Synthesis and Application of Fluorescein-Labeled Pluronic Block Copolymers to the Study of Polymer−Surface Interactions

Fariyal Ahmed, Paschalis Alexandridis, and Sriram Neelamegham*

Department of Chemical Engineering, State University of New York at Buffalo, Buffalo, New York 14260-4200

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We present a novel methodology for the conjugation of Pluronic poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide) block copolymers with a fluorescein derivative, 5-(4,6-dichlorotriazinyl) aminofluorescein (5-DTAF), at room temperature under aqueous conditions. Two Pluronic block copolymers, F68 with molecular weight (MW) 8400 and P105 with MW 6500, were examined. The labeled block copolymers exhibited maximum absorbance at 498 nm, peak fluorescence at 516 nm, and a marked reduction in both absorbance and fluorescence at acidic pH below 7. Flow cytometry was employed to quantify the real-time adsorption kinetics of labeled block copolymers to two types of surfaces: polystyrene microspheres and blood cells, specifically platelets. This methodology allowed detection of Pluronics at low solution concentrations down to 1 μM. In the case of labeled Pluronic F68 and P105 binding to polystyrene beads, a rapid time-dependent adsorption kinetics was observed with 80% adsorption occurring within 30 s and saturation in 2 min. The concentration-dependent adsorption profile for Pluronic F68 binding to polystyrene beads was similar to that of P105, with increasing binding in the range from 1 to 25 μM and saturation at higher concentrations. Surface modification of the polystyrene beads by carboxylation dramatically reduced the binding of both Pluronic F68 and P105. In experiments that examined Pluronic interaction with blood platelets, we observed that Pluronics not only bind the platelets in a dose-dependent fashion but also bind soluble macromolecules in blood plasma. Overall, the labeled block copolymers synthesized in this study were found to exhibit the fluorescence characteristics of the unconjugated 5-DTAF and the binding characteristics of unlabeled Pluronics. Such an approach of labeling hydroxyl-terminated polymers with fluorescein and applying flow cytometry could be used to probe polymer−surface interactions in a variety of chemical and biological systems.

Introduction

Water-soluble triblock copolymers composed of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) blocks linked together in the sequence PEO−PPO−PEO have been developed under the trademark Pluronic (by BASF Chemicals) and under the generic name Poloxamers. Depending on the specific requirements of the application, the amphiphilic nature of these block copolymers can be varied by controlling the length of the PEO and/or PPO blocks.[1] Over the last two decades, Pluronic block copolymers have found numerous applications in the chemical, environmental, and pharmaceutical industries as antifoaming agents, emulsifiers, and colloidal dispersion stabilizers.[2−5] More recently, because of their nontoxic properties and surface-active nature these block copolymers have also found application in the areas of biomaterials,[6,7] drug delivery,[8−10] and cardiovascular therapeutics.[11,12] The binding of Pluronics to hydrophobic surfaces via their PPO segments causes them to extend their hydrophilic PEO chains into the aqueous solvent phase, therefore sterically reducing protein adsorption[13,14] and cell adhesion[15−17] on the surface. This modified surface property enhances its biocompatibility, reduces its thrombogenicity, and allows its application in biomaterial formulations.[18,19] Also, in drug delivery systems Pluronic block copolymers adsorbed onto colloidal carriers have been shown to increase their "stealth" properties, thus preventing their rapid phagocytotic removal by the body's defense system and increasing their life span in circulation.[8,20,21]

A number of techniques have been developed to quantify the adsorption kinetics of Pluronics to surfaces. Because these Pluronics do not contain significant natural ultra-

* Corresponding author. Sriram Neelamegham, Department of Chemical Engineering, State University of New York at Buffalo, 906 Furnas Hall, Buffalo, NY 14260. Phone: (716) 645-2911 ext 2220. Fax: (716) 645-3822. E-mail: neel@eng.buffalo.edu.

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violet or visible light absorbing chromophores, several groups have radioactively modified these polymers and used them as probes to study polymer−surface interaction.[6,22−25] Because of the nature of radioactivity, the handling of such materials requires extensive precautions and the half-life of these reagents is on the order of days. Furthermore, these labeling protocols involve multiple steps; the first step involves substitution of the terminal hydroxyl end of the copolymer with an amino[23] or hydrazine[6,24] group, followed by a second radiolabeling step with ^{125}I. A few limited studies have also developed fluorescently conjugated polymers.[6,26] These methodologies involve multiple steps, long reaction times ($t \geq 12$ h),[26] and the use of organic solvents, for example, tetrahydrofuran (THF).[6,26] Because of the often corrosive or poisonous nature of the intermediates formed during these reactions, the application of these labeled block copolymers in biological systems is limited. Also, the presence of several of these organic solvents has been shown to increase the permeability of cells and decrease their viability. To overcome these limitations, we developed a novel protocol to label Pluronic block copolymers under mild aqueous conditions. Here, the terminal hydroxyl ends of the block copolymers are directly conjugated in a single step with a fluorescein derivative, 5-(4,6-dichlorotriazinyl) aminofluorescein (5-DTAF). As examples, results from labeling two representative members of the Pluronic block copolymer family with varying PEO and PPO block lengths are presented: Pluronic F68 ($EO_{75}-PO_{30}-EO_{75}$) and Pluronic P105 ($EO_{37}-PO_{56}-EO_{37}$).

A wide range of techniques including light scattering,[10] X-ray photoelectron spectroscopy (XPS),[13] total internal reflection fluorescence (TIRF),[26] and surface plasmon resonance (SPR)[27] have been used to study polymer adsorption on surfaces. Light scattering and XPS are both indirect measurement techniques that only estimate Pluronic equilibrium binding levels (and not dynamic adsorption rates) based on quantitation of Pluronics not bound to the surface of beads and the level of atomic oxygen detected on washed surfaces, respectively. TIRF and SPR adsorption data are semiquantitative because they detect light angle shifts and not the absolute binding of polymers on surfaces. Furthermore, these techniques often involve repeated washing of surfaces, which diminishes the accuracy of measurements. The sensitivity of such systems is low and ranges from 0.01 to 1.19 mM.[22] Furthermore, sample preparation and measurement is a cumbersome process involving multiple steps. To overcome these limitations, we have developed a flow cytometric protocol that can be used in conjunction with the novel fluorescent probe to study polymer adsorption kinetics. Similar methodologies that apply flow cytometry have previously found many applications in the analysis of biological interactions.[28−30] This measurement is rapid and direct because particle fluorescence measurements are made in real time. Therefore, the resolution of our measurements and consequently the resolution of polymer binding kinetics is on the order of seconds. Furthermore, as we will demonstrate, these measurements are sensitive to concentrations on the order of 1 μM. In the current study, we demonstrate the application of flow cytometry to the study of Pluronic binding to hydrophobic surfaces including polystyrene beads and blood platelets. In particular, we examine (i) the dynamic time and concentration-dependent copolymer adsorption kinetics, (ii) the effects of surface modification on binding kinetics, and (iii) the influence of block copolymer self-assembly (micelle formation) on adsorption.

Materials and Methods

Materials. Pluronic F68 and P105, PEO−PPO−PEO block copolymers, were obtained as a gift from BASF Corp. (Mount Olive, NJ). Pluronic F68 has a molecular weight (MW) of 8400 and 80% PEO content, and P105 has a MW of 6500 and is 50% PEO by weight. The fluorescent probe 5-DTAF (MW = 495.28) was obtained from Molecular Probes (Eugene, OR). Cobalt nitrate·$6H_2O$ was purchased from J. T. Baker (Phillipsburg, NJ). HEPES buffer containing 110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, and 30 mM HEPES at pH 8.0 was prepared as described earlier.[31] The above chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-50 and G-25 medium-grade beads were purchased from Pharmacia (Uppsala, Sweden). Polybead polystyrene microspheres and Polybead carboxylate microspheres with mean diameters of 2.00 and 1.94 microns, respectively, were obtained from Polysciences, Inc. (Warrington, PA).

Fluorescent Labeling of Pluronic Block Copolymers. Pluronic F68 and P105 were fluorescently conjugated with 5-DTAF in an aqueous medium. Stock solutions of 6 w/v% Pluronic F68 and P105 were prepared by dissolving the polymers in 0.1 M sodium bicarbonate solution at pH = 9.34. A stock solution of 20 g/L 5-DTAF was prepared by dissolving the fluorescein probe in an aprotic solvent, dimethyl sulfoxide (DMSO). The 5-DTAF solution was diluted in 0.1 M sodium bicarbonate solution and added to the Pluronic block copolymer solution such that the molar ratio of 5-DTAF to Pluronic varied from 0.5:1 to 2:1. The reaction was allowed to proceed in the dark at room temperature for 3−5 h.

Purification of Labeled Block Copolymer by Size Exclusion Chromatography and Centrifugation. The reaction of 5-DTAF with Pluronic results in the formation of labeled block copolymer product and excess unreacted 5-DTAF. Size exclusion chromatography was applied to separate the desired labeled Pluronic from the excess 5-DTAF. The separation is based on differences in the molecular weights of the Pluronic copolymers and 5-DTAF. The separation was carried out in the dark to avoid possible photobleaching of the fluorescent tag. Sephadex G-50 beads were used to separate labeled Pluronic P105 from 5-DTAF, and Sephadex G-25 beads were used for labeled Pluronic F68 separation. The Sephadex beads swollen in 0.05 M NaCl solution at 90 °C (1 atm) for 5 h were first packed into a 1 × 10 cm flex-column (KONTES, Vineland, NJ) under gravity. The column was then primed by washing with 0.05 M NaCl solution, followed by passage of 1 bed volume of 6 w/v% unlabeled Pluronic in sodium bicarbonate solution and 2−3 bed volumes of 0.05 M NaCl solution. A 250 μL sample of the reaction mixture was then added to the column, and the labeled product was eluted with NaCl solution. During both the reaction and separation steps, significant dilution of the 5-DTAF occurs and this results in the removal of DMSO from the final product. This chromatographic separation was observed as two distinct yellow bands moving down the length of the column. The column effluent was collected in 1 mL fractionates and set aside for further concentration of labeled product and analysis.

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The labeled Pluronic was concentrated using a Centricon centrifugal filter device (Millipore Corp., MA) with a molecular weight cutoff of 3000. This filtration device used centrifugal force to drive the lower molecular weight 5-DTAF and solvents through an anisotropic membrane filter and retain the labeled copolymers (MW > 3000) above the membrane. Hence, as the retained labeled copolymer solution decreased in volume, the concentration of labeled copolymers increased and the product was concentrated. In our runs, 1 mL of labeled sample was added to the filter tubes and spun at 7000*g* for 30−40 min or until the final volume of the retentate was 300 μL. The labeled Pluronic was recovered and stored for characterization and functional studies. The filtrate containing free unconjugated 5-DTAF was also stored and used in subsequent experiments as a negative control for the fluorescence measurements and flow cytometric studies (discussed later).

Determination of Pluronic Concentration Using Colorimetric Assay. A previously published colorimetry assay[32,33] based on the ability of cobalt thiocyanate to form a complex with Pluronic polymers was modified to determine the concentration of labeled Pluronic F68 and P105 block copolymers. Briefly, in our protocol, a cobalt thiocyanate reagent was prepared by dissolving 3 g of cobalt nitrate and 20 g of ammonium thiocyanate in 100 mL of water. A portion (100 μL) of this reagent was added to 200 μL of ethyl acetate and 200 μL of Pluronic standards in the concentration range 0−0.2 w/v%. The mixture was gently vortexed and centrifuged in an Eppendorf Centrifuge (model no. 5415C, Eppendorf Scientific, NY) at 11500*g* for 8 min or until a clean, blue pellet was formed at the bottom of the tube. This pellet consists of cobalt thiocyanate complexed with Pluronic copolymer standards. The size of the pellet was observed to increase with the concentration of the Pluronic standards. The supernatant was aspirated. The pellet and the walls of the tube were further washed with 200 μL of ethyl acetate to remove excess uncomplexed ammonium thiocyanate with minimal disruption of the pellet. The solution was then centrifuged at 11500*g* for an additional 2 min. A light blue supernatant, formed after "washing the pellet", was removed, and this wash step was repeated 6−8 times until the supernatant appeared colorless. After the final wash, the pellet was dissolved in 1 mL of acetone. A portion (200 μL) of this solution was pipetted to a 96 well plate (Corning Inc., Corning, NY), and the absorbance was measured at 623 nm using a Microplate Spectrophotometer (Spectramax 340, Molecular Devices, CA). Once the calibration standards were established, the assay was repeated with 1 v/v% labeled Pluronic block copolymer. The Pluronic concentration in the labeled sample was determined by comparing its absorbance intensity to the calibration standard previously constructed.

Determination of 5-DTAF Conjugated to Pluronic Using Absorbance and Fluorescence Measurements. The concentration of 5-DTAF conjugated to Pluronic was determined using a combination of absorbance and fluorescence measurements. For these runs, a 5-DTAF stock solution was prepared by dissolving the fluorescein tag in DMSO as described earlier. This was serially diluted in HEPES buffer (pH = 8.0) to concentrations ranging from 3.2×10^{-4} to 5 mM. The absorbance of these standards and of the 1 v/v% diluted labeled Pluronic sample was measured at 498 nm using a spectrophotometer (Spectramax 340, Molecular Devices, CA). These values used in conjunction with the colorimetry assay described above yielded estimates of the concentration of 5-DTAF conjugated to the labeled Pluronic. The fluorescence of the standards and samples was also measured using a spectrofluorometer (Spectramax Gemini, Molecular Devices, CA) with an excitation wavelength of 492 nm and an emission detection wavelength of 516 nm.

Preparation of Polystyrene Beads and Platelets. For our experiments with Polybead polystyrene microspheres (which initially contain detergents), both the plain and carboxylated beads were first resuspended in 1 mL of 0.9 w/v% saline solution. The solution was spun at 11500*g* for 4 min to pellet the beads. The supernatant was then removed to reduce detergent content, and the pellet was resuspended in fresh saline solution. The "pellet and resuspend" protocol was repeated three more times. Following the final resuspension, the beads were sonicated for 10 min to separate any aggregated beads in solution. For experiments with platelets, fresh human blood was obtained from healthy volunteers by venipuncture into a syringe containing 1:9 sodium citrate anticoagulant (Sigma), following protocols approved by the SUNY Institutional Review Board. The blood was centrifuged at 150*g* and room temperature for 12 min to obtain the supernatant which consists of platelets along with the soluble components of blood plasma. This supernatant is called platelet rich plasma (PRP). The remaining blood was further spun at 1900*g* for 15 min to obtain blood plasma that has very few platelets. This later fraction is called platelet poor plasma (PPP). For some experiments, we also obtained washed platelets (WP), which consist of platelets in the absence of any blood plasma. This was achieved by centrifuging the PRP obtained earlier at 190*g* for 5 min. The pellet formed was resuspended in HEPES buffer containing 30 nM ZK36374 (a prostacyclin analogue from Shering AG, Berlin, Germany). The concentration of both the beads (plain and carboxylated) and platelets (in PRP and WP) in suspension was determined using a Coulter Counter (model ZM, Luton, England).

Measurement of Labeled Pluronic Binding to Polystyrene Beads and Platelets using Flow Cytometry. A FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) was used to quantify the binding kinetics of Pluronics to polystyrene bead and platelet cell surfaces. The flow cytometer used here is a standard instrument commonly used in biological/biomedical applications.[28−30] It essentially analyzes the fluorescence of individual particles (beads or platelets in this case). Here, each particle passing through the instrument is individually detected based on its characteristic forward (size) and side (granularity) light scatter. The particle is individually excited with a 488 nm fixed-wavelength argon laser, and the emission of 5-DTAF bound onto the bead surface is detected using a photomultiplier tube (PMT) which detects green fluorescence at 525 nm. It is important to note that this instrument detects only Pluronics bound to the beads, and any background fluorescence due to either free-labeled Pluronic or free 5-DTAF in solution is typically low.

All the experimental runs with beads and platelets were carried out at 37 °C and pH of 7.5−8. In some experiments, where the time-dependent binding of labeled Pluronic to the beads was measured, 0.1 mM labeled Pluronic (F68 or P105) solution was prepared in HEPES buffer. Beads at 2×10^5 particles/mL were then added to the Pluronic solution, and the time-dependent kinetics of Pluronic adsorption onto the polystyrene bead surface was measured at 30 s intervals using flow cytometry. Similar experiments were also performed with blood platelets at 100×10^6 cells/mL, because this concentration is observed in the body. Platelet suspensions at 100×10^6 cells/mL were made either by supplementing PRP with appropriate amounts of PPP to obtain platelets in the milieu of blood plasma or by diluting WP in HEPES buffer to obtain platelets in the absence of blood plasma. In other experiments, carried out to obtain the concentration-dependent adsorption profiles, labeled Pluronic (both F68 and P105) block copolymers were incubated over a range of concentrations from 0 to 2 mM with either the polystyrene beads at 2×10^5 beads/mL or the platelets (PRP or WP) at 100×10^6 cells/mL for 15 min. The saturation geometric mean fluorescence intensity of the single bead/platelet population was then measured using flow cytometry to determine the level of Pluronic adsorption onto the particles. The fluorescence of washed beads/platelets in the absence of labeled Pluronic and in the presence of filtrate obtained during the labeled copolymer concentration step using the Centricon device was used as the negative control for these runs.

Results and Discussion

This manuscript examines two aspects of polymer−surface interactions. First, we discuss a methodology for the synthesis of fluorescent Pluronic probes under aqueous conditions. Second, we describe how flow cytometry may be applied in conjunction with these fluorescent probes, to measure polymer adsorption kinetics onto polystyrene

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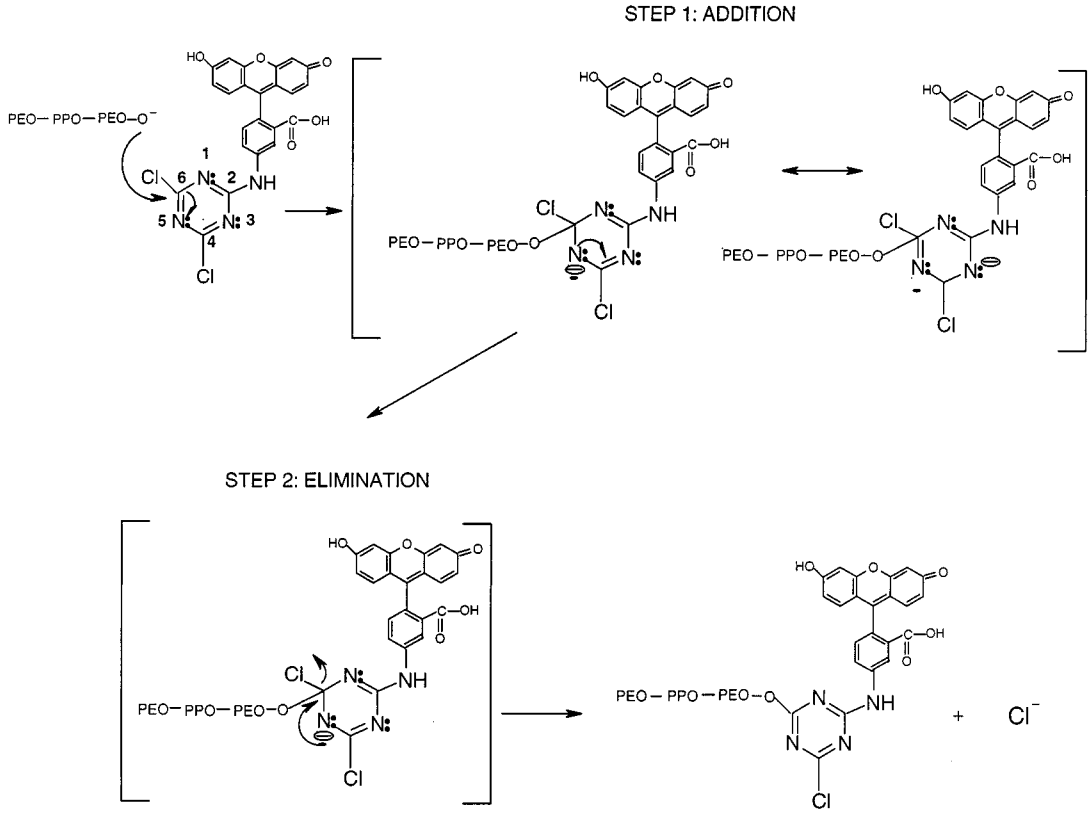

Figure 1. Reaction schematic for labeling Pluronic block copolymer. The detailed reaction mechanism involves nucleophilic aromatic substitution by an addition−elimination pathway. The 2-amino-4,6-dichloro-*s*-triazine ring in 5-DTAF is the reactive site for dye conjugation. The reaction is promoted by strong electron withdrawing groups (N) at the 1, 3, and 5 positions on the *s*-triazine ring. In the addition step (step 1), the polymer hydroxyl attacks either of the chlorine atoms on the *s*-triazine ring, leading to the formation of monosubstituted resonance structures. In the elimination step (step 2), the chlorine atom is released and this results in the final labeled block copolymer product.

beads and blood platelets at low concentrations in real time.

1. Synthesis. *Reaction Mechanism for 5-DTAF−Pluronic Conjugation.* Pluronic polymers react with the fluorescein analogue (5-DTAF) via their terminal hydroxyl group. The conjugation of Pluronic copolymers with the fluorescent tag, 5-DTAF, involves a nucleophilic aromatic substitution mechanism (Figure 1). The terminal hydroxyl groups of the block copolymers are weak nucleophiles that attack the reactive moiety, 2-amino-4,6-dichloro-*s*-triazine, on the 5-DTAF molecule. The reaction is promoted by strong electron-withdrawing groups (N) at the 1, 3, and 5 positions of the *s*-triazine ring. These groups are at the ortho and para positions to the chlorine-leaving group. In the addition step (step 1), the polymer nucleophile attacks a chlorine atom (ipso attack) and this addition produces an anion with a highly delocalized charge. This leads to resonance stabilization. In the elimination step (step 2), the block copolymer removes the chlorine group from the aromatic ring leading to the formation of labeled Pluronic and hydrochloric acid product. Two types of labeled block copolymer products may be formed: those conjugated at one end with 5-DTAF (as in Figure 1) and those labeled with 5-DTAF on both terminal hydroxyl groups. Therefore, the number of fluorescein molecules per polymer (F/P ratio) can at most equal 2. Disubstitution of both chlorine moieties on the same *s*-triazine ring of 5-DTAF by two hydroxyl end groups is not possible at temperatures below 65 °C.[34]

Detection of 5-DTAF Conjugated to Pluronic Block Copolymer. To determine the extent of 5-DTAF reacting with Pluronic polymer, we applied two independent experimental protocols to detect (a) the concentration of Pluronic in solution and (b) the concentration of 5-DTAF conjugated to Pluronic.

The concentration of Pluronic in solution was determined using a colorimetry assay. Experiments were performed with two Pluronic block copolymers, F68 and P105. When mixed with ammonium thiocyanate reagent, both Pluronics formed complexes with the salt. These complexes absorbed light in the range of 540−680 nm with a maximum at 623 nm as shown in Figure 2 (see inset). Negative control experiments that measured Pluronic absorbance in the absence of the cobalt thiocyanate reagent did not exhibit light absorbance (data not shown). Calibration curves were constructed over Pluronic concentrations ranging from 0 to 0.2 w/v% for F68 and P105 (Figure 2). As seen, the concentration-dependent response was linear in the range tested.

The fluorescein tag, 5-DTAF, shows a strong absorbance at 492 nm and a distinct fluorescence profile with a peak emission wavelength of 516 nm.[35] We determined the characteristic absorbance and fluorescence profiles of 5-DTAF when coupled to Pluronic polymer and compared them to that of free, unconjugated 5-DTAF. Figure 3 depicts the characteristic absorbance and emission profiles of labeled Pluronic P105 block copolymer. The fluorescently tagged Pluronic showed a distinct absorbance peak at 498 nm and an emission spectrum with a maximum at 516 nm. The absorbance and emission wavelengths of both the labeled P105 (Figure 3) and F68 (data not shown)

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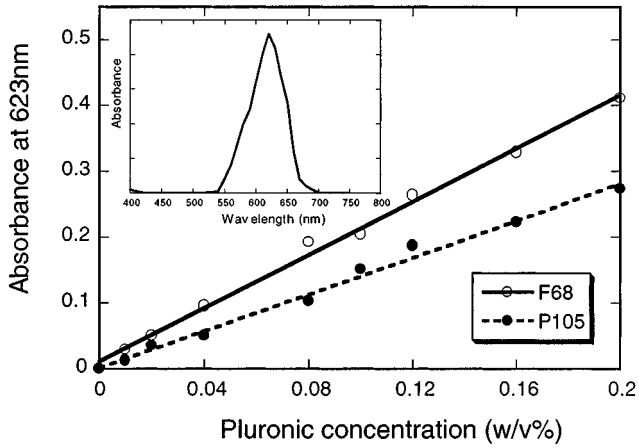

Figure 2. Detection of Pluronic in the labeled DTAF−Pluronic product. Pluronic F68 and P105 were complexed with an ammonium thiocyanate salt as described in Materials and Methods. The inset figure depicts the absorbance profile of the complex. Calibration standards for Pluronic F68 and P105 are also presented over concentrations ranging from 0 to 0.2 w/v%. The data are representative of more than five independent experiments.

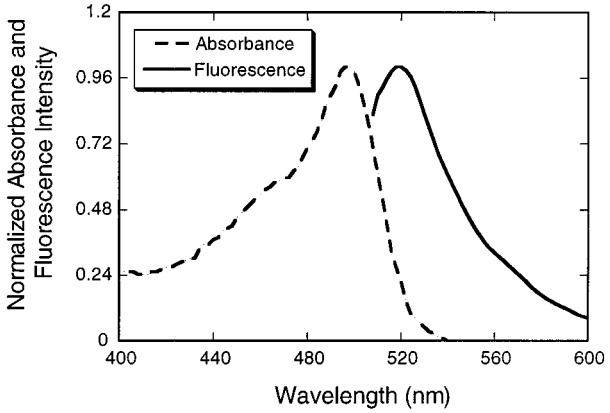

Figure 3. Characteristic absorbance and fluorescence profiles of labeled Pluronic P105. The absorbance of 0.25 mM labeled Pluronic P105 at pH = 8.0 was measured using a spectrophotometer (dotted line). The fluorescence spectrum was obtained using a spectrofluorometer by exciting the polymer at 492 nm (solid line).

were within 1−2% of that of free 5-DTAF. Pluronics not conjugated with 5-DTAF did not exhibit either an absorbance or emission spectrum over the range tested. For the experimental results presented in this paper, we quantified the extent of 5-DTAF conjugated to Pluronic using the absorbance measurements rather than the emission characteristics of the conjugated polymer. This was because although the conjugation of a fluorescent tag to a macromolecule does not typically alter its absorbance value, it may alter the quantum fluorescence yield of the fluorophore.[36]

As seen above, the absorbance wavelength of Pluronic complexed with cobalt thiocyanate (Figure 2 inset) does not overlap with that of 5-DTAF (Figure 3). Therefore, the application of the two independent absorbance measurement protocols described here allows us to distinguish between the concentration of Pluronic and the extent to which it is conjugated with 5-DTAF. These methodologies can be used to quantify the concentration of fluorescein conjugated to the labeled Pluronic.

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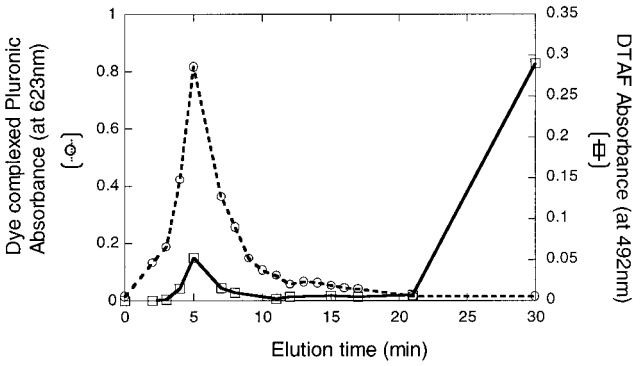

Figure 4. Elution profile of the labeled Pluronic F68 sample through a size exclusion chromatograph. A reaction mixture consisting of labeled DTAF−Pluronic F68, free Pluronic, and unreacted 5-DTAF was passed through a Sephadex G-25 column and separated based on differences in molecular weight. Pluronic F68 and 5-DTAF have MWs of 8400 and 495, respectively. Labeled Pluronic F68 was eluted first, and the Pluronic concentration was detected using the ammonium thiocyanate assay. 5-DTAF, both conjugated to Pluronic F68 and in unreacted form, was detected using absorbance measurements at 498 nm. The data are representative of more than five independent experiments.

Elution of Labeled Pluronic from Size Exclusion Chromatograph. When the reaction mixture containing the 5-DTAF and Pluronic was passed through the size exclusion chromatography column, two distinct yellow bands were formed; the faster-moving band presumably contained the higher molecular weight Pluronic, and the slower-moving band contained the unreacted 5-DTAF. Fractions of samples flowing through the chromatograph were collected, and the two detection assays discussed above were used to quantify the concentration of Pluronic and 5-DTAF in the various fractions. For these measurements, each sample was diluted 100-fold and the concentration of Pluronic was determined using the cobalt thiocyanate assay in conjunction with the calibration curve shown in Figure 2. Simultaneously, the 5-DTAF conjugated to Pluronic was determined from absorbance measurements of the samples at 498 nm in comparison with the free, unconjugated 5-DTAF calibration curve (data not shown). Figure 4 shows the absorbance intensity due to Pluronic and 5-DTAF in samples collected at ∼1−5 min intervals. As seen, there is a clear overlap of Pluronic and 5-DTAF absorbance profiles after 5 min of addition of the reaction mixture to the column. This overlap demonstrates that the higher molecular weight copolymer labeled with 5-DTAF is eluted first in the size exclusion column. A second sharp increase in 5-DTAF absorbance was observed at 21 min. This second, slower band consisted of excess unreacted 5-DTAF.

The degree of separation of the Pluronic band from the free 5-DTAF was found to be a function of both the column height and the bead size. In our experiments, the height of the column used for separation purposes was restricted to a length of 10 cm. This restriction was imposed in order to minimize contact between the labeled copolymer and beads, because this interaction may lead to polymer adsorption and loss of the labeled product. At the same time, we observed that a further reduction in column height resulted in incomplete separation of the eluted components. The typical sample flow rate in these columns was in the range of 0.2−0.5 mL/min. Larger Sephadex G-50 beads were also used for separation of the labeled Pluronic P105 because this polymer self-assembles into micelles at 6 w/v% concentration and room temperature,[37] and the micelles have a larger radius of gyration in

Table 1. Effect of Reactant Molar Ratio on Pluronic Labeling Efficiency

Pluronic labeled	molar ratio of 5-DTAF/Pluronic	F/P ratio ± (SEM)[a]	efficiency (%)[b]
F68	0.5:1	0.012 ± (0.0012)	0.6
	2.0:1	0.063 ± (0.0077)	3.2
P105	0.5:1	0.020 ± (0.0059)	1.0
	2.0:1	0.260 ± (0.0492)	13.0

[a] F/P ratio is the ratio of conjugated 5-DTAF concentration/Pluronic polymer concentration. Standard error mean (SEM) is estimated from 5−7 labeling reactions. [b] Efficiency (%) of labeling Pluronic polymers with 5-DTAF (= F/P × 100/2). The factor of 2 is introduced because each Pluronic molecule has two potential binding sites for 5-DTAF.

comparison to the free polymer.[38,39] In contrast, smaller beads (Sephadex G-25) were used for separation of the labeled Pluronic F68, which remains as a free polymer under these conditions.[37] Overall, we observed that several parameters had to be optimized in order to obtain a cleaner and fast separation.

Degree of Pluronic Conjugation with 5-DTAF. Proton NMR studies (using a Varion Inova-500 MHz NMR spectrophotometer) were performed to examine the conjugation of the Pluronic polymer with 5-DTAF. These studies showed that selective excitation of labeled Pluronic F68 and P105 in the aromatic range caused several peaks to appear between 6.2 and 11.1 ppm, which confirms the presence of the 5-DTAF aromatic molecules in the final product (data not shown). Further, the peaks corresponding to the 5-DTAF associated with the conjugated polymer in the aromatic range were shifted compared to the unconjugated 5-DTAF control, indicating that the fluorescent tag was attached to the polymer. NOE (Nuclear Overhauser Effect) experiments also confirmed the relative proximity of protons in DTAF and Pluronic F68 to within ∼3−4 Å, because excitation at 6.21 ppm (in the aromatic range) resulted in the appearance of a prominent peak at 4.418 ppm in the aliphatic range corresponding to the terminal group of the Pluronic. Although the NMR studies confirmed the linkage of 5-DTAF to Pluronic, size exclusion chromatography studies performed using a Superdex-30 column (Pharmacia, Inc.) did not yield sufficient resolution to resolve between Pluronics conjugated with either one or two 5-DTAF molecules (data not shown).

The efficiency of the labeling reaction depends on the concentration of reactants in the reaction mixture. In our experiments, the molar ratio of 5-DTAF to Pluronic was varied from 0.5:1 to 2:1, and its effect on the labeled product composition was quantified using absorbance and fluorescence measurements as discussed earlier. The ratio of fluorescein (5-DTAF) concentration to the Pluronic concentration (F/P ratio) was determined (Table 1). The tabulated F/P ratio depicts a direct correlation between the concentration of fluorescein reactant 5-DTAF and the labeling efficiency of Pluronic F68 and P105 block copolymers. These results suggest that it may be possible to obtain a higher extent of conjugation by altering the molar ratio of the reactants or increasing the reaction time. Higher labeling efficiencies may also be possible upon addition of organic solvents, but this is not desirable for biological applications. Because the F/P ratio of the labeled product was far less than 1 in all cases, it is likely

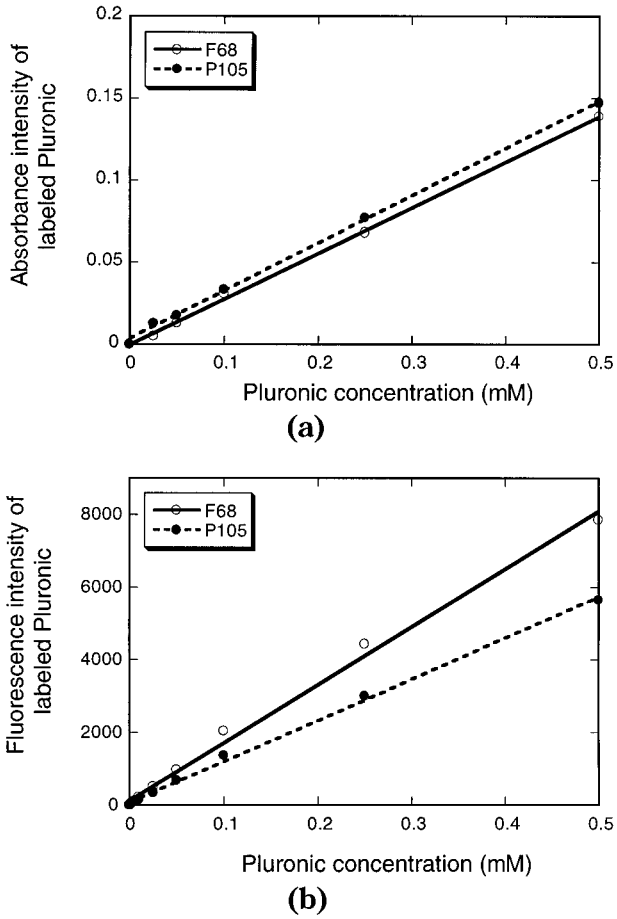

Figure 5. Concentration-dependent absorbance and fluorescence profiles of labeled Pluronic F68 and P105. Labeled Pluronic F68 and P105 were serially diluted in HEPES buffer (pH = 8.0) to obtain concentrations ranging from 1×10^{-3} to 0.5 mM. The absorbance at 498 nm is shown in (a). The fluorescence of the samples measured at 516 nm following excitation at 492 nm is shown in (b).

that only one of the hydroxyl end groups of the block copolymer was typically conjugated with the fluorescein tag and a large number of copolymer molecules remained unlabeled. In our experiments, the overall labeling efficiency of Pluronic block copolymers F68 and P105 was in the range of 0.6−13%.

Absorbance and Fluorescence Properties of Conjugated Pluronic in Solution. Experiments were performed to characterize the absorbance and fluorescence properties of the labeled Pluronic F68 and P105 block copolymer in aqueous solution with respect to changes in polymer concentration and solution pH. To study the dose-dependent behavior, labeled product was serially diluted to obtain concentrations ranging from 1×10^{-3} to 0.5 mM polymer at pH = 8. The absorbance of the labeled Pluronic was measured at 498 nm (Figure 5a). Also, the fluorescence of these polymers was determined at 516 nm (Figure 5b). These features were observed to vary linearly with concentration below 0.5 mM (Figure 5). Further, in comparison to the absorbance measurements the fluorescent detection of labeled Pluronic was more sensitive at low Pluronic concentrations of 1−10 μM (data not shown). Some nonlinear absorbance and fluorescence profiles were observed during the dosage studies at the higher polymer concentrations, especially for the labeled Pluronics with higher F/P ratios. This feature was more prominent for the fluorescence studies compared to the absorbance studies, suggesting that fluorescence quench-

(37) Alexandridis, P.; Holzwarth, J. F.; Hatton, T. A. *Macromolecules* **1994**, *27*, 2414.
(38) Alexandridis, P.; Athanassiou, V.; Hatton, T. A. *Langmuir* **1995**, *11*, 2442.
(39) Alexandridis, P.; Yang, L. *Macromolecules* **2000**, *33*, 5574.

ing may take place for the polymers labeled with higher efficiencies. For this reason, we have preferred to perform experiments with polymers with the lower F/P ratio in this paper. Further, for all the data presented including the cytometry studies in section 2, we have performed extensive studies to confirm that absorbance and fluorescence profiles increase linearly with concentration in the range of our studies.

We also determined the absorbance and fluorescence of the filtrate obtained during Pluronic concentration by centrifugation, to assess (i) if there was a large amount of unreacted 5-DTAF present in the first band eluted from the chromatograph or (ii) if some of the 5-DTAF was released (e.g., via hydrolysis) from the labeled Pluronic after chromatographic separation. In all the experiments conducted, we observed that the filtrate containing free 5-DTAF showed absorbance and fluorescent intensities that were less than 5% in comparison to the labeled Pluronic samples. These results indicate that the labeled Pluronic preparation contained low amounts of 5-DTAF monomer. Also, the labeled products appeared to be stable for time periods >4 weeks, because both the fluorescence and flow cytometric measurements presented in this manuscript were consistently reproducible over this time frame.

Free unconjugated 5-DTAF has been shown to exhibit pH-dependent fluorescence.[35] We examined whether labeled Pluronics also show similar pH-dependent behavior. In these experiments, labeled Pluronics were dissolved in buffers with pH varying from 4 to 9 and the fluorescence of the solution was measured using a spectrofluorometer at 516 nm. We observed that both 5-DTAF and the labeled Pluronic exhibited low absorbance and fluorescence intensities at pH less than 7.0 (data not shown). These parameters increased dramatically at neutral and basic pH. The intensities remained approximately constant (±15%) in a pH range of 7−9. This pH-dependent behavior of the labeled copolymers is an important property because it may allow us to examine the transport of these poly(ethylene glycol) (PEG)-based polymers in the human body/cells. The subcellular components of many human cells exhibit marked variations in pH; for example, the cytosol has a pH of 7.2 whereas the endosomes are acidic (pH < 6).[40] Therefore, the application of our pH-sensitive fluorescent probe in cellular systems may allow the assessment of the transport properties of Pluronics and other PEG-based molecules in biological systems. Such information may be useful in the design of nonviral targeted drug-delivery systems.

2. Application. *Real-time Detection of Labeled Pluronic Binding to Polystyrene Beads Using Flow Cytometry*. We investigated the ability of labeled Pluronic to adsorb onto hydrophobic polystyrene beads using flow cytometry as described in Materials and Methods. Unlike previously used techniques,[22,27] this method does not require a wash or other preparation steps prior to sample analysis. Furthermore, unlike previous studies that estimated the level of bound Pluronic by sampling the supernatant and quantifying the level of unbound polymer,[6,10,41] our methodology allowed direct and real-time detection of Pluronic bound to surfaces.

A typical histogram plot of bead fluorescence intensity obtained from flow cytometric studies is depicted in Figure 6. The plot compares the fluorescence intensity of washed polystyrene beads without labeled Pluronic with that of polystyrene beads with adsorbed labeled Pluronic P105 block copolymer. The geometric mean fluorescence intensity (MFI) was observed to increase dramatically by ~30-fold, from 5.6 for the washed beads alone to 165 for the beads on addition of labeled Pluronic P105. Addition of the filtrate obtained upon concentration of labeled Pluronic (negative control) did not change the baseline fluorescence of 5.6 for polystyrene beads alone by more than 3%. The results demonstrate that it is possible to use flow cytometry in conjunction with fluorescently conjugated polymers to rapidly detect Pluronic adsorption on surfaces.

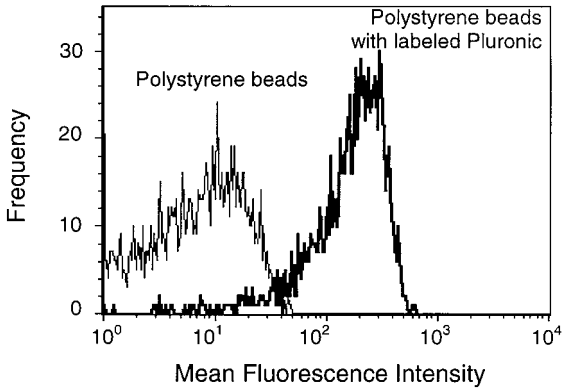

Figure 6. Flow cytometric detection of labeled Pluronic bound to polystyrene beads. Fluorescence intensities of polystyrene beads were detected either in the absence or presence of 0.025 mM labeled Pluronic P105. The MFI of the beads alone was 5.6. On addition of labeled Pluronic P105, the MFI became 165.

Next, we examined whether labeling the Pluronic polymer with 5-DTAF significantly altered the binding characteristics of the Pluronic molecule onto polystyrene surfaces. Two types of treatments were compared. In the first, the polystyrene beads were incubated with 0.5 mM labeled Pluronic F68, and in the second, the beads were incubated with a mixture of 0.25 mM labeled and 0.25 mM unlabeled F68. Therefore, the overall concentration of copolymer F68 was the same in both samples, although the fluorescent fraction in the second case was half that in the first case. After incubation for 10 min at room temperature, the MFIs of both samples were compared. It is anticipated that if the binding characteristics of the fluorescent polymer were identical to that of the unlabeled molecule, then the MFI of the first bead should be twice that of the second. A factor larger than 2 would indicate that the labeled Pluronic binds with a higher affinity to the polystyrene bead than the unlabeled sample and vice versa. In three experiments performed to examine this feature, we observed that the MFI of the first bead was 54.8 ± 2.5 and that of the second sample was 28.3 ± 1.7. The ratio of MFIs for the two cases was ~2. Similar observations were made with labeled and unlabeled Pluronic P105. Together, the above results indicate comparable binding rates for the unlabeled and fluorescein-conjugated Pluronics to polystyrene beads.

Time- and Concentration-Dependent Binding of Labeled Pluronic F68 and P105 to Polystyrene. We examined the time- and concentration-dependent adsorption profiles of Pluronics F68 and P105 onto hydrophobic polystyrene bead surfaces using flow cytometry. In the first series of experiments, we added Pluronic P105 at 0.025 mM to hydrophobic polystyrene beads and quantified the time-dependent adsorption kinetics. This technique allowed time resolution in the order of 5 s between consecutive readings. We observed that the MFI of the beads increased

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(41) Shar, J. A.; Obey, T. M.; Cosgrove, T. *Colloids Surf., A* **1998**, *136*, 21.

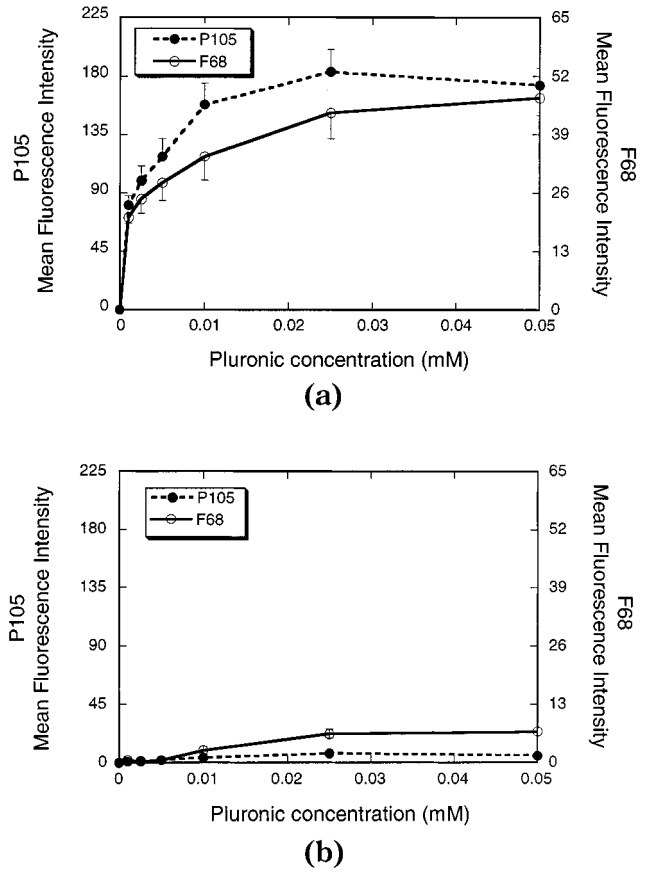

Figure 7. Binding of Pluronics to polystyrene beads. Labeled Pluronic polymers P105 and F68 at concentrations ranging from 0 to 0.05 mM were incubated with 2×10^5 plain polystyrene beads/mL (a) for 15 min at 37 °C in HEPES buffer (pH = 8.0). The MFI of the beads was measured using standard flow cytometry. In (b), experiments similar to that in (a) were performed to quantify the extent to which Pluronic F68 and P105 adsorb onto carboxylated polystyrene microspheres. The data are mean ± SEM from three independent experiments.

rapidly in the first 30 s to 80 ± 5% of its saturation value. Saturation was achieved within 2 min (data not shown). Similar rapid saturation kinetics, within the first 2 min, of polymer−bead mixing was observed for both Pluronic F68 and P105 over the entire range of concentrations tested. Our time-dependent kinetic profile closely followed a similar trend reported by Green et al.[27] using surface plasmon resonance. Here also, the authors report saturation binding of Pluronics to hydrophobic polystyrene surfaces within 1−2 min. Gingell et al. also report in their study that PEO−PBO−PEO block copolymer adsorption on a hydrophobized glass surface reaches a plateau within 2 min.[26]

The concentration-dependent adsorption isotherms of labeled Pluronic F68 and P105 binding to plain polystyrene and carboxylated polystyrene microspheres was examined (Figure 7). Here, labeled copolymers from 1×10^{-3} to 0.05 mM were incubated with polystyrene beads for 10 min and the MFI was measured. As seen in Figure 7a, the isotherms of Pluronic binding to plain polystyrene beads followed a Langmuir type adsorption isotherm, characterized by an initial steep slope at low Pluronic concentrations and a plateau above concentrations of 0.025 mM. We estimated the fraction of bead surface covered on addition of 0.025 mM Pluronic F68 based on our observations and previously reported measurements by Heydegger and Dunning.[42] According to these authors, the interfacial area occupied per molecule of F68 is ∼15.1 nm^2. Therefore, we estimate that the potential area occupied by 0.025 mM Pluronic F68 is 2.27×10^8 $\mu m^2/\mu L$ of solution volume. This value correlates well with our estimate of the total surface area (tube walls and beads) available for polymer adsorption in our experiments, which equals 1.57×10^8 $\mu m^2/\mu L$ of solution volume. Therefore, on the basis of our experimental observation it appears that Pluronic F68 forms an adsorbed monolayer on the surface of polystyrene beads at saturating concentrations of 0.025 mM. Similar calculations were performed to show that P105 also forms a monolayer at 0.025 mM.

The saturation isotherms of both Pluronic F68 and P105 block copolymer were similar (Figure 7a). This is in spite of the fact that Pluronic F68 has been reported to exist as free polymer at 0.025 mM and 37 °C, whereas P105 self-assembles into micelles under these conditions.[37] This observation is in agreement with Green et al.[27] These authors varied the temperature conditions in surface plasmon resonance experiments from 24 to 34 °C to alter the association characteristics of the Pluronic P105 from a free polymer at the lower temperature to micelles at the higher temperature. Under both conditions, they observed a similar level of P105 binding to polystyrene surfaces. On the basis of this, they concluded that the presence of P105 micelles does not affect the rate or extent of adsorption on surfaces.

These observations can be explained from a thermodynamic standpoint. Pluronic polymers under certain conditions of sufficiently high concentration and/or temperature minimize their free energy by spontaneously self-assembling into micelles in an aqueous solvent. However, in the presence of a hydrophobic surface it appears that the binding of the Pluronic to this surface results in a greater reduction in free energy than that due to their self-assembly. Hence, the labeled copolymer P105 unimers first preferentially adsorb onto the available hydrophobic surfaces. Then, depending on the concentration of unabsorbed unimers remaining free in solution, the polymer might find it energetically favorable to self-assemble into micelles.

Alteration in Surface Properties Modifies the Pattern of Block Copolymer Adsorption. To assess the effect of surface modification on polymer adsorption, we determined the concentration-dependent adsorption isotherm of Pluronic block copolymers binding onto carboxylated polystyrene microspheres (Figure 7b) and compared it to the results from plain polystyrene beads (Figure 7a). As seen, the MFI of Pluronic F68 decreased by 10-fold in comparison to that of the plain beads, although it was higher than the negative control. On the other hand, the surface adsorption of P105 on carboxylated beads decreased by ∼100-fold.

There are several possible explanations for the above observation. The most probable scenario suggests that altering the surface properties of the polystyrene bead from a hydrophobic to a hydrophilic surface may alter the nature of polymer adsorption on the surface. On adsorption of Pluronics to hydrophobic surfaces, it has been demonstrated that the block copolymers "anchor" to polystyrene surfaces via their hydrophobic PPO segments, and the hydrophilic PEO segments or "tails" extend into the aqueous solution.[43] Some deviation from this regular anchor and tail conformation, in the forms of "loops" and "trains", may be observed at low polymer concentrations.[44,45] When the surface is altered to a hydrophilic one,

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(43) Andrade, J. D. *Surface properties of aqueous PEO/PPO block copolymer surfactants*; Plenum Press: New York, 1988.
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Pluronics have a markedly reduced tendency to bind to the surface, as seen in the reduced MFI in our experimental results. Further, it is possible that the Pluronics now bind to the surface via the hydrophilic PEO segment rather than their PPO segments. This binding, albeit weak, may take place only under high Pluronic concentrations (above 0.01 mM) when free Pluronics (e.g., F68) in solution repel each other and the system can reduce its free energy by binding some of the polymer to the surfaces. Pluronic P105, on the other hand, may have a greater tendency to self-assemble than to bind to the hydrophilic surfaces. Also, because the PEO chain length of F68 (75 blocks) is longer than that of P105 (37 blocks), F68 is more likely to bind to a hydrophilic surface than P105. Therefore, as seen in Figure 7b there is a tendency (although reduced) for F68 to adsorb to the carboxylated polystyrene surface whereas P105 adsorption was not at all observed.

It is unlikely that changes in the local pH in the microenvironment of the negatively charged carboxylated beads account for the observed decrease in polymer fluorescence. We conjecture this because the concentration of metal anions ($[Na^+]$ and $[K^+]$) in HEPES buffer is high at ~0.12 M compared to the $[H^+]$ ions which are at 10^{-8} M (pH = 8). The $\sim 10^7$-fold higher concentration of the metal anions compared to $[H^+]$ suggests that it is more likely that $[Na^+]$ and $[K^+]$ will surround the negatively charged beads rather than $[H^+]$. The presence of these salt ions does not alter the fluorescence properties of 5-DTAF (data not shown) or affect the results presented here.

Pluronic Binding to Platelet Surface. To provide an example of how the Pluronic probe in conjunction with flow cytometry may be applied to assess polymer interaction in a physiologically relevant biological system, we examined the nature of Pluronic binding to blood cells, specifically platelets. Such interactions may take place under conditions where Pluronics in commercial formulations such as Rheotrx or Flusol are injected in vivo to prevent cell−cell adhesion.[11,12] Figure 8 depicts the saturation level of Pluronic bound to the blood platelets following 15 min of incubation of the cells with the labeled polymer. In these studies, the concentration of platelets is kept at 100×10^6 cells/mL because this is what is observed in the human body. As seen in Figure 8a, the extent of Pluronic F68 bound to platelets (in PRP) increases with concentration, suggesting that the platelet surface is not saturated by the polymer at the highest dosage tested. Further, upon testing with WP (which does not contain any plasma) we observed that the extent of Pluronic bound to platelets is augmented by ~4- to 5-fold at each dosage. The results suggest that when Pluronics are applied in vivo at concentrations below 2 mM (which is typically the highest concentration applied in most biological studies), they bind cells at subsaturating levels. Further, besides binding to blood cells the polymer may also either bind soluble components in blood plasma or compete with plasma components for hydrophobic domains on the platelet surface. Similar results were observed in Figure 8b with Pluronic P105, thus suggesting that Pluronics may assemble with macromolecules (e.g., proteins) in plasma, in addition to binding cells.

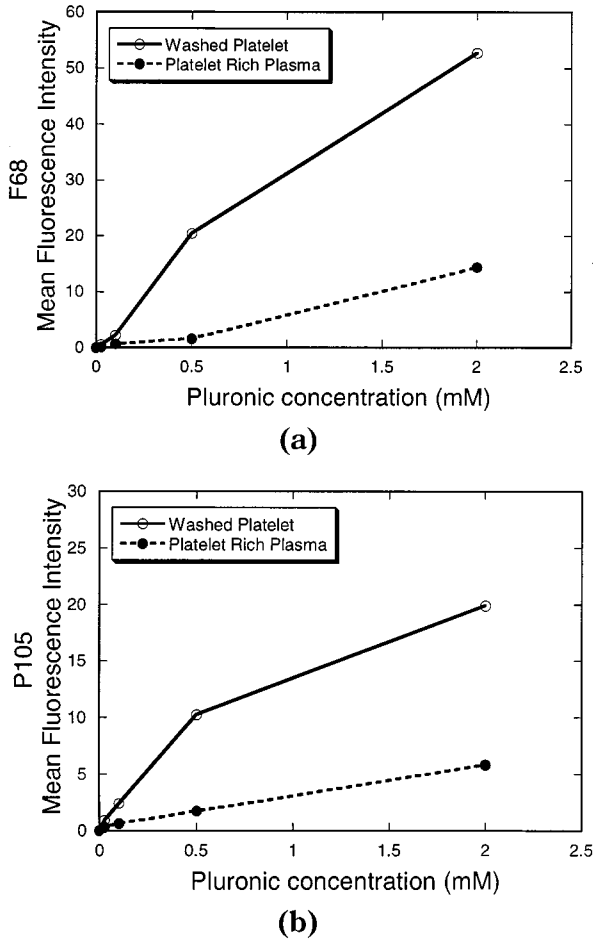

Figure 8. Binding of Pluronics to blood platelets. Labeled Pluronics F68 (a) and P105 (b) at concentrations ranging from 0 to 2 mM were incubated with either PRP or WP at 100×10^6 platelets/mL for 15 min at 37 °C in HEPES buffer (pH = 7.5). The saturation MFI of the cells was measured using standard flow cytometry. The data are representative of at least three independent experiments.

Summary

In this study, we developed a novel methodology to conjugate the hydroxyl end group of Pluronic F68 and P105 block copolymers under room temperature and aqueous conditions with a fluorescein derivative, 5-(4,6−dichlorotriazinyl) aminofluorescein (5-DTAF). The labeling reaction was performed over a range of reactant mixture compositions such that the labeled product exhibited F/P ratios from 0.01 to 0.26. A unique advantage of our conjugation methodology lies in its simplicity and ability to label Pluronics under aqueous conditions. Because of the absence of organic solvent impurities, our fluorescent probes may find numerous applications in biological systems that examine cell−polymer interactions in solution[17] or on surfaces. Such an understanding of polymer properties may further substantiate their application in areas such as the design of novel biomaterials and drug delivery.[46] Although the current paper discusses the fluorescein conjugation of Pluronic polymers, this methodology may also be extended to other water-soluble polymers with terminal or side-chain hydroxyl groups.

The labeled block copolymers formed were characterized in solution using a range of NMR, light absorption, and fluorescence assays. These studies demonstrated that the absorbance, fluorescence, and pH dependence of the labeled polymer were very similar to that of the unconjugated 5-DTAF. Both the free 5-DTAF and conjugated polymers showed peak absorbance at ~498 nm, peak

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(46) Yang, L.; Alexandridis, P. *Curr. Opin. Colloid Interface Sci.*, in press.

fluorescence at ~516 nm, and a marked reduction in these spectrums at lower acidic pH below 7. Such pH-dependent fluorescence properties of the labeled block copolymers could be utilized in studies of copolymer−cell internalization and targeted drug-delivery applications of Pluronic-modified carriers.

A novel methodology was developed using flow cytometry to study the nature of polymer−surface interactions. The system examined the binding of labeled F68 and P105 to both polystyrene beads and blood platelets. This application of flow cytometry enabled the detection of labeled Pluronic adsorption onto particle surfaces when solution concentration was low, down to 1 μM. Because the measurements were performed *in situ* without prior sample preparation steps, the technique allowed us to resolve the binding kinetics of the fluorescent polymer on the particle surfaces in real time with a time resolution on the order of 2−5 s. In these experiments, the mean fluorescence intensity (MFI) of the single population of beads/platelets was indicative of the rate and extent of polymer adsorption. Labeled F68 and P105 copolymers displayed fast binding kinetics and attained saturation adsorption within 1−2 min of their addition to the bead suspension. Furthermore, competitive binding experiments using flow cytometry demonstrated that labeling alone did not noticeably alter the adsorption characteristics of the Pluronic molecules. Such rapid and accurate detection of low concentrations of labeled Pluronics could be advantageous in optimizing the surface modification of colloidal carriers to increase their biocompatibilty and their "stealth" properties.

The methodology of flow cytometry and labeled Pluronic was applied to investigate several aspects of polymer−surface adsorption: (i) the effect of bead surface modification, (ii) the effect of polymer micelle formation in bulk solution, and (iii) the nature of Pluronic binding to blood platelets. In the first case, it becomes apparent that the copolymer adsorption is very sensitive to the bead surface properties. Changing the nature of the bead surface from hydrophobic to hydrophilic markedly reduced the adsorption of both the labeled Pluronic F68 and P105 copolymers. In the second case, we examined the binding kinetics of Pluronic P105 block copolymers, which under our experimental conditions are present in solution as both unimers and micelles in equilibrium. Our flow cytometric observations suggest that upon introduction of hydrophobic beads the free unimers rapidly diffuse and adsorb onto the bead surface. This depletion of unimers in solution causes a rapid breakup of existing micelles, initiating further adsorption onto the available surfaces, before final re-establishment of the unimer−micelle equilibrium according to the closed association model.[47,37] This dynamic process may be interpreted as a tendency of the block copolymers to preferentially adsorb on the polystyrene surface rather than to self-assemble into micelles. In the third case, we demonstrate for the first time that besides binding blood cells in physiologically relevant systems, large amounts of Pluronic (and other such polymers) applied *in vivo* may either remain in solution or bind soluble components in blood plasma.

Overall, the development of an aqueous fluorescein-labeled Pluronic probe along with the application of flow cytometry provides a novel methodology to study polymer−surface interactions. This approach may find diverse applications in the area of colloid and interfacial sciences, as it relates to the study of polymer physicochemical properties and surface interactions in chemical and biological systems.

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