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Dual Use of Amphiphilic Macromolecules As Cholesterol Efflux Triggers and Inhibitors of Macrophage Athero-inflammation

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Abstract

Activated vascular wall macrophages can rapidly internalize modified lipoproteins and escalate the growth of atherosclerotic plaques. This article proposes a biomaterials-based therapeutic intervention for depletion of non-regulated cholesterol accumulation and inhibition of inflammation of macrophages. Macromolecules with high scavenger receptor (SR)-binding activity were investigated for SR-mediated delivery of agonists to cholesterol-trafficking nuclear liver-X receptors. From a diverse feature space of a family of amphiphilic macromolecules of linear and aromatic mucic acid backbones modified with varied aliphatic chains and conjugated with differentially branched poly(ethylene glycol), a key molecule (carboxyl-terminated, C12-derivatized, linear mucic acid backbone) was selected for its ability to preferentially bind scavenger receptor A (SR-A) as the key target. At a basal level, this macromolecule suppressed the pro-inflammatory signaling of activated THP-1 macrophages while competitively lowering oxLDL uptake *in vitro* through scavenger receptor SRA-1 targeting. To further deplete intracellular cholesterol, the core macromolecule structure was exploited to solubilize a hydrophobic small molecule agonist for nuclear Liver-X Receptors, which regulate the efflux of intracellular cholesterol. The macromolecule-encapsulated agonist system was found to reduce oxLDL accumulation by 88% *in vitro* in comparison to controls. *In vivo* studies were designed to release the macromolecules (with or without encapsulated agonist) to injured carotid arteries within Sprague Dawley rats fed a high fat diet, conditions that yield enhanced cholesterol accumulation and macrophage recruitment. The macromolecules lowered intimal levels of accumulated cholesterol (50% for macromolecule alone; 70% for macromolecule-encapsulated agonist) and inhibited macrophage retention (92% for macromolecule; 96% for macromolecule-encapsulated agonist; 4 days) relative to non-treated controls. Thus, this study highlights the promise of designing bioactive macromolecule therapeutics based on scavenger receptor targeting, for potential management of vascular arterial disease.

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Introduction

Atherosclerosis, triggered by macrophage, smooth muscle and endothelial cell interactions with low density lipoproteins (LDL) within the vascular wall, is the major cause of cardiovascular disease and the leading cause of death in developed countries [1]. Elevated LDL plasma levels lead to the accumulation of LDL within the arterial wall, where oxidized LDL activates endothelial cells and recruits circulating monocytes that differentiate into macrophages. This atherogenic inflammatory cycle is believed to trigger foam cell formation and advance atherosclerosis as macrophages endocytose oxidized LDL (oxLDL) through unregulated scavenger receptor uptake. The localized build-up of cholesterol within the vascular intima and the consequent atherogenic inflammatory cascade present a major challenge to current therapeutic strategies.

Major pharmacologic modalities aim to lower systemic levels of cholesterol through liver-based synthetic pathways. Although systemic therapies may have some impact stabilizing atherosclerotic plaques, their ability to rescue the atherogenic inflammatory cascade and restore normal anatomy is limited. Further, they are known to cause adverse side effects (from gastrointestinal complaints to liver enzyme elevation and myopathy) [2, 3]. A number of molecular approaches have been proposed for directed inhibition of atherogenesis, including scavenger receptor knockdown [4, 5] and cholesterol acyl-transferase (ACAT)-1 and ACAT-2 suppression [6]. A marked decrease in the progression of advanced necrotic lesions has been noted in ApoE^{-/-} mice through the targeted deletion of scavenger receptors SR-A and CD36, which are upregulated in inflamed macrophages [7], indicating that scavenger receptors play an important role in disease progression. Atherosclerotic progression may further be controlled through management of atherosclerotic lesion macrophage infiltration [8], which Yamakawa et al. has recently restricted via the administration of dehydroepiandrosterone (DHEA), independent of systemic cholesterol levels [9].

Here a biomaterials-based approach was investigated to target inflamed cells and inhibit their atherogenic progression. Nanoscale amphiphilic macromolecules composed of a sugar backbone derivatized with aliphatic side-chains and poly(ethylene glycol) (PEG) were designed to challenge oxLDL interactions with human macrophages. The amphiphilic macromolecules self-assemble to form nanoscale micelles at very low concentrations, due to their critical micelle concentration (CMC) of 10⁻⁷ M [10], which bind to macrophage scavenger receptors with high affinity and competitively inhibit cellular internalization of oxLDL. Further, the hydrophobic core of the macromolecules supports good loading efficiency enabling the use of the bioactive macromolecules for drug encapsulation and intracellular delivery.

The current study exploits both properties of the macromolecules to reverse key atherogenic inflammatory processes in co-operative ways (Figure 1): suppression of the pro-inflammatory phenotype of macrophages and reduction of cholesterol uptake through binding of scavenger receptors (intrinsic bioactivity); and delivery of encapsulated hydrophobic drug to activate cholesterol efflux channels. The success of the latter approach was demonstrated using a model agonist GW3965, a synthetic small molecular weight agonist to nuclear membrane receptor, liver X Receptor α [11]. This agonist has been shown to alter cholesterol synthesis, influx and efflux causing an overall decrease of cellular cholesterol after 96 hrs as well as regulating inflammation and atherosclerosis in animal models [12–14]. A combination of macromolecule-mediated scavenger receptor binding and intracellular delivery of the GW3965 for lipoprotein metabolism was implemented.

Additionally, the efficacy of this dual-pronged approach to ameliorate both recruitment of inflammatory macrophages and cholesterol accumulation was demonstrated *in vivo*.

Materials and Methods

Materials

A library of amphiphilic macromolecules derived from mucic acid has been previously established [15]. For this study, macromolecules 1cM, 0cM, 2cbM, and 1cP (Figure 2A) were synthesized as previously detailed [16–18] and characterized using established techniques including ^1H NMR-spectroscopy, gel permeation chromatography, differential scanning calorimetry, and dynamic light scattering. The critical micellar concentration, size and charge data has been previously published[15].

Methods

Cell Culture—Human THP-1 monocytes (ATCC) were grown in suspension with RPMI-1640 medium containing 0.4 mM Ca^{2+} and Mg^{2+} , (ATCC) and supplemented with 10 % 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 into macrophage cells by 14 hours incubation in 16nM phorbol myristate acetate. After the 14 hour differentiation period the cells were incubated for an additional 58 hours in RPMI-1640 medium and then tested within three days. Human embryonic kidney (HEK) cells stably transfected with human scavenger receptor A (gift from Dr. Steven R. Post), which are referred to as HEK-SRA. Cells were propagated in high glucose DMEM (Invitrogen) supplemented with 10% FBS, 1% penicillin/streptomycin, 15 ug/mL Blasticidin and 100 ug/mL HygromycinB at 37°C in 5% CO_2 . SR-A expression was induced with addition of 0.5 ug/mL tetracycline overnight and throughout the experiment.

LDL Oxidation—OxLDL was prepared within five days of each experiment. BODIPY-labeled and unlabeled human plasma derived LDL (Molecular Probes, OR) were oxidized by 18 hours of incubation with 10 μM CuSO_4 (Sigma) at 37 °C in 5 % CO_2 [19, 20] After 18 hours the oxidation was stopped with 0.01 % w/v EDTA and the degree of oxidation characterized using standard biochemical assays [21].

Competitive Binding to HEK-SRA Cells—The ability of select compounds from a library of macromolecules to competitively inhibit oxLDL binding was assayed by incubating BODIPY labeled oxLDL (10 $\mu\text{g/mL}$) with HEK-SRA cells for 24 hours at 37 °C and 5% CO_2 . Conditions included 4 macromolecules from our library at 10^{-6} M and a control of RPMI-1640 medium in basal and SR-A induced cells (SR-A expression was induced with addition of 0.5 ug/mL tetracycline overnight and throughout the experiment). The cells were then washed twice with 1xPBS and imaged on a Nikon Eclipse TE2000-S fluorescent microscope to determine fluorescently tagged oxLDL attachment. The images were analyzed with Image Pro Plus 5.1 software (Media Cybernetics, San Diego, CA) and fluorescence was normalized to cell count and medium alone condition.

Polymer Association with THP-1 Macrophages—Polymers and Texas-red conjugated polymers were prepared as previously described [10, 22]. Differentiated THP-1, cells were incubated with carboxy-terminated polymer (1cM) or uncharged control polymer (0cM) at 10^{-6} M for 24 hr in 5% serum RPMI-1640 at 37 °C in both basal and inflamed states (1 ng/ml) with and without 10 ug/ml SR-A antibody blocking (R&D Systems). Cells were washed, fixed with 4% paraformaldehyde and imaged on a Nikon Eclipse TE2000-S fluorescent microscope to determine fluorescently tagged polymer attachment. The images

were analyzed with Image Pro Plus 5.1 software (Media Cybernetics, San Diego, CA) and fluorescence data normalized to cell count.

Macromolecular Encapsulation of Liver X receptor (LXR) Agonist—The LXR agonist, GW3965, was encapsulated within 1cM macromolecule using oil/water emulsion. Specifically, GW3965 in CH_2Cl_2 was added drop wise to a magnetically stirring solution of 1cM in water at a concentration of 1×10^{-4} M 1cM and 1×10^{-5} M GW3965. The solution was stirred continuously for 24 hours in a closed vessel. The vessel was then opened and the solution exposed to ambient atmosphere for 16 hours to facilitate evaporation of the CH_2Cl_2 . The resulting solution was filtered with a 0.20 μm syringe filter (Fisher Scientific) and diluted to the desired concentration. Encapsulated GW3965 concentration was determined by UV absorption on a Beckman DU@520 General Purpose UV/Vis Spectrophotometer at 270 nm. Once encapsulated, the GW absorption peak disappeared due to micelle shielding. The micelles were then disrupted by addition of 50% DMSO and GW3965 concentration quantified by absorption at 270 nm in comparison to a calibration curve.

Cellular Internalization of LXR Agonist—Differentiated THP-1 macrophages were incubated with 1cM at 10^{-6} M and/or 10^{-7} M agonist for 5 hr in serum-free RPMI at 37 °C. Cells were washed and fixed and examined under multiphoton microscopy to detect internalized LXR agonist (agonist) on a Leica TCS SP2 system (Leica Microsystems, Inc., Exton, PA). The cells were illuminated using a titanium: sapphire femtosecond laser with a tunable wavelength set at 780 nm excitation (Mai Tai, repetition rate 80 Mhz, 100 fs pulse duration, 800 mW) and 470–500 nm emission.

Biological Activity of LXR agonist Delivery: Gene Expression Studies—The mRNA levels of key downstream genes targeted by the LXR agonist were quantified using QRT²-PCR based gene expression analysis. The RNA of the samples was extracted with the RNeasy Mini kit from Qiagen. Briefly, cells were lysed with β -mercaptoethanol and QiaShredder before extraction of and purification of the samples. Reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. DNA expansion of lipid transport and inflammatory genes was performed with Quantitative RT²-PCR conducted on a Roche plate reader 480 with a custom PCR array from SA Biosciences and β -actin as a housekeeping gene (SA Biosciences, Frederick, MD).

oxLDL Internalization by Macrophages—The internalization of oxLDL by macrophage cells was assayed by incubating BODIPY labeled oxLDL (10 $\mu\text{g}/\text{mL}$) with cells for 24 hours at 37 °C and 5% CO_2 . Conditions included a control of RMPI-1640 medium, 1cM alone (10^{-6} M), non-encapsulated agonist (10^{-7} M) admixed with 1cM (10^{-6} M), and encapsulated 1cM[agonist] (10^{-6} M/ 10^{-7} M). The cells were then washed twice with 1xPBS and imaged on a Nikon Eclipse TE2000-S fluorescent microscope to determine fluorescently tagged oxLDL attachment. The images were analyzed with Image Pro Plus 5.1 software (Media Cybernetics, San Diego, CA) and fluorescence was normalized to cell count and medium alone condition.

Depletion (Efflux) of oxLDL Pre-loaded Cells—The 1cM[agonist] rescue of macrophage cells was quantified following pre-incubation of cells with excess oxLDL. Macrophage cells were incubated with BODIPY-labeled oxLDL (10 $\mu\text{g}/\text{mL}$) and 5 % FBS serum for 2 hours at 37 °C and 5 % CO_2 . Excess oxLDL solution was then removed from all conditions and the test conditions, in the presence of 5% FBS, were added and incubated for 24, 48, 72, 96 and 120 hours at 37 °C and 5 % CO_2 . A control condition included RMPI-1640 medium with no additional oxLDL added. The remaining conditions each contained additional oxLDL (10 $\mu\text{g}/\text{mL}$); 1cM (10^{-6} M), agonist (10^{-7} M), non-

encapsulated agonist (10^{-7} M) admixed with 1cM (10^{-6} M), and encapsulated 1cM[agonist] (10^{-6} M/ 10^{-7} M). At the desired time point the cells were fixed with 3.7 % formaldehyde and washed twice with 1xPBS and imaged on a Nikon Eclipse TE2000-S fluorescent microscope to determine fluorescently tagged oxLDL attachment. The images were analyzed with Image Pro Plus 5.1 software (Media Cybernetics, San Diego, CA) and fluorescence data normalized with cell number and compared to the control samples.

***In vivo* Studies of Efficacy of Agonist and 1cM Macromolecules**—Four groups of male Sprague Dawley rats at twelve weeks of age were used in this study: 1cM at 10^{-3} M, agonist at 10^{-4} M, 1cM[agonist] at 10^{-4} M agonist and 10^{-3} M 1cM, and a control condition with no agonist or 1cM. All groups were fed a high fat diet (20 % fat Purina chow) for two weeks prior to surgery where they had their right carotid artery injured through the crush method. [23] Briefly, an arterial clip was placed 1 cm below the bifurcation point. A serrated hemostat was then tightly closed on the carotid artery, held for 5 seconds, opened and repositioned so that the entire area between the arterial clip and bifurcation point was injured. After the injury was complete the arterial clamp was removed and an arterial wrap made of collagen type I and parafilm was applied adventitiously around the blood vessel and the wound closed. After 96 hours the animals were sacrificed by formalin perfusion and the injured right artery and non-injured left artery were analyzed for macrophage recruitment and cholesterol accumulation. Oil Red O staining for cholesterol content and CD68 antibody staining for macrophage recruitment was quantified with Image Pro Plus 5.1 software (Media Cybernetics, San Diego, CA) and data normalized with tissue area.

Statistical Analysis—Error bars on graphs indicate standard error of the mean based upon biological triplicate samples in each *in vitro* experiment with three separate experiments for each condition; *in vivo* experiments included two animals per condition with three tissue sections examined per animal. Single factor ANOVA, performed Excel's data package software, was used for statistical analysis. Significance is claimed for differences of $p < 0.05$.

Results

Macromolecule Binding to Scavenger Receptor A

The macromolecules, composed of poly(ethylene glycol) (PEG) conjugated to a mucic acid backbone, derivatized with aliphatic side-chains self-assemble in aqueous media to form nanoscale micelles at the low CMC of 10^{-7} M. Four variants (Figure 2A) that have proven effective in terms of inhibition of oxLDL binding and internalization [15, 22, 24], and have been shown to specifically bind to the macrophage scavenger receptor SR-A via modeling [25], were chosen to investigate further. While the role of SR-A had been implied in past studies, the direct binding of the macromolecules to the receptor *in vitro* had not been explored. Human embryonic kidney (HEK) cells stably transfected with human scavenger receptor A, which are referred to as HEK-SRA, were used to assay SR-A binding. While all of the macromolecules were able to bind to SR-A and prevent the accumulation of oxLDL to some degree, 1cM, the carboxy-terminated amphiphilic macromolecule, was able to reduce oxLDL maximally, and to nearly the same levels seen in basal HEK cells (where the expression of SR-A had not been induced) (Figure 2B). For the remainder of the studies 1cM (abbreviated as “polymer”) was chosen to investigate further as the macromolecule best able to target SR-A and compete with oxLDL.

Macrophage Binding to Activated Macrophages

Two variants of macromolecule chemistries were utilized for binding to *activated* inflammatory cells, macrophages; the carboxy-terminated amphiphilic macromolecule 1cM,

the optimal macromolecule configuration in the 10 macromolecule library as determined by oxLDL uptake, computer modeling and HEK-SRA binding studies,[15, 25] and an uncharged control (denoted as 0cM). Significantly greater 1cM was observed in TNF- α activated macrophage cells (59% increase over basal cells; $P \leq 0.05$; $n = 3$), which have been shown to exhibit increased expression of SR-A (Figure 2C)[26]. When macrophages were pre-treated with an SR-A antibody, the 1cM binding decreased to non-specific levels, nearly equivalent to those seen with the uncharged control. Moreover, when TNF- α treated THP-1 cells were exposed to SR-A blocking antibodies, the levels of 1cM uptake were reduced to non-specific binding levels.

Cellular Uptake of LXR Agonist via SR-A

Carboxy-terminated macromolecule, 1cM, was used to encapsulate GW3965, a liver X receptor agonist (Figure 3A,B). Free agonist and 1cM-encapsulated agonist (1cM[agonist]) were incubated with THP-1 macrophages in order to elucidate the ability of the cells to bind and internalize the drug agonist (Figure 3C). Internalization of agonist at 10^{-7} M in the absence of 1cM was over six-fold lower than that quantified with delivery via encapsulated agonist (82% decrease; $P \leq 0.05$; $n = 3$). The uptake of free agonist by cells remained low and was unaffected by SR-A blocking or TNF- α pretreatment. However, macromolecule-encapsulated agonist delivery increased after TNF- α treatment and was significantly inhibited by the administration of SR-A antibody (37% increase and 88% decrease respectively; $P \leq 0.05$; $n = 3$). This finding paralleled the trends seen for uptake of the macromolecule alone (Figure 2C) and confirmed that agonist delivery is enhanced through macromolecule encapsulation and binding via the scavenger receptor SR-A.

Atherosclerotic Gene Expression

The agonist GW3965 was incubated at various concentrations with THP-1 cells over 24 hours to quantify the dose dependent upregulation of ATP-binding cassette 1 (ABCA1), as a model gene (Figure 3D). When the agonist at 10^{-7} M concentration was presented encapsulated within 1cM, nearly 14-fold upregulation in ABCA1 gene expression was measured compared to 7-fold increase caused by agonist alone ($P \leq 0.05$; $n = 3$). Further, free agonist alone used at even higher concentrations (10^{-5} and 10^{-6} M) failed to elicit the levels of gene upregulation seen with macromolecule-based delivery.

The agonist, both free and 1cM-encapsulated, was incubated with cells over 24 hr in order to profile the variation in THP-1 macrophage gene expression of six anti-atherosclerotic, inflammatory and cholesterol-associated genes (ABCA1, APOA1, APOE, NR1H3, PPARG and RXRA) (Figure 3E). Incubation of 1cM or agonist alone did not cause a significant change in gene expression for basal or TNF- α treated THP-1 macrophages. The addition of 1cM-encapsulated agonist to macrophage cells resulted in a 5-fold or more upregulation of five genes ABCA1, APOA1, APOE, NR1H3 and PPARG ($P \leq 0.05$; $n = 3$). The sixth anti-atherosclerotic gene tested, RXRA, showed a 3-fold upregulation ($P \leq 0.05$; $n = 3$). The administration of 1cM-encapsulated agonist also caused a 6-fold upregulation in SR-A expression ($P \leq 0.05$; $n = 3$).

Oxidized LDL Accumulation in Activated Macrophages

The agonist, free or 1cM-encapsulated, was incubated with TNF- α treated THP-1 macrophages in order to elucidate the ability of the macromolecule-encapsulated agonist to prevent the binding and internalization of oxLDL (Figure 4A). The addition of 1cM to human macrophage cells in the presence of oxLDL caused a significant decrease in cholesterol uptake after 24 hrs in comparison to control condition of no macromolecule or drug intervention (73% less oxLDL than no intervention; $P \leq 0.05$; $n = 3$). Introduction of agonist to cells did not significantly decrease cholesterol content unless 1cM was admixed

with the ligand (55% less oxLDL than no intervention; $P \leq 0.05$; $n = 3$). The most significant decrease was observed when the agonist was encapsulated within 1cM and incubated with macrophages (88% less oxLDL than no intervention; $P \leq 0.05$; $n = 3$).

Macrophage-Preloaded Cholesterol Depletion

The efficacy of 1cM and agonist treatment on cholesterol depletion within macrophages preloaded with oxLDL was assessed (Figure 4B,C). The addition of 10^{-7} M agonist alone to oxLDL-preloaded macrophages did not significantly alter oxLDL content of cells over any of time periods studied (24, 48, 72, 96 and 120 hrs). 1cM alone and 1cM admixed with free agonist each showed an insignificant decrease in overall oxLDL content at all time points. In contrast, after 120 hr, the 1cM-encapsulated agonist reduced the cholesterol content of the cells to significantly lower levels than those observed in control condition, wherein no additional oxLDL was added after the pre-incubation period (45% reduction in cellular oxLDL compared to 60%; $P \leq 0.05$; $n = 3$).

Intimal Cholesterol Accumulation *In Vivo*

An *in vivo* model was established to investigate the efficacy of localized agonist delivery. Male Sprague Dawley rats were raised on a high fat diet before a crush injury to the right carotid artery and a collagen wrap loaded with 1cM (with or without agonist) or free agonist or PBS was inserted (see supplementary Figure S3 for more details). All arteries were noted to be patent at time of harvest. At the early time point of the study, despite vessel injury, no intimal hyperplasia was identified. After 96 hrs, the animals were sacrificed and the injured right artery and non-injured left artery were analyzed for cholesterol accumulation (Figures 5A,C). Animals treated with 1cM exhibited a 50% decrease in cholesterol accumulation relative to non-treated controls (PBS) in injured rat carotid arteries. The addition of the 1cM encapsulated agonist further decreased cholesterol accumulation as compared to the 1cM treated and non-treated controls. (70% less cholesterol than injured PBS control; $P \leq 0.05$; $n = 3$). Notably, 1cM-encapsulated agonist treatment decreased cholesterol content to the same level as non-injured arterial sections.

Macrophage Recruitment *In Vivo*

Using the same *in vivo* model, we examined the impact of 1cM and the encapsulated agonist on macrophage recruitment. The animals were sacrificed after 96 hr and the injured right artery and non-injured left artery were analyzed for macrophage recruitment (Figures 5B,D). Animals treated with 1cM alone and 1cM-encapsulated agonist displayed significantly fewer recruited macrophages in injured arteries (92% and 96% fewer macrophages than injured PBS control; $P \leq 0.05$; $n = 3$). Notably, treatment with 1cM-encapsulated agonist decreases cholesterol content to the same level as non-injured arterial sections. In contrast, free agonist led to no detectable decrease in macrophage recruitment.

Discussion

Effective therapies for cardiovascular disease require the coordinated management of atherogenesis and inflammation [1, 8, 27]. Major factors underlying the chronic progression of atherosclerotic plaques are the activation and recruitment of macrophages to the vascular injury site, followed by uncontrolled scavenger receptor-mediated internalization of oxLDL [8, 28–30]. Several different molecular interventional approaches have been proposed including scavenger receptor inhibitors or knockdowns, enzymatic inhibitors and knockdown, and inhibitors of macrophage recruitment [4–6, 9].

Here we have proposed a dual-pronged approach to attenuate inflammation and cholesterol accumulation. The approach involves the design of macromolecules that exhibit scavenger

receptor-mediated targeting of inflammatory cells combined with activation of cholesterol-efflux via nuclear transcriptional factor LXR [31, 32].

Scavenger receptors are upregulated at sites of atherosclerotic lesions [33, 34]. Carboxy-terminated amphiphilic macromolecules derived from sugar and PEG, can self-assemble into micelles, which bind to macrophage scavenger receptors SR-A and CD36 [35]. The carboxylic acid end groups of the key macromolecule are hypothesized to mediate electrostatic binding to the positive pocket of residues on the SR-A scavenger receptor [36]. The polymer structure shows enhanced binding to SR-A; the carboxylic acid group is able to bind the positive residues of the binding pocket and the aliphatic arms are able to stabilize and enhance binding. These two domains in close proximity, while allowing for flexibility without crowding, provide the most efficient receptor binding, as shown in our previous molecular modeling studies [25]. The increased expression of scavenger receptors in TNF- α activated macrophages further promotes macromolecule binding, allowing for directed delivery of the macromolecule and its encapsulate to activated macrophages. Additionally, the charged macromolecules had intrinsic anti-inflammatory activity on both cytokine and MMP-9 secretion, two of the major inflammatory markers of macrophage activation [37–40]. The unique combination of amphiphilicity, geometry and charge was integral to this behavior as none of the control macromolecules had comparable anti-inflammatory activity. Hence, the charged macromolecule identified could serve as effective therapeutic candidate for inflamed macrophage targeting *in vivo*.

Liver X receptors (LXR) are implicated in the atherogenic process underlying cardiovascular disease [41]. As the LXR- α has been shown to be activated by synthetic agonist GW3965 [14, 42, 43], we examined the ability of the key charged macromolecule to encapsulate and deliver the hydrophobic agonist with specificity to activated cells. This work demonstrates that increased efficacy of agonist, even at lower concentrations, can be achieved via macromolecule encapsulation (Figure S2). Our studies employing direct 2-photon imaging of cell-internalized agonist confirmed that 1cM was internalized within the cytosol; this cellular internalization allows for drug delivery to the nucleus, where ligand binding is essential for enhanced LXR-signaling, as evidenced by the upregulation of key gene targets.

Macromolecule-based agonist delivery elicited a 9-fold increase in LXR α gene and a 15-fold increase in ABCA1 after delivery of 10^{-7} M GW3965, while a study by Albers et al. showed that treatment with free GW3965 even at a higher concentration of 10^{-6} M elicited only modest levels of upregulation of LXR α (3-fold) and ABCA1 (6-fold) [44]. Clearly, macromolecule encapsulation greatly enhanced agonist delivery, via scavenger receptor binding, compared to the non-encapsulated free agonist conditions, resulting in significant alteration in six key atherosclerosis associated genes examined here. Typically, elevated SR-A expression is believed to escalate atherosclerosis progression through increased oxLDL accumulation [45]. Given the enhanced binding of macromolecule-encapsulated agonist to the macrophage SR-A, this system of upregulated receptor expression affords a molecular route for enhanced macromolecule and ligand delivery to inflamed cells. Further, the up-regulation of LXR related genes was also accompanied by enhanced anti-atherogenesis related signaling, and enhanced rescue of cells pre-loaded with oxLDL and under continuing exposure to oxLDL, determined by both cellular concentration of oxLDL, and total cholesterol accumulation (see supplementary Figure S2). These trends illustrate the potential ability of the macromolecule-encapsulated agonist complexes to inhibit atherosclerosis at the gene signaling level as well as via receptor blockage, which opens possibilities for depleting as well as retarding the accumulation of cholesterol.

The *in vivo* model probed the accumulation of cholesterol following localized vascular injury to the carotid artery. Both macromolecule and macromolecule-encapsulated agonist caused significant inhibition of cholesterol accumulation in comparison to the drug and non-treated samples. A notable finding was that the addition of both macromolecule alone and macromolecule-encapsulated agonist significantly inhibited (92% and 96%, respectively) the presence of macrophage cells near the site of injury, which has been reported to be an important atherosclerosis marker independent of cholesterol content [8, 9]. Macrophage recruitment is regulated by factors such as TNF- α and interleukin 1 beta (IL-1 β) [8, 27, 46]. The *in vivo* studies generated bleeding and non-occlusive thrombus formation at the injury site however a marked reduction in macrophage recruitment was observed with macromolecule and macromolecule-encapsulated agonist, suggesting that the macromolecule therapeutics had an inhibitory effect on the activation of recruited macrophages and possibly the further recruitment of monocytes, which is consistent with our *in vitro* studies showing macromolecule-mediated attenuation of TNF- α secretion and IL-1 β secretion (see Figure S4). The changes observed for *in vivo* macrophage recruitment/retention raise the possibility of not only targeting activated macrophages but also modulating their inflammatory phenotype in concert with regulation of atherogenesis.

Conclusions

We demonstrate the feasibility of a dual-pronged design of nanoscale polymers as bioactive materials for targeted therapy to counteract macrophage-engendered atherosclerosis mechanisms. The optimal hydrophobicity and charge of the macromolecules, targeted inflamed macrophages and caused partial blockage of scavenger receptors, which lowered oxidized low density lipoprotein (oxLDL) “influx” within macrophages. Concertedly, the hydrophobic core of the amphiphilic polymers was used to solubilize and deliver a bioactive pharmacologic factor (liver X receptor agonist) to nuclear receptors within inflamed macrophages—this drug delivery approach led to further depletion of accumulated oxLDL within the cells and was significantly more efficacious than the conventional drug controls. A localized release of bioactive nanopolymers *in vivo* showed significant reduction in macrophage recruitment and cholesterol accumulation within areas of athero-inflammation. Thus, our study highlights the possibility of combining synthetic polymer-based therapeutics with drug delivery for targeting multifocal vascular disease, a finding that could be potentially extended to the targeted management of inflammatory and tissue degenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Overview of multifunctional nature of nanosized biomaterials that can be conceived to target inflamed macrophages (via scavenger receptors) and inhibit cholesterol (oxLDL) accumulation by engaging in two cooperative pathways: inhibition of oxLDL uptake; and triggering efflux of internalized oxLDL through intracellular agonist delivery. This article highlights the feasibility of designing a charged amphiphilic macromolecule and its targeted inhibition of macrophage atheroinflammation both *in vitro* and *in vivo*.

Figure 2.

The carboxylic acid-terminated amphiphilic macromolecules 1cM were selected from a subset of the macromolecular library for high degree of scavenger-receptor targeting and anti-inflammatory activity. A) Amphiphilic macromolecules with four diverse features were screened based on differential presentation of anionic charge (location, density, rigidity). 1cM exhibits carboxylic acid display at the mucic acid end; 0cM is neutral, while 1cP exhibits carboxylic acid at the PEG end. 2cbM represents display of two carboxylic acid groups from a rigid, aromatic backbone. B) The 1cM macromolecules showed the greatest SR-A targeted inhibition of oxidized LDL accumulation in HEK-SRA cells induced to express SR-A. The 1cM-inhibition was comparable to that seen in HEK-non expressing cells, indicating the mode of macromolecule action is via SR-A. Thus, the 1cM macromolecule was chosen as the most SR-A specific polymer for the remainder of the study. C) The role for 1cM polymers (Texas red-labeled) in binding to activated macrophages was quantitatively evaluated in THP-1 macrophages in the presence and absence of TNF- α preactivation. Blockage by anti-SR-A antibody confirmed a prominent role for receptor targeting properties of 1cM (but not uncharged control, 0cM) following TNF- α activation.

Figure 3.

Carboxy-terminated macromolecule, 1cM, was used to encapsulate GW3965, a liver X receptor agonist. A) GW3965, when encapsulated in the 1cM polymer, exhibits linear loading and B) does not increase micelle size. In addition, the absorbance of the agonist drug is shielded when encapsulated within the polymer, indicating loading within the micelle core. The 1cM macromolecules enhance delivery and bioactivity of agonist (GW3965) in THP-1 macrophages. C) Agonist showed low levels of cellular uptake within both quiescent and activated THP-1 macrophages; this uptake is not mediated by scavenger receptor SR-A. In contrast, polymer-encapsulation of agonist increased agonist internalization in quiescent macrophages, and enhanced internalization in activated macrophages. D) 1cM macromolecules were used for agonist encapsulation and delivery. Polymer based agonist delivery (10^{-7} M) significantly upregulated ABCA1 gene expression in contrast to the lower efficacy of free agonist evaluated over two orders of magnitude concentrations (14- fold change, $P \leq 0.05$; $n = 3$). The 10^{-7} M agonist dosing for polymer encapsulation was selected and used throughout the study (see Figure S1 Supplementary Information). E) RT-PCR Arrays were used to screen expression of seven key atherosclerotic genes. The delivery of polymer-encapsulated agonist consistently upregulated all genes compared to that elicited by free agonist, free 1cM or admixed 1cM and agonist ($P \leq 0.05$; $n = 3$).

Figure 4.

Polymers (1cM macromolecule), with encapsulated agonist (polymer[agonist]) progressively lowered oxLDL content of THP-1 macrophages. A) OxLDL uptake in THP-1 macrophages: 1cM[agonist]), resulted in a 88% decrease in total oxLDL within cells after a 24 hr incubation period. B) Cells preloaded for 18 hrs were markedly rescued by 1cM[agonist]) over the course of 5 days compared to untreated, oxLDL preloaded cells incubated with media alone (45% reduction in cellular oxLDL compared to 60%; $P \leq 0.05$; $n = 3$). Cell rescue is accomplished through a combination of decreased LDL influx and increased LDL efflux; controlled through polymer attachment and agonist delivery. C) Comparison of BODIPY-oxLDL (green) fluorescence 24 hrs and 120 hrs of 1cM[agonist]) treatment imaged using 2-photon microscopy in THP-1 macrophages counterstained with nuclear DAPI (blue).

Figure 5.

Effect of polymer-encapsulated agonists on cholesterol accumulation and macrophage recruitment *in vivo*. Polymer (1cM macromolecule) was delivered via an adventitial collagen wrap. A and C) a) non-injured control b) PBS control c) agonist d) 1cM e) 1cM[agonist]. Carotid artery cholesterol content 4 days after crush injury and treatment application through adventitial wrap placement, 1cM alone and 1cM[agonist] significantly lowered cholesterol content (50% and 70% reduction respectively compared to injured PBS control; $P \leq 0.05$; $n = 3$). B and D) a) non-injured control b) PBS control c) agonist d) 1cM e) 1cM[agonist]. Macrophage recruitment to the site of injury is greatly reduced by the addition of polymer and to a greater degree with 1cM[agonist] (92% and 96% fewer macrophages than injured PBS control; $P \leq 0.05$; $n = 3$).