Journal of Materials Chemistry B

ARTICLE

Novel zwitterionic polymer for the construction of highly durable anti-biofouling materials and surfaces

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Ryoma Takagi [a], Ayaka Moroto [b], Toshikazu Yamamoto, Tadashi Nakaji-Hirabayashi [a,b,c,], Tatsuya Ichiyama [a,b], Chiaki Yoshikawa [c], Hiromi Kitano [d], Shinpei Yamamoto [e], Yoshiyuki Saruwatari [e]*

Functional materials coated with zwitterionic polymers can significantly suppress the interactions with biological materials. However, the bio-inactivity of existing zwitterionic polymers lacks long-term durability, partly owing to the cleavage of zwitterionic side chains by the hydrolysis of ester bonds. To address this, a novel zwitterionic monomer, "sulfoisobutylbetaine acrylamide (SBBAm)" whose side chain is linked by an amide bond, was synthesized. A copolymer with SBBAm and a monomer with a silane-coupling side chain was synthesized and used to modify a glass substrate to analyze the resultant bio-inactivity and its long-term durability. The performance of the SBBAm copolymer was compared with copolymers comprising existing zwitterionic monomers. The results indicated that the surface modified with the SBBAm copolymer maintained bio-inactivity even after 1 year of incubation in PBS at 37 °C, whereas that of the copolymer with existing zwitterionic monomers mostly deteriorated after 1 week of incubation. Furthermore, the positive effects of the isobutyl linker between the anion and cation units in the SBBAm side chain were demonstrated in comparison with the sulfopropylbetaine polymer. The molecular design of the SBBAm helps to overcome the water-insolubility of sulfobetaine-type polymers, that is, these polymers become water-insoluble with an increase in molecular weight. The results presented here can drive the development of a novel zwitterionic polymer material that causes bio-inactivity with long-term durability.

Keywords: anti-biofouling property, bio-inactive material, bioinert surface, biomaterial design, zwitterionic polymer.

Introduction

Functional materials have been developed using zwitterionic polymers owing to their anti-biofouling properties against various biological materials, including proteins, cells, and bacteria.[1–5] For example, surfaces modified with zwitterionic polymers have been applied to culture well plates for the preparation of embryoid bodies.[6] Furthermore, several different types of zwitterionic polymers have been used as surface coatings to suppress thrombus formation.[7–9] Furthermore, zwitterionic polymers[10,11] and plastics conjugated with zwitterionic polymers[12,13] have been used as coatings to suppress bacterial adsorption and proliferation in a natural environment. Consequently, zwitterionic polymer-based materials are expected to be of great value across fields such as biomaterial development, environmental technology, cell biology, and biomedical devices.

The most well-known zwitterionic monomer is methacryloyloxyethyl phosphorylcholine (MPC)[14,15], and MPC-based polymers are practically used in medical fields.[16] Additionally, various zwitterionic polymers based on carboxymethylbetaine methacrylate (CMBMA) and sulfopropylbetaine methacrylate (SPBMA) have also been developed. They are reported to have the same or similar abilities to MPC-based polymers.[1,17] Zwitterionic polymer-induced bio-inactivity occurs because they do not disturb the water structures in their vicinity, as described previously by Kitano, Takahara and Ishihara et al.[18–22] Additionally, it has also been demonstrated previously that the existence of zwitterionic groups as well as the mechanical property caused by polymer chains, that is, “soft material”, is important for establishing and maintaining bio-inactivity.[1,10,18]

Recently, studies have focused on developing coating methods for catheters to enable prolonged use within the body, as well as for marine materials that can suppress the adsorption of

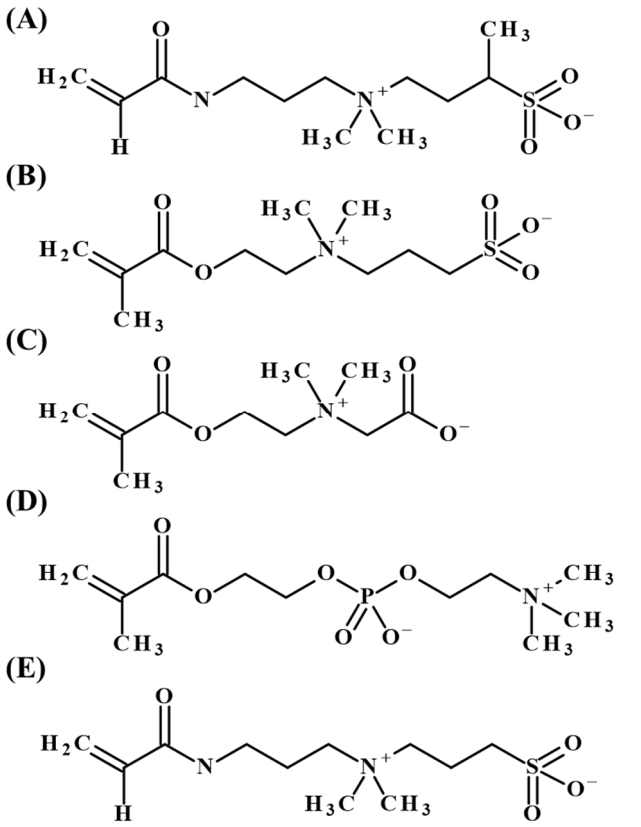
[a] Graduate School of Innovative Life Science, University of Toyama, 3190 Gofuku, Toyama-shi, Toyama 930-8555, Japan
[b] Graduate School of Science and Engineering, University of Toyama, 3190 Gofuku, Toyama-shi, Toyama 930-8555, Japan
[c] International Center for Materials Nanoarchitectnics, National Institute for Materials Science, 1-1 Namiki Tsukuba, Ibaraki 305-0044, Japan
[d] R & D Research Head Office, Institute for Polymer-Water Interfaces, 84 Fukujima, Yatsuo, Toyama-shi, Toyama 939-2376, Japan
[e] R & D Laboratory, Osaka Organic Chemical Industries, 1-7-20 Azuchi-cho, Chuoh-ku, Osaka-shi, Osaka 582-0020, Japan.
[f] * Corresponding Author: T. Nakaji-Hirabayashi, nakaji@eng.u-toyama.ac.jp
† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/b000000x/

bacteria and marine organisms for several years. However, investigations have shown that surfaces modified with existing zwitterionic polymers cannot maintain their induced bio-inactivity for longer periods. This study aimed to develop a novel zwitterionic polymer with high durability by improving monomer design, focusing on the ester side-chain bond of existing zwitterionic monomers. This is because the lack of durable bio-inactivity is ascribed to the hydrolysis of ester bonds and the cleavage of side chains. Therefore, the focus was on an extremely slow hydrolysis of the amide bonds compared to ester bonds, which is caused by the difference in polarization between ester and amide bonds.[23] Additionally, a polymer synthesized using a zwitterionic monomer based on acrylamide was considered to inhibit hydrolysis partly because of the formation of hydrogen bonding between the amide units in the side chain. Therefore, we have aimed to develop a novel zwitterionic monomer comprising acrylamide.

Considering the potential industrial applications, sulfoisobutylbetaine acrylamide (SBBAm), which is a novel zwitterionic monomer, was synthesized using a simple method and purification step (**Scheme 1A**). An isobutyl linker between the anion and cation in the zwitterionic unit was introduced because of the inhibition of association between the zwitterionic side chains by the steric hindrance of the methyl group, which is a special feature of the monomer design. Therefore, it is presumed that the assembly of zwitterionic groups between the side chains or polymers could be suppressed in water, resulting in the dissolution of the SBBAm polymer in water despite its high molecular weight.[24,25]

This study investigated the bio-inactivity and long-term durability of surfaces modified with the SBBAm polymer. To chemically immobilize the SBBAm polymer on a glass substrate, a copolymer comprising SBBAm and a monomer having a silane coupling side chain was synthesized. Additionally, copolymers comprising existing zwitterionic monomers, including SPBMA, CMBMA, and MPC (**Scheme 1B–1D**), were synthesized as controls for comparison with the SBBAm copolymer and to determine its effectiveness. The surfaces modified with the SBBAm polymer and incubated in phosphate buffer saline (pH 7.40) at 37 °C maintained their bio-inactivity for 1 year, whereas the bio-inactivity of the other zwitterionic polymers was maintained only for 1 week, indicating that the SBBAm polymer could maintain long-term bio-inactivity. Moreover, the effect of the isobutyl linker in the zwitterionic unit of SBBAm was investigated by comparing with the copolymer comprising sulfopropylbetaine acrylamine (SPBAm) (**Scheme 1E**), synthesized using a similar process as that of SBBAm.

To the best of our knowledge, the zwitterionic monomer developed in this investigation, which is durable over the long term, is a first of its kind in scientific literature. We consider that MPC and CMB polymers are excellent materials to construct surfaces with bio-inactivity in the short term; however, long-term bio-inactivity will be required in the near future. The findings of this study are expected to improve the design choices for biomaterials using zwitterionic polymers.



Scheme 1. Chemical structures of zwitterionic monomers. (A) Sulfoisobutylbetaine acrylamide (SBBAm), (B) sulfopropylbetaine methacrylate (SPBMA), (C) carboxymethylbetaine methacrylate (CMBMA), (D) methacryloyloxyethyl phosphorylcoline (MPC), and (E) sulfopropylbetaine acrylamide (SPBAm).

Experimental

Synthesis and characterization of sulfoisobutylbetaine acrylamide (SBBAm)

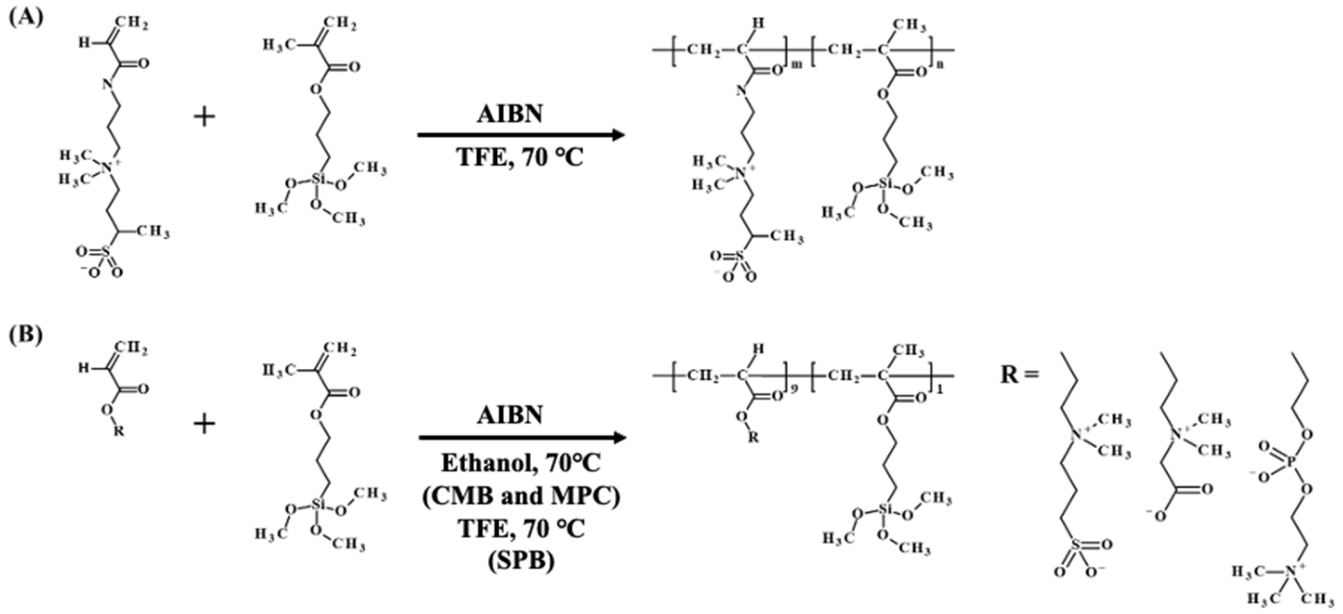
Dimethylaminopropyl acrylamide (DMAPAm, 9.52 g, 60.0 mmol; TCI Co., Ltd., Tokyo, Japan) was dissolved in dry acetone (41.6 mL; Wako Pure Chemical Industries, Osaka, Japan) and stirred vigorously using a magnetic stirrer at 0 °C. Then, 2,4-butanesultone (BSu, 6.34 mL, 63.0 mmol; TCI Co., Ltd.) was slowly added (1.27 mL/min) to the acetone solution containing DMAPAm. After completely adding BSu, the mixture was incubated for 1.5 h at 0 °C. The temperature of the solution was then increased to 25 °C, and the reaction was continued for 24 h. Consequently, a white precipitate was obtained, which was filtered and carefully washed with acetone (300–400 mL) to remove the unreacted BSu and DMAPAm completely. It was then dissolved in methanol (15 mL), and the precipitation process was repeated twice using acetone (500 mL) and dried using a vacuum in the dark. The white SBBAm product had a yield of 16.4 g (92.1%) (**Scheme S1**).[26]

The SBBAm was characterized using ^{1}H NMR (400 MHz; JEOL Ltd., Tokyo, Japan). The chemical shift and integral strength were as follows: δ 1.24 ppm (3H), 1.92 ppm (3H), 2.13 ppm (1H),

2.75 ppm (1H), 3.05 ppm (6H), 3.26 ppm (4H), 3.38 ppm (1H), 3.57 ppm (1H), 5.60 ppm (1H), and 6.15 ppm (2H) (**Figure S1**).

Synthesis of zwitterionic polymers with a silane coupling agent as the side chain

To investigate the potential of the SBBAm polymer in detail, a glass substrate chemically modified with the SBBAm polymer was fabricated. To achieve this, a copolymer with zwitterionic monomer and 3-methacryloyloxypropyl trimethoxysilane



Scheme 2. Synthesis of a copolymer comprising a zwitterionic monomer and MPTMS. (A) Copolymer of SBBAm and MPTMS (PSBBAm*X*; *X* represents the composition ratio of SBBAm). (B) Copolymer of an existing zwitterionic monomer (R = side chain of SPBMA, CMBMA, and MPC) and MPTMS. The composition ratio of these zwitterionic polymers was determined by referencing our previous report.

Table 1. Reaction conditions and characterization of copolymers comprising zwitterionic monomers and MPTMS.

Copolymer	Zwitterionic monomer (ZIM)	MPTMS	AIBN [(a)]	Composition ratio (ZIM:MPTMS)		M_v
	mol	mol	mol	feed	product [(b)]	
PSBBAm50	1.91×10^{-3}	1.91×10^{-3}	2.55×10^{-4} and 5.11×10^{-5}	50:50	no data [(c)]	no data [(c)]
PSBBAm60	2.30×10^{-3}	1.53×10^{-3}		60:40	61:39	1.7×10^4 [(d)]
PSBBAm70	2.68×10^{-3}	1.15×10^{-3}		70:30	74:26	1.6×10^4 [(d)]
PSBBAm80	3.06×10^{-3}	7.66×10^{-4}		80:20	83:17	3.2×10^4 [(d)]
PSBBAm90	3.45×10^{-3}	3.83×10^{-4}		90:10	92:8.2	2.3×10^4 [(d)]
PSBBAm95	3.64×10^{-3}	1.92×10^{-4}		95:5	96:3.8	2.5×10^4 [(d)]
PSBBAm99	3.79×10^{-3}	3.83×10^{-5}		99:1	99:0.97	2.6×10^4 [(d)]
PSPB90	3.45×10^{-3}	3.83×10^{-5}		90:10	89:11	2.4×10^4 [(d)]
PCMB90	3.45×10^{-3}	3.83×10^{-5}		90:10	91:9	1.8×10^4 [(e)]
PMPC90	3.45×10^{-3}	3.83×10^{-5}		90:10	90:10	5.5×10^4 [(e)]
PSPBAm70	2.68×10^{-3}	1.15×10^{-3}		70:30	71:29	2.7×10^4 [(d)]

(a) AIBN at a concentration of 2.55×10^{-4} mol was added in the monomer solution and the solution was reacted for 4 h. After 4 h, AIBN (5.11×10^{-5} mol) was further added in the reaction solution, and the reaction was continued for 4 h. The total reaction time was 8 h.

(b) The composition ratio of the polymers was determined using ^{1}H NMR.

(c) A polymer solution of PSBBAm50 could not be obtained as it formed a gel during polymerization. This is probably owing to the increase of the silane coupling groups in the side chain.

(d) The viscometric average molecular weight (M_v) of PSBBAm*X*s, PSPB90, and PSPBAm70, was determined using the K (6.88×10^{-3} mL/g) and α (0.627) values of the SPBMA homopolymer.

(e) The viscometric average molecular weight (M_v) of PCMB90 and PMPC90 was determined using the K (1.31×10^{-3} mL/g) and α (0.454) values of the CMBMA homopolymer.

(MPTMS, TCI Co., Ltd.) with a silane coupling side chain was synthesized as described in previous studies (**Scheme 2**).[1,10,27] Additionally, copolymers with different SBBAm compositions (SBBAm*X*, where *X* is the composition of SBBAm) were synthesized to determine the optimum composition.

SBBAm and MPTMS with molar ratios shown in **Table 1** were dissolved in dry trifluoroethanol (TFE, 10 mL; TCI Co., Ltd.). After purging with N_2 gas for 15 min, azobisisobutyronitrile (AIBN, 2.55×10^{-4} mmol) was added to the solution and purged with N_2 gas for 30 min to remove oxygen. The solution containing the monomers and the initiator was reacted at 70 °C for 4 h. AIBN (5.11×10^{-5} mol) was further added and continuously reacted for another 4 h.

The control copolymers comprising SPBMA, CMBMA, and MPC, which are P(SPBMA$_{90}$-MPTMS$_{10}$; PSPB90), P(CMBMA$_{90}$-MPTMS$_{10}$; PCMB90), and P(MPC$_{90}$-MPTMS$_{10}$; PMPC90), were synthesized as described previously.[1,17]

All the copolymers were used as coating solutions without purification, as they were crosslinked between the silane coupling groups and aggregated during the purification process, resulting in precipitation.

The viscosity average molecular weight (M_v) of each polymer was determined using the Mark–Houwink-Sakurada's formula (1), and the intrinsic viscosity [η] was obtained from the viscosity measurement.[1]

$$[\eta] = K{M_v}^{\alpha} \qquad (1)$$

The [η] was measured using a Ubbelohde viscometer (viscometer constant = 0.001 cSt/s; SIBATA Scientific Technology, Ltd., Saitama, Japan) in a water bath (VB-3T; SIBATA Scientific Technology, Ltd.) at a constant temperature (37 °C). The viscosity of PSBBAm*X* and PSPB90 was measured using trifluoroethanol as a solvent, whereas ethanol was used for PCMB90 and PMPC90. The values of K (6.88×10^{-3} mL/g) and α (0.627) for PSBBAm*X* and PSPB90 were determined from the viscosity measurements of the SPBMA homopolymer with a known molecular weight (M_w = 3.31 × 104, M_w/M_n = 2.42; determined by the GPC measurement), whereas the values of *K* (1.31×10^{-3} mL/g) and α (0.454) were used for PCMB90 and PMPC90.[1]

Preparation of surfaces chemically modified with zwitterionic polymers

The glass substrate and silicon wafer washed with Piranha solution (sulfuric acid:hydrogen peroxide = 7:3) were incubated for 24 h in 1 w/v % PSBBAm*X* solution (solvent: trifluoroethanol) at 25 °C. The substrates were then carefully washed with trifluoroethanol and ethanol and blow-dried with N_2 gas. Subsequently, the polymer-coated substrates were treated at 80 °C for 12 h to ensure complete reaction with the silane coupling unit (**Scheme S2**). The surface modifications with existing zwitterionic copolymers were performed in trifluoroethanol (PSPB90) or ethanol (PCMB90 and PMPC90) as a solvent.

The glass substrate (20 × 20 mm; Matsunami Glass Ind. Ltd., Osaka, Japan) modified with the polymers was used to investigate the contact angle and cell adhesion, whereas the silicon wafer (20 × 20 mm; Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) modified with the polymers was used to analyze the ellipsometry, ATR-IR, and AFM measurements. In contrast, for protein adsorption measurement using the microBCA method, a large slide glass substrate (76 × 26 mm; Matsunami Glass Ind. Ltd.) was used to increase the detection limit by increasing surface area. A1 w/v % copolymer solution was used during the preparation because a previous study has indicated that the surface coated with PCMB90 and PMPC90 can be coated with a very high coverage rate and that it exhibits non-responsiveness to biological substances .[1]

Water and air bubble contact angle measurements

Water and air bubble contact angles of the copolymer-coated surfaces were measured using a contact angle meter (DMs-401; Kyowa Interface Science Co., Ltd., Saitama, Japan) to determine the surface wettability. A water droplet (1 μL) was placed on the surface modified with the copolymer and incubated for 30 s, and the contact angle was measured at ten points selected randomly, and the data were averaged. Furthermore, five different samples were measured to evaluate the significant differences.

Attenuated total reflection-infrared spectroscopy

The surfaces chemically modified with the zwitterionic polymers were analyzed using an infrared spectrometer equipped with an attenuated total reflection (ATR) accessory (Nicolet iS50 FT-IR; Thermo Fisher Scientific, Waltham, MA, USA). The measuring conditions were as follows: detector, TGS; scan iterations, 512; resolution, 2 cm^{-1}; measuring range, 750–4000 cm^{-1}. The OMNIC software (Thermo Fisher Scientific) was used for peak analyses.

Ellipsometry measurement

The thickness of the zwitterionic copolymers immobilized to the silicon wafer substrates (25 mm × 25 mm) was measured under dry conditions using an ellipsometer (MARY-102SM-TR; Five Lab Co. Ltd., Saitama, Japan). The thickness was determined through curve fitting, which used the Cauchy method for a multi-layered model with reflectivity ratio (ψ) and phase difference (Δ) plots against the incident light (wavelength: 632.8 nm, HeNe laser). The refractive index of the copolymer layer was defined using the same method as that of the PMMA (1.491).[1,10] The data obtained from nine randomly selected points on the substrate were averaged, and measurements from three independent substrates were used to evaluate the significant differences.

Atomic force microscopy

The surfaces modified with zwitterionic copolymers were observed using atomic force microscopy (AFM, Nano Wizard II; JPK Instruments AG, Berlin, Germany) to evaluate the roughness and coverage of the copolymer layer. The measurement conditions of the tapping mode were as follows:

cantilever, FORT-20 (Spring constant k = 1.6 N/m; Applied Nanostructures Inc., CA, USA); scan rate, 100 nm/s; scan range, 30 × 30 μm.

Investigation of anti-biofouling properties

The anti-biofouling properties of the zwitterionic copolymer-modified surfaces were investigated through protein adsorption with bovine serum albumin (BSA; Sigma-Aldrich Inc., St. Loui, MO, USA) as a model protein and fibroblast adhesion using NIH3T3 cells (passage 154) as a model cell.

The protein adsorption was measured using the microBCA method on each substrate, which was previously developed by the authors,[28] and its detection limit is 22.4 ng/cm^2. The average amount of protein adsorption was determined from the results of ten independent substrates. The detailed procedures were in accordance with previous reports.[1,10,28,29]

To determine cell adhesion, the copolymer-modified substrates, sterilized using 70% ethanol solution, were immersed in PBS for 10 min for priming, and in minimal essential medium (MEM; Sigma-Aldrich Co.), which was supplemented with 10% fetal bovine serum (Nichirei Bioscience Inc., Tokyo, Japan) for 30 min. The NIH3T3 cells were seeded on the substrate at a density of 5 × 10^4 cells/cm^2 and incubated for 24 h at 37 °C and 5% CO_2 in an incubator. After removing the non-adhered and weakly-adhered cells by washing with the culture medium, the adherent cells were observed through a phase contrast microscope (IX-71; Olympus Corporation, Tokyo, Japan). Furthermore, the adherent cells were stained with Hoechst33342 (1 μL/mL medium) for 30 min and observed using a fluorescence microscope (IX-71; Olympus Corporation) for quantitative investigation. The cells in the randomly selected ten areas were counted and averaged.

Investigation of long-term durability

To investigate the long-term durability of the zwitterionic copolymers in aqueous solution, the substrate modified with zwitterionic copolymer was immersed in phosphate buffer saline (PBS) at 37 °C for 1, 2, 8, and 12 weeks, 1 year, and 2 years. Thereafter, the anti-biofouling property of the substrates was investigated through cell adhesion.[10] Particularly, the substrates immersed in PBS were carefully washed with water, ethanol, and acetone, and the cells were seeded on the substrates and incubated for 24 h using the previously described method. Furthermore, the substrates immersed in PBS at 70 °C were also investigated using a cell adhesion assay, as it was considered that the long-term durability of the copolymers could be inferred by a short-term evaluation under harsh conditions. The quantitative evaluation of cell adhesion was performed using the previously described method.

Statistical analysis

The data for the contact angle, polymer thickness, protein adsorption, and adherent cell density were all expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) was used to assess the statistical significance, and Turkey–Kramer's honestly significant difference (HSD) test was used for multiple comparisons. The difference was considered significant at $*p < 0.05$ and $**p < 0.01$. All the statistical analyses were performed using the JMP Pro 15.2 software (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Synthesis and characterization of sulfoisobutylbetaine acrylamide

This study aimed to synthesize a sulfoisobutylbetaine acrylamide (SBBAm) as a novel zwitterionic monomer, because acrylamide-based monomers can suppress hydrolytic side chain cleavage. Various biomaterials were developed using a zwitterionic monomer, carboxymethylbetaine methacrylate (CMBMA).[1,10,12,17,27] Although a methacrylamide monomer possessing a carboxymethylbetaine side chain (CMBMAm) was first attempted to be synthesized, the synthesis and purification of SBBAm was found to be simpler than that of CMBMAm, and therefore more suitable for industrial applications. For the reaction, dimethylaminopropyl acrylamide (DAPAm) and 2,4-butanesultone (BuS) were dissolved in acetone, and SBBAm was precipitated owing to its insolubility in acetone. Therefore, the purification of SBBAm was relatively easy as it only required simple washing with acetone.

Sulfopropylbetaine methacrylate (SPBMA) is a zwitterionic monomer with a sulfobetaine side chain. SBBAm is similar to SPBMA; however, it has an isobutyl linker between the sulfonyl and quaternary ammonium groups. This is because of the promotion of charge proximity in the side chain based on the steric hindrance of the methyl groups of the linker. The effects of the isobutyl linker are discussed in the last section of the Results and Discussion (refer to section *Effectiveness of 2-butyl group in sulfoisobutylbetaine side chain*).

The reaction of DAPAm and BuS progressed explosively and generated heat; therefore, BuS was slowly added into the acetone solution containing DAPAm, maintained under 0 °C, while stirring vigorously. Although the white precipitate was carefully washed with acetone after the reaction, ^{1}H NMR analysis indicated that some unreacted BuS remained in the precipitate. Therefore, the product dissolved in methanol was reprecipitated by acetone. The results of ^{1}H NMR indicated that the purity of the SBBAm was satisfactorily high (over 98 %; **Figure S1**), as was its yield (92.4 ± 2.2 %). These results indicate that SBBAm could be synthesized simply and easily and that this procedure could be adapted for industrial applications.

Synthesis of zwitterionic polymers with silane coupling agents

Surfaces modified with a copolymer of SBBAm and MPTMS with a silane coupling side chain were evaluated to investigate their anti-biofouling property and long-term durability. The composition of the SBBAm and MPTMS was varied from the feeding ratio of the polymerization because of the differences in the polymerization behavior of acrylamide in the SBBAm and the methacrylate in MPTMS. Therefore, seven types of copolymers with SBBAm and MPTMS (PSBBAmX; X is the feeding ratio of SBBAm) were synthesized, and the optimum composition for the PSBBAm copolymer for surface coating was determined (**Table 1**). PSBBAm50 formed a gel in the middle of the polymerization process, probably owing to the crosslinking between the silane coupling groups in the side chains; therefore,

it was excluded from the evaluation. The control samples against PSBBAm*X*, PSPB90, PCMB90, and PMPC90 containing 10 mol % of MPTMS were used in this study because previous investigations indicated that PSPB90, PCMB90, and PMPC90 are the optimum composition.[1]

The molecular weights of these polymers were evaluated using viscosity measurements with the Ubbelohde viscometer. As these copolymers possess silane coupling side chains, they can bind to the column packing materials for the GPC measurements. The viscosity average molecular weights (M_v) for all the copolymers were determined to be similar. Therefore, it was inferred that no differences existed in the thickness, coverage, and roughness that resulted from the molecular weight of the copolymers.

Characterization of surfaces modified with zwitterionic copolymers

At first, the average thickness of each copolymer layer immobilized on the substrate was evaluated using ellipsometry (**Figure 1**). Furthermore, the roughness and coverage of the zwitterionic copolymer-modified surfaces were investigated using AFM (**Figure 2** and **Figure S1** in **Supporting Information**).

The thickness of the PSBBAm*X* increased as the SBBAm composition in the polymer chain decreased. In particular, the thickness of the surface with PSBBAm60 was significantly larger than that of the other zwitterionic copolymer-modified surfaces. The thickness of PSBBAm90, PCMB90, PSPB90, and PMPC90 was almost the same, approximately 3 nm, and these data are in agreement with previous literature [Nishida]. Conversely, the thickness of the surface with PSBBAm95 and PSBBAm99 was very thin, below 2 nm. On copolymer-modified surfaces measured by AFM, the surfaces modified with PSBBAm60 (**Figure 2a**), PSBBAm70 (**Figure 2b**), PSBBAm80 (**Figure S1a**), PSBBAm90 (**Figure S1b**), PSPB90 (**Figure S1d**), PCMB90 (**Figure S1e**), and PMPC90 (**Figure S1f**) exhibited a dense roughness compared with bare silicon surface (**Figure 2d**), indicating that their surfaces were densely covered with the copolymers. In contrast, on the surfaces with PSBBAm95 (**Figure 2c**) and PSBBAm99 (**Figure S1c**), the roughness derived from the copolymers was scattered, indicating that the glass surface was partially exposed.

From the results of ellipsometry and AFM measurements, the following was determined. The surface modified by copolymers with a high MPTMS content, particularly PSBBAm60, was completely covered; however, it was multi-layered. This is probably owing to the crosslinking between the silane coupling side chains that remain unreacted to the glass substrate. In a previous work[12], the surface of the CMB-polymer monolayer was constructed by modifying the titanium surface with Phosmer PE (methacrylic penta(ethylene glycol) phosphate) and polymerizing CMB on its surface. It was indicated that the thickness of this surface was 4.4 ± 1.0 nm and had high coverage. Considering the difference in the linker length between Phosmer PE and MPTMS, if the copolymer surface comprising MPTMS is monolayered, it is presumed that the thickness is approximately 3–4 nm. In fact, the thickness of the surfaces modified with PSBBAm70, PSBBAm80, PSBBAm90, PCMB90, PSPB90, and PMPC90 was 2.9–4.6 nm, and these surfaces were assumed to be covered by a monolayer. Furthermore, the AFM measurements indicated that the surface coverage of copolymer-modified surfaces possessing a thickness of over 3 nm was really high. Conversely, the thickness measurements and AFM image clearly indicated that the surface with PSBBAm95 and 99 had low coverage, probably owing to the low contents of silane coupling side chains.

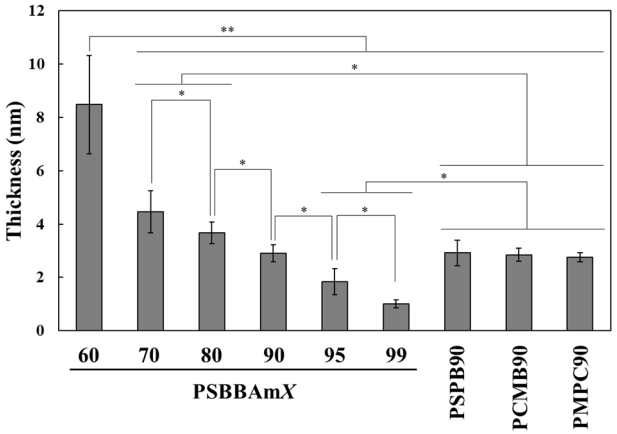


Figure 1. Thickness of the surfaces modified with various zwitterionic polymers that have silane-coupling agents as side chains. Significant difference: * $p < 0.05$, ** $p < 0.01$.

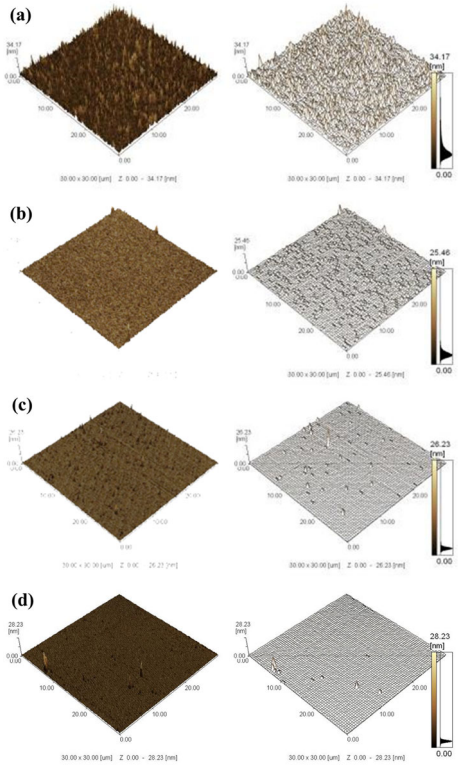


Figure 2. AFM images of zwitterionic polymer-modified surfaces. (a) PSBBAm60, (b) PSBBAm70, (c) PSBBAm80, (d) PSBBAm90, (e) PSBBAm95, (f) PSBBAm99, (g) PSPB90, (h) PCMB90, (i) PMPC90, and (j) bare glass.

The wettability of the surface modified with PSBBAm*X* was investigated by measuring the water and air contact angles. The water contact angle decreased as the composition of SBBAm increased in the copolymer, indicating that the copolymer surfaces with high SBBAm content exhibited high wettability (**Figure 3A)**. In particular, the wettability of the surface modified with PSBBAm90 (14.6 ± 2.2°) was similar to that with PSPB90 (13.8 ± 2.3°). Additionally, the water contact angles on the surfaces modified with PSBBAm95 and PSBBAm98 were small and demonstrated a similar tendency as those of PCMB90 and PMPC90.[1,17] The water contact angles of the surfaces modified with copolymers having low SBBAm content increased because the silane coupling groups, which did not react to the glass substrate, remained on the surface.

Next, the sum of the water and air contact angles of the copolymer-modified surfaces (**Figure 3B**) was evaluated. The sum of the contact angles of the surface modified with PSBBAm60, PSBBAm70, and PSBBAm80 was significantly greater than 180°, indicating that the structure of the copolymer layer changed under the dry and wet conditions. That is, it is inferred that the zwitterionic groups are exposed to water on the surface with PSBBAm60, PSBBAm70, and PSBBAm80.

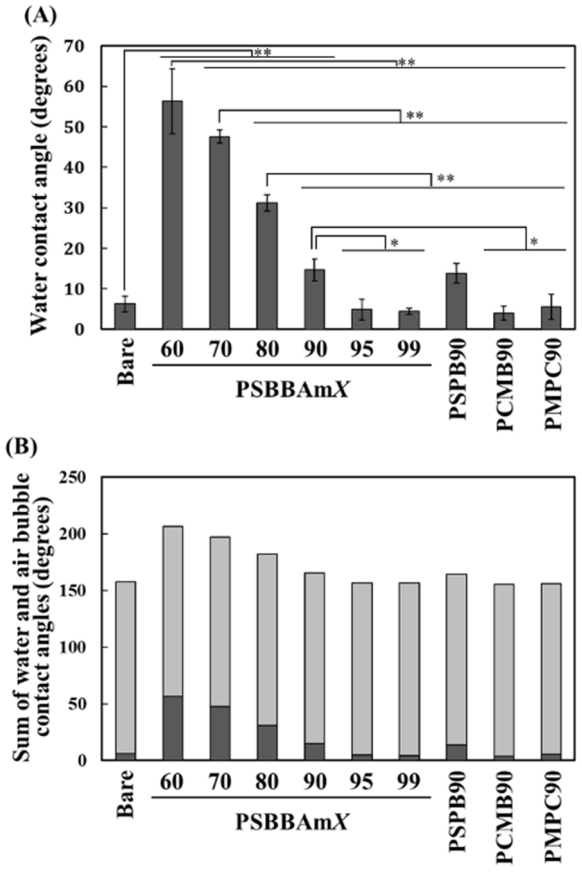


Figure 3. (A) Water contact angles and (B) sum of the water and air bubble contact angles for the surfaces chemically-modified with various zwitterionic copolymers. Significant difference: * $p < 0.05$, ** $p < 0.01$.

The chemical composition of the polymer-modified surface was also evaluated using ATR-IR spectroscopy. The peak of the stretching vibrations derived from S=O (peak top: 1078 cm^{-1}, * in **Figure 4**) in the SBBAm side chain increased with increasing SBBAm composition (**Figure 4** spectra a–d). Conversely, the peak derived from S=O on the surface with PSBBAm95 and PSBBAm98 drastically decreased despite the increase in SBBAm composition in the copolymer (**Figure 4** spectra e–f**)**. The results are in good agreement with those of the surface thickness of the copolymer layer, as the PSBBAm95 and PSBBAm98 surfaces are suggested to have a negligible amount of polymers immobilized on their surfaces. Moreover, the surfaces with PBSP90 (spectrum g) and PCMB90 (spectrum h) exhibited the peak of the stretching vibrations derived from S=O (peak top = 1078 cm^{-1}, * in **Figure 4**) and C=O (peak top = 1108 cm^{-1}, # in **Figure 4**), respectively. Furthermore, the surface with PMPC90 demonstrated the peak of P=O stretching vibration (peak top = 1153 and 1228 cm^{-1}, $ in Figure 2B). These were in good agreement with previous results,[1,30,31] indicating that the surfaces of the control samples were covered with the existing zwitterionic copolymers.

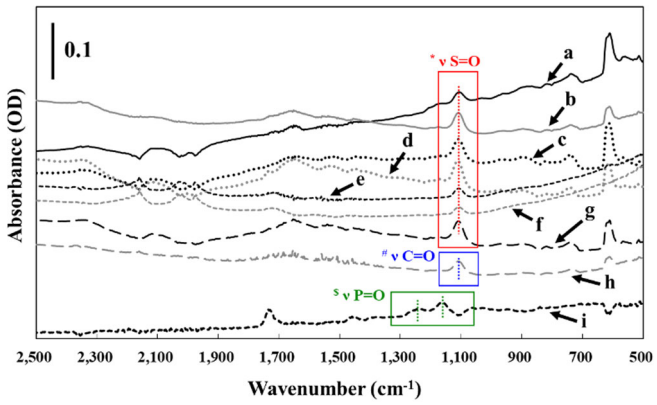


Figure 4. IR spectra of the surfaces modified with PSBBAm60 (a), PSBBAm70 (b), PSBBAm80 (c), PSBBAm90 (d), PSBBAm95 (e), PSBBAm99 (f), PSPB90 (g), PCMB90 (h), and PMPC90 (i). (red *) The peak 1070–1150 cm^{-1} (peak top = 1110 cm^{-1}) represents the S=O stretching vibration of sulfonate groups in PSBBAm and PSPB. (blue #) The peak 1064–1150 cm^{-1} (peak top = 1105 cm^{-1}) represents the C=O stretching vibration of the carbonyl group in PCMB. (green $) The peaks 1100–1290 cm^{-1} (peak tops = 1162 and 1242 cm^{-1}) represent the P=O stretching vibration of the phosphoryl group in PMPC.

Anti-biofouling property of zwitterionic polymer-modified surfaces in the short term

Protein adsorption to the zwitterionic copolymer-modified surfaces was evaluated using the microBCA method. The surfaces with PSBBAm60 and PSBBAm70 significantly suppressed the adsorption of proteins, and the level of suppression was higher compared with PSPB90, PCMB90, and PMPC90 (**Figure 5)**. This was probably because of the thickness of the PSBBAm60 and PSBBAm70 layers, which was greater than

that of the surface layers of PSPB90, PCMB90, and PMPC90. That is, the surfaces with PSBBAm60 and PSBBAm70 had soft interfaces. Notably, the thickness of the surface with PSBBAm90 was almost the same as that of the surfaces with PSPB90, PCMB90, and PMPC90, whereas the amount of protein adsorption to the surface with PSBBAm90 was larger than that with PSPB90, PCMB90, and PMPC90-modified surfaces. We hypothesized that this is because the surfaces with the PSBBAm copolymer were relatively hard, due to hydrogen bonding between the amide groups.

Additionally, the anti-biofouling property of the SBBAm copolymers was investigated using fibroblast adhesion (**Figure 6**). The cell adhesion to the surfaces modified with PSBBAm60–80 was suppressed to an extent equal to or higher than that with PSPB90, PCMB90, and PMPC90. In contrast, most cells adhered to the surfaces with PSBBAm95 and PSBBAm99, compared to the surfaces with the other zwitterionic copolymers. The trend of this result is consistent with that of the protein adsorption results. However, the amount of protein adsorption on the surfaces with PSBBAm95 and PSBBAm99 was similar to that with the glass substrate, whereas cell adhesion on these surfaces significantly decreased compared with that on the bare glass surface. This is because of the excluded volume effect of polymers partially immobilized on the surface. The results of the protein adsorption and cell adhesion analyses showed that the surfaces with PSBBAm60 and PSBBAm70 possess high levels of the anti-biofouling property. Although the optimum composition for the existing zwitterionic monomers (PSPB, PCMB, and PMPC) and MPTMS was 90:10, for the copolymer of SBBAm and MPTMS, the copolymer with a slightly larger MPTMS ratio exhibited excellent anti-biofouling property. It was inferred that the high levels of the anti-biofouling property on the PSBBAm60- and PSBBAm70-modified surfaces were because “their chemical properties were derived from the zwitterionic units” and “their physical properties were derived from a large thickness”. In previous works,[10,18] the authors indicated that the anti-biofouling property disappeared on hard surfaces even though they were covered with zwitterionic groups. PSBBAm is an acrylamide-based polymer, and the amide groups in the polymer chains form hydrogen bonds with the neighboring amide groups. Therefore, it is inferred that the main chains of the acrylamide-based polymer are comparatively harder than those of the methacrylate-based polymers. Consequently, the PSBBAm-modified surface becomes a hard interface compared with the surfaces of the PSPB, PCMB, and PMPC. The PSBBAm-modified surfaces require sufficient thickness to express the anti-biofouling property. To evaluate the long-term durability of the PSBBAm-modified surface, the SBBAm70-modified surface was used as an optimum, because the silane coupling groups that remain on the SBBAm60-modified surface may affect the anti-biofouling property.

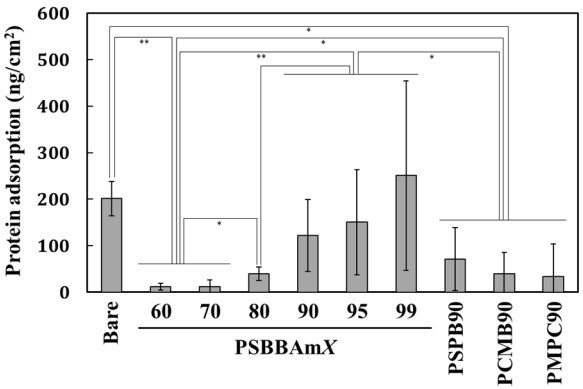


Figure 5. Quantitative evaluation of protein adsorption to surfaces modified with PSBBAm60, PSBBAm70, PSBBAm80, PSBBAm90, PSBBAm95, PSBBAm99, PSPB90, PCMB90. and PMPC90 (n = 9). Significant difference: *$p < 0.05$, **$p < 0.01$. As a positive control, the protein adsorption to bare glass surface was also investigated in this evaluation.

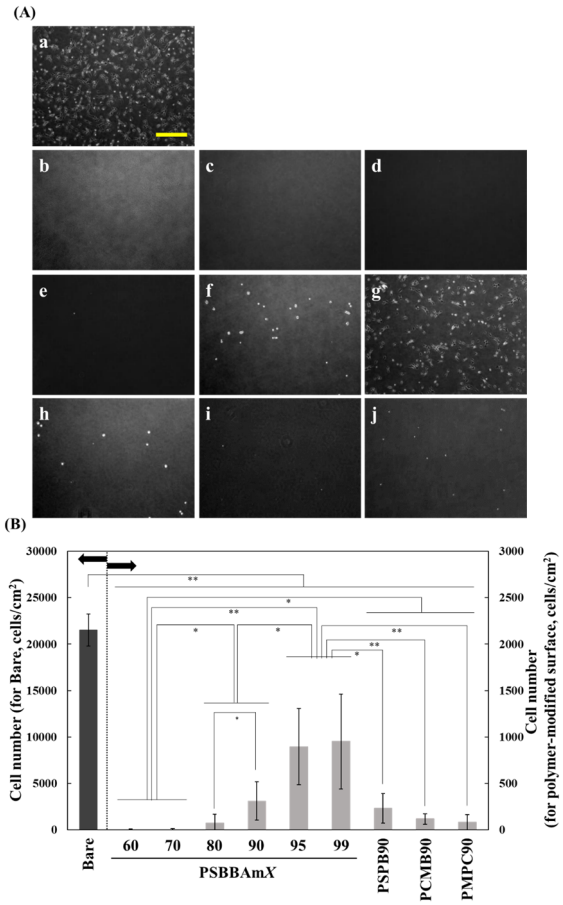


Figure 6. (A) Phase contrast images of cells cultured for 3 days on (a) a bare glass surface and surfaces modified with (b) PSBBAm60, (c) PSBBAm70, (d) PSBBAm80, (e) PSBBAm90, (f) PSBBAm95, (g) PSBBAm99, (h) PSPB90, (i) PCMB90, and (j) PMPC. Scale bar: 500 μm. **(B)** The number of cells adhered to bare glass and zwitterionic polymer-modified surfaces (n = 6). Significant difference: *$p < 0.05$, **$p < 0.01$.

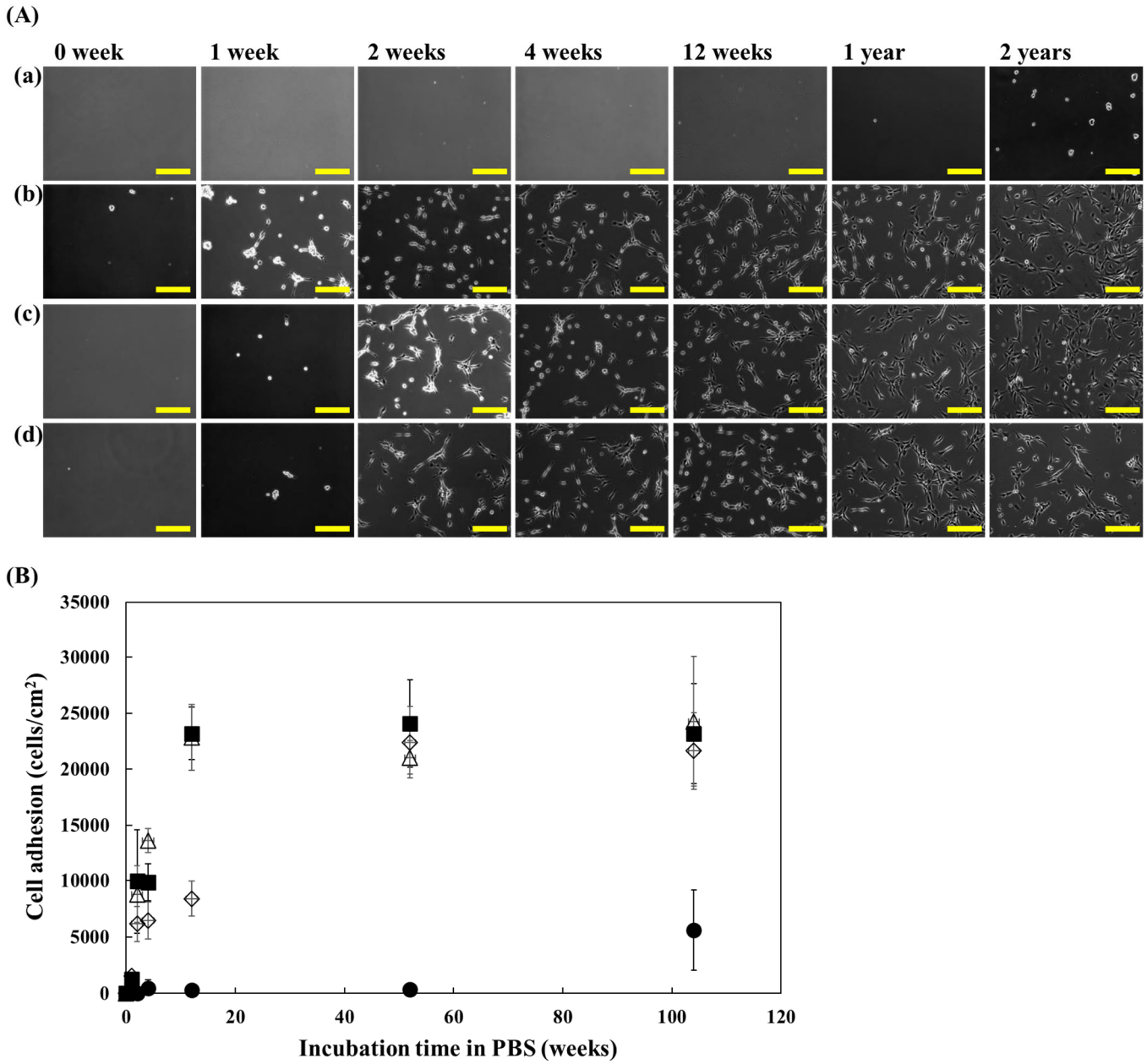


Figure 7. Durability evaluation of PSBBAm70-, PSPB90-, PCMB90-, and PMPC90-modified surfaces. Substrates modified with PSBBAm70, PSPB90, PCMB90, and PMC90 were incubated for 1, 2, 4, 8, and 12 weeks in PBS at 37 °C. The cells were then seeded on the substrates and cultured for 1 day. (A) Phase contrast images of cells cultured for 1 day on (a) PSBBAm70-, (b) PSPB90-, (c) PCMB90- and (d) PMPC-modified surfaces incubated in PBS at 37 °C. (B) The number of cells adhered to the surfaces with (●) PSBBAm70-, (■) PSPB90-, (△) PCMB90- and (◊) PMPC90-incubated in PBS at 37 °C (n = 6).

Long-term durability of anti-biofouling property in zwitterionic polymers

The long-term durability of surfaces modified with PSBBAm70 was evaluated by incubating for various durations in PBS. The surfaces modified with PSPB90, PCMB90, and PMPC90 were used as the control samples. The substrates were immersed for 1, 2, 4, 12, 52, and 104 weeks in PBS at 37 °C, and the maintenance of the anti-biofouling properties of these substrates was investigated using a cell adhesion assay (**Figure 7**).

The results indicated that the cells adhered to PSPB90-, PCMB90-, and PMPC90-modified surfaces incubated for 1 week in PBS. For these three samples, cell adhesion increased with increasing incubation period in PBS. Almost all the cells seeded on the substrate adhered to the PSPB90-, PCMB90-, and PMPC90-modified surfaces incubated for 52 weeks in PBS. Conversely, the anti-biofouling property of the PSBBAm70-modified surface was maintained when incubated for 1 year in PBS. This was because the hydrolytic cleavage of the zwitterionic side chains was suppressed by (1) extremely slow hydrolysis of amide groups compared to the ester groups[23] and (2) the formation of hydrogen bonding between the amide

groups in the acrylamide backbone. However, the cells slightly adhered to the surface with PSBBAm70 when incubated for 1 year and 2 years in PBS (Average cell density: (1 year) 339 ± 129 cells/cm^2, and (2 years) 5603 ± 3581 cells/cm^2). This is presumably because a part of the SBBAm copolymer detached from the surface because of the hydrolysis of the ester groups in the MPTMS. To support our speculation, the disappearance of copolymers was investigated using XPS and ATR-IR measurements. Unfortunately, the data for the PSBBAm70-modified surface incubated for 1 year were almost the same as those for the fresh surface with PSBBAm70, indicating that our surmise could not be confirmed. Although we tried to synthesize an acrylamide monomer with the silane coupling side chain, the target monomer could not be obtained. Therefore, the above consideration is a topic for speculation. Nevertheless, this study clarifies that the SBBAm polymer maintained the anti-biofouling property over a long period.

We aimed to develop a novel coating material that maintains its anti-biofouling properties for several years. Consequently, a method was evaluated to reduce the overall time required to assess the long-term durability of the anti-biofouling property. Particularly, the substrates modified with zwitterionic copolymers were incubated in PBS at 70 °C, and these samples were then investigated using a cell adhesion assay (**Figure S2**). Low levels of cell adhesion were observed on the surfaces modified with PSBBAm70 incubated in PBS at 70 °C for 4 weeks. Additionally, the number of cells that adhered to the surface modified with PSBBAm70 incubated in PBS at 70 °C for 12 weeks (**Figure S2**, average cell density: 268 ± 26 cells/cm^2) was similar to that of the PSBBAm70-modified surface incubated in PBS at 37 °C for 1 year. From these results, it was estimated that the time required to investigate the long-term durability of zwitterionic copolymers could be reduced by approximately 2.5–3 times. Future studies will focus on investigating the long-term durability for a few years using high-temperature incubations.

The current results clearly indicate that the novel zwitterionic polymer with an acrylamide backbone could continuously express the anti-biofouling property over a long period. To the best of our knowledge, SBBAm is the first zwitterionic monomer ever reported to have bio-inactivity and long-term durability, and will contribute to the further development of biomaterials and anti-biofouling materials. The currently used MPC and CMB monomers are excellent and important for manufacturing materials that require anti-biofouling properties. This was evidenced by this investigation, which demonstrated that the surfaces modified with PMPC and PCMB exhibited high anti-biofouling property levels for short periods (**Figure 5–7)**. However, the findings indicate that a better choice of zwitterionic monomers could also be developed to cater to application needs and incorporate factors such as fabrication cost, characterization of the coating surfaces (e.g., thickness and stiffness), and stability and duration of the anti-biofouling property.

Effectiveness of the 2-methyl group in the sulfoisobutylbetaine side chain

A sulfopropylbetaine polymer (PSPB) becomes insoluble in water depending on the increase in molecular weight, and only dissolves in salt solution. Furthermore, PSPB with high molecular weight cannot be dissolved in salt solution. It has often been highlighted that this is owing to the aggregation of polymers with associations between the zwitterionic groups.[24,25,32] To suppress the ionic association between neighboring zwitterionic groups, SBBAm with an isobutyl group between the sulfonyl and quaternary ammonium groups was designed.

To clarify the validity of the design concept for SBBAm, the water solubility of the SBBAm homopolymer was investigated by comparing it with that of a sulfopropylbetaine acrylamide (SPBAm) homopolymer. SPBAm was synthesized using a similar method as that of SBBAm, with propanesultone instead of 2-butanesultone (**Figure S3**). The viscosity average molecular weight for the SBBAm and SPBAm homopolymers synthesized with the free radical polymerization was 5.0×10^4 and 3.6×10^4, respectively. The SBBAm homopolymer completely dissolved in water, whereas the aqueous solution containing the SPBAm homopolymer became clouded (**Figure S4)**. Consequently, it was inferred that the water solubility of the SBBAm homopolymer was enhanced, probably because of (1) the difference in conformation of the zwitterionic group associated with the existence of the methyl group and (2) the suppression of the interaction between the neighboring zwitterionic groups ascribable to the steric hindrance of the methyl group. To support our assumption of (1), the distance between sulfonyl and quaternary ammonium groups was evaluated using a molecular model (**Figure S7**). The results indicated that the distance between the sulfonyl and quaternary ammonium groups of SBBAm became shorter compared with that of SPBAm. Additionally, to demonstrate the enhancement of the anti-biofouling property by the proximity of oppositely charged ions and steric hindrance in the side chain, the cell adhesion on the PSBBAm70-modified surfaces was compared with that of PSPBAm70-modified surfaces, which was polymerized with SPBAm and MPTMS at a molar ratio of 7:3. The surface conditions for PSPBAm70 were the same as those for PSBBAm70, as shown in **Figure S5**. For the PSBBAm70- and PSPBAm70-modified substrates incubated for 1–4 weeks in PBS at 37 °C, cell adhesion was investigated by evaluating the maintenance of the anti-biofouling property. Cell adhesion was significantly suppressed on both copolymer-coated surfaces, and the anti-biofouling property was maintained for 4 weeks (**Figure S6**). However, a few cells adhered to the surface modified with PSPBAm70. This indicates that the anti-biofouling property on the surface with the PSPBAm copolymer slightly disappeared, partly because of the aggregation of the copolymer chains, depending on the association between the sulfopropylbetaine groups.[24,25,32] These results clearly indicate that the steric hindrance of the branched methyl group is important for the suppression of the proximity and aggregation between the zwitterionic side chains. Therefore, it was

demonstrated that the design concept used for the SBBAm was reasonable.

Finally, the construction of the carboxymethylbetaine methacrylamide (CMBAm, **Figure S8**) was evaluated as well, which required a complicated purification process and had a low yield. The expression of the anti-biofouling property was investigated using the PCMBAm70-modified substrate, which was polymerized with CMBAm and MPTMS at a molar ratio of 7:3. The preliminary results showed that the PCMBAm-modified surface possessed anti-biofouling property (**Figure S9)**. If an easy method for the synthesis of CMBAm can be developed, it can be expected as another zwitterionic monomer for use in novel biomaterial manufacturing.

Conclusions

We developed a novel zwitterionic monomer, sulfoisobutylbetaine acrylamide (SBBAm), for use in the construction of anti-biofouling materials, as it enables their functional properties to be maintained over a long period. Surfaces chemically modified with SBBAm copolymer demonstrated both anti-biofouling property in the short term and a yearly high durability. This was because the hydrolytic cleavage of zwitterionic side chains was suppressed owing to the introduction of an acrylamide backbone. Furthermore, the steric hindrance of the methyl group derived from an isobutyl linker between the sulfonyl and quaternary ammonium groups promoted both the proximity of positive and negative charges in the zwitterionic side chains and the suppression of neighboring sulfobetaine side group aggregation, which are also considered to contribute significantly to the anti-biofouling property and high durability. Although MPC and CMB polymers are excellent anti-biofouling materials, the SBBAm-based polymer is a new candidate that expands the current options for designing and customizing anti-biofouling materials to fit specific objectives, applications, and cost-performance aims. The results of this investigation will likely have a significant impact on academic and industrial fields, as SBBAm and its polymer can be used to generate bio-inactive materials with long-term durability, which was previously not possible.

Author Contributions

Ryoma Takagi: Methodology, Validation, Investigation, Formal analysis, Data curation, Writing – original draft, Visualization.

Ayaka Moroto: Investigation, Formal analysis, Validation, Data curation.

Toshikazu Yamamoto: Methodology, Investigation, Validation, Formal analysis.

Tadashi Nakaji-Hirabayashi: Conceptualization, Project administration, Supervision, Formal analysis, Writing – review & editing, Funding acquisition.

Chiaki Yoshikawa: Conceptualization, Methodology, Writing – review & editing.

Hiromi Kitano: Conceptualization, Supervision, Writing – review & editing.

Shinpei Yamamoto: Conceptualization, Investigation.

Yoshiyuki Saruwatari: Conceptualization, Investigation, Writing – review & editing.

Data Availability Statement

All data supporting the findings of this study are available within the article and its Supplementary Information files.

Conflicts of interest

In this research, there are no conflicts to declare.

Acknowledgements

This study was supported by the Grant-in-Aid (17KK01130, 18K19907, 22H03951) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Tokyo, Japan; the Hisaharu AME Charitable Research Foundation, Toyama, Japan; the Academic Research Grant from the Yamaguchi Educational and Scholarship Foundation; and THE HOKURIKU BANK Grant-in-Aid for Young Scientists. Additional support was provided by the Joint Research Hub Program of the National Institute for Materials Science (NIMS) and by JST SPRING (Grant Number JPMJSP2145). The authors are also grateful to Editage for English language editing.

References

1 M. Nishida, T. Nakaji-Hirabayashi, H. Kitano, K. Matsuoka and Y. Saruwatari, *J. Biomed. Mater. Res. A*, **2016**, *104*(8), 2029–2036.
2 K. Ishihara, T. Kitagawa and Y. Inoue, *ACS Biomater. Sci. Eng.*, **2015**, *1*(2), 103–109.
3 W. Zhao, Y. Zhu, J. Zhang, T. Xu, Q. Li, H. Guo, J. Zhang, C. Lin and L. Zhang, *J. Mater. Sci.*, **2018**, *53*(19), 13813–13825.
4 Y. Wei, J. Zhang, X. Feng and D. Liu, *J. Biomater. Sci. Polym. Ed.*, **2017**, *28*(18), 2101–2116.
5 Y. Higaki, K. Hatae, T. Ishikawa, T. Takanohashi, J.-I. Hayashi and A. Takahara, *ACS Appl. Mater. Interfaces*, **2014**, *6*(22), 20385–20389.
6 T. Nakano, S. Ando, N. Takata, M. Kawada, K. Muguruma, K. Sekiguchi, K. Saito, S. Yonemura, M. Eiraku and Y. Sasai, *Cell Stem Cell*, **2012**, *10*(6), 771–785.
7 S. Tada, C. Inaba, K. Mizukami, S. Fujishita, M. Gemmei-Ide, H. Kitano, A. Mochizuki, M. Tanaka and T. Matsunaga, *Macromol. Biosci.*, **2009**, *9*(1), 63–70.
8 X. Lin, K. Fukazawa and K. Ishihara, *ACS Appl. Mater. Interfaces*, **2015**, *7*(31), 17489–17498.
9 S. Fujishita, C. Inaba, S. Tada, M. Gemmei-Ide, H. Kitano and Y. Saruwatari, *Biol. Pharm. Bull.*, **2008**, *31*(12), 2309–2315.
10 Y. Yamazawa, H. Kato, T. Nakaji-Hirabayashi, C. Yoshikawa, H. Kitano, K. Ohno, Y. Saruwatari and K. Matsuoka, *J. Mater. Chem. B*, **2019**, *7*(27), 4280–4291.
11 K. Ishihara, K. Mitera, Y. Inoue and K. Fukazawa, *Colloids Surf. B Biointerfaces*, **2020**, *194*, 111205.
12 M. Nishida, T. Nakaji-Hirabayashi and H. Kitano, *Colloids Surf. B Biointerfaces*, **2017**, *152*, 302–310.
13 I. Dueramae, M. Nishida, T. Nakaji-Hirabayashi, K. Matsumura and H. Kitano, *J. Mater. Chem. B*, **2016**, *4*(32), 5394–5404.

14 K. Ishihara, N. P. Ziats, B. P. Tierney, N. Nakabayashi and J. M. Anderson, *J. Biomed. Mater. Res.*, **1991**, *25*(11), 1397–1407.
15 T. Ueda, A. Watanabe, K. Ishihara and N. Nakabayashi, *J. Biomater. Sci. Polym. Ed.*, **1991**, *3*(2), 185–194.
16 T. Moro, Y. Takatori, M. Kyomoto, K. Ishihara, M. Hashimoto, H. Ito, T. Tanaka, H. Oshima, S. Tanaka and H. Kawaguchi, *J. Orthop. Res.*, **2014**, *32*(3), 369–376.
17 H. Suzuki, L. Li, T. Nakaji-Hirabayashi, H. Kitano, K. Ohno, K. Matsuoka and Y. Saruwatari, *Colloids Surf. B Biointerfaces*, **2012**, *94*, 107–113.
18 K. Nomura, S. Mikuni, T. Nakaji-Hirabayashi, M. Gemmei-Ide, H. Kitano, H. Noguchi and K. Uosaki, *Colloids Surf. B Biointerfaces*, **2015**, *135*, 267–273.
19 H. Kitano, H. Suzuki, K. Matsuura and K. Ohno, *Langmuir*, **2010**, *26*(9), 6767–6774.
20 T. Kondo, K. Nomura, M. Gemmei-Ide, H. Kitano, H. Noguchi, K. Uosaki and Y. Saruwatari, *Colloids Surf. B Biointerfaces*, **2014**, *113*, 361–367.
21 K. Ishihara, M. Mu, T. Konno, Y. Inoue and K. Fukazawa, *J. Biomater. Sci. Polym. Ed.*, **2017**, *28*(10–12), 884–899.
22 M. Mukai, D. Ihara, C.-W. Chu, C.-H. Cheng and A. Takahara, *Biomacromolecules*, **2020**, *21*(6), 2125–2131.
23 M. L. Bender, *Chem. Rev.*, **1960**, *60*, 53–113.
24 L. Xu, P. Ma, Q. Chen, S. Lin and J. Shen, *Prog. Chem.*, **2014**, *26*(2–3), 366–374.
25 G. T. M. Silva and F. H. Quina, *Curr. Opin. Colloid Interface Sci.*, **2019**, *44*, 168–176.
26 T. Nakaji-Hirabayashi, Y. Saruwatari, K. Matsuoka and S. Yamamoto, *JP Patent*, 2020-108234, **2020**.
27 L. Li, T. Nakaji-Hirabayashi, H. Kitano, K. Ohno, T. Kishioka and Y. Usui, *Colloids Surf. B Biointerfaces*, **2016**, *144*, 180–187.
28 T. Nakaji-Hirabayashi, K. Kato, Y. Arima and H. Iwata, *Biomaterials*, **2007**, *28*(24), 3517–3529.
29 T. Nakaji-Hirabayashi, K. Kato and H. Iwata, *Bioconjugate Chem.*, **2009**, *20*(1), 102–110.
30 S. Tone, M. Hasegawa, L. Puppulin, G. Pezzotti and A. Sudo, *Acta Biomater.*, **2018**, *66*, 157–165.
31 Y. Zhu, R. Batchelor, A. B. Lowe and P. J. Roth, *Macromolecules*, **2016**, *49*(2), 672–680.
32 Z. Li, B. Hao, Y. Tang, H. Li, T. C. Lee, A. Feng, L. Zhang and S. H. Thang, *Eur. Polym. J.*, **2020**, *132*, 109704.