol, 6.41 g) in DCM (43 mL) Et3N (6 equiv, 18.3 mmol, 2.55 mL) was added dropwise followed by succinic anhydride (3 equiv, 9.17 mmol, 917 mg) portion wise. The mixture was stirred at rt for 72 h. The mixture was washed with a 1 M solution of HCl in water (50 mL) and brine (50 mL), dried over MgSO4, filtered and concentrated in vacuo to afford (48) as a brown oil (3.06 mmol, 6.61 g, 87 %). The analytical data were in accordance with previously reported data.162

1H NMR (400 MHz, Chloroform-d) δ 7.13 (s, 1H), 3.73 – 3.58 (m, 18H), 3.54 (dt, J = 4.6, 2.2 Hz, 2H), 3.49 – 3.43 (m, 2H), 2.70 – 2.61 (m, 2H), 2.58 – 2.50 (m, 2H).
To a solution of 48 (1 equiv, 2.65 mmol, 5.72 g) in DCM (85 mL) EDCI (4 equiv, 10.6 mmol, 1.65 g) and 3-aminopropyltriethoxysilane (APTES) (2 equiv, 5.3 mmol, 1.23 mL) were added and the mixture was stirred at rt for 48 h. The mixture was concentrated under reduce pressure and the crude product was purified by FCC (DCM/MEOH 8:1) to afford PEG-N₃ as a yellow solid (2.65 mmol, 6.26 g, 93 %). The analytical data were in accordance with previously reported data.¹⁶²

¹H NMR (400 MHz, Chloroform-d) δ 6.47 (s, 1H), 6.30 (s, 1H), 3.80 (qd, J = 6.9, 2.0 Hz, 6H), 3.63 (d, J = 1.8 Hz, 180H), 3.42 (ddt, J = 18.6, 11.2, 5.5 Hz, 4H), 3.22 (dt, J = 13.0, 6.6 Hz, 2H), 2.48 (dt, J = 15.9, 5.6 Hz, 4H), 1.59 (p, J = 7.2 Hz, 2H), 1.21 (td, J = 7.0, 1.8 Hz, 9H), 0.61 (td, J = 8.3, 7.8, 4.1 Hz, 2H).
To a solution of 49 (1 equiv, 0.42 mmol, 1 g) in DCM/EtOH (1:1, 20 mL) 10 % Pd/C (1 mol %) was added and the mixture was stirred under H₂ atmosphere at rt for 24 h. The mixture was filtered through a pad of Celite® and concentrated in vacuo to afford PEG-NH₂ as a white solid (0.42 mmol, 989 mg, quant.). The analytical data were in accordance with previously reported data.

\[ ^1H \text{ NMR (400 MHz, Chloroform-d)} \delta 7.91 \text{ (s, 2H), 6.92 (s, 1H), 6.73 (s, 1H), 3.87 - 3.80 (m, 2H), 3.77 - 3.67 (m, 6H), 3.70 - 3.59 (m, 180H), 3.56 (ddd, } J = 9.1, 4.2, 2.5 \text{ Hz, 2H), 3.49 - 3.37 (m, 2H), 3.31 - 3.19 (m, 2H), 2.61 - 2.45 (m, 4H), 1.65 (d, } J = 6.7 \text{ Hz, 2H), 1.28 - 1.18 (m, 9H), 0.72 - 0.61 (m, 2H). \]
5.2.2 Synthesis of APTES derivatives

Scheme 19: Synthesis of APTES-N₃ derivative. i- NaN₃, MeOH/H₂O, reflux, 7 h; ii- KOH, MeOH/H₂O, rt, 6 h; iii- DCC, NHS, DCM, rt, 4 h; iv- APTES, Et₃N, DCM, rt, 5 h.

Ethyl 4-azidobutanoate (33-a)
Experimental section

To a solution of ethyl 4-bromobutanoate (1 equiv, 15.4 mmol, 2.2 mL) in MeOH/H₂O (4:1, 30 mL) NaN₃ (2 equiv, 30.8 mmol, 2 g) was added and the mixture was refluxed for 7 h. MeOH was evaporated under reduce pressure and the aqueous solution was diluted with H₂O (100 mL). The aqueous layer was extracted with DCM (3 X 100 mL) and the combine organic layers were dried over MgSO₄, filtered and concentrated in vacuo to afford ethyl 4-azidobutanoate (33-a) as a colorless oil (15.4 mmol, 2.55 g, quant.). The analytical data were in accordance with previously reported data.¹⁴⁹

¹H NMR (400 MHz, Chloroform-d) δ 4.14 (q, J = 7.1 Hz, 2H), 3.35 (t, J = 6.7 Hz, 2H), 2.40 (t, J = 7.2 Hz, 2H), 1.91 (p, J = 7.0 Hz, 2H), 1.26 (t, J = 7.1 Hz, 3H).

¹H NMR spectrum of 33-a

4-Azidobutanoic acid (33-b)

To a solution of ethyl 4-azidobutanoate (1 equiv, 15.4 mmol, 2.55 g) in MeOH/H₂O (55:45, 60 mL) KOH (5 equiv, 77.0 mmol, 4.32 g) was added at 0 °C. The mixture was warmed up to rt
and stirred for 6 h. MeOH was evaporated under reduce pressure and the aqueous solution was
diluted with H2O (50 mL). The aqueous layer was extracted with DCM (2 X 100 mL), then
acidified to pH = 1 with a 1 M solution of HCl in water and extracted with Et2O (5 X 100
mL). The combine organic layers were dried over MgSO4, filtered and concentrated in vacuo
to afford ethyl 4-azidobutanoic acid (33-b) as a colorless oil (11.8 mmol, 1.52 g, 73 %). The
analytical data were in accordance with previously reported data.249

1H NMR (400 MHz, Chloroform-d) δ 5.72 (s, 1H), 3.37 (t, J = 6.7 Hz, 2H), 2.46 (t, J =
7.2 Hz, 2H), 1.91 (p, J = 7.0 Hz, 2H).

1H NMR spectrum of 33-b

2,5-Dioxopyrrolidin-1-yl 4-azidobutanoate (33)

To a solution of 4-azidobutanoic acid (1 equiv, 11.8 mmol, 1.52 g) in dry DCM (65 mL) NHS
(1.2 equiv, 14.1 mmol, 1.63 g) and DCC (1.2 equiv, 14.1 mmol, 2.92 g) were added and the
mixture was stirred at rt for 4 h. Sat. KCl solution (50 mL) was added and the organic layer was washed with H₂O (50 mL), dried over MgSO₄, filtered and concentrated under reduce pressure. The crude product was purified by FCC (DCM/MeOH 100:1) to afford 33 as a white solid (8.5 mmol, 7.93 g, 72%). The analytical data were in accordance with previously reported data.²⁴⁹

¹H NMR (400 MHz, Chloroform-d) δ 3.45 (t, J = 6.6 Hz, 2H), 2.84 (s, 4H), 2.73 (t, J = 7.2 Hz, 2H), 2.01 (p, J = 6.9 Hz, 2H).

¹H NMR spectrum of 33

4-Azido-N-(3-(triethoxysilyl)propyl)butanamide (APTES-N₃)
To a solution of 2,5-dioxopyrrolidin-1-yl 4-azidobutanoate (1 equiv, 0.44 mmol, 100 mg) in dry DCM (5 mL) APTES (1 equiv, 0.44 mmol, 0.1 mL) and Et$_3$N (2 equiv, 0.88 mmol, 0.12 mL) were added and the mixture was stirred at rt for 5 h. The mixture was concentrated under reduce pressure and the crude product was purified by FCC (PE/EtOAc 3:2) to afford APTES-N$_3$ as a colorless oil (0.42 mmol, 140 mg, 95 %).

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 5.79 (s, 1H, 1-H), 3.82 (q, $J$ = 7.0 Hz, 4H, CH$_2$OSi), 3.72 (q, $J$ = 7.0 Hz, 2H, CH$_2$OSi), 3.35 (t, $J$ = 6.6 Hz, 2H, 3-H), 3.26 (td, $J$ = 6.9, 5.8 Hz, 2H, 6-H), 2.25 (t, $J$ = 7.2 Hz, 2H, 5-H), 1.93 (p, $J$ = 6.9 Hz, 2H, 4-H), 1.68 – 1.58 (m, 2H, 7-H), 1.23 (td, $J$ = 7.0, 5.1 Hz, 9H, 10-H, 12-H, 14-H), 0.66 – 0.60 (m, 2H, 8-H).

$^{13}$C NMR (101 MHz, Chloroform-d) $\delta$ 171.7 (C$_2$), 58.7, 58.6 (CH$_2$OSi), 51.0 (C$_3$), 42.0 (C$_6$), 33.4 (C$_5$), 25.0 (C$_4$), 23.0 (C$_7$), 18.6, 18.5 (CH$_3$), 7.9 (C$_8$).

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

IR (neat): 3327 (w), 2947 (m), 2087 (s), 1644 (s), 1561 (m), 1283 (w), 1201 (w), 1087 (s), 764 (m)

$^1$H NMR spectrum of APTES-N$_3$
5.3 Synthesis of tetraethylene glycol linkers

Scheme 20: Synthesis of linker 19. i- TsCl, NaOH, THF, rt, 1 h; ii- NaN₃, DMF, 90 °C, 16 h, iii- PPh₃, H₂O, THF, rt, 6 h; iv- Et₃N, DMF, rt, 16 h; v- TsCl, NaOH, THF, rt, 1 h; vi- NHS, EDCI, DMF, rt, 16 h.
Experimental section

2,5-Dioxopyrrolidin-1-yl 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (51)

To a solution of biotin (1 equiv, 6.13 mmol, 1.5 g) in DMF (50 mL) EDCI (1.5 equiv, 9.21 mmol, 1.76 g) and NHS (2 equiv, 12.26 mmol, 1.41 g) were added and the reaction mixture was stirred at rt for 16 h. The solution was poured into ice/water (200 mL) and the precipitate was collected by filtration and co-evaporated with toluene (3 x 10 mL) to give (51) as a white solid (4.35 mmol, 1.49 g, 71 %). The analytical data were in accordance with previously reported data.

\[^1H\text{NMR (400 MHz, DMSO-}d_6\text{)}\]: \(\delta\) 6.41 (s, 1H), 6.35 (s, 1H), 4.31 (dd, \(J = 7.7, 5.1\) Hz, 1H), 4.18 – 4.10 (m, 1H), 3.09 (dd, \(J = 8.0, 4.8\) Hz, 1H), 2.86 – 2.77 (m, 4H), 2.66 (t, \(J = 7.4\) Hz, 2H), 2.58 (d, \(J = 12.5\) Hz, 1H), 1.70 – 1.57 (m, 3H), 1.55 – 1.35 (m, 3H).

\[^1H\text{NMR spectrum of 51}\]
Experimental section

2-(2-(2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (6-a)

To a solution of tetra(ethylene glycol) (10 equiv, 500 mmol, 97.1 g) in THF (10 mL) was added a 50 % solution of NaOH in water (2.4 equiv, 120 mmol, 9.6 mL) at 0 °C. A solution of TsCl (1 equiv, 50 mmol, 19.5 g) in THF (20 mL) was then added dropwise. The reaction mixture was stirred at rt for 1 h and then concentrated in vacuo. The residue was dissolved in chloroform (400 mL), washed with water (200 mL), brine (200 mL), dried over MgSO₄, filtered and concentrated in vacuo. The crude product was purified by FCC (EtOAc/ MeOH 95:5) to afford 6-a as a yellow oil (30.5 mmol, 10.6 g, 61 %). The analytical data were in accordance with previously reported data.250

1H NMR (400 MHz, Chloroform-d) δ 7.80 (d, J = 8.0 Hz, 2H), 7.34 (d, J = 8.0 Hz, 2H), 4.19 – 4.13 (m, 2H), 3.73 – 3.57 (m, 14H), 2.45 (s, 3H).

1H NMR spectrum of 6-a
2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethan-1-ol (6)

To a solution of 2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethyl 4-methylbenzenesulfonate (1 equiv, 0.29 mmol, 100 mg) in DMF (0.44 mL) was added NaN₃ (1.2 equiv, 0.35 mmol, 22.8 mg) and the reaction mixture was stirred at 90 °C for 16 h. The volatiles were evaporated *in vacuo* and the residue was redissolved in a sat. solution of NaHCO₃ in water and extracted with EtOAc (3 x 5 mL). The combined organic layers were washed with brine (5 mL), dried over MgSO₄, filtered and concentrated *in vacuo* to afford 6 as a yellowish oil (0.27 mmol, 60 g, 94 %). The analytical data were in accordance with previously reported data.²⁵⁰

¹H NMR (400 MHz, Chloroform-d) δ 3.77 – 3.70 (m, 2H), 3.72 – 3.64 (m, 10H), 3.65 – 3.58 (m, 2H), 3.40 (t, *J* = 5.1 Hz, 2H).

¹H NMR spectrum of 6
2-(2-(2-(2-Aminoethoxy)ethoxy)ethoxy)ethan-1-ol (23)

To a solution of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-ol (1 equiv, 4.31 mmol, 945 mg) in THF (63 mL) was added triphenylphosphine (1.5 equiv, 6.47 mmol, 1.7 g) portion wise. The reaction mixture was stirred at rt for 1 h. Distilled water (6.5 mL) was then added and the reaction mixture was stirred at rt for 24 h. The volatiles were evaporation under reduce pressure and the aqueous layer was washed with PE/EtOAc 1:1 (100 mL) and concentrated \textit{in vacuo}. The residue was co-evaporated with toluene (3 times) to dryness to afford 23 as a yellow oil (4.09 mmol, 791 mg, 95 %). The analytical data were in accordance with previously reported data.\textsuperscript{251}

\textbf{1H NMR (400 MHz, Methanol-d\textsubscript{4})} $\delta$ 3.69 (dtt, $J = 12.7$, 6.7, 3.5 Hz, 12H), 3.58 (q, $J = 4.1$ Hz, 2H), 3.12 (t, $J = 5.1$ Hz, 2H).

\textbf{1H NMR spectrum of 23}
N-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (18)

To a solution of 51 (1 equiv, 1.75 mmol, 600 mg) in DMF (8 mL) was added a solution of 2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethan-1-ol (1.1 equiv, 1.93 mmol, 373 mg) and Et₃N (2.5 equiv, 4.37 mmol, 0.61 mL) in DMF (8 mL). The mixture was stirred at rt for 16 h and completion of the reaction was monitored by ESI-MS. The volatiles were evaporation in vacuo and the residue was purified by FCC (DCM/MeOH 6:1) to give (18) as a white solid (95.2 mmol, 700 mg, 95 %). The analytical data were in accordance with previously reported data.²⁵¹

¹H NMR (400 MHz, Chloroform-d) δ 7.40 (s, 1H), 6.31 (s, 1H), 4.55 (dd, J = 7.8, 4.8 Hz, 1H), 4.37 (dd, J = 7.8, 4.4 Hz, 1H), 3.75 – 3.68 (m, 4H), 3.68 – 3.59 (m, 8H), 3.55 (t, J = 4.8 Hz, 2H), 3.46 – 3.42 (m, 2H), 3.17 (q, J = 7.2 Hz, 1H), 2.92 (dd, J = 12.9, 4.7 Hz, 1H), 2.77 (d, J = 12.9 Hz, 1H), 2.25 (t, J = 7.4 Hz, 2H), 1.79 – 1.59 (m, 4H), 1.45 (p, J = 7.3 Hz, 2H).

¹H NMR spectrum of 18
13-Oxo-17-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-3,6,9-trioxa-12-azaheptadecyl 4-methylbenzenesulfonate (19)

To a solution $N$-(2-(2-(2-hydroxyethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (1 equiv, 1.19 mmol, 500 mg) in DMF (6 mL) were added dropwise at 0°C a 50 % solution of NaOH in water (2 equiv, 2.38 mmol, 0.19 mL) and a solution of TsCl (2 equiv, 2.38 mmol, 453.7 mg) in DMF (4 mL). The reaction mixture was stirred at rt for 16 h, the volatiles were evaporated in vacuo and the crude product was purified by FCC (MeOH/DCM 1:6) to give 19 as a yellow oil (0.98 mmol, 561 mg, 82 %). The analytical data were in accordance with previously reported data.252

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 7.80 (d, $J = 8.0$ Hz, 2H), 7.35 (d, $J = 8.0$ Hz, 2H), 6.48 (s, 1H), 5.87 (s, 1H), 5.06 (s, 1H), 4.54 – 4.47 (m, 1H), 4.33 (d, $J = 7.9$, 4.7 Hz, 1H), 4.16 (t, $J = 4.8$ Hz, 2H), 3.69 (t, $J = 4.8$ Hz, 2H), 3.62 (d, $J = 5.0$ Hz, 8H), 3.56 (t, $J = 5.1$ Hz, 2H), 3.43 (q, $J = 5.0$ Hz, 2H), 3.15 (td, $J = 7.2$, 4.4 Hz, 1H), 2.91 (dd, $J = 12.8$, 4.9 Hz, 1H), 2.73 (d, $J = 12.8$ Hz, 1H), 2.45 (s, 3H), 2.21 (td, $J = 7.4$, 2.1 Hz, 2H), 1.76 – 1.59 (m, 4H), 1.44 (p, $J = 7.6$ Hz, 2H).
Experimental section

^1H NMR spectrum of 19

Scheme 21: synthesis of tetraethylene glycol linker for conjugation to FAP\(\alpha\) inhibitor. Reagents and conditions: i- NHS, DCC, CHCl\(_3\), rt, 16 h; ii- Propargylamine, Et\(_3\)N, DCM, rt, 1.5 h; iii- N-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide, CuSO\(_4\), NaAsc, THF/H\(_2\)O (4:1), rt, 16 h; iv- 2-(2-(2-azidoethoxy)ethoxy)ethanol-1-ol, CuSO\(_4\), NaAsc, THF/H\(_2\)O (4:1), rt, 16 h.

2,5-Dioxopyrrolidin-1-yl acrylate (1)
To a solution of acrylic acid (1 equiv., 14.57 mmol, 1 mL) and NHS (1 equiv., 14.57 mmol, 1.68 g) in anhydrous CHCl₃ (29 mL), under argon atmosphere, DCC (1 equiv., 14.57 mmol, 3 g) was added at 0 °C. The mixture was warmed up at rt stirred for 16 h. The mixture was filtered and the filtrate was washed with CHCl₃ (5 mL). The aqueous layer was concentrated in vacuo and the crude product was purified by FCC (DCM) to afford 1 as a white solid (11.7 mmol, 1.97 g, 81%). The analytical data were in accordance with previously reported data.²⁵³

¹H NMR (400 MHz, Chloroform-d) δ 6.70 (d, J = 17.3 Hz, 1H), 6.32 (dd, J = 17.3, 10.7 Hz, 1H), 6.16 (d, J = 10.6 Hz, 1H), 2.85 (s, 4H).

Una NMR spectrum of 1

N-(prop-2-yne-1-yl)acrylamide (2)

To a solution of 2,5-dioxopyrrolidin-1-yl acrylate (1 equiv., 4.52 mmol, 764 mg) in anhydrous DCM (30 mL), under argon atmosphere, Et₂N (5 equiv., 23.05 mmol, 3.2 mL) and propargylamine (2 equiv., 9.22 mmol, 0.6 mL) were added dropwise and the mixture was stirred
Experimental section

at rt for 1.5 h. The mixture was washed with a sat. solution of NH₄Cl in water (25 mL) and brine (25 mL) and the organic layer was dried over MgSO₄, filtered and concentrated \textit{in vacuo}. The crude product was purified by FCC (PE/EtOAc 1:1) to afford 2 as a white solid (0.78 mmol, 85 mg, 17%). The analytical data were in accordance with previously reported data.²⁵⁴

\textbf{¹H NMR (400 MHz, Chloroform-d)} \( \delta \) 6.34 (d, \( J = 17.0 \) Hz, 1H), 6.16 (d, \( J = 10.3 \) Hz, 1H), 6.12 (d, \( J = 10.3 \) Hz, 0H), 6.07 (s, 1H), 5.70 (d, \( J = 10.3 \) Hz, 1H), 4.15 (dd, \( J = 5.4, 2.5 \) Hz, 2H).

\textbf{N-(2-(2-(2-(2-(4-(acrylamidomethyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (5)}

To a solution of \( N \)-(prop-2-yn-1-yl)acrylamide (1 equiv., 0.46 mmol, 50 mg) and \( N \)-(2-(2-(2-(2-(azidoethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (1 equi., 0.46 mmol, 205 mg) in THF (2 mL), was added a solution of CuSO₄ (10 mol\%, 0.1 mmol, 7 mg) and sodium ascorbate (30 mol\%, 0.3 mmol, 28 mg) in water (0.2 mL) and the mixture was stirred at rt for 16 h. The solvent was evaporated \textit{in vacuo} and the crude product was purified by FCC (DCM/MeOH 10:1) to afford 5 as a white solid (0.36 mmol, 201 mg, 79%).

\textbf{¹H NMR (400 MHz, Chloroform-d)} \( \delta \) 8.03 (s, 1H, N-H), 7.89 (s, 1H, 5-H), 6.93 – 6.70 (m, 2H, 2 x N-H), 6.26 (d, \( J = 2.3 \) Hz, 1H, 11-H), 6.20 (dd, \( J = 17.1, 9.6 \) Hz, 1H, 10-H), 5.61 (dd, \( J = 9.5, 2.2 \) Hz, 1H, 11-H), 5.19 (s, 1H, N-H), 4.72 – 4.61 (m, 1H, 23-H), 4.58 – 4.43 (m, 4H, 24-H, 7-H), 4.36 (dd, \( J = 7.8, 4.7 \) Hz, 1H, 19-H), 3.89 (t, \( J = 5.0 \) Hz, 2H, 3-H), 3.69 – 3.53 (m, 10H, 1-H, 2-H, 25-H), 3.55 – 3.29 (m, 2H, 26-H), 3.14 (q, \( J = 6.3 \) Hz, 1H, 18-H), 2.92 (dd, \( J = 12.9, 4.8 \) Hz, 1H, 24-H), 2.74 (d, \( J = 12.8 \) Hz, 1H, 24-H), 2.10 – 1.91 (m, 2H, 14-H), 1.75 – 1.47 (m, 4H, 16-H, 17-H), 1.47 – 1.35 (m, 2H, 15-H).

\textbf{¹³C NMR (101 MHz, Chloroform-d)} \( \delta \) 173.4 (C₉), 166.1 (C₈), 164.0 (C₇), 134.9 (C₆), 131.1 (C₁₀), 126.6 (C₁₁), 123.8 (C₃), 70.8 (C₁, C₂, C₂₅), 70.7 (C₁, C₂, C₂₅), 70.6 (C₁, C₂, C₂₅), 70.3 (C₁, 78)
C2, C25), 70.0 (C1, C2, C25), 69.5 (C3), 62.3 (C10), 60.2 (C4), 55.9 (C18), 50.5 (C9), 40.7 (C24), 39.5 (C26), 35.6 (C14), 34.7 (C23), 28.4 (C15 and C16), 25.5 (C17).

**HRMS:** $m/z$ [M + Na]$^+$ Calcd for C24H39N7NaO6S$^+$ 576.2575; Found 576.2588

**IR:** ($\nu_{\text{max}}, \text{cm}^{-1}$) 3300 (m), 2925 (m), 2870 (m), 2110 (w), 2099 (w), 1940 (w), 1887 (w), 1801 (w), 1656 (s), 1544 (m), 1440 (m), 1323 (m), 1307 (m), 1265 (m), 1088 (m), 1037 (m), 976 (w), 920 (w), 867 (w), 834 (w), 760 (w)

$^1$H NMR spectrum of 5
Experimental section

$^{13}$C NMR spectrum of 5

2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethan-1-ol (3)

To a solution of $N$-(prop-2-yn-1-yl)acrylamide (1 equiv., 1.3 mmol, 142 mg) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-ol (1 equiv., 1.3 mmol, 285 mg) in THF (5.2 mL), was added a solution of CuSO$_4$ (10 mol%, 0.13 mmol, 21 mg) and sodium ascorbate (30 mol%, 0.39 mmol, 77 mg) in water (1.3 mL) and the mixture was stirred at rt for 16 h. The solvent was evaporated in vacuo and the crude product was purified by FCC (DCM/MeOH 10:1) to afford 3 as a yellow oil (1.3 mmol, 427 mg, quant.).

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 7.80 (s, 1H, 7-H), 6.79 (s, 1H, 10-H), 6.29 (dd, $J$ = 17.1, 1.3 Hz, 1H, 12-H), 6.13 (dd, $J$ = 17.1, 10.2 Hz, 1H, 13-H), 5.64 (dd, $J$ = 10.2, 1.3 Hz, 1H, 12-H), 4.59 (d, $J$ = 5.2 Hz, 2H, 9-H), 4.52 (t, $J$ = 5.0 Hz, 2H, 6-H), 3.86 (t, $J$ = 5.0 Hz, 2H, 5-H), 3.75 – 3.69 (m, 2H, 1-H), 3.67 – 3.63 (m, 2H, 2-H), 3.62 – 3.56 (m, 8H, 3-H, 4-H).
**Experimental section**

$^{13}$C NMR (101 MHz, Chloroform-d) $\delta$ 165.7 (C$_{11}$), 144.2 (C$_5$), 130.7 (C$_{13}$), 126.9 (C$_{12}$), 123.8 (C$_7$), 72.7 (C$_2$, C$_3$, C$_4$), 70.7 (2 x C$_2$, C$_3$, C$_4$), 70.5 (C$_2$, C$_3$, C$_4$), 70.4 (C$_2$, C$_3$, C$_4$), 69.5 (C$_5$), 61.7 (C$_1$), 50.4 (C$_6$), 35.0 (C$_9$).

**HRMS:** $m/z$: [M + Na]$^+$ Calcd for C$_{14}$H$_{24}$N$_4$NaO$_5^+$ 351.1639; Found 351.1641.

**IR:** ($\nu_{\text{max}}$, cm$^{-1}$) 2922 (m), 2877 (m), 3314 (m), 1789 (w), 1663 (s), 1625 (m), 1542 (m), 1245 (m), 1466 (w), 1352 (w), 1118 (s), 1068 (s), 985 (w), 808 (w), 758 (m), 720 (w)

$^3$H NMR spectrum of 3
Experimental section

$^{13}$C NMR spectrum of 3

Scheme 22: Synthesis of linker 52. Reagents and conditions: i- TsCl, Et$_3$N, DMAP, DCM, rt, 16 h; ii- NaN$_3$, DMF, 90°C, 16 h, iii- PPh$_3$, HCl, toluene, rt, 6 h; iv- 51, Et$_3$N, DMF, rt, 16 h; v- EtOAc, Pd/C, H$_2$, rt, 5 h.

$^{((Oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate)}$ (4-a)

To a solution tetra(ethylene glycol) (1 equiv, 102.8 mmol, 17.8 mL) in DCM (200 mL), cooled at 0 °C under Ar atm, p-toluenesulfonyl chloride (3 equiv, 308.9 mmol, 58.8 g), Et$_3$N (3 equiv,
Experimental section

308.9 mmol, 44 mL) and DMAP (5 mol %, 5.1 mmol, 622 mg) were added. The reaction mixture was warmed to rt and stirred for 16 h and then washed with water. The organic layer was dried over MgSO₄, filtered and concentrated *in vacuo*. The crude product was purified by FCC (PE/EtOAc 1:1) to afford 4-a as a yellow oil (93.6 mmol, 47.1 g, 91%). The analytical data were in accordance with previously reported data.²⁵⁵

¹H NMR (400 MHz, Chloroform-d) δ 7.79 (d, *J* = 8.1 Hz, 4H), 7.33 (d, *J* = 8.0 Hz, 4H), 4.15 (t, *J* = 4.8 Hz, 4H), 3.67 (t, *J* = 4.8 Hz, 4H), 3.56 (s, 8H), 2.44 (s, 6H).

¹H NMR spectrum of 4-a

1-Azido-2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethane (4-b)

![Structural formula of 4-b](image)

To a solution of ((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate) (1 equiv, 45.76 mmol, 23 g) in DMF (175 mL) NaN₃ (2.4 equiv, 109.82 mmol, 7.14 g) was added and the mixture stirred at 90 °C for 16 h. The volatiles were evaporated *in vacuo*. The residue was dissolved in DCM (150 mL) and washed with a sat.
solution of NaHCO$_3$ in water. The aqueous phase was extracted with DCM (3 x 50 mL) and the combined organic layers were dried over MgSO$_4$, filtered and concentrated \textit{in vacuo}. The crude product was purified by FCC (PE/EtOAc 7:3) to afford 4-b as a yellow oil (35.2 mmol, 8.6 g, 74 %). The analytical data were in accordance with previously reported data.$^{256}$

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 3.66 (t, $J = 1.4$ Hz, 12H), 3.37 (t, $J = 5.0$ Hz, 4H).

$^1$H NMR spectrum of 4-b

2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethan-1-amine hydrochloride (4-c)

$\text{N}_3\text{O}O_\text{NH}_2\cdot\text{HCl}$

To a solution of 1-azido-2-(2-(2-azidoethoxy)ethoxy)ethane (1 equiv, 32.6 mmol, 8 g) in a mixture of 5 % solution of HCl in water (40 mL) and toluene (40 mL), cooled at 0 °C, triphenylphosphine (0.95 equiv, 3.9 mmol, 1.03 g) was added portion wise. The reaction was warmed to rt and was stirred for 6 h. The organic phase was separated and the aqueous layer was concentrated under reduced pressure, followed by co-evaporation with toluene (3 X) to
afford 4-c as a white solid (27.2 mmol, 6.9 g, 83 %). The analytical data were in accordance with previously reported data.256

1H NMR (400 MHz, Chloroform-d) δ 8.25 (s, 2H), 3.84 (t, J = 4.9 Hz, 2H), 3.73 – 3.63 (m, 10H), 3.46 (t, J = 5.0 Hz, 2H), 3.30 – 3.20 (m, 2H).

1H NMR spectrum of 4-c

N-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (4)

To a solution of biotin-OSu (1 equiv, 2.34 mmol, 801 mg) in DMF (6 mL) a solution of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-amine hydrochloride (1.1 equiv, 2.57 mmol, 801 mg) and Et3N (2.5 equiv, 5.85 mmol, 0.81 mL) in DMF (6 mL) was added and the reaction mixture was stirred at rt for 48 h. Completion of the reaction was monitored by ESI-MS. The volatiles were evaporated in vacuo. The residue was redissolved in EtOAc (20 mL) and washed with a
sat. solution of NH₄Cl in water (10 mL). The aqueous phase was extracted with EtOAc (3 X 10 mL) and the combined organic layers were dried over MgSO₄, filtered and concentrated \textit{in vacuo} to afford 4 as a white solid (1.17 mmol, 521 mg, 53 %). The analytical data were in accordance with previously reported data.²⁵⁷

\textbf{¹H NMR (400 MHz, Chloroform-d)} \( \delta \) 6.58 (t, \( J = 5.5 \) Hz, 1H), 6.06 (s, 1H), 5.17 (s, 1H), 4.53 – 4.47 (m, 1H), 4.35 – 4.30 (m, 1H), 3.71 – 3.61 (m, 10H), 3.57 (t, \( J = 5.0 \) Hz, 2H), 3.47 – 3.42 (m, 2H), 3.40 (t, \( J = 5.0 \) Hz, 2H), 3.15 (td, \( J = 7.4, 4.5 \) Hz, 1H), 2.91 (dd, \( J = 13.0, 5.1 \) Hz, 1H), 2.77 – 2.65 (m, 1H), 2.23 (td, \( J = 7.3, 2.5 \) Hz, 2H), 1.72 – 1.61 (m, 4H), 1.45 (p, \( J = 7.6 \) Hz, 2H).

\textbf{¹H NMR spectrum of 4}

\[ N-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (52) \]
A solution of \( N-(2-(2-(2-(2\text{-azidoethoxy})ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-\text{oxohexahydro-1H-thieno[3,4-d]}\text{-imidazol-4-yl})\text{pentanamide} \) (1 equiv, 1.06 mmol, 472 mg) in EtOAc (12 mL) was stirred under 1 atm of H₂ in presence of 10 % Pd/C (0.1 mol %) at rt for 5 h. The catalyst was removed by filtration on celite. The filtrate was concentrated \textit{in vacuo} to afford \( 52 \) as a yellow oil (1.03 mmol, 430 mg, 97 %). The analytical data were in accordance with previously reported data.\(^{257}\)

\( ^1\text{H NMR (400 MHz, Chloroform-d)} \delta 7.36 \text{ (s, 1H)}, 6.51 \text{ (s, 1H)}, 5.36 \text{ (s, 1H)}, 4.49 \text{ (dd, } J = 7.9, 4.5 \text{ Hz, 1H)}, 4.36 - 4.28 \text{ (m, 1H)}, 3.70 - 3.53 \text{ (m, 14H)}, 3.42 \text{ (dq, } J = 9.1, 4.7 \text{ Hz, 2H)}, 3.15 \text{ (td, } J = 7.3, 4.3 \text{ Hz, 1H)}, 2.91 \text{ (dd, } J = 21.1, 7.7 \text{ Hz, 1H)}, 2.78 - 2.50 \text{ (m, 3H)}, 2.23 \text{ (t, } J = 7.1 \text{ Hz, 2H)}, 1.80 - 1.57 \text{ (m, 4H)}, 1.44 \text{ (p, } J = 7.9 \text{ Hz, 2H}).

\( ^1\text{H NMR spectrum of 52} \)
5.4 Synthesis of DIBO derivative

Scheme 23: Synthesis of DIBO derivative. Reagents and conditions: i- Me3SiI, anhydrous DCM, 5 °C, 7 days; ii- BuLi, anhydrous THF, rt, 4 h; iii- Br2 CHCl3, rt, 2 h; iv- LDA, anhydrous THF, rt, 1 h; v- 4-nitrophenylchloroformate, pyridine, DCM, rt, 16 h; vi- ethylenediamine Et3N, anhydrous DCM, rt, 1 h; vii- 23, Et3N, DMF, rt, 3 h; viii- 50 % NaOH in water, TsCl, DMF, rt, 24 h.

5,6,11,12-Tetrahydro-5,11-epoxydibenzo[a,e][8]annulene (20-a)

To a solution of 2-phenylacetaldehyde (1 equiv, 90 mmol, 10 mL) in anhydrous DCM (45 mL), cooled to 0 °C under argon, was added dropwise trimethylsilyl iodide (1.02 equiv, 92 mmol, 13 mL). The mixture was warmed to 5 °C and stirred for 7 days. A sat. solution of Na2S2O3 in water (30 mL) and DCM (20 mL) were added and the reaction mixture was stirred until the iodine color vanished. The aqueous phase was extracted with DCM (2 x 25 ml) and the combined organic layers were dried over MgSO4, filtered and concentrated in vacuo. The crude product was purified by FCC (PE/EtOAc 50:1 then 1:1) to afford 20-a as a light brown solid (20.5 mmol, 4.56 g, 45%). The analytical data were in accordance with previously reported data.258

1H NMR (400 MHz, Chloroform-d) δ 7.18 – 7.05 (m, 6H), 7.02 – 6.93 (m, 2H), 5.31 (d, J = 6.1 Hz, 2H), 3.56 (dd, J = 16.2, 6.2 Hz, 2H), 2.78 (d, J = 16.2 Hz, 2H).
Experimental section

\(^1\)H NMR spectrum of 20-a

(Z)-5,6-dihydrodibenzo[a,e][8]annulen-5-ol (20-b)

\[
\begin{align*}
\text{OH} & \\
\end{align*}
\]

To a solution of 5,6,11,12-tetrahydro-5,11-epoxydibenzo[a,e][8]annulene (1 equiv, 16 mmol, 3.6 g) in anhydrous THF (180 mL) under argon was added dropwise a 2.1 M solution of \(n\)-BuLi (2 equiv, 32 mmol, 15 mL) in pentane. The reaction mixture was stirred at rt for 4 h. The reaction was quenched by addition of water and THF was removed under reduce pressure. The aqueous phase was extracted with DCM (3 x 25 mL) and the combined organic layers were washed with brine (40 mL), dried over MgSO₄, filtered and concentrated \textit{in vacuo}. The crude product was purified by FCC (PE/EtOAc 5:1) to afford 20-b as a white solid (13.5 mmol, 2.99 mg, 83%). The analytical data were in accordance with previously reported data.\(^{259}\)

\(^1\)H NMR (400 MHz, Chloroform-d) \(\delta\) 7.48 – 7.43 (m, 1H), 7.26 – 7.07 (m, 7H), 6.88 (d, \(J = 12.2\) Hz, 1H), 6.82 (d, \(J = 12.3\) Hz, 1H), 5.29 (dt, \(J = 10.3, 5.2\) Hz, 1H), 3.46 (dd, \(J = 13.8, 6.2\) Hz, 1H), 3.33 (dd, \(J = 13.8, 10.1\) Hz, 1H).
Experimental section

$^1$H NMR spectrum of 20-b

11,12-Dibromo-5,6,11,12-tetrahydrodibenzo[a,e][8]annulen-5-ol (20-c)

To a solution of (Z)-5,6-dihydrodibenzo[a,e][8]annulen-5-ol (1 equiv, 3.8 mmol, 843 mg) in CHCl$_3$ (11 mL) was added dropwise bromine (1.5 equiv, 5.7 mmol, 0.29 mL) and the reaction mixture was stirred at rt for 2 h. The reaction was quenched by addition of sat. solution of Na$_2$S$_2$O$_3$ in water. The aqueous phase was extracted with CHCl$_3$ (3 x 5 mL) and the combined organic layers were washed with brine (5 mL), dried over MgSO$_4$, filtered and concentrated \textit{in vacuo}. The crude product was purified by FCC (PE/DCM 2:1) to afford 20-c as yellow oil (1.88 mmol, 718 mg, 50%). The analytical data were in accordance with previously reported data.\textsuperscript{260}

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 7.68 (dd, $J = 7.9, 1.3$ Hz, 0.5H), 7.64 – 7.54 (m, 0.5H), 7.39 (d, $J = 6.9$ Hz, 0.5H), 7.23 – 6.84 (m, 6H), 5.87 (d, $J = 5.5$ Hz, 0.5H), 5.82 (d, $J = 2.2$ Hz, 0.5H), 5.76 (dd, $J = 2.2, 1.1$ Hz, 0.5H), 5.36 – 5.25 (m, 0.5H), 3.79 – 3.71 (m, 0.5H),
Experimental section

3.59 (dd, $J = 16.4$, 6.2 Hz, 0.5H), 3.09 (dd, $J = 16.1$, 3.8 Hz, 0.5H), 2.85 (d, $J = 16.4$ Hz, 0.5H).

$^1$H NMR spectrum of 20-c

DIBO (20-d)

To a solution of 11,12-dibromo-5,6,11,12-tetrahydrodibenzo[a,e][8]annulen-5-ol (1 equiv, 1.4 mmol, 518 mg) in anhydrous THF (17 mL), freshly prepared LDA (0.8 M in THF, 5.8 equiv, 8.1 mmol, 10 mL) was added dropwise at 0°C. The mixture was warmed up to rt and the reaction was stirred for 1 h. The reaction was quenched with water (10 mL) and the THF was evaporated under reduce pressure. The aqueous layer was extracted with DCM (3 x 10 mL) and the combined organic layers were washed with water (10 mL), brine (10 mL), dried over MgSO$_4$, filtered and concentrated in vacuo. The crude product was purified by FCC (DCM/PE
Experimental section

3:1) to afford 20-d as white solid (1.05 mmol, 231 mg, 75 %). The analytical data were in accordance with previously reported data.260

1H NMR (400 MHz, Chloroform-d) δ 7.75 (d, J = 7.9 Hz, 1H), 7.48 – 7.27 (m, 7H), 4.67 – 4.61 (m, 1H), 3.11 (dd, J = 14.7, 2.2 Hz, 1H), 2.94 (dd, J = 14.8, 3.7 Hz, 1H).

1H NMR spectrum of 20-d

11,12-Didehydro-5,6-dihydrodibenzo[a,e][8]annulen-5-yl 4- nitrophenyl carbonate (20)

To a solution of DIBO (20-d) (1 equiv, 2.3 mmol, 500 mg) in DCM (66 mL) was added 4-nitrophenyl chloroformate (2 equiv, 4.6 mmol, 915 mg) and pyridine (5 equiv, 11.4 mmol, 0.92 mL) and the reaction mixture was stirred at rt for 16 h. The solution was washed with brine
Experimental section

(2 x 10 mL) and the organic layer was dried over MgSO₄, filtered and concentrated *in vacuo*. The crude product was purified by FCC (PE/EtOAc 5:1) to afford 20 as a white solid (2.03 mmol, 784 mg, 90 %). The analytical data were in accordance with previously reported data.²⁶⁰

¹H NMR (400 MHz, Chloroform-d) δ 8.32 – 8.25 (m, 2H), 7.62 (dd, J = 7.7, 1.0 Hz, 1H), 7.50 – 7.28 (m, 9H), 5.64 – 5.52 (m, 1H), 3.34 (dd, J = 15.4, 2.2 Hz, 1H), 3.05 (dd, J = 15.4, 4.0 Hz, 1H).

¹H NMR spectrum of 20

To a solution of 11,12-didehydro-5,6-dihydrodibenzo[a,e][8]annulen-5-yl 4- nitrophenyl carbonate (1 equiv, 0.26 mmol, 100 mg) in anhydrous DCM (8 mL) was added Et₃N (3 equiv, 0.78 mmol, 0.11 mL) and ethylenediamine (5 equiv, 1.3 mmol, 87 μL) and the reaction mixture
was stirred at rt for 1 h. The mixture was concentrated in vacuo and the crude product was purified by FCC (PE/EtOAc 5:1) to afford 17 as a yellow solid (0.2 mmol, 61 mg, 76%). The analytical data were in accordance with previously reported data.261

To a solution of functionalized tetraethylene glycol 23 (8 equiv, 4.16 mmol, 803.9 mg) in DMF (10 ml), was added a solution of 11,12-Didehydro-5,6-dihydridibenzo[a,e][8]annulen-5-yl 4-nitrophenyl carbonate (1 equiv, 0.52 mmol, 200 mg) in DMF (2mL). Et3N (3 equiv, 1.56 mmol, 0.22 mL) was added dropwise and the reaction mixture was stirred at rt for 4 h. The solvent was removed in vacuo and the residue was dissolved in DCM. The pH was corrected to 5 with a sat. solution of NH4Cl in water and the aqueous layer was extracted with DCM (3 x 5 mL). The combined organic layers were dried over MgSO4, filtered and concentrated in vacuo. The crude product was purified by FCC (DCM/MeOH 50:1) to afford 21 as a yellow oil (0.52 mmol, 229 mg, quant.)

1H NMR (400 MHz, Chloroform-d) δ 7.58 (d, J = 7.7 Hz, 1H, Ar-H), 7.42 – 7.26 (m, 7H, 7 x Ar-H), 6.43 (t, J = 5.7 Hz, 1H, 1-H), 5.53 (d, J = 3.4 Hz, 1H, 8-H), 3.83 (t, J = 4.4 Hz, 2H, 5-H), 3.78 (d, J = 4.3 Hz, 2H, 3-H, 4-H), 3.76 – 3.67 (m, 8H, 3-H, 4-H), 3.59 (t, J = 5.1 Hz, 2H, 3-H, 4-H), 3.42 (q, J = 5.2 Hz, 2H, 2-H), 3.20 (dd, J = 15.0, 2.1 Hz, 1H, 9-H), 2.92 (dd, J = 15.0, 4.1 Hz, 1H, 9-H).

13C NMR (101 MHz, Chloroform-d) δ 155.9 (C6), 152.6 (C9), 151.3 (C9), 130.1 (C7), 128.2 (C12), 128.0 (C13), 127.1 (2 x C13), 126.3 (C8), 126.1 (C7), 124.0 (2 x C7), 121.5 (C10), 113.0 (C4), 110.2 (C4), 76.7 (C7), 72.8, 70.8, 70.6, 70.5, 70.3, 70.2 (CH3O), 61.8 (C5), 46.4 (C5), 41.1 (C2).


IR: (νmax, cm⁻¹) 3421 (w), 3325 (w), 3062 (w), 2920 (w), 2863 (w), 1951 (w), 1718 (s), 1602 (w), 1533 (w), 1474 (w), 1451 (w), 1351 (w), 1251 (m), 1208 (w), 1123 (s), 1101 (s), 1033 (s), 944 (w), 887 (w), 834 (w), 760 (s) cm⁻¹.
$^1$H NMR spectrum of 21
To a solution of 21 (1 equiv, 0.12 mmol, 50.4 mg) in DMF (1 mL), cooled at 0 °C, was added dropwise a 50 % solution of NaOH in water (2 equiv, 0.24 mmol, 19.2 µL). Tosyl chloride (3 equiv, 0.36 mmol, 68.6 mg) was then added portion wise at 0 °C and the mixture was warmed up to rt and stirred for 48 h. The mixture was concentrated \textit{in vacuo} and the residue was dissolved in DCM (5mL) and washed with a sat. solution of NH₄Cl in water (5 mL). The aqueous phase was extracted with DCM (3x 5mL) and the combined organic layers were dried over over MgSO₄, filtered and concentrated \textit{in vacuo}. The crude product was purified by FCC (DCM/MeOH 50:1) to afford 22 as a white oil (67.4 µmol, 40 mg, 56 %).
**Experimental section**

**1H NMR (400 MHz, Chloroform-d)** δ 7.79 (d, J = 8.3 Hz, 2H, H\textsubscript{Ar}), 7.55 – 7.46 (m, 1H, H\textsubscript{Ar}), 7.39 – 7.26 (m, 9H, H\textsubscript{Ar}), 5.54 – 5.44 (m, 1H, 8-H), 4.17 (t, J = 4.8 Hz, 2H, 5-H), 3.71 (t, J = 4.8 Hz, 3-H, 4-H), 3.69 – 3.60 (m, 8H, 3-H, 4-H), 3.58 (t, J = 5.2 Hz, 2H, 3-H, 4-H), 3.39 (q, J = 5.2 Hz, 2H, 2-H), 3.16 (dd, J = 15.0, 2.2 Hz, 1H, 9-H), 2.88 (dd, J = 14.9, 4.0 Hz, 1H, 9-H), 2.43 (s, 3H, 30-H).

**13C NMR (400 MHz, Chloroform-d)** δ 155.63 (C\textsubscript{q}), 152.30 (C\textsubscript{q}), 151.17 (C\textsubscript{q}), 144.96 (C\textsubscript{q}), 133.11 (CAr), 130.07 (CAr), 129.96 (CAr), 129.10 (CAr), 128.17 (CAr), 128.10 (CAr), 128.05 (CAr), 127.18 (C\textsubscript{q}), 127.15 (CAr), 126.35 (CAr), 126.06 (CAr), 125.11 (C\textsubscript{q}), 123.96 (CAr), 123.91 (CAr), 121.42 (CAr), 113.04 (C\textsubscript{q}), 110.10 (C\textsubscript{q}), 76.92 (C\textsubscript{q}), 70.96, 70.76, 70.66, 70.44, 70.18 (CH\textsubscript{2}O), 69.35 (C\textsubscript{q}), 68.88 (CH\textsubscript{2}O), 46.30 (C\textsubscript{q}), 41.08 (C\textsubscript{q}), 21.78 (C\textsubscript{q}).

**HRMS (ESI):** m/z Calcd for C\textsubscript{32}H\textsubscript{35}NO\textsubscript{8}S ([M + Na]\textsuperscript{+}) 616.1976; Found 616.1985

**1H NMR spectrum of 22**
$^{13}$C NMR spectrum of 22
5.5 Synthesis of FAPα inhibitors

5.5.1 Preparation of (S)-1-(2-bromoacetyl)pyrrolidine-2-carbonitrile (12)

Scheme 24: Synthesis of FAPα inhibitor containing biotin. Reagents and conditions: i- Boc₂O, Et₃N, CHCl₃, rt, 16 h; ii- HOBt, EDCI, NH₃, THF, rt, 16 h; iii- Et₃N, TFAA, DCM, 0 °C, 16 h; iv- 2-bromoacetyl bromide, reduced pressure, MeCN, rt, 1 h.²⁶²

(Tert-butoxycarbonyl)-L-proline (12-a)

To a suspension of L-proline (1 equiv, 43 mmol, 5 g) and Boc₂O (1.05 equiv, 45.2 mmol, 9.87 g) in chloroform (86 mL), Et₃N (1.3 equiv, 55.9 mmol, 7.8 mL) was added dropwise at 0 °C. The solution was warmed up to rt and stirred for 16 h. The mixture was washed with a 1 M solution of HCl in water. The organic layer was dried over MgSO₄, filtered and concentrated in vacuo to afford 12-a as a yellow oil (43 mmol, 9.3 g, quant.). The analytical data were in accordance with previously reported data.²⁶²

¹H NMR (400 MHz, Chloroform-d) δ 4.40 – 4.18 (m, 1H), 3.52 – 3.39 (m, 1H), 3.42 – 3.27 (m, 1H), 2.13 – 1.82 (m, 4H), 1.49 (s, 9H).
Experimental section

$^1$H NMR spectrum of 12-a

![NMR Spectrum Image](image_url)

$^{13}$C NMR spectrum of 12-a

![C-NMR Spectrum Image](image_url)

**Tert-butyl (S)-2-carbamoylpyrrolidine-1-carboxylate (12-b)**

To a solution of Boc-$L$-Pro (1 equiv, 41.8 mmol, 9 g) in THF (527 mL) HOBt (1 equiv, 41.8 mmol, 6.4 g) and EDCI (1.15 equiv, 48.1 mmol, 7.47 g) were added and the mixture was stirred at rt for 30 min. Aqueous ammonia 25 % (2 equiv, 83.6 mmol, 5.7 mL) was added dropwise and the mixture was stirred at rt for 16 h. The crude mixture was evaporated in vacuo. The residue was dissolved in AcOEt, and washed with sat. solution of NaHCO$_3$ in water (100 mL) and brine (100 mL). The organic phase was dried over MgSO$_4$, filtered and concentrated in vacuo to afford 12-b as a white solid (33.96 mmol, 7.28 g, 81 %). The analytical data were in accordance with previously reported data.$^{262}$

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 4.36 – 4.17 (m, 1H), 3.58 – 3.28 (m, 2H), 2.43 – 1.81 (m, 4H), 1.47 (s, 9H).
Experimental section

1H NMR spectrum of 12-b

**Tert-butyl (S)-2-cyanopyrrolidine-1-carboxylate (12-c)**

To a solution of (S)-tert-butyl 2-carbamoylpyrrolidine-1-carboxylate (1 equiv, 18.7 mmol, 4 g) and Et₃N (4.5 equiv, 84.2 mmol, 11.7 mL) in DCM (120 mL) TFAA (2 equiv, 37.4 mmol, 5.2 mL) was added dropwise at 0 °C. The mixture was warmed up to rt and stirred for 16 h. The mixture was washed with water (50 mL), a 0.5 M solution of HCl in water (50 mL), and sat. solution of NaHCO₃ in water (50 mL). The organic phase was dried over MgSO₄, filtered and concentrated in vacuo to afford 12-c as a yellow oil (18.7 mmol, 3.67 g, quant.). The analytical data were in accordance with previously reported data.²⁶²

1H NMR (400 MHz, Chloroform-d) δ 4.63 – 4.40 (m, 1H), 3.57 – 3.32 (m, 2H), 2.38 – 1.95 (m, 4H), 1.56 – 1.48 (m, 9H).
Experimental section

$^1$H NMR spectrum of 12-c

(S)-1-(2-bromoacetyl)pyrrolidine-2-carbonitrile (12)

To a solution of tert-butyl (S)-2-cyanopyrrolidine-1-carboxylate (1 equiv, 5.09 mmol, 1 g) in MeCN (10 mL), 2-bromoacetyl bromide (2 equiv, 10.2 mmol, 890 μL) was added dropwise and the pressure in the flask was reduced. The mixture was stirred at rt for 2.5 h. The solvent was evaporated *in vacuo* and the crude product was purified by FCC (DCM/MeOH 75:1) to afford 12 as a red oil (4.84 mmol, 1.05 g, 95 %). The analytical data were in accordance with previously reported data.262

$^1$H NMR (400 MHz, Chloroform-d) δ 4.79 – 4.73 (m, 1H), 3.87 – 3.80 (m, 2H), 3.76 – 3.69 (m, 1H), 3.67 – 3.57 (m, 1H), 2.37 – 2.15 (m, 4H).
$^1$H NMR spectrum of 12
5.5.2 Preparation of DIBO-PEG₃-FAPi and Biotin-PEG₃-FAPi

Scheme 25: Synthesis of quinoline derivative. Reagents and conditions: i- Sodium pyruvate, NaOH 2.5 M, reflux, 4 h; ii- water, microwave, 200 °C, 10 min; iii- (COCl)₂, DMF 1 h, then NH₃ 25%, rt, 2 h; iv- KOH, DMF, rt, 1 h; then 12, DMF, rt, 1.5 h; v- 3, Pd(OAc)₂, PPh₃, Et₃N, DMF, 110 °C, 7 h; vi- 4-Nitrophenyl chloroformate, pyridine, DCM, rt, 16 h; vii- 17, Et₃N, DMF, rt, 5 h; viii- 5, Pd(OAc)₂, PPh₃, Et₃N, DMF, 110 °C, 7 h.

6-Bromoquinoline-2,4-dicarboxylic acid (10)

To a suspension of 5-bromoisatin (1 equiv, 22.2 mmol, 5 g) in a 2.5 M solution of NaOH in water (6 equiv, 133.2 mmol, 53 mL) sodium pyruvate (1.2 equiv, 26.6 mmol, 2.92 g) was added and the reaction mixture was refluxed for 4 h. The mixture was cooled to rt and the pH was corrected to 2 with a 6 M solution of HCl in water. The precipitate was filtered and washed with water (100 mL) to afford 10 as a brown powder (21.5 mmol, 6.35 g, 97%).
Experimental section

\(^1\)H NMR (400 MHz, DMSO-d\textsubscript{6}) \(\delta\) 9.09 (d, \(J = 2.2\ \text{Hz}, 1\text{H}, 6\text{-H}), 8.54 (s, 1\text{H}, 9\text{-H}), 8.19 (d, \(J = 9.0\ \text{Hz}, 1\text{H}, 3\text{-H}), 8.07 (dd, \(J = 9.0, 2.2\ \text{Hz}, 1\text{H}, 2\text{-H}).

\(^{13}\)C NMR (101 MHz, DMSO-d\textsubscript{6}) \(\delta\) 166.5 (C\textsubscript{12}), 165.5 (C\textsubscript{11}), 149.1 (C\textsubscript{8}), 146.6 (C\textsubscript{4}), 135.8 (C\textsubscript{10}), 133.8 (C\textsubscript{2}), 132.6 (C\textsubscript{3}), 127.8 (C\textsubscript{6}), 126.7 (C\textsubscript{5}), 123.9 (C\textsubscript{1}), 122.8 (C\textsubscript{9}).

HRMS: \(m/z\) [M + H-1] Calcd for C\textsubscript{11}H\textsubscript{5}BrNO\textsubscript{4} 293.9407; Found 293.9406

IR: (\(\nu_{\text{max}}, \ \text{cm}^{-1}\)) 2840 (m), 2563 (w), 1689 (s), 1596 (m), 1547 (w), 1468 (m), 1442 (w), 1414 (w), 1382 (w), 1347 (w), 1323 (m), 1272 (m), 1244 (m), 1191 (s), 1160 (m), 1072 (w), 1011 (w), 923 (m), 888 (m), 837 (m), 792 (m), 746 (w), 685 (m)

\(^1\)H NMR spectrum of 10
Experimental section

13C NMR spectrum of 10

6-Bromoquinoline-4-carboxylic acid (11-a)

A suspension of 6-bromoquinoline-2,4-dicarboxylic acid (1 equiv, 0.84 mmol, 250 mg) in water (15 ml), in a Pyrex pressure resistant tube, was treated by microwave irradiation at 200 °C for 10 min. The mixture was slowly cool down to rt and the precipitate was filtered and washed with water (15 mL) to afford 11-a as a brown powder (0.71 mmol, 179 mg, 84 %).

1H NMR (400 MHz, DMSO-d6) δ 9.09 (d, J = 4.4 Hz, 1H, 8-H), 9.00 (d, J = 2.3 Hz, 1H, 6-H), 8.07 (d, J = 9.0 Hz, 1H, 3-H), 8.02 (d, J = 4.4 Hz, 1H, 9-H), 7.98 (dd, J = 9.0, 2.3 Hz, 1H, 2-H).

13C NMR (101 MHz, DMSO-d6) δ 167.0 (C11), 151.2 (C5), 147.1 (C4), 134.5 (C10), 132.9 (C2), 131.8 (C9), 127.7 (C6), 125.7 (C8), 123.3 (C7), 121.6 (C1).

HRMS: m/z [M + H-1] Calcd for C10H5BrNO2 249.9509; Found 249.9507
**IR:** ($\nu_{\text{max}}$, cm$^{-1}$) 2419 (w), 1948 (w), 1911 (w), 1707 (m), 1595 (m), 1495 (m), 1431 (w), 1414 (w), 1349 (m), 1322 (m), 1264 (m), 1196 (m), 1145 (w), 1076 (m), 1055 (m), 1018 (m), 987 (w), 925 (m), 876 (m), 839 (m), 820 (m), 788 (m), 746 (s), 723 (s)

$^1$H NMR spectrum of 11-a
Experimental section

$^{13}$C NMR spectrum of 11-a

6-Bromoquinoline-4-carboxamide (11)

To a solution of 6-bromoquinoline-4-carboxylic acid (1 equiv., 0.99 mmol, 250 mg) in anhydrous CHCl$_3$ (5 mL) under argon (COCl)$_2$ (1 equiv., 0.99 mmol, 90 μL) and DMF (cat., 2 drops) were added dropwise and the mixture was stirred at rt for 1 h. Aqueous ammonia 25% (2 equiv., 1.98 mmol, 0.14 mL) were added dropwise and the mixture was stirred at rt for 2 h. The reaction was quenched with water (1 mL) and the CHCl$_3$ was evaporated under reduce pressure. The pH was corrected to 11 with a 3 M solution of NaOH in water and the precipitate was filtered and washed with water (10 mL) to afford 11 as a brown powder (0.87 mmol, 218 mg, 88 %)

$^1$H NMR (400 MHz, DMSO-d6) δ 9.01 (d, $J = 4.4$ Hz, 1H, 8-H), 8.46 (d, $J = 2.3$ Hz, 1H, 6-H), 8.32 (s, 2H, 12-H), 8.03 (d, $J = 9.0$ Hz, 1H, 3-H), 7.94 (dd, $J = 9.0, 2.3$ Hz, 1H, 2-H), 7.65 (d, $J = 4.3$ Hz, 1H, 9-H).
**Experimental section**

$^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 168.0 (C$_{11}$), 150.9 (C$_8$), 146.6 (C$_4$), 140.6 (C$_{10}$), 132.8 (C$_2$), 131.6 (C$_3$), 127.6 (C$_6$), 125.3 (C$_5$), 120.5 (C$_1$), 120.0 (C$_9$).

**HRMS:** $m/z$ [M + H]$^+$ Calcd for C$_{10}$H$_8$BrN$_2$O$^+$ 250.9815; Found 250.9814

**IR:** ($\nu$$_{\text{max}}$, cm$^{-1}$) 3343 (m), 3122 (w), 1945 (w), 1910 (w), 1668 (s), 1622 (m), 1577 (m), 1506 (m), 1496 (s), 1449 (m), 1398 (m), 1337 (s), 1300 (w), 1200 (w), 1135 (w), 1060 (w), 1011 (w), 969 (w), 862 (s), 841 (m), 827 (m), 783 (m), 757 (w), 715 (m), 685 (m)

$^1$H NMR spectrum of 11
Experimental section

$^{13}$C NMR spectrum of 11

(S)-6-bromo-N-(2-(2-cyanopyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (13)

To a solution of 6-bromoquinoline-4-carboxamide (1.05 equiv., 1.98 mmol, 500 mg) in DMF (10 mL) potassium hydroxide (2.5 equiv., 4.95 mmol, 56 mg) was added portion wise and the mixture was stirred at rt for 1 h. 12 (1 equiv., 1.89, 410 mg) in DMF (2 mL) was added dropwise and the mixture was stirred at rt for 1.5 h. The solvent was evaporated \textit{in vacuo} and the residue was redissolved in DCM (10 mL). The pH was corrected to 5 with a sat. solution of NH$_4$Cl in water (20 mL) and the aqueous layer was extracted with DCM (3 x 10 mL). The combined organic layer were dried over MgSO$_4$, filtered and concentrated \textit{in vacuo}. The crude
Experimental section

Product was purified by FCC (DCM/MeOH 25:1) to afford 13 as a brown foam (1.76 mmol, 682 mg, 89%).

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 8.95 (dd, $J = 4.4$, 2.3 Hz, 1H, 8-H), 8.49 (d, $J = 2.2$ Hz, 1H, 6-H), 8.00 (d, $J = 9.0$ Hz, 1H, 3-H), 7.83 (dd, $J = 9.0$, 2.2 Hz, 1H, 2-H), 7.54 (d, $J = 4.3$ Hz, 1H, 9-H), 7.18 (s, 1H, 12-H), 4.79 (d, $J = 5.2$ Hz, 1H, 16-H), 4.41 (ddd, $J = 17.8$, 4.9, 2.8 Hz, 1H, 13-H), 4.26 (ddd, $J = 17.8$, 3.6 Hz, 1H, 13-H), 3.76 – 3.69 (m, 1H, 19-H), 3.59 – 3.51 (m, 1H, 19-H), 2.43 – 2.19 (m, 4H, 17-H, 18-H).

$^{13}$C NMR (101 MHz, Chloroform-d) $\delta$ 167.0 (C14), 166.9 (C11), 150.3 (C8), 147.5 (C4), 139.8 (C10), 133.8 (C2), 131.7 (C3), 127.8 (C6), 125.7 (C5), 122.4 (C1), 119.6 (C9), 117.9 (C20), 46.9 (C16), 45.8 (C19), 42.6 (C13), 30.1 (C17), 25.2 (C18).

HRMS: $m/z$: [M + H]$^+$ Calcd for C17H16BrN4O2+ 387.0451; Found 387.0450

IR: ($\nu_{max}$, cm$^{-1}$) 3297 (w), 3058 (w), 2954 (w), 2168 (w), 2156 (w), 2022 (w), 2880 (w), 1655 (s), 1586 (w), 1537 (w), 1493 (w), 1430 (m), 1407 (m), 1305 (m), 1345 (w), 1263 (m), 1194 (w), 1157 (w), 1043 (w), 978 (w), 913 (w), 867 (w), 846 (w), 830 (w), 783 (w), 730 (m), 723 (m), 702 (w)

HPLC: (stationary phase: C18-bonded silica; mobile phase: 98% water + 0.5% formic acid/2% CAN +0.45% formic acid to 2% water + 0.5% formic acid/98% CAN +0.45% formic acid over 4min) $t_R$ 1.58 min
Experimental section

$^1\text{H}$ NMR spectrum of 13
Experimental section

$^{13}$C NMR spectrum of 13

Analytical UPLC trace of 13
**Experimental section**

\((S,E)-N-(2-(2-cyanopyrrolidin-1-yl)-2-oxoethyl)-6-(3-(((1-(2-(2-(2-\text{hydroxyethoxy})ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methyl)amino)-3-oxoprop-1-en-1-yl)quinoline-4-carboxamide (14)\)

A solution of \((S)-6\)-bromo-\(N-(2-(2-cyanopyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide\) (1 equiv, 0.72 mmol, 280 mg), compound 3 (2 equiv., 1.1 mmol, 355 mg), palladium acetate (10 mol\%, 0.07 mmol, 16 mg), triphenylphosphine (40 mol\%, 0.29 mmol, 76 mg), Et\(_3\)N (5.5 equiv., 4 mmol) in anhydrous degassed DMF (2.8 ml), in a Pyrex pressure resistant tube under argon atmosphere, was stirred at 110 °C for 7 h. The solvent was evaporated in vacuo and the crude product was purified by FCC (DCM/MeOH 25:1 then 10:1) to afford 14 as a white oil (0.29 mmol, 185 mg, 41%).

\(^{1}H\) NMR (400 MHz, Chloroform-\(d\)) \(\delta\) 8.79 (d, \(J = 4.3\) Hz, 1H, Ar-H), 8.40 (d, \(J = 1.5\) Hz, 1H, Ar-H), 7.94 (d, \(J = 8.8\) Hz, 1H, Ar-H), 7.82 (s, 1H, 7-H), 7.72 (t, \(J = 5.2\) Hz, 1H, N-H), 7.68 (dd, \(J = 8.9, 1.8\) Hz, 1H, Ar-H), 7.59 (d, \(J = 15.6\) Hz, 1H, 13-H), 7.42 (d, \(J = 4.3\) Hz, 1H, Ar-H), 7.37 (t, \(J = 5.6\) Hz, 1H, N-H), 6.69 (d, \(J = 15.7\) Hz, 1H, 12-H), 4.84 - 4.79 (m, 1H, 1H, 30-H), 4.55 (dd, \(J = 5.6, 3.7\) Hz, 2H, 9-H), 4.50 (t, \(J = 5.0\) Hz, 2H, 6-H), 4.37 (dd, \(J = 17.3, 5.4\) Hz, 1H, 25-H), 4.26 (dd, \(J = 17.3, 4.8\) Hz, 1H, 25-H), 3.83 (t, \(J = 5.0\) Hz, 2H, 5-H), 3.76 - 3.70 (m, 1H, 29-H), 3.72 – 3.64 (m, 2H, 1-H), 3.62 – 3.48 (m, 11H, 2-H, 3-H, 4-H, 29-H), 2.38 – 2.17 (m, 4H, 27-H, 28-H).

\(^{13}C\) NMR (101 MHz, Chloroform-\(d\)) \(\delta\) 167.51 (C\(_{\text{q}}\)), 167.46 (C\(_{\text{q}}\)), 165.9 (C\(_{\text{Ar}}\)), 150.4 (C\(_{\text{Ar}}\)), 148.8 (C\(_{\text{q}}\)), 144.7 (C\(_{\text{q}}\)), 141.6 (C\(_{\text{q}}\)), 139.8 (C\(_{13}\)), 134.3 (C\(_{\text{q}}\)), 130.2 (C\(_{\text{Ar}}\)), 129.1 (C\(_{\text{Ar}}\)), 125.1 (C\(_{\text{Ar}}\)), 124.4 (C\(_{\text{q}}\)), 123.8 (C\(_{\text{q}}\)), 123.0 (C\(_{12}\)), 119.6 (C\(_{\text{Ar}}\)), 118.4 (C\(_{\text{c}}\)), 72.6, 70.62, 70.60, 70.5, 70.3 (CH\(_{2}\)O), 69.5 (C\(_{5}\)), 61.6 (C\(_{1}\)), 50.4 (C\(_{6}\)), 47.0 (C\(_{29}\)), 45.9 (C\(_{25}\)), 42.5 (C\(_{25}\)), 35.1 (C\(_{5}\)), 29.9 (C\(_{27}, C_{28}\)), 25.3 (C\(_{27}, C_{28}\)).

HRMS: \(m/z\) [M + Na\(^+\)] Calcd for C\(_{31}\)H\(_{38}\)N\(_{8}\)NaO\(_{7}\) 657.2756; Found 657.2762.

IR: \(\nu_{\text{max}}, \text{cm}^{-1}\) 3307 (w), 2929 (w), 2873 (w), 3057 (w), 1711 (w), 1658 (m), 1577 (w), 1535 (w), 1428 (w), 1410 (w), 1366 (w), 1310 (w), 1266 (w), 1210 (w), 1196 (w), 1120 (m), 1103 (m), 1062 (m), 1036 (w), 983 (w), 934 (w), 918 (w), 862 (w), 832 (w), 732 (s), 702 (m), 665 (w)
\[ ^1H \text{NMR spectrum of 14} \]
To a solution of 14 (1 equiv., 0.016 mmol, 10 mg) in anhydrous DCM (0.5 mL) under argon 4-Nitrophenyl chloroformate (2 equiv., 0.032 mmol, 6.5 mg) and anhydrous pyridine (2 equiv., 0.08 mmol, 6.5 μL) were added and the mixture was stirred at rt for 16 h. The solvent was evaporated in vacuo and the crude product was purified by FCC (DCM/MeOH 10:1) to afford 15-a as a white solid (0.011 mmol, 8.7 mg, 68%).

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 8.86 (d, $J = 4.3$ Hz, 1H, Quinoline-H), 8.49 (d, $J = 1.9$ Hz, 1H, Quinoline-H), 8.27 – 8.20 (m, 2H, Nitrophenyl-H), 8.01 (d, $J = 8.7$ Hz, 1H,
Experimental section

Quinoline-H), 7.77 (s, 1H, 7-H), 7.74 (dd, J = 8.8, 1.9 Hz, 1H, Quinoline-H), 7.66 (d, J = 15.6 Hz, 1H, 13-H), 7.47 (d, J = 4.3 Hz, 1H, Quinoline-H), 7.42 (t, J = 5.0 Hz, 1H, N-H), 7.36 – 7.32 (m, 2H, Nitrophenyl-H), 7.04 (t, J = 5.7 Hz, 1H, N-H), 6.73 (d, J = 15.7 Hz, 1H, 12-H), 4.84 – 4.79 (m, 1H, 30-H), 4.58 (t, J = 6.2 Hz, 2H, 9-H), 4.51 (t, J = 5.1 Hz, 2H, 6-H), 4.42 – 4.39 (m, 2H, 1-H), 4.39 – 4.34 (m, 1H, 25-H), 4.29 (dd, J = 17.5, 4.9 Hz, 1H, 25-H), 3.86 (t, J = 5.1 Hz, 2H, 5-H), 3.79 – 3.75 (m, 2H, 2-H), 3.75 – 3.70 (m, 1H, 29-H), 3.68 – 3.64 (m, 2H, 3-H, 4-H), 3.63 – 3.52 (m, 7H, 3-H, 4-H, 29-H), 2.39 – 2.19 (m, 4H, 27-H, 28-H).

$^{13}$C NMR (101 MHz, Chloroform-d) δ 167.39 (C₉), 167.37 (C₉), 165.7 (C₉), 155.6 (C₉), 152.6 (C₉), 150.5 (CQuinoline), 148.9 (C₉), 145.5 (C₉), 144.6 (C₉), 141.4 (C₉), 139.9 (C₁₃), 134.3 (C₉), 130.4 (CQuinoline), 129.4 (CQuinoline), 125.4 (2 x C Nitrophenyl), 124.8 (CQuinoline), 124.4 (C₉), 123.6 (C₇), 123.1 (C₁₂), 121.9 (2 x C Nitrophenyl), 119.7 (CQuinoline), 118.3 (C₉), 70.8, 70.69, 70.66 (CH₂O), 69.5 (C₃), 68.7 (C₃), 68.4 (C₂), 50.4 (C₆), 47.0 (C₃₀), 45.9 (C₂₉), 42.5 (C₂₅), 35.2 (C₉), 29.9 (C₂₇, C₂₈), 25.3 (C₂₇, C₂₈).

HRMS: m/z: [M + H]$^+$ Calcd for C₃₈H₄₂N₉O₁₁+ 800.2998; Found 800.3007.

IR: (ν max, cm$^{-1}$) 3303 (w), 3061 (w), 2948 (w), 2922 (w), 2892 (w), 2869 (w), 2038 (w), 1936 (w), 1911 (w), 1813 (w), 1769 (w), 1730 (w), 1660 (m), 1598 (w), 1526 (m), 1500 (w), 1431 (w), 1412 (w), 1349 (w), 1294 (w), 1264 (m), 1217 (s), 1129 (w), 1110 (w), 1057 (w), 1013 (w), 985 (w), 946 (w), 904 (w), 860 (m), 832 (w), 802 (w), 769 (w), 735 (s), 702 (m), 663 (w)
$^1$H NMR spectrum of 15-a
Experimental section

$^{13}$C NMR spectrum of 15-a

To a solution of 15-a (1 equiv., 0.063 mmol, 50 mg) in anhydrous DMF (2 mL) under argon Et$_3$N (3 equiv., 0.19 mmol, 26 μL) and 17 (1.1 equiv., 0.069 mmol, 21 mg) were added and the mixture was stirred at rt for 5 h. The solvent was evaporated in vacuo and the crude product was purified by FCC (DCM/MeOH 15:1) to afford 15 as a white oil (0.063 mmol, 61 mg, quant.).

$^1$H NMR (400 MHz, Acetonitrile-d$_3$) δ 8.86 (d, $J = 4.3$ Hz, 1H, Quinoline-H), 8.54 (d, $J = 1.9$ Hz, 1H, Quinoline-H), 8.00 (d, $J = 8.8$ Hz, 1H, Quinoline-H), 7.86 (dd, $J = 8.9, 1.9$ Hz, 1H, Quinoline-H), 7.76 (s, 1H, 7-H), 7.63 (d, $J = 15.7$ Hz, 1H, 13-H), 7.55 – 7.44 (m, 3H, DIBO-PEG$_3$-FAPi (15))
Quinoline-H, DIBO-H, Amide-H), 7.39 – 7.24 (m, 7H, DIBO-H), 7.19 (t, J = 5.8 Hz, 1H, Amide-H), 6.77 (d, J = 15.7 Hz, 1H, 12-H), 6.26 (s, 1H, Carbamate-H), 5.92 (s, 1H, Carbamate-H), 5.28 (s, 1H, 38-H), 4.75 – 4.68 (m, 1H, 30H), 4.51 (d, J = 5.8 Hz, 2H, 9-H), 4.44 (t, J = 5.0 Hz, 2H, 6-H), 4.20 (dd, J = 5.7, 2.1 Hz, 2H, 25-H), 4.03 (t, J = 4.7 Hz, 2H, 1-H), 3.79 (t, J = 5.1 Hz, 2H, 5-H), 3.69 – 3.62 (m, 1H, 29-H), 3.53 – 3.39 (m, 11H, 2-H, 3-H, 4-H, 29-H), 3.18 – 3.07 (m, 5H, 34-H, 35-H, 39H), 2.75 (dd, J = 15.0, 4.0 Hz, 1H, 39-H), 2.24 – 2.05 (m, 4H, 27-H, 28-H).

$^{13}$C NMR (101 MHz, Acetonitrile-$d_3$) $\delta$ 168.5 (C$_q$), 168.2 (C$_q$), 166.3 (C$_q$), 156.8 (C$_q$), 153.3 (C$_q$), 152.4 (C$_q$), 151.8 (C$_{Quinoline}$), 149.8 (C$_q$), 145.8 (C$_q$), 143.2 (C$_q$), 140.1 (C$_{13}$), 135.0 (C$_q$), 131.2 (C$_{DIBO}$), 131.1 (C$_{DIBO}$), 129.34 (C$_{Quinoline}$), 129.27 (C$_{DIBO}$), 128.2 (C$_{DIBO}$), 128.1 (C$_{Quinoline}$), 127.1 (C$_{DIBO}$), 126.84 (C$_{DIBO}$), 126.78 (C$_{DIBO}$), 125.5 (C$_{Quinoline}$), 125.0 (C$_q$), 124.3 (C$_q$), 124.20 (C$_{DIBO}$), 124.15 (C$_7$), 121.8 (C$_{12}$), 120.6 (C$_{Quinoline}$), 120.1 (C$_q$), 113.5 (C$_q$), 110.7 (C$_q$), 77.1 (C$_{38}$), 71.2, 71.1, 71.0, 70.1 (CH$_2$O), 70.0 (C$_5$), 64.8 (C$_1$), 50.9 (C$_q$), 47.7 (C$_{35}$), 47.0 (C$_{29}$), 46.7 (C$_{29}$), 43.0 (C$_{23}$), 41.8 (C$_{34}$, C$_{35}$), 41.6 (C$_{34}$, C$_{35}$), 35.8 (C$_9$), 30.6 (C$_{27}$, C$_{28}$), 25.9 (C$_{27}$, C$_{28}$). Two C$_q$ are not resolved.

HRMS: m/z [M + Na]$^+$ Calcd for C$_{51}$H$_{54}$N$_{10}$NaO$_{10}$ $^+$ 989.3917; Found 989.3920.

IR: ($\nu_{\text{max}}$, cm$^{-1}$) 3319 (w), 3061 (w), 2922 (w), 2878 (w), 2159 (w), 1716 (m), 1657 (m), 1521 (m), 1443 (w), 1431 (w), 1288 (w), 1260 (m), 1121 (m), 1105 (m), 1053 (w), 1037 (w), 981 (w), 916 (w), 860 (w), 832 (w), 764 (m), 733 (s), 699 (m), 1970 (w), 1840 (w)
\(^1\)H NMR spectrum of 15
A solution of (S)-6-bromo-N-(2-(2-cyanopyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (1 equiv, 0.1 mmol, 40 mg), compound 5 (2 equiv., 0.2 mmol, 113 mg), palladium acetate (10 mol%, 0.01 mmol, 2 mg), triphenylphosphine (40 mol%, 0.04 mmol, 12 mg), Et₃N (5.5 equiv., 0.56 mmol) in anhydrous degassed DMF (0.4 ml), in a Pyrex pressure resistant tube under argon atmosphere, was stirred at 110 °C for 7 h. The solvent was evaporated in vacuo and the crude product was purified by FCC (DCM/MeOH 6:1) to afford 16 as a white solid (0.03 mmol, 28 mg, 33%).
Experimental section

$^1$H NMR (400 MHz, Chloroform-d) δ 8.87 (d, $J = 4.3$ Hz, 1H, Ar-H), 8.56 (t, $J = 5.5$ Hz, 1H, N-H), 7.91 (s, 1H, 5-H), 7.78 (dd, $J = 8.9$, 1.8 Hz, 1H, Ar-H), 7.67 (d, $J = 15.6$ Hz, 1H, 11-H), 7.47 (d, $J = 4.4$ Hz, 1H, Ar-H), 7.22 (t, $J = 5.5$ Hz, 1H, N-H), 6.75 (d, $J = 15.7$ Hz, 1H, 10-H), 6.63 (s, 1H, N-H), 5.16 (s, 1H, N-H), 4.85 – 4.78 (m, 1H, 39-H), 4.58 (t, $J = 6.7$ Hz, 2H, 7-H), 4.54 – 4.47 (m, 2H, 4-H), 4.32 (dd, $J = 5.5$, 2.3 Hz, 2H, 37-H), 4.14 (t, $J = 6.4$ Hz, 1H 23-H), 3.92 – 3.81 (m, 3H, 3-H, 19-H), 3.82 – 3.74 (m, 1H, 42-H), 3.67 – 3.50 (m, 1H, 1-H, 2-H), 2.93 – 2.86 (m, 1H, 18-H), 2.69 (dd, $J = 13.0$, 4.9 Hz, 1H, 24-H), 2.49 (d, $J = 12.8$ Hz, 1H, 24-H), 2.37 – 2.19 (m, 4H, 40-H, 41-H), 2.07 – 1.93 (m, 2H, 14-H), 1.67 – 1.42 (m, 6H, 15-H, 16-H, 17-H).

$^{13}$C NMR (101 MHz, Chloroform-d) δ 173.6 (Cq), 168.3 (Cq), 168.2 (Cq), 166.2 (Cq), 163.9 (Cq), 150.6 (Cq), 149.0 (Cq), 144.8 (Cq), 141.7 (Cq), 139.8 (Cq), 134.2 (Cq), 130.3 (Cq), 129.6 (Cq), 125.0 (Cq), 124.8 (Cq), 124.0 (Cq), 123.1 (Cq), 119.5 (Cq), 118.3 (Cq), 70.7, 70.6, 70.3, 70.0 (CH2O), 69.4 (Cq), 61.9 (Cq), 60.0 (Cq), 55.6 (Cq), 50.4 (Cq), 47.1 (Cq), 46.1 (Cq), 42.4 (Cq), 40.6 (Cq), 39.5 (Cq), 35.8 (Cq), 34.9 (Cq), 29.8 (Cq), 28.4 (Cq), 28.2 (Cq), 25.6 (Cq), 25.4 (Cq).

HRMS: m/z: [M + Na]$^+$ Calcd for C$_{41}$H$_{53}$N$_{11}$O$_8$Na$^+$ 882.3691; Found 882.3713.

IR: ($\nu_{\text{max}}$, cm$^{-1}$) 3300 (m), 2925 (m), 2870 (m), 2110 (w), 2099 (w), 1940 (w), 1887 (w), 1801 (w), 1656 (s), 1544 (m), 1440 (m), 1323 (m), 1307 (m), 1265 (m), 1088 (m), 1037 (m), 976 (w), 920 (w), 867 (w), 834 (w), 760 (w)

UPLC: (stationary phase: C18-bonded silica; mobile phase: 98% water + 0.5% formic acid/2% CAN +0.45% formic acid to 2% water + 0.5% formic acid/98% CAN +0.45% formic acid over 4min) t$_R$ 1.32 min.
Experimental section

$^1$H NMR spectrum of 16
Experimental section

$^{13}$C NMR spectrum of 16

Analytical UPLC trace of 16
5.6 Synthesis of Erlotinib derivative

Scheme 26: Synthesis of Erlotinib derivative. Reagents and conditions: i- NH₂OH·HCl, AcOH, reflux, 3.5 h; ii- 2-chloroethanol, K₂CO₃, DMF, 150 °C, 20 h; iii- Acetyl chloride, Py, rt, 3 h; iv- HNO₃, rt, 35 min; v- Pd(OAc)₂, PMHS, KF, THF, rt, 1.5 h; vi- DMF-DMA, AcOH, toluene, reflux, 2 h; vii- 3-ethynylaniline, AcOH, reflux, 2 h; viii- K₂CO₃, MeOH, rt, 30 min; ix- NaH, DMF, 0 °C, 1 h, then 19 , rt, 72 h; x- NaH, DMF, 0 °C, 1 h, then 22 , rt, 24 h.

4-Hydroxy-3-methoxybenzonitrile (25)

To a solution of vanillin (1 equiv, 50.3 mmol, 7.7 g) in AcOH (60 mL) hydroxylamine hydrochloride (1.5 equiv, 75.5 mmol, 5.25 g) was added and the mixture was refluxed for 3.5 h. After cooling to rt, the solution was diluted with Et₂O (300 mL). The organic layer was washed with water (300 mL), brine (3 x 100 mL), dried over MgSO₄, filtered and concentrated in vacuo. The crude product was purified by FCC (PE/AcOEt 3:1) to afford 25 as a pale yellow solid (41.5 mmol, 6.2 g, 81 %). The analytical data were in accordance with previously reported data.²⁵²

¹H NMR (400 MHz, Chloroform-d) δ 7.24 (dd, J = 8.2, 1.8 Hz, 1H), 7.09 (d, J = 1.9 Hz, 1H), 6.97 (d, J = 8.2 Hz, 1H), 3.94 (s, 3H).
Experimental section

$^1$H NMR spectrum of 25

4-(2-Hydroxyethoxy)-3-methoxybenzonitrile (26-a)

A mixture of 4-hydroxy-3-methoxybenzonitrile (1 equiv, 40.9 mmol, 6.1 g), 2-chloroethanol (1.1 equiv, 45 mmol, 3.62 mL) and anhydrous K$_2$CO$_3$ (3 equiv, 122.7 mmol, 17 g) in anhydrous DMF (80 mL) under argon was stirred at 150 °C for 20 h. After cooling to rt, DMF was evaporated in vacuo. The reaction mixture was diluted with a sat. solution of NH$_4$Cl in water (50 mL) and extracted with DCM (3 x 50 mL). The combined organic layers were dried over MgSO$_4$, filtered and concentrated in vacuo. The crude product was purified by FCC (PE/AcOEt 1:1) to afford 26-a as a white solid (2.46 g, 13.7 mmol, 34 %). The analytical data were in accordance with previously reported data.$^{252}$

$^1$H NMR (400 MHz, Chloroform-d) δ 7.29 – 7.24 (m, 1H), 7.10 (d, $J = 1.9$ Hz, 1H), 6.93 (d, $J = 8.4$ Hz, 1H), 4.20 – 4.14 (m, 2H), 4.05 – 3.97 (m, 2H), 3.89 (s, 3H).
Experimental section

$^1$H NMR spectrum of 26-a

2-(4-Cyano-2-methoxyphenoxy)ethyl acetate (26)

To a solution of 4-(2-Hydroxyethoxy)-3-methoxybenzonitrile (1 equiv, 10.6 mmol, 1.9 g) in THF (16 mL) acetyl chloride (1.5 equiv, 15.9 mmol, 1.14 mL) and pyridine (1.5 equiv, 15.9 mmol, 1.28 mL) were added dropwise at 0 °C. The reaction was warmed to rt and stirred for 3 h. The reaction mixture was diluted in DCM (100 mL) and washed with a sat. solution of copper sulfate in water (2 x 25 mL) and water (1 x 50 mL). The aqueous phases were extracted with DCM (3 x 50 mL) and the combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure to afford 26 as a white solid (2.33 g, 9.91 mmol, 94 %). Product was used without further purification. The analytical data were in accordance with previously reported data.$^{252}$

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 7.26 (dd, $J = 8.4, 1.9$ Hz, 1H), 7.09 (d, $J = 1.9$ Hz, 1H), 6.91 (d, $J = 8.3$ Hz, 1H), 4.50 – 4.42 (m, 2H), 4.30 – 4.23 (m, 2H), 3.88 (s, 3H), 2.09 (s, 3H).
Experimental section

$^1$H NMR spectrum of 26

2-(4-Cyano-2-methoxy-5-nitrophenoxy)ethyl acetate (27)

HNO$_3$ (7.89 equiv, 42.6 mmol, 1.77 mL) was slowly added at 0 °C to 2-(4-cyano-2-methoxyphenoxy)ethyl acetate (1 equiv, 5.4 mmol, 1.27 g). The reaction mixture was warmed to rt and stirred for 35 min. The solution was poured into ice-water. The resulting precipitate was collected by filtration and recrystallized from AcOEt to afford 27 as a white solid (4.32 mmol, 1.03 g, 80 %). The analytical data were in accordance with previously reported data.$^{252}$

$^1$H NMR (400 MHz, Chloroform-d) δ 7.84 (s, 1H), 7.22 (s, 1H), 4.56 – 4.46 (m, 2H), 4.42 – 4.34 (m, 2H), 4.01 (s, 3H), 2.11 (s, 3H).
Experimental section

$^1$H NMR spectrum of 27

2-(5-Amino-4-cyano-2-methoxyphenoxy)ethyl acetate (28)

To a solution of 2-(4-cyano-2-methoxy-5-nitrophenoxy)ethyl acetate (1 equiv, 0.41 mmol, 116 mg) in dry THF (2.1 ml) palladium acetate (5 mol %, 0.021 mmol, 4.7 mg) and a 1 M solution of KF in water (2 equiv, 0.82 mmol, 0.82 mL) were added. PMHS (4 equiv, 1.64 mmol, 0.1 ml) was added dropwise and the reaction was vigorously stirred at rt for 1.5 h. The solution was diluted with Et$_2$O (5 mL) and water (3 mL). The aqueous phase was extracted with Et$_2$O (3 x 5 mL) and combined organic layers were dried over MgSO$_4$, filtered and concentrated in vacuo. The crude product was purified by FCC (AcOEt) to afford 28 as a brown powder (0.41 mmol, 103 mg, quant.). The analytical data were in accordance with previously reported data.$^{252}$

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 6.81 (s, 1H), 6.26 (s, 1H), 4.44 (t, $J = 4.9$ Hz, 2H), 4.19 (t, $J = 4.9$ Hz, 3H), 3.78 (s, 2H), 2.09 (s, 2H).
Experimental section

$^1$H NMR spectrum of 28

![NMR spectrum image]

2-(4-Cyano-5-(((dimethylamino)methylene)amino)-2-methoxyphenoxy)ethyl acetate (29)

To a solution of 2-(5-amino-4-cyano-2-methoxyphenoxy)ethyl acetate (1 equiv, 0.83 mmol, 206.4 mg) in toluene (2.7 mL) DMF-DMA (2 equiv, 1.66 mmol, 0.22 mL) and AcOH (0.3 equiv, 0.25 mmol, 14 µL) were added and the mixture was refluxed for 2.5 h. The solution was concentrated in vacuo and the resulting oil was triturated with n-heptane and sonicated. Precipitate was filtered and washed with n-heptane to afford 29 as a pale yellow powder (0.80 mmol, 245 mg, 97 %). The analytical data were in accordance with previously reported data.$^{252}$

$^1$H NMR (400 MHz, Chloroform-d) δ 7.57 (s, 1H), 6.95 (s, 1H), 6.50 (s, 1H), 4.45 (t, $J = 4.8$ Hz, 2H), 4.24 (t, $J = 4.8$ Hz, 2H), 3.83 (s, 3H), 3.07 (s, 6H), 2.10 (s, 3H).
1H NMR spectrum of 29

[Image of 1H NMR spectrum]

2-((4-((3-Ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethyl acetate (30)

To a solution of 2-(4-cyano-5-(((dimethylamino)methylene)amino)-2-methoxyphenoxy)ethyl acetate (1 equiv, 0.8 mmol, 244 mg) in AcOH (2.1 mL) 3-Ethynylaniline (1.1 equiv, 0.88 mmol, 0.1 mL) was added and the mixture was refluxed for 2 h. The solution was concentrated in vacuo and the crude product was purified by FCC (PE/AcOEt 1:5) to afford 30 as a beige powder (0.75 mmol, 283 mg, 94 %). The analytical data were in accordance with previously reported data.252

1H NMR (400 MHz, DMSO-d6) δ 9.52 (s, 1H), 8.51 (s, 1H), 7.99 (s, 1H), 7.90 (d, J = 8.3 Hz, 1H), 7.85 (s, 1H), 7.41 (t, J = 7.9 Hz, 1H), 7.24 (s, 1H), 7.21 (d, J = 7.7 Hz, 1H), 4.43 – 4.39 (m, 2H), 4.39 – 4.35 (m, 2H), 4.20 (s, 1H), 3.98 (s, 3H), 2.06 (s, 3H).
Experimental section

$^1$H NMR spectrum of 30

2-((4-((3-Ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethan-1-ol (31-a)

To a solution of 2-((4-((3-ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)ethyl acetate (1 equiv, 0.53 mol, 200 mg) in MeOH (12 mL) K$_2$CO$_3$ (5 equiv, 2.65 mmol, 366 mg) was added. The solution was stirred for 30 min at rt and diluted with H$_2$O (5 mL). MeOH was evaporated in vacuo and the precipitate was removed by filtration and washed with water to afford 31-a as a beige powder (0.53 mmol, 176 mg, 99 %). The analytical data were in accordance with previously reported data.252

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 9.50 (s, 1H), 8.50 (s, 1H), 7.99 (t, $J = 1.9$ Hz, 1H), 7.93 – 7.88 (m, 1H), 7.84 (s, 1H), 7.41 (t, $J = 7.9$ Hz, 1H), 7.23 – 7.19 (m, 2H), 4.94 (t, $J = 5.3$ Hz, 1H), 4.20 (s, 1H), 4.16 (t, $J = 4.9$ Hz, 2H), 3.97 (s, 3H), 3.81 (t, $J = 5.0$ Hz, 2H).
**Experimental section**

**1H NMR spectrum of 31-a**

![NMR Spectrum Image]

N-(17-((4-((3-ethynylphenyl)amino)-6-methoxyquinazolin-7-yl)oxy)-3,6,9,12,15-pentaoxaheptadecyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (31)

To a solution of sodium hydride (1.5 equiv, 0.51 mmol, 20.3 mg) in anhydrous DMF (14 mL) under argon, at 0 °C, was slowly added erlotinib derivative 31-a (1.5 equiv, 0.51 mmol, 170 mg) and the reaction mixture was stirred for 1 h. Compound 19 (1 equiv, 0.34 mmol, 195.1 mg) was added and the reaction was warmed to rt and stirred for additional 72 h. The reaction was quenched by addition of water (1 mL) and solvents were removed *in vacuo*. The crude product was purified by sequential FCC (DCM/MeOH 25:1 + 1% Et₃N) and HPLC (XTerra Prep RP C18, (19 x 150 mm) Waters) to afford 31 as a white solid (33 μmol, 24 mg, 6%). The analytical data were in accordance with previously reported data.²⁵²
Experimental section

$^1$H NMR (400 MHz, Methanol-d4) $\delta$ 8.06 (s, 1H), 7.87 (s, 1H), 7.71 (d, $J = 7.7$ Hz, 1H), 7.53 (s, 1H), 7.51 – 7.44 (m, 2H), 4.82 – 4.77 (m, 2H), 4.48 (dd, $J = 7.9$, 4.9 Hz, 1H), 4.40 (t, $J = 4.5$ Hz, 2H), 4.27 (dd, $J = 7.9$, 4.4 Hz, 1H), 4.10 (s, 3H), 4.02 (t, $J = 4.5$ Hz, 2H), 3.95 (t, $J = 5.6$ Hz, 2H), 3.17 (dt, $J = 3.4$, 2.5 Hz, 3H), 3.56 (q, $J = 3.4$, 2.5 Hz, 3H), 3.17 (dt, $J = 9.9$, 5.3 Hz, 1H), 2.90 (dd, $J = 12.8$, 5.0 Hz, 1H), 2.68 (d, $J = 12.8$ Hz, 1H), 2.14 (t, $J = 7.4$ Hz, 2H), 1.76 – 1.46 (m, 4H).

$^{13}$C NMR (101 MHz, Methanol-d4) $\delta$ 176.01, 158.56, 153.25, 152.71, 137.17, 131.41, 130.34, 129.12, 126.17, 124.58, 109.39, 104.87, 100.74, 83.63, 79.81, 73.62, 72.97, 71.63, 71.54, 71.52, 71.45, 71.30, 71.19, 70.54, 68.83, 63.33, 57.34, 56.97, 41.04, 40.24, 36.64, 29.68, 29.45, 26.83.

HRMS (ESI): $m/z$ calcd for C$_{37}$H$_{48}$N$_6$O$_9$S ($[M + H]^+$): 737.3327; found: 737.3332

IR: ($\nu_{\text{max}}$, cm$^{-1}$) 3282 (w), 3086 (w), 2926 (w), 2863 (w), 1684 (s), 1628 (m), 1571 (w), 1532 (m), 1513 (m), 1457 (m), 1443 (m), 1403 (w), 1368 (w), 1303 (w), 1276 (m), 1248 (w), 1203 (m), 1182 (w), 1130 (m), 1056 (w), 940 (w), 843 (w), 801 (w), 721 (w) cm$^{-1}$.

$^1$H NMR spectrum of 31
Experimental section

13C NMR spectrum of 31

DIBO-PEG3-Erlotinib (32)

To a suspension of sodium hydride (2 equiv, 0.34 mmol, 8.2 mg) in anhydrous DMF (5 mL) was added dropwise a solution of erlotinib derivative 31-a (1.5 equiv, 0.26 mmol, 87.2 mg) in anhydrous DMF (2 mL) at 0 °C and the mixture was stirred at rt for 1 h. 23 (1 equiv, 0.17 mmol, 100 mg) in anhydrous DMF (2 mL) was added dropwise and the reaction mixture was stirred at rt for 24 h. The reaction was quenched with water and the volatiles were removed in vacuo. The crude product was purified by FCC (DCM/MeOH 50:1 to 25:1) to afford 32 as a white solid (44.9 µmol, 34 mg, 26 %).
Experimental section

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 8.64 (s, 1H, H$_{Ar}$), 7.88 – 7.81 (m, 1H, H$_{Ar}$), 7.71 (dd, J = 8.3, 2.2 Hz, 1H, H$_{Ar}$), 7.48 (d, J = 7.7 Hz, 1H, H$_{Ar}$), 7.38 – 7.22 (m, 9H, H$_{Ar}$), 7.19 (s, 1H, H$_{Ar}$), 7.07 (s, 1H, NH), 5.67 (s, 1H, NH), 5.48 (s, 1H, H$_{6}$), 4.27 (t, J = 4.8 Hz, 2H, 20-H, 21-H), 3.94 (t, J = 5.7 Hz, 2H, 22-H), 3.91 (s, 3H, 31-H), 3.80 – 3.73 (m, 2H, 20-H, 21-H), 3.73 – 3.55 (m, 11H, 20-H, 21-H, 40-H), 3.52 (t, J = 5.0 Hz, 2H, 20-H, 21-H), 3.34 (q, J = 5.3 Hz, 2H, 19-H), 3.15 (dd, J = 15.0, 2.2 Hz, 1H, H-6), 2.87 (dd, J = 15.0, 4.1 Hz, 1H, H-6).

$^{13}$C NMR (101 MHz, Chloroform-d) $\delta$ 156.29 (C$_{q}$), 155.85 (C$_{q}$), 154.22 (C$_{q}$), 153.37 (C$_{Ar}$), 152.22 (C$_{q}$), 151.10 (C$_{q}$), 149.84 (C$_{q}$), 147.16 (C$_{q}$), 138.97 (C$_{q}$), 130.00 (C$_{Ar}$), 129.12 (C$_{Ar}$), 128.16 (C$_{Ar}$), 128.06 (C$_{Ar}$), 127.90 (C$_{q}$), 127.20 (C$_{Ar}$), 126.36 (C$_{Ar}$), 126.11 (C$_{Ar}$), 125.18 (C$_{Ar}$), 123.93 (C$_{q}$), 123.86 (C$_{Ar}$), 122.91 (C$_{Ar}$), 122.42 (C$_{Ar}$), 121.40 (C$_{q}$), 113.03 (C$_{q}$), 110.10 (C$_{q}$), 109.26 (C$_{q}$), 108.51 (C$_{Ar}$), 99.98 (C$_{Ar}$), 83.55 (C$_{Ar}$), 77.02 (C$_{q}$), 71.06, 70.84, 70.77, 70.71, 70.67, 70.39, 70.10 (CH$_{2}$O), 69.30 (C$_{22}$), 68.54 (CH$_{2}$O), 56.40 (C$_{31}$), 46.24 (C$_{6}$), 41.08 (C$_{19}$).

HRMS (ESI): m/z calcd for C$_{44}$H$_{44}$N$_{4}$O$_{8}$ ([M + H]$^+$): 757.3232; found: 757.3240

IR: ($\nu_{\text{max}}$, cm$^{-1}$) 3344 (w), 3291 (w), 3064 (w), 2923 (m), 2874 (m), 1703 (s), 1623 (m), 1576 (m), 1532 (s), 1509 (s), 1454 (m), 1426 (s), 1393 (w), 1354 (w), 1346 (w), 1278 (m), 1249 (s), 1212 (m), 1145 (m), 1107 (m), 1025 (w), 950 (w), 912 (w), 854 (w), 794 (w), 762 (m), 733 (m), 686 (w) cm$^{-1}$. 

146
1H NMR spectrum of 32
Experimental section

$^{13}$C NMR spectrum of 32
5.7 Gadolinium chelating agent

5.7.1 Synthesis of L-homoserine derivative (42)

![Scheme 27: Synthesis of L-homoserine derivative. i- NHS, DCC, THF, rt, 2 h; ii- (S)-(−)-α-Amino-γ-butyrolactone hydrobromide, Et₃N, DCM, rt, 2 h; iii- H₂SO₄, MeOH, 24 h, rt; iv- (COCl)₂, DMSO, Et₃N, DCM 0 °C, 2 h.]

2,5-dioxopyrrolidin-1-yl pent-4-ynoate (40)

A solution of pent-4-ynoic acid (1 equiv, 30.58 mmol, 3 g) and N-hydroxysuccinimide (1 equiv, 30.58 mmol, 3.52 g) in dry THF (100 mL) was cooled to 0 °C. DCC (1 equiv, 30.58 mmol, 6.31 g) in dry THF (35 mL) was added dropwise and the mixture was stirred at 0 °C for 45 min. The mixture was warmed up to rt and stirred for 2 h. The mixture was filtered and concentrated under reduce pressure. The residue was redissolved in EtOAc (100 mL) and filtered. The organic layer was washed with sat. solution of NaHCO₃ in water (100 mL) and brine (100 mL), dried over MgSO₄, filtered and concentrated in vacuo to afford 40 as a white solid (28.7 mmol, 5.60 g, 94 %). The analytical data were in accordance with previously reported data.²⁶³

¹H NMR (400 MHz, Chloroform-d) δ 2.93 – 2.84 (m, 2H), 2.84 (s, 4H), 2.62 (td, J = 7.5, 7.0, 2.7 Hz, 2H), 2.05 (t, J = 2.6 Hz, 1H).
Experimental section

$^1$H NMR spectrum of 40

(S)-N-(2-oxotetrahydrofuran-3-yl)pent-4-ynamide (41)

To a solution of (S)-(−)-α-amino-γ-butyrolactone hydrobromide (1.1 equiv, 16.72 mmol, 3.04 g) and Et$_3$N (3 equiv, 45.6 mmol, 6.3 mL) in dry DCM (130 mL) 2,5-dioxopyrrolidin-1-yl pent-4-ynoate (1 equiv, 15.2 mmol, 3 g) was added portion wise and the mixture was stirred at rt for 2 h. The mixture was washed with sat. solution of NH$_4$Cl in water (100 mL) and the aqueous layer was extracted with DCM (8 X 100 mL). The combine organic layers were dried over MgSO$_4$, filtered and concentrated under reduce pressure. The crude product was purified by FCC (PE/EtOAc 1:3) to afford 41 as a white solid (12.3 mmol, 2.23 g, 81 %). The analytical data were in accordance with previously reported data.$^{264}$

$^1$H NMR (400 MHz, Chloroform-d) δ 6.15 (s, 1H), 4.55 (ddd, $J$ = 11.7, 8.5, 5.6 Hz, 1H), 4.51 – 4.42 (m, 1H), 4.29 (ddd, $J$ = 11.4, 9.3, 5.8 Hz, 1H), 2.59 – 2.45 (m, 4H), 2.15 (dtd, $J$ = 12.6, 11.5, 8.8 Hz, 1H), 2.03 (t, $J$ = 2.5 Hz, 1H).
Experimental section

$^1$H NMR spectrum of 41

Methyl pent-4-ynoyl-L-homoserinate (42-a)

To a solution of (S)-N-(2-oxotetrahydrofuran-3-yl)pent-4-ynamide (1 equiv, 1.38 mmol, 250 g) in MeOH (1.4 mL) conc. H$_2$SO$_4$ (cat., 2 drops) was added and the mixture was stirred at rt for 24 h. The mixture was cooled down to 0 °C and the reaction was neutralized by addition of NaHCO$_3$. The mixture was filtered and concentrated in vacuo at 25 °C. The crude product 42-a was used as such in the next step.

Methyl (S)-4-oxo-2-(pent-4-ynamido)butanoate (42)
A solution of (COCl)₂ (2 equiv, 2.76 mmol, 0.24 mL) in dry DCM (8 mL) was cooled to -78 °C. DMSO (4 equiv, 5.52 mmol, 0.39 mL) in dry DCM (4 mL) was added dropwise and the mixture was stirred at -78 °C for 5 min. Methyl pent-4-ynoyl-L-homoserinate (1 equiv, 1.38 mmol, 294 mg) in dry DCM (4 mL) was added dropwise and the mixture was stirred at -78 °C for 30 min. Et₃N (6 equiv, 8.28 mmol, 1.15 mL) was added dropwise and the mixture was warmed up to 0 °C and stirred for 2 h. The reaction was quenched with H₂O (10 mL) and the aqueous layer was extracted with DCM (8 X 10 mL). The combine organic layer were dried over MgSO₄, filtered and concentrated under reduce pressure. The crude product was purified by FCC (PE/EtOAc 1:3) to afford 42 as a yellow oil (0.60 mmol, 126 mg, 43 %).

\[ \text{1H NMR (400 MHz, Chloroform-d):} \delta 9.71 (s, 1H, 1-H), 6.56 (d, J = 7.7 Hz, 1H, 6-H), 4.87 (dt, J = 7.8, 4.6 Hz, 1H, 3-H), 3.75 (s, 3H, 5-H), 3.17 (ddd, J = 18.7, 4.8, 0.7 Hz, 1H, 2-H), 3.12 – 3.04 (m, 1H, 2-H), 2.56 – 2.38 (m, 4H, 8-H, 9-H), 1.99 (t, J = 2.5 Hz, 1H, 11-H). \]

\[ \text{13C NMR (101 MHz, Chloroform-d):} \delta 199.5 (C₁), 171.2 (C₄), 170.9 (C₇), 82.7 (C₁₀), 69.7 (C₁₁), 53.0 (C₅), 47.4 (C₃), 45.7 (C₂), 35.2 (C₈), 14.9 (C₉). \]

\[ \text{HRMS (ESI/QTOF):} \text{ m/z calcd for C}_{10}H_{14}NO₄^+ ([M + H]^+): 212.0917; Found 212.0921. \]

\[ \text{IR: (ν}_{\text{max}}, \text{ cm}^{-1}) 3624 (w), 3361 (w), 3286 (m), 3069 (w), 2955 (w), 2924 (w), 2849 (w), 1737 (s), 1658 (s), 1537 (s), 1437 (m), 1381 (w), 1268 (m), 1219 (s), 1179 (m), 1048 (w), 985 (w), 656 (w) \]
Experimental section

$^1$H NMR spectrum of 42

![NMR Spectrum](image-url)
Experimental section

$^{13}$C NMR spectrum of 42
5.7.2 Synthesis of Gd(III)-complex (44)

Scheme 28: Synthesis of gadolinium chelating agent. i- H₂SO₄ conc. EtOH, reflux, 16 h; ii- NaBH₄, EtOH, reflux, 16 h; iii- CBr₄, PPH₃, DCM, rt, 3 h; iv- TACN·3 HCl, DIPEA, ACN, reflux, 16 h; v- 42, NaBH₃CN, DCE/ACN, rt, 16 h; vi- KOH, THF, rt 4 h, then GdCl₃, H₂O, rt, 1 h.

Diethyl pyridine-2,6-dicarboxylate (35)

To a solution of pyridine-2,6-dicarboxylic acid (1 equiv, 59.8 mmol, 10 g) in EtOH (200 mL) conc. H₂SO₄ (8 equiv, 479 mmol, 25.5 mL) was added dropwise and the mixture was reflux for 16 h. The mixture was concentrated to half volume and poured into sat. solution of Na₂CO₃ in water at 0 °C. The aqueous layer was extracted with DCM (5 X 150 mL) and the combine organic layers were dried over MgSO₄, filtered and concentrated in vacuo to afford 35 as a white solid (49.5 mmol, 11.1 g, 83 %). The analytical data were in accordance with previously reported data.²⁶⁵

¹H NMR (400 MHz, Chloroform-d) δ 8.28 (d, J = 7.8 Hz, 2H), 8.00 (t, J = 7.8 Hz, 1H), 4.49 (q, J = 7.1 Hz, 4H), 1.46 (t, J = 7.1 Hz, 6H).
Ethyl 6-(hydroxymethyl)picolinate (36)

To a solution of diethyl pyridine-2,6-dicarboxylate (1 equiv, 4.48 mmol, 1 g) in EtOH (15 mL) NaBH₄ (0.8 equiv, 3.58 mmol, 136 mg) was added portion wise and the mixture was reflux for 16 h. The mixture was neutralized with a 6 M solution of HCl in water and the aqueous layer was extracted with DCM (5 X 10 mL) and the combine organic layers were washed with water (10 mL), dried over MgSO₄, filtered and concentrated in vacuo to afford 36 as a white solid (2.47 mmol, 447 mg, 55 %). The analytical data were in accordance with previously reported data.²⁴⁰

¹H NMR (400 MHz, Chloroform-d) δ 8.03 (dd, J = 7.7, 1.0 Hz, 1H), 7.84 (t, J = 7.8 Hz, 1H), 7.50 (dd, J = 7.8, 1.0 Hz, 1H), 4.86 (s, 2H), 4.47 (q, J = 7.1 Hz, 2H), 1.43 (t, J = 7.1 Hz, 3H).
Experimental section

$^1$H NMR spectrum of 36

Ethyl 6-(bromomethyl)picolinate (37)

To a solution of ethyl 6-(hydroxymethyl)picolinate (1 equiv, 2.51 mmol, 455 mg) in dry DCM (6 mL) tetrabromomethane (1.2 equiv, 3.06 mmol, 1.02 g) was added. The mixture was cooled down to 0 °C and triphenylphosphine (1.6 equiv, 4.02 mmol, 1.05 g) in dry DCM (2 mL) was added over 1 h. The mixture was concentrated under reduce pressure and the crude product was purified by FCC (PE/EtOAc 4:1) to afford 37 as a yellow oil (1.73 mmol, 421 mg, 69%). The analytical data were in accordance with previously reported data.²⁴⁰

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 8.04 (dd, $J = 7.7$, 1.0 Hz, 1H), 7.86 (t, $J = 7.8$ Hz, 1H), 7.69 (dd, $J = 7.8$, 1.1 Hz, 1H), 4.65 (s, 2H), 4.49 (q, $J = 7.1$ Hz, 2H), 1.44 (t, $J = 7.1$ Hz, 3H).
Experimental section

$^1$H NMR spectrum of 37

Diethyl 6,6’-((1,4,7-triazonane-1,4-diyl)bis(methylene))dipicolinate (38)

To a solution of ethyl 6-(bromomethyl)picolinate (1.05 equiv, 1.64 mmol, 400 mg) in ACN (12 mL) TACN·3 HCl (1 equiv, 1.56 mmol, 372 mg) and DIPEA (3.7 equiv, 5.78 mmol, 0.95 mL) were added and the mixture was reflux for 16 h. The mixture was concentrated under reduce pressure and the residue was redissolved in a minimum amount of DCM and filtered. The mixture was concentrated under reduce pressure and the crude product was purified by FCC (Al$_2$O$_3$ basic III (4.9 % water) DCM/MeOH 1:0 then 20:1) to afford 38 as a brow oil (0.52 mmol, 235 mg, 63 %). The analytical data were in accordance with previously reported data.\textsuperscript{239}

$^1$H NMR (400 MHz, Acetonitrile-$d_3$) $\delta$ 7.97 – 7.89 (m, 2H), 7.85 (t, $J$ = 7.7 Hz, 1H), 7.76 – 7.67 (m, 2H), 7.55 (d, $J$ = 7.8 Hz, 1H), 4.36 (ddd, $J$ = 12.8, 8.5, 6.3 Hz, 4H), 3.94 (s, 2H), 3.83 (s, 2H), 3.13 – 2.66 (m, 12H), 1.35 (td, $J$ = 7.1, 1.4 Hz, 6H).

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Diethyl 6,6'-(1,4,7-triazonane-1,4-diyl)bis(methylene))((R)-dipicolinate (43)

To a solution of diethyl 6,6'-(1,4,7-triazonane-1,4-diyl)bis(methylene))dipicolinate (1 equiv, 0.11 mmol, 50 mg) in DCE/ACN (2:1, 0.6 mL) 42 (2 equiv, 0.22 mmol, 47 mg) in DCE/ACN (2:1, 0.6 mL) was added dropwise followed by NaBH₃CN (5 equiv, 0.55 mmol, 35 mg) and the mixture was stirred at rt for 16 h. The reaction mixture was quenched with a sat. solution of NaHCO₃ in water and the aqueous layer was extracted with DCM (8 X 2 mL). The combine
organic layers were dried over MgSO₄, filtered and concentrated under reduce pressure. The
crude product was purified by FCC (Al₂O₃ basic III (4.9 % water) DCM/MeOH 1:0 then 50:1)
to afford 43 as a yellow oil (0.03 mmol, 17 mg, 24 %).

1H NMR (400 MHz, Acetonitrile-d₃) δ 7.93 (dd, J = 7.6, 1.2 Hz, 2H, Hₘₙ), 7.86 (t, J =
7.7 Hz, 2H, Hₘₙ), 7.73 (d, J = 7.7 Hz, 2H, Hₘₙ), 7.20 (s, 1H, 20-H), 4.49 (td, J = 8.0, 4.6 Hz,
1H, 18-H), 4.36 (q, J = 7.1 Hz, 4H, 14-H, 14'-H), 3.86 (s, 4H, 7-H, 7'-H), 3.64 (s, 3H, 26-H),
2.93 – 2.71 (m, 12H, 1-H, 2-H, 3-H, 4-H, 5-H, 6-H), 2.60 – 2.51 (m, 2H, 16-H), 2.46 – 2.32 (m,
4H, 22-H, 23-H), 2.19 – 2.15 (m, 2H, 17-H), 1.82 – 1.78 (m, 1H, 25-H), 1.36 (t, J = 7.1 Hz, 6H,
15-H, 15'-H).

13C NMR (101 MHz, Acetonitrile-d₃) δ 174.1 (C₉), 171.8 (C₉), 166.2 (2 X C₉), 148.6 (2 X
C₉), 138.4 (2 X C₉), 127.5 (2 X C₉), 126.4 (2 X C₉), 124.2 (2 X C₉), 84.3 (C₉), 70.1 (C₁₇),
65.2 (C₇, C₇'), 62.3 (C₁₄, C₁₄'), 56.4 (C₁, C₂, C₃, C₄, C₅, C₆), 54.5 (C₁₃), 52.6 (C₁₆), 52.0 (C₁₈),
35.3 (C₂₂), 26.3 (C₂₃), 15.1 (C₂₅), 14.6 (C₁₅, C₁₆).

HRMS (nanochip-ESI/LTQ-Orbitrap): m/z caleed for C₃₄H₄₇N₆O₇+ ([M + H]+): 651.3501;
Found 651.3510.

IR: (νmax, cm⁻¹) 3580 (w), 3372 (w), 3289 (w), 3066 (w), 2928 (m), 2820 (w), 1735 (s), 1733 (s),
1671 (s), 1589 (m), 1543 (m), 1454 (m), 1369 (m), 1308 (s), 1226 (s), 1173 (s), 1138 (s), 1023
(m), 993 (m), 762 (m).
$^1$H NMR spectrum of 43
Experimental section

$^{13}$C NMR spectrum of 43

Gd(III)-Complexe (44)

To a solution of 44 (1 equiv, 15 μmol, 10 mg) in THF (0.5 mL) KOH (5 equiv, 75 μmol, 1.8 mg) was added and the mixture was stirred at rt for 4 h. H₂O (0.5 mL) was added and the THF was evaporated under reduce pressure. The mixture was neutralized with a 0.5 M solution of HCl in water and GdCl₃·6 H₂O (1.1 equiv, 17 μmol, 6.3 mg) was added and the mixture was stirred at rt for 1 h (completion of the reaction was monitored by ESI-MS). The mixture was concentrated \textit{in vacuo} to afford 44 as white solid. The product was used without further purification

5.8 Coating and functionalization of NPs

5.8.1 Coating of NPs

General method for coating NPs with PEG-N$_3$ (49) and PEG-NH$_2$ (50)

The protocol was adapted from previous report.$^{28}$

To a suspension of NPs (2 mg) in EtOH (1 mL) was added a mixture of toluene (1 mL) and aqueous NH$_3$ 25% (320 μL). The suspension was ultra-sonicated for 30 min. PEG-N$_3$ (49) and PEG-NH$_2$ (50) (1:1, 21 μmol, 50 mg) were added and the suspension was ultrasonicated at 40 °C for 16 h. The suspension was centrifuged (10 min, 4 700 rpm). The supernatant was discarded and the NPs were resuspended in EtOH/H$_2$O 1:1 (2 mL). The suspension was shaken until emulsification and centrifuged (10 min, 4 700 rpm). The procedure was repeated 4 times.

NP-PEG were stored in EtOH at a concentration of 2 mg/mL.

A sample (20 μL) was diluted with distilled water (1 mL) and ultra-sonicated for 30 minutes. Mean hydrodynamic diameter and zeta potential were measured on a Malvern NanoZ instrument.

General method for coating NPs with APTES derivatives

The protocol was adapted from previous report.$^{122}$

To a suspension of NPs (2 mg) in EtOH (1 mL) was added cyclohexane (1 mL) and was ultra-sonicated for 30 min. TEOS (2 equiv, 10 μmol, 2.0 μL), APTES (1 equiv, 5 μmol, 1.2 μL) and 4-azido-N-(3-(triethoxysilyl)propyl)butanamide (1 equiv, 5 μmol, 1.7 mg) in EtOH (100 μL)
were added and the suspension was ultrasonicated for 30 min. Aqueous NH$_3$ 25 % (100 µL) was added and the suspension was ultrasonicated at 40 °C for 16 h. The suspension was centrifuged (10 min, 4 700 rpm). The supernatant was discarded and the NPs were resuspended in EtOH (1 mL). The suspension was shaken until emulsification and centrifuged (10 min, 4 700 rpm). The procedure was repeated 4 times. NP-PEG were stored in EtOH at a concentration of 2 mg/mL. A sample (20 µL) was diluted with distilled water (1 mL) and ultra-sonicated for 30 minutes. Mean hydrodynamic diameter and zeta potential were measured on a Malvern NanoZ instrument.

5.8.2 Functionalization of coated NPs

Functionalization of BFO-PEG NPs with DIBO-PEG$_{3}$-FAPi (15)

To a suspension of BFO-PEG NPs (4 mg) in EtOH (4 mL) was added distilled water (4 mL) and was ultra-sonicated for 15 min. A solution of DIBO-PEG$_{3}$-FAPi (15) (17.4 µmol, 17 mg) in DMF (200 µL) was added and the suspension was ultra-sonicated at 40 °C for 16 h. The mixture was then divided into eppendorfs and centrifuged (10 min, 13 000 rpm). The supernatant was discarded and the NPs were resuspended in EtOAc (1 mL) and centrifuged (10 min, 13 000 rpm). The procedure was repeated 3 times. BFO-PEG-Erlotinib NPs were stored in EtOH at a concentration of 1 mg/mL. A sample (20 µL) was diluted with distilled water (1 mL) and ultra-sonicated for 30 minutes. Mean hydrodynamic diameter and zeta potential were measured on a Malvern NanoZ instrument.
**Functionalization of BFO-PEG NPs with Erlotinib derivative (32)**

Scheme 32: Functionalization of PEG coated BFO with Erlotinib derivative (32)

To a suspension of BFO-PEG NPs (3 mg) in EtOH (1.5 mL) was added distilled water (3 mL) and was ultra-sonicated for 15 min. A solution of DIBO-PEG₃-Erlotinib (32) (10.6 μmol, 8 mg) in DMF (150 μL) was added and the suspension was ultra-sonicated at 40 °C for 16 h. The mixture was then divided into eppendorfs and centrifuged (10 min, 13 000 rpm). The supernatant was discarded and the NPs were resuspended in EtOAc (1 mL) and centrifuged (10 min, 13 000 rpm). The procedure was repeated 3 times. BFO-PEG-Erlotinib NPs were stored in EtOH at a concentration of 1 mg/mL.

A sample (20 L) was diluted with distilled water (1 mL) and ultra-sonicated for 30 minutes. Mean hydrodynamic diameter and zeta potential were measured on a Malvern NanoZ instrument.
Experimental section

Figure 41: (A) Dynamic light scattering by number of uncoated BFO (black), PEG coated BFO (red) and Erlotinib functionalized BFO (blue); (B) Zeta potential of uncoated BFO (black), PEG coated BFO (red) and Erlotinib functionalized BFO (blue).

**Functionalization of LNO-PEG NPs with Erlotinib derivative (32)**

To a suspension of LNO-PEG NPs (3 mg) in EtOH (1.5 mL) was added DMF (0.5 mL) and was ultra-sonicated for 15 min. A solution of DIBO-PEG₈-Erlotinib (32) (1.5 µmol, 1.1 mg) in DMF (1 mL) was added and the suspension was ultra-sonicated at 40 °C for 16 h. The mixture was centrifuged (10 min, 4 700 rpm). The supernatant was discarded and the NPs were resuspended in DMF (1 mL) and centrifuged (10 min, 4 700 rpm). The supernatant was discarded and the NPs were resuspended in EtOH (1 mL) and centrifuged (10 min, 4 700 rpm). The procedure was repeated 3 times. LNO-PEG-Erlotinib NPs were stored in EtOH at a concentration of 2 mg/mL.

A sample (20 µL) was diluted with distilled water (1 mL) and ultra-sonicated for 30 minutes. Mean hydrodynamic diameter and zeta potential were measured on a Malvern NanoZ instrument.
Functionalization of LNO-APTES NPs with Erlotinib derivative (32)

Scheme 34: Functionalization of APTES coated LNO with Erlotinib derivative (32)

To a suspension of LNO-APTES NPs (2 mg) in EtOH (1 mL) was added DMF (0.5 mL) and was ultra-sonicated for 15 min. A solution of DIBO-PEG₃-Erlotinib (32) (1 µmol, 0.76 mg) in DMF (1 mL) was added and the suspension was ultra-sonicated at 40 °C for 16 h. The mixture was centrifuged (10 min, 4 700 rpm). The supernatant was discarded and the NPs were resuspended in DMF (1 mL) and centrifuged (10 min, 4 700 rpm). The supernatant was discarded and the NPs were resuspended in EtOAc (1 mL) and centrifuged (10 min, 4 700 rpm). The procedure was repeated 3 times. LNO-APTES-Erlotinib NPs were stored in EtOH at a concentration of 2 mg/mL.

A sample (20 µL) was diluted with distilled water (1 mL) and ultra-sonicated for 30 minutes. Mean hydrodynamic diameter and zeta potential were measured on a Malvern NanoZ instrument.
Experimental section

Figure 43: (A) Dynamic light scattering by number of uncoated LNO (black), APTES coated LNO (red) and Erlotinib functionalized LNO (blue); (B) Zeta potential of uncoated LNO (black), APTES coated LNO (red) and Erlotinib functionalized LNO (blue).

Functionalization of LNO-APTES NPs with Gd(III)-complex (44)

Scheme 35: Functionalization of APTES coated LNO with Gd(III)-complex (44)

To a suspension of LNO-APTES NPs (2 mg) in EtOH (1 mL) was added water (1.5 mL) and was ultra-sonicated for 15 min. A solution of 44 (1 equiv, 1 µmol, 0.76 mg) in water (100 µL) and a mixture of copper (II) sulfate (0.5 equiv, 0.5 µmol, 80 µg) and sodium ascorbate (1.5 equiv, 1.5 µmol, 0.3 mg) in water (20 µL) was added and the suspension was ultra-sonicated at 40 °C for 16 h. The mixture was centrifuged (10 min, 4 700 rpm). The supernatant was discarded and the NPs were resuspended in water (1 mL) and centrifuged (10 min, 4 700 rpm). The supernatant was discarded and the NPs were resuspended in water (1 mL) and centrifuged (10 min, 4 700 rpm). The procedure was repeated 3 times. LNO-APTES-Gd NPs were stored in distilled water at a concentration of 2 mg/mL.
A sample (20 μL) was diluted with distilled water (1 mL) and ultra-sonicated for 30 minutes. Mean hydrodynamic diameter and zeta potential were measured on a Malvern NanoZ instrument.

Figure 44: (A) Dynamic light scattering by number of uncoated LNO (black), APTES coated LNO (red) and Gd(III) complex functionalized LNO (blue); (B) Zeta potential of uncoated LNO (black), APTES coated LNO (red) and Gd(III) complex functionalized LNO (blue).

XPS analysis of LNO-APTES-Gd NPs

Initial surveys scans were acquired. From these surveys, the constitutive elements were selected and accurately measured with multiplexes using higher resolution and longer acquisition time. From the multiplexes, and after having removed the background using a Shirley function, the so-called surface atomic concentration, [at%], were calculated based on the peak area compensated for the atomic sensitivity factor of each transition. The chemical state of the elements were evaluated by comparison with reference spectrum. All multiplexes were referenced at 284.8 eV with the C-C bound of the atmospheric carbon contamination and/or carbon present in the sample.
Experimental section

Figure 45: XPS surveys scans of coated and functionalized LNO NPs.

<table>
<thead>
<tr>
<th></th>
<th>Li1s</th>
<th>O1s</th>
<th>Si2p</th>
<th>Zn2p3</th>
<th>Nb3d</th>
<th>Cu2p</th>
<th>Gd3d</th>
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<tr>
<td><strong>LNO-APTES</strong></td>
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<td>32.19</td>
<td>9.91</td>
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<td>2.18</td>
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<td>0.00</td>
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<td><strong>LNO-APTES-Gd</strong></td>
<td></td>
<td></td>
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<tr>
<td>washed with cyclam</td>
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<td></td>
</tr>
<tr>
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<td>1.96</td>
<td>1.86</td>
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Table 8: Measured surface atomic concentration, at%

**Relaxation measurements of LNO-APTES-Gd**

600 μL of a 2 mg/mL suspension of NPs (LNO, LNO-APTES and LNO-APTES-Gd NPs) in water was introduced in a 5 mm NMR tube. The sample was ultrasonicated for 30 min and loaded in the spectrometer. Prior to acquisition, a waiting time of 15 min was set for temperature stabilization at 37.0 °C.

600 μL of a 1 mg/mL (1.36 mM) solution of 44 in water was introduced in a 5 mm NMR tube. The sample was ultrasonicated for 30 min and loaded in the spectrometer. Prior to acquisition, a waiting time of 15 min was set for temperature stabilization at 37.0 °C.

The longitudinal relaxation time (T1) was measured with inversion recovery method. For fast relaxing sample, 20 variable delays were set from 5 ms to 1000 ms with a quadratic increment. The measurement was averaged with 4 scans. The repetition time was set to 2.5 s. For slow relaxing samples, 20 variable delays were set from 50 ms to 10000 ms with a quadratic
increment. The measurement was averaged with 4 scans. The repetition time was set to 10 s. The $T_1$ value was extracted with mono-exponential fit of the signal intensities.

The transverse relaxation time ($T_2$) was measured with CPMG spin echo sequence. For fast relaxing samples 200 echo points with an echo time ($\tau$) of 1 ms were acquired. The measurement was averaged over 4 scans. The repetition time was set to 2.5 s. For slow relaxing samples 400 echo points with an echo time ($\tau$) of 4 ms were acquired. The measurement was averaged over 4 scans. The repetition time was set to 10 s. The $T_s$ value was extracted with mono-exponential fit of the signal intensities.

The relaxation rate $R_i$ was calculated using equation (2).

$$R_i = \frac{1}{T_1} \quad (2)$$

<table>
<thead>
<tr>
<th></th>
<th>$T_1$ (ms)</th>
<th>err (ms)</th>
<th>$T_2$ (ms)</th>
<th>err (ms)</th>
<th>$R_1$ (s$^{-1}$)</th>
<th>err (s$^{-1}$)</th>
<th>$R_2$ (s)</th>
<th>err (s$^{-1}$)</th>
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<td>194.0</td>
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<td>3.74</td>
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<td>5.154</td>
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Table 9: $T_1$ and $T_2$ measurements and $R_1$ and $R_2$ calculations at 30 MHz.

<table>
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<tr>
<th></th>
<th>$T_1$ (ms)</th>
<th>err (ms)</th>
<th>$T_2$ (ms)</th>
<th>err (ms)</th>
<th>$R_1$ (s$^{-1}$)</th>
<th>err (s$^{-1}$)</th>
<th>$R_2$ (s)</th>
<th>err (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO-APTES-Gd</td>
<td>221</td>
<td>2</td>
<td>93.6</td>
<td>0.1</td>
<td>4.52</td>
<td>0.04</td>
<td>10.68</td>
<td>0.01</td>
</tr>
<tr>
<td>44</td>
<td>103.32</td>
<td>0.04</td>
<td>89.6</td>
<td>0.2</td>
<td>9.679</td>
<td>0.004</td>
<td>11.24</td>
<td>0.03</td>
</tr>
<tr>
<td>LNO-APTES</td>
<td>3440</td>
<td>10</td>
<td>1210</td>
<td>20</td>
<td>0.290</td>
<td>0.001</td>
<td>0.82</td>
<td>0.01</td>
</tr>
<tr>
<td>LNO</td>
<td>3790</td>
<td>80</td>
<td>1120</td>
<td>10</td>
<td>0.263</td>
<td>0.005</td>
<td>0.89</td>
<td>0.01</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>3801</td>
<td>8</td>
<td>1570</td>
<td>20</td>
<td>0.263</td>
<td>0.001</td>
<td>0.63</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 10: $T_1$ and $T_2$ measurements and $R_1$ and $R_2$ calculations at 60 MHz.

ICP/MS analysis were performed to obtain the concentration of gadolinium from each sample.
Experimental section

### Table 11: Concentration of gadolinium complex on the NPs measured by ICP/MS.

<table>
<thead>
<tr>
<th></th>
<th>Concentration of Gd in [μg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO-APTES-Gd</td>
<td>105.4</td>
</tr>
<tr>
<td>LNO-APTES</td>
<td>0</td>
</tr>
<tr>
<td>LNO</td>
<td>0</td>
</tr>
</tbody>
</table>

The concentration of gadolinium complex on the NPs was measured by ICP/MS.

The relaxivity per Gd(III)-complex (in mM⁻¹ s⁻¹) of the relaxing agent was calculated using the equation (1).

\[
\frac{1}{r_1} = \frac{1}{T_\|} - \frac{1}{T_{id}} \frac{1}{c}
\]  

(1)

With i=1 the longitudinal relaxation and i=2 the transverse relaxation of water in presence of the paramagnetic relaxing agent, \(T_{id}\) the longitudinal or transverse relaxation of pure deionized water protons (diamagnetic relaxation) and \(c\) the concentration of paramagnetic species in mM.

<table>
<thead>
<tr>
<th></th>
<th>(r_1) (mM⁻¹s⁻¹)</th>
<th>(r_2) (mM⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO-APTES-Gd</td>
<td>5.17</td>
<td>7.06</td>
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</tbody>
</table>

Table 12: Relaxivity per Gd(III)-Complex at 30 MHz.

<table>
<thead>
<tr>
<th></th>
<th>(r_1) (mM⁻¹s⁻¹)</th>
<th>(r_2) (mM⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNO-APTES-Gd</td>
<td>6.35</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 13: Relaxivity per Gd(III)-Complex at 60 MHz.

### 5.9 Biological assays and microscopy

#### 5.9.1 Enzymatic assays

**Inhibition of hrFAP, hrDPP IV and hrPREP**

The human recombinant enzymes were purchased from commercial sources: hrDPP IV and hrPREP (Enzo Life Sciences, Lausen, Switzerland), hrFAP (R&D systems, Abingdon, UK). The enzymatic activities were measured in flat bottom 96-well plates (Costar) containing in each well 0.01 μg of the enzymes and 50 μM of the appropriate substrates: Z-Gly-Pro-AMC for hrFAPα and hrPOP; H-Gly-Pro-AMC for hrDPP IV (both substrates from Bachem, Vionnaz, Switzerland) diluted in their respective assay buffers (50 mM Tris, 1 M NaCl, 1 mg/mL BSA, pH 7.5, for hrFAP; 50 mM Tris, 1 mg/mL BSA, pH 7.5, for hrPREP; 25 mM Tris, 150 mM NaCl, 1 mg/mL BSA, pH 8.0, for hrDPP IV).
Experimental section

Tris and 1 mg/mL BSA, pH 8.0 for hrDPP IV). The enzyme solutions were incubated for 30 min at 37°C with increasing concentrations (5, 10, 20, 50 and 100 nM) of compounds 13 or Biotin-PEG₂⁻FAPi (15). The residual enzymatic activity was determined by measuring fluorescence increase for 60 min at 37°C in a fluorescence multi-well plate reader (λ_{ex}/λ_{em} = 360/460 nm, Synergy HT). Experiments were conducted in triplicate wells and repeated twice. The half maximal inhibitory concentrations (IC₅₀) were graphically determined and the inhibition constants (Kᵢ) were calculated.²²⁷

Evaluation of the association of BFO-PEG-FAPi NPs with hrFAP

Human Fibroblast Activation Protein alpha/FAP Alexa Fluor® 594-conjugated Antibody was purchased from R&D systems (Abingdon, UK). Incubations were carried out in hrFAPα buffer (50 mM Tris, 1 M NaCl, 1 mg/mL BSA, pH 7.5) in 1.5 mL Eppendorf® tubes at 37°C. The following combinations were evaluated in a final volume of 200 µL: 1) anti-FAPα antibody (1:50 dilution), hrFAPα (100 ng) in buffer (control); 2) anti-FAPα antibody (1:50 dilution), BFO-PEG-FAPi NPs (100 µg/mL) in buffer (control); 3) anti-FAPα antibody (1:50 dilution), hrFAPα (100 ng), BFO-PEG NPs (100 µg/mL) in buffer; 4) anti-FAPα antibody (1:50 dilution), hrFAPα (100 ng), BFO-PEG-FAPi NPs (100 µg/mL) in buffer. The Eppendorf® tubes containing the mixtures were incubated under gentle shaking for 2 h. The mixtures were centrifuged (5 min, 10 000 rpm). The supernatants were collected and transferred to a flat bottom 96-well plate. The intensity of the fluorescent emission of the labelled anti-FAPα antibody was measured on a fluorescence multi-well plate reader (λ_{ex}/λ_{em} = 590/645 nm, Synergy HT). Experiments were conducted in five replicates. Results were analyzed using a student’s t-test (all comparisons *** p<0.001).

Imaging of the association of BFO-PEG-FAPi NPs with hrFAP

Target-specific association of BFO-PEG-FAPi NPs to FAP was assessed by multiphoton microscopy. hrFAPα (100 ng) was incubated at 37 °C for 2 h with BFO-PEG-FAPi NPs (100 g/mL) or BFO-PEG NPs (100 g/mL) in the presence of Human Fibroblast Activation Protein alpha/FAP AlexaFluor®594-conjugated Antibody (dilution 1:5). Multiphoton imaging of the samples was performed on a Nikon multiphoton inverted microscope (A1R-MP) coupled with a Mai-Tai tunable Ti: Sapphire oscillator from Newport-Spectra-Physics (100 fs, 80 MHz, 700 – 1000 nm). A Plan APO 20 × WI N.A. 0.75 objective was used to focus the excitation 840 nm and to epi-collect second harmonic and fluorescence. The collected signals were processed by a Nikon A1 descanned grating-spectrometer equipped with an array of 32-photomultipliers. The detection range used here was 410 nm to 650 nm with 6 nm step-size. Four regions (each region: 635 x 635 µm) of both samples were analyzed to calculate the ratio of the area of structures emitting in both SH and fluorescence channels to the area of SH emitting structures.
5.9.2 **Evaluation of cytocompatibility on MucilAir™-HF tissue samples**

Airway cells were obtained from patients undergoing surgical polypectomy. All experimental procedures were explained in full, and all subjects provided informed consent. The study was conducted according to the declaration of Helsinki on biomedical research (Hong Kong amendment, 1989), and received approval from local ethics commission. Airway epithelia co-cultured with fibroblasts were isolated from a mixture of human airway cells from 14 different donors (MucilAir™-Pool-HF) and maintained at the air–liquid interface (ALI) in MucilAir™ culture medium (EP04MM), ready-to-use, chemically defined, serum-free (Epithelix Sàrl, Geneva, Switzerland), in 24-well plates with 6.5-mm Transwell® inserts (cat #3470, Corning Incorporated, Tweksbury, USA). **Biotin-PEG₃-FAPi** was first solubilized in DMSO and diluted to reach targeted concentrations (i.e. 10 µM and 100 µM) in MucilAir™ culture medium (EP04MM) with a fixed 1% DMSO for each dilution.

**Trans-epithelial electrical resistance (TEER) measurement**

After addition of 200 µl of MucilAir™ culture medium to the apical compartment of the tissue cultures, resistance was measured across cultures with an EVOMX volt-ohm-meter (World Precision Instruments, Sarasota, US). Resistance values (Ω) were converted to TEER (Ω.cm²) by using the following formula: TEER (Ω.cm²) = (resistance value (Ω) - 100(Ω)) x 0.33 (cm²), where 100 Ω is the resistance of the membrane and 0.33 cm² is the total surface of the epithelium.

**Cytotoxicity measurement**

For the lactate dehydrogenase assay, 100 µl from the basolateral medium was incubated with the reaction mixture of the Cytotoxicity Detection KitPLUS, following manufacturer’s instructions (Sigma, Roche; ST Louis, USA). To determine the percentage of cytotoxicity, the following equation was used (A = absorbance values): Cytotoxicity (%) = (A (exp value)-A (low control))/A (high control)-A (low control))*100. The high control value corresponds to a 10 % Triton X-100 treatment applied to the culture for 24 hours.

**Cilia Beat Frequency measurement**

Cilia beating frequency set-up system consists of three parts: a Sony XCD V60 camera connected to an Olympus BX51 microscope and PCI card. The cilia beating frequency is expressed as Hz. 256 Images were captured at high frequency rate (125 frames per second) at room temperature, cilia beating frequency was then calculated using CiliaX software. It should be pointed out that CBF values may be subject to fluctuations due to parameters such as temperature, mucus viscosity or liquid applied on the apical surface of MucilAir™-HF.
5.9.3 Imaging of HNPs in cells

General procedures for imaging HNPs in EGFR+/- cells

BFO functionalized NPs

A suspension of functionalized BiFeO₃ in ethanol (2 mg/ml) was US for 15min. An aliquot of the NPs suspension was collected and centrifuged (4700 rpm, 10min). The supernatant was discarded and the NPs were resuspended in cellular media to obtain a concentration of 50/100 μg/ml. The suspension was sonicated for 30 min and incubated with culture of EGFR-positive/negative (HCC and A549, respectively) cells expressing RFP at 37 °C for 24 h. The cells were washed with PBS (1x), incubated with PBS with orbital agitation for 10min (2 X) and fixed with 4 % formaldehyde in PBS. The experiment was performed in triplicate.

LNO-APTES-Erlotinib NPs

A suspension of functionalized LiNbO₃ in ethanol (2 mg/ml) was US for 15min. An aliquot of the NPs suspension was collected and centrifuged (4700 rpm, 10min). The supernatant was discarded and the NPs were resuspended in cellular media to obtain a concentration of 10 μg/ml. The suspension was sonicated for 30 min and incubated with culture of EGFR-positive/negative cells (NCI-H596 and A549, respectively) at 37 °C for 24 h. The cells were washed with PBS (1x), incubated with PBS with orbital agitation for 10min (2x), stained Nile Red and fixed. The experiment was performed in duplicate.

Fluorescent staining of cell membranes

Cell membranes were stained with the fluorescent probe Nile Red. Cell layers were treated with 0.1 μg/mL of the probe in PBS (1 mg/mL stock solution in DMSO) for 5 min. Then, the cell layers were washed with PBS and maintained in PBS at 4°C until the acquisition of images.

Cell imaging

Multiphoton imaging of the samples was performed on a Nikon multiphoton inverted microscope (A1R-MP) coupled with a Mai-Tai tunable Ti: Sapphire oscillator from Newport-Spectra-Physics (100 fs, 80 MHz, 700 – 1000 nm). A Plan APO 20 × WI N.A. 0.75 objective was used to focus the excitation 810 nm and to epi-collect second harmonic and fluorescence. The collected signals were processed by a Nikon A1 descanned grating-spectrometer equipped with an array of 32-photomultipliers.
6 References


References


References


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References


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References


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EDUCATION

PhD study in organic chemistry and nanoparticles functionalization
École Polytechnique Fédérale de Lausanne (EPFL), Switzerland 2015-2019

Master in Molecular and Biological Chemistry

Bachelor in Chemistry and Chemical Engineering
École Polytechnique Fédérale de Lausanne (EPFL), Switzerland 2010-2013

Matura (High School Diploma), Option Biology and Chemistry
Gymnase de Chamblandes (VD), Switzerland 2007-2010

PROFESSIONAL EXPERIENCE

PhD Student in the Group for Functionalized Biomaterials (EPFL; Gerber S.)
December 2015-November 2019
• Design and synthesis of bioactive molecules in collaboration with biologists and physicists.
• Functionalization of nanoparticles; characterization of nanosystems for cancer imaging.
• Practical laboratory assistant for undergraduate chemistry and chemical engineering students.
  Supervision of Bachelor and Masters Projects. Assistant of Bachelor course “Organic functions and reactions I”, “Organic chemistry (for BIO, PHA)” and “Target synthesis of building blocks.”
  (Winner of the awards for best teaching assistant in 2017)

Master Project, Organometallic Chemistry (EPFL; Zhu J.)
September 2014-February 2015
• Design synthetic pathways and apply them to new promising natural products.

Internship, Biochemistry (EPFL; Zhu J.)
August 2014- August 2014
• Development of novel imaging tools to study mitochondria membrane potential.

COMPUTER PROGRAMS

Chemistry: ChemDraw, MestRenova, topspin, ImageJ
Office: Suite MS-Office

LANGUAGES

French Mother tongue
English Fluent
German Basic
Portuguese Fluent

Conferences

**Swiss Summer School 2016 in Chemical Biology**, Villars, Switzerland, August 21-25, 2016. *Functionalization of second harmonic nanoparticles for targeted tumor imaging and multimodal cancer diagnosis.*

*Poster*

**SCS (swiss chemical society) Fall Meeting**, Zurich, Switzerland, September 15, 2016. *Functionalization of second harmonic nanoparticles for targeted tumor imaging and multimodal cancer diagnosis.*

*Poster*

**SCS (swiss chemical society) Fall Meeting**, Bern, Switzerland, August 21-22, 2017. *Functionalization of harmonic nanoparticles for targeted tumor imaging and multimodal cancer diagnosis.*

*Poster* [Award for Best poster]


*Poster*

**SCS (swiss chemical society) Fall Meeting**, Lausanne, Switzerland, September 7, 2018. *Multifunctional harmonic nanoparticles targeting the microenvironment of lung cancer tumor for multimodal imaging and diagnosis.*

*Poster*


*Poster*


*Poster*
LITERATURE CONTRIBUTION

Gd³⁺-Functionalized Harmonic Nanoparticles for Multiphoton and Magnetic Resonance Multimodal Imaging.


Photocontrolled release of the anticancer drug chlorambucil with caged harmonic nanoparticles.


Inhibitor-Conjugated Harmonic Nanoparticles Targeting Fibroblast Activation Protein.

Raphaël De Matos, Jérémy Vuilleumier, Christophe Mas, Samuel Constant, Davide Staedler and Sandrine Gerber-Lemaire. RSC Advances, 2019, 9, 31659-31669.

Two-photon triggered photorelease of caged compounds from multifunctional harmonic nanoparticles.