Optimization of the Biotinylation of DPPC Vesicles for Active Targeting in the Treatment of Pulmonary Artery Disease

The site specificity of intravenously injected drug delivery systems is significant in improving the efficacy and efficiency of drug therapeutics. A high level of site specificity is offered by active targeting, which uses the ligand-receptor relationship between an antibody conjugated to the drug delivery system and the cell receptor of interest. We made actively targeting 100 nm lipid vesicles that will be used to treat pulmonary artery disease. The vesicles, or lipid bilayer systems, were made from mixtures of...
dipalmitoylphosphatidylincholine (DPPC), a natural lipid, and biotin-conjugated dipalmitoylglycerophosphoethanolamine (DPPE), a biotin protein covalently linked to the natural DPPE lipid. The targeting mechanism for our vesicle-based delivery system exploits a biotin-avidin-biotin protein series for the binding of the antibody to the vesicle. However, the tetrameric structure of the avidin protein can lead to the aggregation of vesicles depending upon the surface biotin concentration, resulting in their removal from the blood stream. This study optimizes the surface biotin concentration for the improvement of the active targeting component (biotin-avidin-biotin series) for the site specific delivery of anti-thrombotics to activated platelet cells. Different concentrations of surface biotin were examined to understand the extent of vesicle aggregation. The vesicle sizes were characterized by dynamic light scattering, freeze-fracture transmission electron microscopy and confocal microscopy. The results indicated that the concentration of surface biotin was proportional to the extent of aggregation. The most promising molar ratio was the 0.05% biotin to DPPC lipid, which showed minimal aggregation and maintained a size of 150-225 nm over a period of four days.

Introduction

The tremendous growth of intravenously injected drug delivery systems has led to in depth investigations of strategies for site specific targeting. Targeted therapeutic systems increase the efficacy and efficiency while decreasing the potential side effects of the drug [1]. The two approaches of targeting include passive and active targeting. Passive targeting uses the physical nature of the tissue to enhance location specificity, ie., angiogenesis in tumors. The enhanced permeation and retention (EPR) effect, caused by ‘leaky’ vessels, can trap nanoparticles in the tumor. Active targeting involves the use of antibodies or peptides for the targeting of antigens upon the surfaces of the cells of interest [2].

This paper focuses on actively targeting lipid-based colloidal systems, more specifically natural lipid vesicles. Vesicles of certain lipid compositions are used to encapsulate drugs inside their lipid bilayer system. Through both size control and biomimetic properties, the vesicles can increase the circulation of these drugs in the vasculature [3,4,6,7]. The size and the external components of the vesicle are also important factors in controlling the removal of the vesicle from the blood stream by the reticuloendothelial system (RES). Vesicles and aggregates with sizes above 300 nm will be removed by the RES. Uptake by the RES limits the delivery of drugs to the liver and spleen, associated with Kupffer cells and spleen macrophages. A size of 100 nm theoretically allows the passage of the vesicles through large vasculature fenestrations, ie., hepatic sinusoidal capillaries [10, 14]. The therapeutic benefits of vesicle systems have influenced cancer chemotherapy, ophthalmology, antimicrobial therapy, vaccines, gene therapy and diagnostic applications [5]. Current pharmaceutical products based upon vesicle drug delivery include DOXIL, Daunosome, Abelcet, Amphocid and Ambisome, uses for chemotherapeutics or anti-fungals [6].

These actively targeting vesicles are being exploited for the delivery of anti-thrombotics to activated platelet cells. The vesicles will treat and eliminate vulnerable plaque, the probable cause of death from sudden cardiac arrest. The active targeting component of this drug delivery system uses the non-covalent, almost irreversible binding of biotin to avidin to create a strong linkage between the vesicle and the antibody. Avidin is a tetrameric protein that has a two-fold symmetry with four binding sites for the protein biotin. The isoelectric points of avidin and biotin occur at pH 10 and 3.5, respectively [3]. This charge difference increases the affinity of the avidin and biotin (avidin is positively charged and biotin is negatively charged between the pH of 3.5 and 10). The valeric chain ring of the biotin and a binding pocket on avidin create a strong bond with a dissociation constant of 10-15 M. Biotin conjugated to a dipalmitoyl-glycerophosphoethanolamine (DPPE) lipid is incorporated into the vesicle membrane during vesicle formation, and through the addition of avidin, a biotin-avidin series forms upon the surface. A targeting peptide conjugated to a biotin attaches to the available binding sites upon
the avidin forming an actively targeting vesicle \([7,8,3,9]\).

This biotin-avidin-biotin series can be used as an exchangeable system for other targeting purposes.

The four biotin binding sites on avidin allow for the random aggregation of vesicles, which depends on the concentration of the surface biotin on the vesicle. The concentration of the surface biotin (biotinylation) becomes a large concern because the more biotin on the surface, the higher the possibility of aggregation. The aim of this study was to optimize the biotinylation to increase the amount of the targeting component on the surface of the vesicles while limiting aggregation. The ratio of avidin/biotin was maintained at 2, a value shown to decrease aggregation by Kisak \([3]\). We tested the sizes of vesicles with 2.0%, 0.5% and 0.05% molar ratios of biotin (to the main component of the vesicle, dipalmitoyl-glycero-phosphocholine (DPPC)) after the addition of avidin to measure the extent of aggregation of the vesicles. Vesicle sizes were characterized using dynamic light scattering, freeze-fracture transmission electron microscopy (TEM) and confocal microscopy. The most promising molar ratio was the 0.05% biotin, which showed little to no aggregation.

**Materials and Methods**

**Materials**

1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC), 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Ammonium salt) (DPPE-Rhodamine), 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-(Biotinyl) (Sodium salt) (DPPE-biotin) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. Phosphate Buffered Saline, pH 7.4 was purchased from Sigma in St. Louis, MO. Avidin-FITC was purchased from Invitrogen (San Francisco, CA) and avidin from Molecular Probes/Invitrogen (Eugene, Oregon). All solvents used (analytical grade or better) were purchased from Aldrich Chemical Co. (St. Louis, MO). Ultrapure water (resistivity 18 MV cm) was obtained from a Milli-Q UV Plus system (Millipore, Bedford, MA).

**Vesicle Formation**

Vesicle preparation follows the same procedure described by Kisak et al \([6]\). The DPPC, DPPE-Rhodamine and DPPE-biotin (three different concentrations of DPPE-biotin were added separately at 0.05%, 0.5% and 2.0% mole ratio to the DPPC) were dissolved and mixed in glass vials and the solvent chloroform was evaporated under N2 flow, leaving thin lipid films around the vials. The dry films were hydrated overnight with 150 mM PBS buffer solution (typically 0.01 M Na2HPO4 and KH2PO4 phosphate buffered saline, 0.138 M NaCl, 0.0027 M KCl, 0.02 wt% NaN3) to give the final DPPC concentration of 10 mg/ml at 60°C. The solution was set through a series of 10 freeze-thaw cycles followed by extrusion through 100 nm Nuclepore Track-Etch membranes in an Avanti Polar Lipids Mini-extruder. A 1 ml aliquot of biotinylated vesicles was added dropwise to a solution of avidin (95%)/avidin-FITC (5%) (the avidin solution was set to a ratio of two times the amount of exposed biotin). The reaction was carried out for 24 hours and then diluted to 1 mg/ml. The vesicles were characterized for a period of 1 week by dynamic light scattering, freeze-fracture TEM and fluorescence microscopy.

**Characterization Methods**

Dynamic Light Scattering (DLS) studies were performed with a Brookhaven DLS, Avalanche photodiode detector and MG Vertically polarized 35mm V Helium-Neon 633nm laser. 1 ml of the avidinated-biotinylated vesicles (at a concentration of 1 mg/ml) were added to the vial. The aperture was set at 100, and each sample was allowed to reach a count of 1.00 x 108 with a count rate of 200 – 300 kilo counts per second. In the case that a lower concentration of sample was measured, the aperture was increased to achieve a higher count rate (the opposite situation can be applied similarly).

Freeze-fracture TEM was performed with a RFD-9010 Freeze fracture system. The samples were vitrified in a liquid ethane/propane mixture (-170°C) on copper planchettes, fractured under vacuum below 2.5 x 10-7 mmHg and platinum and carbon were evaporated onto the sample. The copper planchettes were dissolved and
the replicas were placed onto formvar TEM grids, and imaged with a JOEL JSM-6300V TEM instrument.

Fluorescence microscopy was performed with a Nikon F1 Confocal Microscope. The avidinated-biotinylated vesicles were prepared on microscope slides made with 20 μl of the vesicle sample. The samples were viewed using a 10X lens with a 60X objective. The vesicles had rhodamine (Rh) incorporated into the bilayer by way of DPPE-Rh doped at 0.1% mol (excitation at 550 nm and emission at 590 nm), along with an avidin-FITC conjugate for fluorescent viewing (excitation at 490 nm and emission at 525 nm). The intensity values were acquired from a Z-stack of images (from -15.0 to 10.0 nm, zero value at the brightest location).

Results and Discussion

The relatively fluid nature of the vesicle resulted in batch to batch variability in vesicle size and amount of surface biotin. As calculated using the differences in the surface area of the outer and inner layers of the vesicle bilayer, approximately 48% of the biotin-lipids moved to the inner layer of the bilayer and 52% moved to the outer layer during vesicle formation [3]. The biotin-DPPE can diffuse laterally across the fluid membrane layers and (although it is less likely) flip between the two layers of the bilayer and reorient itself on the membrane. This allows for a variation of amounts of surface, or outer layer, biotin on each vesicle (see Table 1).

<table>
<thead>
<tr>
<th>Molar ratio of Biotin-DPPE (%)</th>
<th>Calculated amount of surface biotin</th>
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<tbody>
<tr>
<td>2.00</td>
<td>1234</td>
</tr>
<tr>
<td>0.50</td>
<td>308</td>
</tr>
<tr>
<td>0.05</td>
<td>31</td>
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After the addition of the avidin to the vesicle suspension, the avidin binds to the surface biotin. Avidin is a tetrameric protein with two binding sites on two sides. There are three possible outcomes for the avidin that are dependent upon the surface biotin concentration of the vesicles: avidin can remain unbound in suspension; avidin can bind to a biotin on the surface of one vesicle and remain unbound on the opposite side, or avidin can bind to a biotin on the surface of another vesicle. The optimal concentration of surface biotin will allow for the availability of a binding site facing away from the vesicle, so that the targeting peptide – biotin conjugate can attach. The avidin bound to the surface biotin can be seen in the fluorescent images of Figure 1.

Avidin-FITC and DPPE-Rhodamine allowed for the fluorescent viewing of the binding of avidin to the surface of the biotinylated vesicles. Avidin-FITC was mixed with the avidin (avidin-FITC was only 5% of the avidin added), which was added dropwise to the suspension. DPPE-Rhodamine conjugates were incorporated into the vesicle membrane during vesicle formation. Avidin-FITC fluoresces yellow/green and the Rhodamine fluoresces red. In the images, there are juxtapositions of green and red that show the proximity of the avidin-FITC to the biotinylated vesicles.
Fluorescence images for 100 nm biotinylated vesicles 48 hours after the addition of avidin. (a) Undialyzed unilamellar vesicles and (b) Dialyzed unilamellar vesicles.

Samples were dialyzed in dialysis tubes with 100 kDa pores (100 kDa MWCO) to remove unbound avidin (MW 68 kDa). The fluorescence intensity of the vesicle solutions (dialyzed and undialyzed) was measured using a z-stack of the fluorescence images, allowing for the approximation of the amount of avidin bound to the surface of the vesicles (note that the fluorescence intensity was measured at 548 nm, the wavelength for the color green). The difference in green fluorescence between the dialyzed and undialyzed samples provides a clear indication that avidin-FITC bound to the vesicle and remained bound to the vesicles after dialysis. The intensity measurements showed a 4-fold decrease after dialysis (Table 2). The samples that were tested by other characterization methods, however, were left undialyzed to provide the possibility of more binding of unbound avidin to the unbound surface biotin.

### Table 2

<table>
<thead>
<tr>
<th>Undialyzed</th>
<th>Dialyzed</th>
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<tr>
<td>Mole ratio of Biotin (%)</td>
<td>Average Intensity (au)</td>
</tr>
<tr>
<td>2.00</td>
<td>1925</td>
</tr>
<tr>
<td>0.50</td>
<td>933</td>
</tr>
<tr>
<td>0.05</td>
<td>81</td>
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The main concern regarding the viability of this avidin-biotin system was the aggregation of the vesicles to a size that would be removed by the body. The aggregation of the vesicles were measured using dynamic light scattering (DLS) and freeze-fracture transmission electron microscopy (TEM). The trends for the aggregate formation and morphology of the vesicles provide insight into the optimal surface biotin concentration.

Dynamic light scattering provides an effective diameter of the vesicles and aggregates, which skews to the higher values. The accuracy of DLS is highly concentration dependent, large concentrations will cause multiple scattering events which will skew the diameter value. Prior to the testing of the samples, the scattering events of the DLS were tested for the concentration of sample suspensions. Single scattering events were confirmed by the linear change in scattering intensity (count rate) with sample concentration as seen in Figure 2. At concentrations of 4 mg/mL we observed a deviation from the linear trend. At high concentrations, deviations are a result of multiple scattering events.

The DLS measurements of the biotinylated vesicle suspensions with avidin showed a trend of increasing aggregate size for increasing molar ratios of biotin (Figure 3). The 2.0% biotinylated vesicles had a relative size of 600 nm, 0.5% biotinylated vesicles a relative size of 300 nm and 0.05% biotinylated vesicles a relative size of 220 nm, 96 hours after the addition of avidin. Furthermore, within the first 24 hours after the addition of avidin, there was a noticeable difference between the sizes. The extent of aggregation was time dependent, the aggregates grew larger as time elapsed.

Figure 2

The linear change in scattering intensity (count rate) versus sample concentration proves a single scattering event for vesicles with 0.5% molar ratio of biotin. The vesicles with 2.0% and 0.05% molar ratios of biotin showed similar linear correlations between scattering intensity and sample concentration.
The freeze-fracture TEM images supported the DLS measurements. Freeze-fracture TEM provides a replica of the morphology of the vesicles and aggregates. As seen in Figure 4 (a), the relative size of the aggregates for the vesicles of 0.05% molar ratio biotin was -100 nm, and (b) the aggregate size for the vesicles of 2.0% molar ratio biotin was >1000 nm. As a result of the low concentration of the vesicle solution (1 mg/ml), the vesicle aggregates and unbound vesicles were found to be sparse on the replica's surface. Additionally, the replicas for all three samples showed a large variation of vesicle and aggregate sizes.

Figure 3
Dynamic light scattering results for biotinylated vesicles after the addition of avidin in PBS solution, pH 7.4.

Figure 4
Freeze-fracture TEM images of the biotinylated vesicles 72 hours after the addition of avidin (notice the scales are different). (a) 0.05% molar ratio of biotin showing no aggregation, the entire replica contained unbound vesicles. (b) 2.0% molar ratio of biotin showing a 1.3 µm aggregate.
The dropwise addition of the biotinylated vesicles to an avidin solution was an important factor in ensuring proper exposure of the avidin to the biotin on the vesicles. Trials were performed where the avidin was not added dropwise to the biotinylated vesicles, and the results were skewed with more aggregation occurring in the lowest molar ratio of surface biotin. This may also be influenced by the salt concentration in the PBS buffer solution. Overall, there is a positive correlation between the concentration of surface biotin and extent of aggregation as shown by the characterization methods.

Conclusion

The optimization of the active targeting component of this drug delivery system relies on the concentration of surface biotin, salt concentration of the PBS solution and methodology of the addition of avidin. The biotin-avidin-biotin series can be used as an exchangeable system for targeting other tissues or cells, i.e., tumor cells. The further development of such a system will provide a useful tool for drug delivery.

There are other techniques that can be explored to further optimize the biotin-avidin-biotin system. These techniques include using free floating biotin for the random binding of sites on avidin to decrease aggregation and a monomeric avidin column for the removal of biotinylated vesicles that have available binding sites for avidin. As a result of the tetrameric structure of avidin and steric hindrance, if one biotin molecule binds to the trans side (trans to the vesicle), there is one location available for the biotin-peptide to bind on the same side. By adding free floating biotin, this can decrease the extent of aggregation by limiting the number of available binding sites. The monomeric avidin column will bind (reversibly) biotinylated vesicles that have open sites for avidin binding. This will allow the biotinylated vesicles with avidin to elute through, decreasing the aggregation caused by biotin and avidin.

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References

