Acta Biomaterialia 6 (2010) 3081–3091

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Controllable inhibition of cellular uptake of oxidized low-density lipoprotein: Structure–function relationships for nanoscale amphiphilic polymers

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ARTICLE INFO

Article history:
Received 22 September 2009
Received in revised form 19 January 2010
Accepted 10 February 2010
Available online 17 February 2010

Keywords:
Biomaterials
Polymers
Nanoassemblies
Low-density lipoproteins
Macrophages

ABSTRACT

A family of anionic nanoscale polymers based on amphiphilic macromolecules (AMs) was developed for controlled inhibition of highly oxidized low-density lipoprotein (hoxLDL) 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 hoxLDL 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 hoxLDL 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 hoxLDL internalization, suggesting that selected modifications to the AMs could potentially promote multifunctional characteristics of these nanoscale macromolecules.

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 atherogenisis [7–16].

We previously explored amphiphilic macromolecules (AMs) for inhibiting hoxLDL uptake through competitive inhibition of scavenger receptors on murine macrophages [17–25]. 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: (i) will be amphiphilic, increasing charge density at the scavenger receptors; (ii) will have the greatest amount of anionic charge possible within the hydrophobic portion, to increase

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doi:10.1016/j.actbio.2010.02.019

scavenger receptor binding; and (iii) will 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-2000 × 2 [30].

2.2. Polymer characterization methods

Proton nuclear magnetic resonance (^{1}H NMR) spectra of the products were obtained using a Varian 400 or 500 MHz spectrophotometer. Samples were dissolved in chloroform-d, with a few drops of dimethyl sulfoxide-d_6 if necessary, with tetramethylsilane as an internal reference. Molecular weight (M_w) and polydispersity indices (PDIs) were determined using gel permeation chromatography (GPC) with respect to polyethylene glycol standards (Sigma–Aldrich) on a Waters Stryagel® 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 717 plus 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^{-1} in tetrahydrofuran, filtered using 0.45 μm 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 °C min^{-1} with a two-cycle minimum.

2.3. Polymer synthesis

2.3.1. 2cbM

The carboxylic acid of 1cM (0.56 g, 0.094 mmol) was activated with $SOCl_2$ (50 ml) at 90 °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 h at room temperature under argon. THF and pyridine were 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 h) and under high vacuum (12 h). Yield: 0.52 g, 91%. ^{1}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 °C GPC: M_w: 6.3 kDa; PDI: 1.09.

2.3.2. 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 μl) 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 h). 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 h) and under high vacuum (12 h). Yield: 0.16 g, 78%. ^{1}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 °C. GPC: M_w: 6.4 kDa; PDI: 1.08.

2.3.3. 0cM-2000×2

1cM-2000 × 2 (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 h 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 h) and under high vacuum (12 h). Yield: 0.11 g, 67%. ^{1}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 °C. GPC: M_w: 4.9 kDa; PDI = 1.07.

2.3.4. 2cbM-2000×2

The carboxylic acid of 1cM-2000 × 2 (0.18 g, 0.035 mmol) was activated with $SOCl_2$ (20 ml) at 70 °C for 4 h 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 h at room temperature. The THF and pyridine were then removed via rotary evaporation and the resulting oil dissolved in CH_2Cl_2, washed with 0.1 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 h) and under high vacuum (12 h). Yield: 0.12 g, 67%. ^{1}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, ~0.36 kH, 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.

2.4. 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