Designing Glycoprotein Films and Micelles to Capture and Remove Pathogens from Aqueous Suspensions

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Abstract

This paper describes transparent, detergent-resistant glycoprotein films that have been developed to help characterize disease-causing pathogens, as well as to help prevent infection. Such films are coated on one face with oil, and on the opposite face with branching carbohydrates that bind to complementary adhesive proteins (lectins) on surfaces of pathogens and biotoxins. When agitated, glycoprotein films form stable, oil-filled micelles that float and sort by diameter within a vertical column of aqueous fluid. Glycoprotein film-coated micelles can be used to remove pathogens and biotoxins from contaminated fluids, and represent novel visualization tools that can be color-coded and used to detect the presence of lectin adhesins and hemagglutinins that bind to tissue-specific targets or "glycotopes" during the process of infection. Glycoprotein micelles coated with multivalent lectin molecules constitute another set of tools that bind to complementary carbohydrates on a variety of structures ranging from glycoprotein molecules to bacterial spore coats and animal cell membranes. These flexible structures offer an additional means of capturing and removing pathogens and biotoxin molecules from contaminated fluids and surfaces, and might also be applied to the study of glycosylation patterns on epithelial cells of human tissues.

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I. Introduction

The field of glycobiology has grown rapidly in recent years, with increased appreciation for the specificity and importance of carbohydrate-binding proteins (i.e., lectins) and receptor sugars on cell surface glycoproteins and glycolipids. Lectin-carbohydrate binding events participate in cellular interactions within all complex organisms, and include the adhesion of pathogens and biotoxins to specific host tissues during initial stages of infection [Karlsson, 1995; Sharon and Lis, 2004]. Here, we shall describe a recently developed set of glycoprotein films and oil-filled, glycoprotein-coated micelles that form at an oil-water interface. These flexible glycostructures constitute a new set of tools that utilize specific lectin-carbohydrate bindings to remove human pathogens and biotoxins from aqueous suspensions and damp surfaces. Glycoprotein-coated "biocapture micelles" have been developed in our laboratories through extensive experimentation. The relatively low density and optical transparency of spherical micelles in the millimeter-diameter range are key attributes of tools that facilitate the visualization and handling of captured pathogenic cells and biotoxin molecules. In addition, when color-coding dyes are included within them, glycoprotein micelles bearing specific receptor sugars can be used in adhesion studies designed to characterize lectins and carbohydrates that participate in the binding of pathogens to receptors on host cell membranes.

The key enabler for these innovations is the discovery that a persistent glycoprotein film forms spontaneously when an aqueous solution of glycoprotein comes in contact with a layer or droplet of oil [Mullen, 1998; Mullen, 2003; Mullen, 2006]. Such glycoprotein films appear to be monolayers associated on one face with oil molecules and on the opposite face with water molecules. At least one complex carbohydrate (i.e., an "oligosaccharide" comprising branching sugar chains) is covalently bound to each molecule in the film. See Figure 1.

On a microscopic scale, a glycoprotein film resembles a sheet of transparent plastic wrap sandwiched between the oil and water, with occasional "stretch marks" bearing evidence of tensile and compression forces imposed on flexible intermolecular associations. See Figure 2. When agitated, a glycoprotein film subdivides and the fragments roll into roughly spherical, oil-filled vesicles or "micelles" that divide again, like soap bubbles, when subjected to shear forces. The word, "micelle" is often associated with nanometer-scale, spherical aggregations of phospholipid molecules that spontaneously associate in aqueous solutions under certain conditions of concentration and surface area [Lasic, 1988]. By contrast, the glycoprotein micelles with micrometer-scale diameters that we discuss here are produced only when films are subjected to shear force by methods analogous to those used in the dairy industry to homogenize milk fat globules [Paquin, 1999].

Unlike oil droplets dispersed in water, adjacent glycoprotein micelles do not coalesce. It is likely that the outer coating of relatively large, hydrated oligosaccharides prevents direct contact
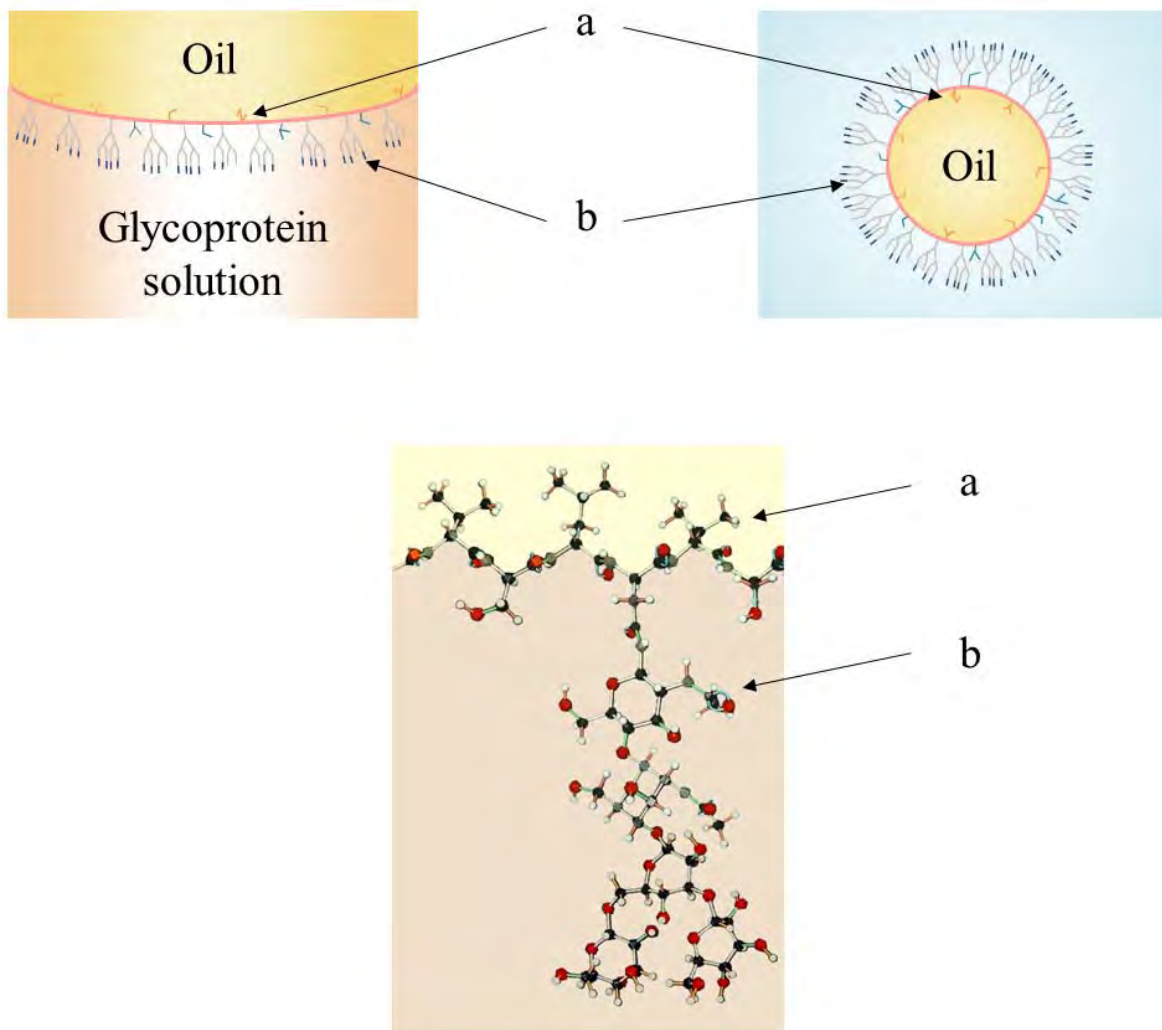
Figure 1. Diagrammatic representations of a floating glycoprotein film (top left and bottom) and micelle (top right) produced at the interface between oil and an aqueous solution of glycoprotein. Hydrophobic amino acid side groups (**a**) project into the oil, while hydrophilic side groups, including oligosaccharides (**b**) remain immersed in the aqueous phase. The blue solution in the diagram on the right calls attention to the fact that micelles can be removed from the fluids in which they were prepared and suspended in a different aqueous medium.

Figure 2. Stretch marks in a glycoprotein film lying at the interface between canola oil and an aqueous solution of chicken egg white glycoproteins, as photographed with an Olympus DP70 Digital Camera through a 10x lens using differential interference contrast (DIC) optics on an Olympus BX51/BX52 microscope.

between the proteins on film faces and the oil molecules sequestered within [Varki, 1993; Imperiali and O'Connor, 1999].

Specific sugar sequences on micelle surfaces can serve as receptors for pathogens and biotoxins that bind to the same structures on human tissue during early stages of an infection. For example, micelles made from the yeast glycoprotein invertase are coated with mannose, a sugar that is also present on human urinary tract epithelium, and serves as a receptor for *E. coli* cells that cause bladder infections [Connell *et al*., 1996]. In the following sections we will use the term "glycotope" to denote the specific sugar sequence or structure that binds to a particular pathogen lectin.

We initially developed biocapture micelles to facilitate the work of scientists who test environmental samples for the presence of pathogens and biotoxins. Subsequently, we discovered that glycoprotein micelles also can serve as visualization tools that can facilitate the study of lectins involved in binding pathogens to tissue-specific sugar structures. Glycoprotein micelles can also facilitate the removal of pathogens and biotoxins from the environment. In Section II, we explain the formation of glycoprotein films and micelles at an oil-water interface and discuss structural attributes that contribute to their suitability as probes for capturing pathogens. We conclude Section II with an overview of discoveries in the fields of glycobiology and microbiology that have elucidated a complex phenomenon analogous to clustering of hooks and loops in VELCRO® products that results in tenacious adhesion of pathogens to host cell membranes and glycoprotein films. Then, in Section III, we introduce a suite of visualization tools based on a macroscopic manifestation of "clustered hook-and-loop" adhesion: agglutination or visible clumping of cells and glycoprotein micelles. The agglutination phenomenon provides visible evidence for the presence of both lectins and glycotopes that are involved in the binding of pathogens to host cell membranes. We conclude Section III with a review of our approach to developing and producing glycoprotein micelles designed to capture specific pathogens.

In Section IV, we describe experimental methods used to expose pathogenic bacteria to micelles suspended in aqueous media, and discuss modifications in standard microbiological protocols that enabled us to compare the concentration of pathogens captured on micelles with the number remaining in an aqueous suspension. Experiments presented in the Appendix demonstrate the capture of uropathogenic *E. coli* on micelles coated with glycotopes of lectin adhesins known to be involved in the establishment of human urinary bladder and kidney infections [Hoschützky *et al.*, 1989; Wu *et al.*, 1996].

Finally, in Section V, we review attributes of glycoprotein films and micelles that contribute to their effective concentration of pathogens and biotoxins from aqueous fluids, and conclude with

suggestions of potential applications related to the removal of pathogens, including antibiotic-resistant strains, from contaminated fluids and surfaces.

We believe that there may be particular utility in this comprehensive presentation of flexible, inexpensive glycoprotein films and micelles that includes examples of how they are prepared and may be used for collecting and characterizing a wide variety of pathogens and biotoxins. Especially, we hope that this paper will be helpful to teams of microbiologists, glycobiologists and carbohydrate chemists who must solve similar problems and seek new, useful tools for doing so.

II. Proposed Structural Basis for Observed Phenomena

In the absence of detailed structural studies that we plan to perform at a later time, here we propose explanations of chemical and structural attributes of glycoprotein films and micelles that appear to underlie their stability and efficacy in applications related to capturing pathogens and biotoxins from contaminated fluids and surfaces. The following explanations represent a synthesis of well-documented theories on glycoprotein structure and chemistry and our own observations of glycoprotein films and micelles produced at an oil-water interface.

II.A Glycoprotein Film Formation, Structure and Properties

Fundamental to the structure, behavior, and function of glycoprotein films and micelles are changes that take place in the three-dimensional conformation of glycoprotein molecules as their amino acid backbones unfold and become entrapped at the two-dimensional oil-water interface. Just as amphiphilic molecules of detergent (having both hydrophilic and lipophilic ends) disrupt hydrophobic and hydrogen bonds that participate in the maintenance of three-dimensional protein structures [Chatterjee *et al.*, 2002], so does an oil-water interface [Lefèvre and Subirade, 2003]. These changes in structural and chemical properties result in the juxtaposition of hydrophilic oligosaccharide substituents or moieties bound to adjacent glycoprotein molecules lying at the oil-water interface. The proximity on film surfaces of intrinsically "multivalent" oligosaccharides underlies the observed tenacious binding of pathogens bearing complementary lectins to glycotopes on glycoprotein micelles [Lindhorst *et al.*, 1998]. (See Section II. C. on "Clustered Hook-and-Loop" Adhesion.)

Normally, in an aqueous solution, the backbone of a glycoprotein molecule is folded in a convoluted manner as a consequence of non-covalent associations (e.g., hydrophobic, ionic and hydrogen bonds) and covalent sulfur bonds between amino acid side groups [Mittal *et al.*, 2010]. In their natural state, many glycoprotein molecules enfold hydrophobic side groups within a globular structure coated with hydrophilic side groups facing the aqueous medium in which they are suspended [Lins and Brasseur, 1995]. We hypothesize that globular glycoprotein molecules become trapped at an oil-water interface and unfold when their hydrophobic amino acid side groups enter the oil phase [Wu *et al.*, 1993]. Hydrophilic side groups, including oligosaccharides, remain in the aqueous phase [Lins and Brasseur, 1995].

A single sugar molecule is approximately the size of an amino acid – about a nanometer in diameter [Einstein, 1906; Fang *et al.*, 2011]. Glycoprotein oligosaccharides can contain as many as twenty sugar moieties covalently linked in branching chains [Hård, 1992]. Thus oligosaccharides protrude from the amino acid backbone far beyond all other side groups. When fully hydrated, these bulky oligosaccharides are unlikely to enter the oil phase [Lehninger *et al.*, 2005] during glycoprotein film formation, and may prevent attached amino acid chains from

doing so. Evidence for this hypothesis lies in our observation that commercially purified and dried glycoprotein molecules that appear to have dissolved, but are not fully rehydrated often produce compound micelles when mixed with liquid oil (i.e., smaller, microscopic "reverse micelles" or oil-coated glycoprotein particles are suspended within the hydrophobic compartment of larger micelles). However, after the same glycoprotein molecules have remained in water for a few days, they produce clear micelles that contain no visible inclusions within the oil. We have found that it is possible, in most cases, to accelerate the rehydration of oligosaccharides by stirring glycoprotein solutions for about an hour at temperatures near the boiling point.

We further hypothesize that hydrogen bonds form between adjacent amino acid strands at the oil-water interface, producing a continuous sheet of glycoprotein [Kowalewski and Holtzman, 1999; Schladitz *et al*., 1999; Lefèvre and Subirade, 2003]. Evidence for this proposed structure lies in the elastic nature of the films, and in our observation that volatile molecules like hexane are sequestered within the hydrophobic micelle compartment until the glycoprotein surface film is ruptured. Thus a glycoprotein film can be viewed as a coherent, amphiphilic monolayer which is lipophilic on the upper face and hydrophilic underneath.

II. B. Glycoprotein Micelle Formation

The sequestering of oil within glycoprotein micelles is facilitated by the well-known hydrophobic effect that causes oil to bead in water. This phenomenon is based on the presence of a network of non-covalent hydrogen bonds between water molecules [Tanford, 1979; Smith *et al*., 2004]. Each of these bonds represents an electrostatic association between a hydrogen atom of one water molecule and the oxygen atom of another. By contrast, the hydrogen atoms in an oil molecule are bound tightly to its carbon backbone and do not form hydrogen bonds with oxygen. Therefore, when oil is mixed with water, it is excluded from the network of hydrogen bonds, and beads into droplets that rise to the surface and coalesce [Chandler, 2002]]. However, when a film of glycoprotein forms around an oil droplet, hydrophilic moieties, including oligosaccharides on the outer surface participate in the network of hydrogen bonds with water molecules and prevent micelles from coalescing [Hoiberg-Nielson et al, 2006; Almond, 2005; Petsko and Ringe, 2004]. Branching oligosaccharides protrude from the spherical hydrophilic face of a glycoprotein micelle, presenting an array of glycotopes that can bind to complementary lectins of pathogens or biotoxins approaching from any direction. The fairly uniform luminance observed around the periphery of micelles coated with fluorescent lectins (see Figure 3) suggests that the distribution of carbohydrates on the film surface is relatively even. Thus a suspension of floating glycoprotein micelles represents a large collective surface area studded with glycotopes that have a high probability of binding to pathogens as they rise through the surrounding fluid [Tu *et al*., 2009; Schengrund, 2003].   In the following section we discuss the importance of clustered hook-and-loop adhesion in the biocapture process.
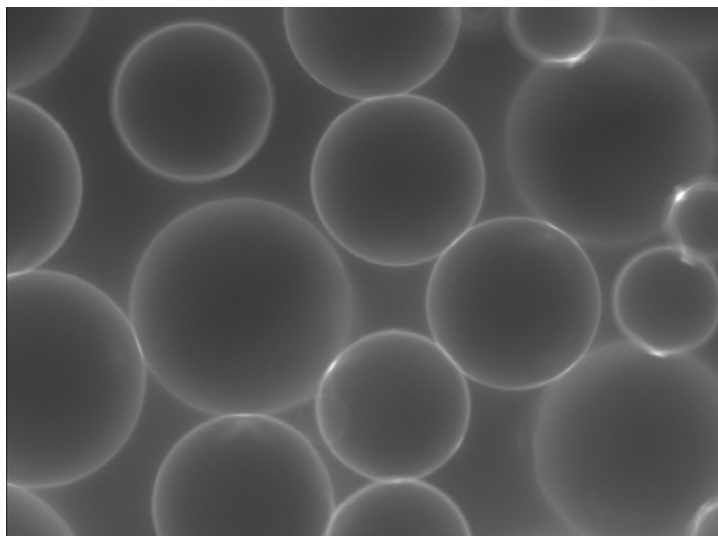
Figure 3. Fluorescein-labeled lectin, GNL (product number FL-1241 purchased from Vector Laboratories, Burlingame, CA), concentrated at 20 micrograms per ml HEPES buffer, binds to terminal mannose on oligosaccharides of glycoprotein micelles coated with yeast invertase (product number I4504, Grade VII purchased from Sigma-Aldrich) and filled with Crisco canola oil (manufactured by Smucker Co.) Photograph was taken with a digital camera (Qlmaging QICAM Fast 1394) at 40x on a fluorescence microscope (Olympus BX51). Excitation lamp (Exfo X-Cite Fluorescence Illumination Source, peak excitation wavelength 490 nm) and digital camera intensity/exposure settings were adjusted to restrict fluorescent signal at micelle edges to a maximum pixel brightness of 256 (white).

Experiments conducted in our laboratory have shown that oil-filled micelles as small as 10 micrometers in diameter can float upward in an aqueous suspension of milk or water fast enough to be gathered from the surface within about 15 minutes after mixing. Microscopic observation and quantification of captured pathogens are facilitated by the transparency and buoyancy of glycoprotein micelles aligned at the top of a drop of fluid. This phenomenon is illustrated in Figure 4.

II.C. "Clustered Hook-and-Loop" Adhesion

Much of the research conducted during the past three decades on lectin-carbohydrate interactions has been associated with attempts to develop anti-adhesive therapeutics that would block the binding of pathogens to host tissues [Simon *et al.*, 1997; Karlsson, 1998; Sharon, 2006]. Scientists involved in the design of "carbohydrate decoys" (synthetic structures that resemble carbohydrate receptors of pathogen lectins) have discovered that clusters of glycotopes are often several orders of magnitude more effective in blocking the binding of pathogens to host cell membranes than the same number of separate, randomly oriented receptor molecules [Weijers *et al*., 2008; Pukin *et al*., 2007; Schengrund, 2003; Simon *et al*., 1997]. One reason for their enhanced effectiveness is that clustered decoys are "multivalent" in the biological sense of the word, presenting several glycotopes that bind to lectins on surfaces of viral or bacterial pathogens [Mulvey, 2001]. Reiko and Yuan Lee first used the term, "glycoside clustering effect" to describe the enhanced adhesive force or avidity that arises from simultaneous binding events involving multiple lectins and carbohydrates on adjacent cell surfaces [Lee *et al*., 1984; Lee and Lee, 2000].

To compensate for the fragility of individual lectin-carbohydrate bonds, multiplicity typically exists on more than one level in the presentation of lectins and carbohydrates that participate in cellular interactions. For example, bacterial cells are often coated with many brittle, hair-like structures called fimbriae or pili, each of which may present several lectins along the shaft [Mulvey *et al*., 2001]. Likewise, human cell membranes are studded with glycolipid clusters and glycoprotein molecules bearing oligosaccharides that often branch into several glycotopes [Butters and Hughes, 1974]. Thus the presentation of multiple molecular binding partners, multivalent in both chemical structure and spatial distribution, creates a "hook-and-loop fastener" effect that results in firm attachment of pathogens to host cell membranes [Mulvey *et al*., 2001; Brewer 2001].

To prevent the adhesion of pathogens to glycoconjugates on cell membranes, tissues often secrete glycoproteins whose oligosaccharides block lectin binding pockets [Gopal, 2000; Morrow, Newburg *et al*., 2005]. For example, kidney cells secrete uromodulin, a glycoprotein that bears several richly-branching oligosaccharide moieties, each of which contains multiple glycotopes of pathogen lectins [Hård *et al*., 1992; van Rooijen *et al*., 1998]. Among these are
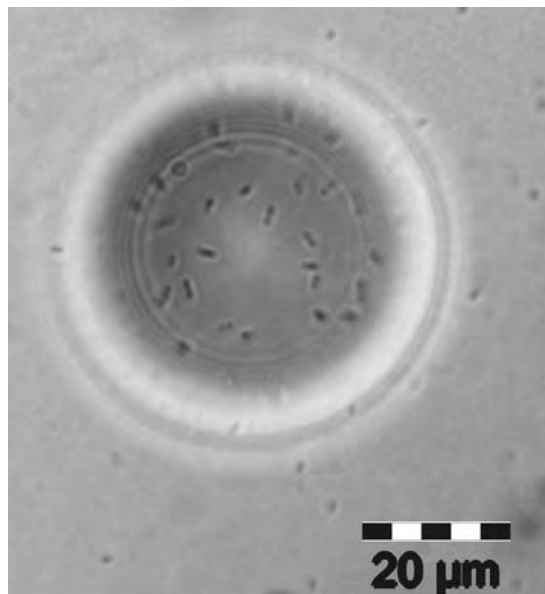
Figure 4. Uropathogenic *E. coli* cells (concentrated at approximately 10^8 cells/ml) adhering to a micelle prepared from the glycoprotein, yeast invertase as photographed with an Olympus DP70 Digital Camera using differential interference contrast (DIC) optics on an Olympus BX51/BX52 microscope. Lens (100x) is focused on the lower surface of the micelle.

sugar sequences that bind to adhesins of *Staphylococcus aureus* [Krivan *et al*., 1988] and *Neisseria gonorrhoeae* [Schengrund, 2003]. Recent studies have demonstrated that uromodulin prevents the binding of uropathogenic *E. coli* cells to urinary bladder epithelium [Pak *et al*., 2005] and that genetic defects associated with uromodulin synthesis are correlated with frequent and severe urinary tract infections [Devuyst *et al*., 2005].

In order to block binding or remove pathogens from infected tissue, carbohydrate decoys must present a sufficient number of glycotopes to compete with cell membrane glycoconjugates for pathogen lectins. We have demonstrated that *E. coli* cells bound to glycoprotein films by lectins at the tips of fragile type 1 fimbriae are not removed by centrifugal force when spun on an AccuSpin Micro R centrifuge for 5 minutes at 13,000 revolutions per minute (about 16,000 G, the force of gravity.) This tenacious adhesion illustrates the avidity of multiple lectin-carbohydrate bonds, and represents a means of separating debris and unbound bacteria from micelles to which pathogens are attached. It also suggests that richly-branched oligosaccharides, covalently bound to glycoprotein micelles, might compete well with loosely-bound glycoconjugates on human cell membranes for pathogen lectins. Thus a suspension of glycoprotein micelles might rinse pathogens from infected tissue. See Figure 5.

Chemical synthesis of multivalent carbohydrate decoys is costly [Boltje *et al*., 2009]. By contrast, glycoprotein films studded with covalently-bound, closely-spaced glycotopes are relatively inexpensive to produce. In the Appendix, we provide examples of raw materials that we have used to produce micelles coated with glycotopes of pathogen lectins, and demonstrate the capture of uropathogenic *E. coli* cells expressing two different lectins involved in the establishment of bladder and kidney infections.

Microscopic observation of bacteria adhering to films prepared from non-sterile glycoproteins [Mullen, 2003] motivated us to conduct a search for information on the chemistry of adhesive structures that bind pathogens and biotoxins to human tissue during the establishment of an infection. In the following sections we discuss the development and implementation of visualization tools based on glycoprotein micelles that facilitate the study of lectins and glycotopes involved in the process of infection. We also describe the development of software tools that enabled us to identify specific glycotopes that could be used to capture pathogens expressing complementary lectin adhesins.
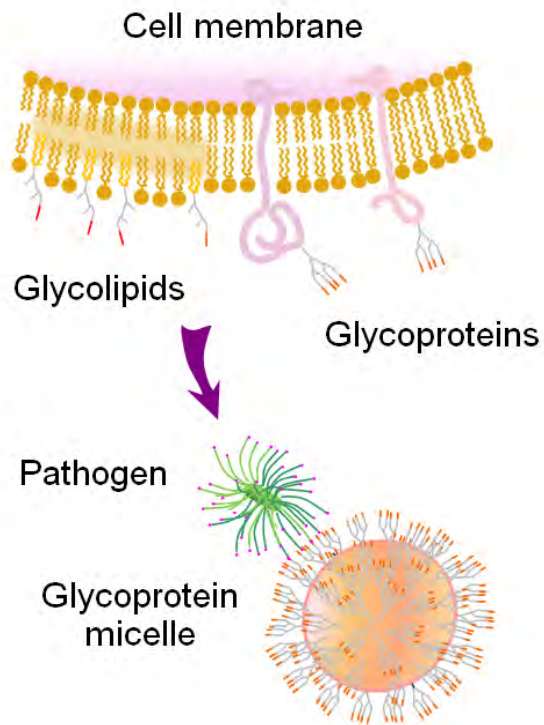
Figure 5. Schematic envisionment (not to scale) of a pathogen (green) being pulled from receptors on a cell membrane by a moving micelle, to which it has adhered through multiple lectin-carbohydrate bonds.

III. Development of Glycoprotein Biocapture Films

Studies reported in the literature had demonstrated that glycotopes bound to solid substrates could capture and remove pathogens from milk and other complex aqueous matrices [Bundy and Fenselau, 2001; Colwell *et al*., 2003]. We hypothesized that floating glycoprotein films coated with glycotopes that are also present on human cell membranes would bind to human pathogens suspended in aqueous fluids and lift them to the surface, where they could be collected and analyzed. Because glycoprotein micelles are slippery and flexible, we also hypothesized that they would be well-suited to the extraction of pathogens and biotoxins from aqueous samples containing debris or sticky proteins that could block a filter. In order to test our hypothesis, we developed the tools described in the sections below.

III.A. Micelle-Agglutination Tools

Agglutination or clumping of glycoprotein micelles represents visible evidence for two sets of nanoscale structures that must be present in order for biocapture to occur: 1) lectin adhesins expressed on the pathogen surface, and 2) glycoprotein films coated with glycotopes that are complementary to the bacterial lectins. See an example of micelle agglutination in Figure 6. A similar phenomenon, hemagglutination (i.e., agglutination of blood cells from a particular animal) has long been used as a clinical diagnostic tool to identify certain viral and bacterial pathogens that express strain-specific lectin "hemagglutinins" [Goldhar, 1995; Ito *et al*., 1997; Shahid *et al*., 2009]. Agglutination of plant and animal cells coated with species-specific glycotopes also provides visible evidence to medical microbiologists that bacterial pathogens are expressing lectin adhesins involved in the establishment of an infection [Hagberg *et al*., 1981; Sharon and Lis, 2004]. In Sections III.A.1 and III.A.2 below, we describe micelle-agglutination tools that have facilitated the development and testing of glycoprotein films designed to capture and remove pathogens and biotoxins from water and from liquid foods. Our experiments suggest that glycoprotein micelles might complement methods currently used to characterize unknown or rapidly-evolving infectious organisms.

III.A.1. Micelle Agglutination Verifies Expression of Pathogen Lectins

It is well-documented in the literature that many bacterial pathogens can express several lectins that bind to different sugar sequences [van der Velden *et al*., 1998; Evans and Evans, 2000; Schengrund, 2003; Shakhsheer *et al.,* 2005]. Further, some bacteria shed or switch lectins in response to specific environmental stimuli [Johnson, 1991]. Therefore, it was necessary to test each bacterial strain to determine which lectins were expressed when cells were cultured under specific conditions and then suspended in the medium from which they would be collected on glycoprotein biocapture films.

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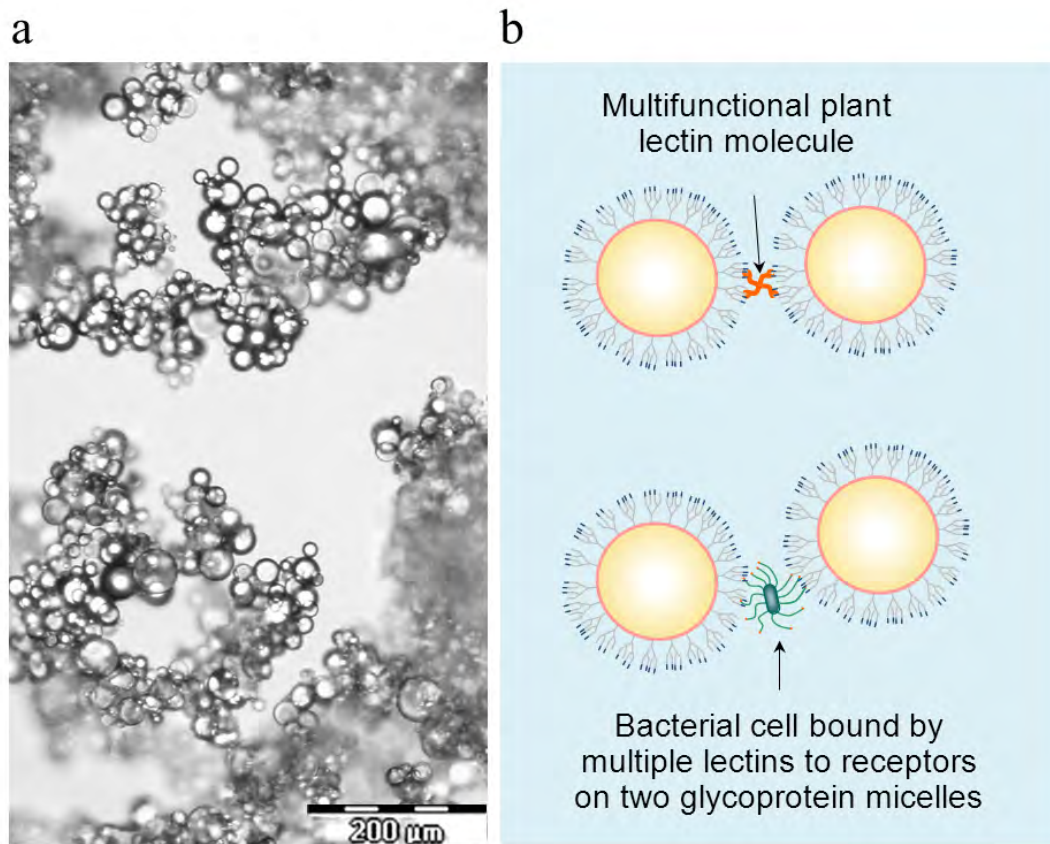
Figure 6. The agglutination process illustrated in this figure involves the coordinated binding of glycotopes on adjacent glycoprotein micelles with lectins attached to opposite sides of intervening bacterial cells or multivalent plant lectin assemblies. (**a**) Mannose-coated micelles prepared from the glycoprotein, yeast invertase are agglutinated by *Salmonella enterica* cells concentrated at approximately 10^8 cells per ml. (**b**) Diagrammatic representation (not to scale) of agglutination resulting from simultaneous binding of multiple lectins projecting from a plant molecule (top) or bacterial cell (bottom) to glycotopes on two micelles.

In some cases, agglutination of plant or animal cells can be used to detect lectin expression. For example, agglutination of guinea pig erythrocytes and baker's yeast cells are used to verify the expression of type 1 fimbrial adhesins and other mannose-binding lectins on cells of pathogenic and commensal bacteria that persist in the human gut [Eshdat *et al*., 1981]. However, not every sugar structure that serves as a receptor for a human pathogen lectin is present on readily-available cells. In an effort to address this issue, we developed glycoprotein micelles that would agglutinate in the presence of bacterial cells present in concentrations higher than 10^6 cells per milliliter.

Prior to conducting biocapture experiments involving bacterial pathogens, we verified lectin expression by mixing a drop of culture broth containing approximately 10^9 colony-forming units (cfu) per ml with a drop of water containing relatively small micelles (ranging from about 5 to 20 micrometers in diameter) coated with complementary glycotopes. Micelle agglutination, which is visible to the unaided eye, provided immediate evidence that pathogens cultured under specific conditions had expressed lectin adhesins by which they could be captured. We observed that some strains of bacteria (*Salmonella enterica* serovar Typhimurium, for example) secreted a sticky substance that also agglutinated micelles. In these cases, it was necessary to rinse and re-suspend bacterial cells prior to conducting an agglutination test for lectin expression.

The addition of translucent colored dyes to the oil sequestered within glycoprotein micelles enhances their visibility in agglutination assays and provides another dimension of lectin discrimination. In some cases, the expression of one or more lectins represents a means of distinguishing a particular pathogen from another closely-related strain [Bertin, 1998]. A bacterial strain that simultaneously expresses two lectins with different binding affinities could agglutinate two sets of micelles coated with different glycotopes and filled with oil containing different dye colors. A mixture of red, yellow and blue micelles would appear greyish to the unaided eye. However, if a strain of bacteria agglutinated only the blue micelles, an observer would see blue dots against an orange background. If it agglutinated both red and blue micelles, purple dots would emerge against a yellow background. Micelle agglutination represents a relatively safe means of determining sugar affinities of lectins associated with pathogens that are unknown or dangerous to handle. However, variation in the decoration of branched oligosaccharides (glycoforms) could present a disadvantage in using natural glycoproteins to characterize pathogen lectins. For example, the absence of a sugar that is usually present at the distal tips of oligosaccharides produced in a particular plant or animal tissue could expose an underlying carbohydrate structure that serves as a receptor for a second lectin expressed by the pathogen under study. One way around this issue is to use synthetic glycoproteins (i.e., neoglycoproteins) covalently bound to synthetic oligosaccharides with well-defined structures.

III.A.2. Micelle Agglutination Verifies Surface Presentation of Glycotopes

Following the preparation of micelles designed to capture pathogens, we used plant lectins to verify that glycotopes were present on the surface of glycoprotein micelles. In an initial proof-of-concept study, we used micelle agglutination to prove that lectins were binding to carbohydrates, and not to amino acids on the hydrophilic face of a glycoprotein film. We conducted an experiment using highly-purified forms of the enzyme RNase isolated from the cow pancreas. One form of the protein known as RNase A is not glycosylated, while the other, RNase B is a glycoprotein bearing a single asparagine-linked oligosaccharide that contains a varying number of mannose residues attached to branching antennae [Puett, 1973]. First we used a Beckman Coulter capillary electrophoresis instrument and molecular weight analysis kit to verify that there was no cross-contamination between the two proteins (purchased from Sigma-Aldrich.) Then we prepared micelles from RNase A and RNase B, and mixed samples from each set with the mannose-binding plant lectin, Concanavalin A (or ConA) purchased from Vector Laboratories. ConA is a tetramer shaped like a pin wheel with four identical arms, each of which can bind to mannose [Reecke *et al*., 1975; Linnemans, 1978; Sharon and Lis, 2004]. As illustrated in the diagram at the top of Figure 6b, we expected ConA to agglutinate micelles prepared from the glycosylated form of the protein, RNase B, but not those prepared from RNase A. Indeed, we found that mixing a drop of RNase B micelles with a drop of plant lectin solution (diluted to about 20 micrograms per ml) produced visible micelle-agglutination. RNase A micelles were unstable, but we saw no agglutination when they were mixed with ConA. In subsequent studies involving biocapture films produced from other glycoproteins, agglutination of micelles by plant lectins demonstrated the presence of specific glycotopes on hydrophilic film surfaces. For example, the plant lectin, GSL II agglutinated chicken egg white micelles coated with oligosaccharides typically terminating in the sugar, N-acetylglucosamine. However, GSL II did not agglutinate yeast invertase micelles coated with oligosaccharides terminating in the sugar, mannose.

Bacterial cells expressing specific lectin adhesins can also be used to verify the presence of glycotopes on micelle surfaces. We found that bacteria in concentrations as low as 10^7 cells per ml could agglutinate micelles ranging from about 2 to 10 microns in diameter. See Figure 6 a. Larger micelles were agglutinated by higher concentrations of bacteria. Thus, in addition to demonstrating the presence of specific glycotopes on micelle surfaces, micelle agglutination could be used as visible evidence of high concentrations of pathogens or biotoxins in turbid water or liquid foods. The addition of dye to the oil sequestered within micelles increases the visibility of agglutinated micelles. The following section describes applications based on this enhancement.

III.B. Glycoprotein Micelles Serve as Tools for Quantifying Captured Cells and Toxins

Glycoprotein micelles represent novel tools for microscopic observation of captured bacterial cells. Because films are transparent, cells attached to upper and lower micelle surfaces (relative to the view through an optical microscope) can be counted separately. This can be accomplished using differential interference contrast microscopy, which facilitates the quantification of bacteria when the stage is slowly moved through the shallow focal plane of a high-powered lens. See Figure 4.

The transparency and spherical form of micelles also enhances visibility of fluorescent tags that are too faint to be detected on a flat surface. Low concentrations of fluorescein-labeled molecules can be detected at the micelle periphery where the film curves sharply at right angles to the focal plane. The enhancement of a fluorescent signal by curved edges is illustrated in Figure 3. Thus glycotope-coated micelles could be used to capture and concentrate biotoxin molecules from dilute solutions, and to enhance the visibility of a fluorescent antibody attached to toxin moieties.

III.C. Specially-Developed Bioinformatics Tools Facilitate the Development of Glycoprotein Films Designed to Capture Specific Pathogens

When we began our research on biocapture films in 2002, our goal was to prepare a set of micelles that could capture any human pathogen from water or liquid food. In order to identify pathogen lectins for which glycotopes had been discovered, we conducted an extensive literature survey. This effort culminated in the production of a searchable database of carbohydrate structures to which particular pathogens and lectins are known to bind. This online bioinformatics resource, SugarBindDB [Shakhsheer *et al.,* 2005] compiles and makes accessible information from studies conducted during the past three decades in several fields, including medical microbiology and glycobiology.

Once we had produced the SugarBindDB database and sorted the contents by carbohydrate ligand, we realized that most of the sugar sequences that had been studied were associated with glycolipid molecules. A notable exception is the sugar mannose which is present in oligosaccharides of glycoproteins, but is rarely found on animal glycolipids. Mannose serves as a receptor for lectins expressed by many bacteria that inhabit the intestines of warm-blooded animals. Mannose-binding bacteria are frequently found in food and water contaminated with human pathogens of fecal origin. Mannose is also involved in binding uropathogenic *E. coli* to urinary bladder cells [Zhou *et al*., 2001]. In the Appendix, we demonstrate the capture of a uropathogenic *E. coli* strain expressing mannose-binding type 1 fimbriae on micelles prepared from the glycoprotein invertase. We also discuss the binding of uropathogenic *E. coli* cells to

oligosaccharides of pigeon egg white glycoproteins that terminate in the disaccharide galabiose, a relatively rare glycotope that is present on glycolipid oligosaccharides in the human kidney.

Despite these exceptions, in most cases, intracellular enzymes add the same tissue-specific sugars to oligosaccharides of both glycolipid and glycoprotein molecules [Bosmann *et al*., 1969; Urdal *et al.*, 1983; Wattenberg, 1990]. These tissue-specific sugars often serve as glycotopes of pathogen lectins. For example, the glycotope, N-acetylgalactosamine (beta 1-4) galactose, present on human respiratory tract epithelium, binds to lectin adhesins of several unrelated respiratory pathogens including *Legionella pneumophila, Klebsiella pneumonia, Streptococcus pneumonia, Pseudomonas aeruginosa* and *Bordetella pertussis* [Thomas and Brooks, 2004; Krivan *et al*., 1988; S de Bentzmann *et al.*, 1996; Brennan *et al*., 1991]. We hypothesized that glycoprotein micelles coated with glycotopes that had first been discovered in glycolipid oligosaccharides would capture pathogens expressing complementary lectins.

By searching the database GlycoSuiteDB [Karlsson *et al.,* 2001], we were able to identify glycoprotein sources of several well-defined glycotopes of human pathogen lectins. The software provides a search capability that allows users to define and locate specific sugar sequences within oligosaccharides listed in the database. In addition, GlycoSuiteDB lists animal and plant sources of oligosaccharides that have been characterized by methods that include enzymatic degradation and mass spectrometry. Using GlycoSuiteDB in combination with SugarBindDB, we were able to select relatively inexpensive sources of glycoprotein molecules that contain one or more glycotope per oligosaccharide moiety. For example, we learned that the glycotope N-acetylgalactosamine (beta 1-4) galactose (see paragraph above) is present on highly-branched oligosaccharides of uromodulin (mentioned previously in Section II.C). These oligosaccharides also bear glycotopes containing the sugar sialic acid that bind to a different set of pathogens, including viruses.

III.D. Production of Micelles Designed to Capture Biotoxins and Pathogens Expressing Lectin Adhesins

We have observed that glycoproteins produced by a variety of organisms can be used to prepare micelles that capture human pathogens. For example, the enzymes "yeast invertase" and "bovine RNase" both bear "high mannose glycans" that bind to FimH lectin adhesins expressed by many pathogenic strains of *E. coli* and *Salmonella* [Kisiela *et al*., 2006; Hung *et al*., 2002]. Glycoprotein micelles designed to collect pathogens from aqueous samples are similar to those produced for the agglutination assays described above in Section III.A, but are larger. Micelles ranging in diameter from about 10 to 100 micrometers – a size that has proved to be ideal for removing pathogens from the surface of an aqueous suspension within a few minutes of mixing – are mechanically stable in aqueous suspensions, and are not degraded by detergents.

To produce floating micelles ranging in diameter from about 10 to 100 micrometers we developed the following production methods. When oil is first introduced into a glycoprotein solution, the mixture is swirled or shaken to produce film-coated droplets that are visible to the unaided eye. Large "micelles" thus formed are mechanically unstable and inefficient in terms of collective surface-to-volume ratio. As micelles rise to the surface of a preparation, they are aspirated into a sterile transfer (Beral) pipette or syringe and forced back through the glycoprotein solution, or against the container wall, to break the micelles into smaller spheres. The shear force required for this process increases as micelle diameter decreases. In an alternative method for breaking up the micelles, the glycoprotein solution is agitated rapidly (for example, by vortexing) as oil is added.

The overall glycoprotein film surface area (within the suspension) increases as micelle diameter decreases; so the concentration of unbound glycoprotein molecules must remain high enough to prevent attenuation of films during the micelle production process. To rapidly produce robust films from inexpensive materials, we have often prepared micelles from sterile-filtered solutions containing one milligram of glycoprotein per milliliter of water, and added one unit of liquid oil to six units of glycoprotein solution. However, glycoproteins differ in length and degree of unfolding during micelle preparation. Therefore, to use materials efficiently, one must determine experimentally an optimal molar ratio of oil to glycoprotein. The volume of micelles produced during this process is roughly equivalent to the volume of oil used in their preparation.

Micelles are rinsed three times to remove free glycoprotein molecules that could block bacterial lectins and prevent them from binding to glycotopes on the film surface. Micelles that rise to the top of a 15-ml tube within 15 minutes are removed and stored in sterile water or buffer for future use in biocapture studies. Smaller micelles that remain in suspension longer than 15 minutes are set aside for use in agglutination studies.

III.E. Lectin-Coated Micelles Capture and Transport Sugar-Coated Cells and Glycosylated Molecules

As illustrated in Figures 3 and 6 b, oligosaccharides on glycoprotein micelle surfaces can be coated with multivalent lectin molecules that can, in turn, bind to complementary sugars on cell surfaces or to oligosaccharides of other glycoprotein molecules. For example, lectin-coated micelles can bind to sugars in the coats of *Bacillus* spores and bacterial cells that shed or fail to express lectin adhesins.

To produce micelles decorated with multivalent lectins that are known to bind to spore coat sugars, glycoproteins are identified that contain receptors for those lectins. Micelles are produced as described above in Section III.D and rinsed to remove excess glycoprotein

molecules. The micelles are then forced through a narrow pipe into a relatively high lectin concentration. Agglutination is prevented during the preparation process by assuring that lectin molecules quickly occupy available receptor sites on micelle surfaces. Lectin-coated micelles are collected from the surface of the suspension, rinsed and stored in HEPES buffer until needed.

Experiments conducted in our laboratory have demonstrated that lectin-coated micelles also can be used to transport biologically-active glycoprotein molecules. For example, micelles were produced from the enzyme invertase (a glycoprotein produced by baker's yeast *Saccharomyces cerevisiae*). Because the three-dimensional (i.e., quaternary) molecular structure is disrupted when the solution is heated and stirred prior to micelle preparation, invertase molecules lying within the surface film are not enzymatically active (unpublished data). Invertase micelles were coated with the plant lectin ConA, rinsed in water then coated again with yeast invertase that had not been heated above 60 degrees centigrade. Intact invertase molecules thus tethered to the micelle surface were able to hydrolize sucrose, as observed by a color change in Benedict's reagent (purchased from Fluka-Chemika-Biochemika).

The use of micelles as transporters can be applied to the retrieval of expensive enzymes and reaction products from a reaction bath. For example, we used the enzyme β-1,4-Galactosyl Transferase I (purchased from Sigma) to add galactose to N-acetylglucosamine on oligosaccharides of micelles produced from the chicken egg white glycoprotein, ovomucoid (purchased from Worthington.) See Figure 7. Following the reaction, redecorated micelles were removed from the surface of the reaction bath, rinsed and mixed with a fluorescent galactose-binding lectin, *Ricinus Communis* Agglutinin (RCA$_{120}$ purchased from Vector Laboratories). Fluorescence microscopy was used to detect the binding of RCA$_{120}$ to micelles redecorated with galactose. As expected, it was found that RCA$_{120}$ did not bind to the original ovomucoid micelles coated with N-acetylglucosamine. This experiment demonstrated our ability to redecorate the oligosaccharides of an inexpensive glycoprotein, and retrieve the product from the reaction bath on floating micelles. Thus, after rinsing, such redecorated micelles could be used to collect pathogens or biotoxins like *Ricinus Communis* Agglutinin that bind to enzymatically-produced glycotopes that are not present on readily available glycoprotein oligosaccharides .

In addition to its utility as a biocapture substrate, yeast invertase serves as an ideal model for the study of glycoprotein film structure and micelle behavior. Unlike most proteins, invertase does not contain intramolecular loops produced by disulfide bridges (i.e., covalent bonds that form between cysteine amino acid constituents of the protein backbone). Therefore, an unfolded invertase molecule can extend at an oil-water interface into an elongated or serpentine chain of about 530 amino acids [Hohmann and Gozalbo, 1988]. Oligosaccharides are fairly evenly distributed throughout the molecule, as are hydrophilic and hydrophobic amino acid side groups. See Figure 8 showing the amino acid sequence of invertase.
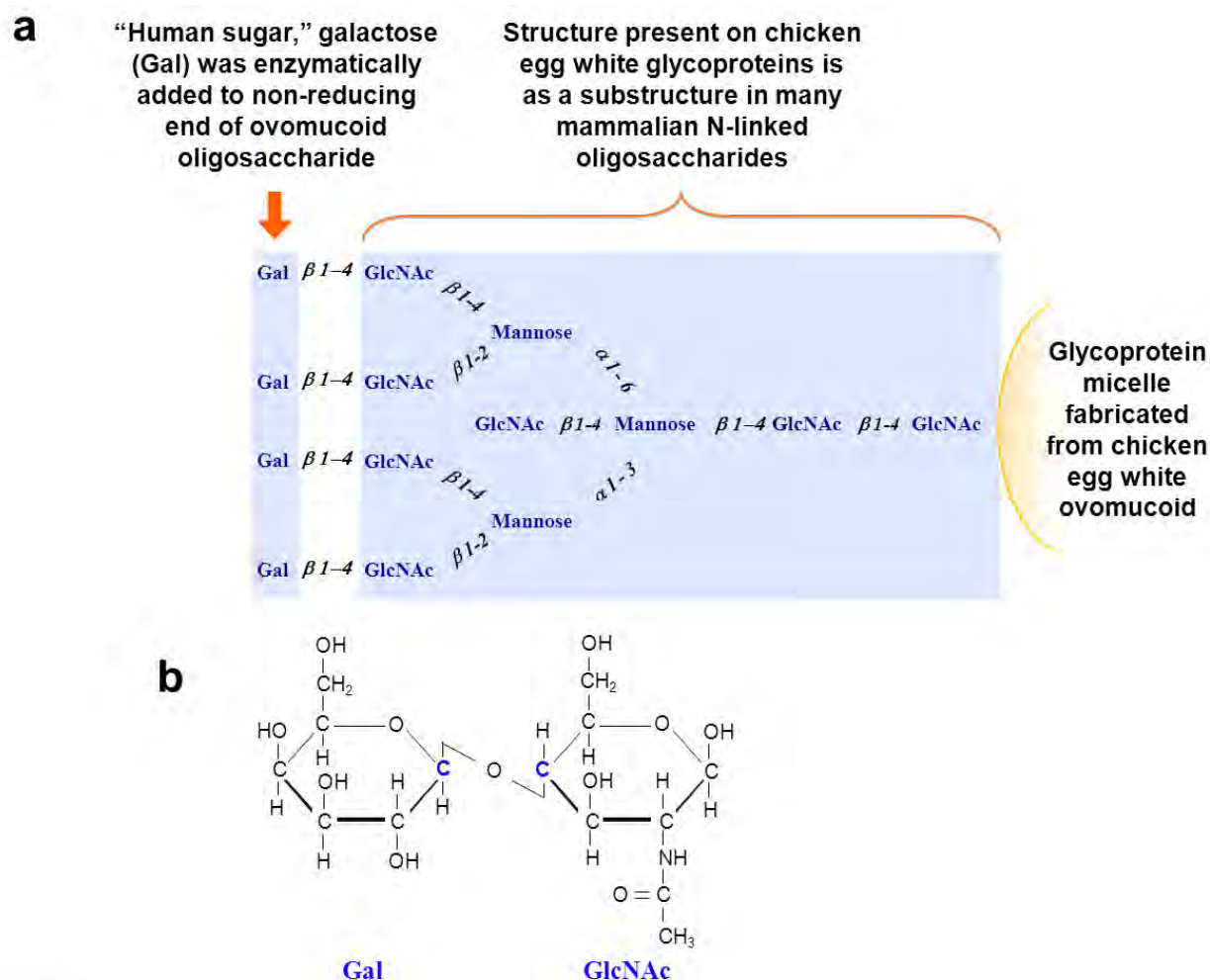
Figure 7. (**a**) Schematic representation of a "redecorated" oligosaccharide attached to a micelle fabricated from the chicken egg white glycoprotein, ovomucoid. The original branching carbohydrate structure (highlighted in blue on the right-hand side of the diagram) is commonly found in N-linked oligosaccharides of both chicken and human glycoproteins. The sugar galactose, abbreviated as Gal and highlighted in blue on the left-hand side of the structure, has been enzymatically added to each N-acetylglucosamine (GlcNAc) residue at the non-reducing end of the chicken oligosaccharide. The structure thus formed is a typically human core structure to which other sugars may be added in a tissue-specific manner. In this case, the enzyme, β-1,4-galactosyl transferase was used to link the anomeric carbon (i.e., carbon number 1) of galactose (provided by a sugar nucleotide molecule) to the fourth carbon in terminal GlcNAc residues of the original ovomucoid oligosaccharide. (**b**) The resulting terminal structure is Gal (b1-4) GlcNAc.

Figure 8. Amino acid sequence of baker's yeast invertase [Hohmann and Gozalbo, 1988] (SUC4 gene, primary accession number: P10596.) Letter codes of hydrophobic amino acids are highlighted in yellow. Positions of oligosaccharide moieties attached to asparagine (N) are indicated by green ovals. (Adapted from UniProKB/Swiss-Prot).

When micelles coated with invertase film are mixed with fluorescein-tagged plant lectin molecules that bind to mannose (e.g., Concanavalin A [ConA] or *Galanthus nivalis* lectin [GNL]), fluorescence microscopy reveals an even distribution of light over the film. See Figure 3. This observation suggests a relatively even distribution of mannose glycotopes on micelle surfaces.

We also have used fluorescein-labeled lectins to prove that sterile invertase micelles are structurally and chemically intact after storage for months at room temperature in distilled water. The binding of invertase micelles to fluorescein-labeled ConA molecules demonstrates a more general application of glycoprotein films and micelles: the removal of toxic lectins from aqueous suspensions.

We have demonstrated in our laboratories that invertase micelles can be used to capture both commensal and pathogenic enteric bacteria that express mannose-binding lectins, including *E. coli* and *Salmonella*. For example, in the Appendix, we describe an experiment in which invertase micelles were used to capture uropathogenic *E. coli*, a pathogen that is benign in the human intestine, but which causes urinary bladder and kidney infections responsible for more than a billion dollars of direct health care costs annually [Russo and Johnson, 2003]. Experimental results suggest that a possible application of glycotope-coated micelles might be the removal of pathogens and biotoxins from contaminated human tissues.

IV. Development of Protocols for Testing the Capture of Human Pathogens on Glycoprotein-Coated Micelles

In this section we describe the development of experiment protocols designed to estimate the number of pathogens that are captured from an aqueous suspension after about 15 minutes of mixing with biocapture micelles of known diameter range and concentration. Pilot studies demonstrated that high concentrations of bacteria agglutinated micelles, making it impossible to use colony counts to estimate the number of bacteria that were captured on biocapture films. Therefore, we modified traditional microbiological methods to increase the accuracy of sample measurements and assumptions on which calculations are based. Experiments presented in the Appendix demonstrate a specific case in which analytical methods introduced in this section were used to quantify the capture of human pathogens on glycoprotein-coated micelles.

Our first biocapture studies were designed to test the capture of *E. coli* expressing type 1 fimbriae on mannose-coated micelles made from inexpensive materials. Experiments were conducted in 15-ml test tubes filled with 6 ml of water or saline solution containing *E. coli* strains "ON" (expressing mannose-binding lectins) or "OFF" (control) in concentrations of approximately 10^7 to 10^6 cfu per ml [Mullen *et al.*, 2006]. Invertase micelles (1 ml prepared as described in Section III.D) were added and continuously mixed into the bacterial cell suspensions by inverting tubes for time intervals ranging from 5 to 20 minutes. Micelles were allowed to rise to the surface for 15 minutes, and then were removed with a transfer pipette and re-suspended in sterile solution to remove unbound bacterial cells. One hundred-microliter samples of aqueous suspension from which cells had been captured were withdrawn and spread on culture plates. After allowing an additional 15 minutes for rinsed micelles and attached bacterial cells to rise to the surface, 100-microliter micelle samples were spread on culture plates. Following overnight incubation at 37 degrees centigrade, visible colonies were counted.

When conventional microbiological methods are used, one can assume that each spot on a culture plate represents a colony that has grown from a single bacterial cell. Therefore, the colony count can be used to estimate the number of living cells (colony-forming units or CFUs) that were present in the sampled fluid. However, through microscopic examination of micelles that had captured bacteria from high concentrations (10^7 to 10^9 cells per ml) we discovered that several bacterial cells weresometimes attached to a single micelle. Consequently, when micelles were spread across the surface of a culture plate, several bacterial cells that remained attached to a single micelle would produce one visible colony. Therefore, colony counts underestimated the number of bacteria captured on micelles. Moreover, bacteria in concentrations as low as 10^7 cfu/ml were found to agglutinate micelles, making it difficult to aspirate small volumes in narrow pipettes. Thus, in order to more accurately quantify the extent of biocapture, we replaced traditional pipette tips with wide-orifice and gel-loading tips. Wide-orifice pipette tips facilitated accurate measurement of small micelle volumes. In order to more accurately count the number

of bacteria that had been removed from a suspension, we used gel-loading tips, which are much longer than traditional pipette tips, to withdraw fluid from lower levels that were unlikely to contain small micelles and attached bacteria that had not risen to the surface. The micelle-rinsing step was omitted from experiments involving lower bacterial cell concentrations (1000 to 10 cfu/ml), after we discovered that the number of cells cultured from the rinse water was insignificant. Figure 9 illustrates this modified experimental procedure.

The process of quantifying biocapture was also complicated by the fact that cells appeared to multiply during the mixing process. Particularly in the control strain that did not express mannose-binding lectins, the total cell count increased dramatically during an experiment, perhaps because strings of adherent cells were separated into individual colony-forming units by turbulent shear stress created when tubes containing air bubbles were inverted. To compensate for this discrepancy, we evaluated experimental results by comparing ratios of colonies grown from the micelle layer to colonies grown from the remaining aqueous suspension.

In low concentrations, the number of bacterial cells in a small sample is extremely variable. Therefore, if the number of colonies on a culture plate was fewer than 30, we considered the experimental results to be statistically insignificant. In order to enumerate cells captured from low concentrations of bacteria (10 to 100 cells per ml), we increased sample sizes and the number of culture plates used to count colonies. Results indicated that, within 10 minutes of mixing micelles with bacterial suspensions, about 50 percent of suspended cells were captured from 6 ml fluid on 1 ml micelles ranging from 10 to 150 microns in diameter. See the discussion of experimental results in the Appendix.

Increasing mixing time tended to increase the total number of cells captured. However, FimH lectins of type 1 fimbriae have been implicated in the initiation of biofilm formation through the binding of bacterial cells to abiotic surfaces, and in auto-aggregation of *E. coli* cells that results in their falling in clumps from a cell suspension [Schembri and Klemm, 2001; Schembri *et al.*, 2001]. Thus prolonged mixing time could increase the likelihood of auto-aggregation and adhesion of cells to container walls – two phenomena that may compete with biocapture on micelle surfaces.

In the Appendix, we discuss our unexpected discovery that *E. coli* cells expressing type 1 fimbriae bind to oligosaccharides that are known to contain little or no terminal mannose. Other experiments conducted in our laboratory (data not shown) demonstrated that micelles made from yeast invertase and water-soluble chicken egg white (CEW) glycoproteins are equally effective in removing type 1-fimbriated *E. coli* cells from aqueous suspensions. In yet-unpublished experiments, we demonstrated that the predominant terminal sugar on CEW oligosaccharides is N-acetylglucosamine, a finding that is consistent with glycoforms of CEW oligosaccharides characterized previously [Yamashita *et al.,* 1982; Harvey et al., 2000]. See Figure 7.

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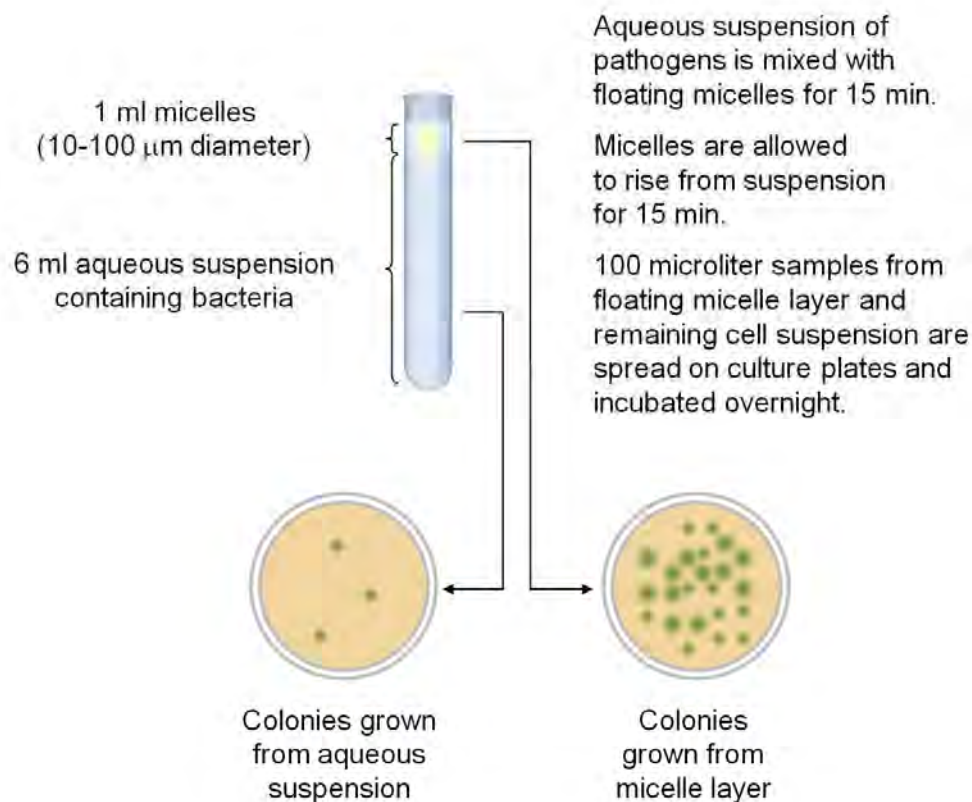
Figure 9. Schematic illustration of a typical biocapture experiment. One-ml aliquots of micelles ranging in diameter between 10 and 100 micrometers are added to 15-ml tubes containing 6 ml of bacterial cell suspensions in concentrations of 10, 100 or 1000 colony-forming units per ml. Tubes are inverted for 10-20 minutes. Micelles are allowed to rise to the surface of the aqueous suspension for 15 minutes. Aliquots (100 or 200 microliters) are withdrawn from the surface into a sterile, wide-orifice pipette tip and distributed over agar culture plates (3 to 5, depending on the original cell concentration) using Lazy L spreaders. Aliquots are withdrawn from the aqueous fluid beneath the micelle layer into a sterile gel-loading pipette tip, and spread onto culture plates (3 to 6, depending on the original cell concentration). After overnight incubation, cell colonies are counted, and concentrations are calculated to determine the number of cells captured on 1 ml micelles, and the number of cells remaining in 6 ml aqueous suspension. Experimental results are expressed as ratios of average cell concentrations: the number of bacteria captured on 1 ml micelles to the number of bacteria remaining in 1 ml aqueous suspension. See Table 3 in the Appendix.

It is now known that type 1 fimbrial lectins expressed by most commensal and pathogenic strains of *E. coli* bind to trimannose [Roe *et al.*, 2001; Schembri and Klemm, 2001], a glycotope that is present in the core of all N-linked oligosaccharides. Therefore, it is likely that inexpensive micelles made from readily available glycoproteins could be used to collect *E. coli* and other enteric bacteria from aqueous samples.

V. Discussion and Summary

The foregoing has described the results of nearly a decade of experimental effort, in which we have refined techniques for making and employing glycoprotein films to both characterize and defend against human pathogens and biotoxins. We have discovered that sheets of glycoprotein film that form at an oil-water interface can be broken into spherical, oil filled micelles coated with covalently-bound oligosaccharides that represent multivalent glycotopes of human pathogen lectins. Because micelles have a long shelf life of several months when stored in sterile water, we hypothesized that they might represent robust biocapture substrates. For example, floating micelles could be used to lift pathogens from fluid samples containing detritus and sticky proteins that would block a filter, and would be especially useful in remote areas where it is not feasible to operate a vacuum-filtration system.

To evaluate the efficacy of using glycoprotein films to collect human pathogens from aqueous suspensions, we used yeast invertase micelles coated with high-mannose glycans to capture uropathogenic *E. coli* cells expressing FimH lectins. We modified standard microbiological protocols to increase the accuracy of quantifying biocapture. However, microscopic examination revealed that a single micelle can collect more than one bacterial cell. Therefore, the number of colonies that grow from an aliquot of micelles spread on a culture plate may not accurately reflect the number of bacterial cells that were captured. Our discovery that bacteria expressing type 1 fimbriae bind to biocapture micelles made from many different glycoproteins (see the Appendix) led to a realization that inexpensive materials could be used to collect these indicators of surface water pollution.

Based on experimental results and on studies cited in GlycoSuiteDB [Karlsson *et al*., 2001] and SugarBindDB [Shakhsheer *et al.*, 2005], we believe that biocapture films prepared from affordable glycoproteins like yeast invertase and human uromodulin bind to a wide assortment of bacterial and viral pathogens. We have demonstrated that, when glycotopes required to capture specific pathogens are not available on affordable glycoproteins, it is possible to produce them on micelle surfaces by enzymatically altering existing oligosaccharides.

We also have demonstrated that, in addition to collecting pathogens that express lectin adhesins, glycoprotein micelles can be coated with multivalent plant lectins that bind to glycotopes in the coats of bacterial spores. Lectin-coated micelles can also be used to transport biologically active glycoprotein enzymes through a chemical reaction, and retrieve them from the reaction bath.

We have expanded our research focus to include the potential application of glycoprotein films and micelles to the prevention and treatment of diseases caused by pathogens and biotoxins that bind to human cell-surface carbohydrates. We propose that the radial presentation of closely-spaced, covalently-bound glycotopes on micelle surfaces underlies their observed tenacious

binding to pathogens bearing complementary lectins. Based on this assumption, we believe that glycotope-coated micelles would compete well with human cell membranes for pathogen lectins, and might be used to remove bacterial cells, virus particles and biotoxin molecules from human tissues.

Another manifestation of the presence of multiple, complementary binding partners on pathogen and glycoprotein film surfaces is micelle agglutination. This phenomenon represents a means of visually detecting high concentrations of pathogens in murky water, liquid foods, and other complex biological samples. The addition of transparent dyes to the oil sequestered within micelles enhances visualization of agglutination, and provides another dimension of discrimination that could be used to facilitate the study of host-pathogen interactions. For example, lectins bound to color-coded micelles could be used to locate specific glycotopes on human tissue samples.

When we began developing biocapture films in 2002, our goal was to prepare a set of micelles coated with glycotopes that could collect any human pathogen from water or liquid food. In the process of searching the literature for glycotopes of lectins associated with human pathogens, we discovered that the human glycome has not been fully characterized [Cummings, 2009]. Therefore, scientists do not yet have a comprehensive list of potential carbohydrate structures to which a human pathogen could bind. In many cases, glycotopes of fimbrial adhesins have not been identified. Further, even in the most thoroughly-studied cases, it is often not clear which environmental conditions induce lectin expression. We believe that color-coded glycoprotein micelles bearing well-defined glycotopes might be used to visualize the expression of specific lectins under different environmental conditions, and would facilitate research on the glycobiology of infectious disease.

Appendix

Example Experiment: Uropathogenic *E. coli* Strains Captured on Glycoprotein Micelles

A. Background

The presence of *E. coli* in food and water serves as an indicator of fecal contamination [Doyle and Erickson, 2006]. Non-pathogenic *E. coli* strains are often accompanied by pathogenic bacteria, viruses, protozoans and other parasites that infect both human and animal populations. Most pathogenic strains of *E. coli*, *Salmonella* and *Shigella* express mannose-binding FimH lectins borne on hair-like type 1 fimbriae [Firon *et al*., 1983; Roe *et al*., 2001]. The experiment discussed in this Appendix was designed to demonstrate and evaluate the efficacy of using floating, mannose-coated glycoprotein micelles to capture *E. coli* from water. For this purpose, we chose the well-studied and highly virulent uropathogenic *E. coli* (UPEC) strain CFT073 as a model organism. The complete genetic sequence of strain CFT073 is known [Welch *et al*., 2002], and genes that encode lectins involved in the establishment of bladder and kidney infections have been identified [Hull *et al*., 1981; Klemm and Christensen, 1987; Strömberg *et al*., 1990; Sokurenko *et al*., 1995; Ishikawa *et al.*, 2004]. Most importantly, mutant UPEC strains have been produced that permit us to separately study cells expressing two lectins known to be involved in urinary tract infection. When "wild type" UPEC cells enter the human urinary tract, they produce FimH lectins at the tips of type 1 fimbriae.   These lectins bind to the sugar mannose on exposed bladder cell membranes [Wu *et al*., 1996]. When they reach the kidneys, UPEC cells shed their type 1 fimbriae and produce pin-like P pili bearing "PapG" lectins that bind to a relatively rare sugar structure known as "galabiose" [Hoschützky *et al*., 1989].

To prepare mannose-coated micelles that would bind to UPEC cells expressing FimH lectins [Hung *et al*., 2002], we chose the commercially-available and relatively inexpensive glycoprotein yeast invertase described above. Each invertase molecule bears thirteen [Hohmann and Gozalbo, 1988; Reddy *et al*., 1999] oligosaccharides that terminate in branching chains of mannose [Trimble and Atkinson, 1992]. See oligosaccharide distribution in Figure 8.

Pigeon egg white (PEW) provides a source of galabiose that binds to PapG lectins typically expressed when "wild type" UPEC cells are grown on agar plates at 37 degrees centigrade [Johnson *et al*., 2001; Johnson *et al.*, 1992; Johnson, 1991]. Galabiose is present at the tips of antennae on oligosaccharides of all major PEW glycoproteins [Suzuki *et al.*, 2001; Takahashi *et al.*, 2001]. Based our research on egg white glycoproteins [Mullen, 1998] and on private conversations with other investigators, [Fenselau, 2001; Lee, 2002; Johnson, 2003], we hypothesized that micelles produced from soluble pigeon egg white glycoproteins would bind to uropathogenic *E. coli* expressing PapG lectins. To ensure that PEW oligosaccharides were

unadulterated, we commissioned Mt. Phoenix in Mineral, VA to collect unfertilized eggs from pigeon hens reared in separate cages from males whose presence is thought to induce ovulation [Harper, 1904].

In order to separately test the binding of UPEC cells expressing FimH and PapG lectins, we obtained two mutant strains from the laboratory of Harry L.T. Mobley, then located at the University of Maryland's School of Medicine. One mutant always expresses type 1 fimbriae bearing mannose-binding FimH lectins, and the other never does. These mutant strains were dubbed "ON" and "OFF," respectively [Gunther *et al*., 2002; Snyder *et al*., 2005]. The "OFF" strain was used as a control in experiments designed to quantify the capture of type 1-fimbriated *E. coli* cells on mannose-coated biocapture micelles.

B. Methods

B.1. Prior to Day of Experiment

B.1.a. Preparation of Bacteria

1. *E. coli* CFT073 "ON" and *E. coli* CFT073 "OFF" strains were grown without shaking at 37°C in 50 ml centrifuge tubes containing 20 ml LB broth for 144 hours to promote expression of type 1 fimbriae in the ON strain.

2. Every 48 hours, bacteria were centrifuged for 10 minutes at 4150 RPM and rinsed once in LB broth prior to replacement of medium.

3. At 144 hours cells were centrifuged and rinsed three times in PBS.

4. Cell concentration was estimated using a Petroff-Hausser bacteria cell counting chamber, and suspensions were diluted to approximately 10^{10} cells per ml.

5. Cell suspensions were mixed gently with an equal volume of 10% DMSO / 10% glycerol / 80% PBS preservation buffer, yielding final concentrations of about 5×10^9 cells/ml.

6. After equilibrating for 30 minutes at room temperature, suspensions were mixed again and allowed to stand for 10 minutes before 1 ml aliquots were placed in microcentrifuge tubes, capped, and stored at -80°C.

7. To determine the number of colony-forming cells that survived preservation and freezing, one day prior to biocapture experiment tubes were removed from freezer and thawed in a bio-safety cabinet.

8. Once thawed, suspensions were spun for two minutes at 14,000 RPM, and fluid containing preservative was removed. Cells were resuspended in 1 ml of PBS and mixed gently with a pipette tip without vortexing, in order to avoid breaking fragile fimbriae. (This process was repeated three times.)

9. Seven serial dilutions (100 microliter bacterial suspension/900 microliter PBS) were performed. 100 microliters of the last three dilutions (containing cell concentrations of approximately 10^5, 10^4, and 10^3 cells per ml) were spread on LB agar culture plates (Gibson Laboratories) using a Fisher Turntable and Lazy L spreaders.

10. Plates were bagged, inverted, and incubated overnight at 37°C.

11. Colonies were counted the following morning.

B.1.b. Preparation of Micelles

1. 200 mg invertase (SIGMA Invertase, Grade VII from Bakers' Yeast, Product Number 14504-1G) was dissolved into 200 ml Millipore-filtered water and stirred for 10-15 minutes.

2. During the stirring process, the solution was heated to 95°C and held at that temperature for 5 min., then allowed to cool to room temperature.

3. Solution was sterile-filtered (SF) using a 0.22 μm Stericup filtration unit.

4. 15 ml aliquots of SF invertase solution were pipetted into 50 ml sterile centrifuge tubes.

5. A sterile, graduated transfer pipette was used to add 5 ml SF Crisco canola oil, dropwise, to each solution while vortexing.

6. 50 ml tubes containing micelle suspensions were inverted with force for five minutes.

7. Micelles were passed through a 21G needle twice and allowed to rise to the surface for 15 minutes.

8. In order to dilute residual glycoprotein, micelles were harvested using a transfer pipette and expelled into 50 ml centrifuge tubes containing 35 ml of sterile DI water.

9. Micelles were allowed to rise to the surface for 15 minutes, then harvested again and transferred to a fresh tube of water. All micelles were then pooled into one 50 ml centrifuge tube containing 20 ml of sterile DI water.

10. Micelles were mixed, and then redistributed into four separate 50 ml centrifuge tubes containing 35 ml of sterile DI water for storage until use on the following day.

11. A sample of micelles was examined under 100 x magnification to ensure that the diameter range was approximately 10-100 micrometers.

B.2. Day of Experiment

B.2.a. Preparation of Bacteria

1. Bacterial colonies were enumerated on Q-count hardware and software in accordance with Spiral Biotech manufacturer's protocol.
2. Once the number of colony-forming units (CFU) in the stock cell suspension was known, dilutions were made to achieve 1000, 100 and 10 CFU/ml PBS cell concentrations, ensuring that appropriate volume was prepared (~40 ml) in accordance with the suggested protocol.
3. Working bacterial suspensions were pipetted into 6 ml aliquots in 15 ml tubes.

B.2.b. Agglutination Tests

1. 500 microliter aliquots of *E. coli* CFT073 "ON" and *E. coli* CFT073 "OFF" bacterial stocks were placed in microcentrifuge tubes and spun for two minutes at 14,000 RPM.
2. Cells were re-suspended in 1 ml (1X PBS) and mixed using a pipette tip.
3. 10 microliters of rinsed *E. coli* cells were mixed with 10 microliters of guinea pig erythrocytes (Lampire Biological Laboratories, 5%, lot 409106 PAI) on a microscope slide.
4. Cell suspension was mixed by gentle rocking and allowed to incubate at room temperature for 5 minutes.
5. Agglutination of erythrocytes by *E. coli* CFT073 "ON" (but not by *E. coli* CFT073 "OFF") cells provided proof that the "ON" stock was expressing mannose-binding FimH adhesins.
6. The procedure outlined in the preceding 5 steps was repeated using invertase micelles.
7. Agglutination of invertase micelles by *E. coli* CFT073 "ON" (but not by *E. coli* CFT073 "OFF") cells provided additional proof that the "ON" stock was expressing mannose-binding FimH adhesins.
8. Agglutination of invertase micelles by *E. coli* CFT073 "ON" also provided proof that invertase micelles were coated with mannose-containing oligosaccharides.

B.2.c. Exposure of Bacteria to Micelles

1. Within a biosafety cabinet, invertase micelles (1 ml) were withdrawn into a sterile transfer pipette and added to bacterial suspensions (6 ml) within sterile polypropylene conical tubes (15 ml capacity) containing the estimated cell concentrations shown in Table 1.

Table 1. Estimated concentrations of cell suspensions and number of replicates used in experiment

E. coli Strain	Estimated Starting *E. coli* Cell Concentration	Number of Tubes Containing Cells suspended in 6 ml PBS
CFT073 ON	1000 CFU/ml	3
	100 CFU/ml	3
	10 CFU/ml	4
CFT073 OFF	10 CFU/ml	3

Table 2. Aliquots of volumes listed below were withdrawn from micelle and aqueous layers, and spread on culture plates as follows.

Micelle Layer			
E. coli Strain	Estimated Starting Concentration	Volume of Aliquot	Number of Culture Plates
CFT073 ON	1000 CFU/ml	100 µl	3
	100 CFU/ml	100 µl	3
	10 CFU/ml	200 µl	5
CFT073 OFF	10 CFU/ml	200 µl	5
Aqueous Layer			
E. coli Strain	Estimated Starting Concentration	Size of Aliquot	Number of Culture Plates
CFT073 ON	1000 CFU/ml	100 µl	3
	100 CFU/ml	500 µl	6
	10 CFU/ml	500 µl	6
CFT073 OFF	10 CFU/ml	500 µl	6

2. Capped tubes were inverted for 10 minutes to mix micelles into cell suspensions. Air traveling through the mixture created turbulence that agitated the suspension and contributed to the mixing process.

3. The micelles were allowed to rise through the cell suspension for 15 minutes. (Micelles exposed to bacteria were not washed in this experiment because, at this cell concentration, an insignificant number of bacterial cells is trapped between micelles.)

4. Aliquots from micelle layers were withdrawn using sterile 200 ml wide-orifice pipette tips and spread onto Gibson LB agar plates using Lazy L spreaders.

5. Aliquots of cells remaining in suspension were withdrawn from the bottom of each tube (using a gel-loading tip) and plated.

6. To account more accurately for cells in lower concentrations, greater volumes were sampled and plated as shown in Table 2.

7. To test sterility, 100 microliters of PBS, oil and invertase micelles were plated in duplicate onto LB agar.

8. After fluid samples had been absorbed into the agar (approximately one hour), the plates were bagged, inverted and incubated overnight at 37°C

B.3. Analysis of Colony Counts on Day After Experiment

Resulting colonies were counted the next morning using QCount hardware and software, and the data were imported to Microsoft Excel for analysis. (See Table 3.)

Colony counts from plates containing partial aliquots of micelles and remaining aqueous suspensions were added to calculate actual numbers of bacteria in each fraction.

C. Experimental Results and Discussion

Experimental results are shown in Table 3. Calculations were based on an assumption that the working stock concentrations were correct. Data demonstrate that approximately 50% of cells expressing type 1 fimbriae (uropathogenic *E. coli* strain CFT073 "ON") were captured from 6-ml suspensions ranging in concentration from about 10 to 1000 cells per ml during 10 minutes of mixing with 1 ml of yeast invertase micelles.

Colony counts suggest that the CFT073 "OFF" strain divided more rapidly than the CFT073 "ON" strain during the experiment; but, when stock concentrations are compared, reproduction rates of the two strains are observed to be roughly equal. A plausible explanation for this anomaly is that loosely-connected strings of bacterial cells come apart when suspensions are subjected to turbulent fluid flow during a biocapture experiment, and that adhesion to micelle

Table 3. Experimental results showing the ratio of the concentration of cells captured on micelles to the concentration of cells remaining in suspension, and the average percentage of cells captured on micelles within 10 minutes of mixing.*

Uropathogenic *E. coli* ON and OFF Strains Captured on Invertase Micelles					
Uropathogenic *E. coli* Strain in Estimated Dilution Concentrations	Cells (CFUs) Captured on 1 ml Invertase Micelles	Cells (CFUs) Remaining in 6 ml Aqueous Suspension	Ratio: Concentration of Cells Captured on 1 ml Micelles to Cells Remaining in 1 ml Suspension	Percentage of CFUs Captured on Micelles	Average Percentage of Captured CFUs
CFT073 ON **1000** CFU/ml	590	760	4.657	43.70%	**50.71%**
	743	520	8.573	58.83%	
	610	620	5.905	49.60%	
CFT073 ON **100** CFU/ml	97	78	7.438	55.35%	**58.41%**
	137	70	5.867	66.13%	
	77	66	6.973	53.75%	
CFT073 ON **10** CFU/ml	7	6	7.000	53.85%	**49.71%**
	15	10	9.375	60.00%	
	12	8	9.231	60.00%	
	6	18	2.000	25.00%	
CFT073 OFF **10** CFU/ml	182	2216	0.493	7.59%	**6.39%**
	177	2496	0.426	6.62%	
	132	2524	0.314	4.97%	

*Table 3 contains data collected by Baddr Shakhsheer and Jason Quizon during a biocapture experiment conducted at Johns Hopkins Applied Physics Laboratory on February 1, 2006. Calculations and analyses were conducted by Baddr Shakhsheer and Elaine Mullen.

surfaces serves to diminish this effect by preserving strings of cells. This phenomenon would increase the number of colony-forming units in the OFF (non-binding) strain more than in the ON strain, and is likely to manifest earlier in higher concentrations of strains that bind to micelles.

As expected, the OFF mutant did not bind to mannose-coated yeast invertase micelles. However, we were surprised by the discovery that OFF mutant cells grown in liquid culture media did bind to pigeon egg white micelles (data not shown), indicating that they expressed P pili. In wild type UPEC cells, expression of type 1 fimbriae tends to repress P pili expression, although an individual cell can express both structures simultaneously [Johnson, 1991; Snyder *et al*., 2005].

The finding that the OFF mutant expresses galabiose-binding P pili when grown in liquid culture suggests that the expression of type 1 fimbriae is inhibited when cells are cultured on solid agar. Perhaps this inhibition permits the expression of P pili when wild type UPEC cells are grown on solid agar.

The FimH binding pocket of type 1 fimbriae is conserved among UPEC isolates [Hung *et al*., 2002] and shows a preference for mannose moieties at the tips of branching oligosaccharides [Schembri and Klemm, 2001]. Therefore, we were surprised that the ON mutant bound to control micelles made from pigeon egg white glycoproteins (data not shown) whose oligosaccharides rarely terminate in mannose [Takahashi *et al*., 2001]. This finding strongly suggests that UPEC FimH lectins bind to the branched "trimannosyl core" of N-linked oligosaccharides, a hypothesis that is supported by research on the binding of type 1 fimbriae to trimannose [Nilsson *et al*., 2006].

These experimental results suggest that it might be possible to use micelles fabricated from sterile pigeon egg white glycoproteins to capture UPEC cells expressing either FimH adhesins or PapG adhesins. In light of the emergence of multidrug-resistant UPEC strains [Rijavec, 2006], we believe that it would be worthwhile to test the efficacy of using pigeon egg white micelles in prophylaxis and decontamination, particularly in hospitals and nursing homes where bedridden patients have recurring bladder and kidney infections. For example, a cleansing agent containing sterile pigeon egg white micelles might be used to disinfect urothelial tissue and other delicate surfaces.

Another positive implication of the apparent affinity of type 1 fimbriae for the trimannosyl core of N-linked oligosaccharides is that micelles designed to capture mannose-binding enteric bacteria could be made from a great number of inexpensive glycoproteins. The presence of bacteria expressing type 1 fimbriae is an indicator of fecal contamination. When these bacteria are present in high concentrations, glycoprotein micelle-agglutination might represent a sentinel for water pollution or food contamination.

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