DEVELOPMENT OF TARGETED NONIONIC SURFACTANT VESICLES FOR TREATMENT OF VASCULAR INJURY

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ABSTRACT

Inflammatory processes play a crucial role in cardiovascular injury and disease. Inflamed endothelial cells express adhesion molecules which are potential therapeutic delivery targets for reducing or disrupting inflammation. We have developed non-ionic surfactant vesicles (niosomes) that are specifically targeted to inflamed vascular cells. Niosomes are self assembly vesicles that can encapsulate therapeutic agents and can be delivered to an injured subject via an injection, through skin or in a gel or patch. In this paper, we will describe the synthesis of the niosomes, results of studies to determine binding to injured/inflamed endothelial cells, efforts to encapsulate anti-inflammatory agents, and initial animal data.

1. BACKGROUND

Non-ionic surfactant vesicles (niosomes) are analogous to, but have advantages over, liposomes as drug carriers. Some advantages include greater chemical stability, lower cost, easier storage and handling, and a reduced likelihood of becoming toxic through oxidation. They are typically composed of synthetic surfactants, cholesterol and dicetyl phosphate (DCP, an electrostatic stabilizer). Niosomes can encapsulate aqueous solutions of drug in the core, and lipophilic molecules can be inserted into the bilayer membrane. Encapsulation of a drug can prolong its circulation time, lower the therapeutic dose, and reduce toxicity to untargeted tissues.

We have developed a method to modify the surface of the niosome to allow for the attachment of monoclonal antibodies (mAbs), which allow for site-specific targeting. A key step in inflammation is the recruitment of leukocytes from the blood stream to the site of injury or disease. This is mediated by cellular adhesion molecules (CAMs) expressed on the cell surface. For the treatment of vascular injury, we have chosen to target the adhesion molecule CD44. Isoforms of CD44 comprise a family of adhesion molecules that mediate leukocyte-endothelial cell adhesion (DeGrendele et al. 1996), and thus play an important role in inflammation. There are at least 10 standard isoforms and 10 variant isoforms of CD44. Certain isoforms are highly expressed in the smooth muscle cells of the intima and media of injured arteries (Libby 2002) and others on the endothelial cells (Koopman et al. 1998). Isoforms of CD44 are also expressed on macrophages (Krettek et al. 2004). All isoforms are highly regulated by cytokines (Krettek et al. 2004).

In this paper we describe the synthesis of the targeted niosome, experiments to verify the binding of the niosome to an inflamed endothelial cell, and an animal study to investigate the effects of targeted niosomes encapsulating an anti-inflammatory agent on atherosclerosis. In early atherosclerosis, CAMs expressed on the vascular endothelium and on circulating leukocytes recruit inflammatory cells and facilitate their transport across the endothelium. Interruption of the inflammatory process has been studied using the cell surface adhesion receptor CD44 blocked by antibody clone IM7. Dramatic suppression of inflammation was achieved through the highly selective binding of the antibody-antigen couple (Mikecz et al. 1999). Expression of CD44 and variants was augmented when exposed to pro-inflammatory cytokines within human atheroma, connecting CD44 expression with arterial diseases (Krettek et al. 2004). CD44 was further implicated in the progression of atherosclerosis when atherosclerotic prone ApoE-deficient mice bred with CD44-null mice showed a 50-70% reduction in aortic lesions versus CD44 heterozygous and wild type mice (Cuff et al. 2001). Although atherosclerosis is a chronic inflammatory process and therefore may be different from that of injury, this study would serve to demonstrate therapeutic delivery to a specific site of inflammation. Furthermore, manipulating the immuno-chemistry of pathogenic inflammatory cells, targeting ligands specific to reducing inflammatory response or disrupting an inflammatory cascade while concomitantly providing pharmaceutical therapeutics, could prove valuable in combating atherosclerosis and cardiovascular disease.

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2. METHODS

2.1 Synthesis of Niosomes

Niosomes are synthesized by a thin film hydration technique described classically (Baillie et al. 1985) using a rotary evaporator followed by agitation and sonication using a bath sonicator. Typically niosomes are composed of a 1.0:1.0:0.1 molar ratio of surfactant: cholesterol: DCP as this forms the most stable particle (Yoshioka et al. 1994). We use a mixture of the surfactants: sorbitan monostearate (Span 60) and polyoxyethylene sorbitan monostearate (Tween 61). To produce the fluorescent images that are presented in this paper, a dye (typically, either 5(6)-carboxyfluorescein or 5(6)-carboxyrhodamine) is encapsulated in the niosome during the hydration step. The encapsulation of other compounds is discussed subsequently.

We have developed a process to modify the Tween 61 molecule to include a linker for the attachment of mAbs, based on analogous work with liposomes (Bendas et al. 1999; Hood et al. 2007). Polyethylene oxide groups on the polar head of Tween 61 are combined with cyanuric chloride (CC) in the presence of di-isopropylethylamine (DIPEA). The resulting molecule, "Tween-CC" is added to the surfactants and lipid mixture prior to forming the thin film. The mAb is incubated with the niosomes containing the Tween-CC linkers. In this way, the mAb is bound to the niosome without prior derivatization or modification to the antibody itself. The niosome containing the mAb is referred to here as an "immuno-niosome".

Niosomes are separated from unencapsulated compounds and unformed lipids by passing the suspensions through a gel exclusion chromatography column. The size of the niosomes is finely controlled by use of an extruder, in which niosomes are forced under pressure through polycarbonate filters with a particular pore size. Based on reports in the literature for cellular uptake of other types of particles, including liposomes, 200 nm is an ideal size for uptake (Chono et al. 2005). Figure 1 presents data from a dynamic light scattering system (Nanosizer S, Malvern Instruments) demonstrating the ability to achieve this size upon several passes through the extruder. The optimal size for our particle is yet to be determined. The results shown in the following sections are for niosomes varying in size from 100 to approximately 400 nm.

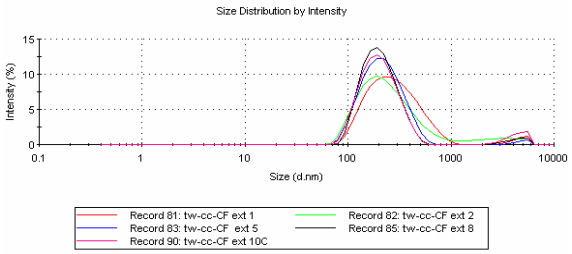


Figure 1. Dynamic light scattering data of extruded niosomes. The number following "ext" is the number of passes through the extruder. Figure from Hood 2007.

A flow diagram summarizing the process for synthesizing immuno-niosomes is depicted in Figure 2.

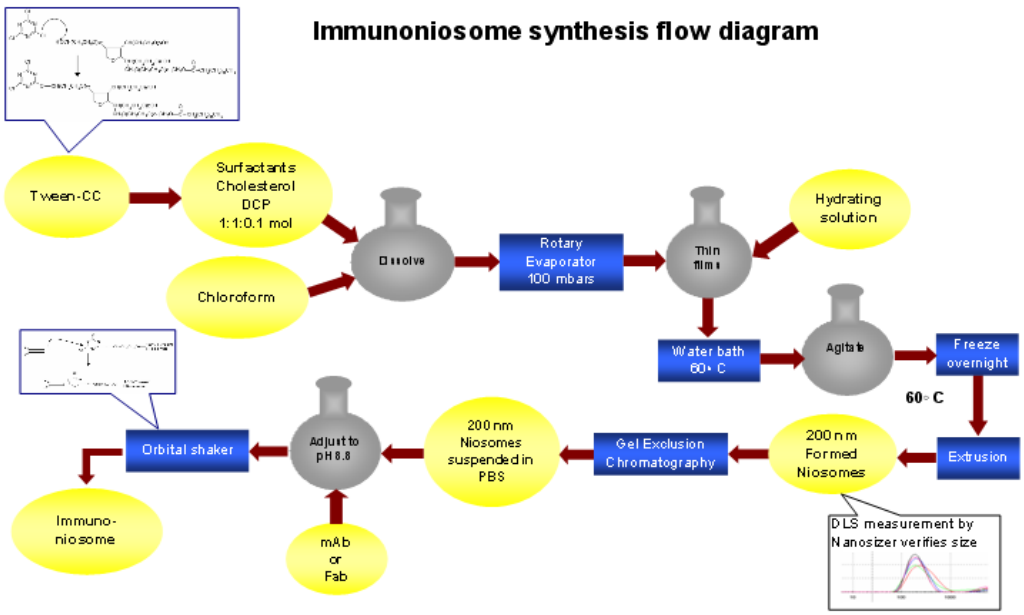


Figure 2. Process flow diagram for synthesis of "immuno-niosomes".

2.2 Binding of Immuno-niosomes to Endothelial Cells

Experiments were performed in static culture and then under physiologic flow conditions to verify binding of the targeted immuno-niosomes to bovine endothelial cells (BAECs) activated by a pro-inflammatory cytokine, tumor necrosis factor (TNF)-α.

In the static cell culture system, BAECs were grown to near confluence using standard sterile tissue culture techniques. A day prior to incubation with immuno-niosomes, fresh media supplemented with 7 ng/ml of TNF-α was added to the culture to activate the cells and induce the expression of cell adhesion receptor CD44. Controls included non-targeted niosomes, and cells in which the targeted receptors were blocked prior to treatment with the niosomes. The cells were fixed overnight with either Histochoice, for immunohistochemical staining, or with paraformaldehyde, for fluorescence imaging. Cell nuclei and membranes were counter-stained with fluorescent dye to show immuno-niosome orientation with respect to the cell nucleus. A subset of cells was incubated with gold-particle containing immuno-niosomes for transmission electron microscope (TEM) imaging.

Next, a flow chamber and pump was built to study relationships between niosome size, flow rate, and amount of binding. This flow chamber is similar to that used extensively to examine the effects of shear stress on endothelial cells (Frangos et al. 1988). Cells are placed in a flow chamber in which laminar, developed flow can be established, at physiologic shear rates. Cells can be exposed to a single-pass, bolus injection of niosomes or to a continuous, recirculating flow.

2.3 Effects of Immuno-niosomes in an Animal Model

All animal research is conducted in compliance with the Animal Welfare Act Regulations and other Federal statutes relating to animals and experiments involving animals and adhere to the principles set forth in the Guide for Care and Use of Laboratory Animals, National Research Council, 1996. Prior to initiation of animal research, approval was obtained from the University of South Florida's Institutional Animal Care and Use Committee as well as the USAMRMC Animal Care and Use Review Office.

Strain B6.129P2-*Apoe*^{tm1Unc}/J (apoE deficient; The Jackson Laboratory) male mice were fed a lipid-rich Western-type diet (Harlan Teklad TD88137) for 23 weeks. We investigated the effect of delivering immuno-niosomes, targeted to CD44, encapsulating a statin. Statins have lipid lowering effects, but also potentially anti-inflammatory effects on the atherosclerotic plaque independent of the lipid lowering effect (Kircher et al. 2008; Zadelaar et al. 2007) Table 1 shows the treatments the mice received.

Table 1

Treatment Group (n=6 for all)	Treatment
Test (low)	1. Surfactant vesicle coated with antibody and containing drug atorvastatin (test substance-low drug concentration)
Test (high)	2. Surfactant vesicle coated with antibody and containing atorvastatin (test substance-high drug concentration)
Control (targeted no drug)	3. Surfactant vesicle coated with antibody and containing buffered saline solution
Control (non targeted with drug)	4. Surfactant vesicle without antibody containing atorvastatin
Control (free drug low)	5. Free drug atorvastatin low concentration (control)
Control (free drug high)	6. Free drug atorvastatin high concentration (control)
Control (vehicle)	7. Buffered saline (control)

Prior to the administration of the first treatment dose, the mice were fasted for four hours (water ad lib.) and blood was collected from the submandibular vein into lithium heparin tubes for plasma isolation. All the mice received treatments by intravenous injection, two times a week for four weeks. At the conclusion of the study, the mice were fasted for four hours (water ad lib.), euthanized by carbon dioxide inhalation and cervical dislocation (USF Division of Comparative Medicine SOP 401.1) and blood was collected by percutaneous cardiac puncture. Plasma was analyzed for total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides on a Cobas Mira Plus analyzer (Yale Mouse Metabolic Phenotyping Center, Yale University School of Medicine, New Haven, CT).

The lungs, heart, liver, kidneys and spleen were dissected and placed in 10% neutral buffered formalin (NBF) followed by standard tissue processing and embedding in paraffin blocks for histo- and immunohistochemical analyses. The heart was embedded in paraffin in the transverse plane and four levels of serial 4 μm-thick sections were cut and collected every 100 μm through the aortic root. The aortic root sections were stained with hematoxylin and eosin (H&E) to determine the histomorphology of the atherosclerotic plaques. Aortic roots sections were also stained for antibodies against granulocytes, macrophages and CD44. The ascending aorta and aortic arch to the descending aorta was fixed in 10% NBF, cryopreserved with sucrose, cut into 6-7 pieces and frozen in transverse cross-sectional orientation with OCT compound. For oil red O staining, 6 levels of serial 10 μm-thick sections were cut and collected every 100

μm and placed on Superfrost/Plus glass slides. The sections were post-fixed in 37-40% formaldehyde, stained with Harris H&E, dehydrated, cleared and mounted with permanent mounting medium.

3. RESULTS

3.1 Interactions between Immuno-niosomes and Cells

In the static cell cultures, immunohistochemical staining verified CD44 expression (Figure 3). These studies demonstrated binding with a high degree of selectivity and specificity (Figure 4). Binding was also verified by TEM (Figure 5). The amount of binding in static cultures was found to vary with incubation time, concentration of immuno-niosomes, and number of antibodies per niosome.

The flow studies demonstrated binding of the immuno-niosomes to TNF-α stimulated endothelial cells at physiologically relevant flow rates. Figure 6 shows fluorescent immuno-niosomes (green) attached to endothelial cells (with nuclei stained blue) under flow. A single bolus injection of immuno-niosomes was introduced into the flow stream, and immuno-niosomes bound to the cells during that single pass.

3.2 Results from the Animal Model of Atherosclerosis

Figures 7 and 8 show representative sections of the aortas and aortic roots of the mice. We are currently analyzing the images for lipid content (oil red O) and total plaque area using Image Pro Plus 6 (Media Cybernetics).

Table 2 shows lipid and body weight data for the animals at the end of the study.

4. CONCLUSIONS

A novel drug delivery particle, an "immuno-niosome" has been successfully synthesized. We have demonstrated that we can use this particle to target a specific site important in the inflammatory process, both in static cell culture and under flow.

The "immuno-niosome" merits further study for its potential as a drug delivery vehicle. Future work includes study of different compositions of the particle, and optimization of the size and antibody concentration on the particle surface. The current animal study will allow us to determine the fate of the drug delivery particle in the biologic system, both untargeted and targeted to specific sites of injury.

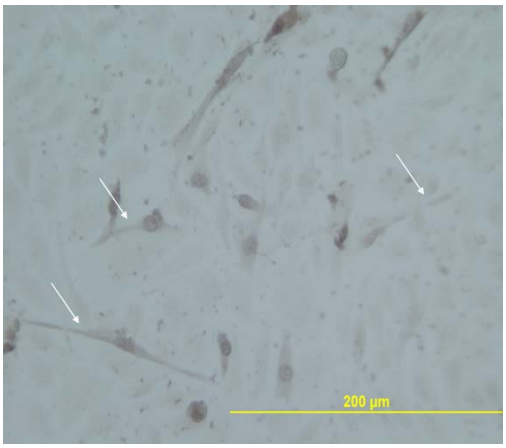


Figure 3. CD44 expression in cell membranes and processes is verified. Figure from Hood 2007.

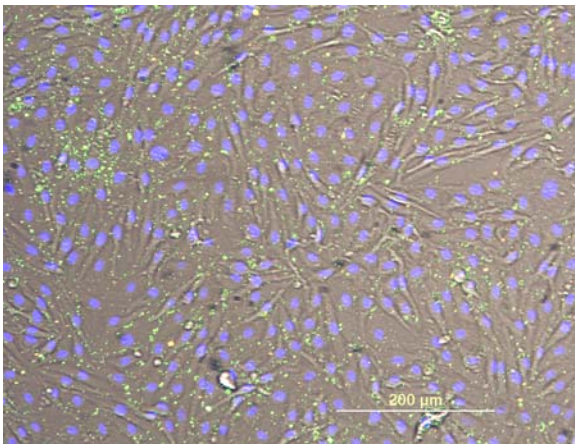


Figure 4. Fluorescent and contrast overlay of immunoniosomes (green) with BAECs (cell nuclei stained blue). Figure from Hood 2007.

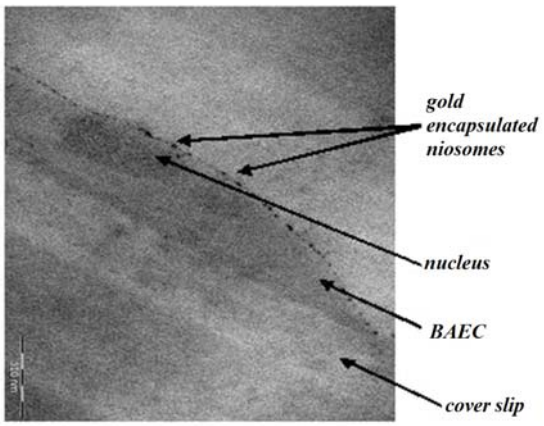


Figure 5. TEM image of immuno-niosomes, encapsulating gold nanoparticles, bound to a BAEC.

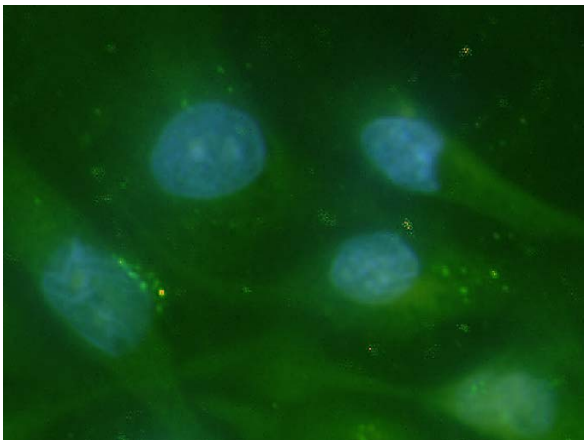


Figure 6. Activated BAECs (nuclei stained blue) in flow. Immuno-niosomes (green) are adhered to cell membranes and processes.

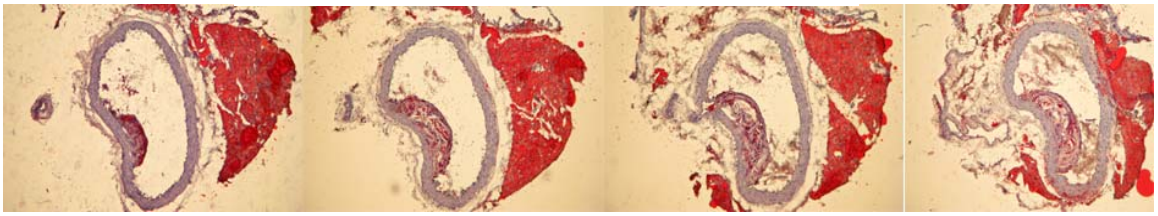
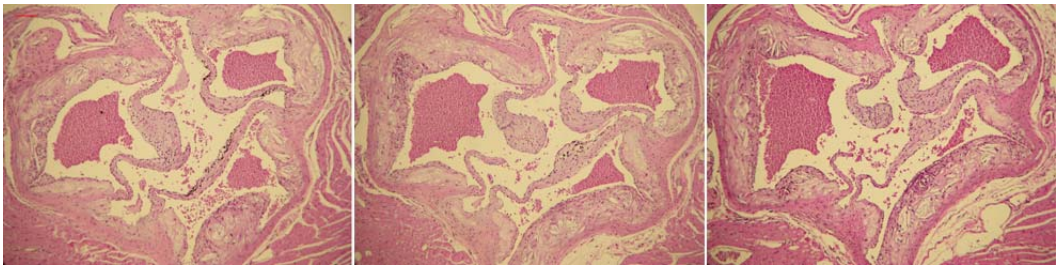


Figure 7: 100x magnification, oil red O staining and hematoxylin counter staining of apoE deficient mouse aorta; 10 µm thick sections cut in the transverse cross-sectional orientation in 100 µm levels (from left to right)



0 µm 50 µm 100 µm

Figure 8: 100x magnification, H&E staining of apoE deficient mouse aortic root; 4 µm thick sections cut in the transverse plane from the apex to base of heart in 50 µm levels (from left to right)

Table 2

Mean ± SD	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Treatment 7
Test	Test (low)	Test (high)	Control (targeted no drug)	Control (non-targeted with drug)	Control (free drug low)	Control (free drug high)	Control (vehicle)
Triglycerides (mg/dl)	134.8 ± 41.71	91.0 ± 83.4	83.4 ± 16.5	79.9 ± 20.2	91.2 ± 60.3	83.20± 41.9	58.3 ± 21.2
N	4	5	5	5	5	5	5
Total cholesterol (mg/dl)	1157.1 ± 445.6	814.7 ± 455.6	792.0 ± 308.7	562.5 ± 274.0	787.6 ± 337.7	832.1 ± 517.3	701.4 ± 276.2
N	4	5	6	5	6	5	5
HDL-direct (mg/dl)	15.8 ± 6.1	11.4 ± 4.1	12.4 ± 3.4	11.7 ± 3.5	10.6 ± 4.1	19.8 ± 11.0	12.5 ± 3.3
N	4	5	6	5	6	5	5
LDL-direct (mg/dl)	337.9 ± 205.8	201.0 ± 198.1	214.5 ± 162.2	166.0 ± 111.9	188.41± 156.3	183.0 ± 215.9	134.4 ± 81.6
N	4	5	5	5	6	5	5
BW (g)	30.3 ± 2.5	28.7 ± 3.3	27.3 ± 2.2	28.4 ± 3.8	26.4 ± 2.3	27.5 ± 6.8	26.3 ± 2.3
N	4	5	6	6	6	6	5

Lipid and body weight values from study end (wk 24)
Lipids analyzed on a Cobas Mira Plus at the Yale Mouse Metabolic Phenotyping Center, Yale University School of Medicine, New Haven, CT
LDL-direct reagents purchased from Roche; triglycerides, total cholesterol, HDL-direct cholesterol reagents purchased from Raichem.

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