Notes

Covalent Tethering of Functional Microgel Films onto Poly(ethylene terephthalate) Surfaces

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Introduction

Recently there has been an increasing interest in developing biotolerant polymeric surfaces that have the ability to support or immobilize biological functionalities tailored for specific biotechnological and medical applications.[1−4] As an important example, it is common to immobilize extracellular-matrix proteins (e.g., collagen and fibronectin) or cell signaling molecules on polymeric surfaces to yield functional biomaterials that have the ability to modulate cell adhesion, proliferation, and differentiation, thus mimicking a natural cellular environment.[3,5−8]

Another interesting class of polymeric materials with desirable properties for biotechnology is the hydrogel microparticle or microgel. The synthesis, characterization and applications of stimuli-responsive microgels have been extensively studied over the past few years.[9−13] Recently, Nolan et al. investigated the performance of poly(*N*-isopropylacrylamide) (pNIPAm) microgels cross-linked with short poly(ethylene glycol) (PEG) chains, as protein adsorption-resistant films.[14] The PEG cross-linked pNIPAm microgels having poly(acrylic acid) as co-monomer were assembled electrostatically by spin-coating onto a cationic glass substrate. The results indicated that glass surfaces coated with microgels showed reduced protein adsorption and cell adhesion in vitro, i.e., nonfouling behavior. However, the potential of these microgel films as nonfouling base coatings for future biomedical implants cannot be probed and realized until they are assembled on a more flexible and biocompatible substrate than glass. This motivated our current goal to design a flexible substrate-based microgel film with potential nonfouling and anti-inflammatory behavior for in vivo studies. Equally important, we also aim to enhance the stability of the adherent microgel films in comparison with the Coulombically assembled film in biological environments by improvements in the surface chemistry.

For in vivo studies of biomaterials, common desirable attributes for a model biomaterial (in this case, a polymer) include good mechanical strength, flexibility, chemical and physical stability in the biological environment, and a surface chemistry/composition that allows for facile biofunctionalization. In view of these properties, poly(ethylene terephthalate) (PET) was chosen as a model biomaterial onto which we could deposit nonfouling microgel coatings in order to enhance its properties. PET has been extensively studied in biomaterial applications such as for sutures, vascular grafts, sewing cuffs for heart valves, and components for percutaneous access devices.[15−18] However, the PET surface is inert and hence not suitable for direct biofunctionalization. Major efforts have therefore been undertaken to introduce various functionalities onto the PET surface, such as amine, carboxyl, and hydroxyl moieties, which can be further employed for the covalent immobilization of biomacromolecules.[6,19,20] It is especially desirable that the methods used for the chemical modification are confined to the polymer surface, without affecting the bulk/mechanical properties of the substrate. A suitable technique in this regard has been the chemical activation of the inert polymer surface by plasma treatment, which has already been employed to render PET surfaces hydrophilic and hence more biocompatible.[21] It is also well-known that the exposure of polymeric surfaces to a plasma along with oxygen treatment generates surface-active hydroperoxide species that can be used for the chemical grafting of desired chemical and biological functional groups.[6,19,22−26]

Our method of functionalizing the PET with polymeric microgel films (Scheme 1) is derived from previous methods based on plasma-induced graft polymerization of poly acrylic acid (pAAc). Plasma- and ozone-induced graft polymerizations of various monomers on PET films, fibers, and fabrics have been demonstrated.[6,25,26] However, in order to make the method more general and to give the adherent microgel film more stability in biological environments, we introduced onto the PET surface a photoaffinity label, viz., aminobenzophenone. Upon excitation with UV irradiation, molecules of the benzophenone family have the ability to abstract an aliphatic hydrogen atom from any nearby polymer chain forming a covalent carbon−carbon bond.[4,27,28] Due to the presence of a microgel in the close vicinity of the benzophenone, it can abstract a hydrogen atom from the microgel and hence covalently attach the particles to the PET surface. Essentially, the benzophenone here serves as a glue between the base PET substrate and the microgel film.

Experimental Section

Materials. All materials were obtained from Sigma Aldrich unless otherwise specified. The monomer NIPAm was recrystallized from hexane obtained from J.T. Baker before use. Poly(ethylene terephthalate) (PET) sheets were obtained from AIN Plastics, Marietta, GA. All other chemicals were used as received. Formate buffer solution (pH 3.47, 10 mM) was prepared from formic acid and NaCl obtained from Fisher Scientific. Poly(ethylene glycol) diacrylate (PEG) (PEG MW 575, Polysciences, Inc.) was used as received. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Pierce. Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker. Phosphate buffered saline (PBS) solution (pH 7.4, 10 mM) was prepared from NaCl (Fisher), Na_2HPO_4 (EM Science), and KH_2PO_4. Water was distilled and then purified using a Barnstead E-Pure system to a

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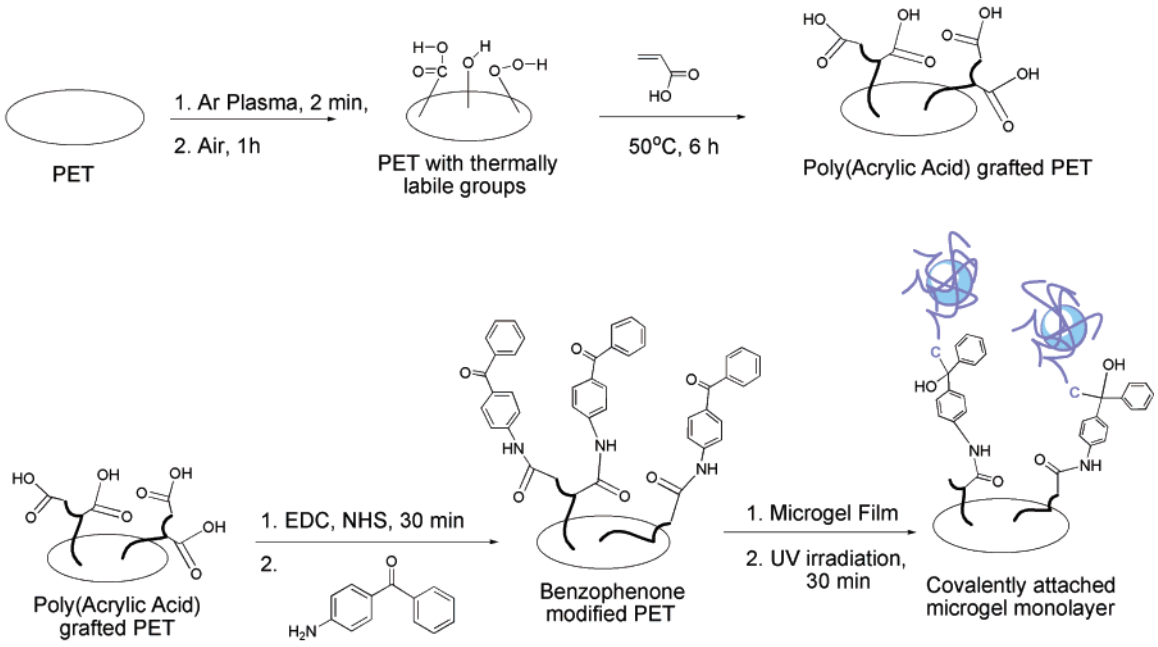
Scheme 1. Strategy for Covalent Tethering of Microgels onto a Poly(ethylene terephthalate) Surface



resistance of 18 MΩ and finally filtered through 0.2 μm membrane filter (Pall Gelman Metricel) before use.

Methods. *Microgel Synthesis.* Poly(*N*-isopropylacrylamide) (pNIPAm) microgel particles (100 mM total monomer concentration) were synthesized with 2 mol % poly (ethylene glycol) (PEG) diacrylate (MW 575) by a free radical precipitation polymerization method. For incorporating functional groups that can be later modified, the microgel particles were synthesized with 10 mol % acrylic acid as a co-monomer. Briefly, 0.4979 g of NIPAm monomer, 0.7011 g of cross-linker PEG-diacrylate, and 0.0025 g of surfactant sodium dodecyl sulfate (SDS) were dissolved in 49 mL of distilled, deionized (DI) water and filtered through a 0.2 μm filter. The solution was transferred to and stirred in a three-neck, round-bottom flask and heated to 70 °C while purging with N_2 gas. After reaching 70 °C and purging for 1 h, 34.3 μL of acrylic acid was added, followed by the addition of 0.0114 g (dissolved in 1 mL of DI water) of ammonium persulfate (APS) to initiate the reaction. The reaction was kept at 70 °C for 4 h. The synthesized microgels were then filtered and cleaned by five cycles of centrifugation at 15 422*g* for 45 min. The supernatant was removed, and the particles were redispersed in DI water. The particles were then lyophilized overnight before being used for deposition onto the PET films.

PET Film Functionalization. PET sheets were cut into 8 mm diameter disks using biopsy punches and briefly rinsed in 70% ethanol to remove contaminants introduced during the manufacturing process. Graft polymerization of acrylic acid (AAc) on 8 mm PET films was done in two steps. PET films were first placed in an 18 W RF Ar plasma (Harrick Scientific) connected to a vacuum pump (5×10^{-4} mbar) for 2 min. Immediately after the Ar treatment, air was introduced into the plasma chamber and maintained at atmospheric pressure for 1 h to generate peroxide and other oxygen-containing functional groups on the PET surface. The films were immediately transferred to a round-bottom flask containing an N_2 purged 25% (v/v) aqueous solution of acrylic acid. The grafting reaction was carried out for 6 h at 50 °C, after which the films were washed in water overnight. The degree of polymer grafting and hence the density of carboxyl groups on the PET surface can be controlled by varying the AAc concentration and reaction time.[22] The pAAc modified PET was further modified with 4-aminobenzophenone (ABP) using carbodiimide coupling.[29] The coupling of 4-aminobenzophenone is done traditionally as a one-step reaction using *N*,*N*′-dicyclohexylcarbodiimide (DCC) in organic media (DMSO). However, we used an aqueous carbodiimide coupling strategy based on activation of carboxyl groups with *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and further reaction with the ABP. This is to avoid the formation of urea precipitate (the byproduct in the DCC reaction), which is difficult to remove completely from the surface being modified.[30] The pAAc modified PET films were first activated by incubation in 2 mM EDC and 5 mM NHS in 10 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer solution (pH 6.0) for 30 min at room temperature. The films were then placed in 20 mM 2-mercaptoethanol solution in DI water to quench the EDC. The activated films were then reacted with ABP in DMSO for 2 h at room temperature. The ABP modified films were washed in DMSO and immersed in 10 mM hydroxylamine solution to quench the reaction. Finally, the films were washed in DI water.

Carboxyl Group Determination. The amount of pAAc grafting on the PET film surface was characterized by a colorimetric method based on Toluidine Blue O staining.[19] Briefly, the grafted film was placed for 6 h at 30 °C in a 0.5 mM Toluidine Blue O solution prepared at pH 10. The film was then removed and thoroughly washed with NaOH (pH 10) to remove any dye nonspecifically adhered to the surface. The bound dye molecules were then desorbed from the film in a 50% acetic acid solution. The final dye content was determined from the optical density (OD) of the solution at 633 nm using a Shimadzu 1601 UV−visible spectrophotometer.

Particle Deposition. A spin-coating process was used to deposit a layer of microgel particles onto the functionalized PET films. The PET film was placed onto a glass slide, and the slide was placed onto the spin coater (Specialty Coating Systems) chuck and held in place by vacuum. The rotor speed was maintained at 500 rpm. Dried microgels were dispersed in a 10 mM formate buffer (pH 3.47) solution and one drop of the microgel solution was deposited onto the PET film while spinning. After keeping the film on the spin coater for 100 s, a second drop of the microgel solution was deposited. The PET film was left on the spin coater for additional 100 s, and the film was allowed to dry. Finally, another drop of microgel solution was deposited on the PET by the same process, and the film was dried after 100 s of spinning. This process was done on both sides of the PET films under dark conditions. Each side of the PET, with the dried microgel film, was irradiated by a 100 W longwave UV lamp (Blak-Ray) for 30 min to covalently attach the microgels onto the PET surface. The microgel-modified PET film was soaked in 10 mM phosphate buffer solution (pH 7.4) for 6 h and then washed with DI water.

Atomic Force Microscopy. All images were obtained in AC mode on an Asylum Research MFP-3D atomic force microscope (AFM).

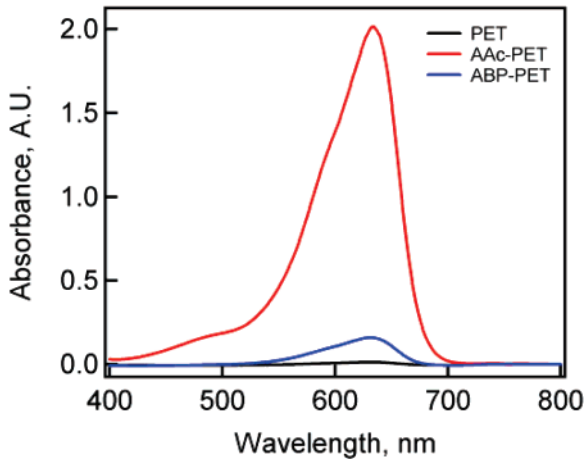


Figure 1. Absorption spectra for desorbed Toluidine Blue O dye from bare PET (black) and poly(acrylic acid) grafted PET before (red) and after modification with 4-aminobenzophenone (blue).

Spring constants were calculated using the thermal method. Imaging and analysis was performed using the Asylum Research MFP-3D software (written in the IgorPro environment, WaveMetrics, Inc., Lake Oswego, OR). An Olympus AC160 cantilever with $k = 42$ N/m, $f_0 = 300$ kHz was used for imaging.

In Vitro Cell Adhesion. The IC-21 murine macrophage cell line (ATCC; Manassas, VA) was used to determine the bioresistant properties of the microgel coated PET in vitro. Cells were seeded at a density of 67 000 cells/cm^2 on unmodified PET and microgel-coated PET disks in 24-well tissue culture-treated polystyrene plates in culture media containing 10% fetal bovine serum. After 48 h, adherent cells were fluorescently stained with calcein-AM (Molecular Probes, Eugene, OR) and imaged using a Nikon TE-300 microscope to determine relative cell numbers and cell spreading on each surface.

Results and Discussion

In order to deposit uniform films of microgels, the PET films had to be rendered amenable to robust particle attachment. The approach described above (Scheme 1) involves surface activation in an Ar plasma followed by the introduction of air to introduce thermally labile groups. These thermally labile groups thermally decompose to form radicals, thus initiating the polymerization of AAc to form pAAc-grafts on the PET surface.[22−24] The carboxyl groups of the pAAc on the PET surface are subsequently used in the functionalization of the surface with photoaffinity label (ABP) using carbodiimide coupling chemistry. We characterized the surface grafting density of pAAc by the Toluidine blue O dye binding assay. Figure 1 shows UV−visible absorbance spectra of Toluidine blue O dye arising from various surface treatments. Based on previous methods, by assuming a 1:1 ratio between the dye and the carboxylic acid groups, the OD at 633 nm gives a measure of the degree of grafting.[19,23] Thus, successful pAAc grafting of the PET surface is evidenced by an increase in the OD from ~0.01 for the bare PET substrate to about 2.02 for the modified surface. The color staining of the dyed films was very uniform across the samples, suggesting relatively uniform coating of the PET (data not shown). For the pAAc grafted PET, we estimate about 1.4×10^{-7} mol of carboxyl groups and following the reaction with

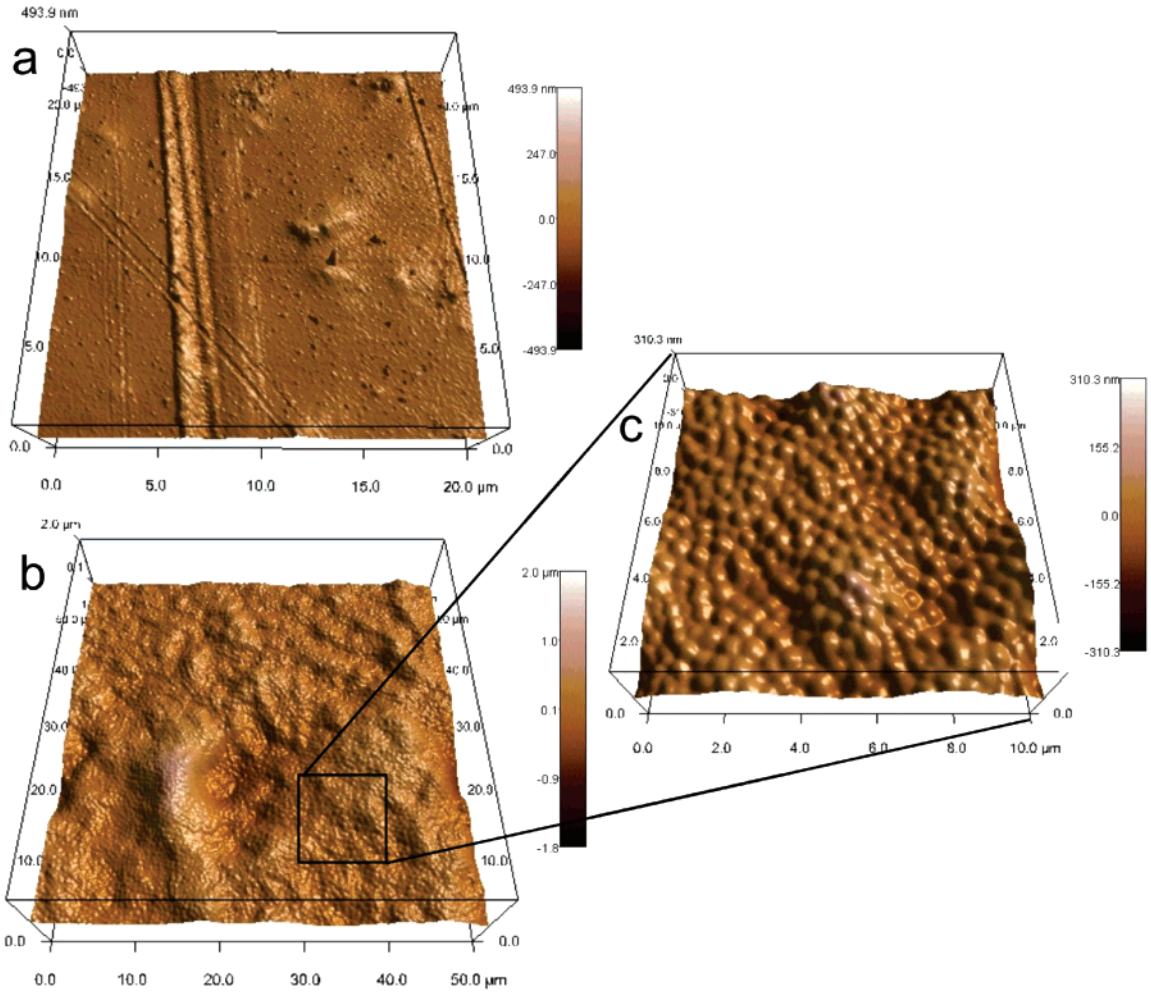


Figure 2. 3D rendering of AFM images for (a) bare PET and (b and c) microgel-modified PET.

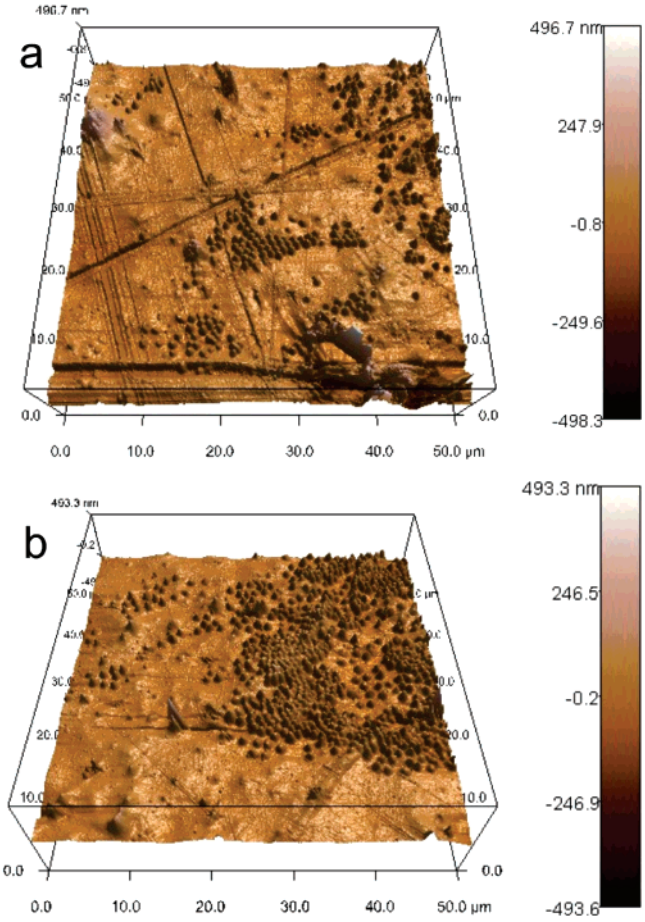


Figure 3. 3D rendering of AFM image of microgels spin coated onto pAAc-grafted PET (a) without benzophenone modification and (b) with benzophenone modification but without UV irradiation.

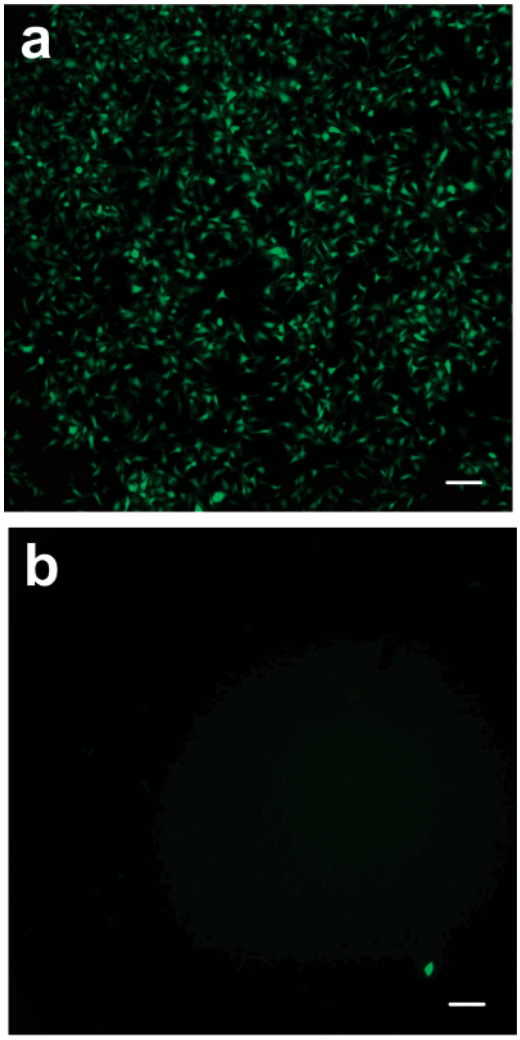


Figure 4. Macrophage adhesion on (a) bare PET and (b) PET covalently functionalized by microgels. Adherent cells were stained with calcein-AM which stains live cells (green). Scale bar = 100 μm.

ABP, only about 1.1×10^{-8} mol of carboxyl groups are left on the surface. Hence, this suggests that the benzophenone modification of the PET results in a loss of ~92% of the carboxyl groups due to their conversion into amide groups.

Our method of surface functionalization of the PET with photoaffinity labels results in a very efficient surface modification with the microgels. Figure 2 shows 3D renderings of AFM images obtained from a representative film. It can be seen from the 50×50 μm scan (Figure 2b) that there are no uncoated areas in the interrogated region. The microgels also form a dense conformal monolayer as indicated by the 10×10 μm scan (Figure 2c). The unevenness in the microgel-coated PET is due to the uneven base surface of the PET as seen in Figure 2a.

The benzophenone modification and photocrosslinking are critically important steps for obtaining a stable monolayer, as suggested by Figure 3. Figure 3a shows an AFM image of a microgel film that was spin-coated onto pAAc-grafted PET without benzophenone modification, followed by extensive washing. It is clear that the coverage is sparse with only a few microgel particles retained on the surface. Since covalent linkages are not possible in the absence of the photoaffinity group, the particles cannot remain adhered to the film during the washing step. This poor coverage is probably also due, in part, to the anionic charge on both the microgels (due to the AAc co-monomers) and the film (due to the pAAc grafts). In the case of benzophenone-modified surface (Figure 3b), slightly more microgels are retained on the PET surface, presumably due to less Coulombic repulsion between the microgels and the modified PET. In this case, the photoirradiation step is omitted, and again, no covalent attachment is possible. However, the best results are found for the pAAc-grafted PET surfaces modified by benzophenone and further photoirradiated (Figure 2b). The photocross-linking is thus shown to provide a microgel film with excellent adhesion to the substrate and hence a presumed stability for use in biological environments.

It is known that one of the key steps in the inflammatory host response to biomaterials is nonspecific protein adsorption, which then mediates cell adhesion and spreading.[31] Recent efforts in the field of biomaterials and medical implants have focused on developing non-fouling surface treatments to prevent this nonspecific protein adsorption and cell adhesion.[4,32−34] Our group has previously shown the efficacy of PEG-containing pNIPAm microgels as protein and cell adhesion-resistant materials.[9,14] In addition to their nonfouling behavior, the facile and well-controlled synthesis of highly monodispersed microgels in a range of sizes, ease of their biofunctionalization using various orthogonal chemical functionalities, and possibility of coassembling varied microgels onto a single substrate to generate complex biointerfaces makes them interesting candidates for biomedical implant coatings for modulation of inflammatory response. We take advantage of these attributes to study and produce model biomaterials incorporating microgels that can be tested for their functionality.

On the basis of the AFM confirmation of a stable uniform monolayer of microgels on the PET surface, we tested the cell

adhesion resistance of these surfaces in vitro. IC-21 macrophages were plated on substrates in culture media containing 10% serum. This provides a rigorous test for bioresistance as cell adhesive proteins present in serum rapidly adsorb onto synthetic surfaces and mediate cell adhesion and spreading. In contrast to bare PET films, which supported high levels of cell adhesion and spreading, microgel-functionalized PET films exhibited no macrophage adhesion over the 48 h test period (Figure 4), indicating a stable cell adhesion-resistant coating. We attribute the lack of cell adhesion to microgel-functionalized surface to the protein-resistant nature of the PEG cross-linked microgels. The ability of microgel-coated surfaces to resist cell adhesion and spreading was distributed throughout the entire sample, indicating uniform distribution of bioresistance. The success of this surface functionalization strategy thus allows the study of the non-fouling behavior of the PEG cross-linked pNIPAm microgels in vivo and also gives us opportunities to develop more complex biomaterials incorporating multifunctional microgel monolayers.

Conclusion

In conclusion, we report a simple, scalable, and reproducible method of functionalizing PET with a conformal, dense film of hydrogel microparticles. The microgel layer is stable due to the covalent attachment of the microgels to the PET surface via a photoaffinity technique. This method can be easily extended for modifying the inert PET surface with any organic species, providing bioactive surfaces possessing excellent stability. Note that the spin coating deposition method is used here mainly for speed, convenience, and potential scalability. However, it cannot be used to coat substrates with complex geometries, and in such cases, other deposition techniques must be employed. We are currently evaluating methods for dip-coating of microgels onto complex substrates. Future studies are also geared toward studying the stability and properties of these microgel coatings in vivo.

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