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Tulane/Xavier Vaccine Development/Engineering Project

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The Tulane/Xavier Biodefense Vaccine Development/Engineering project will develop new vaccines against biological threat agents to aid the war-fighter. Through the innovative use of nanotechnology, researchers and engineers from the Tulane University Schools of Medicine and Science & Engineering and the Xavier College of Pharmacy will fabricate nanoparticulate systems that are effective for transdermal and mucosal delivery of life-saving vaccines. One aim of this project will be to compare different nanocarriers (i.e., nanohydrogels, star copolymers, and spray-dried PLGA nanoparticles) for the ability to incorporate biological threat-relevant vaccine antigens and deliver those antigens through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum. The use of nanocarriers for vaccine delivery is a platform technology, applicable to delivery of a variety of existing and potential vaccines.
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INTRODUCTION

Infectious diseases remain one of the leading causes of death in adults and children world-wide. Each year, infectious diseases kill more than 17 million people, including 9 million children. In addition to suffering and death, infectious diseases impose an enormous financial burden on society. Although antibiotics and vaccines have been effective at reducing the morbidity and mortality of some infectious diseases, new ones such as AIDS, Lyme disease, West Nile fever, Hanta virus, SARS, and Avian Influenza virus are constantly emerging, while others such as malaria and tuberculosis reemerge in drug-resistant forms. Furthermore, we have an aging adult population with diminishing immune function, increased use of immunosuppressive agents for cancer, tissue transplantation, and autoimmune disease, and an upwardly spiraling cost of health care delivery that makes some existing vaccines unaffordable by the populations at greatest risk. In addition, we now face the possibility of bioterrorism with potentially devastating consequences and a limited number of preventative and therapeutic options.

A great deal of effort has been directed towards developing nonparenteral (needle-free) alternatives to traditional vaccine delivery. Nonparenteral vaccines offer a number of potential advantages over traditional vaccines including 1) the potential to confer mucosal as well as systemic immunity, 2) increased stability, 3) increased shelf-life, 4) elimination of needles and the need for specially trained healthcare specialists to administer vaccines, and 5) potentially lower costs. One such approach, transcutaneous immunization (TCI), is a non-invasive, safe method of delivering antigens directly onto bare skin. Immunization is achieved by direct topical application of a vaccine antigen. Despite the attractiveness of TCI, the technology is limited by the relative inefficiency of transport of large molecular weight vaccine antigens across intact skin.

Recent innovations in transdermal delivery of drugs, including chemical enhancers, electricity, ultrasound, and microneedles, demonstrate the feasibility of large-molecule transport through the skin’s permeation-barrier, specifically the stratum corneum. This outer layer of the skin is composed of tightly packed lipid molecules and the dense, crystalline arrangement of these lipids creates the essential barrier to prevent water loss and pathogen entry. Recent evidence has shown that this barrier can be overcome by properly structured nano-sized particles (nanocarriers). This proposal will compare different nanocarriers for the ability to incorporate a model vaccine antigen and deliver that antigen through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum. While the immediate objective will be to deliver vaccines against biological threat agents, the technologies created will have a tremendous impact on health and human welfare around the world because of their applicability to a wide range of infectious diseases and therapeutic treatments, including other infectious diseases that pose threats to the war-fighter and civilian populations.

BODY

Through the innovative use of nanotechnology, researchers and engineers from the Tulane University Schools of Medicine and Science & Engineering and the Xavier College of Pharmacy will fabricate nanoparticulate systems that are effective for transdermal and mucosal delivery of life-saving vaccines. We will compares three different nanocarriers (nanohydrogels, star copolymers, and spray-dried PLGA nanoparticles) for the ability to incorporate a model vaccine antigen and deliver that antigen through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum.
Double Emulsions

This part of our research consisted of sheltering antigens within the safe microenvironment of the internal droplets of double emulsions for prolonged periods of storage, while at the same time enabling their easy release when applied onto a person's skin. The reason for this goal is that the rest of the formulation may contain agents such as “penetration enhancers,” and other nanoparticles and chemicals with the function of harmlessly opening up the skin's pathways; such agents may denature the protein and strip it from its antigenicity. A scheme that will permit long-term storage and easy release foresees a water-in-oil-in-water (W1/O/W2) double emulsion with an oil phase that is solid at storage temperatures and thaws when brought to the temperature of the skin.

Our earlier results from capillary video microscopy experiments had shown that when a stable double emulsion is prepared at a temperature where all three phases – W1, O, W2 – are liquid, and then is brought to a lower (storage) temperature where the oil phase freezes, stability is preserved. However, the crystallization of the oil causes “zone refining” of oil-soluble surfactants responsible for stability, so that when the double emulsion is brought back to temperatures where the oil phase thaws, instability takes place and the previously stored protein is now released. A schematic of this release mechanism is shown below.
We then proceeded to formulate double-emulsions based creams that contain FITC-BSA in their internal W1 phase. A model W1/O/W2 double emulsion was prepared and subsequently subjected to temperature changes that caused the oil phase to freeze and thaw while the two aqueous phases remained liquid. As expected, no phase separation of the emulsion occurred if stored at temperatures below 18°C (freezing point of the model oil n-hexadecane) whereas oil thawing readily caused instability. Crucial variables were identified during experimentation and found to greatly influence the behavior of bulk double emulsions following freeze-thaw cycling. Adjustment of these variables accounted for a more efficient release of the encapsulated protein. In the figure below, we see various double-emulsion cream formulations, which demonstrate varying degrees of instability (phase separation). At 40°C, the cream is totally stable.

**Nanoscale unimolecular reverse micelle (URM) carriers**

The goal of this aspect of the project was to investigate whether discrete nanoscale unimolecular reverse micelle (URM) carriers can act as efficient carrier to bring polar therapeutics across the skin. The predominantly hydrophobic lipids of the stratum corneum act as an effective barrier keeping out most polar compounds, including polar drugs, peptides, proteins, and many potential antigens. The ability to encapsulate these bioactive molecules in “Trojan horse” nanocarriers—with a polar core for encapsulation, and a non-polar corona for skin compatibilization, offers an attractive route permeation enhancement. However, unlike traditional amphiphilic permeation enhancers, this “micellar” structure is covalent reinforced, and therefore their effectiveness cannot be disrupted by dilution, changes in polarity, etc.

We have successfully prepared a family of star-block copolymers in which 3, 6, or 12 amphiphilic block copolymers arms are covalently tethered to a dendritic core. UV spectroscopic studies with polar dyes (e.g. proflavine) have verified these compounds’ ability to encapsulate and solvate small polar dye molecules in hydrophobic solvents in which they are not independently soluble. In comparison with analogous self-assembled micelles, the URMs demonstrate a higher encapsulation efficiency and their covalently reinforced structure is expected to have prevent disaggregation that may occur with self assembled systems. Fluorescent microscopy studies (see next figure) verify that they significantly enhance the transport of polar small molecules (proflavin dye) through porcine stratum corneum, which are otherwise insoluble in the lipids of the extracellular matrix. The inherent modularity of this system is expected to make this approach amenable to the encapsulation of significantly larger payloads, such as peptides and proteins.
Gel systems that are crystalline mesophases

We also continued our previous research on the hydration dynamics of skin to understand lipid conformations. We conducted fundamental studies of gel systems that are crystalline mesophases with the objective of incorporating antigens into the aqueous phase of the gel. During these studies, we developed high resolution NMR and cryo-electron microscopy techniques to characterize crystalline mesophases. The cryo-electron microscopy was also of tremendous help to us in our studies of novel tubular liposomes created by the addition of ceramides to phospholipids. These tubular liposomes can be templated to ceramics. The next figure illustrates some of these liposomes imaged through cryo-transmission electron microscopy, and their ceramized counterparts.

Based on these findings, we prepared twenty different formulations of poly(lactide-co-glycolide) (PLGA) micro/nanocapsules containing BSA. However, none of these formulations were useful for the delivery of vaccine through skin because of their relatively larger size. We then developed five formulations of nanoemulsions containing FITC-BSA with an average particle size of less than 100 nm, which was ideal for permeation through skin.

The nanoemulsion formulation was optimized according to its particle size, viscosity, and temporal stability. The final formulation chosen contained 55% v/v aqueous phase, 40% v/v squalane as the oil phase, and 5% v/v surfactant/co-surfactant blend composed of Tween-80:Span-80 in 1:1 v/v ratio. The permeation profile of each formula is shown below and is summarized in Table 1. As expected, the FITC-BSA solution showed little penetration due to the barrier function of the stratum corneum. As evident in the graph, the nanoemulsion formulation
produced the highest drug permeation after 48 hours. Table 1 shows that the nanoemulsion had the highest steady state flux and a higher enhancement ratio than the macroemulsion solution. Additionally, the nanoemulsion produced consistent results compared to the wide distribution observed for the macroemulsion plot. This result is also reflected in the standard deviation of the steady state flux, which is higher for the macroemulsion. The inconsistency of the macroemulsion is due to the wide range of emulsion particle sizes present in the sample, compared to the small particle size and polydispersity index of the nanoemulsion. Thus, the improved permeation of the nanoemulsion FITC-BSA through the skin compared to FITC-BSA solution can be partly attributed to the size of the nanoemulsion.

Table 1. Permeation Parameters of Different Emulsion Formulations Through Mouse Skin

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle Diameter (nm, ± SD)</th>
<th>Polydispersity Index (± SD)</th>
<th>Jss (µg/cm²hr, ± SD)</th>
<th>Er (relative to FITC-BSA solution)</th>
<th>r² of linear portion of permeation plots</th>
</tr>
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<tbody>
<tr>
<td>Nanoemulsion</td>
<td>144.9 (5.1)</td>
<td>0.207 (0.003)</td>
<td>3.994 (0.223)</td>
<td>36.64</td>
<td>0.9975</td>
</tr>
<tr>
<td>Macroemulsion</td>
<td>62.0 (27.8)</td>
<td>0.256 (0.118)</td>
<td>2.735 (1.767)</td>
<td>25.09</td>
<td>0.9909</td>
</tr>
<tr>
<td></td>
<td>105.2 (263.0)</td>
<td></td>
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1 bimodal size distribution

Permeation profile of cumulative amounts of FITC-BSA permeated through mouse skin for different formulations.

Our custom diffusion cell design allowed us to accurately measure the skin permeability of our optimized nanoemulsion formulation. This study shows that our nanoemulsion formulation has significant potential as a transcutaneous vaccine delivery system with 36 times greater skin diffusion compared to FITC-BSA solution. Additionally, the nanoemulsion formulation shows consistent, superior results compared to the larger particle size emulsion, demonstrating the impact of manufacturing technique and particle size on the efficacy of transcutaneous formulations.

Finally, studies were conducted to determine the exact enhancement effect of the individual components of the nanoemulsion formulation to further optimize the skin permeability of the formulation. Two different studies were conducted with the nano-emulsion formulation NE31a identified in the above study. In the first, the nanoemulsion formulation was compared to its surfactant mixture (1:1 Smix of Span-80 and Tween-80) to see if the enhanced diffusion attributed to the nanoemulsion is merely a product of its excipients which are known permeation enhancers. When compared to a solution containing the same percentage of emulsifiers by volume as nanoemulsion 31A, the nanoemulsion produced an enhancement ratio of 7.44 over the control solution compared to only 1.94 for the emulsifiers formulation (Table 1). Thus, some other property of the nanoemulsion enhances the diffusion of the drug other than the permeation enhancement properties of its excipients. The second study preformed involved improvement of the permeation properties of nanoemulsion 31A with the addition of known permeation enhancers to the formulation. The enhancers investigated were 2% propylene glycol (PG), 2% oleic acid (OA), and 4% PG/OA. While 2% PG did improve the drug diffusion over the nanoemulsion 31A control, the difference was not statistically significant. All of the enhancers preformed better than a control solution of FITC-BSA, but the 2% OA and 4% PG/OA preformed...
the worst, possibly due to their effect on the physical properties of the formulation, specifically viscosity.

Table 2: Comparison of Permeability Parameters for Formulation NE31A and its Surfactants

<table>
<thead>
<tr>
<th>Formulation, Concentration of FITC-BSA</th>
<th>( J_{ss} ) (µg/cm²/h, mean ± SD)</th>
<th>( K_p ) (× 10⁻³ cm/h, mean ± SD)</th>
<th>( E_r ) (mean ± SD)</th>
<th>( r^2 ) (mean ± SD)</th>
<th>Percentage of Drug Recovered at 48 h (^a) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE31A, 2.5 mg/ml</td>
<td>23.439 ± 17.230</td>
<td>9.38 ± 6.89</td>
<td>7.44</td>
<td>0.995 ± 0.004</td>
<td>30.21 ± 18.05</td>
</tr>
<tr>
<td>Emulsifiers, 2.5 mg/ml (^b)</td>
<td>6.102 ± 0.977</td>
<td>2.44 ± 0.39</td>
<td>1.94</td>
<td>0.995 ± 0.002</td>
<td>10.20 ± 1.70</td>
</tr>
<tr>
<td>Control, 2.5 mg/ml</td>
<td>3.152 ± 0.897</td>
<td>1.26 ± 0.36</td>
<td>--</td>
<td>0.987 ± 0.002</td>
<td>3.00 ± 0.67</td>
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\( J_{ss} \): Steady-state flux, \( K_p \): permeability coefficient, \( E_r \): enhancement ratio, \( r^2 \): determination coefficient of linear portion of plots

\(^a\) Relative to amount of drug loaded in donor compartment

\(^b\) 10% \( S_{mix} \) aqueous solution

Other Accomplishments

F1-V Purification
One technical problem we encountered was availability of sufficient quantities of endotoxin free F1-V for the proposed studies. We had obtained the plasmid for production of F1-V from our collaborators at USAMRIID and developed a process for purifying the fusion protein from inclusion bodies in recombinant \( E. \ coli \). However, the yield was insufficient to create a homogenous pool of purified material from which we could remove endotoxin (a confounding variable in immunization studies). Our best production was 5 – 10 mg per batch and we estimated that we would need a minimum of 150 mg for the incorporation and immunization studies. We inquired about purchasing the material from our collaborators at USAMRIID, but the price we were quoted ($1,500 per 0.5 mg) was not supportable. Consequently, we undertook a process improvement project (changing promoters and production strains, improving purification techniques) and were able to make sufficient quantities of purified material for our studies.

F1-V Incorporation
We incorporated F1-V into different nanocarriers in preparation for immunization studies. In each case, incorporation will first be evaluated \( in vitro \) prior to immunization. We initiated these studies with ceramide 3 liposomes encapsulating F1-V. First, a high pH buffer suitable for F1-V was used with FITC-labeled BSA to confirm encapsulation of the protein within the liposome vesicles and to determine the effectiveness of ultracentrifugation to concentrate the dispersion. A significant difference in the fluorescence intensity between the supernatant and the pellet containing the liposome vesicles indicated that the vesicles did indeed contain FITC-BSA. Liposome preparations encapsulating F1-V were prepared and the entrapment and antigenicity of the molecule was indicated by SDS-Page and Western blot analysis, as discussed below.

The following formulations containing F1-V (molecular weight = 53 kDa) were prepared: ceramide 3 liposomes, squalene nanoemulsions, and water-in-oil-in-water double emulsions. These samples were analyzed with SDS-Page gel and Western blot to determine whether any degradation of the protein occurred during the preparation of the formulations and whether the protein retained its antigenicity (the ability to be recognized by antiserum to F1-V). A representative SDS-page gel with these formulations is shown below:
The following components were loaded into each lane:
1) molecular weight marking standard
2) 7.5 µg F1-V in aqueous buffer
3) 3.75 µg F1-V in aqueous buffer
4) 1.5 µg F1-V in aqueous buffer
5) 6.67 µg F1-V encapsulated in water-in-oil-in-water double emulsion formulation
6) 15 µL of liposome pellet collected after ultracentrifugation
7) 3.75 µg F1-V in ceramide 3 liposome (initial preparation before ultracentrifugation) in a total volume of 5 µL
8) 5 µL supernatant after centrifugation of ceramide 3 liposomes
9) F1-V encapsulated in nanoemulsion formulation with protein added prior to emulsification and homogenization
10) F1-V encapsulated in nanoemulsion formulation C with protein added after emulsification and homogenization

From these studies we concluded that 1) There is some slight degradation of the protein once it is lyophilized and resuspended, as indicated by the bands seen at molecular weights less than 53 kDa, the molecular weight of F1-V; 2) None of the preparation/encapsulation techniques resulted in further degradation of F1-V; 3) There is no difference in the different nanoemulsion encapsulation methods as seen in comparing lanes 9 and 10; and 4) There is a significant reduction in the protein concentration between the initial liposome preparation (lane 7) and the supernatant after centrifugation (lane 8), indicating that a significant amount of F1-V is encapsulated in the vesicles (lane 6).

The formulations were then analyzed by Western blot; serum from mice immunized with F1-V was used for staining. A representative membrane is shown below:

From these assays we confirmed that encapsulated F1-V protein retains its antigenicity (i.e., is recognized by mouse anti-F1-V sera). This indicates that the antigenicity of the protein is not hindered and that these formulations should be suitable for use in immunization studies.

We also developed very simple microneedles by coating ball bearings with powdered glass. By simply rolling these bearings onto skin, we create extremely small punctures that allow penetration into the stratum corneum. Below are images indicating penetration of indocyanine green. Tissue imaged using reflectance mode for the surface layer and 16 mm deep into porcine skin is shown in panels (a) and (c), respectively. Panels (b) and (d) show the penetration of indocyanine green at the skin surface and 16 mm deep, captured through fluorescence mode. Traces of indocyanine green are observed both on the surface layer and 16 mm deep into the skin. The arrows in panel d indicated penetration of fluorescent dye in the proximity of the perforations created. (Scale bar: 50 µm). These developments may lead to simple and inexpensive microneedle technologies for antigen delivery.
KEY RESEARCH ACCOMPLISHMENTS

- Using capillary video microscopy, we have shown that when a stable double emulsion is prepared at a temperature where all three phases – W1, O, W2 – are liquid, and then is brought to a lower (storage) temperature where the oil phase – O – freezes, stability is preserved.
- Investigated nanoscale unimolecular reverse micelle (URM) carriers, and gel systems that are crystalline mesophases.
- Conducted incorporation studies with BSA as a model antigen admixed with ceramide liposomes, water-in-oil-in-water (W1/O/W2) emulsions, tubular liposomes, and silica-tube nanogels.
- Prepared twenty different formulations of poly(lactide-co-glycolide) (PLGA) micro/nanocapsules containing BSA.
- Optimized nanoemulsion formulation according to particle size, viscosity, and temporal stability.
- Developed five formulations of nanoemulsions containing FITC-BSA with an average particle size of less than 100 nm, which was ideal for permeation through skin.
- Demonstrated that nanoemulsion formulations show consistent, superior results compared to larger particle size emulsion, demonstrating the impact of manufacturing technique and particle size on the efficacy of transcutaneous formulations.
- Demonstrated that encapsulation of a biologically relevant vaccine antigen (F1-V) in ceramide-3 containing liposomes preserves the antigenicity of the protein and facilitates deliver across intact skin to the immune reactive cells in the epidermis.
• Demonstrated that some property of the nanoemulsion other than the permeation enhancement properties of its excipients enhances the diffusion of incorporated materials.

REPORTABLE OUTCOMES


Ponnsamy, T.; Lawson, L.; Freytag, L.; Blake, D.; Ayyala, R.; John, V. “In Vitro Degradation and Release Characteristics of Spin Coated Thin Films of PLGA with a Breath Figure
CONCLUSIONS

Encapsulating a vaccine antigen within or adsorbing it to appropriate nanocarriers should facilitate transport through the stratum corneum to the targeted dendritic cells of the epidermis and dermis to initiate an immune response. Tailoring the nanocarriers to optimize encapsulation and/or adsorption and permeation efficiency requires an understanding of the interactions between the molecules composing the carrier, the antigen of interest, and the skin components in addition to the potential immune response to the antigen and the possible effect of the carrier or coadministered adjuvants on this response. Antigen-presenting cells show more efficient uptake of antigen incorporated into or onto a vesicular or particulate carrier, suggesting the potential for nanocarriers to enhance not only transport of the antigen through the skin’s barrier but also uptake of the antigen once it reaches the dendritic cells of the viable epidermis and dermis. Nanocarrier-based transcutaneous vaccines represent a promising application of nanotechnology for delivery of vaccines against biological threat agents. Moreover, the technologies created will have a tremendous impact on health and human welfare around the world because of their applicability to a wide range of infectious diseases and therapeutic treatments, including other infectious diseases that pose threats to the war-fighter.