Supporting Information

Site-specific Polymer Attachment to HR2 Peptide Fusion Inhibitors against HIV-1 Decreases Binding Association Rates and Dissociation Rates rather than Binding Affinity

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Propagation of errors calculation

The propagation of error for $k_{off}$ was calculated as follows:

$$
\sigma_{k_{off}} = k_{off} \sqrt{\left( \frac{\sigma_{k_{off}}}{K_D} \right)^2 + \left( \frac{\sigma_{k_{on}}}{k_{on}} \right)^2}
$$

(S1)

MALDI-TOF mass spectrometry

MALDI-TOF MS was carried out on a Shimadzu Axima-CFR™ plus MALDI-TOF mass spectrometer operating in the linear mode in the 500 to 14,000 m/z range. For the characterization of the mPEG-OH and mPEG-acrylates, saturated dithranol matrix in dichloromethane (DCM) with 30 wt % sodium trifluoroacetate (NaTFA), was used. NaTFA-matrix solution was mixed in a 1 : 1 volume ratio with the mPEG-OH or mPEG-acrylate (1 mg·mL⁻¹) dissolved in DCM from which 1 µL was applied to the plate. For analysis of the peptides and the mPEG-peptide conjugates, a 10 mg·mL⁻¹ matrix solution of α-cyano hydroxycinnamic acid matrix dissolved in THF was used. The matrix solution was mixed in a 1 : 1 volume ratio with the mPEG-peptide conjugates dissolved in MilliQ water (1 mg/mL). The samples were placed on the MALDI-TOF plate and allowed to dry. Calibration was achieved using several references: Angiotensin II ([M+H⁺] = 1045.54), adrenocorticotropin hormone_{18-39} (ACTH_{18-39}) peptide fragment ([M+H⁺] = 2464.20) and bovine insulin ([M+H⁺] = 5729.61). Measurements were performed with a laser power of 45-65 (max. laser power: 180).

Gel permeation chromatography (GPC)

All GPC analyses were performed on a Viscotek triple detector array Model 300 equipped with a MetaChem degasser, Viscotek VE 1121 GPC solvent pump, VE 5200 GPC autosampler and Shodex-OH pak 804 and 805 columns. GPC of PEG750, PEG2000, mid-functionalized poly(ethylene glycol)-co-(glycerol) and hyperbranched-poly(glycerol) was performed at 25 °C in 9 : 1 volume ratio of 0.1 M potassium phosphate buffer pH 6.6 and methanol, respectively. The chromatograms were analyzed according to a conventional
calibration against PEG standards. Table S1 presents the molecular weight characteristics obtained via GPC. GPC elugrams are shown in Figure S4.

**NMR spectrometry**

All NMR spectra were recorded using either a Bruker AC 300 (300 MHz) or a Bruker AMX 400 (400 MHz) instrument. The spectra were referenced internally to the residual proton signals of the deuterated solvent (DMSO-$d_6$ or CDCl$_3$).
Table S1. Molecular weight characteristics of the acrylate- and maleimide- end group modified polymers used in this study as determined by $^1$H-NMR and gel permeation chromatography against PEG standards.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$M_n^a$ (g mol$^{-1}$)</th>
<th>$M_w^b$ (g mol$^{-1}$)</th>
<th>$M_n^b$ (g mol$^{-1}$)</th>
<th>$M_w/M_n^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-750-acrylate, 1</td>
<td>790</td>
<td>720</td>
<td>700</td>
<td>1.03</td>
</tr>
<tr>
<td>PEG-2000-acrylate, 2</td>
<td>1960</td>
<td>1900</td>
<td>1800</td>
<td>1.04</td>
</tr>
<tr>
<td>mid-P(EG-co-G)-maleimide, 3</td>
<td>1780</td>
<td>2000</td>
<td>1700</td>
<td>1.16</td>
</tr>
<tr>
<td>hbPG-maleimide, 4</td>
<td>2050</td>
<td>3000</td>
<td>2200</td>
<td>1.36</td>
</tr>
</tbody>
</table>

$a$ Number-average molecular weight determined by comparison of the acrylate or maleimide end-groups versus the polymer backbone in $^1$H-NMR.

$b$ Molecular weight of the acrylate- and maleimide modified polymers determined by gel permeation chromatography against PEG standards using an eluent consisting of 90 vol. % 0.1 M potassium phosphate buffer, pH 6.6 + 10 vol. % methanol.
**Scheme S1.** Synthesis of maleimido functionalized hyperbranched poly(glycerol).
Scheme S2. Synthesis of maleimido functionalized mid-functional poly(ethylene glycol-co-glycerol).
Figure S1. $^1$H-NMR spectrum of maleimido-modified hyperbranched-poly(glycerol) 4.
Figure S2. $^1$H-NMR spectrum of maleimido-modified mid-functional P(EG-co-G) copolymer, 3.
Figure S3. $^1$H-NMR spectra of (a) PEG750-acrylate (1) and (b) PEG2000-acrylate 2.
**Figure S4.** Gel permeation chromatograms of mPEG750 acrylate (1), mPEG2000 acrylate (2), mid-P(EG-co-G)-maleimide (3) and hyperbranched PG (4). The chromatograms are cut-off at 19.6 min due to the presence of the solvent peak which produces a large negative signal.
Figure S5. Analytical reverse phase HPLC chromatograms of the (a) unmodified C41 peptide, (b) N-terminal cysteine modified peptide, (c) S15C peptide, (d) C-terminal cysteine modified peptide and (e) S20C peptide. The insets depict the MALDI-TOF mass spectra obtained for each sample.
Figure S6. Analytical reverse phase HPLC chromatograms of (a) N-terminal-hbPG, (b) N-terminal-mid-functional P(EG-co-G), (c) N-terminal-PEG750 and (d) N-terminal-PEG2000 conjugates. The insets depict the MALDI-TOF mass spectra obtained for each sample.
Figure S7. Analytical reverse phase HPLC chromatograms of (a) S15C-hbPG, (b) S15C-mid-functional P(EG-co-G), (c) S15C-PEG750 and (d) S15C-PEG2000 conjugates. The insets depict the MALDI-TOF mass spectra obtained for each sample.
Figure S8. Analytical reverse phase HPLC chromatograms of (a) C-terminal-hbPG, (b) C-terminal-mid-functional P(EG-co-G), (c) C-terminal-PEG750 (d) and C-terminal-PEG2000 conjugates. The insets depict the MALDI-TOF mass spectra obtained for each sample.
Figure S9. HIV-1\textsubscript{HXB2} infectivity inhibition of the PEGylated S20C conjugates (a) and the S20C – mid-functional P(EG-co-G) and S20C – hyperbranched polyglycerol (b) with HOS CCR5 cells. The error bars indicate standard error of mean (SEM) of 3 or more independent experiments, each performed in duplicate.
**Figure S10.** Illustration of the kinetics exclusion assay (KinExA) using fluorescein modified 5-Helix (5H-f) and C37 modified azlactone beads. (a) Through pre-equilibration of the query peptide or peptide – polymer conjugate with the 500 pM fluorescein modified 5-Helix (5H-f) and subsequent injection of a titration series of these peptide – 5H-f mixtures over C37 modified azlactone beads, the fraction free 5H-f can be derived. (b) Example of the KinExA read out obtained with a titration series of C-terminal PEG2000 with 500 pM 5H-f with I and W indicating injection and buffer wash, respectively. (c) Example of the corresponding KinExA binding curve obtained from the titration of C-terminal PEG2000 conjugate with 500 pM 5H-f over C37 modified azlactone beads. The titration data is fitted using a bimolecular equilibrium binding model (red line).
Figure S11. Poor correlation between affinity $K_D$ and IC$_{50}$. 
Figure S12. Comparison of the kinetic rates of association, $k_{on}$ of HR2-derived C41 and peptide – polymer conjugates with fluorescein modified 5-Helix. The error bars indicate standard error of mean (SEM) of 3 or more independent experiments, each performed in duplicate.
Figure S13. Comparison of the kinetic rates of dissociation, $k_{\text{off}}$ of HR2-derived C41 and peptide – polymer conjugates with fluorescein modified 5-Helix. The error bars indicate standard error of mean (SEM) of 3 or more independent experiments, each performed in duplicate.
**Figure S14.** Affinity of non-PEGylated peptide, C41 towards fluorescein modified 5-Helix (5H-f).
Figure S15. Affinity of (a) C-terminal-PEG750, (b) C-terminal-PEG2000, (c) C-terminal-mid-functional P(EG-co-G), (d) C-terminal-hbPG towards fluorescein modified 5-Helix (5H-f).
**Figure S16.** Affinity of (a) S15C-PEG750, (b) S15C-PEG2000, (c) S15C-mid-functional P(EG-co-G), (d) S15C-hbPG towards fluorescein modified 5-Helix (5H-f).
Figure S17. Affinity of (a) N-terminal-PEG750, (b) N-terminal-PEG2000, (c) N-terminal-mid-functional P(EG-co-G), (d) N-terminal-hbPG towards fluorescein modified 5-Helix (5H-f).