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Determination of non-freezing water in different nonfouling materials by differential scanning calorimetry

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ABSTRACT

Nonfouling materials have attracted increasing interest for their excellent biocompatibility and low immunogenicity. Strong hydration is believed to be the key reason for their resisting capability to nonspecific protein adsorption. However, little attention has been paid to quantifying their strong water binding capacity. In this study, we synthesized four zwitterionic polymers, including poly(sulfobetaine methacrylate) (pSBMA), poly(carboxybetaine methacrylate) (pCBMA), poly(carboxybetaine acrylamide) (pCBAA) and poly(2-methacryloyloxyethyl phosphorylcholine) (pMPC), and compared non-freezing water of these zwitterionic polymers with typical antifouling polymer poly(ethylene glycol) (PEG) using differential scanning calorimetry (DSC). And non-freezing water of their monomers was also investigated. The water binding capacity of the polymers (per unit) is pMPC (10.7 ± 1.4) $\approx$ pCBAA (10.8 ± 1.5) > pCBMA (9.0 ± 0.6) > pSBMA (6.6 ± 0.4) > PEG20000 (0.60 ± 0.04). Similar trend is observed for their monomers. For all studied zwitterionic materials, they showed higher binding capacity than PEG. We attribute the stronger hydration of zwitterionic polymers to their strong electrostatic interactions.

1. Introduction

Nonfouling materials, which have strong resistance to non-specific protein adsorption, cell adhesion, and biofilm development, show significant importance in various fields from drug delivery to surface modification [1–5]. Besides hydrophilic materials (i.e. PEG-based materials), zwitterionic materials have attracted much interest for their excellent biocompatibility and low immunogenicity over the last two decades [6–11]. Lin et al. [12] reported that super-hydrophilic β-cyclodextrin-carboxybetaine polymers exhibit ultra-long half-life in blood circulation (>40 h), and no accelerated blood clearance was found after multiple injections. In the following study,

β-cyclodextrin-sulfobetaine polymers showed obvious shorter circulation time than β-cyclodextrin-carboxybetaine polymers [13]. Shen et al. [14] investigated the antibacterial properties of implants functionalized with polyzwitterionic materials (pCBMA and pSBMA, *via* a single-step photo-grafting process). pCBMA coatings cause a significant reduction in both S. aureus and S. epidermidis adhesion compared to pSBMA coating *in vitro*. *In vivo* antimicrobial properties of pCBMA coating persists during 2-week implantation period. All these results indicate that nonfouling materials are playing crucial roles in many biomedical applications.

PEG and zwitterionic materials, as the most commonly studied nonfouling materials, have been compared in previous reports [15]. For example, Robinson et al. immobilized antibody to pSBMA and PEG, and investigated their difference for antigen detection both *in vitro* and *in vivo*. They found that pSBMA-coated devices showed significantly higher capture efficiency compared to PEG-coated ones, and they suggested this may be due to the higher immobilized antibody density of pSBMA than PEG [16]. Wang et al. grafted pCBMA, pMPC, and PEG to branched polyethyleneimine (PEI), and used them to coat bovine serum albumin-poly(N-3-acrylamidophenylboronic acid) (BSA-PAPBA) nanoparticles. The found that pMPC coated nanoparticles showed the longest blood circulation and tumor accumulation. They suggested the differences should correlate with the chain structure of pCBMA, pMPC, and PEG in physiological and pathological environments [17]. Leng et al. used sum frequency generation (SFG) vibrational spectroscopy to investigate the *in situ* and real time surface hydration of pSBMA and poly[oligo(ethylene glycol) methacrylate] (pOEGMA) upon contact with proteins. And the results indicated that the surface hydration of pSBMA remained unaffected, but the water ordering at the pOEGMA surface was disturbed [18].

The key to nonfouling materials is the tightly bound hydration layer (non-freezing water layer) on the polymers, which is responsible for their resistance to protein adsorption, due to unfavorable change in free energy when removing the tightly bound water in the interface [19–23]. Other than the tightly bound water, other surface characteristics, such as water structure, polymer length, and polymer density, also affect the nonfouling properties [20, 24]. The state of water in a polymer can be measured in many ways, such as differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR), fourier transform infrared spectroscopy (FI-IR), attenuated total reflectance infrared spectroscopy (ATR-IR), and X-ray [25,26]. Differential scanning calorimetry (DSC), as a powerful technique in thermal analysis is used to measure enthalpy changes due to changes in the physical and chemical properties of a material as a function of temperature or time [27]. The wide application of DSC includes investigation of glass transition temperature, melting and crystallization behavior [27], and the states of water can be easily analyzed using DSC [28]. Due to these reasons, DSC was chosen for this study. Tanaka et al. [25] have proposed that water in polymers exists in three states, non-freezing water, freezing-bound (or intermediate) water, and free water. The non-freezing water is non-crystallizable even at a temperature $-100\,°C$, while intermediate water crystallizes at the temperature below $0\,°C$ and free water crystallized at $0\,°C$ [25, 29]. On the other hand, Morisaku et al. [23] and Hirata et al. [30] ~~and~~ classified the water into two states: non-freezing and
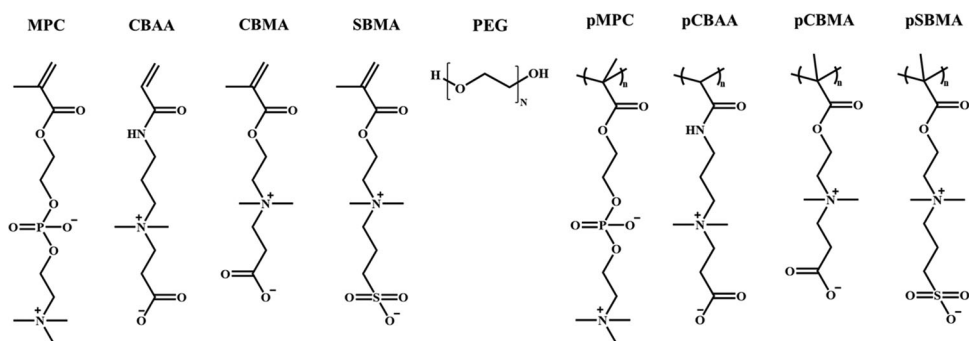
Figure 1. Structure of monomers MPC, CBAA, CBMA, SBMA, their respective polymers, and PEG used for antifouling materials.

freezable water. In this classification, freezable water includes both freezing-bound (or intermediate) water and free water. In this study we are mainly interested in studying the non-freezing water, so we refer the different types of water as non-freezing water and freezable water, otherwise will specify clearly.

Unlike PEG molecules with amphiphilic characteristic that bind water *via* hydrogen bond, zwitterionic polymers, which are superhydrophilic, bind water stronger *via* electrostatic origin [31–33]. Researchers studied the water binding capacity for both PEG and zwitterionic polymers. Wu et al. [31] compared the hydration capacity of pSBMA and PEG using low-field nuclear magnetic resonance and DSC. Zhao et al. [32] studied the hydrogels' properties of four different zwitterionic materials, pCBMA, pMPC, pCBAA, and pSBMA, and DSC was used to study the state of water in hydrogels. Tran et al. [34] studied the relationship between states of water and ion transport properties in hydrated neutral, zwitterionic, cation exchange, and anion exchange polymers. Though researchers have been studying zwitterionic polymers and PEG, and comparing them two, up to date, there is no systemic study comparing their non-freezing water with similar molecular weight and their monomers. In this study, we prepared four most studied zwitterionic polymers, pMPC, pCBAA, pCBMA, and pSBMA (structures of their monomers were shown in Figure 1). The non-freezing water of these zwitterionic polymers and their corresponding were compared using DSC. PEG was also studied as a control.

2. Materials and methods

2.1. Materials

[2-(Methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (SBMA, 97%) was purchased from Sigma-Aldrich (Shanghai, China). 2-(N, N′-dimethylamino)ethyl methacrylate (DMAEMA), 2,2′-azobis-(isobutyronitrile) (AIBN), PEG with an average molecular weight of 550, 5000, 20000 were purchased from Aladdin Reagent (Shanghai, China). Methanol was purchased from Sinopharm Chemical Reagent (Shanghai, China). β-Propiolactone was purchased from J&K (Beijing, China). CBMA was synthesized as previously reported [35,36]. Briefly, β-propiolactone (3.53 mL, 55 mmol) was diluted with 5 mL anhydrous acetone, and the mixture was added dropwise to a solution of DMAEM (8.43 mL, 50 mmol) in 45 mL

anhydrous acetone. The reaction was kept under nitrogen protection with constant stirring at 15 °C for 6 h. Then the reaction mixture was filtrated, and a white solid was collected. The compound was washed with 50 mL anhydrous acetone, and stored at −20 °C before use. MPC was a kind gift from Prof. Jian Ji's group (Department of Polymer Science and Engineering, Zhejiang University).

2.2. Synthesis of zwitterionic polymers

Various polymerization techniques have been used for the preparation of zwitterionic polymers [37–40]. In this study, all polymers are synthesized by conventional free-radical polymerization with AIBN as an initiator. A typical polymerization of pCBMA was carried out as follows. CBMA (0.311 g, 1.35 mmol) and AIBN (0.018 g, 0.11 mmol) were added to a Schlenk tube, dissolved in a mixture of MeOH/H_2O at a volume ratio of 4/1. Then purged with nitrogen for 30 min to remove the oxygen. The monomer concentration was fixed at 5% (w/v), and the polymerisation was carried out at 65 °C for 24 h, then terminated by exposing to air. The polymer was purified by dialyzing using a 3500 MW cutoff membrane against water (purified by a Millipore purification system with a resistivity ≥18.0 MΩ·cm) for 24 h, which was changed every 8 h. The polymer was collected by freeze-drying. The molecular weight was determined by gel permeation chromatography (GPC). pMPC, pCBAA, and pSBMA were prepared in the same method with fixed monomer/initiator ratio and monomer concentration by only changing the monomers.

2.3. GPC measurements

Shimadzu GPC system (Shimadzu Corporation, Japan) equipped with a Waters Ultra-hydrogel column and a Shimadzu refractive index detector was used for all the GPC measurements in this study. The measurements were performed with 0.2 M $NaNO_3$ (in order to suppress the strong interaction between zwitterionic polymer with the column[41,42]) as the eluent at a flow rate of 0.5 mL/min. The column temperature was kept at 40 °C. The molecular weight and polydispersity index (PDI) were calibrated using polyethylene glycol standards.

2.4. DSC measurement

Careful heat calibration of DSC was made by measuring the heats of reference process using aluminum using according to ICTAC Kinetics Committee recommendations [43]. For DSC measurement, an aluminum pan with 6–7 mg polymer mixed with water at different ratio was hermetically sealed. An empty aluminum pan was used as a control. During the cooling and heating experiments, the sample cell was purged with nitrogen gas at a flow rate of 50 mL/min. The samples were initially cooled from room temperature to −60 °C at a rate of 5 °C/min and then heated to 40 °C at the same rate. When all samples were first cooled from room temperature to −60 °C, water mixed would be frozen except for non-freezing water. Then samples were heated to 40 °C, the frozen water (both free and intermediate water) would

undergo an ice-to-water transition, and this was reflected by the endothermic peak. This would allow us to calculate the number of non-freezing water.

3. Results and discussion

3.1. Calculation of non-freezing water molecules bind to each polymer unit

DSC has been used as a well-established way to calculate the freezable and non-freezing water. In this study, we mixed polymers or their monomers with water at different molar ratios (water to polymer unit or monomer), then samples' thermal transition behaviour was monitored by DSC. If water molecules were tightly bound to polymers, no thermal transition would be expected to be observed, and these water molecules are called non-freezing water. Only water molecules that can be frozen would contribute to the thermal transition over the temperature range similar to the ice-to-water transition for bulk water [31]. And we can calculate the enthalpy change of freezable water of the mixture of different polymers with water (ΔH_f) by using TA Instruments Universal Analysis 2000. Because the endothermic peak of intermediate and free water overlap, the ΔHf was the combination of the two peaks [44]. ΔH_f of different polymers or monomers are summarized in Table 1. For example, to calculate the number of non-freezing water molecules (N_{nf}) bind to per polymer unit, we can use equations below,

$$W_c = \frac{w_w - w_d}{w_d} \tag{1;}$$

where W_c is the weight of water relative to the weight of the polymer in a polymer solution. While w_w and w_d are the weight of the polymer solution and the weight of the polymer (freeze-dried).

$$W_f = \frac{\Delta H_f}{\Delta H_{water}} * (1 + W_c) \tag{2;}$$

Table 1. Enthalpy changes of water and number of non-freezing water measured by DSC at different water/polymer unit ratio.

Content	Water/Unit ratio	ΔHf (J/g)	Nnf
pMPCa	15	46.1 ± 14.6	10.7 ± 1.4
pCBAAb	15	51.2 ± 17.6	10.8 ± 1.5
pCBMAc	15	72.8 ± 6.7	9.0 ± 0.6
pSBMAd	15	92.3 ± 4.4	6.6 ± 0.4
MPC	15	40.7 ± 4.6	11.2 ± 0.4
CBAA	15	47.0 ± 2.5	11.1 ± 0.2
CBMA	15	57.5 ± 6.4	10.2 ± 0.5
SBMA	15	62.5 ± 9.3	9.3 ± 0.8
PEG 20000	1.5	76.2 ± 3.5	0.60 ± 0.04
PEG 5000	1.5	81.7 ± 1.3	0.53 ± 0.02
PEG 550	1.5	–	–
PEG 550	5	142.8 ± 3.6	1.8 ± 0.1

[a]: pMPC, M_n=22 kDa, M_w=32 kDa, PDI= 1.4.
[b]: pCBAA, M_n=16 kDa, M_w=29 kDa, PDI = 1.8.
[c]: pCBMA, M_n=33 kDa, M_w=49 kDa, PDI = 1.5.
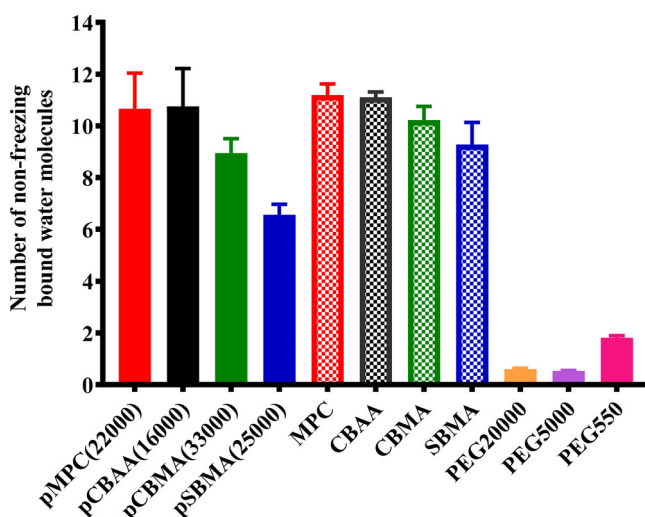: pSBMA, M_n=25 kDa, M_w=40 kDa, PDI = 1.6.

Figure 2. Number of non-freezing water of different nonfouling materials.

where W_f is the weight of freezable water relative to the weight of the polymer in a polymer solution. ΔH_f is the enthalpy change associated with the melting of freezable water and ΔH_{water} is the enthalpy change for the melting of bulk water (333.5 J/g).

$$N_f = W_f * \frac{M_p}{M_{water}} \qquad (3;$$

where N_f is the number of freezable water molecules per polymer repeating unit, M_p and M_{water} are the molecular weight per polymer repeating unit and the molecular weight of water.

$$N_{nf} = N_{water} - N_f \qquad (4;$$

where N_{water} is the total number of water molecules added to per polymer repeating unit, and N_{nf} is the number of non-freezing water molecules per polymer unit. The N_{nf} of each polymer or monomer were summarized in Table 1 and Figure 2 (these da-ta would be discussed in later sections). There was no enthalpy change in the temperature range of the DSC measurements, which means the subtle conformation change of polymers doesn't contribute to the total enthalpy change. Indeed, the glass transition temperatures of these zwitterionic polymers have been measured above 100 °C [45].

3.2. Hydration capacity of different zwitterionic polymers

The zwitterionic materials could bind water molecules tightly through electrostatic attraction, however, different charged groups would grant the materials different properties. We investigated four most studied zwitterionic polymers, namely pMPC, pCBAA, pCBMA, and pSBMA. To investigate the non-freezing water of different zwitterionic polymers, we mixed the four types of zwitterionic polymers with water at
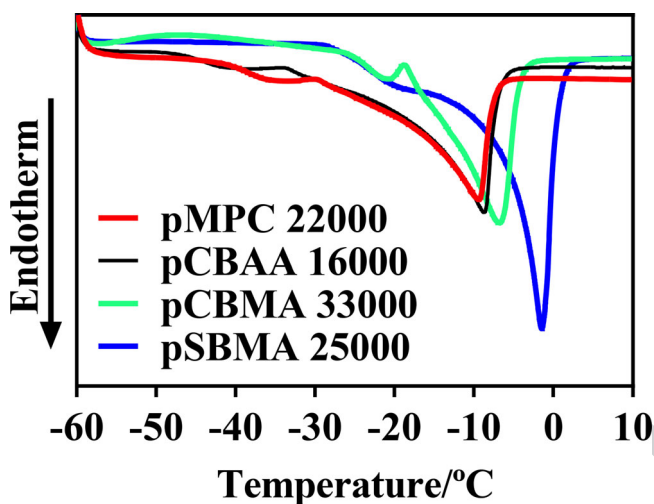
Figure 3. DSC thermograms of water mixed with pCBAA, pCBMA, pMPC, pSBMA separately at a ratio of H2O/repeating unit = 15.

a molar ratio of 1/15 (polymer repeating unit/water molecule). To decide the polymer repeating unit/water molecule ratio, we also tested 1/10, but under this condition, not all the polymers investigated showed endothermic peak (data not shown), then the ratio was increased to 1/15. Since the N_{nf} of zwitterionic materials were almost constant with regard to the water content [23], the N_{nf} of zwitterionic polymers was compared at a single point of added water content in this study. Then using DSC method to calculate the non-freezing water. The non-freezing water numbers and DSC thermograms are shown in Figures 2 and 3. As we can see, pMPC and pCBAA have similar non-freezing water, around 10.7–10.8 water molecules per repeating unit. The number for pMPC is less than what Toshinori et al. [23] reported 23–24 water molecules per repeating unit, which maybe because they were investigating in hydrogel form. The high binding capacity of pCBAA could be attributed to the amide group comparing to the ester group in pCBMA and pSBMA [32]. pMPC is more hydrated than pCBMA and pSBMA, which is consistence with literatures[45–47] and might be due to its unique hydration state [8]. And for pCBMA and pSBMA, they can bind 9.0 and 6.6 non-freezing water molecules respectively, which are similar to what we reported before, 9.3 and 6.0, respectively [13]. Shao et al. discussed the difference in hydration between carboxybetaine and sulfobetaine using molecule simulations [48]. They found out that the positively charged groups in both betaine molecules are similar, and the negatively charged groups in carboxybetaine can bind less water molecules while interact stronger than the negatively charged groups in sulfobetaine. Though the simulation is not for the non-freezing water research purpose, we can still conclude that different charged groups would affect many parameters, for example, dipole orientation distribution and then eventually affect the non-freezing water binding capacity. This also applies to pMPC and pCBAA. Moreover, we need to point out, that though we used polymers with similar molecular weight, but they varied a bit from each other. As the molecular weight also affect the polymers'
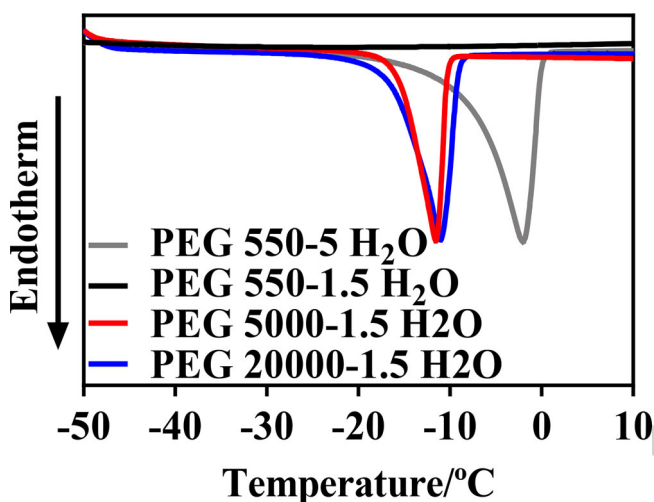
Figure 4. DSC thermograms of water mixed with PEG at a ratio of H2O/EG unit = 1.5 for PEG 20000, PEG 5000, PEG 500, and H2O/EG unit = 5 for PEG 500.

nonfouling properties [49], the relationship between molecular weight and non-freezing water worth further investigating.

Interestingly, we can also see, in Figure 3, the melting peak of the freezable water added to these four polymers, showed a trend that pMPC ≈ pCBAA < pCBMA < pSBMA. This trend is consistent with different polymers' non-freezing water binding capacity. With higher binding capacity, the melting peak of freezable water is lower. This indicates that the freezable water in the pSBMA solution behave more like free water than in the pMPC, pCBAA, and pCBMA ones, which may be attributed to lower water binding capacity of pSBMA. When the water binding capacity of one polymer is weaker, it has a weaker influence on the intermediate water, which will shift to a higher melting peak.

PEG is a well-recognized nonfouling material, so we also include PEG as a comparison. We selected PEG with three different molecular weight, 550, 5000, and 20000. The PEGs were first mixed with water at a molar ratio of 1/1.5 (EG unit/water molecule). The results were summarized in Figure 4. We can see that for PEG550, there was no thermal transition, which means all 1.5 water molecules were bound to PEG 550 as non-freezing water. Therefore, we increased the ratio to 1/5 (EG unit/water molecule). Then we calculated the non-freezing water for all these PEGs. It showed that non-freezing water molecules bind to per EG unit is 0.60 for PEG 20000, 0.53 for PEG 5000, and 1.8 for PEG 550. This result is similar to what has been reported by Wu et al. that PEG 20000 could bind 0.5–1 water molecule per one EG-unit when testing with DSC [31, 50]. And we could also see PEG with a lower molecular weight (PEG 550) could bind more non-freezing water comparing to PEG with higher molecular weights (PEG 20000 and PEG 5000). It is believed that the higher non-freezing water molecules mainly caused by the higher percentage of hydroxyl groups in lower molecular weight PEG. Nonetheless, all the zwitterionic polymers studied showed higher non-freezing water binding capacity than the PEGs in this study. Furthermore, water structure effects on the nonfouling properties of

zwitterionic materials are due to electrostatic effects. It is worth to investigate the salt effect on the N_{nf} of zwitterionic polymers in the future study. Such result may enable us to understand the phenomena found in a recent study more clearly [51], where super-hydrophilic pMPC-stabilized liposomes demonstrated at least one order magnitude low friction than weak hydrated peg-stabilized liposomes in 0.15 M salt solution.

3.3. Hydration capacity of different nonfouling monomers

To further study the hydration difference between different polymers, we investigated the water binding capacity of their monomers. The non-freezing water number and DSC thermograms are shown in Figures 2 and 5. The monomers showed the similar non-freezing water binding trend as their polymers, each MPC molecule can bind 11.2 water molecules, 11.1 for CBAA, 10.2 for CBMA, and 9.3 for SBMA. MPC still has the highest non-freezing water binding capacity. And interestingly, all the zwitterionic monomers showed higher non-freezing water numbers compare to their corresponding polymers, which is attributed to stronger dipole-dipole interaction (mostly) [52,53]. Among these polymer and corresponding monomer pairs, we can find that the largest difference in hydration capacity between polymer and monomer is SBMA/pSBMA (6.6/9.3), which is largely attributed to the large dipole-dipole interactions between sulfobetaine zwitterion groups [22, 54,55].

As expected, the melting peak of the freezable water added to these monomers also showed a consistent trend with their non-freezing water binding capacity. The higher the binding capacity, the lower melting peak of freezable water they have. And also, when we compare the monomer with its polymer (Figure 6), we can see the monomers always have lower melting peak. This is consistent with the conclusion, that higher non-freezing water binding capacity can also increase its influence on its intermediate water.
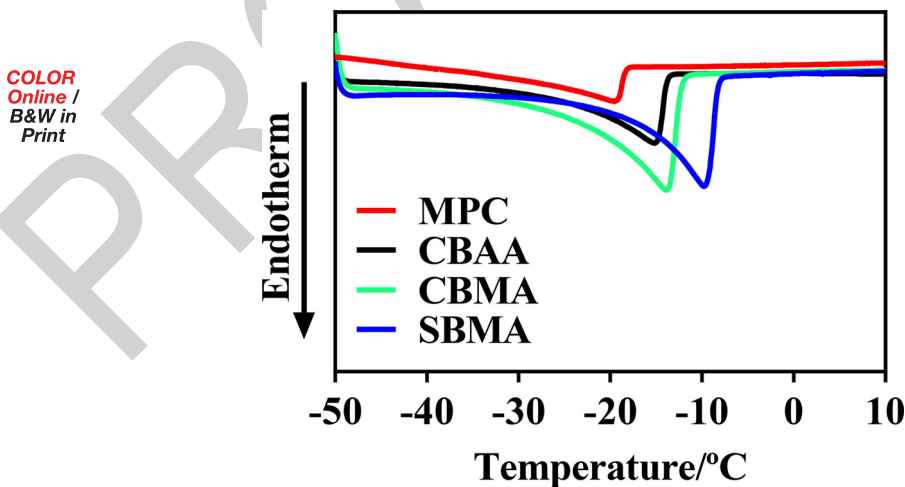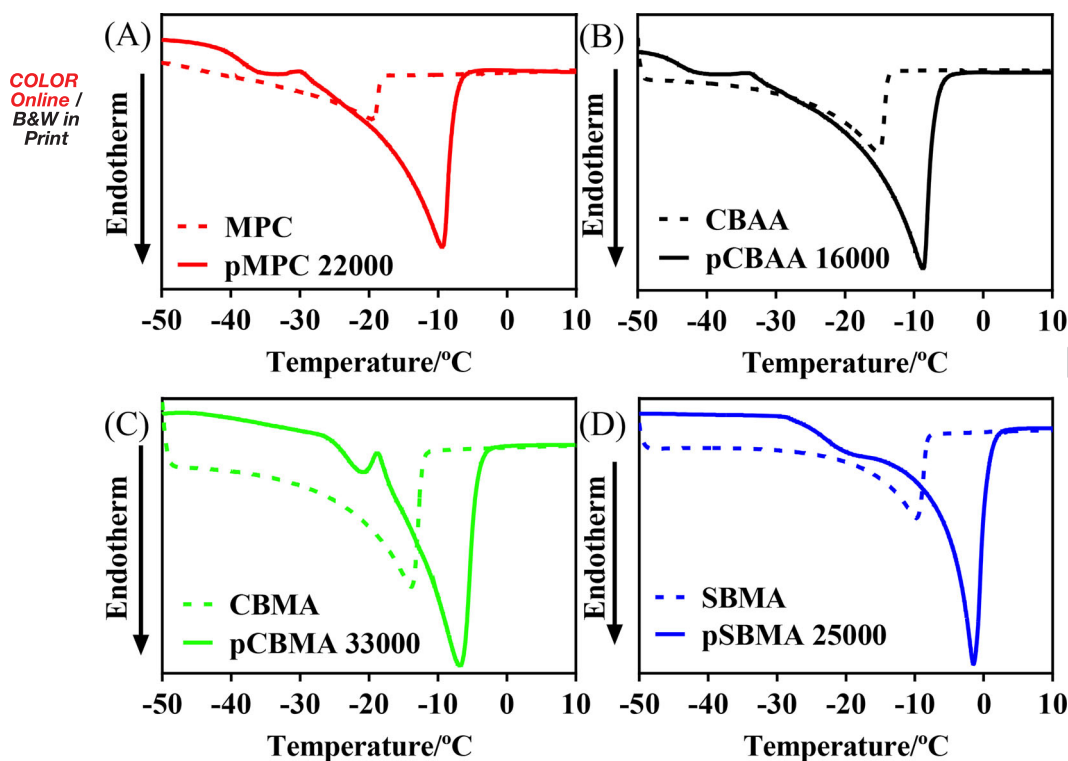
Figure 5. DSC thermograms of water mixed with CBAA, CBMA, MPC, SBMA monomer separately at a ratio of H2O/monomer = 15.

Figure 6. DSC thermograms of water mixed with MPC (A), CBAA (B), CBMA (C), and SBMA (D) polymer (solid line) or monomer (dash line) separately at a ratio of H2O/repeating unit (or monomer) = 15.

4. Conclusions

The non-freezing water is believed to be the key reason for nonfouling materials to resist nonspecific protein adsorption. In this study, we compared four different zwitterionic materials' non-freezing water, and showed the monomers have a non-freezing water binding capacity trend of MPC > CBAA > CBMA > SBMA. Their polymers share the same, but have less non-freezing water comparing to their monomers. And we also confirmed that zwitterionic polymers have higher non-freezing water binding capacity compared to PEG due to their strong electrostatic interactions. This work quantifies non-freezing water of four zwitterionic materials, which may give a valuable evaluation for their nonfouling performance with different molecular groups.

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Disclosure statement

The authors declare no conflict of interest in this research.

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