Membrane for Selective Capture of the Microbial Pathogen *Listeria monocytogenes*

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Introduction

Membrane filtration is widely used for sterile filtration, flow-through immuno-filtration, virus removal, and microbiological analysis.[1,2,3,4,5,6] Prior work has described membranes that retain one protein over others.[7,8] The purpose of this work is to capture a target species of living cells from a mixed population of microorganisms being concentrated in the retentate of solution being pushed through a membrane. We report selective capture of one living microorganism from another by an antibody immobilized on a lysine-modified polycarbonate membrane. Capture of a relatively large microbial entity (*L. monocytogenes* at 1 μm) by a 75 to 150 fold smaller ligand (the antibody) occurs with high efficiency, when a spacer (poly-L-lysine) reacted to the membrane's surface is derivatized with an antibody (P66) specific to *Listeria*. The results show that isolation of the food pathogen *L. monocytogenes* from *E. coli* in less than 2 h is possible.

Materials and Methods

Another study[9] has shown that polycarbonate membrane retains microbes and that subsequent rinsing of the membrane will recover most of the pathogenic *Listeria monocytogenes* in a viable state. The method of Suye et al.[10] was used to coat the polycarbonate surface with poly-L-lysine (Sigma, Cat. #P6516). The amino group at one end of the poly-L-lysine, a 14.6 kD hydrophilic spacer consisting of approximately 113 lysine monomers, is fixed to the membrane surface by reacting with the carbonate group of polycarbonate membrane matrix. The amino group at the other end of the poly-L-lysine is activated by addition of glutaraldehyde. The glutaraldehyde may also react with underivatized sites on the membrane's surface, that is, sites that do not have poly-L-lysine attached to them. The protein links to glutaraldehyde crosslinked to the membrane matrix or to the poly-L-lysine. Soltys and Etzel[11] had previously shown that another hydrophilic spacer, poly-(ethylene glycol) PEG, enhances the activity, binding capacity and stability of sensing layers.

Polycarbonate membrane from Osmonics (0.2 μm, 25 mm dia., Cat. #K02SH02500) was cut into 1.23 cm^2 quarters for batch incubation. Each piece of membrane was placed into a 24-well plate with each well containing the 0.5 mL of poly-L-lysine solution (2 mg/mL in 50 mM sodium carbonate). Following incubation at room-temperature for 24 h, the membrane pieces were rinsed and washed with deionized water that was added and removed from each well. Glutaraldehyde (v/v 1%, 0.5 mL in PBS buffer, which is 10 mM phosphate with 0.2 M NaCl, pH 7.4) was then added into each well and incubated for

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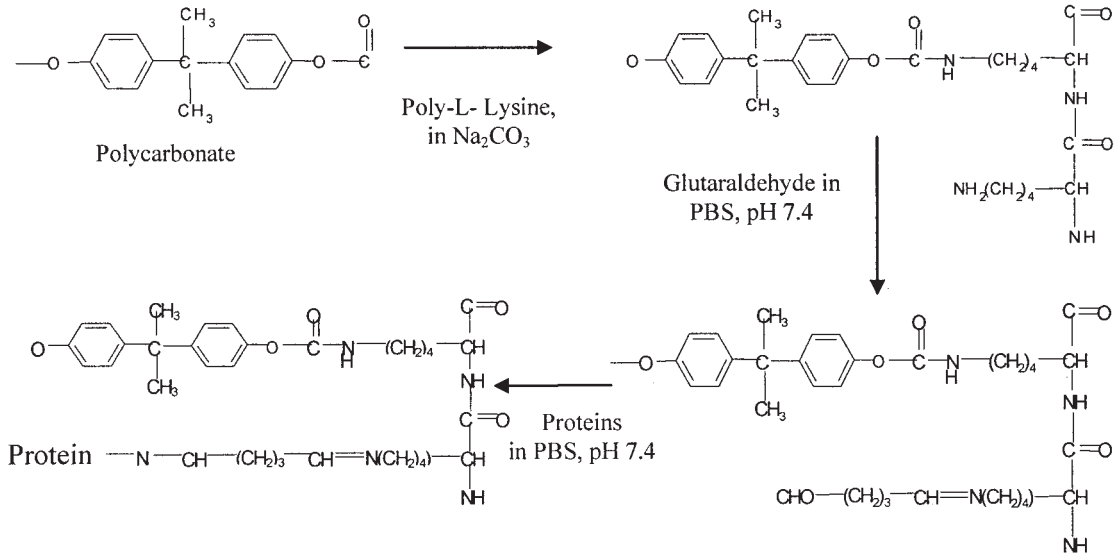

Scheme 1

2 h at room-temperature followed by washing and rinsing with deionized H_2O and finally PBS buffer. Bovine serum albumin (BSA, Sigma, Cat #A2153), antibody P66, or FITC-labeled forms of these proteins were then added (Scheme 1).

The polyclonal antibody (P66) against *L. monocytogenes* was generated in rabbits in our laboratory at a concentration of 0.6 mg/mL.[12] The antibody was immobilized by adding 0.5 mL of 1:50 dilution 0.6 mg/mL P66 or FITC-P66 antibody to the poly-L-lysine/glutaraldehyde treated membranes, incubating overnight in the presence of air, rinsing with PBS, and then finally incubating with BSA (0.5 mL at 1 mg/mL) for 40 min to form the P66 membrane. BSA serves as a blocking agent that prevents nonspecific binding.[13,14] BSA or FITC-BSA was immobilized by adding 0.5 mL of 1 mg/mL BSA to the poly-L-lysine/glutaraldehyde treated membranes and incubating overnight in the presence of air (BSA membrane). The FITC protein emits fluorescence that is readily detected using a fluorescence microscope. Antibody that is not crosslinked was washed off during buffer rinsing. A poly-L-lysine/glutaraldehyde treated membrane covalently binds P66 antibody or BSA which is retained strongly while lack of poly-L-lysine and glutaraldehyde results in unstable binding (Figure 1) . FITC-P66 and FITC-BSA treated membranes glowed intensely, showing that labeled BSA or P66 is immobilized.

Binding of bacteria by batch incubation was tested using *L. monocytogenes* V7 and *E coli* ATCC 52739 at an undiluted concentration of about 7.3×10^8 cells/mL. The cells were labeled with FITC as follows. Fluorescein isothiocyanate 2 mg (FITC, Sigma, Cat. #F4274) was dissolved in 1 mL of carbonate-bicarbonate buffer (67 mM $NaHCO_3$, 32 mM Na_2CO_3) (pH 9.6). One milliliter of freshly cultured bacteria was centrifuged at 10,000 $\times$ rpm for 5 min at room-temperature in the microcentrifuge (Eppendorf, Model 5415D). The supernatant was decanted and 1 mL of FITC solution was added and incubated with the bacteria for 40 min at room-temperature. The cells were then rinsed with 1 mL of carbonate-bicarbonate buffer (pH 9.6) three times.[15]

For batch experiments, a volume of 0.5 mL of FITC-bacteria ($\sim 3.7 \times 10^8$ cells) was added into wells of a 24-well plate containing P66 or BSA membranes and incubated for 40 min. Unbound bacteria were rinsed three times by pipetting 1 mL of PBS (phosphate buffered saline, pH 7.4) solution into each well and then removing the fluid. The membrane was then examined using a fluorescent or scanning electron microscope (SEM). Results are shown in Figure 2.

For flow experiments, 25 mm, P66 derivatized, whole membranes were placed into a syringe holder (Millipore, Cat #SX00002500) and connected to a 60-mL syringe (BD, Cat #309663), which was placed into a syringe pump (Havard apparatus, PHD2000). A volume of 50 mL of bacteria in PBS buffer (pH 7.4) containing $\sim 3.7 \times 10^9$ cells was pushed through the membrane at 5 mL/min. The membrane was then removed and placed in 3 mL of PBS in a Petri dish for 30 min in order to wash off unattached cells. The washed membrane

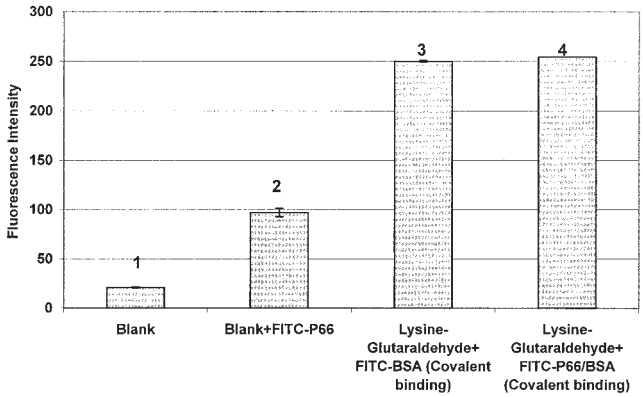

Figure 1. Fluorescence intensity.

(1) Blank: as indicated by the large fluorescence intensity membrane treated with poly-L-lysine and glutaraldehyde shows little fluorescence; (2) membrane binds FITC-P66 by physical adsorption; (3) and (4) membrane treated with poly-L-lysine and glutaraldehyde followed by FITC-BSA and FITC-P66, respectively, give covalent binding of BSA and P66 as indicated by the large fluorescence intensity that is not washed off by rinsing. Fluorescence measured at integration time of 8 ms.

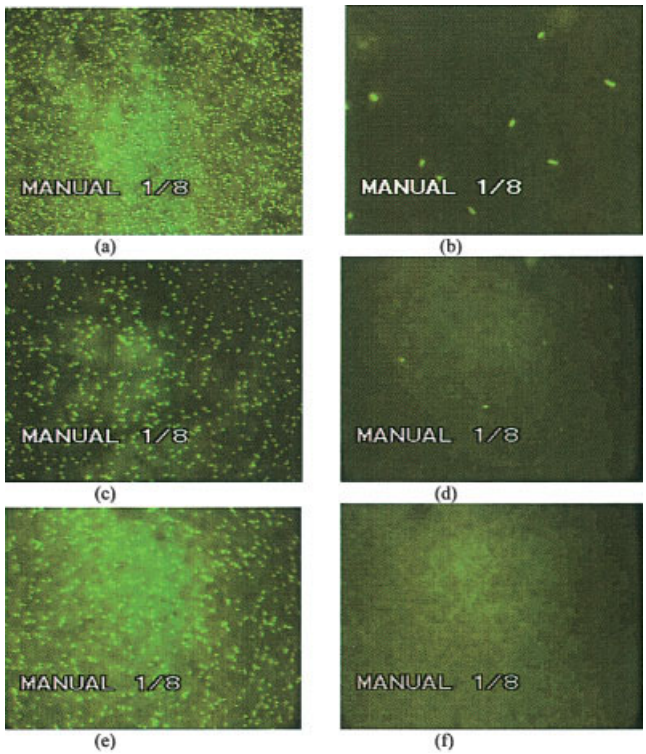

Figure 2. Fluorescence images.

(a) Nonspecific binding of FITC-labeled *L. monocytogenes*; (b) blocking effect of immobilized BSA in preventing binding of FITC-labeled *L. monocytogenes*; (c) capture of *L. monocytogenes* (at concentration of 7.3×10^8 cells/mL) from 0.5-mL sample volume contacted with P66 membrane, with 1 mg/mL BSA in free solution in order to block sites that lack immobilized antibody; (d) absence of *E. coli* capture on P66 antibody immobilized on membrane surface (sample volume of 0.5 mL of containing 7.3×10^8 cells/mL contacted with membrane for 40 min at ambient temperature); (e) mixture of *L. monocytogenes* and *E. coli* incubated with P66 membrane for 40 min (each at 3.7×10^8 cells) at 25°C.

Only *L. monocytogenes* was labeled with FITC in this case. Binding of bacteria are indicated by glowing spots on figures, and (f) Mixture of *L. monocytogenes* and *E. coli* was incubated with P66 membrane for 40 min at 25°C.

Each of the two mixtures of bacteria contained a total number of cells of 3.7×10^8. Only *E. coli* was labeled with FITC in case (f) vs *L. monocytogenes* in case (e). Absence of fluorescence shows absences of adsorption of *E. coli*. Fluorescence measured at integration time of 125 ms.

was then examined using fluorescence or scanning electron microscopy (SEM)[13]. Results are shown in Figure 3.

Results

Fluorescent microscopy from batch experiments show that *L. monocytogenes* attaches nonspecifically to poly-L-lysine/glutaraldehyde membranes and is not removed by washing three times with PBS buffer (Figure 2a). Each dot represents a labeled bacterial cell. BSA covalently bound to the membrane using poly-L-lysine/glutaraldehyde blocks *L. monocytogenes* adsorption[13] (absence of dots in Figure 2b). When *L. monocytogenes* is contacted with P66 membrane, it binds (Figure 2c) while *E. coli* does not (Figure 2d). When a mixture of *L. monocytogenes* and *E. coli* was incubated for 40 min with P66 membrane, at 25°C, and the membrane was then washed three times with 1 mL of PBS buffer at 25°C to remove nonspecifically bound bacteria, FITC labeled *L. monocytogenes* and unlabeled *E. coli* gave the image in Figure 2e. Labeled *E. coli* mixed with unlabeled *L. monocytogenes* gave the image of Figure 2f indicating absence of labeled *E. coli* on the P66 surface.

The flow experiment gave strong differences in fluorescence as indicated by Figure 3a with the mean value of fluorescence intensity corresponding to images in Figure 3b. These showed *L. monocytogenes* exhibits higher retention on P66 membrane than *E. coli* ($P < 0.001$). This was confirmed by the corresponding SEM's on P66 membranes contacted with either *L. monocytogenes* or *E. coli* at a concentration of 7.3×10^7 cells/mL (Figure 3 c). The scanning electron micrographs may

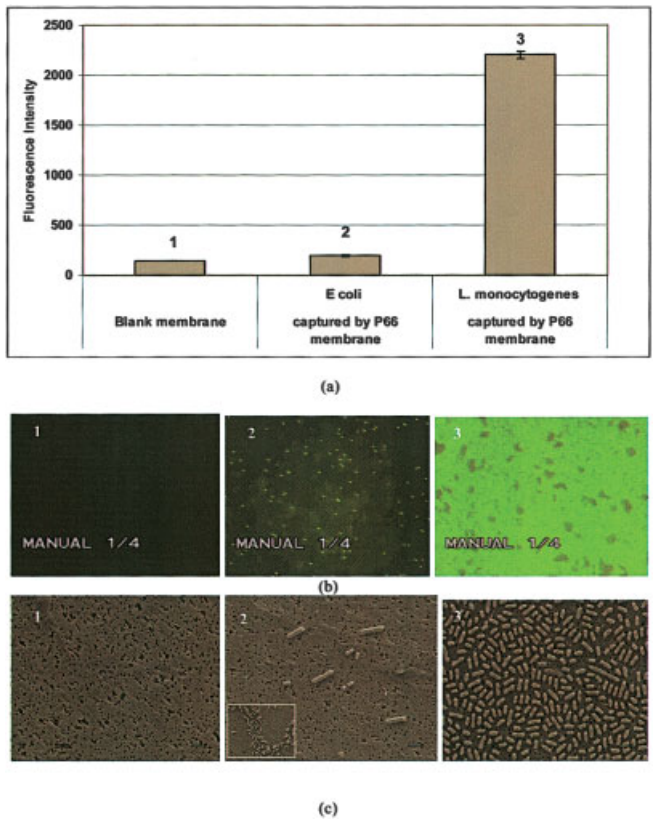

Figure 3. Flow-through experiment with P66 membrane.

(a) Fluorescence Measurements. (1) Blank membrane denotes membrane treated with poly-L-lysine and glutaraldehyde. (2) *E coli* captured by membrane after flow-through experiment followed by membrane rinse. Membranes were removed from membrane holder, placed in 3 mL of PBS in a Petri dish and gently shaken for 30 min. (3) *L. monocytogenes* captured by membrane after flow-through experiment after membrane was removed from holder and gently shaken in 3 mL PBS for 30 min. (a) Fluorescence intensity comparison. This measurement was taken at an integration time 250 ms. Statistically significant intensities are much higher for *L. monocytogenes* than for *E. coli* ($P < 0.001$). (b) Fluorescent images of corresponding membranes after flow-through experiment. Fluorescence intensity is so strong (see panel (b) – 3) that individual dots (microbes) are not discernible for *L. monocytogenes*. This is probably due to monolayer coverage of *L. monocytogenes* as indicated by the SEM. (c) SEM of bacteria on membrane after 50 mL of solution was flowed through at 7.3×10^7 cells/mL followed by a static rinse. *L. monocytogenes* forms a monolayer on membrane. *E. coli* has much lower adsorption density than *L. monocytogenes* (compare middle panel and inset in panel (c) – 2 to panel (c)).

also suggest that the coating that we have placed on the membrane preserves cell viability. The SEM's, show both the *L. monocytogenes* and *E. coli* cells that are dividing (see cells with elongated shape, Figure 3c) although we do not know if the cells adsorbed in this state or if division occurred after adsorption.

We calculated the fraction of cells captured based on the micrographs that represent 336 μm^2 of area, which is 7×10^{-7} of the total membrane area (where the total membrane area is 4.91 cm^2). If there are 7.3×10^8 cells/mL with each cell covering an area of 4×10^{-9} cm^2 (1×0.4 μm), then 2.92 cm^2 of area would be needed if all of the cells were to be retained. The area of the membrane, based on a 25 mm dia. is 4.91 cm^2, so there should be sufficient area to capture all of the cells.

L. monocytogenes forms a monolayer on P66 membrane. The cell density on the membrane's surface (based on 3 different SEM images) corresponds to 10 to 20% recovery of cells from the fluid phase. In comparison, *E. coli* has a much lower retention rate (0.8 to 1.8%) and is not distributed uniformly on the surface of the P66 membrane (Figure 3 c middle panel and insert). While the *E. coli* are readily washed off using PBS buffer, the *L. monocytogenes* cells were retained. Their desorption would require changing pH. Desorption was not addressed in this study.

The use of the antibody-derivatized membrane enables rapid concentration of microorganisms with selective capture of one organism from another. The specific approach described in this article is significant, since it demonstrates how a known and easy-to-use hydrophilic spacer can be applied to a polycarbonate membrane to enable direct and productive binding of the antibody to a microbial cell that is about 75 to 150 $\times$ larger than the F_c or specific binding sites, respectively, of the antibody. Steric hindrance is minimized, and a uniform coating of the target species, *L. monocytogenes* is obtained on the membrane. The capture and retention of *L. monocytogenes* over *E. coli* dominates, with the fluorescent signal for the *L. monocytogenes* being much stronger than that of *E. coli*. The membranes to which antibodies are attached via a poly-L-lysine linker combine rapid cell concentration by microfiltration with selective retention of a pathogenic organism over a nonpathogenic one. This provides another tool that can be used to rapidly detect food pathogens.

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