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NOTE

Bacteriophages immobilized on electrospun cellulose microfibers by non-specific adsorption, protein–ligand binding, and electrostatic interactions

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Abstract Phage therapy has significant potential in specifically targeting bacterial pathogens in food and medicine. There is a significant interest to combine phages with materials to enhance and broaden potential applications of phages. This study compares non-specific adsorption, protein–ligand binding, and electrostatic interactions on cellulose microfibers without any chemical or genetic modification of phages. Success in immobilization of phages on biomaterials without genetic and chemical modification can enable effective translation of naturally occurring phages and their cocktails for antimicrobial applications. The immobilization approaches were characterized by phage loading efficiency, phage distribution, and phage release from fibers. The results indicated that non-specific adsorption and protein–ligand binding had insignificant phage loading while electrostatic interactions yielded approximately 15–25% phage loading normalized to the initial titer of the phage loading solution. Confocal imaging of the electrostatically immobilized phage fibers revealed a random phage distribution on the fiber surface. Phage release from the electrostatically immobilized phage fibers indicated a slow release over a period of 24 h. Overall, the electrostatic immobilization approach bound more active phages than non-specific adsorption and protein–ligand binding and thus may be considered the optimal approach to immobilizing phages onto biomaterial surfaces.

Keywords Bacteriophages · Cellulose · Immobilization · Antimicrobials · Fibers · Electrostatic interaction

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Introduction

As bacterial pathogens gain resistance to more classes of antibiotics, alternative strategies for eliminating bacterial pathogens have gained increased significance over the last decade. Bacteriophages or phages, have been used as a viable alternative antimicrobial strategy to combat bacterial pathogens in both food and medicine by specifically targeting the pathogen of interest (Abuladze et al. 2008; Chan et al. 2013; Coffey et al. 2010; Garcia et al. 2008; Greer 2005). Phages immobilized on biomaterials have significant potential in extending phage therapy applications for food packaging and biomedical applications (Ma et al.

2008; Puapermpoonsiri et al. 2009). One of the key challenges in delivering phages across a large surface area is their slow diffusion rate as compared to smaller molecular weight antimicrobials. In food applications, phages are typically applied in form of a liquid formulation by dipping or spraying on the surfaces of meat and fresh produce (Abuladze et al. 2008; Borie et al. 2008; Leverentz et al. 2001). Many of the food surfaces are hydrophobic, especially in the case of fresh produce, or have significant surface roughness as in the case of meat. It is expected that these surface characteristics significantly limit phage transport, which limits phages from effectively interacting with microbes on a food surface (Deboosere et al. 2012; Kukavica-Ibrulj et al. 2004). The challenges of phage transport in more complex surfaces, such as wounded tissue, can be even more limiting due varying tissue properties and increased surface volume. To address the mass transfer of phages in such an environment, uniformly distributing the phages across the targeted area increases the probability that a phage will come into contact with its target microbe. One of the potential approaches to achieve uniform phage delivery is to immobilize them on contact surfaces such as packaging materials and medical dressing.

Phages may be immobilized on material surfaces by a diversity of approaches including various physical, chemical, and genetic approaches. Non-specific adsorption of phages on surfaces by weak and reversible van der Waals interactions has been reported on gold surfaces for biosensing applications (Nanduri et al. 2007; Singh et al. 2009). Though phages were successfully immobilized on the gold surfaces for their intended applications, non-specific adsorption was less efficient in immobilizing phages compared to chemical crosslinking of phages on the gold substrate via glutaraldehyde activated thiol modified gold surface (Singh et al. 2009). Electrostatic interactions of phages on biomaterials, another form of physical adsorption, rely on the charged properties of phages to deposit on oppositely charged silica and other surfaces including formation of a multiple layer structures on a substrate via layer by layer (LBL) approaches (Cademartiri et al. 2010; Steinmetz et al. 2008; Yoo et al. 2006). Immobilization in LBL coatings is however limited due to the potential phage displacement during the deposition process, and the formation of multilayer structure is dependent on phage geometry (Steinmetz et al. 2008). Chemical immobilization approaches include utilizing self-assembled monolayer (SAM) on gold, glass, and carbon electrodes to form amide bonds with the surface proteins of the phages (Arya et al. 2011; Handa et al. 2008; Shabani et al. 2008). Though this approach improves material stability over a period of days, the phages were coated unevenly on surfaces and their orientation cannot be precisely controlled to expose the phage tail fibers for binding target bacteria (Hagens and Loessner 2007; Shabani et al. 2008). Genetic modifications of the phages require significant effort to modify the head proteins to display affinity binding tags and may reduce virulency of the phage type (Edgar 2006; Tolba et al. 2010). Genetic modification is also limited to purified and well characterized mono species populations of phages, such as M13, T4, and T7, hindering the broader adoption of the more effective and multiple wild type phages in a phage cocktail than single phage formulations (Chan et al. 2013). Genetic modification as an immobilization method is also burdened with regulatory hurdles if applied to food, adding complication for use in commercial products. Physical adsorption of phages as an immobilization method provide broad applicability to phage species, requires little preparation, and reduces inadvertent inactivation of the phage.

Despite prior evaluation of physical, chemical, and genetic modification immobilization approaches of phages on various inorganic and organic substrates, there is limited understanding of how different immobilization approaches compare on the same biopolymer material. To narrow the immobilization approaches, this study aims to compare three types of physical adsorption immobilization approaches, non-specific adsorption, protein–ligand, and electrostatic interactions, as physical adsorption provides better advantages over chemical and genetic modification immobilization approaches. The physical adsorption approaches will be compared on a single cellulose based fibrous biopolymer material. Ultra-fine cellulose fibers were chosen as the biopolymer substrate due to their ultra-high specific surface and versatility to form membranes, paper, bandages, and other solid formulations (Konwarh et al. 2013; Ma et al. 2005; Schiffman and Schauer 2008). T7 phage from the *Podoviridae* family was chosen for this study as it is a virulent lytic phage which produces large and clear plaques. These phage immobilized fibers were characterized by phage loading efficiency, phage

distribution, and phage release. Determining optimal phage immobilization techniques has the potential to improve phage delivery, phage release, and phage density. Optimizing these factors for phage immobilization can potentially improve phage applications in food and medicine.

Materials and methods

Materials

Cellulose acetate (CA) (M_n = 30,000 *Da*, 39.8 wt% acetyl content), chitosan (*(*M_v of 405 k*Da*, 84.7% deacetylated), and poly(ethylenenimine) (PEI, $M_w \sim$ 750 k*Da*) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Cibacron Blue F3GA (CB) was obtained from Polysciences (Warrington, FL, USA). Acetone, *N,N′*-dimethylacetamide (DMAc) (EMD Chemicals, analytical grade), and sodium hydroxide (97.0%, ACS GR) were used without further purification.

Bacteriophage propagation and plaque forming unit (PFU) counting

Escherichia coli BL21 (ATCC # BAA-1025) was used in all experiments. *E. coli* was cultured according to standard methods provided by American Type Culture Collection (ATCC). Bacteriophage T7 (ATCC # BAA-1025-B2) was acquired from ATCC and propagated according to the instructions provided by ATCC. Phage activity, or titer, was measured using a standard soft agar overlay plate counting method and reported in plaque forming units (PFU) before immobilizing on the fibers (Kropinski et al. 2009).

Preparation of cellulose fibers

Cellulose fibrous membrane was prepared by a highly versatile and efficient process of electrospinning of cellulose acetate followed by alkaline hydrolysis as previously reported (Liu and Hsieh 2002). Cellulose fibrous membrane was electrospun from 15 w% cellulose acetate in 2:1 acetone/DMAc, then hydrolyzed in mild (0.05 M.) aq. NaOH to deacetylate the acetate groups back to hydroxyls. The electrospun cellulose acetate fibers were amorphous, then transformed into cellulose II crystalline structure upon hydrolysis. The cellulose fibrous membrane was ultrathin with ca. 60 μm thickness, consisting of submicrometer wide fibers with an average width of 262 ± 79 nm, and negatively charged with zeta potentials of −29.9 mV. The base cellulose membrane was used as is for non-specific adsorption.

The cellulose fibrous membrane was used to prepare Cibacron Blue F3GA dye ligand bound cellulose (cellulose-CB) (76 mg CB/g Cellulose), PEI (cellulose–PEI), and chitosan (cellulose–chitosan) coated cellulose. For the protein–ligand approach, the cellulose-CB membrane was prepared by reacting CB with cellulose at 60 °C for 1 h, then with Na_2CO_3 added at 80 °C for 2 h as previously established (Lu and Hsieh 2009). To prepare membranes for the electrostatic binding approach, cellulose was immersed in PEI or chitosan solutions. For cellulose–PEI, the cellulose membrane (ca. 0.1 g) was immersed in 0.5 wt% PEI solution (50 g) at room temperature for 24 h. The PEI saturated cellulose was removed and dried overnight at room temperature. Cellulose–Chitosan fibrous membranes were modified with positively charged chitosan (Ding et al. 2011; Du and Hsieh 2009). Briefly, chitosan was deposited on cellulose via electrostatic attraction of positively charged chitosan and negatively charged cellulose fiber surfaces. The pH of the chitosan solution was adjusted to 5 with HCl (1 mol/L).

The cellulose fibers prepared by different phage immobilizing strategies are summarized in Fig. 1. For adsorbing phages on the fibers, the originally received phage solutions at concentration levels between 6 log(PFU/mL) and 7 log(PFU/mL) were diluted with pure de-ionized water to 10 mL, the quantity needed to prepare the samples. For each of the four samples, 2 mL of the diluted phage solution, which had a final concentration between 5 log(PFU/mL) and 6 log(PFU/mL) respectively, was added slowly to saturate the membranes. The phage deposited samples were dried at room temperature and then kept at ∼4 °C for the following experiments.

Phage loading efficiency

To determine total loading of phages on the fibers, the fibers were disrupted and the resulting solution was PFU counted to determine the concentration of active phage (PFU/ml). To prepare fiber samples for disruption, 1 cm disks were cut from prepared fibers and

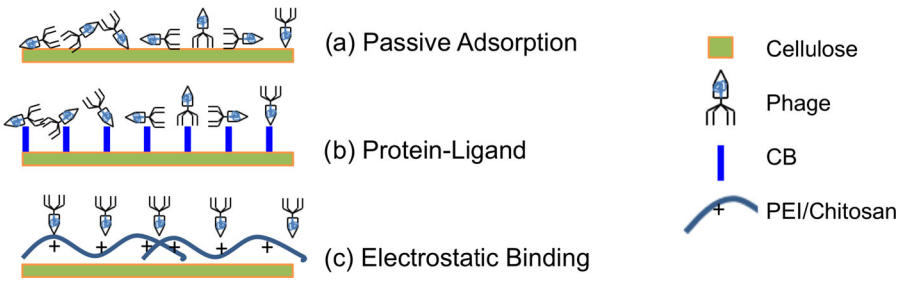

Fig. 1 Pictorial representation of non-specific adsorption, protein–ligand binding, and electrostatic interaction approaches for immobilizing phages on cellulose fibers. In each case, phage orientation is highlighted by the ability of the approach to orient phages

placed in 2 mL of water in a 15 mL plastic conical tube. The samples were then tip sonicated for 7 pulses at 30 s each, with 30 s rest between pulses. The resulting liquid was plate counted, and the results reported as a percentage of initial loading. The procedure was duplicated for each fiber type. In addition, a control experiment to assess the influence of sonication conditions on phage activity was conducted. Phages in solution and an equivalent amount of cellulose fiber at 6 log(PFU/mL) were placed in the same 15 mL plastic tube and sonicated as described above. After sonication, the phage activity was measured by PFU counting. The pre and post sonication effects on phage activity were compared.

Phage labeling and imaging for determining phage distribution

In order to image phages using confocal microscopy, phages were labeled with Alexa Fluor 488 carboxylic acid, succinimidyl ester (Invitrogen, USA) with excitation at 488 nm and emission 500–550 nm. 20 µL of Alexa 488 was added to 500 µL of purified T7 phage at 8 log(PFU/mL) and incubated covered at room temperature for 3 h. The solution was then passed through a Zeba Desalt Column (Fisher Scientific) equilibrated with sterile water to remove excess fluorophores. The fibers were prepared normally with a final 7 log(PFU/mL) loading concentration and covering the fibers to prevent photobleaching of the fluorophore. Fiber disks of 1 cm in diameter were cut and mounted to glass slides. A Carl Zeiss LSM 510 confocal microscope, with a 488 nm laser excitation and a 500–550 nm bandpass emission filter, were used for imaging the fibers. Both Alexa 488 labeled T7 and non-labeled T7 fiber samples were imaged.

Phage release

In order to measure phage release from the fibers, 1 cm disks were cut from fiber samples and placed into 2 mL of water. The samples were shaken at 150 rpm and room temperature in a shaker incubator for a period of 24 h. At 30 min, 6, and 24 h, an aliquot of 100 µL was taken from each sample and plate counted for phage activity as described in the previous section. Phage counts are reported as log(PFU). The procedure was duplicated twice for each fiber type.

Statistical analysis

Results were analyzed using Excel (Microsoft, Redmond WA). To determine significant results, a T test assuming unequal variances was used. Significant results of $p < 0.05$ were noted where appropriate.

Results

Immobilization of phages on cellulose fibers

Phage loading efficiency on cellulose fibers by non-specific adsorption, protein–ligand binding, and electrostatic interactions were measured. The modification of the cellulose membrane with CB is the key difference between the non-specific adsorption and protein–ligand binding of phages on cellulose fibers. CB is an anionic dye ligand that binds proteins by non-covalent means, typically based on a combination of hydrophobic interactions, hydrogen bonds, weak electrostatic interactions, and binding to the proteins' active site and thus is hypothesized to optimize phage binding on to the cellulose fiber (Denizli and Pişkin 2001). The phage loading efficiency comparing the

three binding approaches is summarized in Fig. 2a. Non-specific adsorption showed little to no recoverable phage, at less than 0.001% of the initial loading. Similarly, protein–ligand binding bound less than 0.001% of the initial loading. Electrostatic interaction immobilization by PEI or chitosan modified cellulose phage loading efficiency was approximately 18 and 25% of the initial phage loading, respectively. In order to show that the experimental approach to measure loading efficiency did not influence the phage activity (plaque forming ability), a set of control experiments using sonication were conducted. The results of this control experiment demonstrated that the phages did not lose their activity with sonication for the selected duration and amplitude as shown in Fig. 2b. The results indicate a high loading efficiency of phages on positively charged membranes as compared to non-specific adsorption and protein–ligand binding.

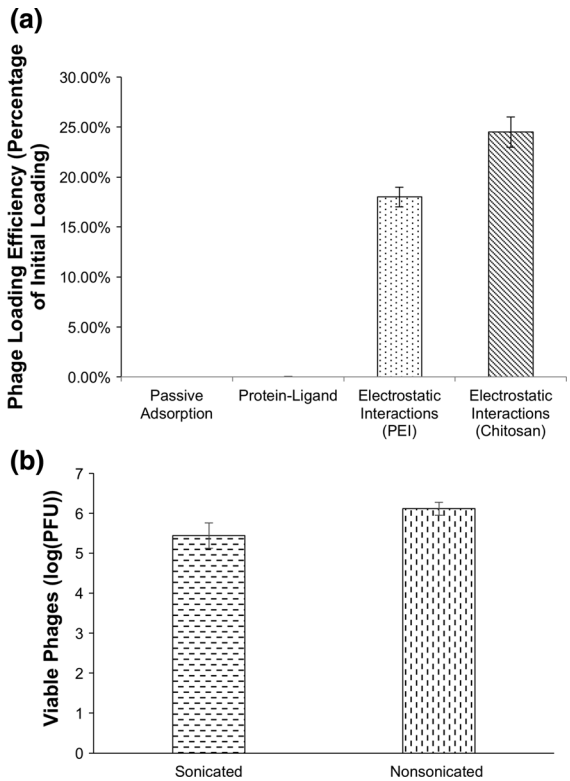

Fig. 2 Phage loading efficiency, normalized to initial loading, for the two electrostatic interaction approaches in **a**. Non-specific adsorption and protein–ligand binding yielded no significant loading at less than 0.001% of initial phage loading. Electrostatically immobilized phages loaded at approximately 16 and 24% for cellulose–PEI and cellulose–chitosan, respectively. **b** Demonstrates the lack of effect of sonication on phage viability

Imaging distribution of immobilized phages on cellulose fibers

To evaluate the distribution of immobilized phages on cellulose fibers, phages immobilized on cellulose fibers were labeled with fluorescent dye molecules and imaged using confocal fluorescence microscopy as shown in Fig. 3. Non-specific adsorption and protein–ligand binding cellulose fibers were excluded from imaging due to low phage binding based on the results from Fig. 2. The control samples with non-labeled phages show negligible autofluorescence under the same set of imaging conditions. For cellulose PEI, the fluorescence signal is localized with higher intensity in regions along the fiber complex. In the case of the cellulose–chitosan fibers, the fluorescence signal appears more diffuse and evenly spread over the fibers. Furthermore, the results also highlight some differences in the surface chemistry of the cellulose–chitosan fibers compared to the cellulose–PEI fibers. These results demonstrate that the phages can be uniformly distributed across a large surface area using electrostatic phage immobilization.

Phage release from suspended fibers

The phage release from cellulose fibers was measured as shown in Fig. 4. Similar to Fig. 3, only the electrostatically immobilized phages were measured for release. Phages were released from the fibers by incubating in water and shaken at 150 rpm over the course of 24 h. In the case of cellulose–PEI, the phage release can be detected within 30 min at approximately 2log PFU/mL. At 6 and 24 h of release, 3.7 log(PFU) and 5.5 log(PFU) of phages were released, respectively. Approximately 34% of the immobilized phages were released from the cellulose–PEI fibers in a 24 h period. In contrast with cellulose–PEI, the phage release from cellulose–chitosan in the first 30 min was not detectable. Over 6 and 24 h, 2.8 and 6.2 log(PFU) were released, respectively, for cellulose–chitosan. These results showed that phages were released more rapidly from cellulose–PEI than cellulose–chitosan in the first 30 min ($p < 0.05$), but were not significantly different at 6 and 24 h ($p > 0.05$). Altogether, these above results show a high phage

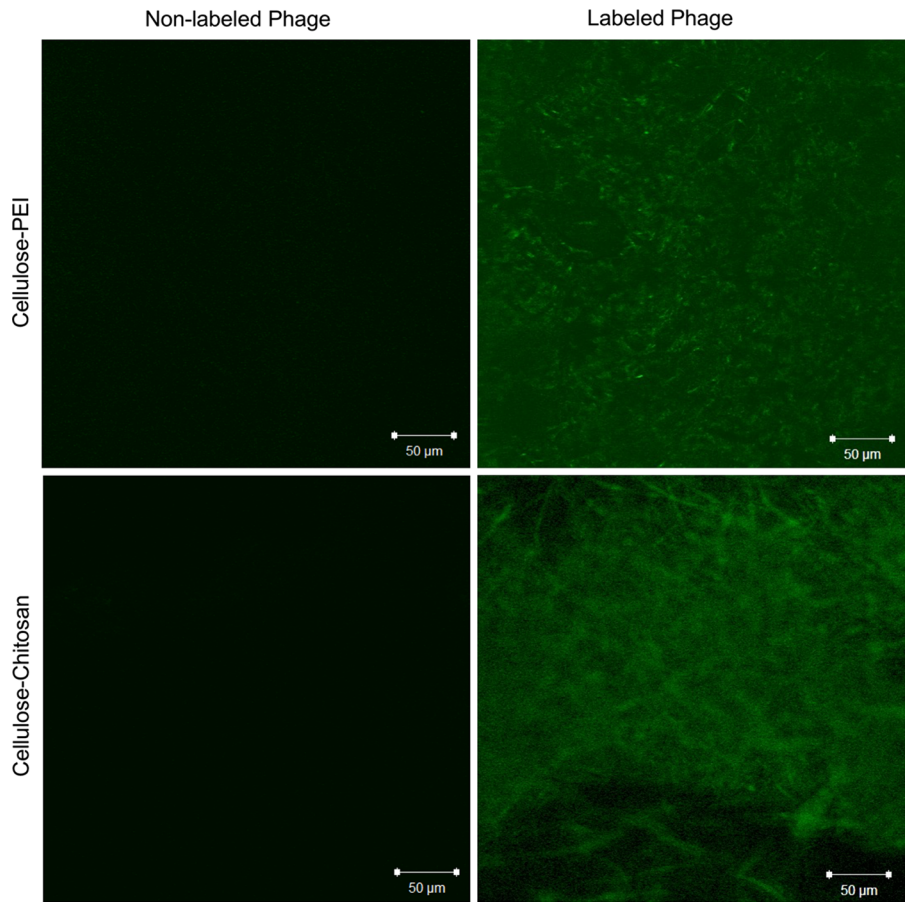

Fig. 3 Confocal images fluorescently labeled and unlabeled phages immobilized on cellulose–PEI and cellulose–chitosan fibers. Fluorescent signal on both labeled phage immobilized fibers indicates a random and uniform distribution on the surface of the cellulose fibers. Non-labeled phage immobilized fibers show little signal background

loading efficiency and uniform coverage on cellulose fibers with positively charged PEI or chitosan surfaces, and these immobilized phages can be slowly released in an aqueous environment with moderate shaking at 150 rpm over 24 h.

Discussion

Non-specific adsorption and protein–ligand binding were not effective for immobilization of phages on the cellulose fiber surfaces. The lack of detectable phages as compared to electrostatic interaction is in agreement with a previous study, though the study used preformed cellulose membranes (Anany et al. 2011). The lack of recovery of phages in the non-specific adsorption and protein–ligand binding approaches may be due to limited adsorption efficiency despite the large surface area of fibrous substrate. Protein–ligand binding takes advantage of many non-covalent binding approaches to immobilize the phage and hypothetically might be more efficient at immobilization due to the protein capsid of the phage. CB, the ligand used in this study, is routinely used in column chromatography for affinity-based purification of proteins (Denizli and Pişkin 2001). This protein–ligand based binding of phages has significant advantages compared to a conventional conjugation approach, such as NHS-Ester or EDC chemistries, as the protein–ligand based approach does not require direct chemical modification of the phages. Conventional conjugation processes require multiple steps, including reaction and multi-step purification. Using the dye ligand approach, the time and labor required for phage binding to cellulose fibers is simplified. However, the data suggests that protein–ligand binding is not as highly

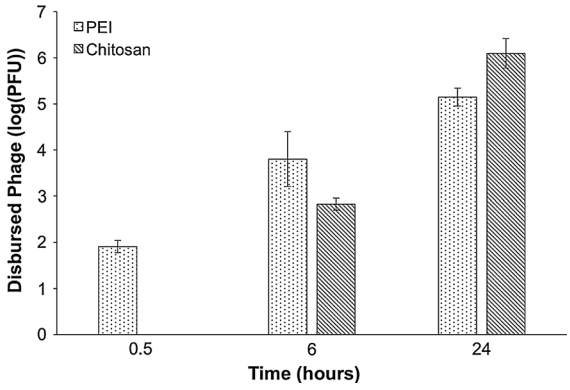

Fig. 4 Phage release profiles from cellulose–PEI and cellulose–chitosan cellulose fibers over 24 h. At 30 min, only phage from cellulose–PEI are detectable. At 6 h, phage from cellulose–PEI and cellulose–chitosan are detectable at approximately 4 log(PFU/mL) and 3 log(PFU/mL) respectively. Release from cellulose-PEI and cellulose–chitosan at 6 and 24 h are not significantly different ($p > 0.05$)

efficient for phage immobilization as originally hypothesized based on past work immobilizing lipase enzyme using the same approach (Lu and Hsieh 2009). Additionally, desiccation of the phage during the immobilization process may reduce active phage numbers (Anany et al. 2011). Further experimentation is needed to elucidate the lack of effective phage immobilization from non-specific adsorption and protein–ligand binding.

In contrast to non-specific adsorption and protein–ligand binding, the electrostatic interaction of phages is the most effective approach to immobilize phages on cellulose among the approaches studied. The electrostatic immobilization approach results are in agreement with previous studies that have demonstrated that phages can be effectively immobilized on inorganic substrates by electrostatic interaction (Anany et al. 2011; Cademartiri et al. 2010; Jabrane et al. 2009). Though Anany et al. had a similar approach to this study using pre-fabricated cellulose membranes, this previous study did not compare the influence of diverse binding mechanisms on cellulose substrate such as protein–ligand binding. In addition, none of the prior approaches has characterized the differences in binding and release of phages from cellulose substrate complexed with different cationic polymers, and similarly phage distribution via confocal imaging was not characterized. For the electrostatic interaction immobilization approach, a uniform distribution of phages on the surface of cellulose fibers was achieved. Such a uniform phage distribution as provided by cellulose–PEI and cellulose–chitosan can enhance better distribution or delivery to and enable higher probabilities of phages interacting with microbes on a surface of interest. It is also important to note that many phages like T7 phages also carry net negative charges (Archer and Liu 2009; Serwer and Hayes 1982). Therefore, this electrostatic immobilization approach may also be applied to different phages to target different pathogens or perform other functions. Successful electrostatic immobilization of model bacteriophage T7 on cellulose fibers can be extended to binding multiple phage strains. Binding multiple phage strains in a cocktail has been shown to enhance the reduction in bacterial pathogens as opposed to a single strain of phage and lower the probability of the bacterial pathogen gaining phage resistance (Leverentz et al. 2001; Sharma et al. 2009).

In addition to the improved phage binding efficiency of the electrostatic interactions approach, the fibers also release a significant number of phages in an aqueous environment. A previous study has shown that electrostatically bound phages do not release to an aqueous environment (Cademartiri et al. 2010), and thus the results in this study is unexpected. In order to explain this study's results, it is hypothesized that the release mechanism from these fibers may be due to fiber disintegration from the membrane, PEI or chitosan dissociation from the fibers, phage dissociation from PEI or chitosan on fiber surfaces, or some combination thereof. In all experiments, the cellulose fiber membranes were observed to be disintegrating and increasing the turbidity of the solution, suggesting that fiber disintegration is the dominating mechanism for phage release. In the case of PEI/chitosan dissociation from the fibers, chitosan is not expected to dissociate from the fibers into solution as chitosan is insoluble in aqueous solution and has been confirmed by a prior study (Ding et al. 2011). PEI is water soluble and likely released from the cellulose fibers into the aqueous solution. PEI's solubility may explain the difference between cellulose–PEI and cellulose–chitosan release data at 30 min.

Conclusion

In this study, phage immobilization by non-specific adsorption, protein–ligand binding, and electrostatic

interactions on cellulose microfibers were examined. These phage immobilized fibers were characterized by their phage loading, distribution, and release to an aqueous environment. High loading efficiency, phage release from the carrier, and uniform distribution of phages across large surfaces are needed to treat the bacterial targets across diversity of applications in the areas of food and biomedical. Based on the results, electrostatic interactions had the most effective phage loading compared to non-specific adsorption and protein–ligand binding. Electrostatically immobilized phages were uniformly distributed over the fiber matrix and were released from the fibers. Overall, the results demonstrate the feasibility of binding phages to cellulose fibers based on electrostatic interactions.

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