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Conjugation of mono-sulfobetaine to alkyne-PPX films via click reaction to reduce cell adhesion

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Abstract. A surface resisting protein adsorption and cell adhesion is highly desirable for many biomedical applications such as diagnostic devices, biosensors and blood-contacting devices. In this study, a surface conjugated with sulfobetaine molecules was fabricated via the click reaction for the anti-fouling purpose. An alkyne-containing substrate (Alkyne-PPX) was generated by chemical vapor deposition of 4-ethynyl-[2,2]paracyclophane. Azide-ended mono-sulfobetaine molecules were synthesized and then conjugated on Alkyne-PPX via the click reaction. The protein adsorption from 10% serum was reduced by 57%, while the attachment of L929 cells was reduced by 83% onto the sulfobetaine-PPX surface compared to the protein adsorption and cell adhesion on Alkyne-PPX. In conclusion, we demonstrate that conjugation of mono-sulfobetaine molecules via the click chemistry is an effective way for reduction of non-specific protein adsorption and cell attachment.

Keywords: anti-fouling; sulfobetaine; cell adhesion; protein adsorption; click chemistry

1. Introduction

Anti-fouling surfaces are highly in demand for some biomedical devices, such as diagnostic devices, biosensors and blood-contacting devices, in order to prevent detrimental clinical complications or device malfunction that are initiated by non-specific attachment of proteins/cells (Ratner 1993). One of the key characteristics of anti-fouling surfaces is the ability to attract tightly bound water molecules. A common strategy for creating anti-fouling surfaces is decorating a surface with hydrophilic materials such as poly(ethylene glycol), poly(2-hydroxyethyl methacrylate), polyacrylamide, dextran and zwitterionic polymers (McArthur *et al*. 2000, Jiang and Cao 2010, Banerjee *et al*. 2011, Kuo *et al*. 2011, Zhao *et al*. 2011, Kuo *et al*. 2012, Liu *et al*. 2012). Recently, zwitterionic materials such as phosphorylcholine, sulfobetaine, and carboxybetaine, which are overall charge-neutral molecules containing both positively and

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negatively charged moieties, have attracted considerable attention for biomedical applications due to their excellent anti-fouling ability (Feng and Zhu 2005, Chang *et al*. 2006, Zhang *et al*. 2006, Jiang and Cao 2010, Ye *et al*. 2010, Kuo *et al*. 2011, Chien *et al*. 2013, Shen and Lin 2013, Diaz Blanco *et al*. 2014, Maeta and Ishihara 2014). The excellent anti-fouling property of zwitterionic materials is ascribed to their high water-binding capacity via electrostatic interactions (Jiang and Cao 2010).

Facile and versatile techniques for surface conjugation of zwitterionic molecules are crucial for their anti-fouling applications. Zwitterionic polymers could be deposited on surfaces physically or chemically via "graft-to" or "graft-from" methods (Jiang and Cao 2010). In general, covalent immobilization of zwitterionic molecules is more desirable for long-term stability. For example, polymerization of sulfobetaine methacrylate from surface-immobilized initiators creates an anti-fouling surface that exhibits undetectable protein adsorption (<0.3 ng cm^{-2}) (Zhang *et al*. 2006). However, many conjugation methods are substrate-dependent, tedious, and time-consuming. Thus, facial and versatile zwitterionization methods benefit the popularity of the anti-fouling applications in biomedical devices.

Chemical vapor deposition (CVD) of poly-*p*-xylylenes (PPX) possesses several unique features such as applicability to a wide range of materials such as polymers, silicon, glass, and ceramics, ultra-thin coating (20-100 nm), solvent-free environment, and the absence of cytotoxic initiators and plasticizers (Chen *et al*. 2008a, b). Thus, PPX coating is especially suitable for surface modification of biomedical devices. One important feature of this technique is that a variety of functionalized *p*-cyclophanes could be used for the fabrication of functional PPX coatings, which can serve as excellent platforms for covalent immobilization of molecules (Chen *et al*. 2008a, b).

Recently, we utilized amino-PPX substrates for conjugation of mono-zwitterionic molecules (Chien *et al*. 2014). Aldehyde-ended carboxybetaine molecules were synthesized and could be conjugated on the amino-PPX via the formation of imine bonds with the primary amines. The conjugation of aldehyde-ended carboxybetaine molecules reduces non-specific cell adhesion and protein adsorption. However, we found that the conjugation of carboxybetaine via the aldehyde-amine reaction is not satisfactorily effective, which deteriorates the applicability of this method. Therefore, we have been seeking more efficient conjugation chemistry in order to improve the conjugation of zwitterionic molecules.

The click chemistry represents a family of powerful and efficient chemical reactions that generate substances quickly and reliably by joining small units together (Kolb *et al*. 2001). The characteristics of click chemistry include modular, wide in scope, with very high chemical yields, relatively insensitive to solvents and pH, and stereospecific. The most widely used click reaction is Huisgen 1,3-dipolar cycloaddition between azides and alkynes in the presence of copper(I) catalyst, often referred as the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (Rostovtsev *et al*. 2002, Tornoe *et al*. 2002). Previously, sulfobetaine molecules containing azides have been conjugated efficiently on the polyurethane containing alkyne groups via the click chemistry (Huang and Xu 2011). Therefore, CuAAC has been applied to bio-functionalization of biomaterial surfaces.

Nandivada *et al.* previously synthesized alkyne-containing *p*-cyclophanes (4-ethynyl-[2,2]paracyclophane) and then created Alkyne-PPX coatings via CVD (Nandivada *et al*. 2006). They showed that Alkyne-PPX is a convenient platform for conjugation of azide-containing molecules via CuAAC. In this study, we utilized this platform and evaluated whether the conjugation efficacy of zwitterionic molecules could be enhanced via CuAAC. We synthesized azide-ended mono-sulfobetaine molecules and conjugated them on the Alkyne-PPX

coating via the click reaction. The anti-fouling capability of the sulfobetaine-modified substrates was then evaluated by protein adsorption and cell adhesion.

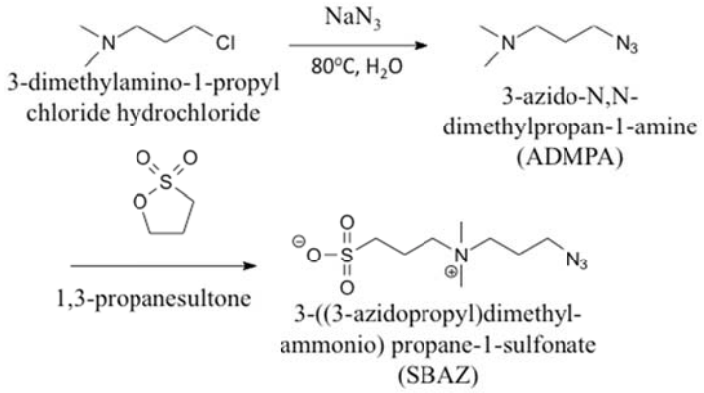
2. Materials and methods

2.1. Materials

Most of the reagents were purchased from Sigma-Aldrich (St. Louis, USA) unless specified otherwise and used as received: copper sulfate (cat# 209198), sodium ascorbate (cat# A4034), sodium azide (cat# S2002), 3-dimethylamino-1-propyl chloride hydrochloride (cat# D145203), and 1,3-propanesultone (cat# P50706). 4-ethynyl[2,2]paracyclophane was synthesized according to a previous protocol (Nandivada *et al*. 2006). L929 mouse fibroblast-like cells were received from Food Industry Research and Development Institute (Hsinchu, Taiwan). L929 cell culture medium contained alpha minimum essential medium (αMEM; HyClone, USA) and was supplemented with 10% fetal bovine serum (Biological Industry, USA), 2 mg/mL $NaHCO_3$, 0.5% fungizone (GIBCO, USA), 0.25% gentamycin (GIBCO) and 0.679% β-mercaptoethanol. Phosphate-buffered saline (PBS) was composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4.

2.2 Synthesis of 3-((3-azidopropyl)dimethyl-ammonio) propane-1-sulfonate)

3-((3-azidopropyl)dimethyl-ammonio) propane-1-sulfonate) was synthesized via a ring-opening reaction of 1,3-popanesultone by 3-azido-N, N-dimethylpropan-1-amine. The synthetic schemes of 3-azido-N, N-dimethylpropan-1-amine (ADMPA) and 3-((3-azidopropyl)dimethyl-ammonio) propane-1-sulfonate (SBAZ) are illustrated in Scheme 1. For the synthesis of ADMPA, sodium azide (2.71 g, 41.65 mmol) and 3-dimethylamino-1-propyl chloride hydrochloride (2.19 g, 13.88 mmol) were dissolved in 50 mL deionized water, and then allowed for 16-h reaction at 80°C. The product was precipitated by diethyl ether. The yield of ADMPA was ~68%. The final product was verified by ^{1}H NMR ($CDCl_3$; Bruker, 500 MHz) (Fig. 1(a)): δ (ppm): 2.32 (d, 2H, CH_2), 2.19 (m, 6H, CH_3), 1.74 (s, 2H, CH_2), 1.68 (m, 2H, CH_2).



Scheme 1 The synthetic route of 3-((3-azidopropyl)dimethylammonio) propane-1-sulfonate (SBAZ)

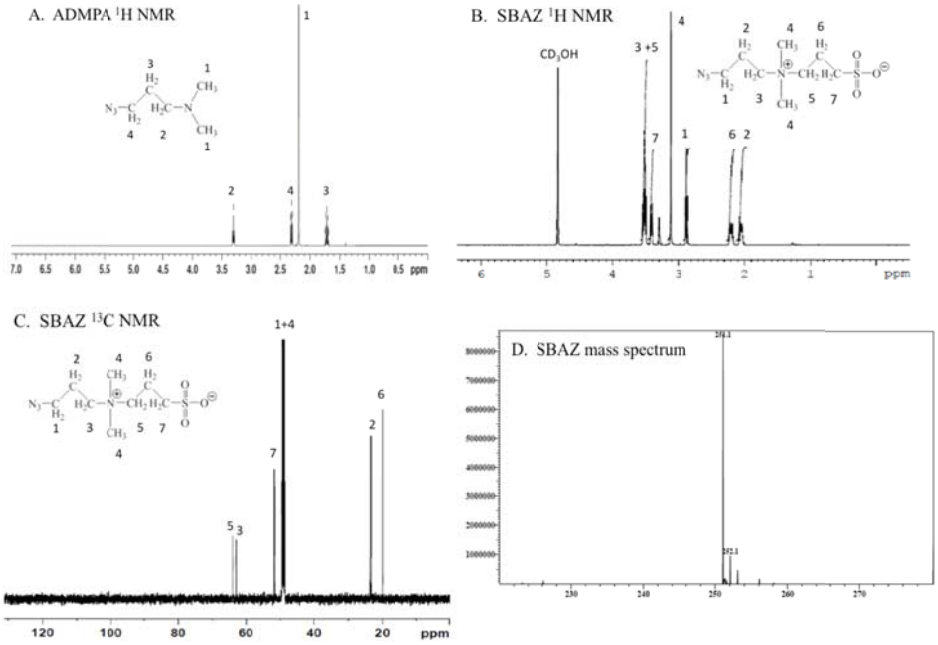


Fig. 1 Characterization of synthesized molecules: (a) ^{1}H NMR spectrum of 3-azido-N,N-dimethylpropan-1-amine (ADMPA); (b) ^{1}H NMR, (c) ^{13}C NMR and (d) mass spectra of 3-((3-azidopropyl)dimethyl-ammonio) propane-1-sulfonate) (SBAZ)

For the synthesis of SBAZ, ADMPA (1.0 g, 7.8 mmol) and 1,3-propanesultone (1.10 g, 9.0 mmol) were dissolved in 50 mL of anhydrous tetrahydrofuran and allowed for 8-h reaction at 0°C under N_2 atmosphere. The product was precipitated by ethyl acetate, followed by collection and drying. SBAZ were immediately stored in a desiccator at -20°C. The yield of SBAZ was ~48%. The final product was verified by ^{1}H NMR (in $CDCl_3$); δ (ppm): 3.51 (d, 2H, CH_2), 3.50 (d, 2H, CH_2), 3.41 (d, 2H, CH_2), 3.12 (d, 2H, CH_2), 2.08 (d, 2H, CH_2), 2.23 (d, 2H, CH_2), 3.29 (d, 6H, CH_3) (Fig. 1(b)), ^{13}C NMR (in $CDCl_3$): δ = 19.9, 23.4, 54.4, 51.5, 51.6, 62.9, and 63.9 ppm (Fig. 1(c)), and mass spectrometry (70eV, ESI-MS, Finnigan, USA): 251.1 [M^{+1}] 83.6% , 252.1 [M^{+1}] 11% , 253.1 [M^{+1}] 5.4 % (Fig. 1(d)).

2.3 Fabrication of SBAZ-modified surfaces

The fabrication of SBAZ-modified surfaces is illustrated in Fig. 2. Alkyne-PPX was deposited on substrates using CVD polymerization of 4-ethynyl-[2,2]paracyclophane (Nandivada *et al*. 2006). Alkyne-PPX was deposited on 96-well tissue culture polystyrene (TCPS) for the experiments of electron spectroscopy for chemical analysis (ESCA) and cell adhesion, on glass slides for water contact angle measurement, and on gold substrates for quantification of protein adsorption.

SBAZ was conjugated on Alkyne-PPX via CuAAC. Briefly, SBAZ was dissolved in deionized water to the final concentrations of 0.4, 4 and 20 mM or 0.1, 1 and 5 mg/mL, hereafter referred to as SBAZ_0.1, SBAZ_1 or SBAZ_5, respectively. Prior to CuAAC, the SBAZ solutions were

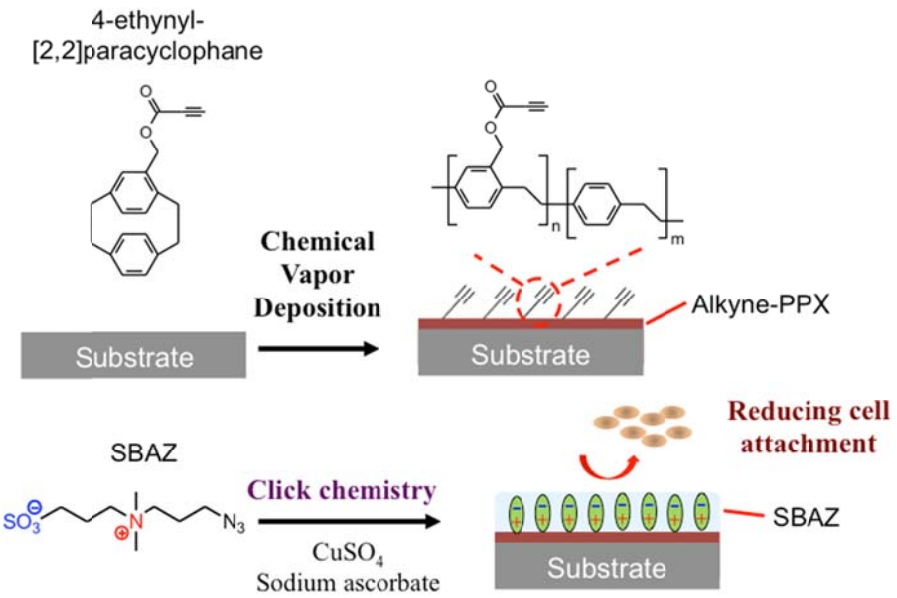


Fig. 2 Illustration of surface conjugation of 3-((3-azidopropyl)dimethyl-ammonio) propane-1-sulfonate) (SBAZ) to a alkyne-substrate. The substrate was first deposited with a thin film of poly(4-ethynyl-*p*-xylylene) (Alkyne-PPX) via chemical vapor deposition polymerization of 4-ethynyl-[2,2]paracyclophane. SBZA was then conjugated on Alkyne-PPX via the copper(I)-catalyzed azide-alkyne cycloaddition. The SBAZ-conjugated substrate is expected to reduce cell attachment

supplemented with copper sulfate and sodium ascorbate at the ratios of 1 and 5 mol% of SBAZ, respectively. 100 μL of each SBAZ solution was then added in 96-well Alkyne-PPX, and then incubated for 8 h at room temperature. The substrates were then rinsed with deionized water to remove residual reactants.

2.4 Surface characterization of the SBAZ-modified surfaces

Surface wettability was evaluated by water static contact angle (WCA) measurement (FTA-125, First Ten Angstroms, USA). The WCA from at least six 5-μL droplets were measured on each sample. The surface chemical compositions of the SBAZ-modified surfaces were characterized by ESCA (VG ESCA Scientific Theta Probe, UK). ESCA spectra were taken with an Al Kα X-ray source radiation (1486 eV) at a takeoff angle of 53°. The atomic compositions of the surfaces were calculated from the high-resolution spectrum of each element.

2.5 The attachment of L929 cells

The SBAZ-modified substrates were sterilized by soaking in 70% ethanol, followed by rinsing with sterilized water and then PBS prior to cell experiments. L929 fibroblasts were inoculated on each substrate at a density of 2×10^4 cells/cm^2 for 6 h. After the unattached cells were rinsed away with PBS, the attached cells were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI, cat #D9542). Fluorescent images were taken using a fluorescence microscope (100×objective

magnification). The DAPI-stained nuclei were counted from the fluorescent photos using Image J software (NIH) for the determination of cell densities (6 images/substrate).

2.6 Determination of protein adsorption

The adsorption of serum proteins to the SBAZ-modified surfaces was determined using a quartz crystal microbalance (QCM, ANTQ300, ANT Inc., Taipei, Taiwan). Alkyne-PPX was deposited on gold substrates of QCM quartz crystal chips (ca. 9 MHz resonance frequency), followed by SBAZ conjugation. Cell culture medium containing 10% fetal bovine serum flew into the QCM chamber at 50 μL/min. After the resonance frequency reached equilibrium, PBS then flew into the chamber to remove loosely bound proteins until the second equilibrium was reached. The mass of adsorbed proteins was calculated from the frequency shift according to the Sauerbrey equation (Babacan *et al*. 2000).

2.7 Statistical analysis

The data was reported as means ± standard deviation (SD). The statistical analyses between different groups were determined using Student's t-test. A probability of $p \leq 0.05$ was considered a significant difference. All statistical analyses were performed using GraphPad Instat 3.0 program (GraphPad Software, USA).

3. Results and discussion

3.1 Surface characterization of SBAZ-modified substrates

SBAZ was successfully synthesized, which is confirmed by the NMR and mass spectra (Fig. 1). We next verified whether SBAZ molecules could be conjugated on Alkyne-PPX. The wettability of SBAZ-modified substrates was first investigated using WCA measurement. The deposition of Alkyne-PPX to TCPS was indicated by an increase of WCA from 68° to 82° ($p<0.05$, Table 1). Since sulfobetaine binds water tightly, we expected that the surface hydrophilicity would be increased after SBAZ immobilization. After the conjugation of SBAZ at 0.1, 1 or 5 mg/mL, the

Table 1 The surface atomic compositions determined by ESCA and water contact angles (WAC) of the SBAZ-modified Alkyne-PPX

Surfaces[a]	Surface atomic percentages				WCA (degree)
	C	N	O	S	
Alkyne-PPX	88.2	0	11.8	0	82.0±4.1
SBAZ_0.1	85.6	0.8	12.6	0.9	61.7±3.9[b]
SBAZ_1	84.6	2.2	11.7	1.5	59.6±2.6[b]
SBAZ_5	82.2	3.4	11.3	3.1	54.7±6.0[b]

a. The Alkyne-PPX surfaces treated with 0.1, 1 or 5 mg/mL of SBAZ were referred to as SBAZ_0.1, SBAZ_1 or SBAZ_5, respectively
b. $p<0.05$ vs. Alkyne-PPX. $n=5$, value=mean±standard deviation

WCA was decreased to 61°, 59° or 54°, respectively (p<0.05 vs. Alkyne-PPX), confirming increased surface hydrophilicity after SBAZ conjugation.

The ESCA measurement was used to characterize the elemental compositions of the SBAZ-modified surfaces (Table 1). Since Alkyne-PPX does not contain nitrogen and sulfur atoms, the conjugation of SBAZ could be indicated by the appearance of the two elements. The conjugation of SBAZ at 0.1, 1 or 5 mg/mL increased the nitrogen content to 0.8%, 2.2% or 3.4%, and the sulfur content to 0.9%, 1.5% or 3.1%, respectively. The ESCA data showed that the amount of surface sulfobetaine was increased with the SBAZ concentrations from 0.1 to 5 mg/mL. The results of WAC and ESCA indicated that SBAZ was successfully deposited on Alkyne-PPX. The positive correlation between the surface hydrophilicity and the sulfobetaine amount demonstrates that the surface hydrophilicity is increased by sulfobetaine conjugation.

3.2 Cell-resistance of the SBAZ-modified PPX

The cell-resistance of the SBAZ-modified PPX was evaluated by the adhesion of L929 cells. The DAPI-stained fluorescent images (Fig. 3(a)) show that the cells attached well on Alkyne-PPX

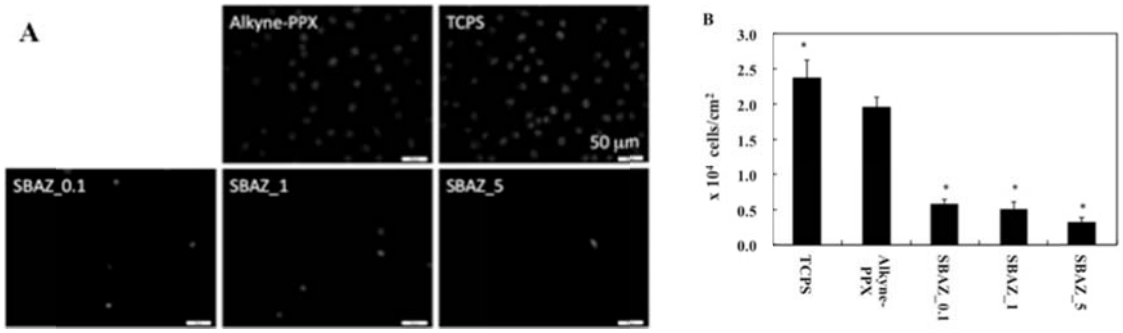


Fig. 3 L929 cells were cultured on the SBAZ-conjugated surfaces for 6 h. (a) The fluorescent images of the DAPI-stained attached cells and (b) the quantification of the attached cells from the fluorescent images. Scale bar=50 m. *, p<0.05 vs. Alkyne-PPX. n=5, value=mean±standard deviation

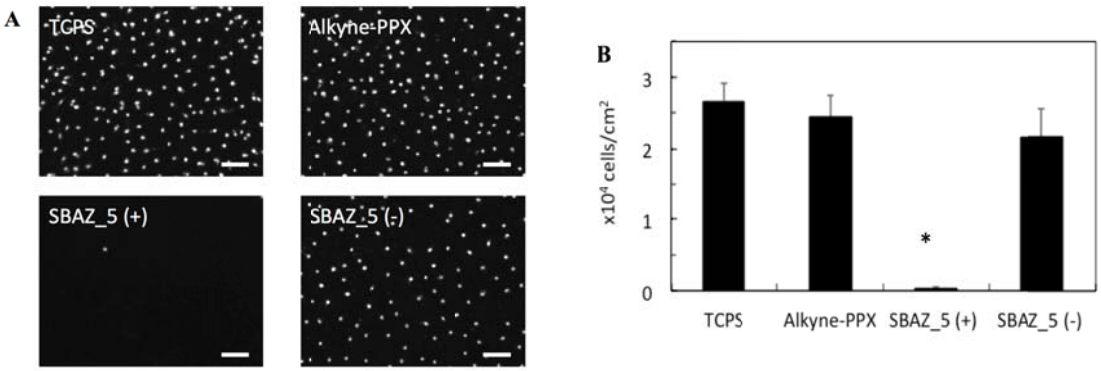


Fig. 4 SBAZ was conjugated onto Alkyne-PPX at 5 mg/mL in the presence (+) or absence (-) of Cu(I) ions. L929 cells were cultured on the surfaces for 6 h. (a) The fluorescent images of the DAPI-stained attached cells and (b) the quantification of the attached cells from the fluorescent images. Scale bar=100 m. *, p<0.05 vs. Alkyne-PPX. n=5, value=mean±standard deviation

and TCPS, while the cell adhesion was reduced on the SBAZ-modified PPX. Quantitatively, cell adhesion to TCPS and Alkyne-PPX was 2.36×10^4 and 1.96×10^4 cells/cm^2, respectively (Fig. 3(b)). The conjugation of SBAZ at 0.1, 1 and 5 mg/mL reduced cell attachment to 30%, 26% and 17% of that on Alkyne-PPX, respectively ($p<0.05$, Fig. 3(b)). The results demonstrate that the conjugation of SBAZ reduces cell adhesion and the extent of cell-resistance is positively correlated to sulfobetaine surface contents. On the other hand, without the addition of Cu(I) ions during the SBAZ conjugation, cell adhesion was not reduced (Fig. 4), indicating that the catalysis of Cu(I) is necessary for the conjugation of SBAZ to Alkyne-PPX, and the surface immobilization of SBAZ is not via physical adsorption. Furthermore, the cell adhesion on SBAZ_5 in Fig. 4 was much smaller than that at the same condition in Fig. 3. We suggest that the difference could be due to batch-to-batch variations.

3.3 QCM analysis of protein adsorption from serum-containing medium

Since protein adsorption to an artificial substrate plays an important role in cell adhesion, the adsorption of serum proteins from the cell culture medium to the SBAZ-conjugated surfaces was evaluated by QCM measurement. The frequency shifts were decreased on the SBMA-modified surfaces in comparison with Alkyne-PPX (Fig. 5), and the lowest frequency shifts appeared on SBAZ_5 as -66.7 Hz. Based on the Sauerbrey equation (Babacan *et al*. 2000), the amounts of the adsorbed serum proteins were calculated as 829, 544, 393 and 364 ng/cm^2 for Alkyne-PPX, SBAZ_0.1, SBAZ_1 and SBAZ_5, respectively. The amount of the surface-bound proteins is decreased with increasing SBAZ concentrations, consistent with the trend of cell adhesion.

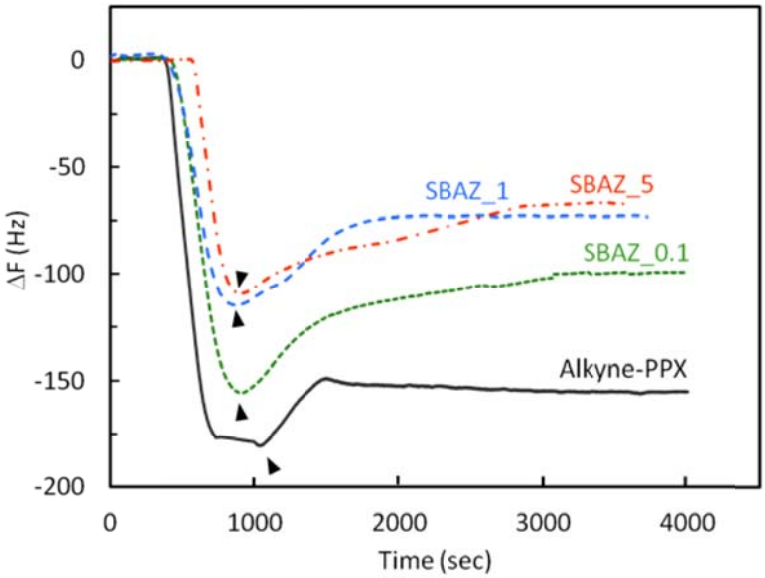


Fig. 5 QCM measurement for the adsorption of serum proteins from the cell culture medium containing 10% fetal bovine serum onto (A) Alkyne-PPX, (B) SBAZ_0.1, (C) SBAZ_1 and (D) SBAZ_5. The cell culture medium flew through the chamber at a flow rate of 50 μL/min. When the resonance frequency decrease reached a steady value, PBS was injected into the QCM chamber in order to remove loosely bound serum proteins (indicated by arrows) until the resonance frequency reached equilibrium again

3.4 Alkyne-PPX as a platform for conjugation of anti-fouling sulfobetaine molecules

We previously conjugated aldehyde-ended carboxybetaine molecules to Amine-containing PPX for anti-fouling purposes (Chien *et al*. 2014). Aldehyde-ended carboxybetaine molecules could be coupled to Amino-PPX, and then reduce cell adhesion and protein adsorption, but a high concentration of aldehyde-ended carboxybetaine molecules (>50 mg/mL) is needed in the conjugation to Amino-PPX in order to reduce a significant amount of cell adhesion and protein adsorption. We suggest that the reason for the requirement for the high concentrations of aldehyde-ended carboxybetaine molecules should be due to low reactivity between aldehydes and amines. Therefore, a highly reactive functionality is needed for efficient conjugation of zwitterionic molecules on a surface.

In this study, the Alkyne-PPX platform is used for the conjugation of zwitterionic sulfobetaine molecules. Cell adhesion and protein adsorption are greatly reduced on the SBAZ-modified surfaces. Due to the effectiveness of CuAAC, a much less amount of SBAZ molecules is sufficient to reach a similar level of protein/cell-resistance that is achieved by the aldehyde/amine system. In this study, 1 mg/mL (~4 mM) of SBAZ was sufficient to achieve significant resistance to protein adsorption and cell adhesion to a level, which is achieved by 50 mg/mL aldehyde-ended carboxybetaine in our previous study (Chien *et al*. 2014). The CuAAC reaction provides a more effective tool for surface conjugation of zwitterionic molecules compared to the aldehyde-amine reaction.

In spite of preliminary success, the overall anti-fouling ability of the SBAZ-conjugated PPX is still inferior to the zwitterionic surfaces that are created using surface-initiatd atom transfer radical polymerization (Yang *et al*. 2009, Inoue and Ishihara 2010, Nguyen *et al*. 2011), or self-assembled zwitterionic monolyaer (Chen et al. 2005, Wu et al. 2010, Huang and Chang 2014). We conjecture that the conjugation density of SBAZ molecules on Alkyne-PPX might not be high enough to completely inhibit protein adsorption and cell adhesion. The conjugation of SBAZ is limited by the amount of surface alkyne groups of Alkyne-PPX. Unfortunately, we could not determine the amount of alkyne groups on Alkyne-PPX. Previously, Chang *et al*. determined the total deposition weight of PPX using QCM, and then estimated the amount of functional groups on PPX as 5.30±0.11 nmol·cm^{-2} (Chang *et al*. 2014). However, the actual density of functional groups on the outmost layer of PPX films should be much lower that value since PPX deposition is not merely a monolayer. Since we only conjugated mono-sulfobetaine molecules instead of polymeric sulfobetaine, the conjugation of mono-sulfobetaine might not fully cover the whole surface. Therefore, we suggest that the density of surface-bound sulfobetaine molecules should be increased by the conjugation of oligo- or poly-zwitterionic molecules instead of mono-zwitterionic molecules, in order to reach ultra-low fouling.

4. Conclusions

The present study displays an effective method for preparation of surfaces to reduce cell adhesion and protein adsorption.

- An azido mono-sulfobetaine molecule was synthesized.
- SBAZ was conjugated to a alkyne-poly-p-xylylene film via copper(I)-catalyzed azide-alkyne cycloaddition.
- Protein adsorption and cell adhesion were greatly reduced on the SBAZ surface.

• Alkyne-PPX is a suitable substrate for effective conjugation of anti-fouling molecules.

Acknowledgements

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