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Controllable Inhibition of Cellular Uptake of Oxidized Low Density Lipoprotein: Structure-Function Relationships for Nanoscale Amphiphilic Polymers

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Abstract

A family of anionic nanoscale polymers based on amphiphilic macromolecules (AMs) was developed for controlled inhibition of highly oxidized low density lipoprotein (oxLDL) uptake by inflammatory macrophage cells, a process that triggers the escalation of a chronic arterial disease called atherosclerosis. The basic AM structure is composed of a hydrophobic portion formed from a mucic acid sugar backbone modified at the four hydroxyls with lauroyl groups conjugated to hydrophilic poly(ethylene glycol) (PEG). The AM structure-activity relationships were probed by synthesizing AMs with six key variables: length of the PEG chain, carboxylic acid location, type of anionic charge, number of anionic charges, rotational motion of the anionic group, and PEG architecture. All AM structures were confirmed by nuclear magnetic resonance spectroscopy and their ability to inhibit oxLDL uptake in THP-1 human macrophage cells was compared in the absence and presence of serum.

We report that AMs with one, rotationally restricted carboxylic acid within the hydrophobic portion of the polymer was sufficient to yield the most effective AM for inhibiting oxLDL internalization by THP-1 human macrophage cells under serum-containing conditions. Further, increasing the number of charges and altering the PEG architecture in an effort to increase serum stabilization did not significantly impair the ability of AMs to inhibit oxLDL internalization, suggesting that selected modifications to the AMs could potentially promote multifunctional characteristics of these nanoscale macromolecules.

Keywords

Biomaterials; Polymers; Nanoassemblies; Low-Density Lipoproteins; Macrophages

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1. Introduction

Elevated levels of cellular and plasma cholesterol have serious consequences on the progression of cardiovascular disease, the leading cause of death in America. [1–3] The major carriers of cholesterol in blood plasma are low-density lipoproteins (LDLs), which enter the arterial walls through injured or leaky endothelial lining on the intima. [3,4] Once LDL enters the intima, it can be oxidized, causing compositional alterations and compromising the ability of native LDL receptors on macrophage cells to recognize the particle. [5] [6] Rapid uptake of highly oxidized LDL (hoxLDL) occurs through scavenger receptors on the macrophage cellular membrane, including scavenger receptor A (SRA), scavenger receptor B (SRB), CD36 and CD68. Scavenger receptors lack a feedback mechanism to control internalization, leading to excess cholesterol accumulation within the cytoplasm and triggering the development of foam cells and fatty streaks, a key characteristic of early atherogenesis. [7–16]

We previously explored AMs for inhibiting hoxLDL uptake through competitive inhibition of scavenger receptors on murine macrophages. [17–21] [22–25] These AMs are biocompatible polymers, [26] composed of poly(ethylene glycol), mucic acid and four aliphatic acid chains, that form nanoscale micelles with an extremely low critical micelle concentration (10^{-7} M) in aqueous media. [27,28] Despite the early promise of the AMs, the structure-activity relations for controlled inhibition of uptake of cholesterol are lacking, particularly under physiological conditions.

In this study, we synthesized AMs with systematic variations to identify the critical elements that contribute to the ability of AMs to inhibit hoxLDL uptake by human THP-1 macrophage cells cultured *in vitro*, under serum-lacking and serum-containing conditions. Six key parameters were varied and the degree of hoxLDL uptake was quantitatively compared: length of the PEG chain, anionic charge location, type of anionic charge, number of anionic charges, rotational motion of the anionic group, and PEG architecture. We hypothesize that the most efficient polymer for hoxLDL inhibition will be: i) amphiphilic, increasing charge density at the scavenger receptors; ii) have the greatest amount of anionic charge possible within the hydrophobic portion, to increase scavenger receptor binding; and iii) have two, short-chain linear PEG chains conjugated to the hydrophobic backbone *via* a branch-point, to better shield the anionic charge and stabilize the nanoassemblies from disruption by serum proteins.

2. Materials and methods

2.1 Synthetic Materials

Unless otherwise stated, solvents and reagents were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO) and used as received. PEG 5 kDa was purchased from Polysciences, Inc. (Warrington, PA) and dried by azeotropic distillation from toluene before use. Specialty, functionalized PEGs were purchased from Laysan Bio, Inc (Arab, AL) and used as received. Several polymers were prepared as previously described: **1cM** [27], **1cM-2000** [27], **0cM** [29], **2cM** [20], **1cP** [18], and **1cM-2000x2** [30].

2.3 Polymer Characterization Methods

Proton nuclear magnetic resonance (^1H -NMR) spectra of the products were obtained using a Varian 400 MHz or 500 MHz spectrophotometer. Samples were dissolved in chloroform- d_3 , with a few drops of dimethyl sulfoxide- d_6 if necessary, with tetramethylsilane as an internal reference. Molecular weights (Mw) and polydispersity indices (PDI) were determined using gel permeation chromatography (GPC) with respect to polyethylene glycol standards (Sigma-Aldrich) on a Waters Strygel® HR 3 THF column (7.8×300 mm). The Waters LC system (Milford, MA) was equipped with a 2414 refractive index detector, a 1515 isocratic HPLC

pump, and 717plus autosampler. An IBM ThinkCentre computer with Waters Breeze Version 3.30 software installed was used for collection and processing of data. Samples were prepared at a concentration of 10 mg/mL in tetrahydrofuran, filtered using 0.45 μ m pore size nylon or poly(tetrafluoroethylene) syringe filters (Fisher Scientific) and placed in sample vials to be injected into the system. Melting points were determined by DSC on a TA DSC Q200. TA Universal Analysis 2000 software was used for data collection on a Dell Dimension 3000 computer. Samples (3–5 mg) were heated under dry nitrogen gas. Data were collected at heating and cooling rates of 10 $^{\circ}$ C /min with a two-cycle minimum.

2.2 Polymer Synthesis

2.2.1 2cbM—The carboxylic acid of **1cM** (0.56 g, 0.094 mmol) was activated with SOCl_2 (50 mL) at 90 $^{\circ}$ C overnight under argon gas. Excess SOCl_2 was removed *via* rotary evaporation and the yellow oil subsequently dissolved in anhydrous THF (15 mL) and anhydrous pyridine (1 mL). 5-Aminoisophthalic acid (0.14 g, 0.75 mmol) in anhydrous THF (16 mL) and anhydrous pyridine (2 mL) was then added to the reaction flask and allowed to react for 48 hours at room temperature under argon. THF and pyridine were removed *via* rotary evaporation and the resulting oil dissolved in CH_2Cl_2 , washed with 1N HCl and brine, dried over MgSO_4 , and concentrated. The desired product was then precipitated from CH_2Cl_2 by addition of 10-fold diethyl ether and the solid collected by centrifugation. Solvent was removed by decanting and the resulting yellow solid was dried under ambient atmosphere (12 hrs) and under high vacuum (12 hrs). Yield: 0.52 g, 91 %. $^1\text{H-NMR}$ (CDCl_3): δ 8.61 (m, 1H, ArH), 8.17 (m, 2H, ArH), 5.70 (m, 2H, CH), 5.20 (m, 2H, CH), 4.24 (m, 2H, CH_2), 3.60 (m, ~0.45 kH, CH_2O), 3.38 (s, 3H, CH_3), 2.44 (m, 4H, CH_2), 2.29 (m, 4H, CH_2), 1.60 (m, 8H, CH_2), 1.26 (m, 64H, CH_2), 0.88 (t, 12H, CH_3). T_m = 56 $^{\circ}$ C GPC: M_w : 6.3 kDa; PDI: 1.09.

2.2.2 1sM—2-Aminoethyl hydrogen sulfate (7.0 mg, 0.050 mmol) was dissolved in DMSO (2 mL) by warming over medium heat on a stir plate for 15–30 min. After cooling to room temperature, 0.5 M NaOH (101 μ L) was added and the solution stirred for 30 min. In a separate flask, **0cM** (0.20 g, 0.033 mmol) was dissolved in CH_2Cl_2 (6.0 mL) and subsequently added to the solution of 2-aminoethyl hydrogen sulfate dropwise and the reaction stirred overnight (12 hrs). The CH_2Cl_2 was then removed *via* rotary evaporation then the DMSO removed *via* lyophilization. The resulting solid was dissolved in CH_2Cl_2 and the solution filtered to remove excess 2-aminoethyl hydrogen sulfate and the N-hydroxysuccinimide by-product. The desired product was precipitated from CH_2Cl_2 by addition of 10-fold diethyl ether and the solid collected by centrifugation. Solvent was removed by decanting and the resulting yellow solid was dried under ambient atmosphere (12 hrs) and under high vacuum (12 hrs). Yield: 0.16 g, 78 %. $^1\text{H-NMR}$ (CDCl_3): δ 5.83 (m, 2H, CH), 5.48 (m, 2H, CH), 3.67 (m, ~0.45 kH, CH_2O), 3.38 (s, 3H, CH_3), 2.32 (m, 8H, CH_2), 1.60 (m, 8H, CH_2), 1.22 (m, 64H, CH_2), 0.88 (t, 12H, CH_3). T_m = 55 $^{\circ}$ C. GPC: M_w : 6.4 kDa; PDI: 1.08.

2.2.3 0cM-2000x2—1cM-2000x2 (0.18 g, 0.035 mmol) was esterified with N-hydroxysuccinimide (0.040 g, 0.34 mmol) with DCC (1.0 mmol) as the dehydrating reagent in anhydrous CH_2Cl_2 (20 mL) and anhydrous DMF (10 mL). The reaction was stirred for 12 hrs at room temperature under argon gas before the DCU byproduct was removed by vacuum filtration. The filtrate was then washed with 0.1 N HCl and brine, dried over MgSO_4 , and concentrated. The desired product was then precipitated from CH_2Cl_2 by addition of 10-fold diethyl ether and the solid collected by centrifugation. Solvent was removed by decanting and the resulting yellow solid was dried under ambient atmosphere (12 hrs) and under high vacuum (12 hrs). Yield: 0.11 g, 67 %. $^1\text{H-NMR}$ (CDCl_3): δ 8.42 (s, 1H, ArH), 8.38 (s, 2H, ArH), 5.75 (d, 2H, CH), 5.51 (m, 1H, CH), 5.14 (d, 1H, CH), 4.43 (t, 4H, CH_2), 3.68 (m, ~0.36 kH, CH_2O), 2.81 (t, 4H, CH_2), 2.37 (m, 4H, CH_2), 2.25 (m, 4H, CH_2), 1.59 (m, 8H, CH_2), 1.24 (m, 64H, CH_2), 0.84 (t, 12H, CH_3). T_m = 45 $^{\circ}$ C. GPC: M_w : 4.9 kDa; PDI = 1.07.

2.2.4 2cbM-2000x2—The carboxylic acid of **1cM-2000x2** (0.18 g, 0.035 mmol) was activated with SOCl_2 (20 mL) at 70 °C for four hours under argon gas. Excess SOCl_2 was removed *via* rotary evaporation and product dried under high vacuum overnight. The product was then dissolved in CH_2Cl_2 (5 mL) and was added to a solution of 5-aminoisophthalic acid (0.06 g, 0.35 mmol) in anhydrous THF (10 mL) and anhydrous pyridine (2 mL) and stirred for 6 hours at room temperature. The THF and pyridine were then removed *via* rotary evaporation and the resulting oil dissolved in CH_2Cl_2 , washed with 0.1N HCl and brine, dried over MgSO_4 , and concentrated. The desired product was then precipitated from CH_2Cl_2 by addition of 10-fold diethyl ether and the solid collected by centrifugation. Solvent was removed by decanting and the resulting yellow solid was dried under ambient atmosphere (12 hrs) and under high vacuum (12 hrs). Yield: 0.12 g, 67 %. $^1\text{H-NMR}$ (CDCl_3): δ 8.42 (s, 2H, ArH), 8.38 (s, 4H, ArH), 5.75 (d, 2H, CH), 5.51 (m, 1H, CH), 5.14 (d, 1H, CH), 4.43 (t, 4H, CH_2), 3.68 (m, $\sim$ 0.36 kHz, CH_2O), 2.37 (m, 4H, CH_2), 2.25 (m, 4H, CH_2), 1.59 (m, 8H, CH_2), 1.24 (m, 64H, CH_2), 0.84 (t, 12H, CH_3). $T_m = 50$ °C. GPC: M_w : 4.4 kDa; PDI = 1.05.

2.3 Cell Culture

Human THP-1 monocytes (ATCC), were grown in suspension, at a concentration of 100,000 cells/ cm^2 with RPMI medium containing 0.4 mM Ca^{2+} and Mg^{2+} (ATCC) and supplemented with 10 % fetal bovine serum (FBS), in an incubator with 5% CO_2 at 37 °C and split every four days through centrifugation. The cells were seeded at a concentration of 100,000 cells/ cm^2 and differentiated from monocytes into adherent macrophage cells by the addition of 16 nM phorbol myristate acetate. [31] Once differentiated, the macrophage cells were not propagated and were used within one week of differentiation.

2.4 LDL Oxidation to hoxLDL

Highly oxidized LDL (hoxLDL) was prepared by oxidation of LDL within five days of each experiment. BODIPY-labeled human plasma derived LDL (Molecular Probes, OR) was oxidized by incubation with 10 μM CuSO_4 (Sigma) for 18 hrs at 37 °C with 5% CO_2 before oxidation was stopped with the addition of 0.01% w/v EDTA (Sigma). [32,33] Characterization of hoxLDL has been previously reported. [18]

2.5 hoxLDL Internalization

Internalization of hoxLDL by macrophage cells was assayed, either with or without 5% FBS, by incubating fluorescently labeled hoxLDL (10 $\mu\text{g}/\text{mL}$) and the various polymer conditions, at 10^{-6} M, with cells for 24 or 48 hours at 37 °C and 5 % CO_2 . Test conditions also included two controls: RPMI medium and polymers alone to ensure that no cell or polymer auto-fluorescence was observed. The cells were then washed twice with PBS and imaged for cell-associated fluorescence. Images were analyzed using Image Pro Plus 5.1 software (Media Cybernetics, San Diego, CA) and fluorescence was normalized to cell number before being compared to the sample with only hoxLDL, the negative control run in every experiment. An SRA antibody (10 $\mu\text{g}/\text{mL}$; clone 351620; R&D Systems, Minneapolis, MN) was used as a positive control for the assay for 24 hours in serum free conditions.

2.6 Statistical Analysis

Error bars on graphs indicate standard error of the mean based upon biological triplicate samples in each experiment and three separate experiments for each condition. Single factor ANOVA with Tukey's post-hoc analysis, performed on SPSS software, was used for statistical analysis. Significance is claimed for differences of $p < 0.05$.

3. Results and discussion

3.1 Synthesis & Characterization of AMs

A range of AMs were designed (see Table 1) based upon results from previous work [17–21, 30] and synthesized to determine the most effective inhibitor of hoxLDL uptake. Several AMs were previously synthesized for a variety of applications.[17–21,26,27,29,30,34–36] New AMs were synthesized from these previously published compounds as summarized in Scheme 1.

The parent compounds, **1cM** and **1cM-2000x2**, served as building blocks for subsequent modifications. Specifically, the carboxylic acid on the mucic acid backbone of **1cM** and **1cM-2000x2** was activated with N-hydroxysuccinimide or thionyl chloride to functionalize the polymers with a small linker molecule containing the desired functionality, such as a sulfate to yield **1sM** or di-carboxylic acid to yield **2cbM** and **2cbM-2000x2**. Polymer structure was verified by ¹H NMR and molecular weight determined by GPC relative to PEG standards. Due to the abundance of PEG in the polymers (~83 % of protons), it was often difficult to detect the presence of new protons in the ¹H NMR, particularly from 0.8 to 2.4 ppm where the methylene protons of the hydrophobic arms make up the majority of that region. Thus, spectra were monitored for the disappearance of the protons of the activating group (N-hydroxysuccinimide), 2.8 ppm, or the appearance of aromatic protons (**2cbM** and **2cbM-2000x2**), ~ 8.4 ppm.

In total, ten AMs were tested for their ability to inhibit hoxLDL uptake with and without serum. The characteristics tested were amphiphilicity, branching of the hydrophobic region, length of the PEG chain, anionic charge location, type of anionic charge, number of anionic groups, rotational motion of the charged group, and PEG architecture. Structures of all polymers, schematics representing the placement of anionic charge, and information regarding specific variable parameters are shown in Table 1. It was hypothesized that by altering each of these key parameters, the optimal AM structure for inhibiting hoxLDL uptake would be determined.

3.2.1 Role of Amphiphilicity

To examine the role of the amphiphilicity, the efficiency of control polymers, **PEG** and **PEG-COOH**, to inhibit hoxLDL internalization was tested. **PEG** is a common hydrophilic polymer chosen for its biocompatibility and ability to evade excretion from the bloodstream by the reticuloendothelial system (RES). [37–39] The PEGs tested here were chosen because they have the same average molecular weight as the PEG of **1cM**. **PEG-COOH** was chosen due to the presence of the anionic charge, which has been found to be a key parameter for AM inhibition of hoxLDL uptake. [17–21] Additionally, in modeling studies, Plourde et. al. observed the binding energy of a **PEG-COOH** model when docked with an SR-A collagen-like domain homology model was favorable, but with two-times more energy than **1cM**. [21] Based on these modeling results, **PEG-COOH** was anticipated to inhibit hoxLDL better than **0cM**, but to a lower extent than **1cM**, while **PEG** was anticipated to behave like **0cM** or have no effect at all.

As shown in Figure 1, the amphiphilic polymers, **0cM** and **1cM**, consistently inhibited hoxLDL internalization to a greater extent than both anionically terminated or unfunctionalized hydrophilic **PEGs** under all conditions. Notably, **1cM** was the only polymer that showed significant inhibition of uptake at all conditions, whereas **0cM** showed modest levels of effect, suggesting that the combination of amphiphilicity and anionic charge presentation may be operative in early competitive studies. Additionally, as **PEG-COOH** had little effect with or without serum, this suggests that the anionic group on the hydrophobic portion of the polymer may be more effective, which is discussed later.

3.2.2 Role of Branching of the Hydrophobic Region

To examine the effect of the branched hydrophobic region of AMs, the well-known amphiphiles **Pluronic P85** and **Cremophor EL** were tested for their ability to effect internalization of hoxLDL. **Pluronic P85** and **Cremophor EL** were chosen based on their structural similarity to AMs with respect to their hydrophilic PEG regions. Additionally, like AMs, they are amphiphilic, biocompatible and widely studied for pharmaceutical applications. [40–43]

As shown in Figure 1, compared with **Pluronic P85** and **Cremophor EL**, the AMs, specifically **1cM** and **0cM** shown here, competed far more effectively with hoxLDL uptake whereas **Pluronic P85** and **Cremophor EL** had little effect on hoxLDL uptake. These results highlight the importance of the branched architecture of the hydrophobic component of the AMs, which allows them to significantly reduce hoxLDL uptake.

3.2.3 Role of Length of the PEG Chain

The ability of PEG to evade excretion by the RES system is generally attributed to PEG's ability to shield the system from disruption by serum proteins. [37–39] Based upon literature, a longer PEG chain length was anticipated to yield increased stability in serum caused by additional PEG density resulting in more chain entanglements, providing enhanced micelle stability and protection of the critical anionic charge. [37–39] This expectation was confirmed in testing AMs with hydrophilic PEG molecular weights of 2 kDa (**1cM-2000**) and 5 kDa (**1cM**). As shown in Figure 2, at the 24 hour time point with and without serum, **1cM** and **1cM-2000** have comparable levels of hoxLDL inhibition. However, after 48 hours in the presence of serum, the longer PEG chain length was most effective at hoxLDL inhibition. This result confirms the hypothesis that increased PEG length results in increased chain entanglements and better micelle stability and protection of the anionic charge from serum protein disruption.

3.2.4 Role of Carboxylic Acid Location

As previously stated, AM inhibition of hoxLDL uptake by macrophage cells occurs by competitive binding between AMs and hoxLDL for positively charged scavenger receptors. [19,21] Thus, it is necessary for the anionic group of AMs to be readily available and easily bound in the receptor pocket. Therefore, it was initially anticipated that the anionic group on the hydrophilic, or external, portion of the micelle would be more readily available for binding to scavenger receptors and increased binding would lead to better inhibition of hoxLDL uptake. However, modeling studies and *in vitro* testing in the absence of serum demonstrated that the anionic charge in the hydrophobic region of the polymer was more effective at inhibiting hoxLDL uptake than having the anionic charge within the hydrophilic region. [19–21] These results were confirmed herein and, expanding on previous work, in the presence of serum both at 24 and 48 hours **1cM** performed significantly better than **1cP**, as shown in Figure 3. This effect is likely due to the interaction of serum proteins with the carboxylic group, which is not shielded by PEG when it is positioned on the hydrophilic portion of the micelle. For serum and non-serum containing conditions it is postulated that the greater charge density that results from having the anionic charge within the hydrophobic interior when micellization occurs leads to greater hoxLDL inhibition. In addition, the hydrophobic core is conformationally rigid, such that the carboxylic acid has less freedom of rotation and a better ability to lock into the active site on scavenger receptors. [44]

3.2.5 Role of Type of Anionic Charge

Like carboxylic acids, sulfates have also been shown to bind to scavenger receptors and thus inhibit hoxLDL uptake.[45] To investigate this possibility with the AM structure, a sulfate

moiety (**1sM**) was incorporated into the hydrophobic portion of the polymer. In the absence of serum, both **1cM** and **1sM** inhibited hoxLDL uptake more than the non-acidic system (**0cM**), but **1cM** did so to a far greater extent than **1sM**, as shown in Figure 4. However, in serum-containing conditions, at 24 and 48 hours, **1sM** inhibited hoxLDL uptake on a similar level to **0cM**, significantly less than **1cM**. This result may be due to the decreased steric hindrance surrounding the sulfate, making it more readily available for interactions such as hydrogen bonding with other residues of the scavenger receptor. Additionally, the methylene spacer increases the rotational motion available to the **1sM**, which may result in inadequate binding to the active receptor site. The role of decreased steric hindrance surrounding the anionic group and increased rotational motion will be discussed in later sections.

3.2.6 Role of Number of Anionic Charges

The number of anionic charges was investigated by comparing **1cM**, **0cM**, and **2cbM**. It was hypothesized that increasing the number of charge groups would result in greater charge density which would result in more significant attraction of the AMs to the positively charged binding pocket on scavenger receptors and therefore greater hoxLDL uptake inhibition. Additionally, it was anticipated that the addition of a spacer molecule would reduce the steric hindrance surrounding the carboxylic acid and allow better access to the binding site, which would decrease hoxLDL uptake. However, as shown in Figure 5, the addition of an additional carboxylic acid with an aromatic spacer, **2cbM**, shows hoxLDL uptake inhibition comparable to that of **1cM** for all time points.

We postulate that this result is due to the ability of only one carboxylic acid to bind to scavenger receptors at a time. Thus, the addition of more carboxylic acids does not provide any additional benefit. In fact, in modeling studies by Plourde et al. additional carboxylic acids actually lowered the overall AM binding affinity to a scavenger receptor model due to hydrogen bonding of the carboxylic acids with residues in the binding pocket that inhibited binding of the AM to the active site. [21] This result suggests that the carboxylic acids are too readily available and accessible for the first interaction they encounter, which is hydrogen bonding rather than binding in the active site. Further, this data indicates that despite the steric hindrance surrounding the carboxylic acid of **1cM**, the carboxylic acid is available for binding and that adding a spacer molecule to increase availability may potentially hinder binding.

3.2.7 Role of Rotational Motion of the Anionic Group

To analyze the effect of the rotational motion of the carboxylic acid charge, the aromatic dicarboxylic acid-containing **2cbM** was compared with the aliphatic dicarboxylic acid-containing **2cM**, which would allow the carboxylic acids more freedom for rotation and movement. We anticipated that changing the rotational motion would have a significant impact on scavenger receptor ability to bind the carboxylic acid. As **2cM** has aliphatic carboxylic acids, the rotational motion is less restricted than the aromatic carboxylic acids of **2cbM**. As shown in Figure 6, the aromatic carboxylic acids on **2cbM** result in significantly greater hoxLDL uptake inhibition than **2cM** for all time points with and without serum. Also, **2cM** behaves like **1cP** with respect to the extended time point in serum, both of which did not result in lower percent hoxLDL per cell than the 24 hour time point in serum. Although the carboxylic acids are in different regions of the polymer, in both **1cP** and **2cM** the carboxylic acids have significantly more rotational motion as aliphatic components. These results indicate that restriction of rotational motion of the anionic group is essential for the carboxylic acid to effectively bind to the active site on scavenger receptors. Additionally, this result also suggests that the steric hindrance surrounding the carboxylic acid of **1cM** restrict the rotational motion of the carboxylic acid such that the carboxylic acid is better capable of binding to the active site.

3.2.8 Role of PEG Architecture

Finally, it was hypothesized that utilizing a structural branching point to which two, short-chained PEGs were attached would provide increased stabilization against serum proteins in the blood stream. [30,46] To maintain overall PEG content, each PEG arm was synthesized to be 2 kDa, compared to the linear AMs with PEG 5 kDa. AMs with zero, one, and two carboxylic acids in the hydrophobic core were synthesized with this architectural branching point and tested for their ability to inhibit hoxLDL uptake. As shown in Figure 6, all AMs with the PEG branching point inhibited hoxLDL more significantly than their linear counterparts without serum. In the absence of serum, **2cbM-2000x2** results in the lowest percent of hoxLDL per cell overall, edging out **1cM** which typically outperforms all other polymers. In serum-containing conditions for both 24 and 48 hours, the AMs with a PEG branching point inhibited hoxLDL at similar levels to their linear counterparts except for the **2cbM-2000x2** and **2cbM** polymers. At 48 hours, the **2cbM-2000x2** significantly inhibited hoxLDL internalization more than **2cbM**. However, while **2cbM-2000x2** outperforms its linear counterpart, inhibition of hoxLDL uptake is statistically the same as **1cM**.

Generally, utilizing two, short-chain PEGs emanating from a branch-point resulted in more efficient hoxLDL inhibition compared to their linear counterparts under serum-free conditions. However, providing a PEG branching point did not significantly alter the polymer's hoxLDL inhibitory capacity compared with the linear polymers in serum-containing conditions. While a PEG branching point likely increases shielding of the micelle hydrophobic core, it is proposed that the linear PEG 5 kDa results in significant chain entanglements, as seen with the **1cM-2000** and **1cM** polymers, such that utilizing a branching point with lower molecular weight PEGs did not significantly passivate against serum proteins, and consequently did not significantly alter the hoxLDL inhibition.

3.2.9 Cumulative Comparison of hoxLDL Uptake Inhibition via AM Library of Polymers

The combined results for hoxLDL uptake normalized to respective cell number across all polymer configurations synthesized are shown in Figure 7 (**top**). Additionally, fluorescent images of BODIPY-labeled hoxLDL uptake for the two most efficient AMs (**1cM** and **2cbM-2000x2**) compared with hoxLDL uptake alone are shown in Figure 7 (**bottom**). It should be noted that the highly efficient AMs perform comparably to positive control (SRA antibody blockage), with hoxLDL uptake inhibited by about 20% after 24 hours in serum free conditions. It can also be noted that the early serum free data appears to correlate well with the 48 h data in serum conditions. These trends suggest that it takes longer (more than 24 h) to reverse the serum protein-mediated competitive blockage of polymer-receptor binding via serum proteins. Thus, behaviors of polymer binding to macrophages at early times in serum free conditions, which may be primarily scavenger receptor mediated, manifest similarly to the behaviors seen at longer times in serum conditions.

We note that the inhibitory results found were not due to polymer cytotoxicity as judged by cell morphology and cell counts for each polymer. We have previously reported using IC21 murine macrophage cultures that these polymers are highly biocompatible in vitro, even at two orders of magnitude higher concentrations than those used in the current study [17]. Recent reports show that these polymers are non-cytotoxic in human umbilical endothelial cells (HUVEC), umbilical arterial smooth muscle cells (UASM), and normal dermal human fibroblasts (NDHF). [26]

4. Conclusions

Overall, we report that AMs exhibit rich structure-function relationship behaviors regarding the inhibition of hoxLDL uptake in macrophages. To optimize the AM properties, several

structural characteristics were analyzed: length of the PEG chain, carboxylic acid location, type of anionic charge, number of anionic charges, rotational motion of the anionic group, and PEG architecture. The parent AM with its rigid, hydrophobic carboxylic acid and linear PEG chain of 5 kDa was the most efficient at inhibiting hoxLDL internalization by THP-1 human macrophage cells in the presence of serum. Thus, merely one rotationally-restricted carboxylic acid within the hydrophobic core was sufficient for effective inhibition of hoxLDL uptake. Polymers with increased charge and altered PEG architecture based on two, short-chain, linear PEGs emanating from a branch point, showed equivalent levels of inhibition of hoxLDL uptake but not any further enhancement over that elicited by the simpler parent AM structure. In general, longer PEG chains were found to be more efficient at protecting AMs in serum-containing conditions. These findings could have implications for the design of polymers for improved competitive inhibition of intimal cholesterol uptake under physiological as well as pathologic conditions.[47,48] Future efforts will need to dissect the role of receptor-mediated processes that effect hoxLDL inhibition. Further translational research will be necessary to examine the role of the selected AM in vivo within animal models of atherogenesis and inflammation.

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Figure 1. Role of amphiphilicity and branching of the hydrophobic region on degree of inhibition of hoxLDL uptake in THP-1 macrophages. Levels of hoxLDL were measured and quantified in comparison to corresponding hoxLDL treated cells that were not treated with any polymers.

Figure 2.
Role of PEG chain length on degree of inhibition of hoxLDL uptake in THP-1 macrophages.

Figure 3.
Role of carboxylic acid location on degree of inhibition of hoxLDL uptake in THP-1 macrophages.

Figure 4. Role of the type of anionic group on degree of inhibition of hoxLDL uptake in THP-1 macrophages.

Figure 5.
Role of number of anionic charges and rotational motion on degree of inhibition of hoxLDL uptake in THP-1 macrophages.

Figure 6.
Role of PEG architecture on degree of inhibition of hoxLDL uptake in THP-1 macrophages.

Figure 7.

Internalization of hoxLDL by THP-1 macrophage cells after incubation with 10^{-6} M AMs for 24 or 48 hours with and without 5% FBS; (A) Comparison of all polymers tested in comparison to hoxLDL alone (negative control), and hoxLDL with scavenger receptor antibody pre-treatment (positive control); (B) Fluorescent images of BODIPY-labeled hoxLDL uptake following hoxLDL treatment, hoxLDL with **1cM**, and hoxLDL with **2cbM-2000x2** (the two most efficient polymers at inhibiting hoxLDL uptake).

SCHEME 1.

Synthetic scheme for newly synthesized polymers **2cbM**, **1sM**, **0cM-2000x2**, and **2cbM-2000x2**

h (where 2 kDa is indicated by “short”

	A schematic diagram showing a long, blue, wavy polymer chain. At one end of the chain, there is a red circular component, possibly representing a bead or a specific functional group. The chain is shown in a coiled or folded state.	PEG Length Long	Charge Location Hydrophobic	Charge Number 1	Type of Charge Carboxylic Acid	PEG Architecture 1 Linear
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Schematic		PEG Length	Short	Charge Location	Hydrophobic	Charge Number	1	Type of Charge	Carboxylic Acid	PEG Architecture	1 Linear

PEG Length	Charge Location	Charge Number	Type of Charge	PEG Architecture
Long	None	0	None	1 Linear
Long	Hydrophilic	1	Carboxylic Acid	1 Linear

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		Long	Hydrophobic	1	Sulfate	1 Linear
		Long	Hydrophobic	2	Carboxylic Acid	1 Linear

Schematic	PEG Length	Long
	Charge Location	Hydrophobic
	Charge Number	2
	Type of Charge	Carboxylic Acid
	PEG Architecture	1 Linear

Schematic	PEG Length	Short x2
	Charge Location	Hydrophobic
	Charge Number	1
	Type of Charge	Carboxylic Acid
	PEG Architecture	2 Linear from a branch point

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PEG Length	Charge Location	Charge Number	Type of Charge	PEG Architecture
Short x2	None	0	None	2 Linear from a branch point
Short x2	Hydrophobic	2	Carboxylic Acid	2 Linear from a branch point

Non-amphiphilic		PEG Length Long	Charge Location None	Charge Number 0	Type of Charge None	PEG Architecture 1 Linear