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Understanding the effect of hydration on the bio-inert properties of HEMA copolymers with small amounts of amino- or/and fluorine-containing monomers

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Dedicated to the 100 years' birthday (01.01.2020) **of prof. Teiji Tsuruta**

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Abstract: Materials exhibiting "bio-inert properties" are essential for developing medical devices, because they are less recognized as foreign substances by proteins and cells in the living body. We have reported that the presence of intermediate water (*IW*), the water molecules loosely bound to a polymer, is a useful index of the bio-inertness of materials. Here, we analyzed the hydration state and the responses to biomolecules of poly(2-hydroxyethyl methacrylate) (PHEMA) copolymers including small amounts of 2-(dimethylamino)ethyl methacrylate (DMAEMA) (N-series) or/and 2,2,2-trifluoroethyl methacrylate (TFEMA) (F-series). The hydration structure was analyzed by differential scanning calorimetry (DSC), the molecular mobility of the produced copolymers by temperature derivative of DSC (DDSC), and the water mobility by solid ^{1}H pulse nuclear magnetic resonance (NMR). Although the homopolymers did not show bio-inert properties, the binary and ternary PHEMA copolymers with low comonomer contents showed higher bio-inert properties than PHEMA homopolymer. The hydration state of PHEMA was changed by introducing small amount of comonomers. The mobility of both, water molecules and hydrated polymers was changed in the N-series, nonfreezing water (*NFW*), the water molecules tightly bound to a polymer, was shifted to high mobility *IW* and free water (*FW*), the water molecules scarcely bound to a polymer. On the other hand, in the F-series, *FW* turned to *IW* and *NFW*. Additionally, a synergetic effect was postulated when both comonomers coexist in the copolymers of HEMA, which was expressed by widening the temperature range of cold crystallization, contributing to further improvement of the bio-inert properties.

KEYWORDS Intermediate water, bio-inert, poly(2-hydroxyethyl methacrylate), hydration state, fluorinated polymers, amino and fluorine.

Introduction

When any materials are implanted in the body, nonspecific protein adsorption may occur as a reaction to the foreign body. A number of different cells, such as platelets, adhere to these material surfaces and as a result this may lead to the upregulation of cytokines and subsequent proinflammatory processes.[1] To develop the implantable medical devices (e.g., stents, artificial blood vessel, artificial lung oxygenator, etc.), many efforts have been made to elucidate the expression mechanisms of bio-inert properties of materials – namely the mechanisms of low protein adsorption and cell adhesion inhibition.

Poly(ethylene glycol) (PEG)[2] and zwitterionic polymers[3] are the typical bio-inert polymers with the above mentioned properties. There are so many proposed mechanisms describing the properties originate from; it deems that the materials with high hydrophilicity often exhibit the properties. However, these homopolymers are limited in application because of their poor water resistance. Therefore, the polymers are generally used by improving the water resistance by copolymerizing with hydrophobic monomers or covalently grafting to a substrate, while these methods sacrifice the properties to some extent.

Han et al.[4] reported that poly(ethylene oxide)-grafted polyurethane surface showed lower fibrinogen and albumin adsorption than that of conventional polyurethane. The protein adsorption resistance property of the PEG-coated surface increases with increasing the density and length of the chains on the surface.[5,6] Nagasaki et al.[7] reported that the PEG graft density was enlarged by adding a filler layer made of low molecular weight PEG (2kDa) in the preconstructed high molecular weight PEG (5kDa) layer, which contributed to low protein adsorption. Similarly, Ishihara et al. reported that 2-methacryloyloxyethyl phosphorylcholine (MPC) can be used as a coating material by insolubilization either copolymerizing with hydrophobic butyl methacrylate[8]

or covalently bonding the MPC polymer to the surface.[9] PMPC-coated surfaces exhibit excellent nonthrombogenicity to human whole blood.[10]

On the other hand, numerous investigations have been made to deepen the discussion not only on the chemical structure of polymers but also on the polymer behavior at the water/polymer interface including the interaction of the polymer with water. Poly(2-methoxyethyl acrylate) (PMEA) is a water insoluble homopolymer with excellent blood compatibility, presumably due to the presence of intermediate water (*IW*) – the water molecules that loosely bound to the polymer chain in the hydrated state.[11,12] The blood compatibility of PMEA coated artificial lung (oxygenator) was demonstrated in *in vitro* test and the compatibility was equivalent to that of heparin-immobilized oxygenator because of its low plasma protein adsorption and denaturation.[13] PMEA coated medical devices have thus been approved by the Food and Drug Administration (FDA) have achieved clinical applications such as artificial lungs and catheters. In addition, systematic studies were conducted with a variety of PMEA derivatives on clarifying the relationship between the polymer structure, the *IW* contents (IWC), and the interactions with biomolecules.[14,15,16] Consequently, the amount of *IW* in a hydrated polymer is gaining acceptance as a useful index of the bio-inertness of the materials[17] rather than non-freezing water and free water.

Poly(2-hydroxyethyl methacrylate) (PHEMA) is one of the typical bio-inert materials, and many researches have been conducted to study its properties as biomaterial. Since PHEMA has a hydroxyl group in each monomer unit, it exhibits high hydrophilicity thanks to a high equilibrium water content (EWC) of about 40 wt%. It has been utilized as a grafting material to the surfaces or a hydrophilic unit in copolymers to give high hydrophilicity to materials. As examples of the surface grafting with HEMA, silastic surfaces by Ratner et al.[18] and polyurethane film by Martins et al.[19] are given. For random copolymers, ethyl methacrylate copolymer by Ratner and Hoffman

et al.[20], and block copolymer with styrene by Okano et al.[21] and Senshu et al.[22,23] are given as examples.

Kikuchi et al.[24] investigated the retention behavior of blood cells (erythrocytes, lymphocytes, and platelets) using the surface of poly[*N*-methyl-*N*-(4-vinylphenethyl)ethylenediamine-*co*-2-hydroxyethyl methacrylate] (HAV copolymer). The authors showed that the adhesion of lymphocytes was suppressed by incorporating a few mol% of *N*-methyl-*N*-(4-vinylphenethyl)ethylenediamine (MVEDA) into HEMA. In one of his last publications, Professor Tsuruta[25] postulated a mechanism on the suppression. That is, the introduction of extraneous comonomers would loosen the intermolecular hydrogen-bonding network of PHEMA and allow water molecules to readily infiltrate into the polymer matrix; so that makes the PHEMA chains as well as water molecules more mobile to form the soft biological surface, where *IW* predominates and blood compatibility increases. This suggestion encouraged Tanaka et al.[26] to push their research employing small amounts of comonomers, not only in HEMA[27 28 29] but also in MEA polymers,[30] in order to understand the contribution of such monomers to increasing the bio-inert properties of the copolymers. In addition to introducing nitrogen atoms (MVEDA contains amino groups), a variety of elements have been incorporated into HEMA copolymers and their contribution to bio-inert properties was investigated. Zhao et al.[31] investigated the adsorption behavior of bovine serum albumin (BSA) and human plasma fibrinogen (HFg) using the surface coated by poly(2-perfluorooctylethyl methacrylate (FMA)-*co*-HEMA). The adsorption behavior on FMA-*co*-HEMA copolymer depends on the type of protein, the lowest adsorption amount of BSA and HFg were observed with 7.56 and 2.45 mol% of FMA loading, respectively.

Furthermore, there are reports on random[27], block[28] and graft[29] copolymers of HEMA with MEA; the protein-fouling resistance enhancement was also detected by incorporating a small amount of

MEA into HEMA. Although there was no significant difference in the number of adhered platelets, the largest amount of *IW* was expressed when HEMA content was 20 mol% for the random, 9.6 mol % for the block, and 40 mol% for the graft copolymers of HEMA and MEA. Recently, we reported MEA copolymers containing small amount of 2,2,2-trifluoroethyl methacrylate (TFEMA), in which the IWC was increased and the copolymers showed improved bio-inert properties.[30] These results suggest that controlling the chemical structure of polymer leads to control of the hydration structure. Therefore, in this study, we dared to select non-bio-inert comonomers for the syntheses of HEMA copolymers and evaluated the bio-inertness to examine the above hypothesis. Whitesides et al.[32 33] reported that surfaces which resist protein adsorption have the following characteristics: 1) they are hydrophilic; 2) they do not contain hydrogen-bond donors but hydrogen-bond acceptors; 3) they are overall electrically neutral.

One of the monomers we chose is an amino-functionalized monomer, 2-(dimethylamino)ethyl methacrylate (DMAEMA) which has a -NCH_3 group. The pKa of its polymer was reported to be 8.4 and more than 90% becomes cationic under the physiological environment.[34] In addition, polyDMAEMA is one of the well-known biomaterials used as non-viral gene delivery vectors.[35,36]

Another one is fluorine-containing monomer, TFEMA. We have already reported the bio-incompatibility of the obtained polymer, PTFEMA-coated substrate exhibited the water in air contact angle of 96.9 degrees, and it displayed a very high degree of fibrinogen adsorption and denaturation as well as a large number of adhered platelets.[30] This monomer is also classified as a non-bioinert monomer that deviates from the above-mentioned property of hydrophilic. Notably, the homopolymer PTFEMA is soluble in non-fluorine solvent such as DMF and the hydrophilic PHEMA is also soluble in DMF; the characteristic solubility of PTFEMA leads us to obtain the

amphiphilic copolymers. It is therefore expected that TFEMA can be copolymerized with hydrophilic HEMA to give a copolymer with a composition that reflects the feed monomer ratio.

In this work, we synthesized HEMA binary and ternary copolymers containing small amounts of DMAEMA, TFEMA, and a combination of both monomers to evaluate their responses to proteins and platelets. We discuss the expression mechanisms of bioinert properties on the copolymers in terms of their hydration states.

Experimental section

Experimental Part

1. Materials

HEMA, DMAEMA, and TFEMA (all from TCI chemicals) were used as received without further purification. All other solvents and chemicals were used as received unless otherwise stated.

2. Synthesis of the HEMA copolymers

The HEMA copolymers were synthesized by performing free radical homo- and copolymerizations of HEMA with DMAEMA and TFEMA. The molar ratios of HEMA to DMAEMA and TFEMA are shown in Table 1. The following describes the reaction conditions with a ratio of 80:10:10 as a representative example. To a pressure glass vessel, 1.70 mL of HEMA (80 mmol), 0.95 mL of DMAEMA (10 mmol), 0.249 mL of TFEMA (10 mmol), 11.88 mL of DMF, and 24.0 mg of 2,2'-azobis(isobutyronitrile) (AIBN, 0.15 mmol) as an initiator were added, nitrogen was purged to eliminate the air, and the reaction was conducted at 80 °C for 15 hours. The copolymer was obtained as a white solid precipitate, by pouring the DMF solution to diethyl ether. After decantation, the precipitate was dissolved in ethanol, precipitated in hexane for purification, and dried at 40 °C under reduced pressure.

3. Analyses.

3-1) Characterization of the chemical structure of obtained polymers

The HEMA copolymers were characterized by ^{1}H NMR, using a JEOL 500 MHz JNM-ECX spectrometer at room temperature in DMSO-d_6 as the solvent. The chemical shifts are reported in δ ppm downfield from TMS.

Molecular weights were determined by gel permeation chromatography (GPC) employing an integrated SEC unit of a Tosoh HLC-8320 chromatograph equipped with three Styragel HR columns connected in series (WAT044229, WAT044241, and WAT044235) and a refractive index (RI) detector. Measurements were performed with DMF as an eluent, containing 10 mM lithium bromide at 40 ^{o}C with a 1.0 mL/min flow. Molecular weights were calculated using PEG standards with narrow molecular weight distribution in the range of 1.96 x 10^3 – 1.02 x 10^6 g/mol.

3-2) Thermal property evaluation and quantification of the interactions between water and (co)polymers.

Differential Scanning Calorimetry (DSC) measurements were executed using Q1000 from TA Instruments in a temperature range of –80 to 100 ^{o}C at a heating rate of 5 ^{o}C min^{-1} under nitrogen. Dry T_g was determined automatically by the instrument from the second heating trace and is reported as the midpoint of the thermal transition. The hydrated T_g was defined by the peak position of DDSC that is temperature derivative of DSC during the heating process. The preparation of hydrated sample and the analysis of the amount of hydration water in the hydrated polymers were performed with a previously

reported method.[14] The hydrated samples were prepared by immersing in ultrapure water for more than 3 days before performing the measurements.

Based on the interactions of water molecules with the polymer chains, we classified three types of water in hydrated polymers; non-freezing water (*NFW*), intermediate water (*IW*), and free water (*FW*) those are tightly, loosely, and scarcely bound to the polymer, respectively. Their content is given as NFWC, IWC, and FWC, respectively. The DSC measurements ~~was~~ were performed with 3 to 7 mg weight of the samples. The sample weight was checked before and after the DSC measurement to ensure the proper seal of the sample pan. The data from samples with weight change were omitted.

The water content of the polymer was adjusted by changing the drying time and temperature before sealing. Under the applied measurement's conditions, pure water shows a sharp exothermic peak at about -18 °C in the cooling process on the DSC thermogram and an endothermic peak at about 1 °C in the heating process. The maximum water content at which these peaks do not appear was defined as equilibrium water content (EWC), and is given by the following Equation (1):

$$\text{EWC (wt\%)} = ((W_1 - W_0) / W_1) \times 100 \quad (1)$$

where W_0 and W_1 are the weights of the dry and hydrated samples, respectively.

The amounts of the different types of water in the hydrated polymers are given by the following Equations:

$$\text{EWC (wt\%)} = NFW\ \text{(wt\%)} + IW\ \text{(wt\%)} + FW\ \text{(wt\%)} \quad (2)$$

$$IW\ \text{(wt\%)} = \Delta H_{cc} / 334(\text{Jg-}^1) \quad (3)$$

$$FW\ \text{(wt\%)} = (\Delta H_m / 334(\text{Jg-}^1)) - IW \quad (4)$$

ΔH_{cc} and ΔH_m are enthalpy changes during cold crystallization and the melting of ice, respectively.

3-3) Quantification of the mobility of the polymers in the hydrated state

Water mobility at the hydrated state of the polymers was quantified by a low-field NMR spectrometer (Bruker Minispec mq20) at 20 MHz using ^{1}H NMR. Spin-spin relaxation time (T_2) was measured using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence at 40 °C. Approximately 400 mg of the samples were used. The hydrated samples were prepared by immersing in ultrapure water for more than 3 days before measurements. The presence of the two types of water molecules, interacting or non-interacting water molecules, can be reproduced using a linear combination of a following exponential Equation (5): [37]

$$\exp(-t / T_2) = a \exp(-t / T_{2\ \text{interacting water}}) + b \exp(-t / T_{2\ \text{non-interacting water}}) \quad (5),$$

where the corresponding weight ratio, a and b are normalized as $a + b = 1$. In this case, $_{\text{interacting}}$water consists of *NFW*, *IW*, and *FW*. To evaluate $T_{2\ \text{interacting water}}$, a (that is the weight ratio of the $_{\text{interacting}}$ water, equal to EWC) was determined from the DSC measurement. $T_{2\ \text{non-interacting water}}$ was determined by conducting the same experiment for water only. b was calculated after drying the sample used for the DSC measurement.

3-3) Preparation and characterization of the polymer surfaces

Film samples were prepared as follows and were used for contact angle (CA), X-ray photoelectron spectroscopy (XPS) measurement, and the human blood platelet adhesion test. The

polymer samples were dissolved in ethanol or methanol to prepare a 0.2 wt /vol % solution and filtered. The solution was spin-coated onto round-shaped 14 mm ϕ substrates that was washed by methanol prior to coat the surface. The substrates were coated twice with the solution using a Mikasa spin coater MS-A100 at rates of 500 rpm for 5 s, 2000 rpm for 10 s, slope for 5 s, 4000 rpm for 5 s, and slope for 4 s; and then the substrates were dried at room temperature.

CA of water on the polymer coated surface was measured at 25 °C by the sessile drop method. The captive bubble method was used to evaluate the hydrated surface. 2 μL of air bubbles are injected beneath the hydrated surface immersed in water.[14,15,16,30] The reported values for both measurements are the average of three measurements made at different positions of the substrate.

XPS measurement was carried out on an ESCA-5500 (Physical Electronics, Inc.) equipped with a monochromated Al Kα X-ray source. The measurements were performed at a photoelectron take-off angle (measured with respect to the plane of the sample) of 45 degrees to the polymer-coated substrates. Charge compensation was achieved with a low energy electron flood gun.

3-4) Protein Adsorption and Deformation evaluation by micro BCA and ELISA

The micro BCA was performed in order to measure the amount of adsorbed proteins on the polymer surfaces. Fibrinogen (Sigma) was used as the protein, and the analyses were performed as published before.[14,15,16,30] ELISA was executed to determine the amount of the exposed gamma

chains in fibrinogen. The polymer surfaces were prepared for both micro BCA and ELISA by the following manner: 100 μL of the polymer solution (0.2 wt /vol%) in ethanol was added to the polypropylene (PP) 96-well plate. The plate was slowly air-dried over 3 days at room temperature.

Two types of polymer-casted 96-well PP plates were prepared. One is the water pre-soaked (priming) substrate, which was immersed in 200 μL PBS for 16 hours at 37 °C, and after that the PBS was removed. The other is the non-priming substrate that was not subjected to PBS prior to perform micro BCA and ELISA analyses.

3-5) Preparation of Polymer Substrates and Human Platelet Adhesion Test

The polymer substrates used in the human platelet adhesion tests were prepared as follows: The polymer-coated substrates were cut into 8 × 8 mm squares. The substrates were then fixed on an Scanning Electron Microscope (SEM) specimen stage using double-sided tape. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained from human blood by two stages of centrifuging.[14,15,16,30]

SEM was used to quantify the number of platelets adhered to the substrates. For statistical purposes, the platelet adhesion test was performed three times with human blood from different donors. The reported values are the average of 5 measured points on 3 different films, excluding the maximum and the minimum value.

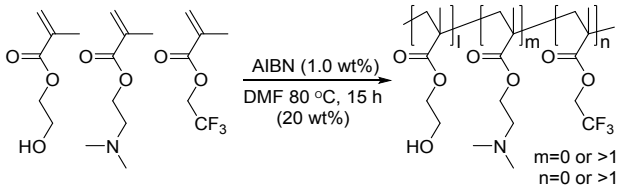
3-6) Statistical analysis

All the data are expressed as means ± SD. The significance of the differences between two samples was determined using an unpaired Student's t-test using Microsoft Excel 2013. Differences with P values less than 0.05 were statistically significant.

Results and Discussion

HEMA copolymers were prepared using either one or both of the comonomers DMAEMA and TFEMA. The synthetic route is outlined in Scheme 1.

Scheme 1. Synthetic route to HEMA copolymers



1. Synthesis and characterization of the HEMA copolymers

Binary and ternary copolymers were synthesized as shown in Scheme 1: poly(HEMA-*co*-DMAEMA) (N-series), poly(HEMA-*co*-TFEMA) (F-series), and poly(HEMA-*co*-DMAEMA-*co*-TFEMA) (N&F-series).

The composition of copolymers was estimated by ^{1}H NMR analysis of the peak for –O*H* in HEMA at δ 4.80 ppm, –CH_2–N$(CH_3)_2$ in DMAEMA at δ 3.30 ppm, and –O–CH_2–CF_3 in TFEMA at δ 4.30 ppm (Figure S1). The monomer composition shown in Table 1 is in good agreement with the monomer ratio in the reaction mixture. PDMAEMA shows high water solubility and this property can be imparted to the HEMA copolymers by copolymerizing 50 or more mol% of DMAEMA. The molecular weight and molecular weight distribution of all polymers are very similar. Figure S2 shows the GPC data of all polymers. The GPC trace of PHEMA has a shoulder at the low MW region, and all HEMA copolymers possess this characteristic. Additionally, higher amount incorporation of DMAEMA (in N10 and N25) cause a shoulder in the high MW region. The RI of fluorine is low and close to that of DMF, and therefore F50 and F100 showed weak intensity peaks despite the same concentration.

Table 1. Composition, yield, and the molecular weight characteristics of all the synthesized HEMA copolymers.

Category	Code	Mol. ratio (l/m/n)		Yield (%)	M_n [b] (g/mol)	M_w/M_n [b]
		Feed	Found [a]			
Control	PHEMA	100 / 0 / 0	100 / 0 / 0	79.5	14500	2.44
N-series	N1	99 / 1 / 0	99 / 1 / 0	94.9	18700	2.02
	N3	97 / 3 / 0	97 / 3 / 0	80.3	15600	2.44
	N5	95 / 5 / 0	95 / 5 / 0	84.3	15500	2.08
	N7	93 / 7 / 0	93 / 7 / 0	90.7	15500	1.92
	N10	90 / 10 / 0	90 / 10 / 0	89.7	14600	3.26
	N25	75 / 25 / 0	75 / 25 / 0	68.8	15500	4.90
	N100	0/ 100 / 0	0/ 100 / 0	33.4	27300	1.26
F-series	F1	99 / 0 / 1	99 / 0 / 1	91.6	19400	2.04
	F3	97 / 0 / 3	97 / 0 / 3	89.3	17400	2.28
	F5	95 / 0 / 5	95 / 0 / 5	94.7	17300	2.44
	F7	93 / 0 / 7	93 / 0 / 7	75.4	17100	2.22
	F9	90 / 0 / 10	91 / 0 / 9	88.2	16500	2.32
	F26	75 / 0 / 25	74 / 0 / 26	80.6	17500	2.07
	F100	0/ 0 / 100	0/ 0 / 100	68.5	10200	1.71

N&F -series	N3F4	94 / 3 / 3	93 / 3 / 4	97.5	18200	2.29
	N5F5	90 / 5 / 5	90 / 5 / 5	99.8	18000	2.20
	N7F8	86 / 7 / 7	85 / 7 / 8	85.3	15200	2.54
	N10F10	80 / 10 / 10	80 / 10 / 10	89.1	15200	2.75

[a] The copolymer composition was identified by ^{1}H-NMR. [b] Molecular weight was measured by GPC carried out in DMF using PEGs as standards.

2. Protein adsorption/denaturation behavior on the surface of HEMA copolymers.

Figure 1 summarizes the results of fibrinogen adsorption and the exposure degree of the gamma chain of fibrinogen. The synthesized N-series and F-series were coated on 96 well plate, and the surface was analyzed for the samples with or without priming. The values showed the minimum in both adsorption amount and denaturation degree when a small amount (3-10 mol%) of the comonomer was incorporated. Although the chemical structure is different from the comonomers in this work, a similar trend was reported for HEMA copolymers with MVEDA[24] and FMA.[31] It is safely assumed that there is an optimal copolymer composition to achieve high bio-inert properties in HEMA copolymers. Since

this trend appeared to be more pronounced with substrate priming, the hydration structure of the copolymers was further investigated using DSC.

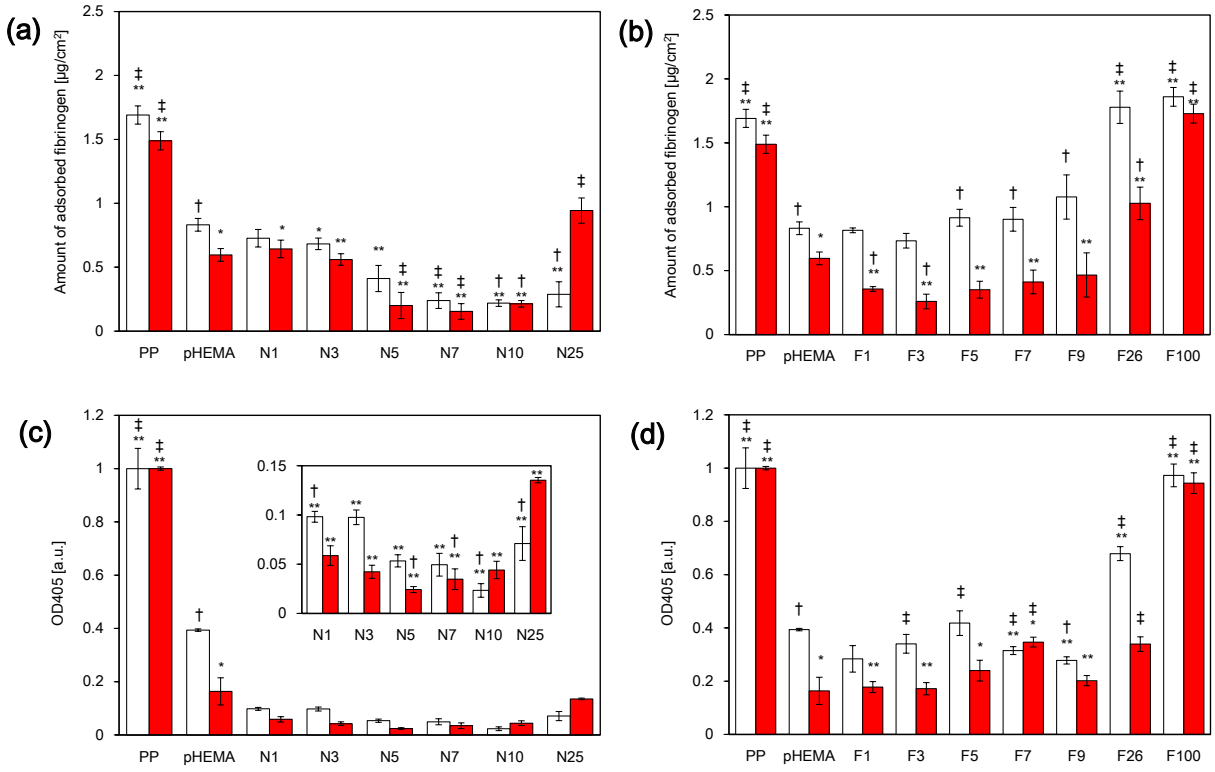


Figure 1. The amount of fibrinogen adsorption (a, b), the exposure of gamma chain of fibrinogen (c, d) onto the surfaces of HEMA copolymers using without (white bar) and with priming (red bar). PP is included as control surface. The inset in (c) is an enlarged graph for N1 to N25. The data represent the means ± SD (n = 3) of fibrinogen adsorption and denaturation *: P < 0.05, **: P < 0.01 vs. PHEMA without priming and †: P < 0.05, ‡: P < 0.01 vs. PHEMA with priming substrate.

3. Hydration characteristics of HEMA copolymers

3-1) Analysis of the hydration structure by DSC.

The hydration structure was evaluated by DSC, and the water content relative to the comonomer composition was summarized in Figure 2. Detailed evaluation results for both N- and F-series, and the DSC profiles at the EWC and different water content are in supporting information (see Table S1 and Figure S3-S20). Biomolecules such as proteins and platelets interact with materials in the aqueous medium, so it can be considered that they are interacting with each other under EWC conditions. Therefore, quantification of EWC is important to consider the interaction between these molecules. The EWC was found to increase with DMAEMA and decreased with TFEMA, depending on the type and amount of comonomer. It deems that the variation of EWC is brought about by the characteristics of each comonomer that are more hydrophilic or hydrophobic than HEMA. Additionally, EWC was roughly additive and correlated with the composition of the copolymerized monomers. However, each water content was not changed additively in

the range up to about 10 mol%. The N-series showed a decrease in *NFW* despite an increase in EWC. On the other hand, although the F-series maintained a certain amount of *NFW*, *FW* decreased more rapidly than EWC.

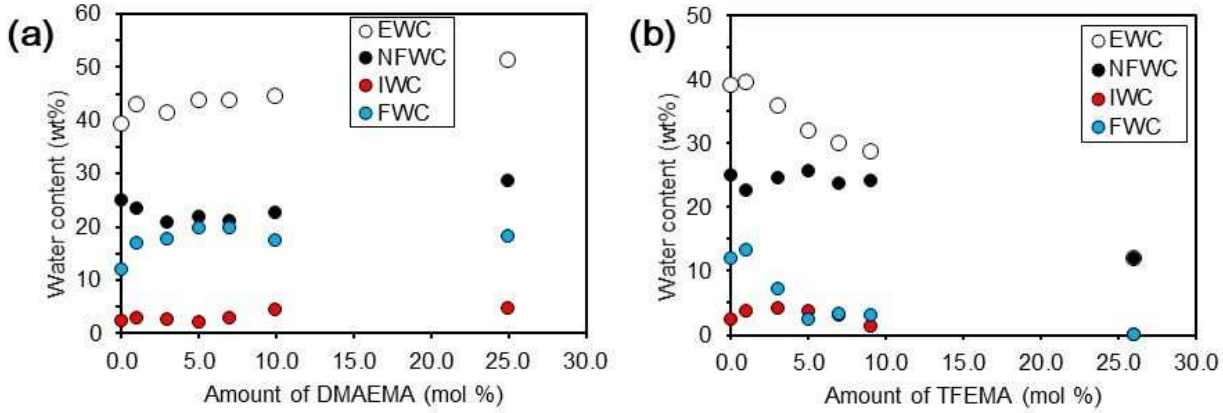


Figure 2 Dependence of the water content on the amount of the introduced DMAEMA (a) and TFEMA (b) at EWC.

3-2) Molecular mobility analysis in hydrated state by DDSC.

Since PHEMA has a -OH group in the side chain, it can form a hydrogen-bonding network through intra- and inter-molecular interactions. Therefore, PHEMA exhibits a higher T_g than that of poly(propyl methacrylate) with a similar side chain length. Morita et al.[38] investigated the hydrogen-bonding structure in PHEMA by infrared spectroscopy, and OH ⋯ O=C and OH ⋯ OH types of hydrogen bonds were observed. Table 2 shows T_g of synthesized polymers for both

experimental and predicted values. Fox's equation was used to calculate the predicted values, the reciprocal of the copolymer's T_g is given by the sum of the reciprocal of each homopolymer's T_g multiplied by its weight fraction. However, as can be seen from Table 2, N10 and F26 showed almost the same T_g as PHEMA. Furthermore, the value was almost constant regardless of the comonomer content. Thus, the incorporation of a small amount of comonomer into PHEMA is not sufficient to break-up the hydrogen bonding network in the dry state (For DSC thermograms, see Figure S21 in supporting information).

In general, plasticizers are known to work by weakening the polymer-polymer interaction and thus reduce the T_g of polymers. Water molecules sorbed into PHEMA interact strongly with hydroxy group at the side chain termini and exhibit plasticizing effect by disrupting the hydrogen-bonding polymer network. Consequently, the molecular mobility of PHEMA drastically changed upon hydration and the high mobility of polymer chain can be observed as a T_g reduction on DSC thermogram. Hatakeyama et al.[39] reported the estimation of T_g for a copolymer of styrene and 4-hydroxystyrene using Fox's equation. Additionally, they also reported that when a small amount of water molecules is present, the hydroxyl groups in the polymer interact with water to break the inter-/intra-polymer hydrogen bonding. Further increase of the water content plasticizes the polymer additionally.[40]

Meakin et al.[41] reported that the T_g of PHEMA dropped to about 50 °C from 113 °C by about 8 wt% hydration from the dry state. They estimated the T_g of hydrated PHEMA by applying the

Fox's equation with their EWC, −136 °C was used as the T_g of water.[42] Kim et al.[43] calculated the hydrated T_g of Nafion by substituting EWC into the Kelley-Bueche Equation,[44] which was in a good agreement with the observed T_g at low water content, but not at high water content. From this result, the authors concluded that freezable water molecules, which crystallize at temperatures higher than -80 °C, do not significantly affect T_g in hydrated state. Tran et al.[45] also reported estimations for the hydrated T_g of polyethylene glycol diacrylate copolymers with poly(ethylene glycol) methyl ether acrylate, sulfobetaine methacrylate, 2-acrylamido-2-methyl-1-propanesulfonate sodium, and [2-(acryloyloxy)ethyl] trimethyl ammonium chloride. The estimation showed good agreements with the observed T_g by using the NFWC rather than EWC.

Since the T_g of HEMA copolymers in the hydrated state was difficult to observe by DSC clearly, the peak top of DDSC in the heating scan was used as the T_g. The DDSC curve for the N- and F-series polymers are shown in Figure 3. For the F-series copolymers with more than 7 mol% TFEMA, no apparent T_g was observed in DDSC. From the Δ^{NFWC} in Table 2, the hydrated T_g tends to be predicted as 10 °C lower than the observed value. We are now speculating that the DDSC peaks of F7 and F9 were overlapped with the endothermic peaks of ice-melting at about 0 °C because the hydrated T_g tends to be predicted at 10 °C lower as in the case of a fluorine amount less than in F5. Additionally, the DDSC peaks for F26 and F100 were predicted to appear at above 30 °C, and were not detected below 50 °C.

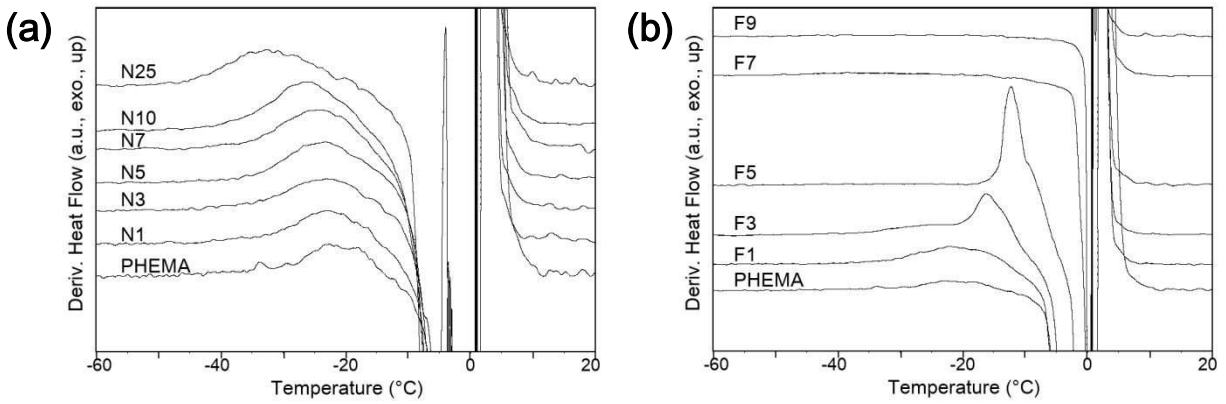


Figure 3. Analyses of hydrated T_g of N-series (a) and F-series (b) by DDSC.

The measured dry and hydrated T_gs will be further discussed together with the theoretical values estimated from Fox's equation. These values are presented in Table 2 and the hydrated T_g is calculated from EWC and NFWC. The value for the hydrated T_g is found to be closer to that of the experimental values when using NFWC instead of EWC as the interacting water fraction. These results indicate that only strongly bound water, *NFW*, acts to disrupt the stable hydrogen bonding network in PHEMA, while freezable *IW* and *FW* molecules do not affect plasticization and T_g reduction.

Table 2 T_g's from actual DSC measurements and calculated by the Fox equation

Category	Code	Dry T_g (°C)			Hydrated T_g (°C)				
		Observed	Predicted [a]	Δ Dry [b]	Observed	Predicted EWC [c]	Δ EWC [d]	Predicted NFWC [e]	Δ NFWC [f]
Control	PHEMA	94.6	94.6	0.0	-22.7	-54.6	31.9	-29.0	6.3
N-series	N1	93.5	93.4	0.1	-23.3	-63.1	39.8	-29.3	6.0
	N3	93.3	91.2	2.1	-23.6	-59.5	35.9	-21.3	-2.3
	N5	94.0	88.9	5.1	-20.2	-64.5	44.3	-26.2	6.0
	N7	94.0	86.7	7.2	-24.3	-64.3	40.0	-23.6	-0.7
	N10	96.8	83.5	13.3	-26.8	-65.4	38.6	-27.7	0.9
	N25	90.9	68.9	22.0	-32.6	-79.3	46.7	-50.0	17.4
	N100	18.3	18.3	0.0	- [g]	- [g]	- [g]	- [g]	- [g]
F-series	F1	94.4	94.5	0.0	-22.3	-55.6	33.3	-24.0	1.7
	F3	93.8	94.2	-0.4	-17.2	-47.1	29.9	-25.3	8.1
	F5	96.0	93.9	2.1	-12.3	-36.4	24.1	-23.6	11.3

	F7	90.7	93.6	-2.9	- [h]	-32.5	- [h]	-19.2	- [h]
	F9	95.4	93.3	2.1	- [h]	-27.7	- [h]	-17.7	- [h]
	F26	94.5	91.1	3.4	- [h]	30.8	- [h]	30.8	- [h]
	F100	83.6	83.6	0.0	- [h]	74.4	- [h]	74.4	- [h]

[a] Estimated from the Fox's equation. [b] Δ^{Dry} defined as dry T_g (Observed) –dry T_g.(Predicted). [c] Estimated from the Fox's equation by using EWC as the water content. [d] Δ^{EWC} defined as hydrated T_g (Observed) –hydrated T_g.(Predicted ^{EWC}). [e] Estimated from the Fox's equation by using the NFWC as the water content. [f] Δ^{NFWC} defined as hydrated T_g (Observed) –hydrated T_g.(Predicted ^{NFWC}). [g] Not defined as water soluble. [h] Not detected clear peak below 50 ° C.

3-3) Analysis of water mobility by solid-state ^{1}H pulse NMR.

The water molecules in a polymer matrix also change their mobility by molecular interaction with polymer chains. According to the Bloembergen-Purcell-Pound theory,[46,47] the spin-spin relaxation time, T_2, decreases continuously as the correlation time, τ_c, increases. Tanzawa[48] roughly classified the water molecules into three types based on the deference in τ_c of 10^{-6} sec, 10^{-9} sec, and 10^{-11} sec, as *NFW*, *IW,* and *FW*, respectively - the longer the T_2 of water, the higher the mobility. In other words, the mobility of water decreases by interacting with the polymer, and T_2 decreases. The T_2 of water was measured by solid-state ^{1}H pulse NMR[43] using hydrated samples with water content close to their EWC quantified by DSC. The results are presented in Figure 4, together with the hydrated T_g of the materials.

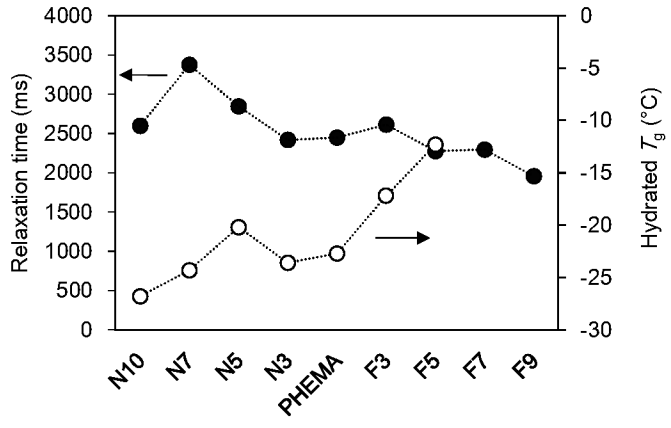


Figure 4. Relaxation time of water and hydrated T_g of the HEMA copolymers.

Compared to hydrated homo- PHEMA, the relaxation time of water in copolymers with a small amount of foreign elements (N or F) was decreased with the introduction of TFEMA, and increased with DMAEMA. The relaxation time of pure water was found to be about 3100 msec, close to the previously reported value of 3195 msec ($1/T_2 = 0.313$ sec $^{-1}$).[49] Relaxation spectra of water in all (co)polymers are shown in Figure S22 (see supporting information).

Considering the result of T_2 analysis together with the result of the hydrated structure analysis by DSC (Figure 2), by copolymerizing small amounts of foreign monomers with HEMA, the following changes in hydration state can be caused by the changes in intra- and inter- molecular interactions. Introducing a small amount of DMAEMA into HEMA copolymers reduces the amount of hydroxyl groups that can strongly interact with the hydrogen-bonding site. This reduction in hydroxy groups reduces the interaction between polymer chains; thus the free volume increases. Furthermore, since the number of functional groups that have a role in polymer-water interaction is reduced, resulting in an increase in *IW* and *FW* with less interaction with the polymer. As a result, the strong hydrogen-bonding network of water due to the interaction between water and PHEMA is loosened, and a part of *NFW* changes to *IW* or *FW*. However, when the DMAEMA composition is as high as 50 mol%, the polymer became water-soluble because of the predominance of the interaction between water and $-NCH_3$. Conversely, by introducing a small amount of TFEMA into HEMA copolymers, the hydrophobic fluorine groups are agglomerated due to hydrophobic interaction upon the hydration of PHEMA matrix. This aggregate acts as a physical cross-linking point, thereby reducing the free volume, and consequently increasing *NFW* that strongly interacts with the polymer. Fluorinated side chains are highly hydrophobic and

their affinity with water is weak. Therefore, it can also be interpreted that T_g, the temperature at which micro Brownian motion of polymer starts, was increased by the promotion of dehydration from the polymer chains. Thus, the stable network between water and PHEMA gets stronger, and a part of *FW* changes to *IW* and *NFW*.

4. Relationship between hydration structure and biological response

The relationship between the fibrinogen adsorption/denaturation to the hydration state is shown in Figure 5. The comonomer composition is also shown in the figure. The role of *IW* is interpreted to shield the blood component from direct contact with *NFW*, and it has been reported that the blood component is not activated if a sufficiently thick *IW* is present.[50] Therefore here, IWC is used as an index of the hydration structure.

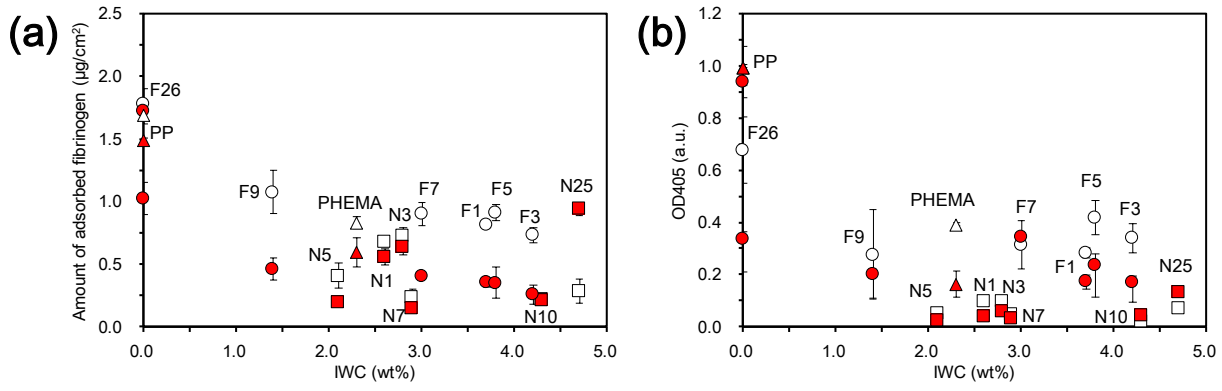


Figure 5. Correlation between the ratio of IWC and the biological response: fibrinogen adsorption (a), and the exposure of gamma chain of fibrinogen (b). The data represent the means ± SD (n = 3). PP is included as control surface. Squares, circles, and triangles indicate N-series, F-series, and controls, respectively, and white and red indicate without and with priming, respectively.

As can be seen in Figure 5, the substrates coated with the HEMA copolymers showed lower fibrinogen adsorption and denaturation as the content of IWC increases. In particular, this trend became more prominent with priming, and the relationship between IWC and the biological response is in good agreement with previous works.[14,30] However, although IWC of F9 was about half that of N25, the amount of adsorbed fibrinogen was less than half. This discrepancy seems to be due to the surface hydration structure; thus the surface was therefore further investigated by contact angle and XPS measurements.

5. Contact angle measurements of dry and hydrated HEMA copolymer films

The water in air contact angles (WCA, sessile drop method) and the air in water contact angles (ACA, captive bubble method) of the HEMA copolymers are summarized in Table 3. The WCA results show that the contact angle of sessile drop changes over time. Immediately after the water droplet contact with the surface, WCA of the copolymers is higher than homo- PHEMA in most of the samples. This is probably due to the segregation of the copolymerized side-chain functional groups in HEMA copolymers, $–NCH_3$ groups of DMAEMA and the $–CF_3$ groups of TFEMA, are segregated at the air/polymer interface. However, after 60 seconds, all the F-series samples were more hydrophobic than PHEMA, whereas the N-series were more hydrophilic. The time

dependence of WCA after placing a water droplet on polymer-coated substrates is shown in Figure S23. F9 presented the highest hydrophilicity in water among all samples in the F-series. The functional group containing fluorine is segregated to the air/polymer interface because of its low cohesive force. However, upon contact with water, the fluorine-containing functional groups translocate into the more hydrophobic environment, the polymer matrix. Consequently, the surface is more easily reconstructed with a hydrophilic surface.[51] From the evaluation results by the captive bubble method as shown in Table 3, both F- and N-series were hydrophilized at the water/polymer interface. Therefore, the elemental analysis by XPS was conducted to determine the elemental composition at the interface.

Table 3. Contact angle[a] of all the HEMA copolymers used in the investigation.

Category	Code	Sessile drop (WCA)		Captive bubble (ACA)
		1s	60 s	24 h
Control	PHEMA	39.2 ±1.3	22.9 ±0.5	132.0 ± 6.5
N-series	N1	40.9 ±3.6	19.3± 1.0	146.6 ± 0.4
	N3	41.3 ±2.3	15.0 ±3.7	143.9 ± 0.8
	N5	44.7 ±6.4	15.1 ±3.4	150.0 ±1.5
	N7	43.0 ±2.6	10.1 ±0.3	146.6 ±1.5

	N10	49.3 ±4.6	14.4 ±4.5	149.2 ±0.4
	N25	48.3 ±5.0	13.5 ±2.8	146.9 ±1.9
F-series	F1	47.2 ±1.8	25.5 ±0.7	134.0 ±1.4
	F3	51.4 ±1.6	31.2 ±2.5	133.7 ±6.2
	F5	59.7 ±1.3	47.2 ±1.4	136.4 ±2.7
	F7	65.4 ±1.1	57.5 ±2.8	137.4 ±0.8
	F9	66.9 ±1.6	58.5 ±3.9	141.2 ±1.0
	F26	84.6 ±2.1	82.1 ±1.7	131.8 ±1.2
	F100	92.9 ±1.2	91.6 ±0.8	100.9 ±2.6

[a] WCA (sessile drop method) and ACA (captive bubble method). The data represent the means ± SD (n = 3).

6. XPS analyses of dry and hydrated HEMA copolymers.

The N25 coated substrate shows higher hydrophilicity in water (Table 3) and also the IWC of N25 is about twice higher than that of F9 (Table S1). However, N25 adsorbs twice as much amount of fibrinogen as compared to F9. This result for N25 was considered a singularity compared to other copolymers. In order to analyze the molecular orientation at the water/polymer interface, XPS measurement was performed using freeze-dried polymer-coated PET substrates from the water contact environment.

We have previously reported that PMEA copolymerized with a high T_g component maintains the hydrophilized surface even after freeze-drying and the vacuum process of XPS measurement.[30] PHEMA has a high T_g in a dry state and the 8 wt% hydrated PHEMA shows T_g at about 50 °C. As the water content in hydrated PHEMA decreases during the freeze-drying process, the T_g is likely to be maintained above room temperature. Therefore, surface reconstruction was expected to be sufficiently slow to obtain the information reflecting the state of hydrated surface. Thus, the surface of novel HEMA copolymers that treated by the freeze-drying process was analyzed by XPS. The samples were immersed in water for 1 hour before freeze-drying at room temperature and kept at –30 °C for 16 hours under a vacuum condition of 20 Pa during freezing. To compare the distribution of the elements on the surfaces with and without priming, the samples without freeze-drying were used as a control. The results are shown in Figure 6. In the case of HEMA copolymers with DMAEMA, the N/C ratio of copolymers were found to be higher than the theoretical ones in the hydrated state, probably due to the segregation of $–NCH_3$ group to the polymer/water interface. On the other hand, in the case of the copolymers containing TFEMA, the F/C ratio was lower than the theoretical value. Therefore, this can be interpreted that the functional groups containing fluorine atoms have translocated from the air/polymer interface into the polymer

matrix. Based on the above results, the reason for a large amount of fibrinogen adsorbed on N25 surface is because the positively charged $–NCH_3$ group was segregated at the interface in the physiological environment. It is noteworthy that there are reports describing the mechanisms of fibrinogen adsorption onto the positively charged surface with electrostatic interaction. The core of fibrinogen is negatively charged and the core of fibrinogen attaches onto the surface by side-on adsorption.[52] In particular, the degree of adsorption and denaturation on N10 and N25 were increased. This is probably because the effect was prominently expressed by priming.

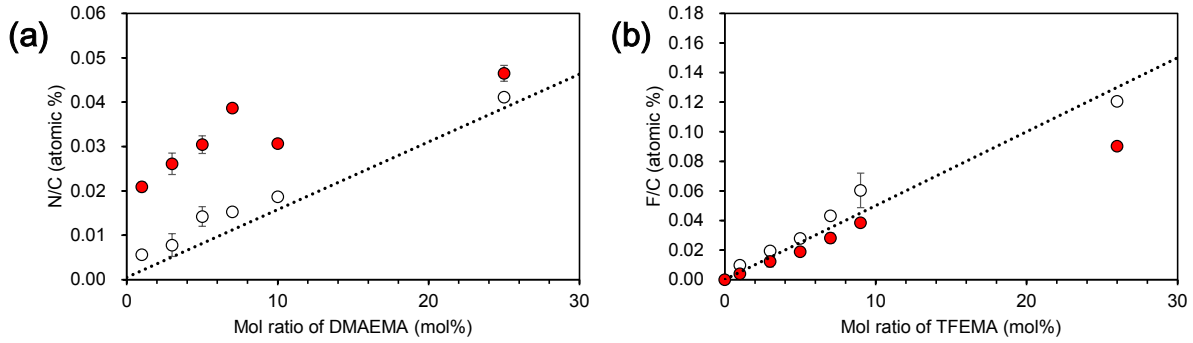


Figure 6. Surface elemental analysis of N-series (a) or F-series (b) by XPS. The dotted line shows the theoretical value calculated from the composition ratio. The data represent the means ± SD (n = 3). White and red indicates before and after freeze drying, respectively.

7. Novel molecular design of bio-inert hydrophilic polymers

Up until this section, the discussion has been focused on binary HEMA copolymers. Hydration state was changed with changes in mobility of hydrated polymer and water by copolymerizing a small amount of either amino or fluorine monomers. The change in the hydration state is

considered to be brought about by the changes in intra- and intermolecular interactions and was represented by the opposite effect in the case of N- and F-series. In order to verify whether this effect can coexist in a single copolymer, ternary copolymers (denoted as NxFy) were synthesized and their physical properties were evaluated as shown in Table 1. Since NxFy exhibited an intermediate amount of EWC between Nx and Fy, the copolymers were likely affected by both TFEMA and DMAEMA (Table S1). Figure 7 shows the DSC heating profile of N5F5 under EWC condition as a typical example of a ternary copolymer, and the results for its binary analogs (N5 and F5) are summarized in Table 4. Water molecules in N5 and F5 began to cold crystallize at −31.1 and −17.1 °C, and their exothermic peaks appeared at −12.8 and −8.1 °C. On the other hand, cold crystalliziation of N5F5 started at a lower temperature than that of N5, −35.9 °C, whereas its exothermic peak at −9.9 °C was close to that of F5. Based on these results, we can conclude that the copolymers incorporating both amino and fluorine monomers simultaneously exhibit the effects observed independently for each binary HEMA copolymers. From the results of the surface elemental analysis (Figure S24), before and after freeze-drying, both N/C and F/C of NxFy showed the same behavior as that of the binary HEMA copolymers. Therefore, it can be considered that the copolymer containing both monomers reflects the properties of each copolymer in the hydrated state, not only in bulk but also in the water/polymer interface. Thus, the cold crystallization of water in the ternary copolymer appeared as the sum of water crystallization behavior in both binary copolymers, the crystallization of IW appeared in a wider temperature range as shown in Figure 7 and Table 4. Therefore, the relationship between IWC and biological responses is examined in the following section. With the increase of IWC, improvement in the bio-inert properties is expected.[14,27,29]

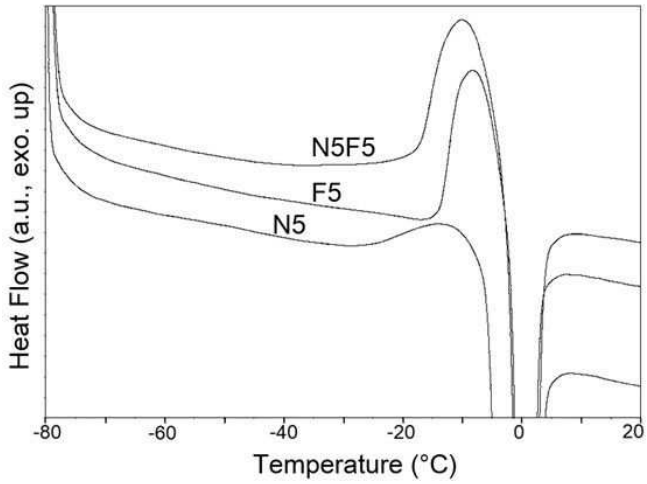


Figure 7. DSC heating profiles of N5, F5 and N5F5 at EWC conditions

Table 4. Temperature range of cold crystallization and IWC in the heating process of N5, F5, and N5F5 at EWC condition.

	PHEMA	N5	N5F5	F5
Start of the cold crystallization (℃) [a]	-34.7	-31.1	-35.9	-17.1
Exothermic peak (℃)	-13.3	-13.7	-9.9	-8.1
IWC (wt%)	2.3	2.1	5.2	3.8

[a] The temperature of the beginning of the cold crystallization was identified from the inflection temperature.

Figure 8 shows the evaluated results for the amount of adsorbed fibrinogen, its denaturation degree, and the number of adhered human platelets. In almost all cases, the ternary copolymers containing the same amount of one or both foreign monomers were more bio-inert than the corresponding

binary copolymers. Similar effects were observed for N3F3 and are shown in Figure S25. These results indicate the importance of introducing foreign monomers and the synergetic effect of IWC increasing from different mechanisms of amino and fluorine groups in PHEMA. This IWC increment may be the reason for the improvement of bio-inert properties of copolymers synthesized herein.

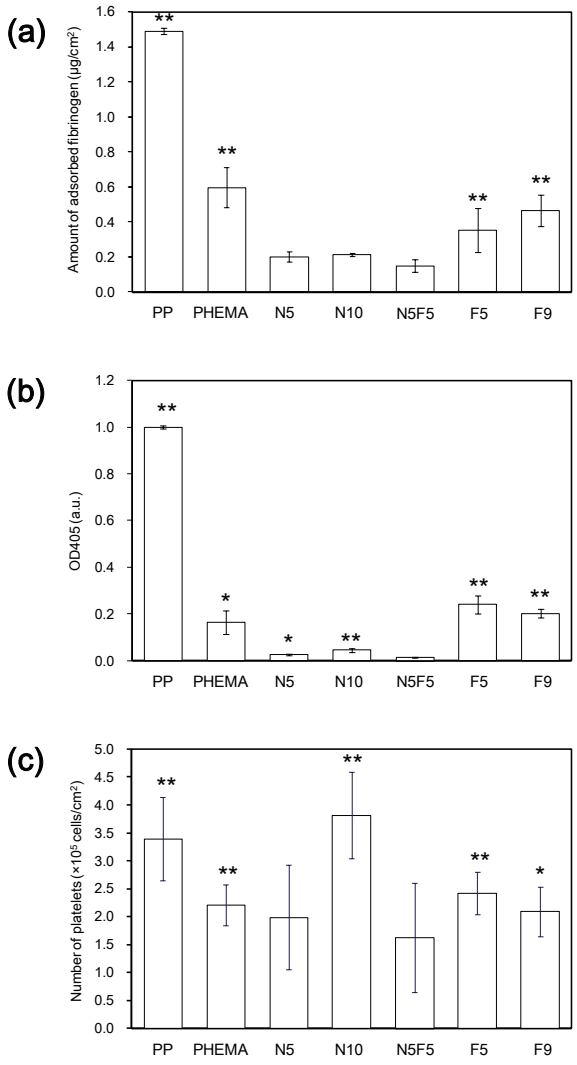


Figure 8. The amount of fibrinogen adsorption (a), the exposure degree of gamma chain of fibrinogen (b), and the number of human platelet adhesion (c) onto the surface of HEMA

copolymers and using surfaces with priming. PP is included as control surface. The data represent the means ± SD (n = 3) of fibrinogen adsorption and denaturation and ± SD (n = 11) of the platelet adhesion. *: $P < 0.05$, **: $P < 0.01$ vs. N5F5 substrate with priming.

Conclusions

We have synthesized binary and ternary HEMA copolymers containing small amounts of DMAEMA and TFEMA by free radical polymerization. Although the homopolymers obtained from these two monomers did not show bio-inert properties, HEMA copolymers containing a small amount of these two monomers showed higher bio-inert properties than homo-PHEMA. By incorporating a small amount of amino or fluorine monomers into HEMA, the hydration state was changed.

From the evaluation results of polymer and water mobility in the hydrated state, it is revealed that the incorporation of small amounts of foreign monomers loosens or fastens the water-polymer interaction in HEMA copolymers. This result can be interpreted as follows: When hydrophilic DMAEMA was introduced to HEMA, *NFW* changed to *IW* and *FW*. Conversely, introduction of hydrophobic TFEMA to HEMA, *FW* changed to *IW* and *NFW*. Since

DMAEMA is positively charged in physiological environments, segregation of the charged amino group to the water-polymer interface leads to more protein adsorption, denaturation, and platelet adhesion. For TFEMA, the fluorine with low cohesive energy was found to segregate rapidly to the air-polymer interface, effectively switch to the hydrophilized surface at the water-polymer interface upon hydration. Increased IWC and effective shielding of the *NFW* by *IW* contributes to improving the bio-inert properties of these copolymers. This interpretation of presented results is fully consistent with our previous investigations for MEA copolymers with small amount of fluorine monomers[30]. In addition, ternary copolymers containing TFEMA and DMAEMA exhibited an intermediate amount of EWC between N- and F-series. The ternary copolymer showed better bio-inert properties than that of binary copolymers having the same amount of foreign comonomers. This result can be interpreted as the higher IWC was achieved by the synergetic effect of DMAEMA and TFEMA in the ternary copolymers with HEMA. More investigations to confirm these findings are already on going in our laboratory.

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publication website at DOI:…

Detailed characterization data, DSC profiles, NMR spectra and SEC data for the discussed polymers.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

Materials

PEG	Poly(ethylene glycol)
MPC	2-methacryloyloxyethyl phosphorylcholine
PMPC	copolymer with 2-methacryloyloxyethyl phosphorylcholine and butyl methacrylate
PMEA	Poly(2-methoxyethyl acrylate)
HEMA	2-hydroxyethyl methacrylate

PHEMA	Poly(2-hydroxyethyl methacrylate)
TFEMA	2,2,2-trifluoroethyl methacrylate
DMAEMA	2-(dimethylamino)ethyl methacrylate
PP	Polypropylene
PET	polyethylene terephthalate

Analyses

NMR	Nuclear Magnetic Resonance
GPC	Gel Permeation Chromatography
DSC	Differential Scanning Calorimetry
DDSC	temperature derivative of differential scanning calorimetry
CA	Contact Angle
WCA	Water in air Contact Angle
ACA	Air in water Contact Angle
XPS	X-ray Photoelectron Spectroscopy
SEM	Scanning Electron Microscope

Interactions between water and polymers

NFW(C)	non-freezing water (content)
IW(C)	intermediate water (content)
FW(C)	free water (content)
EWC	equilibrium water content

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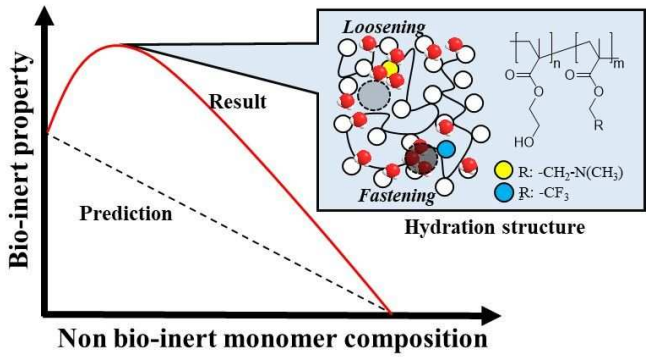

Understanding the effect of hydration on the bio-inert properties of HEMA copolymers with small amounts of amino- or/and fluorine-containing monomers

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