Cell Fouling Resistance of Polymer Brushes Grafted from Ti Substrates by Surface-Initiated Polymerization: Effect of Ethylene Glycol Side Chain Length

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This paper presents a comparative study on the antifouling properties of poly(ethylene glycol) (PEG)-based polymer coatings prepared by surface-initiated polymerization (SIP). Three types of poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMEMA) polymer thin films of approximate 100 nm thickness were grafted from a catechol initiator that was immobilized on a Ti substrate. OEGMEMA monomers containing side chains of 4, 9, and 23 EG units were used in surface-initiated atom transfer radical polymerization (SI-ATRP) to form POEGMEMA-4, -9, and -23 polymer brushes. The chemical composition, thickness, and wettability of the polymer brushes were characterized by X-ray photoelectron spectroscopy (XPS), ellipsometry, and static water contact angle measurements, respectively. The dependence of antifouling performance on EG side chain length was systemically tested and compared by 3T3 fibroblast cell adhesion assays. Results from 4-h cell culture experiments revealed the complete absence of cell attachment on all the grafted Ti substrates. Excellent cell fouling resistance continued with little dependence on EG side chain length up to three weeks, after which long-term antifouling performance depended on the EG chain length as the grafted samples reached confluent cell coverage in 7, 10, and 11 weeks for POEGMEMA-4, -9, and -23, respectively.

Introduction

Ti and its alloys are among the most attractive biomaterials due to their high specific strength, corrosion resistance, and biocompatibility.[1] However, the oxide surface of Ti is prone to spontaneous nonspecific protein adsorption and cell adhesion when exposed to biological tissues or fluids.[2] In some instances, nonspecific protein/cell adsorption can be detrimental to device performance, such as in the case of in vivo biosensor and cardiovascular stent surfaces.[3] Strategies for Ti surface modification for control of the biointerface include grafting with nonfouling biopolymers such as poly(ethylene glycol) (PEG),[4,5] formation of self-assembled monolayers (SAMs),[6] monolayer/multilayer polyelectrolyte adsorption,[7] formation of supported phospholipid bilayers,[8] interpenetrating polymer networks (IPN),[5] and photochemical attachment of polymer thin films.[9] Recently, the technique of surface-initiated polymerization (SIP) of vinyl monomers from surface-bound initiators has been applied with increasing frequency to modify surfaces with grafted polymer brushes for both medical and nonmedical applications.[10,11] As compared to other methods for grafting polymers, SIP has the advantage of readily producing covalently anchored polymer coatings of high density and thickness,[12] which are important parameters for antifouling performance.[13]

Atom transfer radical polymerization (ATRP) is one technique that has been extensively employed for SIP.[14] ATRP can be carried out in aqueous media with a wide range of hydrophilic monomers,[15] and there has been considerable interest in employing aqueous ATRP to polymerize water-soluble methacrylate macromonomers that contain oligo(ethylene glycol) (OEG) side chains.[16−18] Although OEG macromonomers of various lengths have been used in these previous studies, the effect of OEG side chain length on the long-term antifouling behavior of the grafted polymer coatings has not been systematically investigated.

In this paper, we report a comparative study of polymer coatings grafted on Ti substrates by surface-initiated ATRP (SI-ATRP) of OEG methyl ether methacrylate (OEGMEMA) macromonomers with average EG lengths of 4, 9, and 23 (OEGMEMA-4/-9/-23). Grafted polymer films (∼100 nm thickness) were prepared by SI-ATRP, and their successful grafting was confirmed by a series of surface analytical techniques. Coated samples were then subjected to in vitro cell adhesion assays in which mammalian cells were cultured up to 2.5 months with frequent challenge by cells and fresh serum. The goal was to investigate the dependence of both short- and long-term antifouling behavior on the OEG chain length of these polymer coatings.

Experimental Section

Materials. Silicon wafers with surface orientation (100) were purchased from MEMC Electronic Materials (St. Peters, MO). Ultrapure water (resistivity =18.2 MΩ, pH = 6.82) used in all experiments was obtained from a NANOpure Infinity System from Barnstead/Thermolyne Corporation (Dubuque, IA). OEGMEMA monomers (Aldrich, Figure 1a) were passed through an activated basic alumina (Aldrich) column to remove the inhibitor. The SI-ATRP initiator (Figure 1b) was synthesized using previously published procedures.[19] Other reagents for polymerization were purchased from commercial sources and used without further purification.

Substrate Preparation and Modification. Silicon wafers were coated with 100 nm Ti in an Edwards FL400 electron beam evaporator at $<10^{-6}$ Torr. The coated wafer was then cut into 1 cm × 1 cm pieces and subsequently cleaned by ultrasonication in the following media:

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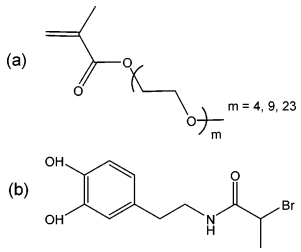

Figure 1. Structures of (a) OEGMEMA monomers and (b) catechol initiator for SI-ATRP.

5 vol % Contrad70 (Fisher), ultrapure water, acetone, petroleum ether, and 2-propanol. The substrates were further cleaned in an oxygen plasma chamber (Harrick Scientific) at <200 mTorr and 100 W for 3 min. Freshly cleaned Ti substrates were immersed in a 1 mg/mL aqueous solution of initiator for 12 h in the dark. The modified substrates were rinsed with copious amounts of ultrapure water to remove unattached initiator and dried under a stream of dry N_2.

SI-ATRP. In a typical polymerization, modified substrates were placed in a two-necked flask under Ar flow. OEGMEMA-4 monomer (2 mL, 7 mmol), ultrapure water (2 mL), *N*,*N*,*N*′,*N*′,*N*″-pentamethyldiethylenetriamine (PMDETA, 1.45 uL, 7 μmol), and CuBr (1 mg, 7 μmol) were mixed and purged with Ar flow for 1 h and then charged into the flask via a degassed syringe. The polymerizations were performed at room temperature under Ar protection. The mixture remained light blue and stable throughout the reaction. The substrates were taken out of the polymerization solution after overnight reaction and washed with copious amounts of ultrapure water, followed by drying with a stream of dry N_2. The polymer-grafted samples were further dried under vacuum overnight before further analysis.

Surface Characterization. X-ray photoelectron spectroscopy (XPS) spectra were collected on an Omicron ESCALAB (Omicron, Taunusstein, Germany) configured with a monochromated Al Kα (1486.8 eV) 300 W X-ray source, 1.5 mm circular spot size, a flood gun to counter charging effects, and an ultrahigh vacuum ($<10^{-8}$ Torr). All binding energies (BEs) were calibrated using the C 1s peak (284.8 eV). Analysis consisted of a broad survey scan (50.0 eV pass energy) followed by a 10 min high-resolution scan (22.0 eV pass energy) at 270−300 eV for C 1s. Surface chemical compositions were determined from spectral area ratios after correcting with individual elemental sensitivity factors, which were calibrated using stable binary compounds of known stoichiometries. Ellipsometry measurements were performed on a variable angle spectroscopic ellipsometer (model ESM-300, J. A. Woolam, Inc., Lincoln, NE) at 60°, 65°, and 70° incident angles and a wavelength range from 370 to 1000 nm. Ellipsometric thickness was obtained by fitting the measured Δ and Ψ data with fixed A_n (1.46) and B_n (0.01) values using the Cauchy model and the WVASE32 software module integrated with the system. For each sample, the thickness value is reported as the average of measurements on at least three different spots. A custom-built contact angle goniometer (components from Rame-Hart, Inc., Mountain Lakes, NJ) was used to measure the static water contact angles of experimental substrates. At least three spots on each sample were measured, and the average was reported.

Cell Culture and Adhesion Assay. 3T3-Swiss albino fibroblasts obtained from ATCC (Manassas, VA) were maintained at 37 °C and 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Cellgro, Herndon, VA) containing 10% fetal bovine serum (FBS) and 100 μg/mL of penicillin and 100 U/mL of streptomycin. Immediately before use, fibroblasts of passages 12−16 were harvested using 0.25% trypsin-EDTA, resuspended in DMEM with 10% FBS, and counted with a hemocytometer. Bare and modified substrates were pretreated in 12-well tissue culture polystyrene (TCPS) plates with 1.0 mL of DMEM containing FBS for 30 min at 37 °C and 10% CO_2. Cells were seeded onto the sample substrates at a concentration of 1×10^4 cells/mL twice weekly and maintained in DMEM with 10% FBS at 37 °C and 10% CO_2. Adherent cells were stained with 2.5 μM Calcein-AM (Molecular Probes, Eugene, OR) in complete phosphate buffered saline (PBS, Invitrogen, Grand Island, NY) for 50 min. Quantitative cell attachment data were obtained by acquiring 5−9 images (depending on substrate size) from random locations on each substrate using a Leica epifluorescent microscope equipped with a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI). The resulting images were quantified in terms of total projected cell area using thresholding in Metamorph software (Universal Imaging, Downington, PA). Statistical data analysis (Oneway ANOVA) was performed using SPSS software (SPSS, Inc., Chicago, IL).

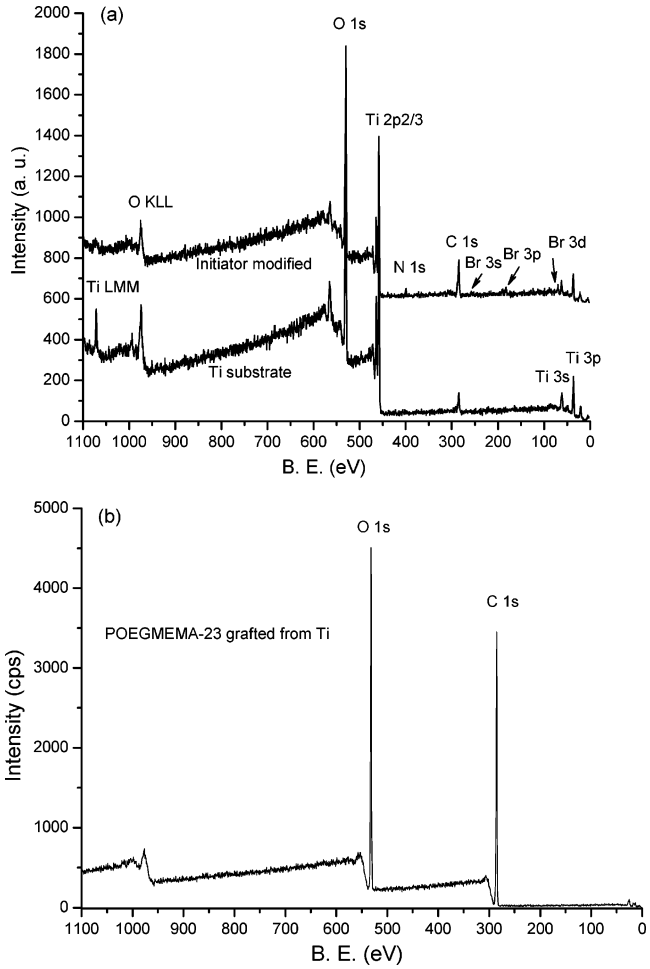

Figure 2. (a) XPS survey spectra of bare and initiator-modified Ti substrates. (b) POEGMEMA-23 thin film grafted from Ti substrate.

Results and Discussion

The use of catechols for surface modification of Ti and TiO_2 has been well-documented.[20,21] The initiator used in the present study contained a catechol functional group as well as an alkyl bromine for initiation of ATRP.[19] The bifunctional initiator was immobilized on freshly cleaned and oxidized Ti substrates via adsorption from aqueous solution, and initiator-modified Ti substrates were then submerged at room temperature in an aqueous solution of ATRP catalysts and OEGMEMA macromonomer. Following polymerization, the chemical composition, wettability, thickness, and cell fouling resistance of grafted poly(OEGMEMA) (POEGMEMA) layers were respectively analyzed by XPS, contact angle goniometry, ellipsometry, and cell adhesion assays with 3T3-Swiss albino fibroblasts.

Characterization of Initiator- and POEGMEMA-Modified Ti Surfaces. The presence of initiator and polymer layers was first ascertained by XPS survey scan measurements. As shown in Figure 2a, bare Ti substrate showed strong signals from Ti

Table 1. XPS Surface Composition, Contact Angle, and Ellipsometric Thickness Data of POEGMEMA Polymer Layers Grafted from Ti Substrates

	XPS chemical composition (%)					[O]/[C]	[O]/[C]	contact angle	thickness
sample	Ti	O	C	N	Br	measured	theoretical	(±1°)	(nm)
bare	59.8	30	10.2			2.94		10	
initiator	37.3	24.5	34.3	2.6	1.3	0.71	0.36	54	0.5 ± 0.1
POEGMEMA-4	0	29.4	70.6			0.42	0.46	52	99 ± 3
POEGMEMA-9	0	29.1	70.9			0.41	0.48	49	109 ± 5
POEGMEMA-23	0	30.6	69.4			0.44	0.49	40	94 ± 2

Table 2. BEs and Ratios of C 1s Components of POEGMEMA-4/-9/-23 Samples

sample	ester (288.9 eV)	ether (286.4 eV)	aliphatic (284.8 eV)	theoretical ratio
POEGMEMA-4	1	8.9	3.2	1:9:3
POEGMEMA-9	1	17.8	2.5	1:19:3
POEGMEMA-23	1	41	2.7	1:47:3

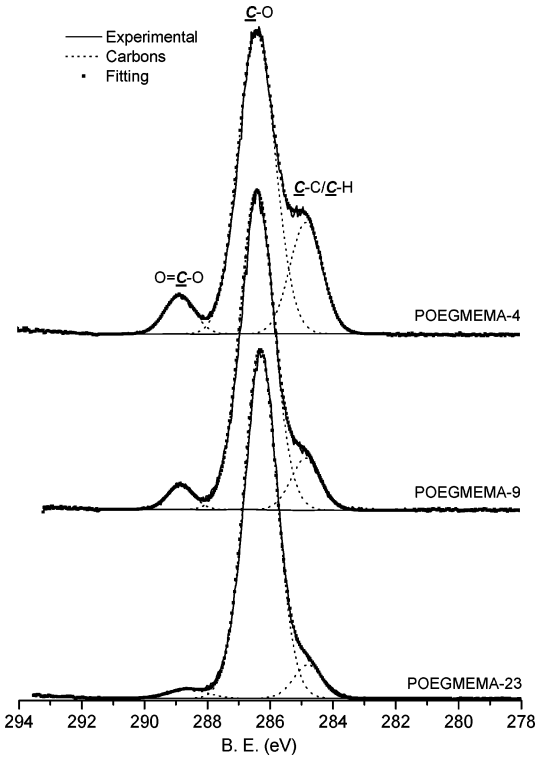

Figure 3. XPS C 1s high-resolution scan spectra of POEGMEMA-4/-9/-23 surfaces.

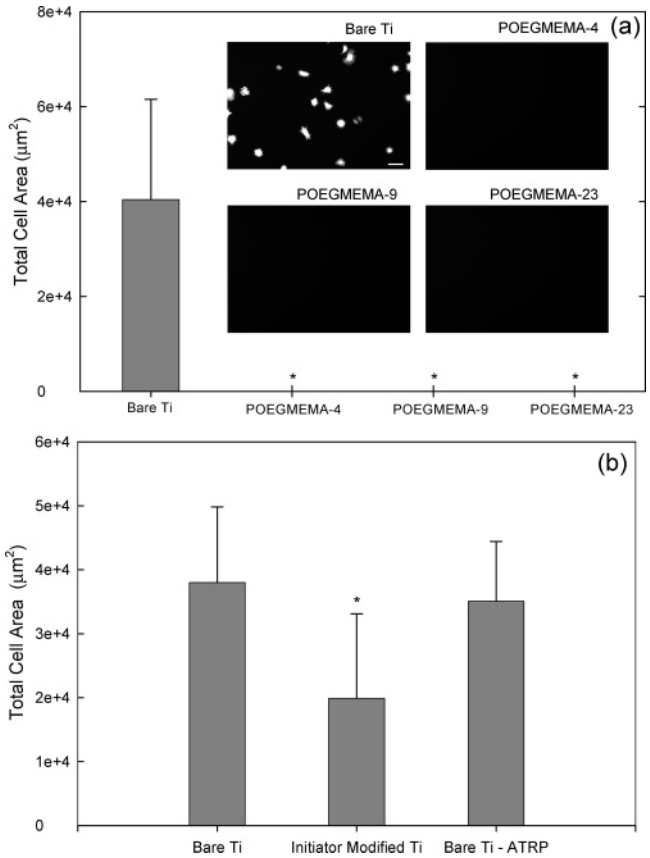

Figure 4. (a) 4-h cell adhesion assay of bare and grafted Ti substrates (*, $p < 0.05$ versus bare Ti). Inset pictures represent typical fluorescence micrographs of each sample. Scale bar in the picture of bare Ti represents 100 μm for all pictures. (b) 4-h cell adhesion assay of control samples. (Initiator-modified Ti = Ti substrate treated by initiator adsorption but without subsequent SI-ATRP. Bare Ti-ATRP = Ti substrate subjected to SI-ATRP conditions without surface bound initiator. *, $p < 0.05$ versus bare Ti.)

2p and O 1s as well as a small C 1s peak resulting from hydrocarbon contamination. In addition, the initiator-modified sample also displayed a N 1s peak, Br 3s, 3p, 3d peaks, and a more intense C 1s peak originating from the adsorbed initiator. Quantitative surface composition data shown in Table 1 obtained from XPS survey scans revealed that the bare Ti surface was mainly composed of Ti and O from native surface oxide as well as 10.2% of C owing to adventitious hydrocarbon contamination. Successful initiator anchoring was demonstrated by a significant increase in C concentration (34.3%) and the presence of N (2.6%) and Br (1.3%) signals. It is believed that the initiator complexes with the native titanium oxide surface through reaction between OH groups of the hydroxylated oxide and the phenolic OH groups in catechol, resulting in strong bidentate charge-transfer complexes.[22]

Figure 2b shows a typical survey scan spectrum of a Ti surface after grafting POEGMEMA by SI-ATRP. As compared to those of bare and initiator-modified samples, a substantial increase in C concentration (69.4%, Table 1) was detected and the Ti signal from the substrate was absent, indicating the polymer layer thickness (~94 nm for POEGMEMA-23, see discussion on ellipsometry measurements below) exceeded the probe depth of XPS. Very similar spectra (not shown) were obtained for POEGMEMA-4 and -9 surfaces, the chemical composition data for which are shown in Table 1. It should be noted that the [O]/[C] ratios for the POEGMEMA-modified surfaces measured by XPS are close to the theoretical values of the three POEGMEMA polymers. Detailed analysis of the high-resolution XPS C 1s spectra of the grafted POEGMEMA polymers also revealed the dependence of surface spectra on OEG chain length. C 1s core-level scans shown in Figure 3 clearly demonstrate three distinct features corresponding to *C*—C/*C*—H, *C*—O, and O═*C*—O functional groups in POEGMEMA structure. All high-resolution XPS spectra could be

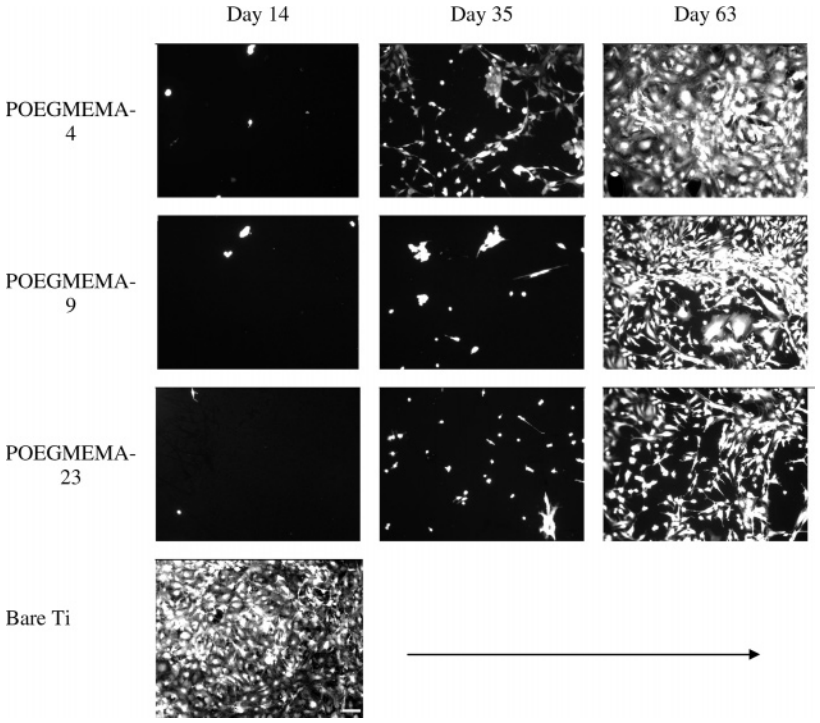

Figure 5. Typical fluorescence images showing 3T3 cell attachment on POEGMEMA surfaces at selected time of long-term cell adhesion assay. Scale bar in the picture of bare Ti represents 100 μm for all pictures.

fitted with three peak components at BEs of 284.8, 286.4, and 288.9 eV, consistent with the BEs of aliphatic, ether, and ester carbons, respectively.[23] It can be seen that the intensity of the *C*—O increases with respect to the *C*—C/*C*—H and O═*C*—O peaks as the length of the OEG side increases. The spectral area ratios of the three components were calculated and found to be in good agreement with the theoretical ratios for all three POEGMEMA polymers (Table 2). The XPS results are in good agreement with those observed by Yu et al. for PEG-based polymer coatings grown from Si substrates by SI-ATRP[24] and by Li et al. for OEG-terminated alkanethiol SAMs on Au.[25] The OEGMEMA macromonomers used in this study were determined to be polydisperse (data not shown), leading to the possibility that the deviations from theoretical ratios can be explained by preferential polymerization of low molecular weight fractions of the monomer mixture.

Static water contact angle measurements further confirmed the presence of initiator and POEGMEMA on the Ti substrates. O_2 plasma cleaned Ti substrates were hydrophilic with a contact angle of ~10°, which increased to an average value of 54° after initiator immobilization, indicating a decrease in hydrophilicity due to the adsorbed initiator. After SI-ATRP, the contact angle further decreased to ~52°, 49°, and 40° for grafted POEGMEMA-4, -9, and -23 coatings, respectively. Contact angle of grafted polymer brushes with OEG side chains have been observed to be in the 35−45° range,[13,24] and the observed trend is consistent with the increasing hydrophilicity of the grafted polymer with increasing OEG side chain length.

The ellipsometric thickness data of the POEGMEMA coatings used in this study are shown in the rightmost column of Table 1. As several previous studies have shown,[17,26] thickness of polymer brushes grafted by aqueous SI-ATRP of hydrophilic monomers increases approximately exponentially with time. We carried out SI-ATRP of all three OEGMEMA monomers to extended polymerization times with the intention of creating polymer coatings with maximum thickness for optimal antifouling performance. Under these conditions, the final thickness of all grafted polymer coatings was approximately 100 nm, allowing us to focus on OEG side chain length in comparisons of antifouling performance.

Short-Term Cell Adhesion Assays. Figure 4a exhibits the comparative results from short-term cell adhesion assays in which 3T3 fibroblast cells were cultured in 10% FBS for 4 h at 37 °C on bare and POEGMEMA-grafted Ti. While cells readily attached and spread on the bare Ti surface with an average cell adhesion area of approximately 4×10^4 μm^2, exceptional short-term fouling resistance was demonstrated by the complete elimination of cellular adhesion for all grafted samples. The fouling resistance of grafted POEGMEMA is illustrated qualitatively in the representative fluorescence microscopy images shown as insets of Figure 4a, in which no cells were found on the three grafted samples, whereas they easily adhered on bare Ti. This result is consistent with other short-term studies of cell adhesion on polymer brush coatings formed by SI-ATRP of OEG-based macromonomers.[17,18]

In a separate experiment, we explored the possibility that either initiator or ATRP of OEGMEMA monomer directly from bare Ti surface (in the absence of initiator) could explain the observed antifouling properties. As demonstrated in Figure 4b, average cell adhesion area on initiator-modified Ti was about half of that on unmodified surface, while bare Ti that was subjected to ATRP showed a similar level of cell adhesion as compared to the unmodified sample. Thus, we conclude that the markedly improved cell fouling resistance exclusively resulted from grafted POEGMEMA coatings formed by SI-ATRP from surface-bound initiator.

Our results indicate that short-term cell resistance of the POEGMEMA coatings does not depend on the length of the OEG side chains. Andruzzi et al. also found that their OEG-containing polymer brush coatings markedly reduced short-term cell adhesion regardless of OEG chain length (4 vs 7),[13] and similar OEG length insensitivity was also observed by Tugulu et al. for the antifouling polymer coatings that contained OEG-6 and -10 moieties grafted by SI-ATRP.[27] The primary difference

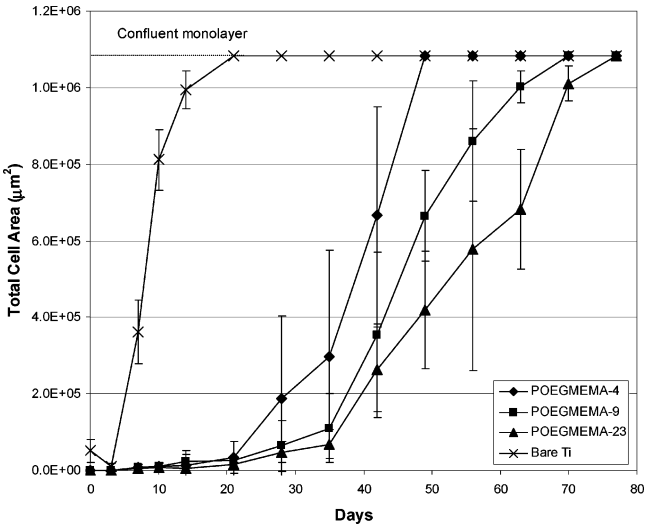

Figure 6. Long-term cell adhesion assay of Ti substrates grafted by POEGMEMA polymers.

between our study and previous SIP systems lies in using the catechol initiator to anchor polymer brushes on Ti, rather than employing more common silane initiator designs for Si.

Long-Term Cell Adhesion Assays. To evaluate the long-term antifouling performance of grafted and unmodified Ti substrates, fresh cells were seeded on the sample surfaces twice weekly, providing a rigorous in vitro test of cell fouling resistance up to 2.5 months. Fluorescence microscopy imaging was performed at selected times, and representative pictures are shown for comparison in Figure 5. Total cell adhesion area was quantified by image analysis and plotted with respect to time of culture in Figure 6. Throughout the first three weeks of culture, all POEGMEMA samples resisted cell attachment, reducing cell area by 98% or more regardless of the OEG side chain length compared to control bare Ti substrate. However, after three weeks of culture, the influence of OEG chain length on antifouling behavior began to emerge. Starting from day 28, POEGMEMA-4 surface showed significantly higher cell adhesion than POEGMEMA-9 and -23 samples, gradually rising to confluence by day 49. POEGMEMA-9 and -23 demonstrated low levels of cell fouling through day 35, after which POEGMEMA-23 showed lower cell adhesion area and reached confluence on day 77, a week after full cell coverage for POEGMEMA-9 was observed. These results are qualitatively supported by the fluorescence images shown in Figure 5. From the results of our long-term in vitro cell adhesion assay, it is generally concluded that the antifouling performance of POEGMEMA thin films was similarly effective for the first three weeks independent of their OEG chain length, after which cell fouling resistance depended on OEG chain length. Both initiator-modified Ti and bare Ti without initiator that was subjected to ATRP demonstrated virtually identical long-term cell adhesion behavior compared to the unmodified Ti (data not shown), confirming the effect of grafted POEGMEMA coatings on cell fouling resistance.

Several possible explanations exist to account for the observed decrease in fouling resistance with time. The gradual loss of fouling resistance for our POEGMEMA coatings may be explained by PEG degradation. PEG is prone to thermal/oxidative chain scission,[28] which has been proposed as the underlying cause of compromised antifouling performance over extended periods of time.[29] It is also conceivable that POEGMEMA chains detached from the surface during culture due to cleavage of the Ti−catechol bond anchors. However, we believe this to be unlikely, considering the robust and water-resistant nature of the Ti-catechol bond and the fact that the catechol anchor should be protected from aggressive culture media components, being deeply buried beneath a dense and thick polymer coating. Furthermore, using essentially identical methodology, we recently demonstrated resistance to in vitro cell attachment for over 5 months for a catechol-containing peptidomimetic polymer immobilized on Ti,[21] suggesting that the catechol−Ti bond is stable for long periods of time under cell culture conditions.

We believe a more likely explanation for the long-term increase in cell attachment could be related to the hydrolysis of the ester group which links the OEG side chain to methacrylate backbone in the comb-shaped POEGMEMA brushes. Gradual hydrolysis of these ester bonds would lead to a loss of antifouling OEG side chains and an increase in protein and cell adsorption. However, more studies are needed to fully explain the mechanism of long-term fouling of the modified surfaces.

Conclusions

Three polymer coatings, POEGMEMA-4, -9, and -23 were grafted from Ti substrates by aqueous SI-ATRP from a surface-bound catechol initiator. Both XPS and static water contact angle measurements confirmed the successful grafting of the polymer coatings. Thin films (∼100 nm thickness) were obtained for all the three POEGMEMA samples as measured by spectroscopic ellipsometry. Grafted and unmodified Ti samples were then subjected to in vitro 3T3 fibroblast cell culture experiments which proceeded for 2.5 months. Greatly improved short-term antifouling properties for all three POEGMEMA modified samples were demonstrated by substantially reduced cell attachment for a period of three weeks as compared to bare Ti. Afterward, coatings with shorter OEG side chain length succumbed to cell fouling more rapidly than those with longer OEG side chains. Finally, POEGMEMA-4, -9, and -23 samples reached complete cell coverage in 7, 10, and 11 weeks, respectively.

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