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Immuno-targeting of nonionic surfactant vesicles to inflammation

Elizabeth Hood [a], Monica Gonzalez [a], Anna Plaas [b], Joel Strom [a,c], Michael VanAuker [a,*]

[a] *Biomedical Engineering Program, Department of Chemical Engineering, University of South Florida, Tampa, FL, United States*
[b] *Division of Rheumatology, College of Medicine, University of South Florida, Tampa, FL, United States*
[c] *Department of Medicine, College of Medicine, University of South Florida, Tampa, FL, United States*

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Abstract

Niosomes composed of sorbitan monostearate (Span 60), polyoxyethylene sorbitan monostearate (Tween 61), cholesterol, and dicetyl phosphate were conjugated with a purified monoclonal antibody to CD44 (IM7) through a cyanuric chloride (CC) linkage on the polyoxyethylene group of the Tween 61 molecule. Inclusion of small amounts of Tween 61 within the surfactant component of niosomes formed using thin film hydration techniques and sonication did not hamper vesicle stability as compared to Span 60 niosomes. Conjugation was verified by UV absorbance of fluorescently tagged IM7 in non-fluorescing niosomes and fluorescent micrographs. The immuno-niosomes were incubated with synovial lining cells expressing CD44. Attachment of niosomes was evident and showed selectivity and specificity compared to controls. These findings suggest that the resulting immuno-niosomes may provide an effective method for targeted drug delivery.

Keywords: Niosomes; Drug targeting; Surfactants; Antibodies

1. Introduction

1.1. Benefits of drug encapsulation and targeted drug delivery

Normal administration of drugs or therapeutic agents does not allow for concentrated accumulation of drug at diseased sites due to an essentially uniform distribution of drug throughout the body. In order to adequately treat affected sites using traditional systemic administration high dosages of drug must be delivered. This not only increases costs, but also can create toxic side effects as normal tissues and organs are needlessly exposed to pharmaceuticals (Torchilin, 2000). Encapsulation of drugs for passive targeting, either by liposome (Chono et al., 2005; Park et al., 2004; Lasic and Martin, 1995), niosome (Lu et al., 2003; Balasubramaniam et al., 2002; Gianasi et al., 1997; Baillie et al., 1986; Collins et al., 1993), or polymeric (Rapoport et al., 2003) media has shown increased retention time, decreased therapeutic dose, and reduced toxicity to unspecified tissues.

Drug targeting was originally conceived at the beginning of the 20th century by Paul Erlich who discovered antibodies and their role humoral immunity. He proposed the 'magic bullet' concept of using antibodies to send therapeutic agents to target cells (Torchilin, 2000). Not until the development of monoclonal antibody (mAb) production could his 'magic bullet' concept be realized. In the 1970s the B cell melanoma was identified as producing a single type of antibody (Waldmann, 2003) and the process of producing monclonal antibodies was developed (Kohler and Milstein, 1975). Later techniques were developed to humanize monoclonal antibodies for therapeutic uses (Riechmann et al., 1988).

1.2. Vesicular drug delivery

Niosomes are self-assembled vesicles composed primarily of synthetic surfactants and cholesterol (Baillie et al., 1985; Torchilin, 2000). They are analogous in structure to the more widely studied liposomes formed from biologically derived phospholipids. Vesicular drug delivery has been studied widely as a means to increase efficacy and reduce systemic toxicity in tumor targeting and cancer therapies (Xiong et al., 2005; Uchegbu et al., 1996; Sapra and Allen, 2003; Fonseca et al., 2005). Echogenic liposomes have been developed for

* Corresponding author.
E-mail address: vanauker@eng.usf.edu (M. VanAuker).

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cardiovascular applications that can perform targeted imaging of atheroma (Dayton and Ferrara, 2002; Hamilton et al., 2004) and deliver drugs to the site of plaque development (Tiukinhoy et al., 2004). Liposomes have been used to deliver fibrinolytics, such as streptokinase, and tissue plasminogen activator, and prostaglandin E1 (Feld et al., 1994; Heeremans et al., 1995; Nguyen et al., 1989).

1.3. Niosomes as drug carriers

Niosomes behave similarly to liposomes *in vivo* by prolonging circulation time of the encapsulated drug and altering chemical distribution within the body (Baillie et al., 1986; Azmin et al., 1985; Ruckmani et al., 2000). However, niosomes have advantages over liposomes as drug carriers, including greater chemical stability, lower cost, easier storage and handling, and are less likely than liposomes to become toxic (Uchegbu and Florence, 1995). Niosomal encapsulation reduces toxicity of drugs in many different applications and therapies. Niosomal drug delivery has been studied using various methods of administration (Blazek-Welsh and Rhodes, 2001) including intramuscular (Arunothayanun et al., 1999), intravenous (Pillai and Salim, 1999; Namdeo and Jain, 1999), peroral (Rentel et al., 1999), and transdermal (Uchegbu et al., 1995; Yoshioka et al., 1994). Nebulized surfactants entrapping all-*trans*-retinoic acid (ATRA) were delivered as an inhaled aerosol reducing the drug toxicity and altering the pharmacokinetics (Desai and Finlay, 2002). In addition, as drug delivery vesicles, niosomes have been shown to enhance absorption of some drugs across cell membranes (Lasic, 1993), to localize in targeted organs (Jain and Vyas, 1995; Namdeo and Jain, 1999) and tissues (Baillie et al., 1986; Azmin et al., 1985), and to elude the reticuloendothelial system (RES) (Gopinath et al., 2001). Cellular uptake of niosomes can be via endocytosis (Baillie et al., 1986); however they have been shown to bind and fuse with cell plasma membranes via cellular receptors when vesicle surface charge is sufficiently negative (Oommen et al., 1999).

1.4. Immuno-targeting and targeted drug delivery

Active drug targeting is generally described as the use of a vector molecule with a high specific affinity toward the affected tissues bound to a drug or drug carrier (Torchilin, 2000). The use of monoclonal antibodies or antibody fragments bound to drug carriers using differing carriers (Paukner et al., 2005; Dinauer et al., 2005; Balthasar et al., 2005; Sapra and Allen, 2003; Torchilin, 2001), and immuno-conjugates (Volkel et al., 2004; Murciano et al., 2003; Park et al., 2001) has been explored for varied medical applications (Dinauer et al., 2005; Sapra and Allen, 2003; Gaidamakova et al., 2001; Mastrobattista et al., 1999; Torchilin, 1995). Liposomal immuno-targeting has been used extensively for cancer and cardiovascular applications. Antibody–vesicle conjugation chemistries are varied but there are similar physical configurations that result in increased efficacy of antigen binding when the ligand is attached distal to the vesicle surface. This increases rotational freedom of the targeting moiety and decreases hindrance by the polyethelyne glycol (PEG) chains grafted on the surface of a 'stealthy' liposome (Zalipsky et al., 2004). The addition of polyethylene glycol to a liposome to elude the RES is well documented (Lasic and Martin, 1995; Uster et al., 1996). Attachment of ligand distal to the vesicle on a PEG terminus was found to have increased binding to target cells compared to attachment on the surface (Hansen et al., 1995; Ishida et al., 1999). Development of a PEG–PE end group functionalized with cyanuric chloride allows for attachment of antibodies without prior derivatization of antibodies (Bendas et al., 1999). Active targeting of niosomes was shown using glucose targeting with the inclusion of a glucose–palmitoyl glycol chitosan conjugate in a sorbitan monostearate niosome (Dufes et al., 2004). Improved tumor targeting was shown using niosomes with PEG–glucose conjugates using a paramagnetic agent encapsulant (Luciani et al., 2004). To our knowledge there is no literature on antibody targeting of niosomes.

1.5. Adhesion molecules

Inflammatory processes play a role in vascular disease, rheumatoid and osteoarthritis, chronic obstructive pulmonary disease, and inflammatory bowel disease, lupus, among others. The inflammatory process is characterized by accumulation of inflammatory cells, leukocytes and macrophages, that perpetuate the process and contribute to tissue destruction. Inflammatory cells are recruited by cellular adhesion molecules (CAMs), which are glycoproteins expressed by the endothelium. CAMs mediate blood cell–endothelial cell interactions common to all segments of the vasculature under physiological or pathological conditions (Guray et al., 2004). Increased development of atherosclerosis in rheumatoid arthritis patients without the traditional risk factors point to common mechanisms and the systemic implications of inflammatory pathologies (Hürlimann et al., 2004). 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 CD44 blocked by antibody IM7 (anti-CD44) (Gee et al., 2004). Expression of CD44 and its variants was augmented when exposed to pro-inflammatory cytokines within human atheroma, implicating CD44 expression with the pathogenesis of arterial diseases (Krettek et al., 2004). CD44 was further implicated in the progression of atherosclerosis. Atherosclerotic prone ApoE-deficient mice bred with CD44-null mice showed a 50–70% reduction in aortic lesions compared to CD44 heterozygous and wild type mice (Cuff et al., 2001). These results suggest that CD44 promotes atherosclerosis by both mediating inflammatory cell recruitment to atherosclerotic lesions and by altering smooth muscle function (Cuff et al., 2001). The primary ligand of CD44 is hyaluronan (HA), a principal glycosaminoglycan of the extracellular matrix. HA was shown, in a low molecular weight form, to stimulate vascular cellular adhesion molecule (VCAM-1) and proliferation of smooth muscle cells (SMC), whereas high molecular weight forms of HA inhibit SMC proliferation. Thus, manipulating the immuno-chemistry of pathogenic inflammatory cells and targeting ligands specific to reducing inflammatory

response or disrupting an inflammatory cascade while concomitantly providing pharmaceutical therapeutics could prove valuable in combating inflammatory diseases.

In this paper, we describe a novel technique to conjugate monoclonal antibodies targeted to cell receptors to niosomes. In this study, conjugation of the IM7 antibody to the CD44 receptor was demonstrated using cultured fixed synovial lining cells.

2. Materials and methods

2.1. Materials

Niosome preparations and surfactant derivitizations were made from sorbitan monostearate (Span 60), polyoxyethylene sorbitan monostearate (Tween 61), cholesterol, and dicetyl phosphate (DCP), diisopropylethylamine (DIPEA), cyanuric chloride (CC), and fetal bovine serum (FBS) all came from Sigma Chemical, St. Louis, MO. Fluorescent dyes, 5(6) carboxyfluorescein (CF) and 5(6) carboxyrhodamine (CR) were obtained from Biotium, Hayward, CA. Phosphate buffered saline (PBS), borate buffer pH 11.0, Sephadex G50, Dulbecco's modified Eagle medium (DMEM), Histochoice tissue fixative, Hank's balanced saline (HBS), and goat serum were obtained from Fisher Scientific, Suwannee, GA. Alexa Fluor 488 (AF) came from Molecular Probes, Carlsbad, CA. IM7 antibodies were provided by Dr. K. Mikecz of Rush University Medical Center, Chicago, IL. Collagenase P was obtained from Roche Applied Science, Indianapolis, IN. Bovine synoviocytes where obtained by primary culture described below. Immunohistochemical staining kit was obtained through Vector Labs, Burlingame, CA.

2.2. Immuno-niosome synthesis

To enable site-specific targeting, niosomal surface modification is needed. The overall scheme includes first chemically modifying a surfactant component, polyethelyne sorbitan monostearate to create a linker, then incorporating the linker within the niosome membrane, and then incubating the functionalized niosomes with monoclonal antibodies to achieve conjugation. The resulting vesicle is modeled in Fig. 1.

2.2.1. Surfactant derivatization

In order to conjugate a targeting moiety to a niosome, we needed to conceive of a linking agent to be either attached or inserted into the niosomes after they are formed or to be incorporated within the membrane during formation. The last approach would not require an additional process step or potentially affect vesicle stability. Niosomes composed of sorbitan monoesters have been widely studied, with those composed of Span 60 reported as forming the most stable vesicle (Yoshioka et al., 1994). The surfactant Tween 61 shown in the right side of Fig. 2 is nearly identical in structure to Span 60 except for the additional incorporation of polyethylene branches on the hydrophilic head group. The polyethylene oxide (PEO) groups on the polar head of Tween 61 surfactant potentially could be exploited as a linker for antibody conjugation. This linkage is analogous to antibody coupling on the distal end of PEG groups added to immuno-liposomes (Allen et al., 1995; Bendas et al., 1999; Sapra and Allen, 2003; Torchilin, 2001; Zalipsky et al., 2004).

Tween 61 was functionalized prior to niosome synthesis by activation of the hydroxyl groups on the ends of the PEO chains. In the presence of diisopropylethylamine (DIPEA), Tween 61 and cyanuric chloride are incubated in a nitrogen environment. The overall mechanism is shown in Fig. 2. The cyanuric chloride undergoes nucleophilic substitution binding to the terminal hydroxyl group of a PEO chain on the Tween 61 molecule. The molar ratio of Tween:CC:DIPEA was 1:0.8:2 (Bendas et al., 1999) and a 0.2 g/ml solution was made by combining 1 g Tween 61, 0.124 g CC, 0.274 ml DIPEA, and 5 ml chloroform. The Tween 61 and chloroform were combined in a round bottom flask. Cyanuric chloride was added and the DIPEA was withdrawn from a sealed flask using a long sharp metal syringe tip and added directly into the mixture. The flask was rotated in a nitrogen environment for 36 h. The excess solution was stored and remained stable at −4 °C for several months. The resulting functionalized Tween–CC solution was added to the surfactants and lipids in chloroform prior to forming a thin film.

2.2.2. Niosome synthesis

Niosomes were synthesized by thin film hydration techniques using both agitation and bath sonication during the hydration

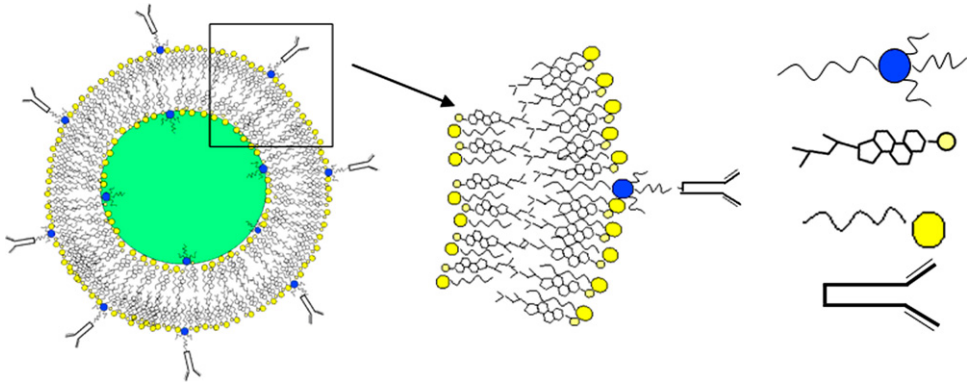


Fig. 1. Representation of a cross-section of a surfactant functionalized niosome membrane conjugated to monoclonal IgG antibodies. Components on right: top, cyanuric chloride functionalized Tween 61 and top middle, cholesterol molecule. Bottom middle, Span 61 and bottom, monoclonal IgG antibody.

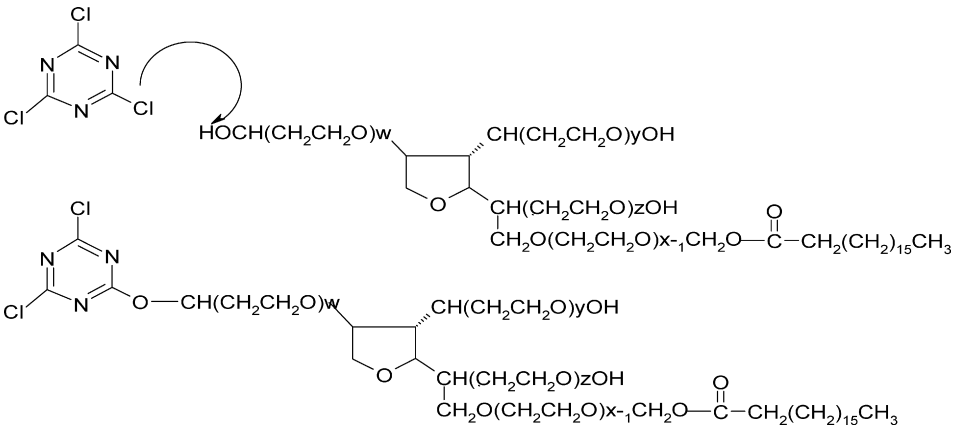


Fig. 2. Mechanism of cyanuric chloride binding to Tween 61.

phase. Vesicles are composed of a 1.0:1.0:0.1 molar ratio of surfactant:cholesterol:DCP at a lipid concentration of between 0.0144 and 0.144 M. Optimal molar ratios of vesicle components of sorbitan monoester niosomes have been well described (Yoshioka et al., 1994). The lipids were dissolved in chloroform and dried in a 50 ml round bottom flask rotating under a 3 l/min steady stream of nitrogen gas to form a thin film on a rotary evaporator (Büchi Rotovapor R200, Brinkmann Instruments, Westbury, NY). Once dried, the thin films were hydrated with either 0.01 M PBS, 5.0 mM CF, or 1.0 mM CR rotating for 1–2 h in a 60 °C water bath. At regular intervals during the hydrations the solutions were agitated on a vortex mixer (Touch Mixer 231, Fisher Scientific, USA). Once complete, the solution was sonicated for 30–60 min in a bath sonicator (G1125PIG Laboratory Supplies CO, Hicksville, NY) at 80 kHz and 80 W. Residual chloroform after 24 h was measured to be less than 0.35% of original solvent by mass before hydration.

Niosomes were separated from unencapsulated dye and unformed lipids by passing the vesicles through a Sephadex G50 gel exclusion chromatography (GEC) column (Superdex HiLoad XK 16/60, GE Healthcare, Piscataway, NJ) at 1 ml/min with 0.01 M PBS as the elution buffer. Due to the high concentration of the lipids in the 0.144 M niosome preparations, those suspensions are reheated to 60 °C prior to injection into the column which is integrated with a fraction collection and monitoring liquid chromatography instrument (ÄKTAprime, Amersham Biosciences, GE Healthcare, Piskataway, NJ). Entrapment of dye was assessed in two ways, first by disrupting vesicles and measuring fluorescence of entrapped dye in a fluorescence spectrometer (LS-3B, Perkin-Elmer, Boston, MA) relative to a standard curve, and secondly, by monitoring the UV absorption of the CF during GEC as shown in Fig. 3. Niosomes were disrupted using a 10% Triton X 100 solution in deionized water at a 1:1 dilution. The final fluorescence intensity was used to obtain the entrapment of dye. Entrapment overall is defined as the amount of dye, in moles, initially added in the formation of niosomes in the hydrating fluid to that recovered after purification multiplied by a dilution factor. Niosomes elute in the void volume (v_0) and free dye in the latter peak. Formation of vesicles was assessed by light and fluorescent microscopy. Mean particle size and distribution of formed vesicles was determined by light scattering and obscuration techniques (Accusizer 780A, Particle Sizing Systems, Santa Barbara, CA). While providing accurate particle counts, this instrument has a lower detection limit of approximately 500 nm. Therefore, in addition, niosome preparations were also analyzed with a Malvern Zetasizer Nano-S (Malvern Instruments Limited, Malvern, UK), which provides semi-quantitative data for smaller particle sizes.

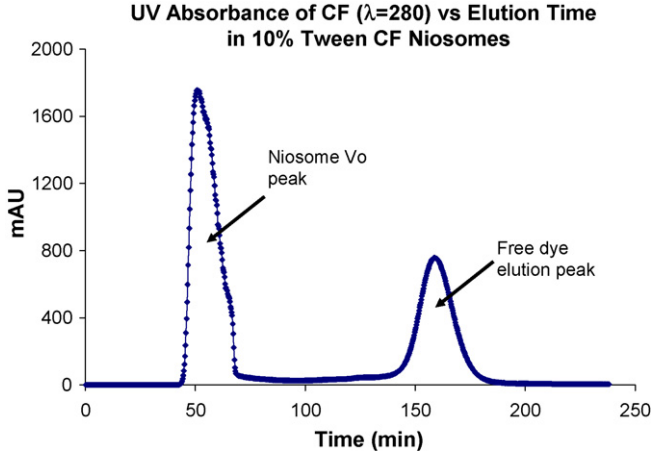


Fig. 3. UV absorbance elution profile of a 10% Tween GEC purification. The v_0 peak represents 280 nm absorbance of CF entrapped in the niosomes.

2.2.3. Antibody conjugation

The GEC purified niosome solutions were adjusted to pH 8.8 using borate buffer (Ultrabasic UB10, Denver Instruments, Denver, CO). IgG monoclonal antibodies (either fluorescently tagged Alexa-488 (AF)-IgGs or anti-CD44 IM7) were incubated with the niosomes at a concentration of and gently shaken for 16 h in the dark. At pH 8.8 the antibody binds to the cyanuric chloride linker distal to the vesicle surface shown in Fig. 4. After antibody conjugation the immuno-niosome solution pH is restored to 7.4 using 0.1 M PBS. Concentration of antibodies incubated was 5 μg protein/ml niosomes which is equivalent to 2.78 μg protein/μmol lipid. Concentration of total lipids in the post GEC niosome solution is 1.8 mM, found by calculation from the original hydration concentration of 0.0144 M accounting for the 6× dilution factor and particle retention

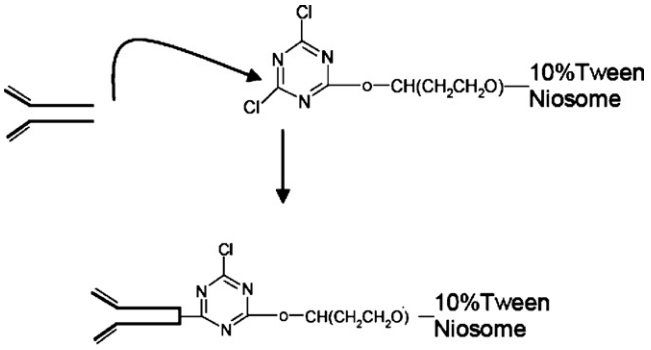


Fig. 4. Cyanuric chloride links an IgG antibody to the functionalized Tween 61 molecule incorporated in the niosome membrane.

efficiency during GEC. Viable binding groups of Tween–CC linkage are 52% of the Tween component by calculation. This value is based on the reaction molar ratio (1:0.8 of Tween:CC) and a published binding efficiency (65%) of the reaction of CC with 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine polyethylene glycol (DPPE-PEG) (Bendas et al., 1999). The overall molar percentage of Tween in the total lipid concentration of the niosomes was 4.76%, making the Tween–CC linker 2.47% of total lipids, or 0.045 μmol Tween–CC/ml niosomes. This provides 2.68×10^{16} binding sites/ml niosomes. Antibody incubation of 5 μg antibodies/ml of niosomes relates to a ratio of greater than 1300:1 Tween–CC binding sites to antibodies.

2.3. Cell culture

Primary cell cultures of bovine synoviocytes were used in niosome incubation experiments. Synovial membranes were harvested from the metacaralphalengeal joints of 3-month-old bovines. After washing with PBS and the tissues were dispersed in 0.1% collagenase P in 4% BSA. Isolated cells were washed with PBS suspended in DMEM containing 10% fetal calf serum at a concentration of 10^5 cells/ml. The cells were plated in Labtek 8 well microslides. Cells were allowed to attach overnight. The media was removed, and the cells washed with PBS and fixed for 2 h in Histochoice. A subset of fixed cells was immuno-stained with IM7 using standard immuno-histochemical techniques.

2.4. Experimental methods

2.4.1. Niosome antibody conjugation

Prior to immuno-niosome cell binding experiments, the coupling of the Tween–CC linker incorporated within the niosome to an antibody was tested by conjugating fluorescently tagged AF rat IgGs to non-fluorescing (PBS containing) niosomes. Structurally similar to CF, AF absorbs UV at 280 nm and the monitoring of absorbance during GEC can be used to verify the presence of the AF-tagged antibodies conjugated to non-fluorescing niosomes during the elution profile. To further verify the antibody binding to the niosomes, the post GEC purified AF-immuno-niosomes were examined with an Olympus 1X71 inverted fluorescent microscope. Approximately 50 μl of immuno-niosome suspension was pipetted onto a glass microscope slide and viewed at 10× and 40× using a FITC filter to verify the presence of fluorescent spherical particles. Fluorescent images where captured using DP-BSW software.

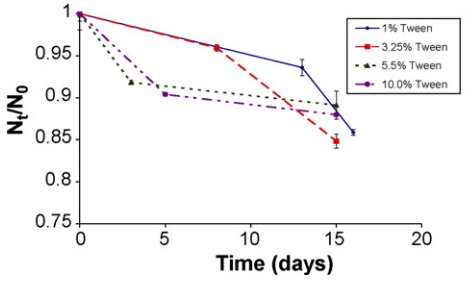
2.4.2. Immuno-niosome fixed cell binding

Once coupling was verified, binding of immuno-niosomes to target antigen in a fixed cell model was assessed. The fixed cell layers were pre-incubated in 0.01 M PBS with 2% goat serum with or without soluble IM7 antibody for 1 h at room temperature prior to incubation with immuno-niosomes. Cells were rinsed with PBS and incubated for 1 h at 37 °C with fluorescent niosomes or fluorescent niosomes derivatized with IM7. The cells were well rinsed to remove unbound niosomes and examined by fluorescent microscopy. For a given image, a phase contrast picture was captured, and then the light and filters were changed to fluorescent mode to image the cell nuclei using the DAPI filter, and the FITC filter to image the fluorescent niosomes bound to the cells. These images were captured and combined using DP-BWR image analysis software to overlay them.

3. Results

The effect of the inclusion of a range of small molar percentages of Tween 61 in a Span 60 niosome on vesicle entrapment capacity and membrane stability was evaluated and measured by retention of entrapped dye over time at 4 °C in a PBS suspension. Although Tween 61 niosomes are reported to have a greater entrapment capacity (Manosroi et al., 2005), we observed that niosomes whose surfactant component was entirely composed of Tween 61 lost three times more encapsulated dye relative to niosome formulations whose surfactant component was purely Span 60 under static conditions holding cholesterol and DCP molar ratios constant (Fig. 5). This figure also shows that the inclusion of up to 10 mol.% of Tween 61 showed no statistical effect on retention.

Based on the Accusizer 780A data, the mean size of the niosomes with a lipid concentration of 0.0144 M after antibody binding was 720 nm, and the modal size was 570 nm. The concentration of these particles was $3.8 \times 10^7 \pm 1.0 \times 10^6$ with $n = 3$ measurements. The Zetasizer confirmed the distribution of niosomes in this range, but also revealed significant numbers of niosomes at a size of 100 nm.



%Tween	% CF Retained
0	88.0%
1	85.9%
3.25	84.8%
5.5	89.1%
10	88.0%
100	62.2%

Fig. 5. Stability of niosomes, as defined as the fraction of dye remaining entrapped, over time suspended in PBS. *Y*-axis represents the ratio of the moles of dye entrapped at time *t* over the moles of dye entrapped at time $t = 0$. "%Tween" is the percentage of total surfactant that is Tween 61. The remaining surfactant is Span 60. The error bars represent the standard error of the mean of three fluorescent measurements.

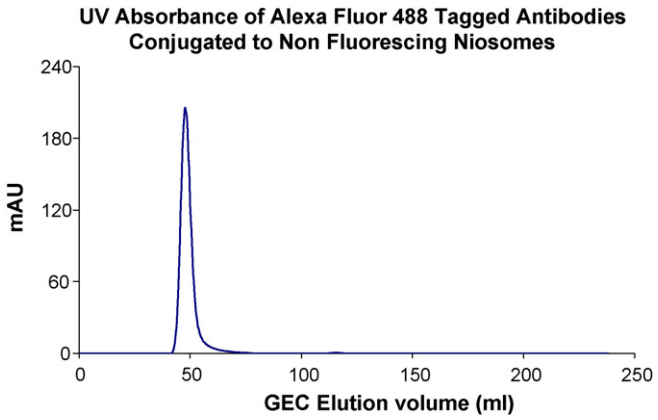


Fig. 6. Absorption of Alexa Fluor 488-tagged IgG conjugated to niosomes is evident in the v_0 elution peak.

Successful attachment of Alexa Fluor-tagged IgGs to PBS containing niosomes is demonstrated by the observance of UV absorbance at 280 nm at the GEC elution void volume in Fig. 6. A very slight signal at 120 ml indicates that few of the AF antibodies were left unbound since the protein and the bound dye would both contribute to the signal at that wavelength. Further observation of the conjugation of AF antibodies was evident when distinct fluorescent spheres were evident under a fluorescent microscope using a FITC filter. The discrete fluorescent spheres appear to be of the same size and relative size distribution of niosomes. These two independent measures indicate successful antibody conjugation of the AF antibodies to non-fluorescing niosomes. Antibodies bound per niosome, by calculation based on the particles counted by the Accusizer 780A, are $5.28 \times 10^5 \pm 1.45 \times 10^4$.

Binding of immuno-niosomes to target antigen in a fixed cell model is confirmed as follows. The fluorescent and light micrographs demonstrate the specificity and selectivity of immuno-niosome binding to target antigens (Fig. 7). The upper micrograph figures of cells (A, C, and E) correspond exactly to the fluorescent micrographs below them (B, D, and F). Parts A and B of Fig. 7 correspond to the cells incubated with IM7-tagged niosomes and show binding of the immuno-niosomes evident by the bright spherical shapes attached at cell processes and cell membranes. Whereas the cells pre-incubated with free IM7, shown in C and D, do not show the small spherical attachments due to blocking of the targeted binding sites. This demonstrates the targeting selectivity of antibody antigen binding. In parts E and F, cells have been incubated with unconjugated niosomes. Additionally, the absence of binding in the cells incubated with untagged niosomes, parts E and F, demonstrates the specificity. In all of the fluorescence images some autofluorescence of cells is evident but clearly distinct from the brighter point-like images of the fluorescent niosomes. In Fig. 8, CD44 expression at the cell processes and at the cell membranes shown using IHC staining techniques. Correspondingly binding of IM7 immuno-niosomes in Fig. 9 is seen at cell processes and membranes.

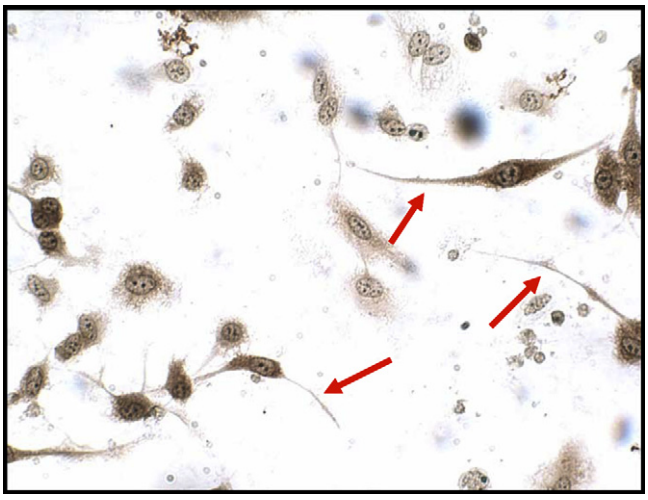


Fig. 8. SL cells stained for CD44 with nuclear counterstaining. Arrows indicate CD44 expressed on cell processes.

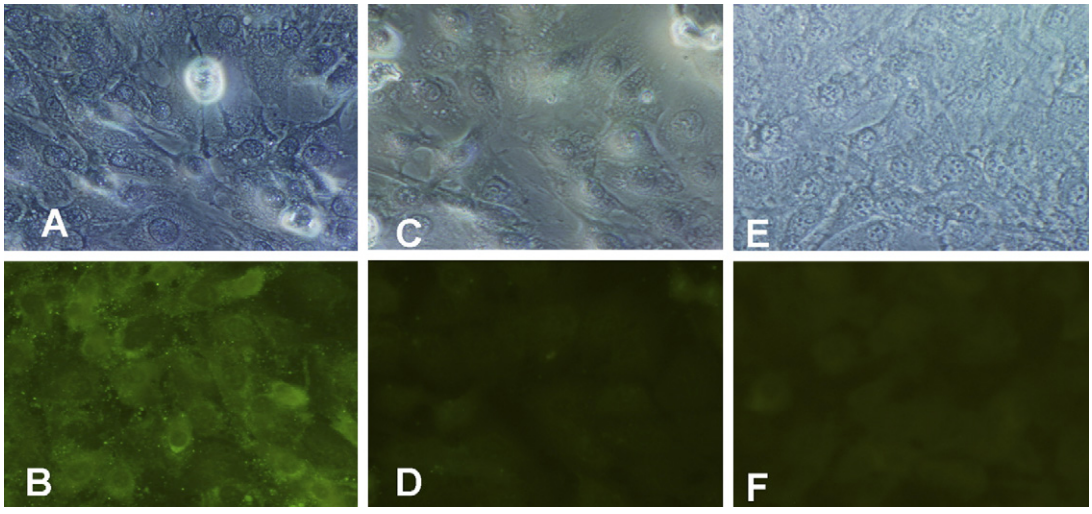


Fig. 7. The upper slides (A), (C), and (E) show contrast micrographs and the lower slides (B), (D), and (F) are the corresponding fluorescent micrographs of those above them captured using a FITC filter. (A) and (B) show SL cells incubated with IM7-tagged niosomes containing 1 mM CR dye. (C) and (D) SL cells pre-incubated with free IM7. (E) and (F) SL cells incubated with untagged niosomes.

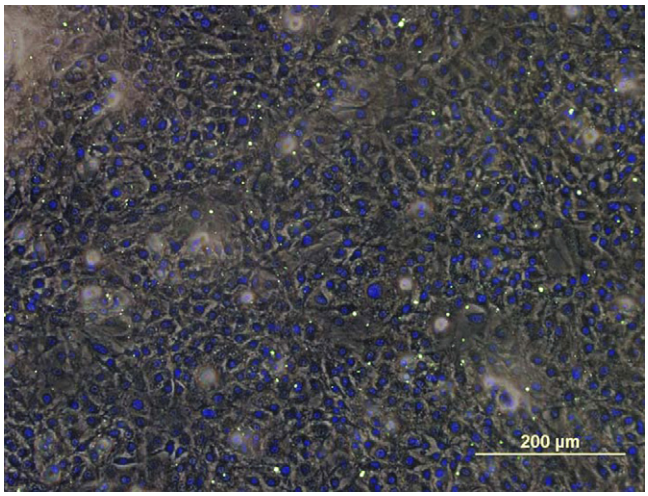


Fig. 9. SL cells incubated with IM7 conjugated niosomes. Fluorescent image is an overlay of green (FITC) and blue (DAPI) fluorescence and contrast images showing attachment of IM7-tagged niosomes.

4. Discussion and conclusions

These results confirm the capacity to develop monoclonal antibody conjugated niosomes targeted to specific cell receptors. Sorbitan monostearate based niosomes can be functionalized through inclusion of a cyanuric chloride derivatized polyoxyethylene monostearate to conjugate monoclonal IgG antibodies to the vesicle surfaces without requiring derivatization of the antibody. The coupling of antibodies to vesicles is efficient. The resulting 'immuno-niosome' can bind to target antigens in fixed cells. In the fixed cell model targeting shows high selectivity and specificity. Further studies will be conducted to investigate uptake by inflamed endothelial cells *in vitro* and *in vivo*. Since the attachment of antibodies is independent of the type and generic to any IgG antibody, the system's therapeutic targeting is flexible and may include more than one targeting vector if desired.

The size and particle distribution of immuno-niosomes will be addressed in future work. We recognize the necessity to maintain appropriate vesicle size for *in vivo* applications. We are currently establishing size reduction protocols using extrusion, monitored by sub-micron particle sizing, that will modify and verify vesicles of size conducive to cellular uptake (Chono et al., 2005). Additionally, measuring optimal antibody coating density is being pursued using activated fixed endothelial cells conducted prior to live cell uptake studies. In liposome applications, as few tens of antibodies or antibody fragments (Kirpotin et al., 1997; Hansen et al., 1995) conjugated per vesicle have shown binding and cellular uptake, necessitating the optimization of our immuno-conjugation.

In this report, the anti-CD44 antibody IM7 was conjugated to sorbitan ester based niosomes via a cyanuric chloride linkage and targeted to fixed cells know to express CD44. Further exploration of the capacity of vesicle binding and subsequent uptake in endothelial cells of the immuno-niosomes will test the potential of the system to not only target inflammatory disease but also to deliver anti-inflammatory agents. Further *in vitro* studies will involve quantifying immuno-niosome binding to endothelial cells and then assessing uptake into cells. The implications for therapeutic treatment of inflammatory diseases is significant not only in the capacity to target chemical therapy to affected tissues but also by blocking receptors of inflammatory pathways and interrupting the perpetuating effect of the process (Pure and Cuff, 2001).

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