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# Multivalent Display of Quinic Acid Based Ligands for Targeting E-Selectin Expressing Cells

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The site-specific expression of molecular markers on endothelial cells of blood vessels during inflammatory response and angiogenesis provides an opportunity to target drugs and imaging molecules to the vascular endothelium of diseased tissues. This paper describes an innovative strategy for selective delivery of polymer conjugates to E- and P-selectin expressing cells using a series of quinic acid (Qa) based non-carbohydrate analogues of the natural ligand sialyl Lewis$^x$ (sLe$^x$) as targeting moieties. We demonstrate that such analogues antagonize the adhesion of sLe$^x$ expressing HL-60 cells to both E- and P-selectin. Significantly, the apparent avidity of polymer conjugates carrying multiple Qa copies has increased by 3 orders of magnitude relative to their monomeric forms. Furthermore, we found that the major mechanism of copolymer entry and delivery into E-selectin expressing cells is endocytosis. These selectin-targetable copolymers provide the foundation to support controlled delivery of anticancer drugs and imaging agents to tumor vasculature for therapeutic and diagnostic applications.

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## Introduction

The selectins comprise a family of carbohydrate binding proteins (lectins) that are expressed on cytokine-activated endothelial cells (E- and P-selectin), platelets (P-selectin), and leukocytes (L-selectin).[1,2] Like most lectins, selectins bind to a range of glycoconjugates with terminal components that include α2,3-linked sialic acid and α1,3-linked fucose, both characterized by the sialyl Lewis$^x$ (sLe$^x$)[a] determinant (NeuAcα2,3Galβ1,3[Fucα1,3]GlcNAcβ1-R; Figure 1). Studies of selectins in animal models have revealed their importance in inflammation,[3,4] immune responses,[5] and tumor metastasis.[6,7] E and P selectins are not expressed on endothelial cells in vivo unless stimulated by an inflammatory cytokine such as interleukin 1β or tumor necrosis factor α (TNFα), secreted at the sites of inflammation and by tumor cells in cancer patients. This site-specific expression of E- and P-selectin on activated endothelial cells could be exploited for targeting drugs to the tumor vasculature to block the tumor blood flow.[8] Thus, vascular specific carriers, such as liposomes,[9−11] biodegradable polymer microspheres,[12,13] and protein conjugates containing sLe$^x$ or similar carbohydrates that display sLe$^x$ residues have been created for the selective delivery of drugs and genes to sites of chronic inflammation and angiogenic blood vessels in tumors that overexpress E-selectin. Yet, since the selectin−sLe$^x$ interaction is weak ($K_d$ in the millimolar range), such interactions alone could not support firm binding of targeted drug delivery carrier to the endothelium. Furthermore, the carbohydrate-based molecules are prone to suffer from enzyme degradation, possess poor pharmacokinetic, and often rely upon complex synthesis, which limits their use as targeting ligands.

The identification of the essential binding motifs on sLe$^x$ structure has paved the way for the development of small non-carbohydrate mimetics of sLe$^x$.[14−19] Such mimetics are attractive as pharmaceutical leads, since they tend to exhibit better pharmacokinetic profiles and are generally less difficult to synthesize. The search for non-carbohydrate mimetics of sLe$^x$ had led to the identification of quinic acid (Qa) derivatives as a novel sLe$^x$ analogue. The Qa scaffold was utilized to mimic the three hydroxyl groups of fucose on sLe$^x$, while the carboxylic acid on the Qa cyclohexane ring served as an anchoring point to introduce peptides with a free carboxyl terminus as an anionic subsistent to replace sialic acid (inset to Figure 1).[20,21] Nevertheless, such small Qa-based mimetics of sLe$^x$ (Qa ligands) have been shown low affinity for both E- and P-selectin ($IC_{50}$ > 50 mM). This low affinity could probably be due to the inaccurate distance and position of the Qa hydroxyls relative to the carboxylic acid of the terminal amino acid on these compounds.

Since the selectin−ligand interactions are multivalent, analogues presented in multiple copies should possess increased potency to E- and P-selectins, relative to their

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[a] Abbreviations: AIBN, 2,2′-azobis(isobutyronitrile); CRD, carbohydrate recognition domain, DIPEA, diisopropyleethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; FITC, fluorescein 5-isothiocyanate; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HPMA, *N*-(2-hydroxypropyl)methacrylamide; HUVEC, human umbilical vein endothelial cells; IVEC, human immortalized vascular endothelial cells; MA, methacryloyl; MAP, methacryloylaminopropyl; MA-Gly-Gly-OH, methacryloylglycylglycine; MA-Gly-Gly-ONp, methacryloylglycylglycine *p*-nitrophenyl ester; MALDI-TOF MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; $M_w$, weight average molecular weight; NeuAc, *N*-acetylneuraminic acid, sialic acid; ONp, *p*-nitrophenoxy; P, *N*-(2-hydroxypropyl)methacrylamide copolymer backbone; PD, polydispersity, PNp, *p*-nitrophenol; Qa, quinic acid; SEC, size-exclusion chromatography; sLe$^x$, sialyl Lewis$^x$; TFA, trifluoroacetic acid; TNFα, tumor necrosis factor α.


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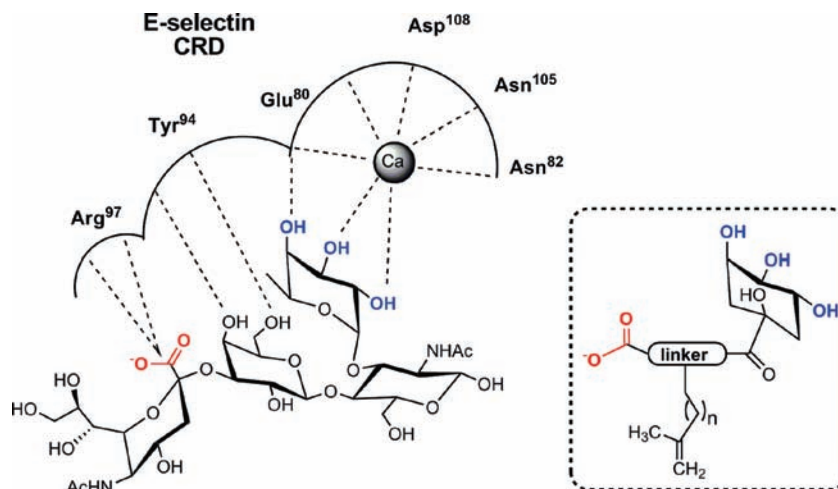


**Figure 1.** Schematic description of sLe$^x$ binding to E-selectin CRD emphasizing the primary intermolecular interactions. Inset: general structure of the Qa ligands that are assayed in this study (linker = variable peptide sequence), which show structural analogy to sLe$^x$.

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monomeric counterparts. We thus hypothesized that a biologically relevant recognition of Qa ligands could be achieved by coupling multiple copies of such analogues (with an appropriate distance between the Qa scaffold and the $CO_2H$ terminus) to synthetic water-soluble polymers. To this end, various types of *N*-(2 hydroxypropyl)methacrylamide (HPMA) copolymers with multivalent display of Qa ligands were synthesized as potential drug delivery and diagnosis system to tumor vascular endothelium. The HPMA copolymer has been previously used as a carrier molecule to modify the in vivo biodistribution of chemotherapeutic agents.[22,23] Even without a specific targeting ligand, HPMA copolymers can accumulate passively through blood vessel leakiness in tumor tissues by a process called "enhanced permeability and retention" (EPR).[24] The EPR effect has been used to deliver a variety of macromolecular bioactive agents to solid tumors,[25] including antiangiogenic drugs.[26] This passive localization can be greatly improved by active mechanism involving receptor−ligand interactions.[22,27] We have previously exploited multivalent carbohydrate ligands to increase the targeting of HPMA−doxorubicin copolymers to human hepatocellular carcinoma and colon-adenocarcinoma cells.[28−30] A multivalent copolymer conjugate of RGD4C peptides has also been shown to increase tumor vascular uptake and reduce extravascular migration in normal organs compared to free RGD4C.[31] This may significantly enhance antitumor effect and decrease normal tissue toxicity relative to a free drug.

In this paper we describe several steps in the construction of HPMA copolymers that take advantage of the selectivity of E- and P-selectins as markers of tumor vascular endothelium. First, we achieved the design, synthesis, and copolymerization of a set of new peptide-Qa entities that can serve as targeting ligands. Then, binding of these ligands to the selectins, either in monomeric form or as HPMA copolymers, was assayed in vitro, and we found that the polymers bind to both receptors with high affinity ($IC_{50}$ values in the micromolar range), about 3 orders of magnitude stronger than the monomers, and with affinity that is comparable to or higher than that of synthetic, commercially available, nonbiocompatible sLe$^x$-bearing polyacrylamide. Finally, the subcellular fate of the fluorescein isothiocyanate (FITC) labeled copolymers was followed as a means to assess targeting of the endothelium of tumor vessels, showing that indeed targeted copolymers were internalized by E-selectin and localized at the lysosomal compartment of human immortalized vascular endothelial cells (IVEC). To the best of our knowledge, this is the first attempt for testing water-soluble copolymers as potential drug carrier for actively targeting E- and P-selectin expressing cells.

## Results and Discussion

Tumor-associated endothelial cells are excellent targets for directed chemotherapy,[32] since they express molecular markers that are distinct from those of normal mature vessels. These markers of endothelial cell adhesion molecules, including ICAM-1, VCAM-1, and E- and P-selectin, are frequently overexpressed in the blood vessels of a variety of human cancers. Targeting the tumor-associated endothelial cells offers a number of critical advantages over direct tumor targeting, namely, (1) accessibility (endothelial cell surface markers are directly accessible from the circulation), (2) stability (endothelial cells present a genomic target with stable surface receptor properties), (3) potency (eliminating a single endothelial cell may translate into the death of hundreds of tumor cells), and (4) applicability (the treatment should be broadly applicable, i.e., independent of tumor type or dedifferentiation).[33]

As discussed in the Introduction, HPMA copolymers equipped with targeting moieties are considered promising entities for chemotherapy, as they show specific active targeting and nonspecific passive accumulation in solid tumors due to the EPR effect.[23] Furthermore, the HPMA polymer backbone may simply mask the antigenic determinants of conjugated ligands and decrease the overall immunogenicity.[34] In fact, HPMA copolymers carrying monoclonal antibodies and Fab′ antibody fragments as targeting moieties have demonstrated previously minimal side effects in both preclinical and clinical settings.[35,36]

As part of our interest to actively target and deliver drug substances to sites of tumor angiogenesis, we have studied HPMA copolymers carrying Qa ligands for targeting E- and P-selectin. The structural and functional characteristics and the effect on the subcellular fate relative to nontargeted copolymers in E-selectin expressing cells were evaluated. Four peptide Qa ligands were synthesized. Each contains a Qa at the N-terminus and free carboxyl groups at the C-terminus, and each is also branched by a short methacryloyl chain suitable for polymerization (Figure 2). The ligands differ in

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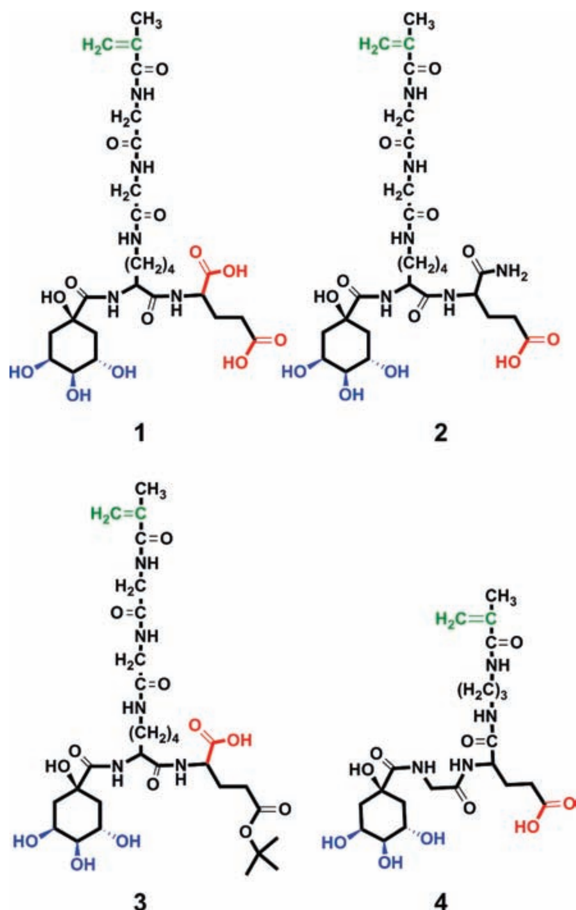

**Figure 2.** Molecular structure of Qa ligands synthesized as sialyl Lewis$^x$ (sLe$^x$) mimetic. For each ligand the functional Qa hydroxyl groups are marked in blue, the free carboxyl groups are in red, and methacryloyl chain suitable for polymerization is in green.

the number of exposed carboxyl groups, either 1 (ligands **2**, **3**, and **4**) or 2 (ligand **1**), by the distances that separate the carboxyl groups from the Qa, either 7 or 9 atoms, and by the length of the methacryloyl chains, forming spacers of either 7 or 12 atoms. The ligands were synthesized on solid phase or by combinations of solid and solution phase syntheses (Figure 3) that afforded the coupling of the all-acetylated quinic acid (Qa(OAc)$_4$) and of the branching methacryloyl linker at Lys or Glu sites. The Qa protecting groups had been removed by saponification either right after the synthesis, when the monomeric form of the ligands were used for the binding assays, or after copolymerization with HPMA (Figure 4), when polymer binding assays were performed. All peptides were purified by preparative HPLC, and their identity and purity were analyzed by analytical HPLC, MS (MALDI-TOF or LCMS), and NMR.

To evaluate the Qa ligands binding to E- and P-selectin, their inhibition of the adhesion of sLe$^x$ expressing HL-60 cells to recombinant soluble protein-coated plates was determined, using *o*-phenylenediamine as a substrate for myeloperoxidases released from lysed cells. The results are summarized in Table 1, where binding of the new ligands to the selectins is also compared to the binding of natural sLe$^x$. The results allowed us to estimate the significance of mounting a specific distance between the Qa cyclohexane ring and the terminal carboxyl groups for the binding affinity. We found that monomeric ligands in which the distance between the Qa and the COOH group was nine atoms in length, compounds **2**

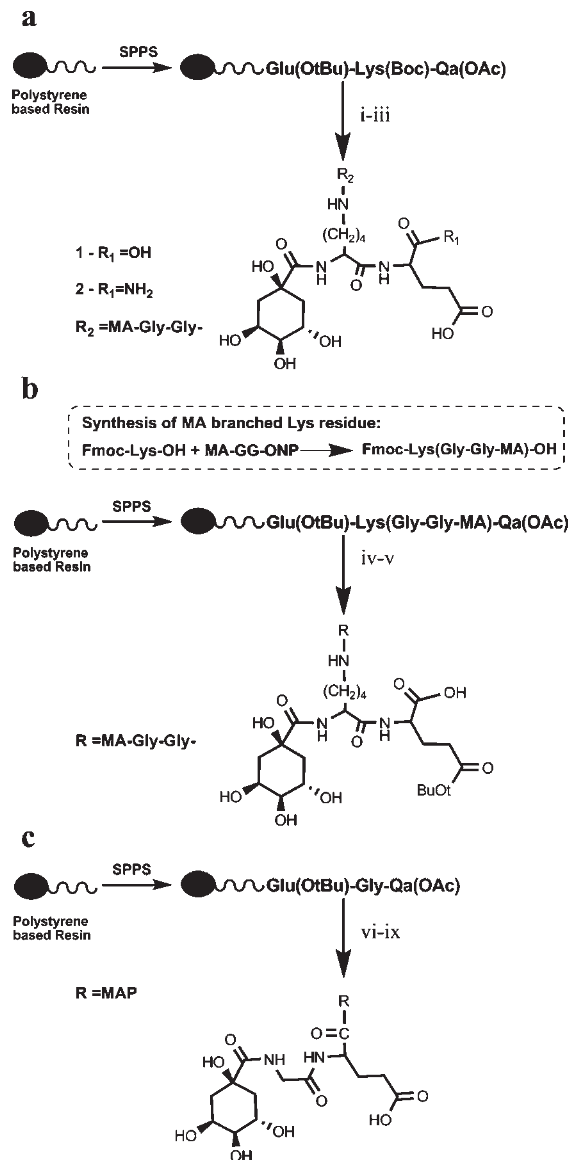


**Figure 3.** Solid phase peptide synthesis (SPPS) of the Qa ligands **1**–**4**. All peptides were prepared using the Fmoc based method and characterized by LCMS and $^1$H NMR. Qa(OAc)$_4$ was attached to the N-terminus in the same way as the amino acids. (a) Synthesis of **1** on 2-Chlorotrityl chloride resin and of **2** on Rink amide MBHA resin: (i) TFA/TIS/$H_2O$ (95:2.5:2.5) 2 h, room temp; (ii) MA-GG-ONP, TEA, DMF 5 h, room temp; (iii) $CH_3ONa$ in $CH_3OH$, 0.5 M, 3 h, room temp, and then Amberlite IR200. (b) Synthesis of **3** on 2-chlorotrityl chloride resin: (iv) AcOH/TFE/DCM (6:2:2), 2 h, room temp; (v) $CH_3ONa$ in $CH_3OH$, 0.5 M, 3 h, room temp, and then Amberlite IR200. (c) Synthesis of **4** on 2-chlorotrityl chloride resin: (vi) AcOH/TFE/DCM (6:2:2), 2 h, room temp; (vii) MAP, Pybop, DIPEA, DMF, 4 h, room temp; (viii) TFA/TIS/$H_2O$ (95:2.5:2.5), 2 h, room temp; (ix) $CH_3ONa$ in $CH_3OH$, 0.5 M, 3 h, room temp, and then Amberlite IR200.

and **4**, demonstrated the greater affinity toward E-selectin, 6 and 10 mM, respectively. This binding affinity is comparable to that of natural sLe$^x$ ligand (1 mM), perhaps because of almost accurate three-dimensional orientation and spacing. The analogue in which the Qa and the carboxyl group were linked by peptide chain of seven atoms (compound **3**), and the analogue terminated with the dicarboxyl functionality of seven and nine atom spacing (**1**) demonstrated low binding toward E-selectin ($IC_{50}$ > 50 mM). The general observed

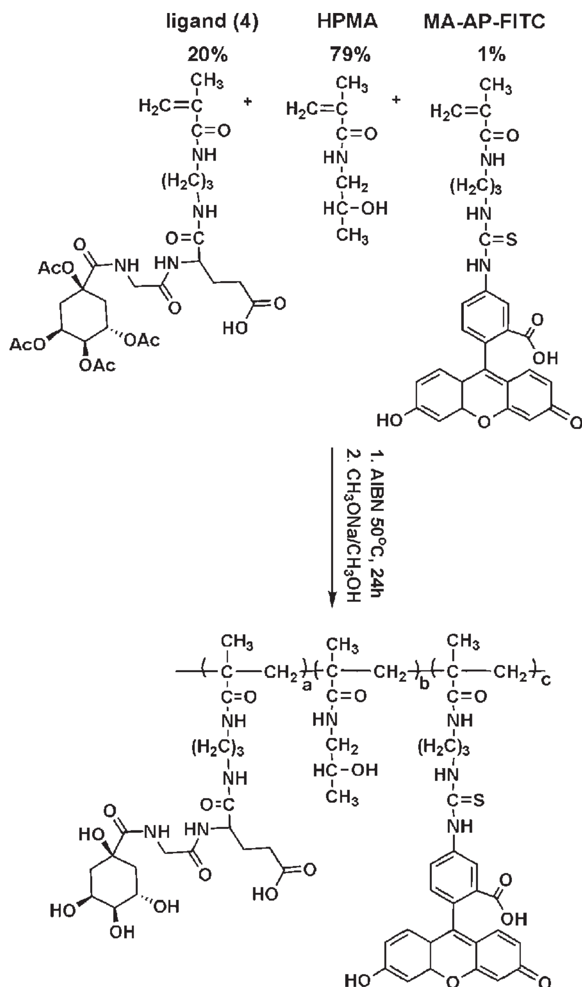


**Figure 4.** Reaction scheme for the synthesis of FITC-labeled HPMA copolymer carrying multiple copies of a Qa ligand (example is shown using compound **4**).

**Table 1.** $IC_{50}$ Values (in mM) for Monomeric Qa Ligands Binding to E- and P-Selectin

| | | | $IC_{50}$ (mM) | |
|---|---|---|---|---|
| ligand | sequence | Qa to carboxyl distance | for E-selectin | for P-selectin |
| $sLe^x$ | NeuAcα2,3Galβ1, 3[Fucα1,3]GlcNAcβ1 | | 1 ± 0.2 | 10 ± 0.4 |
| **1** | Qa-Lys(-Gly-Gly-MA)-Glu−OH | 7, 9 | > 50 | > 50 |
| **2** | Qa-Lys(-Gly-Gly-MA)-Glu-$NH_2$ | 9 | 6 ± 0.3 | 25 ± 0.9 |
| **3** | Qa-Lys(-Gly-Gly-MA)-Glu($O^tBu$)-OH | 7 | > 50 | > 50 |
| **4** | Qa-Gly-Glu-MAP | 9 | 10 ± 0.2 | > 50 |

trend of ligand and analogues affinity to E-selectin was thus $sLe^x$ > compound **2** > compound **4** ≫ compounds **1** and **3**. All analogues and the $sLe^x$ have demonstrated lower binding affinity to P-selectin when compared with E-selectin. Out of the four tested ligands, only compound **2** exhibited comparable inhibition of HL-60 binding to both E- and P-selectin, with $IC_{50}$ values of 6 and 25 mM, respectively.

It has been demonstrated previously in other systems that multivalent display of carbohydrate ligands often results in higher affinity for target proteins.[15,29,37] To investigate relevant aspects of multivalency of the targeting moiety, we tested

**Table 2.** Characteristics of Qa−HPMA Copolymers and Their $IC_{50}$ Values for Targeting E-Selectin

| copolymer | $M_w$ (kDa)[a] | $M_n$ (kDa) | PD | Qa ligand (mol %)[b] | $IC_{50}$ (mg/mL)[c] | equivalent $IC_{50}$ (μM)[d] |
|---|---|---|---|---|---|---|
| $sLe^x$ | | | | | 1.5 ± 0.1 | 1000 |
| PAA-$sLe^x$ | 30 | | | 20 | 0.4 ± 0.08 | 50 |
| **P-1**[e] | 56 | 40 | 1.42 | 25 | 1.8 ± 0.1 | 273 |
| **P-2** | 30 | 22.3 | 1.29 | 15 | 0.1 ± 0.07 | 20 |
| **P-3** | 37 | 22.1 | 1.61 | 20 | 4.3 ± 0.4 | 1600 |
| **P-4** | 48 | 26.4 | 1.81 | 15 | 0.5 ± 0.1 | 58 |
| P-Gly-Gly-OH | 28 | 22 | 1.40 | | > 5 | > 2000 |

[a] Determined by SEC. [b] Determined by $^1H$ NMR in $D_2O$. [c] Each point represents the mean ± SD of duplicates. [d] Calculated using the formula [(ligand content in mol %)($IC_{50}$)]/$M_w$. [e] P designates the HPMA copolymer backbone.

**Table 3.** Characteristics of Qa−HPMA Copolymers and Their $IC_{50}$ Values for Targeting P-Selectin

| copolymer | $M_w$ (kDa)[a] | $M_n$ (kDa) | PD | Qa ligand (mol %)[b] | $IC_{50}$ (mg/mL)[c] | equivalent $IC_{50}$ (μM)[d] |
|---|---|---|---|---|---|---|
| PAA-$sLe^x$ | | | | | > 5 | |
| **P-1**[e] | 36 | 23 | 1.56 | 7 | 2.3 ± 0.8 | 162 |
| **P-2** | 36 | 23 | 1.56 | 9 | 1.3 ± 0.3 | 95 |
| **P-3** | 19 | 10 | 1.9 | 16 | > 5 | > 2000 |
| **P-4** | 24 | 15 | 1.6 | 22 | 1.0 ± 0.2 | 23 |
| P-Gly-Gly-OH | 28 | 22 | 1.4 | | > 5 | > 2000 |

[a] Determined by SEC. [b] Determined by $^1H$ NMR in $D_2O$. [c] Each point represents the mean ± SD of duplicates. [d] Calculated using the formula [(ligand content in mol %)($IC_{50}$)]/$M_w$. [e] P designates the HPMA copolymer backbone.

the affinity of copolymers exhibiting multiple copies of Qa ligands toward E- and P-selectin. The Qa ligands were copolymerized with HPMA, using the radical precipitation method with a feed ratio of 20 to 80 mol %, respectively, to yield the targeted copolymers (Figure 4). The 7- or 12-atom spacer arms between the polymeric backbone and the Qa ligand reduce steric hindrance and retain the peptide analogues accessible for selectin binding after conjugation to the polymer. The estimated weight average molecular weight ($M_w$), molecular weight distribution (polydispersity, PD), and side chain content of copolymers that were used for E- and P-selectin are shown in Tables 2 and 3. The $M_w$ values of the polymers were found by size exclusion chromatography to vary between 30 and 56 kDa, with a polydispersity index ranging from 1.2 to 1.8, similar to other polymeric conjugates reported in the literature.[38,39] The incorporation of Qa ligands varied between 75% and 100% of the feed content. The copolymers thus contained approximately 20−30 analogue units per polymer backbone. To confirm Qa ligand binding to E- and P-selectin after covalent coupling onto the HPMA backbone, binding affinity to human recombinant proteins was tested as described above. The affinity of the copolymers to the selectins is shown in Tables 2 and 3, and we use the data to compare their affinity to that of the respected monomer ligands, natural $sLe^x$, and commercial polyacrylamide bearing $sLe^x$ (PAA-$sLe^x$) and to control polymer with a nonfunctional linker (P-Gly-Gly-OH).

The results for E-selectin binding (Table 2) indicated no loss of Qa ligand binding activity due to conjugation with HPMA. The binding affinity of the copolymers carrying multiple copies of the Qa ligands has increased up to 3 orders of magnitude relative to their monomeric counterparts. In correlation with monomer binding affinity, we found that multivalent displays of Qa ligands separated by nine atoms between the cyclohexane ring of Qa and the Glu carboxyl (**P-2** and **P-4**)

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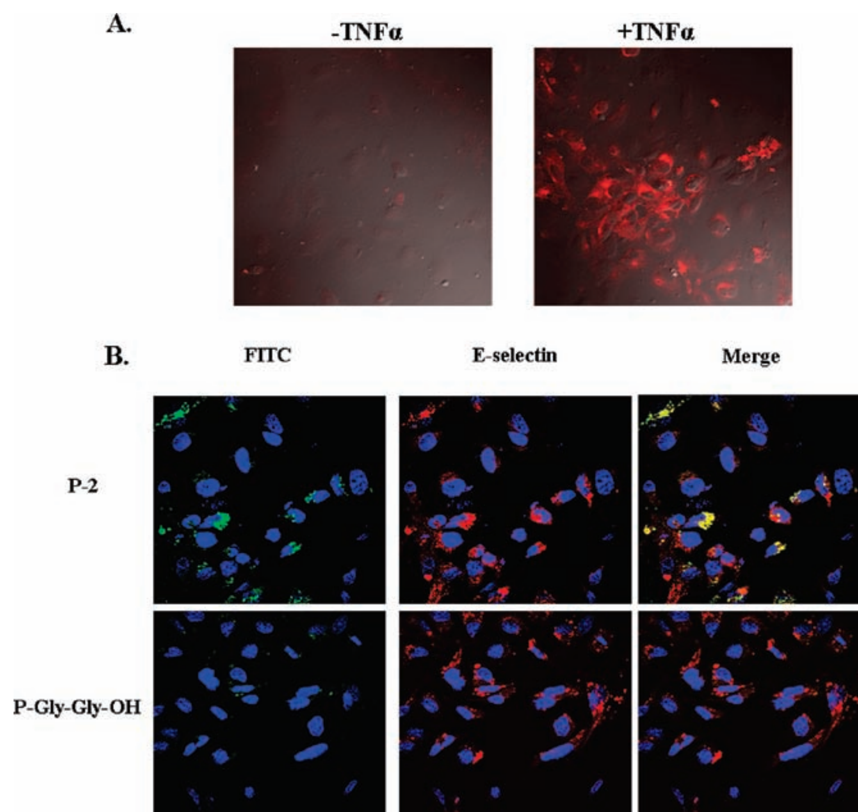


**Figure 5.** (A) Confocal images of E-selectin expression on nonactivated (left) and TNFα-activated (right) IVEC. Cells were treated with 10 ng/mL TNFα for 4 h and then stained with antihuman E-selectin antibody in red (CD62E, R&D Systems). (B) Colocalization images of E-selectin antibody with targeted (**P-2**) and nontargeted (P-Gly-Gly-OH) copolymers (100 $\mu$g/mL) on TNFα activated IVEC after 15 h of incubation at 37 °C: green, fluorescence of FITC; red, fluorescence of Cy3-labeled secondary antibody; blue, DAPI staining.

are more effective inhibitors than polymers carrying analogues with seven atom distance (**P-1** and **P-3**). Polymers **P-2** and **P-4** bind 20–50 times more tightly to E-selectin than sLe$^x$. The commercially available PAA-sLe$^x$ copolymer carrying 20 mol % of sLe$^x$ inhibited E-selectin binding with IC$_{50}$ of 50 $\mu$M. Notably, our results show that the new copolymers may improve binding affinity to immobilized E-selectin even relative to PAA-sLe$^x$; **P-2** was about 2.5-fold better inhibitor to E-selectin than PAA-sLe$^x$, and **P-4** had similar affinity. No inhibition of cell adhesion was caused by the control HPMA copolymer carrying 20 mol % of a nonspecific dipeptide (glycyl–glycine) attached to the polymeric backbone (P-Gly-Gly-OH).

For P-selectin binding (Table 3), three out of the four copolymers, **P-1**, **P-2**, and **P-4**, have shown improved affinity relative to the binding of their respected monomeric ligands. Generally speaking, these three copolymers have similar affinity for binding E- and P-selectin. No inhibition of cell adhesion was caused by the PAA-sLe$^x$ copolymer in our system. Interestingly, copolymer **P-4** had shown increased binding, despite the low affinity exhibited by the monomeric ligand **4**. Furthermore, its affinity to P-selectin was about 4-fold greater than that of **P-2** and higher than its affinity to E-selectin. The higher affinity of **P-4** for P-selectin can be due to the small differences in the structure of P-selectin CRD.[40,41] In P-selectin, Arg$^{97}$ is substituted for serine, eliminating an ion–ion interaction with the sialic acid of sLe$^x$. The superior interactions of sialic acid with E-selectin CRD may explain the 10-fold greater affinity of sLe$^x$ for E-selectin over P-selectin, reported here. This is in agreement with previous literature reports.[15,20] As expected, no inhibition of cell adhesion was caused by the control P-Gly-Gly-OH.

Most of the work reported to date focused on the incorporation of sLe$^x$, or related naturally occurring carbohydrates, into polymeric displays. It is important to note that the polymer–carbohydrate conjugates are generally difficult to synthesize and are susceptible to glycosidic cleavage by glycosidases. The resulting glycoconjugates generally exhibit moderate binding to E-selectin with IC$_{50}$ values in the micromolar range.[42] The inhibitory activity of PAA-sLe$^x$ in the E- and P-selectin assay described here is in line with earlier reports.[15,20] Another aspect that should be noted is that the synthetic PAA-glycopolymers generally have several problems as drug delivery systems for in vivo use, such as low solubility, significant cytotoxicity,[43] and immunogenicity.[44]

The nontoxic and nonimmunogenic HPMA copolymers possess clear advantages for their use as drug carriers. Our results show that the HPMA polymer carrying Qa ligand (**P-2**) binds 50 times more strongly to E-selectin than sLe$^x$. This is significant, although the enhancement is less remarkable than observed in other instances of the cluster effect.[45–47] The relatively low $M_w$ of our copolymers (30–50 kDa) might explain these results.

A detailed study of the subcellular trafficking of newly designed copolymers is an important part of polymeric drug evaluation. The use of a water-soluble HPMA copolymer as a platform for drug targeting limits cellular uptake to the endocytic route. Polymers lacking targeting ligands are usually internalized slowly as a solute by fluid-phase pinocytosis. The conjugation of an adequate targeting ligand may further promote enhanced uptake by receptor-mediated endocytosis.[48]

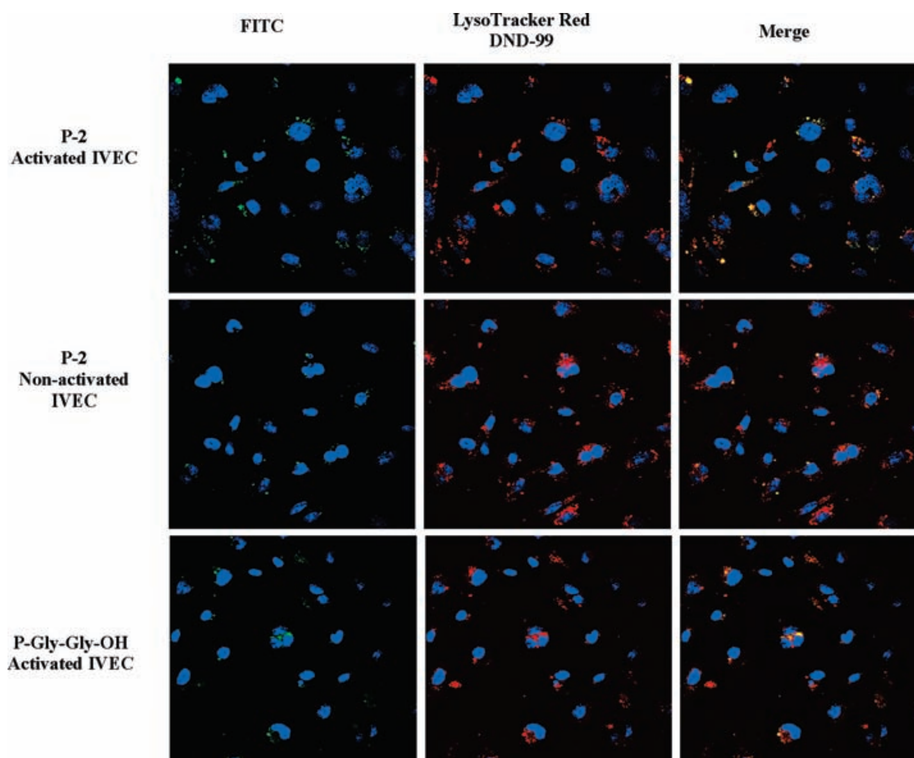


**Figure 6.** Confocal images of activated (+TNFα, first and third rows) and nonactivated (−TNFα, second row) IVEC after 15 h of incubation at 37 °C with 100 μg/mL of the nontargeted (P-Gly-Gly-OH) and targeted (**P-2**) copolymers: green, fluorescence of FITC; red, fluorescence of Lysotracker red DND-99; blue, DAPI staining.

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To learn about fate of HPMA polymer carrying Qa ligand in cells, a stable immortalized vascular endothelial cell line was selected as a model for human E-selectin expressing endothelial cells. IVEC were obtained by transfection of human umbilical vein endothelial cells (HUVEC) and retain many normal differentiated endothelial characteristics.[49] When it becomes activated by TNFα, induction of intracellular adhesion molecules (including E-selectin) takes place, as was reported and as illustrated using our data in Figure 5A. This induction in E-selectin expression by TNF-α is, however, lower by 2-fold on IVEC relative to HUVEC. Here, we compare the binding of the targeted (Qa-based) and nontargeted copolymer to TNFα activated and nonactivated cells, namely, probing the possible interaction with cells possessing tumor and normal endothelial characteristic, respectively. IVEC were cultured in a medium containing the fluorescently labeled targetable (**P-2**) and nontargetable (P-Gly-Gly−OH) copolymers for 15 h before confocal images were taken. The intrinsic fluorescence of FITC attached to HPMA copolymers permitted us to follow the subcellular fate of the copolymers. A nondegradable (aminopropyl, AP) spacer was used to link the FITC to the polymeric backbone so that the images observed correspond to the localization of the copolymers. Our results demonstrate that the fluorescently labeled **P-2** was actively internalized by the E-selectin expressing endothelial cells (IVEC, Figure 5B). Confocal colocalization images of E-selectin antibody with **P-2** copolymers confirmed that the polymer and E-selectin are localized to the same intracellular compartment. A clear enhancement in the rate of **P-2** internalization relative to the nontargeted P-Gly-Gly-OH was observed (Figure 5 and Figure 6, left).

The purpose of binding a drug to a polymeric carrier is to confer its property of endosomal or lysosomal accumulation to facilitate drug release. The colocalization of the lysosomal marker (Lysotracker red) and of fluorescently labeled **P-2** (Figure 6, right) indicated that Qa ligand promotes enhanced uptake and lysosomal trafficking of the copolymers relative to nontargeted controls. We thus conclude that Qa facilitates E-selectin-mediated endocytosis of the delivery system. This lysosomotropism may initiate the release of drugs introducing degradable polymer−drug linkages (e.g., GFLG or pH-sensitivity linkage) and thus be transported via the cytoplasm into the cell nuclei.

## Conclusions

Our studies demonstrate that specific targeting of Qa ligands to E-selectin using a polymeric platform is feasible. The flexible design of the HPMA copolymers allows multivalent display of Qa ligands to bind selectins with high affinity and facilitates specific subcellular (lysosomal) localization. As discussed above, our system includes many of the appealing characteristics of targeted delivery systems, such as biocompatibility, nonimmunogenicity, easy synthesis, and low costs.

The outcome of this paper shows that it is possible to use polymers with multivalent display of Qa ligands to target E- and P-selectin expressing cells. We believe that this report provides a foundation that should support targeted delivery of chemotherapeutics and imaging agents to tumor vasculature for diagnostic and therapeutic applications. HPMA−doxorubicin copolymers carrying Qa ligands as targeting agent to selectins are currently being synthesized and will be tested for their in vitro and in vivo cytotoxicity toward activated human vascular endothelial cells and in a prostate cancer mouse model.

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## Experimental Section

**1. Synthesis. General. Materials.** Unless otherwise mentioned, all chemicals were of reagent grade and obtained from Sigma-Aldrich (Rehovot, Israel). Fmoc protected amino acids and resins were purchased from Novabiochem and used without further purification. HBTU was purchased from GL Biochem (Shanghai, China). HOBT was purchased from Luxembourg industries. MAP was purchased from Poly-Sciences (Warrington, PA). FITC was purchased from Fluka. Quinic acid, *p*-nitrophenol, and *o*-phenylenediamine were purchased from Acros Organics. Endothelial Cell Growth Medium 2 Kit was purchased from PromoCell. LysoTracker Red DND-99 was purchased from Molecular Probes (Leiden, The Netherlands). E- and P-selectin and sLe$^x$ were purchased from Calbiochem (EMD Biosciences, Darmstadt, Germany). Human TNFα was purchased from Peprotech Asia (Rehovot, Israel).

**Cell Line.** HL-60 (kindly provided by Prof Yoav Sharoni, Ben Gurion University, Beer-Sheva, Israel) was grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and penicillin/streptomycin (100 U/mL, 100 $\mu$g/mL) (all from Biological Industries, Kibbutz Beit-Haemek, Israel).

The human immortalized vascular endothelial cells, IVEC,[49] were cultured on gelatin precoated dishes in endothelial cell growth medium (PromoCell) supplemented with 2% fetal calf serum (PromoCell), growth factors (0.5 ng/mL vascular endothelial growth factor, 5 ng/mL epidermal growth factor, 0.2 $\mu$g/mL hydrocortisone, 10 ng/mL basic fibroblast factor, 20 ng/mL R3 IGF-1; all from PromoCell), 1 $\mu$g/mL ascorbic acid (PromoCell), 22.5 mg/mL heparin (PromoCell), and penicillin/streptomycin (100 U/mL, 100 $\mu$g/mL).

**Methods.** All monomer peptides were synthesized using the Fmoc based procedures. Peptide synthesis grade solvents (DMF and $CH_2Cl_2$), coupling reagent (HBTU), and base (DIEA) were used for all syntheses. The peptides were purified by semipreparative HPLC (Thermo-Finnegan), and their identity and purity (>95%) were confirmed by analytical HPLC (Dionex), NMR (Bruker 500 MHz), and LCMS (Thermo).

Copolymerization of the peptide−MA conjugates, HPMA, and MAP-FITC was achieved using radical precipitation. The weight average molecular weights of the copolymers were determined by size exclusion chromatography (SEC) on a fast protein liquid chromatography (FPLC) system (GE Healthcare) equipped with UV and RI (Shodex) detectors. SEC measurements were carried out on Sephacryl 16/60 S-200 column (GE Healthcare) with PBS buffer, pH 7.4, calibrated with fractions of known molecular weight HPMA copolymers.

All-acetylated quinic acid Qa(OAc)$_4$,[20] MA-GG-ONp,[50] HPMA,[51] and MAP-FITC[50] were synthesized as described previously.

**Monomer Synthesis. Qa-Lys(-Gly-Gly-MA)-Glu-OH, 1.** The peptide conjugate Qa(OAc)$_4$-Lys(Boc)-Glu-OH was synthesized on a chlorotrityl chloride resin in 0.1 mmol scale. Qa-(OAc)$_4$ was attached to the N-terminus in the same way as the amino acids. The peptide conjugate was cleaved of the resin using TFA/TIS/$H_2O$ (95:2.5:2.5) during 2 h at room temperature. TFA was removed, and Qa(OAc)$_4$-Lys-Glu-OH was precipitated in cold diethyl ether. The presence of the desired product was confirmed with MALDI-TOF MS ($M^+$ = 617).

Qa(OAc)$_4$-Lys-Glu-OH (50 mg, 0.08 mmol) was conjugated with MA-Gly-Gly-ONp (30 mg, 0.09 mmol) in DMF (3 mL) with TEA (30 $\mu$L) as base. After 3 h at room temperature all the amine was consumed (TLC; ethyl acetate/isopropanol/$H_2O$, 5:3:2). The product Qa(OAc)$_4$-Lys(-Gly-Gly-MA)-Glu-OH (**1**(OAc)$_4$) was precipitated in cold acetone/ether (1:1) mixture, purified by preparative HPLC, and characterized with analytical HPLC, LCMS, and $^1$H NMR. $^1$H NMR (500 MHz, $D_2O$): 1.1 (s, 1H), 1.25 (s, 2H), 1.5 (s, 2H), 1.6 (s, 1H), 1.80 (d, 2H), 1.98 (m, 3H), 2.05 (s, 2H), 2.21 (m, 12H), 2.33 (s, 2H), 3.1 (s, 2H), 3.8 (s, 2H), 4.1 (m, 2H), 4.55 (m, 1H), 5.0 (s, 1H), 5.70 (s, 1H), 5.79 (s, 1H). MS calculated for $M^+$: 799. Found: 799.4 and $(M + Na)^+$ 822. 69% Yield.

**1**(OAc)$_4$ (24 mg) was dissolved in MeOH (0.5 mL) and cooled in an ice bath, and $CH_3ONa$ (0.5 M, 1 mL) was added. The solution was stirred for 3 h at room temperature. The mixture was treated with prewashed (MeOH and then THF) Amberlite IR-120($H^+$) until pH 3 was reached. The solution was filtered, evaporated under reduced pressure, and lyophilized. The product was isolated as a white fluffy hygroscopic solid. $^1$H NMR (500 MHz, $D_2O$): 1.1 (s, 1H), 1.25 (s, 2H), 1.5 (s, 2H), 1.6 (s, 1H), 1.80 (d, 2H), 1.98 (m, 3H), 2.05 (s, 2H), 2.33 (s, 2H), 3.1 (s, 2H), 3.25−3.33 (m, 3H), 3.8 (s, 2H), 4.1 (m, 2H), 4.55 (m, 1H), 5.50 (s, 1H), 5.79 (s, 1H). MS calculated for $M^+$: 630. Found: 629.1. Yield, 86%.

**Qa-Lys(-Gly-Gly-MA)-Glu-NH$_2$, 2.** The protected conjugate peptide **2**(OAc)$_4$ was prepared in the same manner as compound **1**(OAc)$_4$ except that a Rink amide MBHA resin was used to afford amidation of the α-carboxylate of the C-terminus Glu residue. The product was purified by preparative HPLC and characterized with analytical HPLC, LCMS, and $^1$H NMR. $^1$H NMR (500 MHz, $D_2O$): 1.1 (s, 1H), 1.25 (s, 2H), 1.5 (s, 2H), 1.6 (s, 1H), 1.80 (d, 2H), 1.98 (m, 3H), 2.05 (s, 2H), 2.21 (m, 12H), 2.33 (s, 2H), 3.1 (s, 2H), 3.8 (s, 2H), 4.1 (m, 2H), 4.55 (m, 1H), 5.0 (s, 1H), 5.70 (s, 1H), 5.79 (s, 1H). MS calculated for $M^+$: 799. Found: 799.4 and $(M + Na)^+$ 822. Yield, 74%.

**2**(OAc)$_4$ (17 mg) was dissolved in MeOH (0.5 mL) and cooled in an ice bath, and $CH_3ONa$ (0.5 M, 1 mL) was added. The solution was stirred for 3 h at room temperature. The mixture was treated with prewashed (MeOH and then THF) Amberlite IR-120($H^+$) until pH 3 was reached. The solution was filtered, evaporated under reduced pressure, and lyophilized. The product was isolated as a white fluffy hygroscopic solid. $^1$H NMR (500 MHz, $D_2O$): 1.1 (s, 1H), 1.25 (s, 2H), 1.5 (s, 2H), 1.6 (s, 1H), 1.80 (d, 2H), 1.98 (m, 3H), 2.05 (s, 2H), 2.33 (s, 2H), 3.1 (s, 2H), 3.25−3.33 (m, 3H), 3.8 (s, 2H), 4.1 (m, 2H), 4.55 (m, 1H), 5.50 (s, 1H), 5.79 (s, 1H). MS calculated for $M^+$: 630. Found: 629.1. Yield, 90%.

**Qa-Lys(-Gly-Gly-MA)-Glu(O$^t$Bu)-OH, 3.** Fmoc-Lys-OH (250 mg, 0.67 mmol) was coupled to MA-GG-ONp (210 mg, 0.68 mmol) in 50 mL of DMF/DCM (20:80) in the presence of TEA (100 $\mu$L, 0.7 mmol). The reaction product Fmoc-Lys(Gly-Gly-MA)-OH was extracted with hexane, precipitated in water, and recrystallized twice in EtOAc. Its formation was confirmed by MALDI-TOF MS ($M^+$, 550; found, 550.2). Fmoc-Lys(Gly-Gly-MA)-OH was then conjugated to chlorotrityl resin (0.1 mmol) preloaded with O$^t$Bu-Glu-NH$_2$, followed by the addition of Qa(OAc)$_4$ as the N-terminus residue of the sequence. The peptide conjugate was cleaved of the resin with DCM/AcOH/TFE (6:2:2) for 2 h at room temperature, and the solvent was evaporated to result in a white powder. The product Qa(OAc)$_4$-Lys(-Gly-Gly-MA)-Glu(O$^t$Bu)-OH (**3**(OAc)$_4$) was purified by preparative HPLC and characterized by analytical HPLC, MALDI-TOF MS, and $^1$H NMR. $^1$H NMR (500 MHz, $D_2O$): 1.1 (s, 1H), 1.25 (s, 2H), 1.40 (s, 9H), 1.5 (s, 2H), 1.6 (s, 1H), 1.80 (d, 2H), 1.98 (m, 3H), 2.05 (s, 2H), 2.21 (m, 12H), 2.33 (s, 2H), 3.1 (s, 2H), 3.8 (s, 2H), 4.1 (m, 2H), 4.55 (m, 1H), 5.0 (s, 1H), 5.70 (s, 1H), 5.79 (s, 1H). MS calculated for $M^+$: 855. Found: 855.3.

**3**(OAc)$_4$ (14 mg) was dissolved in MeOH (0.5 mL) and cooled in an ice bath, and $CH_3ONa$ (0.5 M, 1 mL) was added. The solution was stirred for 3 h at room temperature. The mixture was treated with prewashed (MeOH and then THF) Amberlite IR-120($H^+$) until pH 3 was reached. The solution was filtered, evaporated under reduced pressure, and lyophilized. The product was isolated as a white fluffy hygroscopic solid. $^1$H NMR (500 MHz, $D_2O$): 1.1 (s, 1H), 1.25 (s, 2H), 1.4 (s, 12H), 1.5 (s, 2H), 1.6 (s, 1H), 1.80 (d, 2H), 1.98 (m, 3H), 2.05 (s, 2H), 2.33 (s, 2H), 3.1 (s, 2H), 3.25−3.33 (m, 3H), 3.8 (s, 2H), 4.1 (m, 2H), 4.55 (m, 1H), 5.50 (s, 1H), 5.79 (s, 1H). MS calculated for $M^+$: 687. Found: 687.6. Yield, 50%.

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**Qa-Gly-Glu-MAP, 4.** The peptide conjugate $Qa(OAc)_4$-Gly-Glu($O^tBu$)-OH was synthesized on chlorotrityl chloride resin (0.1 mmol). It was cleaved of the resin with AcOH/TFE/DCM (2:2:6) mixture for 2 h at room temperature, and the mixture was evaporated to give a white powder. The existence of the product in the crude material was verified by MALDI-TOF MS (M, 602; found, 602 and $(M + Na)^+$ 625). $Qa(OAc)_4$-Gly-Glu($O^tBu$)-OH (40 mg, 0.07 mmol) was conjugated with 3-aminopropylmethacrylamide (MAP) (12 mg, 0.072 mmol) in dry DMF (5 mL), using DIC (10 $\mu$L, 0.072 mmol) and DIEA (100 $\mu$L, 0.072 mmol), for 2 h at room temperature. The reaction completion was monitored by TLC (etyl acetate/acetonitrile 4:1, $R_f = 0.79$). The mixture was evaporated under vacuum, and the crude mixture was redissolved in 2 mL of TFA/TIS/$H_2O$ for 30 min to remove the $O^tBu$ protecting group. TFA was removed, and the product was precipitated in cold ether. The desired product $Qa(OAc)_4$-Gly-Glu-MAP (**4**$(OAc)_4$) was purified by HPLC and characterized with analytical HPLC, LCMS, and $^1H$ NMR. $^1H$ NMR (500 MHz, $D_2O$): 1.78 (m, 2H), 1.93 (m, 3H), 2.05 (s, 2H), 2.21 (m, 12H), 2.33 (s, 2H), 2.92 (d, 2H), 3.22 (m, 2H), 4.1 (m, 2H), 5.4 (s, 1H), 5.79 (s, 1H). MS calculated $M^+$: 670. Found: 670 and $(M + Na)^+$ 693. Yield, 53%.

**4**$(OAc)_4$ (22 mg) was dissolved in MeOH (0.5 mL) and cooled in an ice bath, and $CH_3ONa$ (1 mL) was added. The solution was stirred for 3 h at room temperature. The mixture was treated with prewashed (MeOH and then THF) Amberlite IR-120(H+) until pH 3 was reached. The solution was filtered, evaporated under reduced pressure, and lyophilized. The product was isolated as a white fluffy hygroscopic solid. $^1H$ NMR (500 MHz, $D_2O$): 1.78 (m, 2H), 1.93 (m, 3H), 2.05 (s, 2H), 2.33 (s, 2H), 2.92 (d, 2H), 3.20−3.22 (m, 3H), 3.35 (m, 1H), 4.1 (m, 2H), 5.4 (s, 1H), 5.79 (s, 1H). Yield, 88%.

**Synthesis and Characterization of Copolymers.** The targeted copolymers were synthesized by copolymerization of the acetylated Qa based ligand monomers with HPMA. Radical precipitation copolymerization was performed in a sealed vial in acetone/DMSO mixture at 50 °C for 24 h using AIBN as the initiator. The feed molar ratio of the monomers was 20%:80% for Qa based ligand and HPMA, respectively. In synthesis of the FITC labeled copolymers the feed ratio of monomers was 20%:79%:1% for the Qa ligand, HPMA, and MAP-FITC, respectively. The concentration of monomers, with respect to initiator and solvent, was at a ratio of 12.5:0.6:86.9 mol %, respectively. After 24 h at 50 °C the solvent was evaporated and polymers were dissolved in methanol and precipitated in cold acetone/ether (1:2). The solvent was discarded and the precipitant dried in a desiccator. After polymerization, the acetyl Qa protecting groups were removed by dissolving in methanol containing a catalytic amount of sodium methoxide. The crude polymer was dissolved in $H_2O$ and purified on Sephadex G-25 (PD-10) column. The weight average molecular weight of the copolymers was determined by SEC on FPLC system with Sephacryl 16/60 S-200 column. The characteristics of the polymers and the methods used for characterization are summarized in Table 1.

**2. Evaluation of E-Selectin Binding with HL-60 Inhibition Assay.** Affinity of monomers and copolymers to E- and P-selectin was evaluated by an inhibition assay of HL-60 ($sLe^x$ expressing) cells binding to E-selectin coated plate, as described previously.[52] Briefly, human recombinant E- or P-selectin (Calbiochem) was coated onto 96-well plates (Costar, 9018 high binding polystyrene) using 50 $\mu$L of a 3 $\mu$g/mL solution (~150 ng per well) of the selectin in PBS buffer overnight at 4 °C. The wells were washed three times with PBS buffer containing 1% of bovine serum albumin and then treated with 200 $\mu$L of the same solution for 1 h to block the uncoated surfaces. The blocking solution was removed, and an amount of 40 $\mu$L of inhibitor solutions (monomers or copolymers), prepared from a 10 mM PBS stock solution diluted in Hanks solution containing 20 mM Hepes (pH 7.4), 0.2% glucose, and 1% BSA, was added, followed immediately by addition of 20 $\mu$L of a $10^5$ HL-60 cell suspension. After 15 min of incubation time, the solution was removed and the wells were washed three times with 200 $\mu$L of Hanks solution, 20 mM Hepes (pH 7.4), 0.2% glucose, 1% BSA, and 1 mM $CaCl_2$. Lysis buffer was then added (50 $\mu$L of citric acid, 24 mM; dibasic sodium phosphate, 51 mM; and 0.1% Nonidet P-40) and the plates were shaken for 5 min. Myeloperoxidase liberated during the lysis process was then detected by addition of *o*-phenylenediamine as a substrate (50 $\mu$L of citric acid, 24 mM; dibasic sodium phosphate, 51 mM; 0.1% *o*-phenylenediamine; and 0.03% hydrogen peroxide). After 1 h, the reaction was stopped by addition of 40 $\mu$L of 4 N sulfuric acid, and the absorbance of the solution was measured at 492 nm. The inhibition percentage was calculated by comparison to the absorbance of a positive control in which only HL-60 was added (no inhibitor). The $IC_{50}$ values are shown in Tables 1−3.

**3. Immunofluorescence Analysis.** IVEC were plated on 0.2% gelatin-coated coverslips and incubated for 24 h. Cells were activated with 10 ng/mL TNF-α for 4 h and rinsed in PBS at room temperature, fixed in 3% paraformaldehyde, and then incubated in 3% BSA for 1 h to block nonspecific binding, followed by incubation for 1 h with antihuman E-selectin antibody (CD26E, R&D systems) ($^1/_{1000}$) in PBS, 1% BSA at room temperature. Coverslips were washed and incubated with Cy3-labeled secondary antibody ($^1/_{100}$ in PBS with 1% BSA) containing DAPI (1:500) for 30 min at room temperature. Finally, the coverslips were rinsed with PBS and mounted in Mowiol-DABCO (Aldrich Chemical Co., Milwaukee, WI; Sigma, St. Louis, MO). The cells were visualized using an Olympus FV1000-IX81 confocal microscope. Images were acquired with a red filter analysis (excitation at 543 nm, emission collected with a 570 nm barrier filter). Autofluorescence background was ascertained using control (untreated) cells.

**4. Colocalization Analysis.** Confocal microscopy was used to evaluate the subcellular fate of the FITC labeled copolymers by human immortalized vascular endothelial cells, IVEC. Lysotracker red DND-99 was selected to visualize lysosomes. Cells ($3 \times 10^4$) were seeded onto gelatin coated coverslips in a 24-well plate with 500 $\mu$L of endothelial cells culture medium and incubated for 24 h. The cells were then activated with TNFα in medium for 4 h to induce surface expression of E-selectin. Control cells were left untreated. Subsequently, 100 $\mu$g/mL of the FITC-labeled copolymers was added to control and TNF-α treated cells and incubated for 15 h. Cells were subsequently rinsed three times with media and exposed to Lysotracker (50 nM, 60 min, 37 °C), after which they were rinsed three times with cold PBS, fixed in 3% paraformaldehyde, and mounted in Mowiol-DABCO mounting medium. Images were acquired with a Fluor filter block (excitation at 488 nm, emission collected with a 515 nm barrier filter), followed by a red filter analysis (excitation at 543 nm, emission collected with a 570 nm barrier filter). Autofluorescence background was ascertained using control (untreated) cells. Quantitative analysis was performed using Image-Pro Plus 4.0 (Media Cybernetics, Silver Spring, MD). Fluorescence intensities were expressed in arbitrary units per square micrometer. Data were normalized to the number of cells per unit field.

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