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Functionalization of Hybrid Poly(*n*-isopropylacrylamide) Hydrogels for *Escherichia coli* Cell Capture via Adsorbed Intermediate Dye Molecule

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ABSTRACT: Poly(*n*-isopropylacrylamide) Laponite (PNIPAM-*Lap*) hybrid hydrogels, which use the synthetic clay Laponite as a crosslinker, permanently adsorb cationic laser dyes out of solution. This proof-of-concept expounds on this capability by adsorbing an intermediate dye molecule and using it as the foundation for successfully conjugating microbial antibodies to the surface of a PNIPAM hydrogel. The study involves using acriflavinium chloride molecules, adsorbed by a PNIPAM-*Lap* hydrogel from an acriflavine laser dye solution, as an intermediate molecule to attach antibodies raised against *E. coli* to the hydrogel and demonstrate cell capture. Furthermore, this system exemplifies a novel biotechnological platform for greatly expanding PNIPAM hydrogels' capabilities and applicability through conjugation chemistry to surface-bound molecules. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41557.

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INTRODUCTION

Poly(*n*-isopropylacrylamide) (PNIPAM) hydrogels have found wide applicability in a variety of fields such as biotechnology and soft tools.[1–10] Several studies have been done to modify traditional PNIPAM hydrogels and expand their range of functionality. These modifications have resulted in optimized shrinking/swelling kinetics, enhanced structural stability, and improved conductivity, among other characteristics.[2–6,8,10–18]

A standard method of synthesizing PNIPAM hydrogels involves using a *N*,*N*′-methylenebis(acrylamide) (BIS) crosslinker for the free radical polymerization of PNIPAM molecules in the presence of ammonium or potassium persulfate as an initiator with tetramethylethylenediamine (TEMED) as the catalyst.[1,3,11] The use of alternate crosslinkers can have a profound effect on the functionality of the hydrogel.[2,3,12–16] For instance, Massad-Ivanir et al. used the BIS analog bis(acryloyl) cystamine (BIS-CA), which contains disulfide bonds, as a method to functionalize a hydrogel with reactive thiol groups.[14] Consequently, thiol-reactive biotin was used as the foundation for a biotin-streptavidin bridge to an *Escherichia coli* (*E. coli*) antibody.[14] Another crosslinking agent, a synthetic clay called Laponite, has been previously used by our research team to strengthen the structural stability and improve the shrinking/swelling kinetics of PNIPAM hydrogels.[2,3,6] These hybrid PNIPAM-Laponite (PNIPAM-*Lap*) hydrogels have also been shown to adsorb cationic molecules out of solution.[6,18] The adsorption of cationic molecules occurs rapidly, which concentrates the adsorbed molecules at or near the surface of the hydrogel.[6,18] This characteristic was used to use PNIPAM-*Lap* hydrogels as a mechanism for separations,[18] as well as a way to absorb laser irradiation for remote-induced volume phase transitions.[6]

The structural integrity, reliability, versatility, and multifunctionality are, collectively, unique capabilities of PNIPAM-*Lap* hydrogels, offering a vast potential for these hybrid hydrogels in biotechnology. In this work, we take advantage of PNIPAM-*Lap* hydrogels' adsorption capabilities to develop them into a novel biotechnological platform with a wide range of applicability. Specifically, the PNIPAM-*Lap* hydrogel's surface was permanently functionalized with a cationic dye thus enabling a new range of characteristics and increasing the applicability of this sturdy hybrid hydrogel, as surface-bound dye molecules can further be used as the foundation for conjugation chemistry. To our knowledge, this is the first system in which a PNIPAM hydrogel's functionality was expanded via conjugation to surface-bound molecules. As a proof-of-concept system for the utilization of this biotechnological platform, we directly conjugated antibodies raised against *Cryptosporidium* and *E. coli* to the surface of a PNIPAM-*Lap* hydrogel, with the ultimate goal of demonstrating whole-cell capture. Only the *E. coli* results are detailed below, because heat-killed fluorescent-labeled *Cryptosporidium* cells were not readily available to display the ability of the attached antibodies to capture cells.

Table I. Description of Three Hydrogels Used for the Antibody Conjugation and Verification Experiment

	Protocols for Each Hydrogel		
	Dye adsorption	*E. coli* primary antibody (raised in goat)	Donkey antigoat Alexa-647 secondary antibody
Hydrogel A	X	X	X
Hydrogel B	X		X
Hydrogel C		X	X

EXPERIMENTAL

Cylindrical PNIPAM-*Lap* Synthesis

Glass tubes of approximately 1 cm in length with an inner diameter of 1.2 mm were made hydrophobic by submerging them in solution of distilled water, 1.0% volume acetic acid (Sigma Aldrich), and 0.5% volume dimethoxysilane (Sigma Aldrich). After the tubes were allowed to soak in the solution for approximately 15 min, they were removed and dried under vacuum in a vacuum oven at 113°C (approximately 30 min).

The protocol for synthesizing PNIPAM hydrogels is described elsewhere.[3] Briefly, 400 mg of Laponite-XLG (Southern Clay) was added to 28.5 mL of distilled water and stirred for approximately 30 min until the solution returned to clear. 3.5 g of PNIPAM (Sigma Aldrich) was added to the solution and was stirred for approximately 15 min until the solution returned to clear. A solution of 30 mg of potassium persulfate dissolved in 1.5 mL of distilled water was added to the stirring PNIPAM and water solution, followed immediately by 24 μL of *N,N,N′,N′*-TEMED. The hydrophobic glass tubes were immersed in the resulting solution, which was then allowed to polymerize for 24 h at 50°C. These hydrogels were synthesized in their shrunken state at a temperature above the critical temperature of 33°C, so the cylindrical hydrogels were easily removed from the glass tubes and allowed to hydrate in distilled water for 24 h at room temperature. After full hydration from the swollen state in which the gel was synthesized, the diameter of the gel would increase to approximately 5 mm.

Acriflavinium Chloride Adsorption

PNIPAM-*Lap* hydrogels were submerged in a 5 mg mL^{-1} solution of acriflavine (Sigma Aldrich, 33% acriflavinium chloride) and distilled water for 5 min. Acriflavinium chloride molecules are cationic and, consequently, are capable of being adsorbed on to the hybrid hydrogel's surface. Additionally, the acriflavinium chloride molecules each contain two primary amine groups that were used for the conjugation experiments described below. After dye adsorption, the hydrogels were rinsed with and submerged in fresh distilled water repeatedly until the unbound acriflavine molecules had been removed from the hydrogel, which was determined by when dye molecules were no longer leeching in to fresh water.

Table II. Description of the Two Hydrogels Used for *E. coli* Cell Capture Experiment and Verification Protocol

	Protocols for Each Hydrogel		
	Dye adsorption	*E. coli* antibody conjugation	*E. coli* cell exposure
Hydrogel X (control)	X		X
Hydrogel Y	X	X	X

Primary Antibody Conjugation and Verification

Table I describes three hydrogels that went through the *E. coli* antibody conjugation and verification experiments described below. For each hydrogel, an approximately 1.5 mm thick and 5 mm in diameter cross section of the hydrogel was used for the conjugation and verification experiments.

After dye adsorption, the hydrogels were rinsed with and submerged in fresh distilled water repeatedly until the unbound acriflavine molecules had been removed from the hydrogel, which was determined by when dye molecules were no longer leeching in to fresh water. Hydrogels A and C were crosslinked to 150 μg of O157:H7 *E. coli* antibodies (goat IgG, KPL) with glutaraldehyde (Sigma Aldrich) in 2 mL of phosphate buffered saline (PBS) 1× for 2 h at 4°C. After crosslinking of antibodies to the hydrogel, the O157:H7 *E. coli* would bind specifically to the antibody crosslinked to the hydrogel with noncovalent

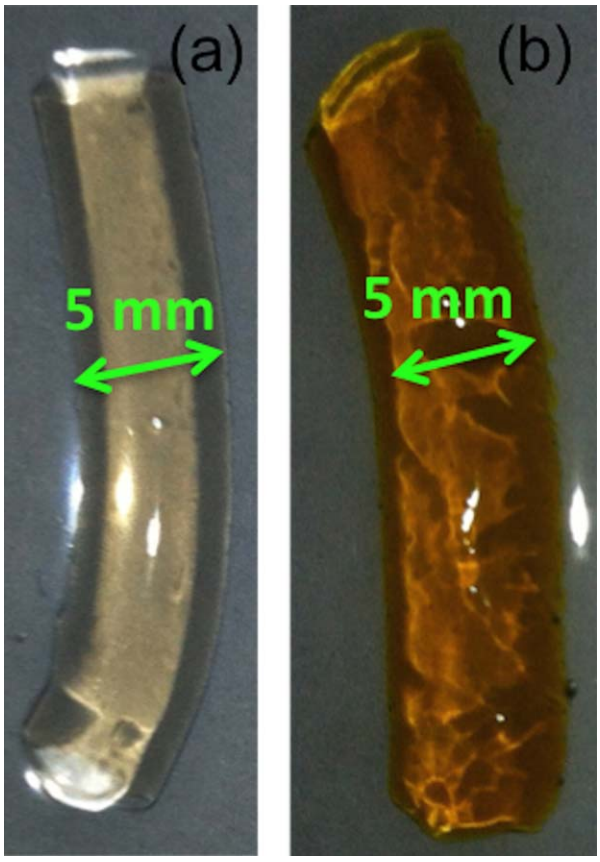

Figure 1. (a) Hydrogel after synthesis and hydration. (b) Hydrogel with adsorbed acriflavinium chloride molecules. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

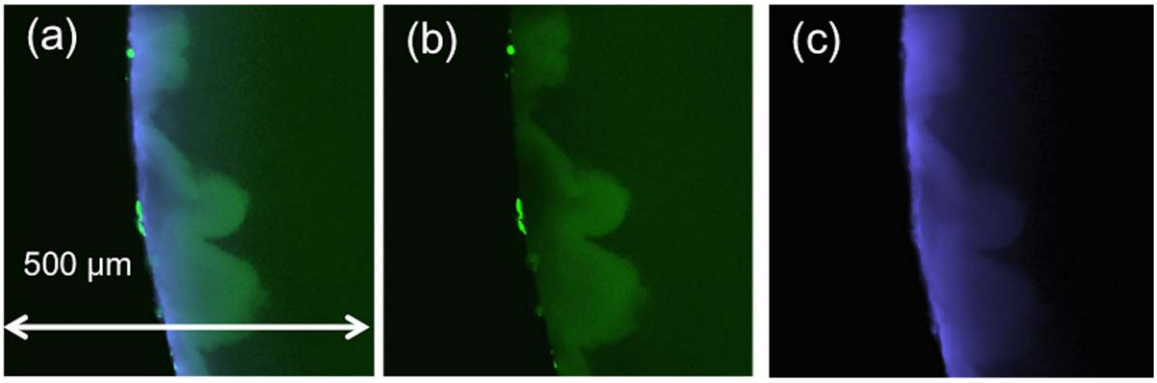

Figure 2. Corresponds to Hydrogel A. (a) Fluorescent image of both the green (488 nm) and purple (640 nm) channels, while (b) displays only the green channel and (c) displays the purple channel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

interactions. The hydrogels were then washed for 30 min four times in PBS 1× to remove unconjugated antibodies.

To verify *E. coli* antibody attachment, Hydrogels A, B, and C were each exposed to 20 μg of Alexa-647 labeled donkey anti-goat antibodies (KPL) in a 9:1 PBS 1× to horse serum solution. Each of the hydrogels was washed with 3 mL of PBS 1× four times to remove unconjugated secondary antibodies. These three hydrogels were then mounted on a microscope slide and analyzed with a 3024 Nikon A1R upright confocal microscope for presence of the fluorescent secondary antibody, which has a strong affinity for the *E. coli* antibody.

E. coli Cell Capture and Verification

Prior to exposing hydrogels X and Y to *E. coli* cells, the cells were fluorescent labeled for detection via fluorescence microscopy. One mg of heat-killed, positive control O157:H7 *E. coli* cells (KPL) were suspended in 150 μL of sodium bicarbonate (Sigma Aldrich) and then centrifuged at 8500 RPM for 5 min. The supernatant sodium bicarbonate was removed and the cells were resuspended in 100 μL of sodium bicarbonate. 100 μg of Alexa-555 *N*-hydroxysuccinimide esters (Life Technologies) were suspended in 30 μL of dimethyl sulfoxide, and 5 μL of that solution was added to the suspended *E. coli* cell mixture. The mixture was allowed to react for 45 min at room temperature while protected from light. Following the reaction, 50 μL of PBS 1× was added and the mixture was centrifuged at 8500 RPM for 5 min. The supernatant was removed, and the labeled cells were resuspended in 200 μL PBS 1× and centrifuged to remove unreacted esters. This wash step was repeated four times. The labeled cells were diluted to a concentration of 1 mg mL^{-1} in PBS 1× and frozen.

Table II describes the two hydrogels that went through the subsequent *E. coli* cell capture experiment and verification protocol described below. As shown in the table, Hydrogel Y went through the primary antibody conjugation protocol as described previously.

Hydrogels X (Control) and Y were each exposed to a concentration of 100 μg of *E. coli* cells in 500 μL PBS 1×. The gels and *E. coli* cells were exposed in a 3 mL vial on a rocker for 1 h, and then were washed in 2 mL of PBS 1× for 30 min. The wash step was repeated once. The hydrogel cross sections were then mounted on microscope slides and analyzed with a 3024 Nikon A1R Multi-Photon Upright Confocal microscope for presence of the fluorescent-labeled *E. coli* cells.

RESULTS AND DISCUSSION

Acriflavinium Chloride Adsorption

Acriflavine dye used in this study is comprised of approximately 33% acriflavinium chloride molecules, which are positively

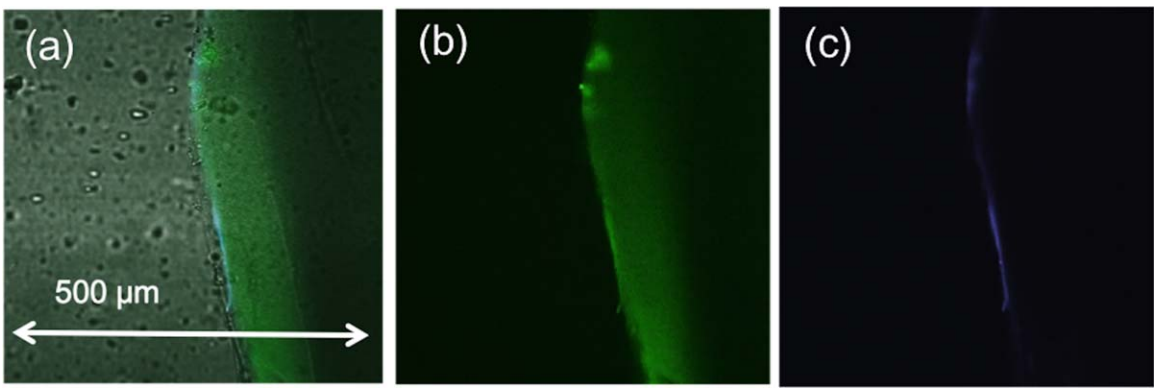

Figure 3. Corresponds to Hydrogel B. (a) Fluorescent image of the green (488 nm), purple (640 nm), and transmitted light channels, while (b) displays only the green channel and (c) displays the purple channel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

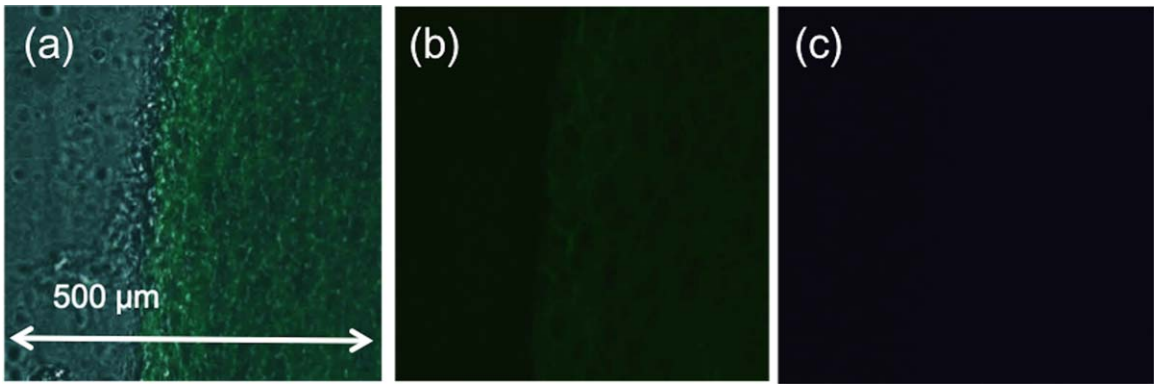

Figure 4. Corresponds to Hydrogel C. (a) Fluorescent image of the green (488 nm), purple (640 nm), and transmitted light channels, while (b) displays only the green channel and (c) displays the purple channel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

charged due to a quaternary amine and provide two primary amine groups in their structures. The primary amines that the adsorbed acriflavinium chloride molecules provide to the surface of the hydrogel are used as the foundation for conjugation chemistry in our proof-of-concept system, in which we directly conjugate anti-*E. coli* antibodies to the hydrogel via the amine-to-amine crosslinker glutaraldehyde. Figure 1 displays a hydrogel that has and has not been dyed with acriflavinium chloride via adsorption of acriflavine. The adsorbed molecules shown in Figure 1(b) are those that are still bound after extensive washing. The mechanical properties of the hydrogels were not noticeably affected by dye adsorption. As shown in our previous study, volume phase transitions induced in PNIPAM-*Lap* hydrogels with adsorbed dye molecules are sufficient for peristaltic locomotion.[6] Microanalysis of the shrinking and swelling kinetics of PNIPAM-*Lap* hydrogels after dye adsorption is outside the scope of this article and will be addressed in future work.

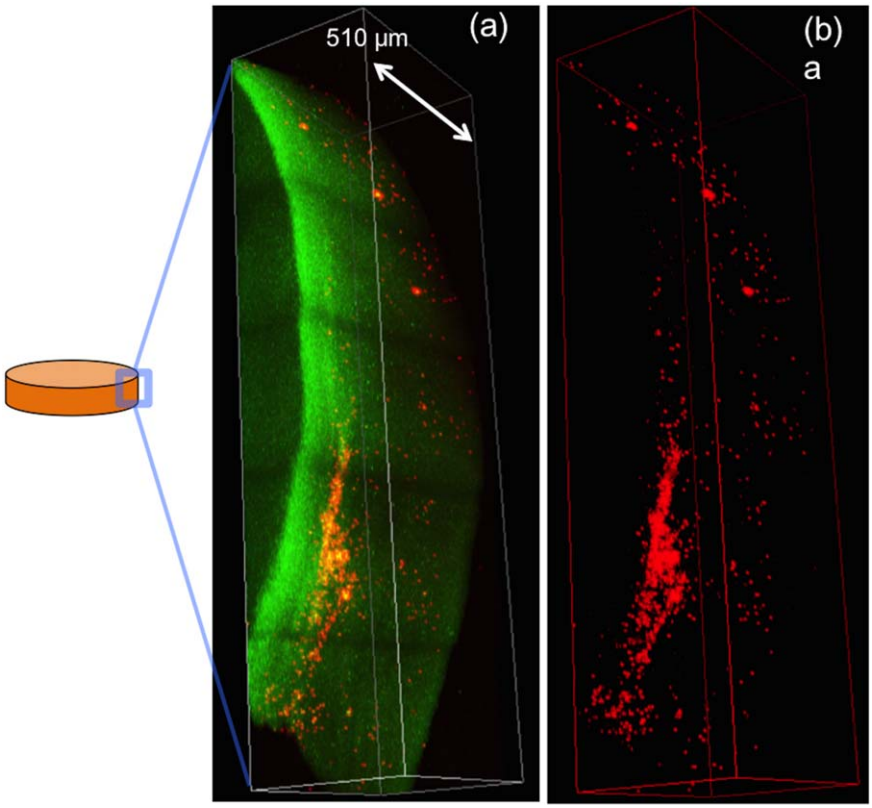

Figure 5. (a) Fluorescent image for Hydrogel Y. A 3D fluorescent image of a portion of the surface is shown. Red dots are *E. coli* cells with Alexa-555 fluorescent labels that are attached to the surface of the hydrogel. (b) Duplicate of the fluorescent image for Hydrogel Y showing only the *E. coli* cells on the red channel. The representation of the 1-mm thick hydrogel cross section is included to assist with orientation of the images to the hydrogel sample. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

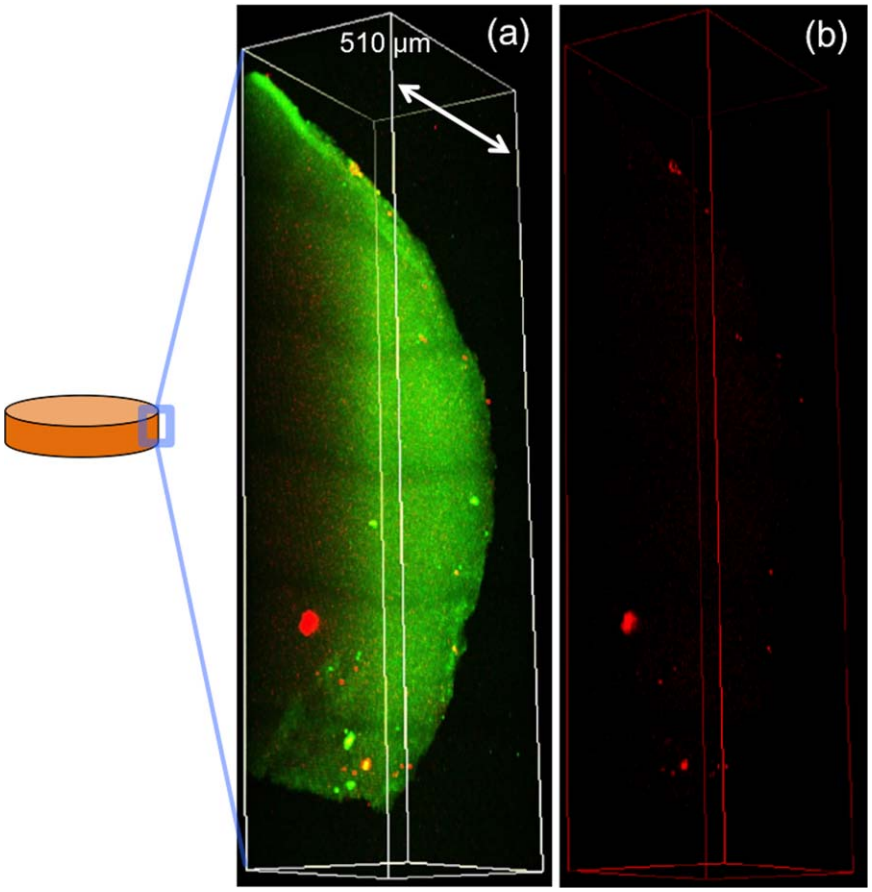

Figure 6. (a) Fluorescent image for Hydrogel X (control). A 3D fluorescent image of a portion of the surface is shown. Red dots are *E. coli* cells with Alexa-555 fluorescent labels that are attached to the surface of the hydrogel. (b) Duplicate of the fluorescent image for Hydrogel X showing only the *E. coli* cells on the red channel. The representation of the 1-mm thick hydrogel cross section is included to assist with orientation of the images to the hydrogel sample. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Primary Antibody Conjugation and Verification

Figures 2, 3, and 4 show the fluorescent images of Hydrogels A, B, and C, respectively, at 488 nm emission and 640 nm emission. Figures 2(a), 3(a), and 4(a) display green (488 nm) and purple (640 nm) channels. Figures 2(b), 3(b), and 4(b) display only the green channel, while Figures 2(c), 3(c), and 4(c) display only the purple channel. The green channel shows acriflavinium chloride fluorescence and the purple channel shows the fluorescence of Alexa-647, which is the fluorescent label attached to the secondary antibodies. Figures 3(a) and 4(a) also use the transmitted light channel to better define the surface of the gel. The green in Figure 4(a,b) is the autoflourescence of the hydrogel and not the presence of acriflavinium chloride. Figure 2 shows a significant amount of purple fluorescence, indicative of secondary antibodies attached at and near the surface of the hydrogel. The minimal amount of purple fluorescence in Figure 3 is indicative of what is likely nonspecific binding of secondary antibodies to the acriflavinium chloride molecules adsorbed by Hydrogel B. Figure 4 shows no purple fluorescence. The presence of a significant amount of secondary antibodies on Hydrogel A and not on Hydrogels B and C indicates that the only hydrogel that has secondary antibodies, and consequently, *E. coli* antibodies, present is Hydrogel A. This verifies that the conjugation protocol was successful.

E. coli Cell Capture and Verification

Figure 5 shows a three-dimensional (3D) z-stack image of a portion of the outer surface of Hydrogel Y. The fluorescent image was taken with the 488 nm and 550 nm emission channels. The Alexa-555 labeled *E. coli* cells are shown as the red ovals that are approximately 2–3 μm in width. These figures show that the hydrogel with attached primary antibodies has captured a significant number of *E. coli* cells.

Figure 6 shows Hydrogel X and the corresponding 3D z-stack image of a portion of the outer surface of the hydrogel. As these figures show, this hydrogel has significantly fewer *E. coli* cells attached to its surface. However, nonspecific binding has resulted in a small number of *E. coli* cells on the surface of the hydrogel. The difference in the amount of *E. coli* cells attached to the surface of Hydrogel X (the control) compared to Hydrogel Y verifies that the hybrid PNIPAM hydrogels were successfully functionalized to capture *E. coli* cells.

DISCUSSION

In this proof-of-concept system, we have succeeded in functionalizing hybrid PNIPAM hydrogels with the ability to capture *E. coli* O157:H7 cells. We independently verified that *E. coli* antibodies may be conjugated to the hydrogel via an intermediate

adsorbed cationic molecule, and that the resulting hydrogels can capture *E. coli* cells in water. Using the ability to capture *E. coli* cells as an example system, we have explored a new method for the functionalization of hybrid PNIPAM hydrogels. This method may be used as an alternative method to synthesize known hybrid hydrogels or as a method to synthesize new hybrid hydrogels via conjugation to surface-bound molecules.

The ability to attach a wide variety of molecules to the surface of the hydrogel through this biotechnological platform may broaden the usage of hydrogels as drug delivery systems.[7–9,19] This open-ended conjugation system could be used to attach and carry cargo.[2] Our method may also be used to attach carbon nanotubes to the surface of the hydrogel, with potential significance in the improvement of hydrogel mechanics, as well as in areas of biosensing and biotechnology.[20,21] For instance, carbon-based hybrid PNIPAM hydrogels have been shown to increase conductivity and improve shrinking/swelling kinetics in hydrogels.[22–28] Magnetic Fe_3O_4 powder has been used to synthesize magnetic hybrid hydrogels that are capable of being easily manipulated structurally, which has ramifications in biotechnology, specifically in the areas of direct drug delivery, hypothermia treatment and microvalve construction.[26–28] Other microbe capturing materials such as immunomagnetic beads may be attached directly to the surface of the hybrid hydrogel using this method.[29–33] Furthermore, this platform could be used to incorporate emerging technologies and materials in to the structure of PNIPAM hydrogels.[34–40]

CONCLUSION

In conclusion, our method of using an adsorbed cationic dye as the basis for conjugating antibodies to the hybrid hydrogel has created a new biotechnological platform for their functionalization. PNIPAM hydrogels, due to their mobility, superior shrinking/swelling kinetics, and structural reliability, are valuable tools across a number of fields including biosensors, soft tools, and biomedical tools for drug delivery and cavity exploration, among others. By utilizing our method of functionalization, the applicability of hybrid PNIPAM hydrogels in each of these fields can be expanded greatly.

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