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Antimicrobial sponge prepared by hydrophobically modified chitosan for bacteria removal

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ABSTRACT

Hydrophobically modified chitosan (HMCS) prepared by reacting chitosan with dodecyl aldehyde can generate very stable foam when dissolved in mild acidic condition under vigorous mechanical stirring. A durable and lightweight (density of 32 mg/ml) sponge was obtained by freeze-drying the stably formed HMCS foam. In addition to the cationic nature of chitosan, the grafted C_{12} alkyl chains were also able to help HMCS sponge for capturing *E. coli* cells ($\sim 4.0 \times 10^8$ cells/mg sponge) by intercalating into the outer membrane of *E. coli* cells. *E. coli* cells captured on HMCS sponge were found to be mostly dead and easily released into the bulk solution so that the active surface could be continuously regenerated for capturing and killing the rest of alive cells. In comparison with its counterpart (chitosan sponge), HMCS sponge maintained a higher operational stability for the removal of *E. coli* cells. After 5 repeated cells removal operation, the removal capacity of HMCS sponge could be regenerated back to > 90% by thorough washing with ethanol.

1. Introduction

Microbial contamination of water has been a major threat to human health (Ashbolt, 2004). The pathogens in water can be eliminated by chlorine, chloramines, ozone and chlorine dioxide (Jin, Liu, Shan, Tong, & Hou, 2014; Richardson, 2003). However, these chemicals could react with various constituents in natural waters to generate more than 600 kinds of disinfection byproducts, many of which are carcinogenic (Jin et al., 2014; Richardson, 2003). Moreover, the conventional antibiotic methods are becoming less efficient as the emergence of antibiotic-resistant strains (Jin et al., 2014; Sayah, Kaneene, Johnson, & Miller, 2005). Development of new bacteria decontamination strategy is therefore of great urgency and importance (Jin et al., 2014).

Capture and separation of bacteria from water with bacteria-adhesive materials can provide an alternative to eliminate the pathogens from water. In previous literatures, some materials that can effectively capture the bacteria from water such as carbon nanotubes, resin-linked oligoacyllysine bead, carbohydrate functionalized graphene derivatives have been reported (Qi et al., 2015). However, implementation of these materials for bacteria removal operation would be inhibited by the difficulty in recovery due to their relatively small sizes. Whereas, magnetic nanoparticles (MNPs), which can be conveniently separated from water by applying a magnetic field, have been used for target bacteria detection and water decontamination after proper surface functionalization (Ambashta & Sillanpaa, 2010; Huang, Wang, & Yan, 2010; Jin et al., 2014).

Hydrophobically modified chitosan (HMCS) is one of many useful derivatives of chitosan prepared by grafting a hydrophobic chain to the amino group of chitosan via Schiff-base reaction (Desbrières, Martinez, & Rinaudo, 1996). HMCS has not only the properties of chitosan such as biocompatibility, antibacterial activity, and many other features (Dutta, Tripathi, Mehrotra, & Dutta, 2009; Kong, Chen, Xing, & Park, 2010), but also its own hydrophobic interaction capability (St. Dennis et al., 2011). HMCS has demonstrated its strong interaction with red blood cells (Dowling, Keibler et al., 2011; Dowling, MacIntire et al., 2015), liposomes (Zheng et al., 2014), vesicles (Arora et al., 2015; Dowling, Javvaji, Payne, & Raghavan, 2011), and human cells (Javvaji, Dowling, Oh, White, & Raghavan, 2014). Moreover, the hydrophobic chains grafted to HMCS can insert into the lipid bilayer of *E. coli* outer membrane (Vo, Whiteley, & Lee, 2015). Unlike water soluble derivatives of chitosan such as carboxymethyl chitosan (Vo, Sabrina, & Lee, 2017) and *N*-alkyl chitosan derivatives with quaternary ammonium salts (Kim, Choi, Chun, & Choi, 1997), the hydrophobic chains protrudes from water insoluble HMCS film has been demonstrated can anchor *E. coli* cells by piercing into their cell membrane under neutral pH condition (Vo & Lee, 2017b). The presence of hydrophobic chains in its structure makes HMCS an amphiphilic compound and becomes surface active that a very stable foam can be formed when intensive mechanical stirring is applied to HMCS solution. A durable sponge has been obtained by simply freeze-drying the stable HMCS foam as demonstrated

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in our previous work (Vo & Lee, 2017a). In this study, HMCS sponge prepared from stable HMCS foam was shown to be very effective not only for bacterial cells capture also for contact-killing bacteria. Gram-negative *E. coli* strain and Gram-positive *S. aureus* were employed as model bacteria. The bacterial cells capture capacity and reusability of as-prepared HMCS sponge were investigated.

2. Experiments

2.1. Materials

Chitosan from shrimp shells of molecular mass ~512 kDa (calculated by intrinsic viscosity using the Mark–Houwink–Sakurada equation) with deacetylation degree of approximately 72% was purchased from Sigma-Aldrich. Sodium cyanoborohydride ($NaCNBH_3$, 95%), ninhydrin, dodecyl aldehyde (92%), sodium phosphate, monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$, 99%), sodium phosphate, dibasic heptahydrate ($Na_2HPO_4{\cdot}7H_2O$, 99%), sodium chloride (NaCl, 99.5%) were purchased from Acros. Acetic acid glacial (analytical grade) and sodium hydroxide were obtained from Fisher Scientific. *E. coli* BL21 was obtained from Novagen (Madison, WI, USA). *S. aureus* (ATCC6538P) was provided by Taiwan Textile Research Institute. LIVE/DEAD cells fluorescent assays (Thermo Fisher Scientific, Baclight Bacterial Viability Kit) was employed to check the viability of bacteria.

2.2. Preparation of HMCS sponge

The hydrophobically modified chitosan (HMCS) was prepared according to our previous work (Vo et al., 2015). Briefly, chitosan (4 g) was dissolved in acetic acid (220 ml, 0.2 M) and diluted with ethanol (150 ml) followed by pH adjustment to pH 5.1 using 1N NaOH. The homogeneous chitosan solution was treated with dodecyl aldehyde solution prepared in ethanol (10 ml, 0.05 g/ml). An excess amount of sodium cyanoborohydride (3 mol per chitosan monomole) was then added and the reaction was last for 24 h at room temperature. At the end of reaction, pH was adjusted to 7.0 using 1 N NaOH. The product was precipitated from the solution by adding an excess amount of ethanol. After thorough washing with ethanol to remove the unreacted dodecyl aldehyde and sodium cyanoborohydride, the precipitate was dried under vacuum at room temperature and designated as HMCS.

For sponge preparation, HMCS (0.5 g) was dissolved in acetic acid (50 ml, 0.2 M) and vigorously stirring was applied using a mechanical stirrer for 24 h to generate stable foam. The HMCS foam was then transferred into a plastic centrifuge tube for freeze-drying. HMCS sponge was then obtained after 1 day freeze-drying. It was further dried under vacuum at 40 °C for 2 days to ensure the volatile acetic acid was completely removed. Chitosan (CS) sponge was also prepared following the same procedure. Either CS or HMCS sponge before used for bacteria capture was first wetted in ethanol to remove the trapped air followed by thorough washing with deionized water.

2.3. Sponge characterization

The water contact angle of sponge samples was measured by using Contact Angle Goniometer (Sindatek Model 100SB). The density of sponge was determined by measuring the weight gain after completely filling the sponge structure with absolute ethanol to calculate its volume. All samples measurements were triplicated in the experiment. The degree of swelling of CS or HMCS sponge in phosphate buffered saline (PBS, 0.01 M, pH 7.4) at 37 °C was determined at predetermined time intervals by measuring their weight gains after removing the surface adhered solution by blotting with filter paper. The degree of swelling (DS) was calculated by the following formula (Liu et al., 2014)

$$DS(\%) = \frac{W_w - W_d}{W_d} \times 100\%$$

where, W_w and W_d are the wet and dry weight of sponges, respectively.

2.4. Bacteria removal

A single colony of *E. coli* and *S. aureus* on LB and TSB nutrient agar plates, respectively was picked and placed into 5 ml medium, which was then incubated at 37 °C for 12–16 h. The cell suspension was then centrifuged at 8000 rpm for 3 min. The obtained cells pellet was washed with PBS twice and re-suspended in PBS (0.01 M, pH 7.4) to achieve an optical density of 0.1 or 1.0 at 600 nm. The bacterial suspension (1 ml) was then added into Eppendorf tube to mixed with wetted CS or HMCS sponge (1 mg) (37 °C, 200 rpm). The cell concentration in the mixture was measured with time at 600 nm by using an UV/Vis spectrophotometer (Jasco, Model V-730). In addition, the colony-forming unit of bacteria suspension after 36 h mixing with sponges was also measured.

2.5. Contact-Killing activity of sponges

Sponges with bacteria captured at different time (0.5 h, 2 h, 4 h, 12 h and 36 h) were taken out from bacteria suspension and thoroughly rinsed with PBS (0.01 M, pH 7.4). The sponge samples were then fixed in 5% (v/v) glutaraldehyde (GA) solution prepared in PBS solution for 30 min at room temperature. The GA fixed samples were immersed in 25%, 75%, and 100% ethanol stepwise for dehydration and dried at room temperature. The bacteria attached sponge was observed by field-emission scanning electron microscopy (JEOL, Model JSM-6500F) (Vo et al., 2015). On the other hand, the PBS rinsed sponges were also stained using the LIVE/DEAD cells assay kit to visualize the intensity of red and green fluorescence displayed on sponge surfaces using a fluorescence microscope (Vo & Lee, 2017b; Vo et al., 2017). LIVE/DEAD staining was also applied to the bacteria in the suspensions to check their viability after contacting with the sponges.

2.6. Reusability

The *E. coli* captured sponges were regenerated by thoroughly washing followed by immersing in PBS solution (0.01 M, pH 7.4) under shaking (1 h, 200 rpm). The regenerated sponges were immediately applied for the next cycle of bacteria capture. After 5 cycles of bacteria capture operation, the sponges were activated by washing and immersing in 70% ethanol solution for 36 h. After activation, the sponges were washed with deionized water and applied for next reused cycles. The bacteria removal efficiency was evaluated by measuring the decrease of optical density of *E. coli* suspension at 600 nm after in contact with the sponges.

3. Results and discussion

3.1. Hydrophobically modified chitosan sponge

The hydrophobic nature of HMCS sponge can be well demonstrated when compared with its counterpart (CS sponge) as shown in Fig. S1 (Supporting information). CS sponge is easily wetted and submerges into water while the HMCS sponge can only float on water surface if without ethanol wetting. Evidently, the pore surface of HMCS sponge is hydrophobic that prevents water from uptake into HMCS sponge structure via capillary action. In contrast, ethanol can easily wet HMCS sponge due to its hydrophobic nature. Once wetted by ethanol followed by in contact with water solution, the pore-filled ethanol can be then effectively replaced by water that leads HMCS sponge submerge into water solution (Fig. S1b). Moreover, the water contact angle can only be measured on the surface of HMCS sponge to be 122° (Fig. S1c, d). In contrast, the water droplet applied on the surface of CS sponge and wetted HMCS sponge for contact angle measurement was soaked into the sponge immediately before measurement could be carried out.