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Ionic starch-based hydrogels for the prevention of nonspecific protein adsorption

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ABSTRACT

Non-fouling materials bind water molecules via either hydrogen bonding or ionic solvation to form a hydration layer which is responsible for their resistance to protein adsorption. Three ionic starch-based polymers, namely a cationic starch (C-Starch), an anionic starch (A-Starch) and a zwitterionic starch (Z-Starch), were synthesized via etherification reactions to incorporate both hydrogen bonding and ionic solvation hydration groups into one molecule. Further, C-, A- and Z-Starch hydrogels were prepared via chemical crosslinking. The non-fouling properties of these hydrogels were tested with different proteins in solutions with different ionic strengths. The C-Starch hydrogel had low protein resistance at all ionic strengths; the A-Starch hydrogel resisted protein adsorption at ionic strengths of more than 10 mM; and the Z-Starch hydrogel resisted protein adsorption at all ionic strengths. In addition, the A- and Z-Starch hydrogels both resisted cell adhesion. This work provides a new path for developing non-fouling materials using the integration of polysaccharides with anionic or zwitterionic moieties to regulate the protein resistance of materials.

1. Introduction

Biofouling or nonspecific protein adsorption remains a challenging problem in biomedical applications, such as bio-separation membranes, biosensors, bio-implants, and drug carriers (Anderson, Rodriguez, & Chang, 2008; Brault et al., 2010; Sun, Yue, Huang, & Meng, 2003). This ubiquitous problem can result in the adhesion of microorganisms or cells in the formation of biofilms on the surface of biomedical devices which can cause microbial infections or thrombosis. About 45% of nosocomial infections are thought to be due to biomedical devices-associated infections (Schierholz & Beuth, 2001). Recently, some non-fouling materials have been used to prevent nonspecific protein adsorption. Poly(ethylene glycol) (PEG), which can prevent nonspecific protein adsorption via a steric repulsion mechanism, is the most widely employed non-fouling material (Gol & Jewrajka, 2014; Herrwerth, Eck, Reinhardt, & Grunze, 2003; Matsumoto, Matsusaki, & Akashi, 2014). However, PEG is unstable in the presence of oxygen and transition metal ions which results in a loss of function in most biological media (Herold, Keil, & Bruns, 1989; Rodriguez-Emmenegger et al., 2011). In fact, there are few materials that can prevent nonspecific protein adsorption in biomedical applications.

In order to design new non-fouling materials, many researchers have studied the mechanisms for protein resistance. It has been proposed that the formation of a hydration surface layer on a non-fouling material prevents protein adsorption by forming an energetic physical barrier (Herrwerth et al., 2003; Hower, He, Bernards, & Jiang, 2006; Zheng et al., 2005). The strength of this hydration layer is determined by many factors but the surface chemistry of the material is one of the most important factors. In general, a hydration layer can be formed through hydrogen bonding or by ionic solvation. Typical examples of hydrogen bonding-based non-fouling materials are PEG (Gol & Jewrajka, 2014; Herrwerth et al., 2003; Matsumoto et al., 2014), sugars (Ederth et al., 2011; Fyrner et al., 2011; Luk, Kato, & Mrksich, 2000), and polyamide (Statz, Meagher, Barron, & Messersmith, 2005). Ionic solvation-based non-fouling materials include polyzwitterionic materials like poly(sulfobetaine methacrylate) (PSBMA) (Sin, Sun, & Chang, 2014) and poly(carboxybetaine methacrylate) (Carr, Xue, & Jiang, 2011).

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Notably, studies have found that ionic solvation-based non-fouling materials bind more water molecules and bind them more tightly than hydrogen bonding-based materials and that materials with ionic solvation hydration capabilities often have better nonspecific protein resistance (He et al., 2008; Wu, Lin, Wang, Chen, & Chang, 2012). Ladd, Zhang, Chen, Hower, and Jiang (2008) found that polyzwitterionic surfaces have better protein resistance than PEG in human plasma or serum. In addition, polyzwitterionic surfaces greatly suppress bacterial adhesion over the course of ten days, whereas PEG surfaces fail to resist long-term bacterial formation (Cheng, Zhang, Chen, Bryers, & Jiang, 2007). However, polyzwitterionic non-fouling materials exhibit poor protein resistance at low ionic strengths (Chang et al., 2010; Holmlin, Chen, Chapman, Takayama, & Whitesides, 2001). Therefore, it is necessary to develop non-fouling materials which can prevent nonspecific protein adsorption even at low ionic strengths.

Most polysaccharides, which are rich in hydroxyl groups and negatively charged, are low-fouling materials. They prevent protein adsorption via binding water through both hydrogen bonding and ionic solvation. In fact, several polysaccharides with negative charges (such as hyaluronic acid (Ombelli et al., 2011), heparin (Chen, Chen, Sheardown, & Brook, 2005), and gellan gum (Lee, Tsai, Wen, & Huang, 2012)) have demonstrated good protein resistance at physiological pH.

Starch, one of the most common polysaccharides, is universally available and non-toxic. Since it is a neutral polysaccharide, starch can resist protein adsorption by forming a hydrogen-bonded hydration layer. The protein resistance of starch should be improved by incorporating ionic solvation hydration groups. Therefore, in this study, in order to improve the protein resistance of starch, ionic starch-based polymers with different charges were synthesized (Fig. 1). These anionic, cationic, and zwitterionic starch-based ionic polymers (abbr: A-, C- and Z-Starch respectively) can bind water molecules via both hydrogen bonding and ionic solvation. Then, A-, C- and Z-Starch hydrogels were prepared via chemical crosslinking using poly(ethylene glycol) diglycidyl ether (PEGDE) as the cross-linker. Their non-fouling properties were then assessed using neutrally charged HRP-conjugated goat antihuman IgG (HRP-IgG), positively charged lysozyme and negatively charged pepsin. Moreover, the protein resistance mechanisms of the A-, C- and Z-Starch hydrogels were discussed. Their biocompatibility and cell resistance properties were also investigated using human umbilical vein endothelial cells (HUVECs).

2. Materials and methods

2.1. Materials

Starch (from potato, amylose; Mw 1.01×10^6) was obtained from Jiangtian (Tianjin, TJ, CN). 1,3-Propanesultone, 1-chloro-3-dimethylaminopropane hydrochloride (CDMAP•HCl) (98%), 3-chloro-2-hydroxypropyltrimethyl ammonium chloride (CHPAC, 69%, w/v) and 3-chloro-2-hydroxypropanesulfonic acid sodium salt (CHPSNa) were purchased from Mengde (Danyang, JS, CN), Alfa Aesar (Haverhill, MA, USA), Guofeng (Dongying, SD, CN) and Xingye (Yangzhou, JS, CN), respectively. PEGDE and [2-(methacryloxy)ethyl] dimethyl-3-sulfopropylammonium hydroxide (SBMA) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and N,N′-methylenebis (acrylamide), sodium metabisulfite and ammonium persulfate were supplied by Guangfu (Tianjin, TJ, CN). Pepsin from porcine stomach mucosa, and lysozyme from chicken egg white were obtained from Sigma–Aldrich (St. Louis, MO, USA). HRP-IgG was purchased from Biosynthesis (Beijing, BJ, CN). HRP-conjugated rabbit anti lysozyme antibody and HPR-conjugated goat anti pepsin antibody were purchased from Abcam (Cambridge, Cambs, UK). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). HUVECs were obtained from the Collection for Type Cultures of Chinese Academy of Sciences (Beijing, BJ, CN). Deionized water was purified to 18.2 mΩ on a Millipore water purification system. PSBMA hydrogel was synthesized using the method in the literature (Carr, Cheng, Xue, & Jiang, 2010). Brifly, 400 mg SBMA monomer was dissolved in 1 mL water. N,N′-Methylenebis (acrylamide) was added at a cross-linker ratio of 4% (w/w). Then, 10.9 μL of 15% sodium metabisulfite and 10.9 μL of 40% ammonium persulfate were added. Polymerization was initiated at 60 °C for 30 min. Then the hydrogel was immersed in water for use.

Table 1
Preparation of A-, C- and Z-Starches and their DSs.

Sample	Charge type	Amount of chemicals (g)			DS
		Starch	NaOH	Etherifying agent[a]	
Z-Starch	+ and −	1.60	1.00	4.91	0.38
A-Starch	−	1.60	1.00	10.01	0.40
C-Starch	+	1.60	1.00	1.90	0.42

[a] Etherifying agents: DCAPS, CHPSNa or CHPAC for A-, C- and Z-Starches, respectively.

2.2. Synthesis of zwitterionic etherifying agent

CDMAP•HCl (15.8 g) was dissolved in 10 mL water and then 10 mL NaOH solution (40%, w/v) was added dropwise to the CDMAP•HCl solution. After 30 min, CDMAP was obtained using a separatory funnel. Then, DCAPS was synthesized by reacting CDMAP with 1,3-propanesultone (18.3 g) in 1,2-dichloroethane (100 mL) at 70 °C for 6 h. The crude product, a white precipitate, was washed with 1,2-dichloroethane and dried in vacuum. Yield: 93%. The structure of the zwitterionic etherifying agent, 3-dimethyl(chloropropyl) ammonium propanesulfonate (DCAPS), was identified by ^{1}H NMR spectra (Figure S1).

2.3. Synthesis of A-, C- and Z-Starches

For the Z-Starch, the desired amounts of starch and NaOH (see Table 1) were dissolved in water at 40 °C for 1 h. Then 50% (w/w) DCAPS solution was added dropwise and the etherification was performed at 60 °C for 6 h. The product was collected after precipitation from a methanol solvent. The remaining solvent was removed via vacuum drying to give a white powder.

A- and C-Starches were synthesized using a similar method except that the etherifying agents were CHPAC or CHPSNa, respectively. The detailed reaction parameters are listed in Table 1.

The chemical characteristics of the A-, C- and Z-Starch polymers were determined using ^{1}H NMR (Bruker Avance III spectrometer). The degrees of substitution (DS) of the zwitterionic groups in the Z-Starch, the anionic groups in the A-Starch and the cationic groups in the C-Starch were calculated from the ^{1}H NMR data. DS is defined as the number of ionic groups per anhydroglucose group.

2.4. Preparation of A-, C- and Z-Starch hydrogels

Hydrogels were prepared by chemical crosslinking using PEGDE as the cross-linker. Briefly, 400 mg of starch, A-Starch, C-Starch or Z-Starch was dissolved in 1 mL NaOH solution (pH = 11) and then 76 μL of PEGDE was added to each solution with stirring. The mixtures were then poured into a mold and gelled at 37 °C for 8 h. Finally, the hydrogels were immersed into deionized water for four days to remove any unreacted chemicals.

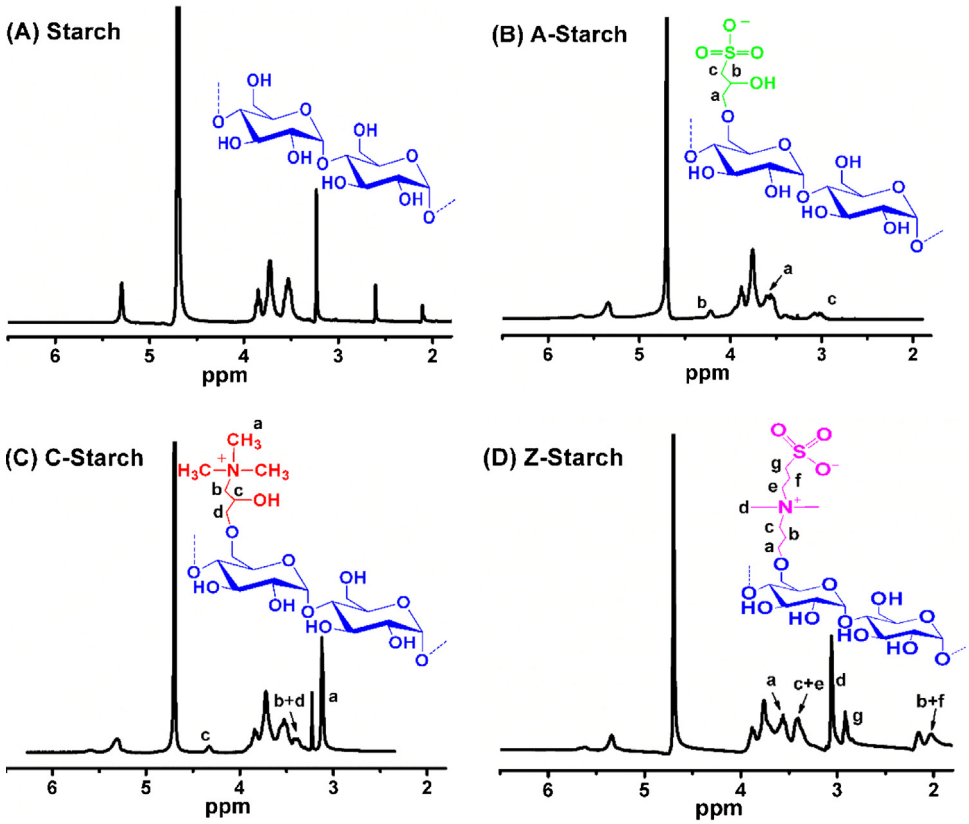

Fig. 1. ^{1}H NMR spectra of (A) Starch, (B) A-Starch, (C) C-Starch and (D) Z-Starch.

2.5. Measurement of zeta potentials

First, the hydrogels were crushed in water with an Ultrasonic Cell Crusher (JY92-IIN, SCIENTZ) at 800 W for 5 min. Then the zeta potentials of the hydrogels were measured using NanoSizer apparatus (Malven, UK) which was equipped with an electrophoretic light-scattering spectrophotometer. Six measurements were applied to each sample.

2.6. Equilibrium swelling studies

Freshly prepared hydrogels were soaked in water for four days. Then the hydrogels were cut into disks with diameter of 6.5 mm and height of 1.5 mm. The disks were carefully taken out and wiped with filter paper to remove surface water, then they were weighed (m_w). After the hydrogel disks were lyophilized, the weights of dry disks were measured again (m_d). The equilibrium water content (EWC) was determined based on the change in weight relative to the initial sample weight according to Eq. (1). All tests were done in triplicate.

$$EWC = \frac{m_w - m_d}{m_w} \times 100\% \quad (1)$$

2.7. Assessment of protein adsorption

The protein adsorption behaviors of the different hydrogels were evaluated using an enzyme-linked immune sorbent assay (ELISA) which has been described elsewhere (Chang, Chang, Shih, Wei, & Hsiue, 2011; Cheng, Xue, Li, & Jiang, 2010; Dobbins, McGrath, & Bernards, 2012). HRP-IgG, lysozyme and pepsin were used as model proteins. Each of them was dissolved in water to obtain protein water solution. A certain amount of phosphate was added to the protein water solution to obtain protein phosphate buffer solution (PBS) with ionic strength of 10 mM PBS. Then, NaCl was added to the protein solution of 10 mM PBS to prepare protein solutions with ionic strength of 0.2 or 1.0 M NaCl. Hydrogel disks (46.5–47.6 mg) with diameter of 6.5 mm and height of 1.5 mm were used.

For the test of HRP-IgG adsorption, the hydrogel disks were soaked in 500 μL of HRP-IgG solution (1 μg/mL) with medium of water or 10 mM PBS at 37 °C for 90 min. The protein solutions were then removed and the hydrogels were washed five times with 500 μL of water or 10 mM PBS to remove the non-adherent proteins. The hydrogels were transferred into a new container and immersed in 500 μL of 0.1 M citrate-phosphate buffer (pH 5.0) containing 1 mg/mL o-phenylenediamine and 0.03% hydrogen peroxide at 37 °C. After 15 min, the enzyme induced color reaction was stopped by adding 500 μL of 1 M H_2SO_4 and the absorbance at 490 nm was determined by a microplate reader.

For the test of lysozyme, the hydrogel disks were soaked in 500 μL of lysozyme solution (1 mg/mL) with medium of water, 10 mM PBS, 0.2 M NaCl or 1.0 M NaCl at 37 °C for 90 min. The protein solutions were then removed and the hydrogels were washed five times with 500 μL corresponding medium (water, 10 mM PBS, 0.2 M NaCl or 1.0 M NaCl) to remove the non-adherent proteins. The hydrogel disks were then incubated for another 90 min in 500 μL of HRP-conjugated rabbit anti lysozyme antibody solution (10 μg/mL) with the corresponding medium. They were then rinsed five times again with 500 μL corresponding medium. All of the hydrogels were transferred into a new container and the rest was conducted using the same method as HRP-IgG.

For the test of pepsin adsorption, the experiment was conducted using the similar method with lysozyme adsorption except that pepsin solution (1 mg/mL) and HPR-conjugated goat anti pepsin antibody (10 μg/mL) solution were used. All tests were done in triplicate.

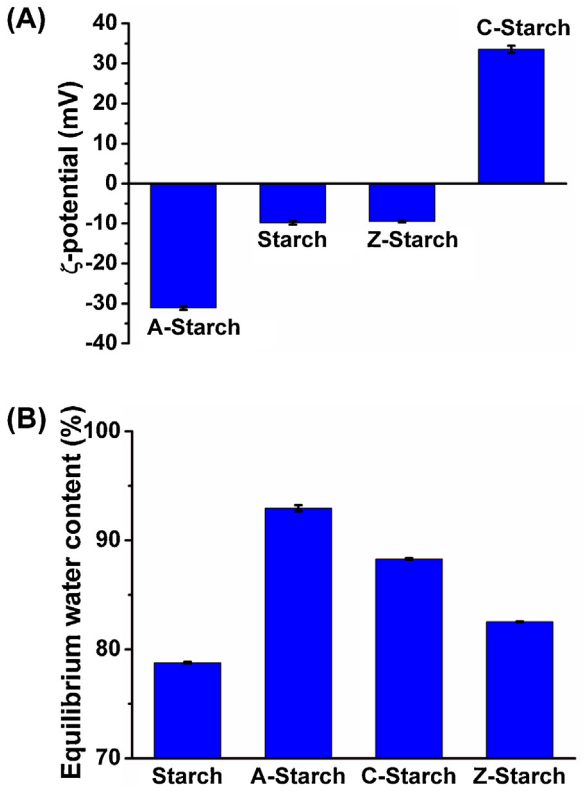

Fig. 2. (A) Zeta potentials of different hydrogels. (B) Equilibrium water contents of different hydrogels.

2.8. Cytotoxicity evaluation

HUVECs were used to investigate the biocompatibility of the different hydrogels. Hydrogel disks (113.1–114.6 mg) with diameter of 10.0 mm and height of 1.5 mm were sterilized with 75% ethanol solution. The sterile hydrogels were then immersed in 2 mL of DMEM culture medium with 10% FBS and 1% penicillin-streptomycin at 37 °C. After 48 h, the hydrogels were gently removed and the extract solutions were retained for the evaluation of cytotoxicity.

Cytotoxicity evaluation was performed using an MTT [3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium] assay. A culture medium (100 μL) containing about 7500 HUVECs was added to 96 wells. After incubation for 24 h, the culture medium was replaced with the hydrogel extract solutions. After incubation for 1, 3 or 5 days, the cell viability was evaluated using the MTT assay. Briefly, 100 μL of MTT solution was added to each well and incubated at 37 °C for 4 h. After removal of the medium, the formazan crystals produced were dissolved by adding 100 μL of DMSO. After shaking for 10 min, the optical density of the formazan solution at 595 nm was measured using a microplate reader. Triplicate samples were analyzed for each experiment.

2.9. Cell adhesion assay

An HUVEC suspension (1 mL) containing 7.5×10^4 cells was seeded in 24-well tissue culture plates containing starch, C-Starch, A-Starch or Z-Starch hydrogel disks. The cells were allowed to grow on the hydrogels at 37 °C in a humidified atmosphere with 5% CO_2. At day 1, 100 μL of fluorescein diacetate (5 μg/mL) was added to each well and allowed to incubate for 5 min under ambient conditions. Then, the samples were rinsed with DMEM to remove the unattached cells. The morphologies of the cells that had attached onto the surface of the hydrogels were observed by a fluorescence microscope (IX81, Olympus). The number of adherent cells was counted and divided by the area of the image in order to calculate the adherent cell density. Three test surfaces of each sample were used.

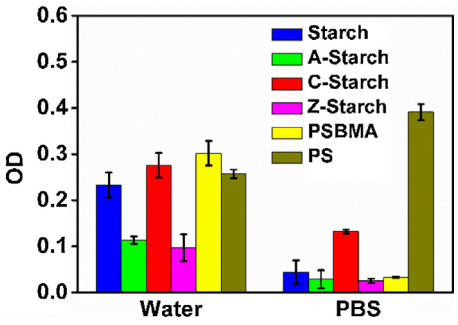

Fig. 3. HRP-IgG adsorption on different samples in the mediums of water and PBS.

3. Results and discussion

3.1. Characterization of the A-, C- and Z-Starches

Three ionic starch-based polymers with different charges were designed and synthesized in order to incorporate hydrogen bonding and ionic solvation hydration moieties into one molecule. Fig. 1 shows the ^{1}H NMR spectra of starch, A-Starch, C-Starch and Z-Starch. Compared to starch, there are three new peaks (a, b and c) in the A-Starch spectrum (Fig. 1(B)), which belong to the anionic side-chain ($-CH_2CH(OH)CH_2SO_3$). Similarly, peaks a, b, c and d in the C-Starch spectrum (Fig. 1(C)) are characteristic of the cationic side-chain ($-CH_2CH(OH)CH_2N(CH_3)_3$) and peaks a, b, c, d, e, f and g in the Z-Starch spectrum (Fig. 1(D)) are due to the zwitterionic side-chain ($-CH_2CH_2CH_2N(CH_3)_2CH_2CH_2CH_2SO_3$). These results indicated that ionic starch-based polymers with different charges were successfully synthesized via the etherification reactions. In Fig. 1(B), the peaks of $-CH_2SO_3$ (ppm 3.0) and C_1-H anhydroglucose (ppm 5.3) were used to determine the DS of the anionic groups in A-Starch; in Fig. 1(C), the peaks of $-N(CH_3)_3$ (ppm 3.0) and C_1-H anhydroglucose (ppm 5.3) were used to determine the DS of the cationic groups in C-Starch; and in Fig. 1(D), the peaks of $-N(CH_3)_2-$ (ppm 3.0) and C_1-H anhydroglucose (ppm 5.3) were used to determine the DS of the zwitterionic groups in Z-Starch. All DS values were around 0.40 (see Table 1).

3.2. Characterization of the A-, C- and Z-Starch hydrogels

The surface charge on a hydrogel surface can impact its protein resistance. The zeta potentials of the hydrogels were measured to determine the relative levels of surface charge on the ionic starch-based hydrogels. As shown in Fig. 2(A), native starch is neutral and its hydrogel surface had a zeta potential of -9.5 ± 0.2 mV. The Z-Starch hydrogel had a zeta potential of -9.6 ± 0.5 mV. This is comparable with that of the neutral native starch hydrogel and suggests that the Z-Starch hydrogel also has a neutral surface.

The positively charged C-Starch hydrogel and the negatively charged A-Starch hydrogel had zeta potentials of $+32.2 \pm 0.9$ and -32.1 ± 0.5 mV, respectively. The signs of the zeta potential values are in agreement with the expected charge properties of the corresponding polymers. The absolute values of the zeta potentials of the C- and A-Starches are approximately equal, indicating that they have similar amounts of charge.

The EWCs of the hydrogels are shown in Fig. 2(B). The starch hydrogel which only binds water molecules via hydrogen bonding had a EWC less than 80%. The A-, C- and Z-Starch hydrogels all

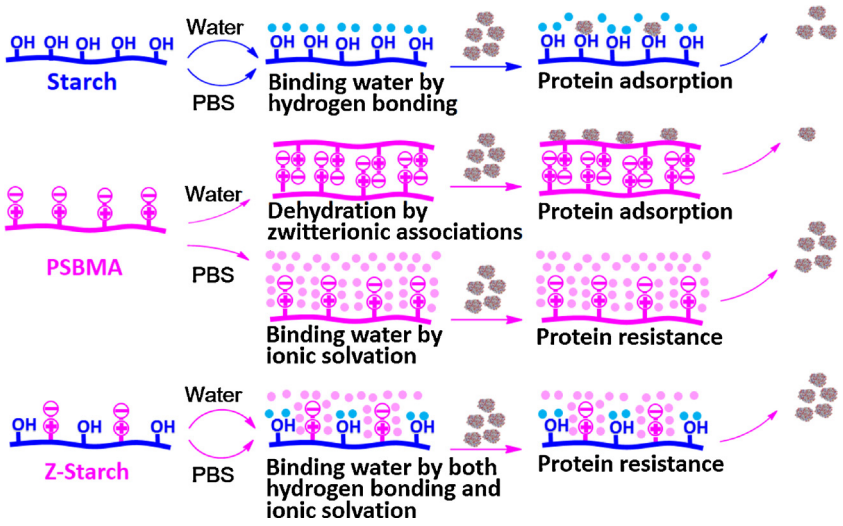

Fig. 4. Schematic diagram for protein adsorption on starch, PSBMA and Z-Starch hydrogels in water and PBS. : Protein; : water bound by hydrogen bonding; : water bound by ionic solvation.

had higher EWCs than the starch hydrogel because they all retain water molecules by both hydrogen bonding and ionic solvation. Since the hydrogels all had the same degree of crosslinking, the higher EWCs mean that the three ionic hydrogels possess better hydration capacities than the starch hydrogel.

3.3. Non-fouling characterization

3.3.1. HRP-IgG adsorption on the A-, C- and Z-Starch hydrogels

The A-, C- and Z-Starch hydrogels were first exposed to the neutral protein HRP-IgG. Starch and PSBMA were used as the hydrogen bonding and ionic solvation based non-fouling controls respectively, and polystyrene (PS) was used as a positive control. As shown in Fig. 3, a great deal of HRP-IgG adsorbed onto the starch, C-Starch and PSBMA hydrogels and PS in deionized water, whereas little HRP-IgG adsorbed on the A- and Z-Starch hydrogels. When the ionic strength was elevated, all of the samples except PS showed improved protein resistance; among all hydrogels, the A-Starch, Z-Starch and PSBMA hydrogels had the fewest protein adsorption; and the protein adsorption on the PSBMA hydrogel showed the most obvious dependence on ionic strength. Fig. 4 shows a schematic of the protein resistance mechanisms (hydrogen bonding or ionic solvation) for PSBMA, Z-Starch and starch. In water, electrostatic attraction-induced zwitterionic associations cause the dehydration of PSBMA which leads to its poor protein resistance. For Z-Starch, the density of the zwitterionic groups is low (ca. 40%), resulting in fewer zwitterionic associations. However, the zwitterionic groups in Z-Starch still can participate in ionic solvation hydration. So Z-Starch possesses both hydrogen bonding and ionic solvation hydrations in both deionized water and PBS. In contrast, PSBMA only has ionic solvation hydration in PBS. The strong hydration layer formed in Z-Starch by both methods (hydrogen bonding and ionic solvation) creates better protein resistance for Z-Starch hydrogel.

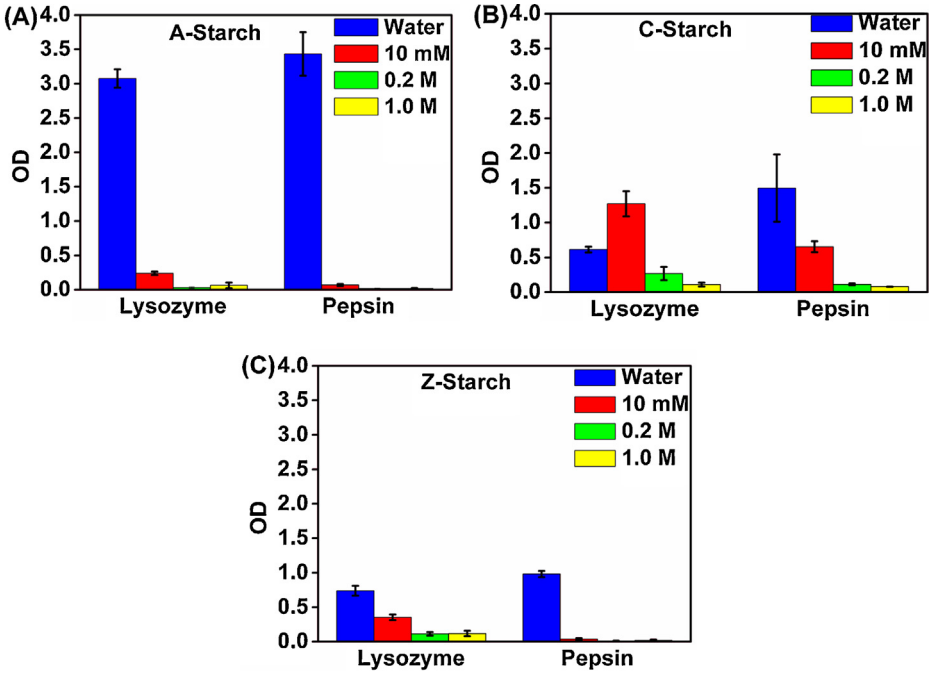

Fig. 5. Pepsin and lysozyme adsorption on different samples in water, 10 mM PBS, 0.2 M NaCl and 1.0 M NaCl. (A) A-Starch, (B) C-Starch, and (C) Z-Starch.

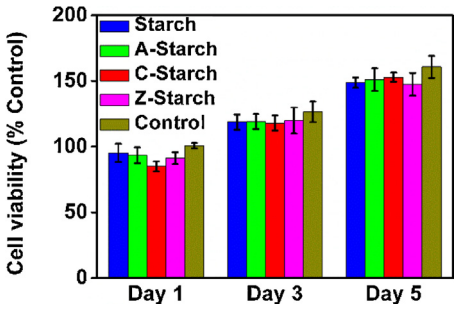

Fig. 6. Viability of HUVECs cultured in the extracts of different hydrogels for 1, 3, and 5 days with DMEM as control.

Even though the A-, C- and Z-Starches all have the same amounts of ionic groups, their protein resistance performances are quite different. Compared with starch, the introduction of zwitterionic or anionic groups can improve the protein resistance, whereas the introduction of cationic groups decreases the protein resistance. This implies that the nature of the ionic hydration has an influence on protein resistance.

3.3.2. Pepsin and lysozyme adsorption on the A-, C- and Z-Starch hydrogels

In addition to HRP-IgG, two other model proteins, lysozyme and pepsin, were used to investigate the protein resistance of the ionic starch-based hydrogels (Fig. 5). These two model proteins are

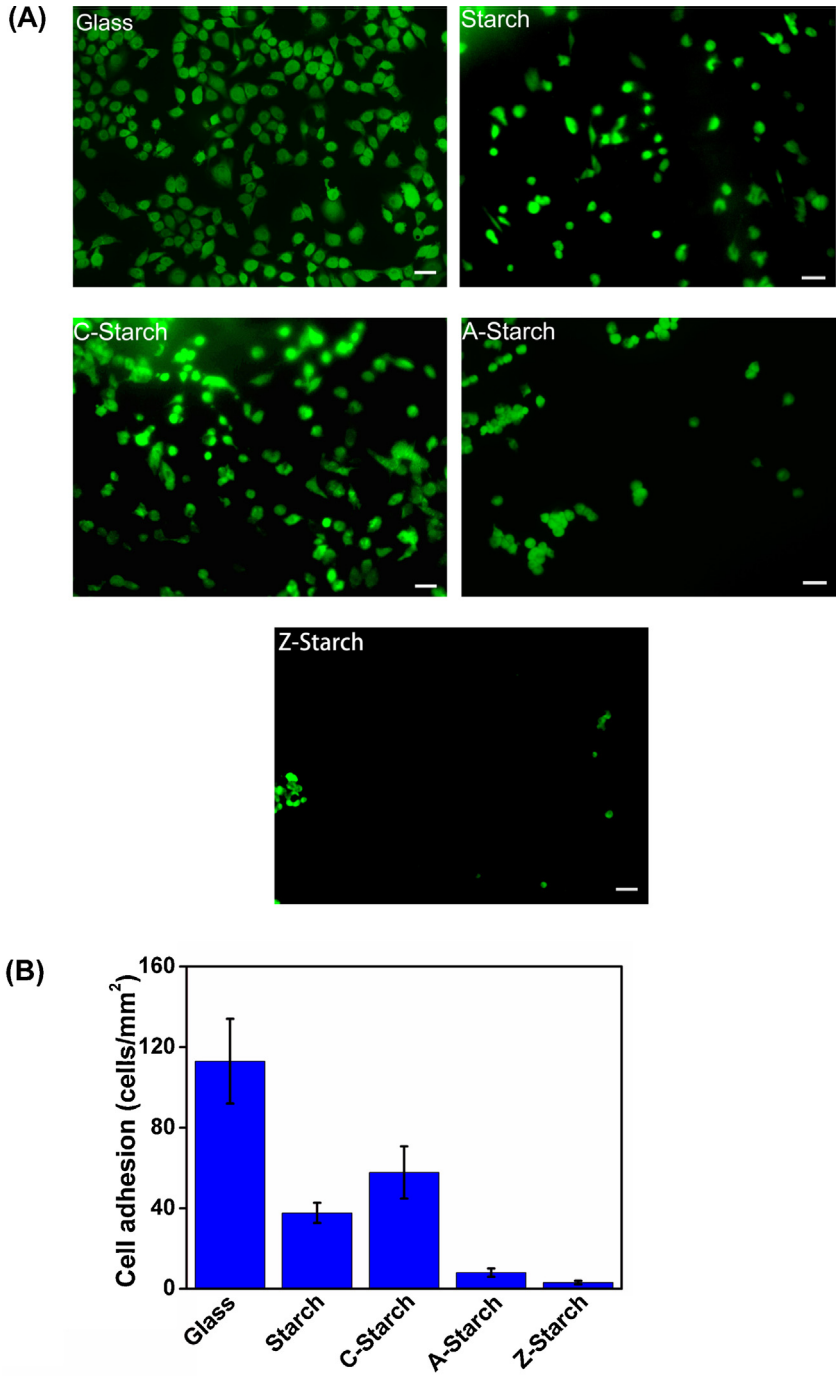

Fig. 7. (A) Microscopic images of HUVECs cultured on the surfaces of hydrogels and glass for one day. Scale bar represents 100 μm. (B) Quantification of HUVECs adhesion to the surfaces of hydrogels and glass.

frequently used in protein resistance assays. Under the experimental conditions, lysozyme has an isoelectric point of 11.0, is positively charged and behaves like a hard particle. The isoelectric point of pepsin is 1.2 and it is negatively charged and regarded as a soft protein.

For all three modified starches, the charged groups were favorable for protein resistance due to their excellent hydration capacities. On the other hand, the charged groups also improved the protein adsorption capacity owing to electrostatic interactions between the hydrogels and the proteins. In deionized water, the sulfonate groups of A-Starch were "bare", so for both positively charged lysozyme and negatively charged pepsin, a great deal of protein was adsorbed through electrostatic interactions (Fig. 5(A)). When the ionic strength was elevated to 10 mM, the Debye length (the average distance over which two charges sense each other), significantly decreased, which caused the electrostatic attractions between the protein and the A-Starch to almost disappear. However, a strong hydration layer still existed in the A-Starch resulting in improved protein resistance. Since most applications use ionic strengths above 10 mM, the A-Starch should be an excellent protein resistant material. Previous studies have also found that other sulfonated polymers (e.g. heparin-like materials) exhibit similar protein resistant behaviors. For example, Kim, Han, Park, and Kim (2003) found sulfonated PEG has a better antithrombogenicity and biostability than PEG in vivo.

For C-Starch, the protein resistance depended much less on ionic strength than for A-Starch. The quaternary ammonium groups of C-Starch were weakly electropositive and the hydration was weak because of hydrophobic and steric effects which originated from the three methyl groups. Thus the electrostatic interactions between proteins and C-Starch were lower than that between proteins and A-Starch. As depicted in Fig. 5(B), in water, there was much less protein adsorbed on C-Starch than on A-Starch. However, C-Starch had the lowest protein resistance in PBS due to weak hydration. This data is consistent with the results reported by Dobbins et al. (2012) which showed that the protein resistance of materials containing positive charges was much poorer than that of materials with negative charges.

The protein resistant properties of the zwitterionic Z-Starch (Fig. 5(C)) are more similar to A-Starch than to C-Starch. This is because the negatively charged sulfonate groups are located in the outer portion of the side chains. In addition, the Z-Starch has uniform charge distribution and charge neutrality since it contains two oppositely charged moieties. Therefore, the Z-Starch hydrogel can maximize surface hydration which reduces electrostatic interactions with protein molecules (Chen, Li, Zhao, & Zheng, 2010; Chen, Zheng, Li, & Jiang, 2005; He et al., 2008). These features give Z-Starch a better protein resistance than A-Starch not only in water but also in PBS. In summary, the protein resistance of the ionic starch-based hydrogels is in the order: Z-Starch $\geq$ A-Starch > C-Starch.

3.4. Cytotoxicity assay

Biocompatibility is a prerequisite for biomedical protein resistant materials. So the cytotoxicities of the ionic starch-based hydrogels were measured by an MTT assay. As shown in Fig. 6, the number of HUVECs cultured in the extracts of the ionic starch-based hydrogels increased with culture time. Moreover, their proliferation behavior was similar to that of the cells cultured in DMEM. These results suggest that the ionic starch-based hydrogels are biocompatible and have low cell cytotoxicities.

3.5. Cell adhesion

It is generally believed that protein adsorption on a surface is the initial stage of cell adhesion. In view of the excellent protein resistance of the ionic starch-based hydrogels, they should also have good resistance to cell adhesion. HUVECs were employed to evaluate the cell adhesion resistance of the ionic starch-based hydrogels. Fig. 7(A) shows the fluorescence images of HUVECs seeded on the different ionic starch-based hydrogel surfaces and on glass. For glass, a large number of HUVECs were uniformly adhered to the surface. The starch and C-Starch hydrogels also had many cells adhered to their surfaces. Fewer cells adhered to the A-Starch hydrogel and Z-Starch hydrogel had the fewest cells. All of cells that adhered on the A- and Z-Starch hydrogel surfaces were round. The results of the quantitative analysis are shown in Fig. 7(B). As anticipated from the images, the quantitative assessment confirms that there were a greater number of cells on the glass, starch and C-Starch samples as compared to the A- or Z-Starch samples. These results indicate that the A- and Z-Starch hydrogels do not support the adhesion of cells and are consistent with the protein adsorption behaviors; namely the A- and Z-Starch hydrogels exhibited good protein resistance, whereas the C-Starch hydrogel adsorbed a great deal of proteins in 10 mM PBS. Therefore, due to their excellent cytocompatibility and protein resistance, the A- and Z-Starches are expected to be promising non-fouling materials for a wide range of applications such as coatings for biomedical implants, drug delivery carriers, and biosensors.

4. Conclusions

Ionic starch-based hydrogels with different charges were synthesized via etherification reactions. Their protein resistance behaviors depended on the ionic strength of the medium. The C-Starch hydrogel had low protein resistance at all ionic strengths; the A-Starch hydrogel resisted protein adsorption at ionic strengths of more than 10 mM; and the Z-Starch hydrogel prevented protein adsorption at all ionic strengths. The protein resistance of the different materials follows the order: Z-Starch $\geq$ A-Starch > C-Starch. Compared to PSBMA, the Z-Starch hydrogel possessed non-fouling properties even in water medium. In addition, the Z- and A-Starch hydrogels both resisted cell adhesion and these ionic starch-based hydrogels showed excellent biocompatibility. This investigation confirms that the ionic starch-based hydrogels with negative or zwitterionic groups are promising materials for non-fouling applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.09.077.

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