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Tulane/Xavier Vaccine Development/Engineering Project

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**ABSTRACT**

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.

**SUBJECT TERMS**

Vaccines, Bioterrorism, Biothreat, Nanocarrier, Immunity, Prophylaxis

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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.
During this period of the grant, we were scheduled to begin Specific Aim 1 (Examine the ability of different nanocarriers to incorporate F1-V while preserving the ability of the molecule to be recognized by anti-F1 and anti-V antibodies). In addition, studies were performed with fluorescently labeled bovine serum albumin (FITC-BSA) as a model protein.

We began by examining the permeation of fluorescently-labeled proteins and commercially-available fluorescent microspheres in an in vitro porcine skin model. Confocal and fluorescence microscopy enabled detection of solubilized protein molecules, microspheres, and protein molecules adsorbed to microspheres as they travel through the stratum corneum and deeper tissue layers of the skin over time. Microscopic imaging of skin sections after six hours of exposure to an aqueous solution of FITC-BSA indicated that there was accumulation of the protein within the stratum corneum and along hair follicles deeper in the tissue. Fluorescent microspheres were present in the stratum corneum of pig skin after 15 minutes; the microspheres were shown to enter the viable epidermis, the layer just below the outer stratum corneum, to a limited extent after 45 minutes and to a larger extent after two and six hours. These results establish a foundation for comparing the permeation of nanocarriers specifically tailored for transcutaneous delivery of F1-V and other biodefense relevant antigens.

The most common technique for facilitating transcutaneous immunization through intact skin is hydration with water. As a first step in development of nanocarriers to enhance delivery of F1-V, we needed to understand the role of hydration in opening the hydrophobic and hydrophilic pathways through the stratum corneum. In these studies, we were able to demonstrate that hydration for an extended period (up to 4 hours) can lead to a significant enhancement of permeation of macromolecules, including proteins. Moreover, such hydration is entirely innocuous with full repair of the skin occurring upon removal of hydration. Of particular interest are the fundamentals of this process, which we have captured on cryoelectron microscopy. The skin lipids transform from sheetlike lamellar structures to what appear to be multilamellar vesicles, opening up hydrophilic pathways. The stratum corneum dilates threefold with water uptake, further increasing inter-corneocyte dimensions and allowing proteins to pass through.

The following figures show these phenomena.

The cryo SEM on the upper left illustrates native Stratum Corneum (SC) while the one on the upper right illustrates fully hydrated SC showing the “cisternae” that result upon swelling (a threefold swelling is seen here).

Furthermore, penetration of a model protein (FITC-BSA) is significantly enhanced upon hydration as shown in the cross-section confocal micrographs below.
The bottom series of progressively magnified images (left to right) show the lack of penetration of FITC-BSA (green fluorescence) in native porcine skin, while the top images show the penetration in skin that has been hydrated for 6 hours.

This study demonstrates the underlying mechanism through which hydration facilitates penetration of intact skin and establishes a baseline and analytical technique for evaluating the different nanocarrier technologies. In addition, we can better understand the hydrophilic and hydrophobic pathways we are attempting to exploit with the different nanocarrier formulations.

One of those formulations we are examining utilizes star-block copolymers, nanoparticles of a spherical shape with two domains, an internal domain that will be hydrophilic and encapsulate antigens, and an external domain which is lipophilic and will compatibilize the carrier with the skin lipids. It is expected this architecture will enable the rapid transport of polar peptides through the skin, which alone would demonstrate negligible transcutaneous transport.

In this reporting period, efforts have been focussed primarily on synthetic methods for star-block copolymer formation. These involve 1) the synthesis of dendritic initiators which will act as the core of the nanocarriers, 2) the synthesis of amphiphilic block copolymers, and 3) the combination of these two techniques to yield star polymers and star block-copolymers. The synthesis of the dendritic core molecules has been completed and is presently being scaled up to greater than a 10 g scale. Though not fully optimized, this technique offers the promise of a high through-put synthetic route involving simplified purification techniques (precipitation and filtration) which improve the likelihood of commercial viability.

The synthesis of amphiphilic block copolymers has also been successful, using the well researched Atom Transfer Radical Polymerization (ATRP) method. We have prepared polymer samples which demonstrate the narrow polydispersity (molecular weight distribution) required for biomedical applications. Synthetic techniques have been optimized for the preparation of
blocks copolymers consisting of both a polar domain (oligoethylene glycol methacrylate) and a non-polar domain (lauryl methacrylate.)

The preparation of the desired star-block copolymer architecture has been successful. Using dendritic initiators with 3 and 6 initiating groups, 3- and 6-arm star molecules have been synthesized with polar internal blocks and non-polar external blocks. As verification that the external properties are dominated by the external block, these molecules demonstrate solubility in non-polar solvents, such as hexane. We are currently pursuing the synthesis of 12- and 24-arm initiators and the resultant star polymers.

We also investigated the incorporation of ceramide lipids into liposome vesicles for protein encapsulation and transcutaneous transport. Phospholipid vesicles with an average diameter of 1000-1470 nm were initially prepared; an extrusion device in our laboratory enabled reduction of the size of these vesicles to approximately 178-560 nm. Liposomes composed of ceramide 3, a lipid prevalent in the stratum corneum of both human and porcine skin, were prepared and imaged using cryo-scanning electron microscopy to visualize the structure and size-distribution of the vesicles. Fluorescently-labeled proteins were encapsulated within these liposomes and work is underway to quantify and optimize encapsulation efficiency. Further work will be done to confirm the antigenicity of encapsulated F1-V and to determine if the incorporation of ceramides within lipid vesicles will enhance permeation through the skin and improve antigen delivery.

Other work in our laboratories has focused on the development of double emulsions for vaccine delivery. We have refined the technology of conducting microscopy inside cylindrical capillary tubes, while observing microscopic objects with dimensions comparable to the tube’s diameter. This largely strips the objects that are under observation of two out their three degrees of freedom of movement, thus forcing them to stay on the same focal plane and in the view of a recording camera while phenomena occur. Using this technology, we have gained insight on the stability of double emulsions with potential uses in vaccine delivery. We are continuing to evaluate the “double carrier” concept, where a nano-scale delivery vehicle is being transported by a micro-scale delivery vehicle in order to simultaneously take advantage of both systems. We are completing studies on using temperature stimuli to release encapsulated proteins from double emulsions. Such research examines the potential of double emulsions to (i) store vaccines at temperatures below the oil freezing point thus protecting them from denaturation and (ii) promote antigen release by letting the system melt. This work will be important for formulations containing F1-V and other antigens with penetration enhancers for double emulsions that are temperature sensitive.

We have also optimized the process for purifying a non-his-tagged version of F1-V, expressed by E. coli BLR(DE3)/pPW731, and isolated F1-V to 99% purity by gel filtration chromatography. Briefly, following overnight growth, protein in inclusion bodies from lysed cells is denatured with 6 M urea on ice. F1-V is then purified by size exclusion chromatography on a Superdex™ 75 gel filtration column (Amersham). Purified F1-V is analyzed on SDS-PAGE gel using the Novex gel system (Invitrogen) and stained with Coomassie dye to detect the presence of the recombinant protein. Western blots are performed using sera from goats immunized with purified F1 and V to confirm the presence and quantification of the recombinant protein.

KEY RESEARCH ACCOMPLISHMENTS

- Developed the analytical techniques to fully characterize permeation through the skin.
- Reached a full understanding of microstructural changes in the stratum corneum (SC) upon hydration and have begun to develop techniques to enhance penetration.
- Demonstrated that hydration of porcine skin tissue for a period of 4-10 hours causes a three-fold expansion in the SC. Confocal microscopy studies show distinct enhancement
in penetration of a large biomacromolecule, fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) through the skin when excessively hydrated (4-10 hours).

- Demonstrated that overall protein permeation was increased by 12% when ceramide 3 liposomes were applied to the skin, confirming our hypothesis that the incorporation of lipids prevalent in the skin will enhance liposome permeation, thereby increasing protein transport.

- Demonstrated that 6-arm, amphiphilic star polymers enhance encapsulation of polar guests relative to 1 arm block co-polymer.

- Demonstrated 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.

- Optimized the process for purifying a non-his-tagged version of F1-V, expressed by E. coli BLR(DE3)/pPW731, and isolated F1-V to 99% purity by gel filtration chromatography.

REPORTABLE OUTCOMES


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.