Immuno-Targeting of Non Ionic Surfactant Vesicles

Elizabeth Hood[1]*, John Elliott*[1]*, Monica Gonzalez*[1]*, Joel Strom*[2]*, Anna Plaas*[2]*, and Michael D. VanAuker*[1]*. (1) Chemical Engineering, University of South Florida, 4202 E Fowler Ave, ENB 118, Tampa, FL 33620, (2) Medicine, University of South Florida, 4202 E Fowler Ave, ENB 118, Tampa, FL 33620*

Abstract

Non ionic surfactant vesicles (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. Cell binding density was determined to be 2.29 +/- 0.26 immunoniosomes per cell at a cell density of 2.20×10^5 cells/cm^2. These findings suggest that the resulting immuno-niosomes may provide an effective method for targeted drug delivery.

Introduction

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. Increased dosages not only increase costs, but can create toxic side effects as normal tissues and organs are exposed to unneeded pharmaceuticals [1].

Targeted Drug Delivery

By definition **targeted drug delivery is a strategy aiming at the delivery of a compound to a particular tissue in the body**. Ideally, drug targeting would provide a high local concentration of drug at the site of disease and a concentration below levels of toxicity in healthy tissues. This strategy allows increased drug effectiveness by decreasing therapeutic dose, reducing toxicity to untargeted tissues, and reducing the negative side effects of drugs with systemic complications. Drug targeting has been shown to control rate of release with encapsulated drugs providing a more continuous blood plasma level [2,3] and facilitates uptake of drug by tissues. Myriad drug delivery systems have been developed to maintain greater control of therapeutic levels and focused concentration of drugs at target locations through either passive or active delivery. Passive drug delivery effects occur as drug accumulates at sites of increased permeability of the vasculature. Active targeting schemes usually encompass the use of ‘vector’ molecules with high specific affinity toward the targeted tissues bound to a drug carrier [4].

Niosomes as Drug Carriers

Niosomes are self assembled vesicles composed primarily of synthetic surfactants and cholesterol. They are analogous in structure to the more widely studied liposomes formed from biologically derived phospholipids. Niosomes behave similarly to liposomes *in vivo* by prolonging circulation time of the encapsulated drug and altering chemical distribution within the body [5-7]. However, niosomes have advantages over liposomes as drug carriers, including greater chemical stability, lower cost, easier storage and handling, and decreased likelihood of oxidation into toxic forms [8]. Niosomal drug delivery has been studied using various methods of administration [9] including intramuscular[10], intravenous [3,11], peroral [12], and transdermal [13,14]. In addition, as drug delivery vesicles, niosomes have been shown to enhance absorption of some drugs across cell membranes [15], to localize in targeted organs [3,16] and tissues [5,6], and to elude the reticuloendothelial system [2]. Active targeting of niosomes was shown using glucose targeting with the inclusion of a glucose-palmitoyl glycol chitosan conjugate in a sorbitan monostearate niosome [34]. Improved tumor targeting was shown using niosomes with PEG-glucose conjugates using a paramagnetic agent encapsulant [35].

Immunotargeting

In active drug targeting schemes the use of monoclonal antibodies or antibody fragments bound to drug carriers using differing carriers [17-21], and immunoconjugates [22-24] has been explored for varied medical applications [18,20,25-27]. Liposomal immunotargeting has been used extensively for cancer and cardiovascular applications. . Numerous linking chemistries exist but successful antigen binding schemes have a common physical configuration with the ligand attached distal to the vesicle surface. For example , the addition of polyethylene glycol to a liposome to elude the reticuloendothelial system (RES) is well documented [29,30]. 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 [31,32]. This increases rotational freedom of the targeting moiety and decreases hindrance by the polyethelyne glycol at the surface of a ‘stealthy’ liposome [28]. Development of a PEG-PE end group functionalized with cyanuric chloride allows for attachment of antibodies without prior derivatization of antibodies [33]. To our knowledge, no studies exist that use monoclonal antibodies as active targeting vectors with niosomes.

Inflammation and 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, which perpetuate the process and contribute to tissue destruction. Drug delivery specifically targeted to vascular inflammation has numerous therapeutic cardiovascular applications including atherosclerosis and coronary artery disease. Using vesicular targeted drug delivery specific to inflammatory cells could interrupt disease progression while also providing drug therapy.

In order to specify targeted drug delivery an appropriate receptor on the affected tissues must be identified. Cellular adhesion molecules (CAMs) are glycoproteins expressed by the endothelium, which initiate the cellular response to injury, and provide a potential therapeutic target when expressed by pathologically inflamed tissues. Interruption of the inflammatory process has been studied using the cell surface glycoprotein CD44 blocked by antibody IM7.

Dramatic suppression of inflammation was achieved through the highly selective binding of the antibody-antigen couple [36].

The aim of our studies was to investigate the viability of an immuno-conjugated niosome drug delivery vesicle to bind to specific targeted cellular adhesion molecules in a fixed cell model.

Experimental Methods

Immunoniosome Synthesis

The overall scheme in synthesizing an immunoniosome drug carrier was to first chemically modify a surfactant component, polyoxyethylene sorbitan monostearate (Tween 61), to create a linker, and then to incorporate the surfactant-linker within the niosome membrane, and finally to incubate the functionalized niosomes with monoclonal antibodies to achieve conjugation. Niosomes were synthesized by classical thin-film hydration methods [14] with a mixture of biocompatible sorbitan ester surfactants and cholesterol using both agitation and sonication during the hydration phase. We conjugated the formed vesicles to IM7 antibodies through a novel polyoxyethylene sorbitan monostearate (Tween 61)-cyanuric chloride (CC) linker incorporated in the vesicle membrane. Tween 61 was functionalized prior to niosome synthesis by activation the hydroxyl groups on the ends of the polyethylene oxide (PEO) chains. In the presence of diisopropyl ethyl amine (DIPEA), Tween 61 and cyanuric chloride are incubated in a nitrogen environment. The cyanuric chloride undergoes nucleophilic substitution binding to the terminal hydroxyl group of a PEO chain on the Tween 61 molecule as shown in Figure 1.

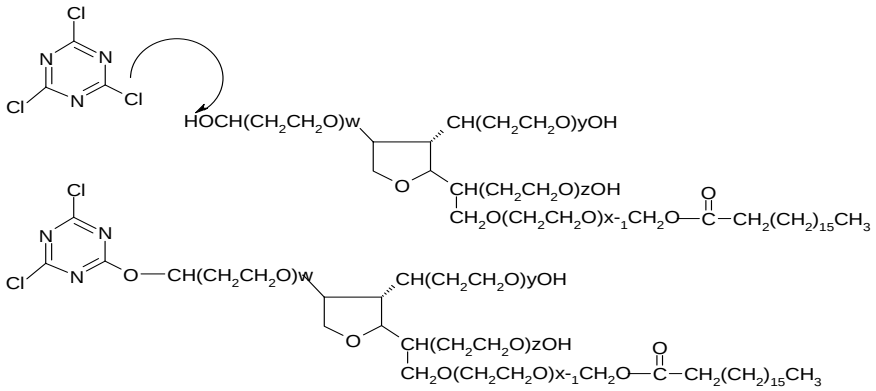

Figure 1. The nucleophilic addition of cyanuric chloride to polyoxyethylene sorbitan monostearate.

The molar ratio of Tween:CC:DIPEA was 1:0.8:2. The resulting functionalized Tween-CC solution added to the surfactants and lipids in chloroform prior to forming a thin film. Vesicles are composed of a 1.0:1.0:0.1 molar ratio of surfactant:cholesterol:DCP at a concentrations between 0.0144 and 0.144 M. Niosomes were separated from unencapsulated dye and unformed lipids using gel exclusion chromatography by passing vesicles through a Sephadex G50 column with a 0.01M PBS mobile phase at a flow rate of 1.0 ml/min.

Entrapment of fluorescent dye used as a drug model was measured using both UV absorbance during the purification step, and fluorescence intensity of disrupted vesicle suspensions relative to a standard curve. 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.

Once formed, niosome solutions were adjusted to pH 8.8 and were incubated with monoclonal anti-CD44 IM7 antibodies. At pH 8.8 the binding of a terminal carboxyl group on the antibody is preferred over that of an amino group at the antigen binding terminus. The resulting 'immunoniosomes' bind selectively and specifically to CD44 antigen targets on synoviocytes, our initial cell model, at IgG concentrations far lower than advocated by traditional immunoliposome literature [37]. Concentration of antibodies incubated was 5 μg protein /ml niosomes which is equivalent to 2.78 μg protein/ μmol lipid. Antibody-niosome binding was evaluated by UV absorbance and fluorescence microscopy.

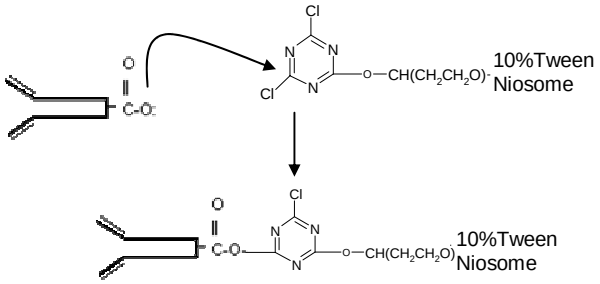

Figure 2. Antibody-niosome binding at the antigen binding terminus.

Cell Binding Studies

Once the conjugation was verified, binding of immunoniosomes to target antigen in a fixed cell model was assessed. Bovine synoviocytes were used in niosome incubation experiments. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum and subcultured at a concentration of 10^5 cells per ml into 8 well microslides for niosome incubation. Cells were allowed to attach overnight. The media was removed, and the cells washed with PBS and fixed for 2 hours in Histochoice. The fixed cell layers were pre-incubated in 0.01 M PBS with 2% goat serum with or without soluble IM7 antibody for 1 hour at room temperature prior to incubation with immunoniosomes. Cells were rinsed with PBS and incubated for 1 hour at 37 ºC with fluorescent niosomes or fluorescent niosomes derivatized with IM7. The cells were well rinsed to remove unbound niosomes and examined by fluorescent microscopy. The cell study's two controls included cells preincubated with IM7 to block binding sites, and cells incubated with non immono-tagged niosomes. Both phase contrast and fluorescent images of post incubated cells were captured with an Olympus 1X71 inverted fluorescent microscope and combined using DP-BSW image analysis software to overlay images. Original images were retained for digital analysis to quantify binding. Some cells incubated with immunoniosomes were also DAPI stained to visualize the cell nuclei and the resulting blue and green fluorescent images were combined with the light image.

Digital Image Analysis

To quantify the amount of adherence of fluorescent immunoniosomes to cells we developed an image analysis program using Matlab ® version 7.1. The program prompts the user to import the fluorescent images representing the number of cells (blue DAPI image), and the number of immunoniosomes (green FITC images). From the micrograph scale bar the program creates a scale. The user is prompted to crop the total image. Each blue cell image is identified as an object, and then measured and counted based on the average area of all the objects. Very small objects are filtered out of the cell count, and very large objects (closely packed cell membranes) are divided by the mean area overall to determine the number of cells represented. The program computes a cell count and a cell density based on number per unit area. The FITC image is processed similarly; each individual object is identified, analyzed, and counted. The immunoniosome binding density is a simple ratio of number of green objects identified to the blue objects.

Results and Conclusions

A contrast and fluorescence overlay micrograph of IM7 conjugated immuno-niosomes shows adherence to the surfaces of CD44 expressed cells in the left image of Figure 3. The image is the overlay of the contrast image with the DAPI (blue) image of the cell nuclei, and the FITC (green) image of the immunoniosomes. Results are for a 1 hour incubation of immunoniosomes at a concentration of approximately 3.0×10^7 #/ml, and 720 nm as measured by light scattering and obscuration at a limitation of particles measured at diameters greater than 500 nm. Niosome cell binding density was determined to be 2.29 +/- 0.26 immunoniosomes per cell at a cell density of 2.20×10^5 cells/cm^2 with a standard error of the mean of less than 3% at an n=12 measurements taken per image. Controls did not show any non specific binding of immunoniosomes or any untargeted interactions of non-immunoniosomes to cell surfaces.

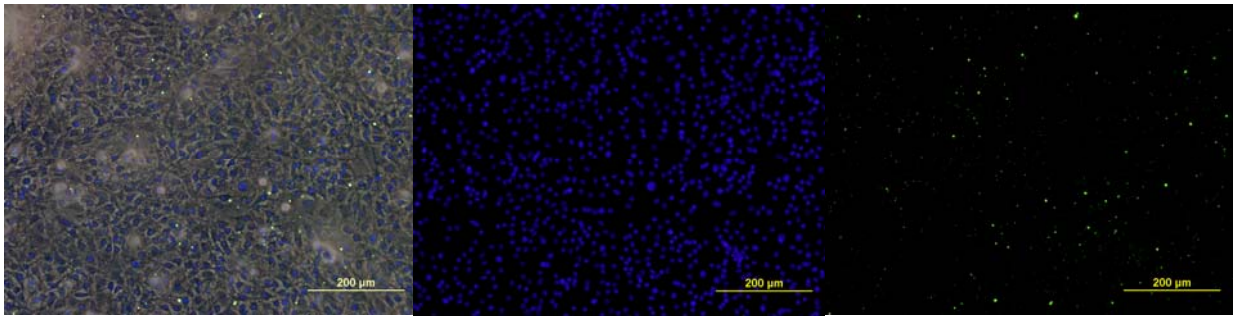

Figure 3. Left: Overlay of contrast micrograph of SL cells with the fluorescent DAPI (middle) stain of cell nuclei, and FITC (right) immunoniosome images.

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 resulting ‘immunoniosome’ 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 the optimal particle concentration and antibody density.

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 antiCD44 antibody IM7 was conjugated to sorbitan ester based niosomes via a cyanuric chloride linkage and targeted to fixed cells known 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.

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References

1. Torchilin VP: Drug targeting. European Journal Of Pharmaceutical Sciences 2000;11:S81-S91.
2. Gopinath D, Ravi D, Karwa R, Rao BR, Shashank A, Rambhau D: Pharmacokinetics of zidovudine following intravenous bolus administration of a novel niosome preparation devoid of cholesterol. Arzneimittel-Forschung-Drug Research 2001;51:924-930.
3. Namdeo A, Jain NK: Niosomal delivery of 5-fluorouracil. Journal Of Microencapsulation 1999;16:731-740.
4. Nobs L, Buchegger F, Gurny R, Allemann E: Current methods for attaching targeting ligands to liposomes and nanoparticles. Journal Of Pharmaceutical Sciences 2004;93:1980-1992.
5. Baillie AJ, Coombs GH, Dolan TF, Laurie J: Nonionic Surfactant Vesicles, Niosomes, As A Delivery System For The Antileishmanial Drug, Sodium Stibogluconate. Journal Of Pharmacy And Pharmacology 1986;38:502-505.
6. Azmin MN, Florence AT, Handjanivila RM, Stuart JFB, Vanlerberghe G, Whittaker JS: The Effect Of Non-Ionic Surfactant Vesicle (Niosome) Entrapment On The Absorption And Distribution Of Methotrexate In Mice. Journal Of Pharmacy And Pharmacology 1985;37:237-242.
7. Ruckmani K, Jayakar B, Ghosal SK: Nonionic surfactant vesicles (niosomes) of cytarabine hydrochloride for effective treatment of leukemias: Encapsulation, storage, and in vitro release. Drug Development And Industrial Pharmacy 2000;26:217-222.
8. Uchegbu IF, Florence AT: Nonionic Surfactant Vesicles (Niosomes) - Physical And Pharmaceutical Chemistry. Advances In Colloid And Interface Science 1995;58:1-55.
9. Blazek-Welsh AI, Rhodes DG: Maltodextrin-based proniosomes. AAPS pharmSci [electronic resource] 2001;3:E1.
10. Arunothayanun P, Turton JA, Uchegbu IF, Florence AT: Preparation and in vitro in vivo evaluation of luteinizing hormone releasing hormone (LHRH)-loaded polyhedral and spherical tubular niosomes. Journal Of Pharmaceutical Sciences 1999;88:34-38.

11. Pillai GK, Salim MLD: Enhanced inhibition of platelet aggregation in-vitro by niosome-encapsulated indomethacin. International Journal Of Pharmaceutics 1999;193:123-127.
12. Rentel CO, Bouwstra JA, Naisbett B, Junginger HE: Niosomes as a novel peroral vaccine delivery system. International Journal Of Pharmaceutics 1999;186:161-167.
13. Uchegbu IF, Double JA, Turton JA, Florence AT: Distribution, metabolism and tumoricidal activity of doxorubicin administered in sorbitan monostearate (Span 60) niosomes in the mouse. Pharmaceutical Research 1995;12:1019.
14. Yoshioka T, Sternberg B, Florence AT: Preparation And Properties Of Vesicles (Niosomes) Of Sorbitan Monoesters (Span-20, Span-40, Span-60 And Span-80) And A Sorbitan Triester (Span-85). International Journal Of Pharmaceutics 1994;105:1-6.
15. Lasic DD: Liposomes: from physics to applications / D.D. Lasic. Amsterdam; New York, Elsevier, 1993.
16. Jain CP, Vyas SP: Preparation And Characterization Of Niosomes Containing Rifampicin For Lung Targeting. Journal Of Microencapsulation 1995;12:401-407.
17. Paukner S, Kudela P, Kohl G, Schlapp T, Friedrichs S, Lubitz W: DNA-loaded bacterial ghosts efficiently mediate reporter gene transfer and expression in macrophages. Molecular Therapy 2005;11:215-223.
18. Dinauer N, Balthasar S, Weber C, Kreuter J, Langer K, von Briesen H: Selective targeting of antibody-conjugated nanoparticles to leukemic cells and primary T-lymphocytes. Biomaterials 2005;26:5898.
19. Balthasar S, Michaelis K, Dinauer N, von Briesen H, Kreuter J, Langer K: Preparation and characterisation of antibody modified gelatin nanoparticles as drug carrier system for uptake in lymphocytes. Biomaterials 2005;26:2723.
20. Sapra P, Allen TM: Ligand-targeted liposomal anticancer drugs. Progress In Lipid Research 2003;42:439-462.
21. Torchilin VP: Structure and design of polymeric surfactant-based drug delivery systems. Journal Of Controlled Release 2001;73:137-172.
22. Volkel T, Holig P, Merdan T, Muller R, Kontermann RE: Targeting of immunoliposomes to endothelial cells using a single-chain Fv fragment directed against human endoglin (CD105). Biochimica Et Biophysica Acta-Biomembranes 2004;1663:158-166.
23. Murciano JC, Muro S, Koniaris L, Christofidou-Solomiclou M, Harshaw DW, Albelda SM, Granger DN, Cines DB, Muzykantov VR: ICAM-directed vascular immunotargeting of antithrombotic agents to the endothelial luminal surface. Blood 2003;101:3977-3984.
24. Park JW, Kirpotin DB, Hong K, Shalaby R, Shao Y, Nielsen UB, Marks JD, Papahadjopoulos D, Benz CC: Tumor targeting using anti-her2 immunoliposomes. Journal Of Controlled Release 2001;74:95-113.
25. Gaidamakova EK, Backer MV, Backer JM: Molecular vehicle for target-mediated delivery of therapeutics and diagnostics. Journal Of Controlled Release 2001;74:341-347.
26. Mastrobattista E, Storm G, van Bloois L, Reszka R, Bloemen PGM, Crommelin DJA, Henricks PAJ: Cellular uptake of liposomes targeted to intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cells. Biochimica Et Biophysica Acta-Biomembranes 1999;1419:353-363.
27. Torchilin VP: Targeting Of Drugs And Drug Carriers Within The Cardiovascular-System. Advanced Drug Delivery Reviews 1995;17:75-101.
28. Zalipsky S, Mullah N, Qazen M: Preparation of poly(ethylene glycol)-grafted liposomes with ligands at the extremities of polymer chains. in Liposomes, Pt D. Methods In Enzymology. 2004, vol 387, 50-69.

29. Lasic DD, Martin FJ: Stealth Liposomes. CRC Press, 1995.
30. Uster PS, Allen TM, Daniel BE, Mendez CJ, Newman MS, Zhu GZ: Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time. Febs Letters 1996;386:243-246.
31. Hansen CB, Kao GY, Moase EH, Zalipsky S, Allen TM: Attachment Of Antibodies To Sterically Stabilized Liposomes - Evaluation, Comparison And Optimization Of Coupling Procedures. Biochimica Et Biophysica Acta-Biomembranes 1995;1239:133-144.
32. Ishida T, Iden DL, Allen TM: A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs. Febs Letters 1999;460:129-133.
33. Bendas G, Krause A, Bakowsky U, Vogel J, Rothe U: Targetability of novel immunoliposomes prepared by a new antibody conjugation technique. International Journal Of Pharmaceutics 1999;181:79-93.
34. Dufes C, Gaillard F, Uchegbu IF, Schatzlein AG, Olivier J-C, Muller J-M: Glucose-targeted niosomes deliver vasoactive intestinal peptide (VIP) to the brain. International Journal Of Pharmaceutics 2004;285:77.
35. Luciani A, Olivier JC, Clement O, Siauve N, Brillet PY, Bessoud B, Gazeau F, Uchegbu IF, Kahn E, Frija G, Cuenod CA: Glucose-receptor MR imaging of tumors: study in mice with PEGylated paramagnetic niosomes. Radiology 2004;231:135-142.
36. Gee K, Kryworuchko M, Kumar A: Recent advances in the regulation of CD44 expression and its role in inflammation and autoimmune diseases. Archivum Immunologiae Et Therapiae Experimentalis 2004;52:13-26.
37. Allen TM, Brandeis E, Hansen CB, Kao GY, Zalipsky S: A New Strategy For Attachment Of Antibodies To Sterically Stabilized Liposomes Resulting In Efficient Targeting To Cancer-Cells. Biochimica Et Biophysica Acta-Biomembranes 1995;1237:99-108.