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Carboxybetaine methacrylate polymers offer robust, long-term protection against cell adhesion

Goher Mahmud¹, Sabil Huda¹, Wei Yang³, Kristiana Kandere-Grzybowska^{1,*}, Didzis Pilans¹, Shaoyi Jiang³, and Bartosz A. Grzybowski^{1,2,*}

¹Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208

²Department of Chemistry, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208

³Department of Chemical Engineering, University of Washington, Seattle, Washington 98195

Abstract

Films of poly(carboxybetaine methacrylate), poly(CBMA), grafted onto microetched gold slides are effective in preventing non-specific adhesion of cells of different types. The degree of adhesion resistance is comparable to that achieved with the self-assembled monolayers, SAMs, of oligo(ethylene glycol) alkanethiolates. In sharp contrast to the SAMs, however, substrates protected with poly(CBMA) can be stored in dry state without losing their protective properties for periods up to two weeks.

Keywords

Bio-resistance; polymer coatings; biofouling; micropatterning; Wet Etching

Introduction

Bio-resistant surfaces (e.g., surfaces that resist adsorption of proteins and adhesion of cells) have been used in fundamental studies of protein adsorption¹⁻³ and cell adhesion,⁴⁻⁶ and have proven important for tissue engineering,⁷⁻¹⁰ and reducing biofouling.¹¹⁻¹³ With the advent of soft-lithographic^{5,14-17} and wet etching¹⁸⁻²² techniques, it became possible to combine cell-resistant and cell-adhesive sites on the same substrate. These cell micropatterning methods have proven particularly powerful in conjunction with the so-called self-assembled monolayers^{23,24} – there, the use of oligo(ethylene glycol) alkanethiolates, EGs, to render select regions of metal surfaces bioresistant enabled cell micropatterning,^{14,25} study of cell growth and apoptosis under well-defined geometrical constraints,^{5,26-29} imaging of cytoskeletal organization and dynamics,³⁰⁻³⁴ or separation of cancerous from non cancerous cells via the so-called geometrical ratchets.³⁵ While SAMs based on EG thiols – notably, hexa(ethylene glycol) alkanethiolates, EG6 – have become a standard for biologically inert surfaces,³⁶ they are not without limitations due to relatively high cost (hundreds of dollars per 100 mg from companies like ProChimia, Asemblon, or Sigma Aldrich) and, above all, limited stability in cell culture or in solution.^{17,37-39}

*Correspondence to: K.K.G. (kanderek@northwestern.edu) or B.A.G. (grzybor@northwestern.edu).

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Consequently, ready-to-go EG6/SAM/metal “chips” cannot be stored, sold and shipped but, instead, every researcher must prepare their own monolayer immediately prior to experiment. Therefore, for the popularization and commercialization of micropatterned surfaces for cell studies, other types of surface chemistries need to be developed. Recently, zwitterionic polymers⁴⁰ of sulfobetaine and carboxybetaine such as poly(carboxybetaine methacrylate) poly(CBMA)⁴¹ have been grafted onto surfaces and have shown to highly resist non-specific protein adsorption from undiluted blood plasma and serum (superlow fouling)^{42,43} and to resist bacterial biofilm formation.⁴⁴ Here, we tested poly(CBMA) compatibility with cell micropatterning and fluorescence imaging, and compared the performance of these surfaces (in terms of cell-adhesion-resistance) with the performance of EG6 SAMs. We found that for multiple cell lines (B16F1, Rat2, and CHO-k1) and for the times ranging from 8 hours to two weeks, the substrates coated with poly(CBMA) – even if kept dry for prolonged periods of time – resisted cell adhesion just as well as substrates coated with EG6 SAMs. These results indicate that poly(CBMA) substrates are a robust alternative to the traditional EG6 surface functionalization schemes used in cell patterning.

Experimental Section

CBMA was synthesized by the reaction of 2-(N,N'-dimethylamino)ethyl methacrylate and β -propiolactone using a method published previously.⁴¹ Standard glass coverslips (VWR, catalog # 48366-227, #1.5, 22 mm $\times$ 22 mm) covered with a thin film of Ti 5nm / Au 30 nm were microetched using the Wet Etching method^{25,30,31,35} to give substrates with transparent islands surrounded by opaque Ti/Au regions (see Fig. 1a). These coverslips were grafted with poly(CBMA) following a method published previously.⁴⁴ Briefly, CuBr (28.6 mg, 0.2 mmol), CuBr₂ (4.4 mg, 0.02 mmol) and 2,2'-bipyridine (68.6 mg, 0.44 mmol), and glass coverslips with immobilized initiators prepared from ω -mercaptoundecyl bromoisobutyrate were placed in a nitrogen-purged reaction tube. The degassed solution (pure water and methanol in a 1:4 volume ratio, 4 mL) with 0.6 g of CBMA was transferred to the tube using a syringe. The substratum was reacted for 24 hrs at 25 °C under nitrogen protection. After reaction, the coverslips were removed, rinsed with ethanol, water, and phosphate buffered saline (PBS) solution, and dried with a stream of nitrogen before shipment. Thus prepared substrates were kept dry for one to two weeks (including exposure to various environmental changes during shipping from the Jiang lab in Seattle, WA, to the Grzybowski lab in Evanston, IL). Immediately prior to experiments, the substrates were rinsed with PBS and coated with fibronectin (25 μ g mL⁻¹) (Sigma-Aldrich, catalog # F1141) at room temperature for 1 hr.

For comparison, we used the similarly etched coverslips but with the gold regions protected with SAMs of hexa(ethylene glycol) alkane thiols (HS(CH₂)₁₁(OCH₂CH₂)₆ OH, EG6, from ProChimia Surfaces, www.prochimia.com). The SAMs were prepared by immersing the etched substrates in a 5 mM ethanolic solution of EG6 for 12 hrs. Protected substrates were thoroughly washed with 200-proof ethanol (Sigma-Aldrich, catalog # E7023) and dried under a stream of nitrogen. The substrates were then rinsed with PBS and incubated with fibronectin, as described above for the CBMA polymer substrates.

Altogether, three cell types were tested: B16F1 mouse melanoma cells, Rat2 fibroblasts, and Chinese Hamster Ovary (CHO) cells. These cells were plated onto both the poly(CBMA) and the EG6 substrates coated with fibronectin at typical densities of 10,000–20,000 cells cm⁻². B16F1 and Rat2 cells were cultured at 37 °C and 10% CO₂ in a DMEM cell culture medium (Cellgro, catalog # 10-017-CV) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, catalog # S11550). CHO-K1 cells were cultured at 37 °C and 5% CO₂ in a F-10 cell culture medium (Invitrogen, catalog # 12390) supplemented with 10% FBS. The cells were allowed to fully spread onto the substrate's microislands for ~8 hrs for B16F1

and Rat2 cells and >24 hrs for CHO cells. Subsequently, cells were fixed and detergent-permeabilized (as previously described in ref³⁰), and were stained with phalloidin coupled to Alexa Fluor 488 (Invitrogen, catalog # A12379) to visualize actin filaments, and with Hoechst 33342 dye (Invitrogen, catalog # H3570) to visualize the nuclei.

Coverslips were mounted using the so-called “sandwich” mounting approach. That is, an etched coverslip with fixed/stained cells was first attached to a clear, thin glass coverslip (#1.5, 22 mm × 22 mm) by using PolyAquaMount mounting medium. Then, these two coverslips together were mounted onto thick microscopic slide so that etched glass coverslip was “sandwiched” between thin transparent glass slide and thick support slide. In this method, imaging of cells is done through glass and both cells on islands and on gold are visible.

Results and Discussion

Typical results are illustrated in Figure 1. Figure 1a has a low-magnification image of an etched substrate protected with EG6 SAM (images look similar for polymer coatings) and after cell plating. In the phase-contrast image, the etched islands are transparent while gold regions are opaque. Cells spread only unto etched microislands and assume their circular shapes. In the corresponding fluorescent image (Hoechst/nucleus), cell nuclei appear as oval and bright spots (more than one spot per island indicates several cells localized onto one island) and etched patterns can be visualized clearly but conveniently with lower fluorescence intensity values. Because in Figure 1a the gold regions are protected from cell adhesion, almost all cells adhere to the etched islands.

To quantify the efficiency of the cell-adhesion protection of the gold layer by either EG6 SAM or poly(CBMA), we defined parameter η_{islands} as the ratio of the cell nuclei within the boundaries of circular islands to the total number of nuclei. If the nuclei were difficult to classify as “on” or “off” an island, higher magnification images were analyzed and the cell was counted as on-island, if >50% of the nucleus' area was within island's boundaries. We note that the nucleus-based measure is easier and less ambiguous than classifying whole cells, since cell contours are often irregular and even in cells localized onto the islands, the cells can form ruffling protrusions onto the surrounding, protected gold.³⁰ On unprotected substrates, the values of the η_{islands} parameter were low (see Fig. 1b). In such cases, the cells are expected to distribute randomly over the substrate's surface such that η_{islands} should be roughly equal to the percentage of substrate's area occupied by the islands. For the micropatterns we used, this percentage is $\eta_{\text{random}} \sim 20\%$. It follows that any percentage greater than this value indicates some degree of bioresistance. For appropriately protected surfaces (both by EG6 SAM or poly(CBMA)), the values of η_{islands} are typically above 90% (see Table 1 and also Table S1 in the Supporting Information for the statistical significance tests)

We first tested the adhesion-protection of EG6 vs. poly(CBMA) substrates for short times (8 hrs) after cell plating. To this end, we plated B16F1 mouse melanoma and Rat2 fibroblast cells and obtained ten to twenty phase-contrast (Fig. 1a) and corresponding fluorescent (Fig. 1a and Fig 2a,b,f,g) images using low magnification objective (10×). Quantification of relatively large numbers of cells (600 to over 2000 for each treatment/cell type, see Table 1) indicates that poly(CBMA) substrates offer cell-adhesion protection comparable to that of EG6 SAMs. In addition, actin cytoskeleton organization is comparable on micropatterned islands surrounded by gold regions derivatized with EG6 SAMs or poly(CBMA) (Fig. 2c,d and h,i). High-resolution imaging of actin cytoskeleton with confocal microscopy (using 63× objective) shows that for both types of substrates, cells spread out completely and occupy the etched islands fully. Also, on both types of substrates, B16 cells display mostly

peripheral actin bundles around the circular perimeter, while Rat2 cells feature more pronounced, straight actin stress fibers. These are typical cell type differences.

For some cell micropatterning applications such as long-term-use biosensors³⁸ or for studies of neuron-based logical circuits,⁴⁵ it may be useful to culture cells on micropatterned surfaces over long periods of time (days rather than hours). To test whether polymer-coated surfaces offer long-term resistance to cell adhesion, we performed experiments with slow growing CHO-k1 cells. The experimental details were similar to those described above for short-term experiments. The only difference was that we initially plated cells sparsely and cultured them on poly(CBMA) and EG6 SAM substrates for 2, 6, and 14 days followed by cell fixation, staining, and imaging. Images in Figure 3 and pertinent data in Table 1 indicate that poly(CBMA) protects the gold regions from cell adhesion as well as EG6 SAM for up to 6 days (98% for poly(CBMA) vs. 94% for EG6 SAM), and at 14 days polymer protected surfaces are only slightly less effective than EG6 SAMs (~87% for poly(CBMA) vs. 96 % for EG6 SAM). Despite this difference, 87% is still an excellent degree of protection (see Fig.3)

Finally, we note that poly(CBMA) layers have performance superior to the EG6 SAMs when both types of substrates are stored under 200 proof ethanol for several days (ethanol is chosen because it is a suitable solvent for shipping samples via most commercial carriers). For example, after four days, η_{islands} is only 68.3 % for EG6 SAMs compared to 89.4% for the poly(CBMA) kept under identical conditions. In contrast, under nitrogen storage, this difference is much less pronounced (e.g., after a week under N₂, η_{islands} = 95.6 % for EG6 vs. η_{islands} = 98.6 % for poly(CBMA)), suggesting that in solution the EG6 SAMs are more prone to equilibrate with the surrounding solvent than the poly(CBMA) layers.⁴⁶

Conclusions

In summary, poly(CBMA) coated gold surfaces are highly resistant to cell adhesion and suitable for cell micropatterning. While their degree of cell-adhesion-resistance is similar to that of EG6 SAMs, poly(CBMA) films can be stored in dry or wet state without a marked loss of properties. These characteristics suggest that poly(CBMA) substrates can become more applicable than EG6 SAMs in industrial applications where the substrates for cell-based assays should be easy to handle and robust.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Quantification of the resistance to cell-adhesion on micropatterned substrates

a) Phase contrast image shows a pattern of etched microislands (light circles) surrounded by gold (dark) protected with appropriate surface chemistry (here, EG6 SAM). Upon staining with Hoechst dye, the cells localize almost exclusively onto the microislands and can be conveniently identified by their nuclei. b) The effectiveness of bioprotection of the gold surface can be quantified by the ratio of the cell nuclei within islands' contours to the total number of nuclei (here, nuclei/cells on the islands are colored green—and nuclei/cells outside of the islands are colored red). For totally unprotected substrate s, $\eta_{\text{islands}} = 20.7 \pm 3.8\%$ based on the analysis of $n = 7$ substrates). Of course, on efficiently protected substrates such as those in a), η_{islands} is much higher, ~94-99%. Scale bars are 200 μm , and same for the two images in a).

Figure 2. Testing of the protected micro-etched gold surfaces for their resistance to cell-adhesion and applicability to cell micropatterning

Representative fluorescent images (Hoechst/nucleus) showing nuclei stained with Hoechst dye (oval bright spots) of B16F1 mouse melanoma cells (a,b) or Rat2 cells (f,g) on micro-etched substrates derivatized with EG6 SAM (a,f) or poly(CBMA) (b,g). Although the poly(CBMA) substrate was kept dry for 10 days before cell patterning, it protected gold from cell adhesion to a degree similar to the EG6 SAM. For a given cell type, organization of actin cytoskeleton (visualized by fluorescent phalloidin staining in (c,d) and (h, i) was similar irrespective of the chemistry used to render gold cell-resistant. Scale bars are 200 μm for (a,b,f,g) and 20 μm for (c,d,h,i). Quantification of the proportion of cells on etched islands (e,j); red line indicates statistically expected result for an unprotected surface onto which the cells attach randomly.

Figure 3. Long-term bioresistance of EG6 SAMs vs. poly(CBMA)-coated substrates
 CHO-k1 cells were cultured on the substrates for 2, 6 or 14 days. a,b,c) shows representative Hoechst/nucleus images. d) Quantifies the results in terms of the η_{islands} parameter. Red line indicates statistically expected result for an unprotected surface (i.e., for random distribution of the cells over the entire surface). Note that in addition to the times indicated, the poly(CBMA) substrates were kept dry, under ambient atmosphere for ~ 2 weeks. Scale bar is $200 \mu\text{m}$ and the same for all images.

Table 1

Quantification of the percentages, η_{islands} , of cells on cell-adhesive islands of the micropatterned substrates protected with either EG6 SAMs or poly(CBMA) films.

Cell Type, Spread Time	Surface	η_{islands} (%)	Number of Cells (substrates/images), standard deviation of η_{islands}
B16F1 cells, 8 hours	EG6 SAM	99.4	1610 ($s=6$, $i=28$); $\sigma = 1.21$
	CBMA polymer	98.9	1942 ($s=6$, $i=40$); $\sigma = 1.86$
Rat2 cells, 24 hours	EG6 SAM	98.7	2815 ($s=5$, $i=44$); $\sigma = 1.83$
	CBMA polymer	98.5	1964 ($s=7$, $i=52$); $\sigma = 2.99$
CHO-k1 cells, 2 days	EG6 SAM	87.0	678 ($s=2$, $i=16$); $\sigma = 5.91$
	CBMA polymer	98.6	2395 ($s=2$, $i=40$); $\sigma = 4.47$
CHO-k1 cells, 6 days	EG6 SAM	94.1	2205 ($s=2$, $i=4$); $\sigma = 1.37$
	CBMA polymer	98.8	1341 ($s=2$, $i=3$); $\sigma = 2.5$
CHO-k1 cells, 14 days	EG6 SAM	96.6	647 ($s=2$, $i=30$); $\sigma = 2.4$
	CBMA polymer	87.4	2364 ($s=2$, $i=7$); $\sigma = 2.81$