Chemical Modification of Surface Active Poly(ethylene oxide)−Poly(propylene oxide) Triblock Copolymers

Jenq-Thun Li,[†] Jan Carlsson,[‡] Jinn-Nan Lin,[§] and Karin D. Caldwell*[,†]

Center for Biopolymers at Interfaces, Department of Bioengineering, University of Utah, Salt Lake City, Utah 84112, Pharmacia Diagnostics AB, Uppsala 75182, Sweden, and Diagnostic Products Company, Los Angeles, California 90045. Received January 5, 1996[⊗]

A general route has been developed to chemically modify a series of poly(ethylene oxide)−poly(propylene oxide) triblock copolymers with molecular weights from 6500 to 14 600. It is initiated by the introduction of *p*-nitrophenyl groups; such nitrophenyl conjugated copolymers are stable in an organic milieu and in a dry state but are seen to react easily with amino-containing molecules including small peptides. Among them, introduction of 2-pyridyl disulfide groups after coupling with 2-(2-pyridyldithio)ethylamine enables the selective attachment of thiol-containing molecules. The released thiopyridone in such thiol−disulfide reactions can be used to quantify the content of 2-pyridyl disulfide groups. In addition, a new type of modified copolymers was developed for the radioisotope (^{125}I) labeling purpose that consists of a reaction of nitrophenyl conjugated copolymers with hydrazine and a subsequent coupling with *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton−Hunter reagent). Adsorption studies of ^{125}I-labeled and 2-pyridyl disulfide conjugated copolymers on polystyrene particles are consistent with previous determinations of surface coverage using other technologies, in turn indicating that this new chemical modification does not alter their surfactant properties on hydrophobic solid phase. The coating of common hydrophobic surfaces with 2-pyridyl disulfide conjugated copolymers has been demonstrated as a general and robust immobilization method to generate a high-sensitivity bioactive surface with low nonspecific binding. The optimal space between immobilized ligands can also be controlled by incubating the solid phase with solutions containing mixtures with different ratios of unmodified and modified copolymers.

INTRODUCTION

Pluronic (BASF Corp.) surfactants, a group of poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide) (PEO/PPO/PEO), triblock copolymers also known as poloxamers, have been shown to possess many unique properties leading to numerous valuable applications (*1*). Generally, the poloxamers are utilized as delivered, and a broad range of relative block compositions is available from the manufacturer. Thus, low molecular weight, liquid poloxamers are used as washing detergents, while high molecular weight surfactants with high ethylene oxide contents may be selected for the purpose of dispersion stabilization. Several members of the Pluronic family are known to adsorb to hydrophobic plastic surfaces in a pseudo-irreversible way, forming complexes that are stable for long periods of time following suspension in a surfactant-free medium.

Pluronic compounds are of considerable interest in the biotechnological and pharmaceutical industry because of their unique surfactant abilities and their extremely low toxicity and immunogenic response. In fact, Pluronic compounds are among a small number of surfactants that have been approved by the U.S. Food and Drug Administration for direct use in medical applications and as food additives (*1*). Recently, surface treatments with Pluronic have been found to efficiently reduce platelet adhesion (*2*), protein adsorption (*3*−*6*), bacterial adhesion (*7*), and phagocytosis (*8*−*10*.) Furthermore, several Pluronic compounds have been found to enhance the therapeutic effect of drugs, both *in vitro* and *in vivo* (*11, 12*). In addition, surfactants of this type have seen a variety of uses including serving as vehicles for the introduction of antisense oligonucleotides to inhibit the proliferation of vascular smooth muscle cells (*13*) and as a drug administered to reduce myocardial injury (*14*).

Despite the numerous sophisticated applications of this class of surfactants, relatively few attempts have been made to subject them to chemical derivatization. Here, to broaden their versatility, we will introduce a simple and reliable means for their modification, which is based on the introduction of reactive intermediates for conjugating other functional groups, e.g., for radioisotope labeling or solid phase immobilization. Through these reactions, quantification of Pluronic can be simplified by a direct probing of labeled surfactants, and biological molecules can be tethered to surfaces of interest after physical adsorption of the conjugated surfactants.

Nonspecific immobilization of biological molecules, such as enzymes or antibodies, can be achieved by simple adsorption onto a solid phase. For example, polystyrene (PS) in the form of microtiter plates is a common solid phase in many heterogeneous enzyme immunoassay systems. Since such assays are based on the specific interactions between reagents and analytes, the preparation of a bioactive surface is a critical factor in developing a testing assay with high sensitivity and selectivity. The effectiveness of a passive coating is based on the retention of a specific biological activity upon adsorption, be it an antibody−antigen interaction or an enzymatic reaction. The fact that antibodies spontaneously bind to hydrophobic surfaces and in most cases preserve their immunochemical activity is the basis for the common use of PS as the solid phase in heterogeneous immunoassays (*15, 16*).

* Author to whom correspondence should be addressed.
[†] University of Utah.
[‡] Pharmacia Diagnostics AB.
[§] Diagnostic Products Co.
[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1996.

S1043-1802(96)00048-1 CCC: $12.00

Yet, sensitive monoclonal antibodies sometimes lose their activities due to conformational changes caused by their sharing hydrophobic interactions with the surface, and such a physical adsorption method appears to be unsuitable for the immobilization of many enzymes on PS surfaces. Indeed, Sandwick and Schray (*17*) demonstrated that enzymes such as alkaline phosphatase, β-galactosidase, lysozyme, horseradish peroxidase, catalase, and glucose-6-phosphate dehydrogenase lose their catalytic activity when they adsorb onto small PS latex particles from low concentration ($<1 \times 10^{-3}$ mg/mL) protein solutions. Because of the tendency of proteins, particularly in low concentration, to denature on hydrophobic surfaces, adsorption is of limited applicability as an immobilization method. Here, we will introduce a new technique for immobilizing proteins and their ligands to hydrophobic surfaces. This technique, although still based on adsorption, involves precoating of surfaces with a 2-pyridyl disulfide derivatized Pluronic surfactant and a subsequent attachment of thiol-containing ligands.

As discussed previously (*4, 5*), it is believed that a Pluronic surfactant adsorbs to hydrophobic PS surfaces via its hydrophobic PPO center block, while leaving its terminal hydrophilic PEO chains to extend from the surface into the hydration surrounding, thus rendering it hydrophilic with a much reduced tendency to nonspecifically adsorb proteins from the environment. A derivatized Pluronic coating layer may then ideally serve the dual purpose of providing reactive groups for specific coupling of a desired ligand in a preferred orientation as well as creating a protein-compatible environment for the immobilized molecules. Since most molecules can be easily thiolated by the modification of suitable functional groups, this new technique can be applicable to a large array of molecules of interest. In addition, the coating layer, which is rich in dynamic PEO chains, offers very little opportunity for nonspecific adsorption of biomolecules.

The most attractive chemical modification of Pluronic surfactants involves the hydroxyl ends of the PEO blocks, and the PEO chemistry developed by other groups (*18*−*20*) may thus be applied also to the PEO/PPO/PEO triblock copolymers. We have found that an introduction of reactive carbonate structures into the Pluronic with the aid of 4-nitrophenyl chloroformate, previously used to activate polyols for biomolecule binding (*21, 22*), is a straight-forward and reliable starting step for derivatizing the functional groups of interest. Here, we will first discuss a general synthetic procedure for radioisotope labeling of all types of Pluronic and will then specifically focus on Pluronic F108, which has the highest molecular weight and highest PEO content in a series of PEO−PPO triblock copolymers examined in this study for further introduction of functional groups.

EXPERIMENTAL PROCEDURES

Materials. The Pluronic surfactants, P105, F68, F88, and F108, with molecular weights of 6500, 8400, 11 400, and 14 600 corresponding to numbers of monomer units in each PEO block of 37, 76, 104, and 129, respectively, were kindly donated by BASF Co. PS latex particles with a diameter of 0.272 μm were from Seradyn, and porous monodisperse PS beads (10 μm) in dry form were kindly supplied by Dr. P.-Å. Pernemalm at Pharmacia-BTG-LKB, Uppsala, Sweden. The 4-nitrophenyl chloroformate and 1,3-aminopropane were obtained from Aldrich, hydrazine was from EM Science, *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton−Hunter reagent) was from Pierce, chloramine T was from Eastman Kodak, $Na^{125}I$ (100 mCi/mL) was from Amersham, sodium metabisulfite was from Fisher, triethylamine (TEA) was from Baker, and dithiothreitol (DTT) was from Bio-Rad. Mercaptoethylamine hydrochloride, 2,2′-dithiopyridine, 2-aminoethanesulfonic acid (taurine), glycyltryptophan (Gly-Trp), β-galactosidase (grade 8 from *Escherichia coli*) were all purchased from Sigma. Disposable prepacked PD-10 columns (Sephadex G-25 M with an approximate bed volume of 8.5 mL) were from Pharmacia, and the dialysis membranes (Spectra/Por) were purchased from Spectrum Medical.

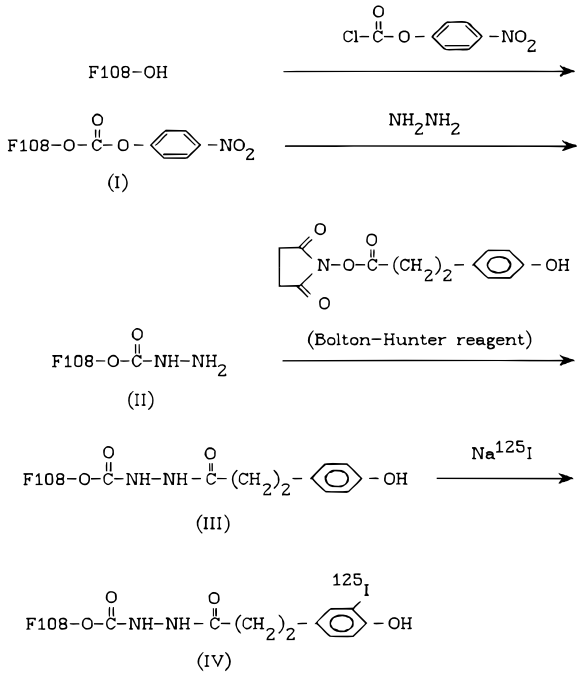

Figure 1. Modification of Pluronic F108 for radioisotope ^{125}I labeling.

General Methods for Activation of Pluronic with 4-Nitrophenyl Chloroformate. Two grams of Pluronic of either type was first dissolved in 6 mL of benzene, and this solution was slowly added to a stirred solution of 4-nitrophenyl chloroformate in 6 mL of benzene. The content of 4-nitrophenyl chloroformate varied with the molecular weight of Pluronic as the ratio of −OH/nitrophenyl was kept at 1:3. After 24 h of shaking, the reaction product was precipitated with ether, recovered by filtration, and redissolved in benzene. This procedure was repeated at least three times and the product (**I**) (Figure 1, where F108 represents the Pluronic F108 molecule) was finally recovered by evaporation of the remaining solvent under vacuum overnight. For Pluronic P105, the precipitation was done by using an 80 times excess petroleum ether (bp 35−60 °C). The degree of substitution was determined spectrophotometrically by measuring the amount of *p*-nitrophenolate ions released in an alkaline solution. For this purpose, an accurately weighed sample of the 4-nitrophenyl chloroformate-activated Pluronic (product **I** in Figure 1) was dissolved in 0.1 M NaOH. After 1 h of mixing under rotation, its absorbance was measured at 402 nm and converted to concentration, using a molar extinction coefficient of 18400 cm^{-1} M^{-1} (*19*).

Radioisotope ^{125}I Labeling of Pluronic. The modification of Pluronic for radioisotope labeling is schematically shown in Figure 1. A 1.5 g portion of activated Pluronic, product **I** in Figure 1, was dissolved in 3 mL of methanol and mixed with 97% hydrazine; the molar ratio of hydrazine to Pluronic was kept as 100:1. After an 8 h reaction time, the product **II** was precipitated with ethyl

ether. Redissolution in methanol followed by precipitation was repeated at least three times, and the final product was dried under vacuum overnight. For Pluronic P105, the recovery of product **II** was not so straightforward, and after considerable trial and error the following dialysis process was found to efficiently remove all possible traces of the strongly colored *p*-nitrophenolate ions. The mixture after reaction was concentrated in a rotating evaporator for 1−1.5 h and was then dissolved in 6 mL of methanol. This solution was transferred to a dialysis bag with a molecular weight cutoff of 1000 and was dialyzed against a 30% methanol solution. The methanol/water mixture (500 mL) was changed three times in 24 h and was then replaced by 0.1 M $NaHCO_3$ solution (500 mL). This weak alkaline solution was changed every 8 h, and the procedure was repeated three or four times. Then, deionized water was used as incubation solution and was changed three times in 24 h, whereupon product **II** was recovered by lyophilization.

The Bolton−Hunter reagent was found to be an efficient chemical for coupling to the introduced $-NH_2$ groups and for subsequent labeling with radioisotope ^{125}I (*23, 24*). One volume of a 17 mM solution of product **II** in dimethyl sulfoxide was vigorously mixed with 4 volumes of a 43 mM Bolton−Hunter reagent also in dimethyl sulfoxide. After 4 h, product **III** was precipitated from the solution using ether and then dried under vacuum overnight. For Pluronic P105, tetrahydrofuran replaced dimethyl sulfoxide as the solvent. After 4 h of reaction, this mixture was concentrated in a rotating evaporator and was then dissolved in 3 mL of methanol. This solution was transferred to a dialysis bag with a molecular weight cutoff of 1000 and dialyzed against methanol for 8 h and subsequently against deionized water for 8 h. The incubation solution was then replaced by fresh deionized water, and after 8 h, the product was sent to lyophilization. The substitution rate of this reagent can be calculated by measuring the ratio of conjugated phenol groups to the moles of Pluronic molecules present. A series of samples with Bolton−Hunter reagent in various concentrations were prepared in methanol, and their UV absorbances were determined at a wavelength of 270 nm using a Perkin-Elmer Lambda 6/PECSS spectrophotometer. From these measurements a molar extinction coefficient of the Bolton−Hunter phenol group was calibrated as 1270 cm^{-1} M^{-1}. Finally, 0.5 mL of a solution containing 0.6 mg/mL of product **III** in PBS buffer (0.01 M phosphate, 0.14 M NaCl, pH 7.4) was treated with 3 μL of $Na^{125}I$, and 50 μL of 4 mg/mL chloramine T was added as an oxidizing reagent. This iodination reaction was terminated after 4 h by using 50 μL of a solution containing 4.8 mg/mL sodium metabisulfite. The ^{125}I-labeled Pluronic and free $Na^{125}I$ were then separated by passing through a PD-10 column using PBS as the mobile phase, and the eluate was fractionationed into 12 culture tubes. Twenty microliters of each 1 mL fraction of the eluate was transferred into a counting vial, and the radioactivity was determined by a radioisotope detector (Beckman 170M).

Coupling of Amines to Pluronic. *(A) 1,3-Diaminopropane.* 1,3-Diaminopropane (3.3 g) was mixed with 5 mL of deionized water. After the pH was adjusted to 8.2 with concentrated HCl, the solution was mixed with a solution of 0.5 g of 4-nitrophenyl chloroformate activated Pluronic F108 (prepared as described above) in 5.0 mL of deionized water. The reaction mixture, which immediately turned yellow, was kept at 25 °C for 15 h. This solution was transferred to a dialysis tubing (with a molecular weight cutoff of 3500) and was dialyzed against 4 L of deionized water. During the 48 h dialysis process,

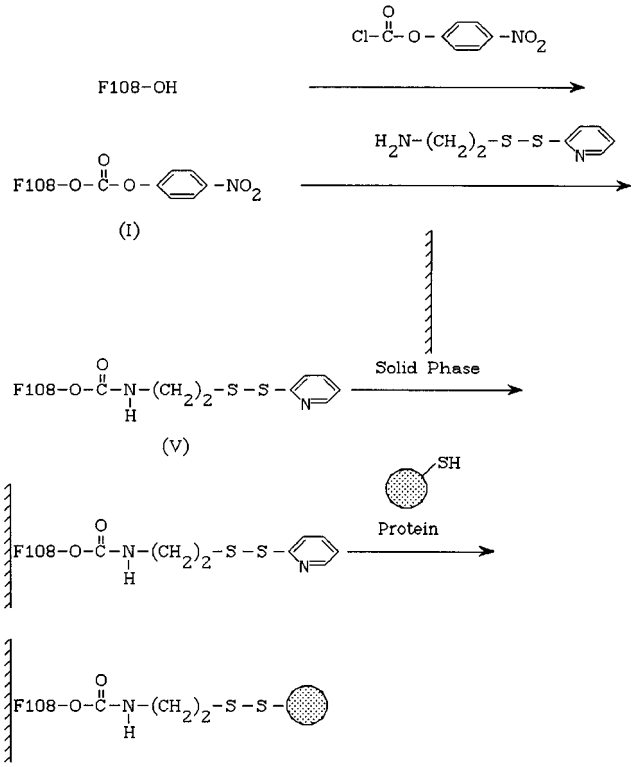

Figure 2. Preparation of a F108−PDS surface for protein specific binding.

water was changed five times until the low molecular weight material was assumed to be completely removed. The product was then recovered by lyophilization. The degree of substitution was determined by elemental nitrogen analysis. In this calculation the nitrogen content determined per a given mass of product was taken to exclusively derive from the attached diamine.

(B) 2-Aminoethanesulfonic Acid (Taurine). Taurine (3.4 g) was dissolved in 7 mL of deionized water, and the pH of the solution was adjusted to 9.4 with 2 M HCl. The solution was mixed with a 5 mL water solution of 0.5 g of 4-nitrophenyl chloroformate activated Pluronic F108. The resulting reaction mixture was kept at 25 °C overnight, and the product was obtained after dialysis and lyophilization as described previously. The degree of substitution was determined through sulfur and nitrogen analysis of a known amount of product; its molar taurine content was then readily calculated.

(C) Glycyltryptophan (Gly-Trp). Two 11 mg portions of Gly-Trp were each dissolved in a vial with 2 mL of methanol. One of the vials contained 0.05 mL of 1.2 M TEA. To both vials was added 11 mg of 4-nitrophenyl chloroformate activated Pluronic F108, and the final solutions were kept at 25 °C overnight. The reaction mixtures were then passed through PD-10 Sephadex G-25 columns, and the void fractions were pooled. The amount of bound Gly-Trp was determined by dry weight determination and photometric analysis using a molar extinction coefficient of 6170 cm^{-1} M^{-1} for the tryptophan residue (*25*).

Preparation of F108−2-Pyridyl Disulfide Derivative. 2-(2-Pyridyldithio)ethylamine was prepared first for conjugating the pyridyl disulfide group to Pluronic F108 (Figure 2). In this step, 1.13 g of mercaptoethylamine hydrochloride was dissolved in a mixture of 1.2 mL of methanol and 0.8 mL of acetic acid and was then added dropwise to a stirred solution of 6.74 g of 2,2′-dithiopyridine in 30 mL of methanol. The yellow reaction mixture was stirred for 30 min at room temperature and

was then slowly poured into a beaker with 200 mL of stirred ether, where it precipitated. The product that was separated from the ether phase was dissolved in a small volume of methanol and was again precipitated by ether. This procedure was repeated until the crystals appeared white, and the product was then dried under vacuum. A 0.6 g portion of this 2-(2-pyridyldithio)ethylamine hydrochloride was dissolved in 3 mL of methanol, and 300 mg of TEA was added (mole ratio is 1:1). After the addition of TEA, 2-(2-pyridyldithio)ethylamine hydrochloride becomes 2-(2-pyridyldithio)ethylamine and is ready for the next reaction. Subsequently, 1 g of 4-nitrophenyl chloroformate activated F108 (product **I**) was dissolved in 3 mL of methanol and was added to the above stirred solution. The reaction mixture, which immediately turned yellow, was left at room temperature for 15−20 h. The TEA was then neutralized by adding 0.2−0.25 mL of 10 M HCl. Four milliliters of deionized water was subsequently added to this mixture, and the whole was transferred to a dialysis tubing (3500 cutoff) and was dialyzed against 4 L of deionized water. Water was changed five times during the 48 h dialysis process, and the F108−2-pyridyl disulfide (F108−PDS) product (**V**) in Figure 2 was finally recovered by lyophilization. The derivative was stored in a desiccator over P_2O_5 at room temperature. The determination of its content of 2-pyridyl disulfide groups was essentially as described by Carlsson et al. (*26*). Exactly weighed F108−PDS was dissolved in phosphate−NaCl buffer (0.05 M phosphate, 0.15 M NaCl, pH 7.3). The UV absorbance at a wavelength of 343 nm was measured before as well as 10 min after the addition of 0.1 mL of 25 mM DTT to both the reference cuvette, containing pure phosphate−NaCl buffer, and the sample cuvette containing F108−PDS. The concentration of released 2-thiopyridone, which is identical to the original concentration of F108−PDS, was calculated using a molar extinction coefficient of 8060 cm^{-1} M^{-1}.

Pluronic Adsorption to Polystyrene Latex Particles. A suspension containing 2.5% (w/v) of PS particles (0.272 μm in diameter) was incubated with 4% Pluronic (^{125}I-labeled and plain surfactants were mixed with a ratio of 1:50) in PBS for 24 h with end-over-end shaking at room temperature. The free surfactants were then removed after coated PS spheres were pelleted in a Fisher microcentrifuge, Model 235A. Particles were resuspended in PBS and were again centrifuged. These washing cycles were repeated until the supernatant was free from measurable radioactivity. The radioactivity of the particles was then measured, and the surface density was therefore calculated on the basis of the known amount of particles present in the examination. For the sake of comparison, the surface density was also determined by a nonlabeling method, namely sedimentation field-flow fractionation (sedFFF). The instrumentation as well as the operation and use of sedFFF in characterizing colloidal adsorption complexes have been described in detail elsewhere (*27, 28*). Briefly, sedFFF is a colloid separation technique that causes selective retention of colloidal particles based on difference in buoyant mass. In the case of Pluronic adsorption, the increases in particle retention associated with uptake of surfactants can be directly related to the mass adsorbed per particle. From this information one can easily obtain the surface density provided the size of the spherical particles had been well characterized in a previous run.

F108−2-Pyridyl Disulfide Coating of Porous Polystyrene Particles. Twenty milligram of dry porous PS particles with a size of 10 μm were incubated with 1.0 mL of ethanol. The suspension was mixed by end-over-end rotation for 2 h at 25 °C. After being settled by centrifugation, the porous PS particles were separated from the ethanol and incubated in 1 mL of 0.2 M phosphate−NaCl buffer. The PS particles were again centrifuged, the buffer was removed, and the particles were resuspended in 1 mL of phosphate−NaCl buffer. After five consecutive washings, the PS particles were incubated in 2.0 mL of 4% F108−PDS solution, which was prepared in 0.2 M phosphate−NaCl buffer, and the mixture was rotated end-over-end for 20 h at 25 °C. The coated beads were then washed eight times using phosphate−NaCl buffer as described above, and the amount of coated modified Pluronic F108 was determined by measuring the disulfide reactive groups. The coated PS particles were first incubated in 25 mM DTT for thiolation. After 10 min of incubation, the coated particles were pelleted by centrifugation and the supernatant containing the released 2-thiopyridone was carefully transferred to a new centrifuge tube and was subjected to one more centrifugation to remove possible remaining particles. The 2-thiopyridone content was then determined photometrically as described above. After the weight of the PS particles that were trapped on a preweighed Millipore filter and dried extensively over P_2O_5 was determined, the content of reactive disulfide groups per dry weight of PS porous particles was finally calculated.

Immobilization of β-Galactosidase on Porous Polystyrene Coated with F108−2-Pyridyl Disulfide. Eleven milligrams of β-galactosidase was dissolved in 1.0 mL 0.2 M phosphate−NaCl solution containing 30 mM reduced glutathione. After 30 min of reduction, the solution was passed through a PD-10 column equilibrated with phosphate−NaCl buffer, and 2.0 mL of the reduced β-galactosidase containing solution was collected, of which 1.0 mL was mixed with a 0.5 mL suspension containing 10 mg of F108−PDS-coated porous particles and the other 1.0 mL was reserved for determination of its thiol content. To measure the amount of thiol groups present in this sample, 0.2 mL of a saturated solution of 2,2′-dithiopyridine in water (1.5 mM) was added to both the reference cuvette with 1 mL of 0.2 M phosphate−NaCl buffer and the sample cuvette with the above reduced β-galactosidase. After 10 min of incubation, the thiol concentration was calculated by measuring the concentration of released 2-thiopyridone as described above. The mixture of coated particles and β-galactosidase was rotated end-over-end for 48 h at 4 °C, and the particles were then washed with the 0.2 M phosphate−NaCl buffer as described previously. The amount of immobilized β-galactosidase was determined by amino acid analysis. The amino acid analysis based on the phenyl isothiocyanate derivatization method has been detailed elsewhere (*5*).

RESULTS AND DISCUSSION

To develop a general procedure for activation of a Pluronic surfactant to enable its conjugation with other functional groups of interest, we selected Pluronic P105, F68, F88, and F108 in this investigation. The conjugation of *p*-nitrophenyl groups to the PEO ends of these surfactants was found to provide an efficient coupling chemistry and was independent of molecular weight. The coupling yield was readily determined from the release of yellow *p*-nitrophenolate ions in alkaline solution. In this calculation, the degree of substitution was based on the assumption of two available −OH groups in each Pluronic molecule and was generally found to be around 80% (Table 1). The degree of substitution of each Pluronic type reported in this table is the average value

Table 1. Chemical Modification of Pluronic Surfactants

	degree of substitution (%)	
	4-nitrophenyl chloroformate	Bolton−Hunter reagent
Pluronic P105	82	64
Pluronic F68	78	84
Pluronic F88	85	93
Pluronic F108	83	93

of at least three repeated experiments performed under the same conditions (−OH:nitrophenyl was kept at 1:3) and was not found to increase significantly when a larger excess of reagents was used. Such activated Pluronic surfactants can be stably stored in a desiccator over P_2O_5 at room temperature and have been found not to lose their activity after months of storage.

Radioisotope ^{125}I Labeling of Pluronic. It is difficult to quantify a Pluronic surfactant in solution or in its adsorption complexes precisely because it does not have any chromophore or other functional groups that can be specifically determined by a common instrument. Although the turbidity measurement of Pluronic is the most common technique for its quantification, this method usually has many limitations and is only applicable to polymers in a dust-free solution (*29*). Our approach is to modify the *p*-nitrophenyl-activated Pluronic molecules by means of the introduction of phenol groups. Through the iodination of such groups, biomolecules or polymers have been found to be easily labeled with radioactive iodine (*30*). The Bolton−Hunter reagent, with its succinimidyl and phenol groups, has been found to be a good reagent for coupling to $-NH_2$ groups in proteins, and such conjugated biological molecules can subsequently be radioiodinated with high specific activity. By this technique, proteins in nanogram quantities can be detected and quantified (*21*). To provide available $-NH_2$ groups for attachment of the Bolton−Hunter reagent to Pluronic surfactants, hydrazino groups were introduced by reacting hydrazine with the *p*-nitrophenyl-activated Pluronic (product **I**, Figure 1). This reaction usually went to completion, and measurements of the amount of released *p*-nitrophenol were in close agreement with the amounts of conjugate **I** used for the reaction. In Table 1, a high degree of substitution is also indicated for the reaction of the Bolton−Hunter reagent's hydroxylsuccinimide ester with $-NH_2$ groups of the modified Pluronic (product **II**, Figure 1). The purified phenol-conjugated Pluronic was then ready for labeling with radioactive iodine. Following iodination by means of the chloramine T method, ^{125}I-labeled Pluronic and free Na^{125}I were separated using a disposable PD-10, Sephadex G-25 column. The radioisotope-labeled polymer was collected and passed through the PD-10 column a second time to check for the presence of free Na^{125}I in the solution. By this procedure all free Na^{125}I was proven to be completely removed from the labeled polymer. Although the labeling efficiency cannot be calculated by a general precipitation method such as the trichloroacetic acid method used in protein labeling (*3*), the efficiency estimated from the second GPC profile was around 95−96%. In the above three-step reaction, Pluronic F68, F88, and F108 were all modified chemically in organic solvents and were successfully recovered by precipitation with ether. The product yield for each step was from 75% to 80% as shown in Table 1. By contrast, the Pluronic P105 analog was not easily separated from unreacted reagents and often had to be recovered by other elaborate schemes. This solubility difference in ether may be the result of differences in chemical composition between P105 and the other selected Pluronic surfactants; P105 contains 50% PEO, whereas the others contain up to 80% of PEO. The ^{125}I-labeled surfactants have been used to quantify the amounts adsorbed on PS colloids (see below) and to trace the stability of such coated colloids in various environments (*4*).

Table 2. Coupling of Amino-Containing Molecules to Pluronic F108

reactive groups	reaction milieu	degree of substitution (mol/mol F108)
hydrazine	CH_3OH	1.7
1,3-diaminopropane	aqueous pH 8.2	1.4
2-aminoethanesulfonic acid	aqueous pH 9.4	1.5
Gly-Trp	CH_3OH	1.0
Gly-Trp	CH_3OH/TEA	1.1
2-(2-pyridyldithio)ethylamine	CH_3OH/TEA	1.0

Coupling of Amines. In addition to the isotope labeling described above, the *p*-nitrophenyl conjugated derivative is a useful intermediate for the introduction of various other substituents into Pluronic surfactants. By this route it has for instance been possible to introduce groups that carry a negative or a positive charge at physiological pH, by reacting the surfactant derivative with bifunctional molecules containing a primary amine. Activated Pluronic F108 (product **I** in Figure 1) was thus chosen to show this useful route by reacting with 2-aminoethanesulfonic acid (taurine), 1,3-diaminopropane, and the dipeptide Gly-Trp, respectively (Table 2). The coupling can be performed in both organic and aqueous environments. In water the coupling should be performed at an alkaline pH (between 8 and 10) in which milieu most primary amino functions are at least partially deprotonated. In organic solvents deprotonated amines seem to react well even without a base catalyst. Thus, the same degree of substitution was obtained when the dipeptide Gly-Trp was reacted with *p*-nitrophenyl-activated F108 in methanol with or without the base TEA (Table 2).

A particularly interesting derivative is the reactive disulfide derivative of Pluronic obtained by reacting 4-nitrophenyl chloroformate activated surfactant with 2-(2-pyridyldithio)ethylamine (Figure 2). The 2-pyridyl disulfide group reacts specifically with thiols in a so-called thiol−disulfide exchange reaction leading to the release of 2-thiopyridone (Figure 2) and the formation of a more stable disulfide. This reaction occurs at both acidic and alkaline pH and usually goes to completion even under equimolar concentrations of the reactants. Thiol−disulfide exchange is a specific and reversible reaction in which the hydrolysis rate of the reactive disulfide is negligible at pH <8.5. This reaction has been the base for several useful applications, such as covalent chromatography, reversible protein immobilization, and protein−protein conjugation (*31*−*33*). When the disulfide functional groups have been immobilized on a solid phase designed to bind thiol-containing molecules, the bound molecules can subsequently be easily released and removed from the solid surface by the addition of low molecular weight thiol-containing reagents (Figure 2). Here, the 2-pyridyl disulfide was substituted into Pluronic F108 by the reaction of *p*-nitrophenyl-activated F108 and 2-(2-pyridyldithio)ethylamine. The new F108, modified to contain a reactive disulfide (F108−PDS), can be stably stored in solid form at room temperature in a desiccator over P_2O_5 for months. As described by Rydén and Carlsson (*34*), the 2-pyridyl disulfide group is very stable both in solid form and in aqueous solutions under physiological conditions of pH and temperature. We found that each F108 molecule can only be linked with one disulfide active group even though it contains two

Table 3. Surface Density of Pluronics on Polystyrene Latex Particles (0.272 μm) Determined by ^{125}I Labeling or Sedimentation Field-Flow Fractionation

	surface density (nm^2/molecule)	
	^{125}I labeling	sedFFF
Pluronic P105	7.4	7.8
Pluronic F68	9.4	10.9
Pluronic F88	8.6	9.9
Pluronic F108	7.9	7.0

Table 4. Porous Polystyrene Particles (10 μm) Coated with F108−2-Pyridyl Disulfide and Its Specific Binding of β-Galactosidase

amount of bound F108−2-pyridyl disulfide (μmol/g of particle)	45
thiol content of β-galactosidase (mol/mol of protein)	12
amount of bound β-galactosidase (mg of protein/g of particle)	
on particles coated with F108−2-pyridyl disulfide	34
on particles coated with unmodified F108	<1

active *p*-nitrophenyl groups (Table 1). In Table 2, we summarize the degrees of substitution of 2-pyridyl disulfide as well as different amino-containing molecules to Pluronic F108. The reactions of *p*-nitrophenyl-activated F108 with hydrazine, 1,3-diaminopropane, and 2-aminoethanesulfonic acid are all seen to be reasonably efficient. Yet, as in the attachment of disulfide groups to F108, a low degree of substitution was obtained when the dipeptide glycyltryptophan was conjugated to F108, whether the coupling was performed with or without the base TEA. The yield in the dipeptide reaction was low, even though a large excess of reagents was used in the reactions. This finding indicated that the introduction of the first amine might alter the availability of the second activated PEO end group in the block copolymer.

Pluronic Adsorption to Polystyrene Latex Particles. Polymeric colloids with a large surface area to volume ratio are commonly used as the solid phase in a variety of fields, especially for therapeutic purposes or as medical diagnostic products. In previous studies, we have found that plasma protein uptake on PS colloids can be minimized by using a Pluronic coating and that the blood circulation half-life of such treated colloids can be more than 13 h compared to just a few minutes for the corresponding uncoated particles (*4, 5*). Quantification of adsorbed surfactants on colloidal particles is one of the important tasks to provide an understanding of their surface properties and their relationship to biocompatibility. By using monodispersed PS latex particles as the adsorbent, we have determined the surface density of several Pluronic surfactants by using both ^{125}I labeling and sedFFF methods as shown in Table 3. In addition, to a good agreement with an independent measurement based on the use of fluorescent probes (*5*), modification of the triblock copolymers appears not to have altered the characteristics of the surfactants in terms of their adsorption on PS colloids.

Protein Coupling to Pluronic F108−PDS-Coated Surfaces. As mentioned before, there is always a risk of denaturation associated with the use of hydrophobic materials for protein immobilization, although these solid surfaces may provide many conveniences. In addition, a nonspecific protein binding, such as is usually observed on hydrophobic surfaces, may produce misleading results when these solid phases are used in quantitative assays based on specific binding. This leads to the idea of covering the hydrophobic surfaces with biocompatible polymers, while also providing functional groups for the binding of specific biological molecules (*35*). Our study has shown that by coating a Pluronic surfactant, for example F108, onto hydrophobic materials such as PS colloids, these materials will turn highly hydrophilic and become virtually resistant to protein uptake (*4, 5*). The reduced protein adsorption is predominantly due to steric repulsion and polymer chain dynamics (*36*). Here, we extend this approach by examining the attachment of a model protein, namely the naturally thiol-containing β-galactosidase, to Pluronic-coated highly porous PS particles. β-Galactosidase from *E. coli* is one of the most extensively investigated enzymes and is a commonly used component in enzyme-linked immunosorbent assays (*13*).

Monodisperse porous PS particles (10 μm in diameter) provide both an extremely large surface area of approximately 350 m^2/g and the advantage of easy sedimentation for removal of unbound proteins. By measuring released 2-thiopyridone, the amount of F108−PDS adsorbed to these particles, and the resulting thiol content, can be determined as listed in Table 4. Compared to the F108−PDS coating on other particles with similar hydrophobicity (*37*), the surface concentration found for these particles is very similar, and the coating procedure can therefore be considered useful also for other hydrophobic materials. It was not surprising to find an extremely small amount of β-galactosidase adsorbed on the porous PS particles when coated with the unmodified F108 (see Table 4). By contrast, results in Table 4 demonstrate that the protein binding increased significantly when the same PS particles were coated by F108−PDS. From this it is clear that β-galactosidase can be specifically immobilized on the particles through the thiol−disulfide exchange between F108−PDS and proteins.

Potentially, one of the most interesting applications of this F108−PDS coating is its use in enzyme-linked immunosorbent assay (ELISA), where it may enhance the immunoaffinity of immobilized antibody by site-specific chemical coupling. Although many factors are important in the preparation procedure, the sensitivity and the specificity of an assay are usually enhanced by a robust solid phase capture surface. Because of partial inactivation of the antibody or the shielding of its antigen binding sites as a result of the surface attachment, antibody immobilization through passive adsorption or random chemical coupling always leads to a reduced capacity to bind antigen. To overcome these drawbacks, an orientation control of the immobilization has been studied by many authors, exemplified by the improved specific binding through immobilization of Fab′ fragments (*38*) and the alteration of the chemical nature of the solid phase to promote a directional antibody immobilization (*39*). In recent work, we have also demonstrated the potential of the present approach by immobilizing thiolated anti-IgE to F108−PDS-coated PS microtiter plates (*33*). Preliminarily, our data indicate that antigen binding by the site-specifically immobilized antibody is 8 times higher than that of passively coated antibody. Another potential approach is to utilize the free thiol of the antigen binding Fab′ fragment for direct attachment at the surface. Although other ligands of interest may not contain thiol groups, a number of techniques, e.g. modification through a nonessential amino group, have been derived to thiolate proteins and peptides without changing their activity or specificity (*32*). With respect to preparation of the substrate, we have demonstrated that the content of adsorbed F108−PDS on the PS surface can be controlled by incubating the solid phase with mixtures of modified and unmodified Pluronic F108 in different stoichiometric ratios (*34*). This approach can be used to create a suitable spacing between the im-

mobilized biological molecules to give an optimal microenvironment for their biological function. It is generally very difficult to create such an adjustable distribution of the reactive molecules by direct chemical modification of hydrophobic materials. In summary, surface coating with Pluronic containing these reactive disulfide groups is a general, yet specific, immobilization technique that may prove to to be a promising approach in developing stationary phases for affinity chromatography, cell separation, DNA binding, heterogeneous testing assay, immunonephelometry, etc.

CONCLUSION

By employing a reaction of the hydroxyl ends of Pluronic surfactants with 4-nitrophenyl chloroformate, we can efficiently introduce reactive carbonate groups to such PEO/PPO/PEO triblock copolymers. These activated Pluronic surfactants are stable in an organic milieu and in a dry state but react relatively easily with primary amines. Hydrazine, 2-pyridyl disulfides, peptides, and other amino-containing chemicals were thus conjugated to the activated Pluronic. Using hydrazino groups as the bridge, tyrosyl groups for radioisotope labeling purpose were subsequently coupled to the Pluronic by a reaction with the Bolton−Hunter reagent.

On the basis of the specific thiol−disulfide reaction and the well-known surfactant properties of Pluronic, the coating of hydrophobic surfaces with 2-pyridyl disulfide conjugated Pluronic provides a new technique to render hydrophobic surfaces much more protein compatible while at the same time allowing the immobilization of desired molecules with a controlled spacing. As mentioned above, the rate of hydrolysis of the 2-pyridyl disulfide groups at physiological pH is almost negligible in comparison with the rate of the thiol−disulfide exchange reaction, and therefore such surface coatings can be sensitively activated at very low concentrations of thiol-containing biomolecules such as proteins. Since the thiol−disulfide exchange is a reversible reaction, the bound molecules can subsequently be released from the solid phase by adding thiol-containing reagents of low molecular weight. Thiol groups can be substituted into biomolecules without inactivation by a number of general methods, and the solid phase can be a hydrophobic surface in the form of particles (porous or nonporous) or plates. This surface coating technique is therefore very general in nature and has potential for use in diversified fields; it is a particularly attractive alternative when covalent immobilization techniques are complicated to carry out because of a need for prior surface activation.

ACKNOWLEDGMENT

Support by Grant GM 38008-05 from the National Institutes of Health and Pharmacia Diagnostics AB is gratefully acknowledged.

LITERATURE CITED

(1) BASF (1990) *Pluronic & Tetronic Surfactants*, BASF Co., Mount Olive, NJ.

(2) Amiji, M., and Park, K. (1992) Prevention of protein adsorption and platelet adhesion on surfaces by PEO/PPO/PEO triblock copolymers. *Biomaterials 13*, 682−692.

(3) Lee, J. H., Kopecek, J., and Andrade, J. D. (1989) Protein-resistant surfaces prepared by PEO-containing block copolymer surfactants. *J. Biomed. Mater. Res. 23*, 351−368.

(4) Li, J.-T., Caldwell, K. D., and Tan, J. S. (1991) Size analysis of a block copolymer-coated polystyrene latex. *Particle Size Distribution II: Assessment and Characterization* (T. Provder, Ed.) pp 247−262, American Chemical Society, Washington, DC.

(5) Tan, J. S., Butterfield, D. E., Voycheck, C. L., Caldwell, K. D., and Li, J.-T. (1993) Surface modification of nanoparticles by PEO/PPO block copolymers to minimize interactions with blood components and prolong blood circulation in rats. *Biomaterials 14*, 823−833.

(6) Gombotz, W. R., Guanghui, W., Horbett, T. A., and Hoffman, A. S. (1992) Protein adsorption to and elution from polyether surfaces. In *Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications* (J. Milton Harris, Ed.) pp 247−261, Plenum Press, New York.

(7) Bridgett, M. J., Davis, M. C., and Denyer, S. P. (1992) Control of staphylococcal adhesion to polystyrene surface by polymer surface modification with surfactants. *Biomaterials 13*, 411−416.

(8) Watrous-Peltier, N., Uhl, J., Steel, V., Brophy, L., and Merisko-Liversidge, E. (1992) Direct suppression of phagocytosis by amphipathic polymeric surfactants. *Pharm. Res. 9*, 1177−1183.

(9) Illum, L., Jacobsen, L. O., Müller, E. M., and Davis, S. S. (1987) Surface characteristics and the interaction of colloidal particles with mouse peritoneal macrophages. *Biomaterials 8*, 113−117.

(10) Davis, S. S., and Illum, L. (1988) Polymeric microspheres as drug carriers. *Biomaterials 9*, 111−117.

(11) Cappel, M. J., and Kreuter, J. (1991) Effect of nonionic surfactants on transdermal drug delivery: II. Poloxamer and poloxamine surfactant. *Int. J. Pharm. 69*, 155−168.

(12) Müller, R. H. (1991) *Colloidal Carriers for Controlled Drug Delivery and Targeting*, CRC Press, Boca Raton, FL.

(13) Villa, A. E., Guzman, L. A., Poptic, E. J., Labhasetwar, V., D'Souza, S., Farrell, C. L., Plow, E. F., Levy, R. J., DiCorleto, P. E., and Topol, E. J. (1995) Effects of antisense c-myb oligonucleotides on vascular smooth muscle cell. *Circ. Res. 76* (4), 505−513.

(14) Schaer, G. L., Hursey, T. L., Abrahams, S. L., Buddemeier, K., Ennis, B., Rodriguez, E. R., Hubbell, J. P., Moy, J., and Parrillo, J. E. (1994) Reduction in reperfusion-induced myocardial necrosis in dogs by RheothRx injection (poloxamer 188 N.F.), a hemorheological agent that alters neutrophil function. *Circulation 90* (6), 2964−2975.

(15) Tijssen, P. (1985) *Laboratory Techniques in Biochemistry and Molecular Biology: Practice and Theory of Enzyme Immunoassays*, Elsevier, New York.

(16) Brillhart, K. L., and Ngo, T. T. (1991) Use of microwell plates carrying hydrazide groups to enhance antibody immobilization in enzyme immunoassays. *J. Immunol. Methods 144*, 19−25.

(17) Sandwick, R. K., and Schray, K. J. (1987) Inactivation of enzymes upon interaction with a hydrophobic latex surface. *J. Colloid Interface Sci. 115*, 130−138.

(18) Zalipsky, S. (1995) Functionalized poly(ethylene glycol) for preparation of biological relevant conjugates. *Bioconjugate Chem. 6*, 150−165.

(19) Woghiren, C., Sharma, B., and Stein, S. (1993) Protected thiol-polyethylene glycol: a new activated polymer for reversible protein modification. *Bioconjugate Chem. 4*, 314−318.

(20) Harris, J. M., Sedaghat-Herati, M. R., Sather, P. J., Brooks, D. E., and Fyles, T. M. (1992) Synthesis of new poly(ethylene glycol) derivatives. *Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications* (J. M. Harris, Ed.) pp 371−381, Plenum Press, New York.

(21) Lääne, A., Haga, M., Aaviksaar, A., Chytry, V., and Kopecek, J. (1983) Activation of poly[*N*-(2-hydroxypropyl)-methacrylamide] for the binding of bioactive molecules: 1. Activation with 4-nitrophenyl chloroformate. *Makromol. Chem. 184*, 1339−1344.

(22) Miron, T., and Wilchek, M. (1987) Immobilization of proteins and ligand using chlorocarbonates. *Methods Enzymol. 135*, 84−90.

(23) Bolton, A. E., and Hunter, W. M. (1973) The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J. 133*, 529−539.

(24) Raja, R. H., LeBoeuf, R. D., Stone, G. W., and Weigel, P. H. (1984) Preparation of alkylamine and ^{125}I-radiolabelled derivatives of hyaluronic acid uniquely modified at the reducing end. *Anal. Biochem. 139*, 168−177.

(25) Sober, H. A. (1968) *Handbook of Biochemistry Selected Data for Molecular Biology*, The Chemical Rubber Co., Cleveland, OH.
(26) Carlsson, J., Drevin, H., and Axén, R. (1978) Protein thiolation and reversible protein-protein conjugation, *N*-succinimidyl 3-(2-pyridyldithio)propionate: a new heterobifunctional reagent. *Biochem. J. 173*, 723−737.
(27) Li, J.-T., and Caldwell, K. D. (1991) Sedimentation field-flow fractionation in the determination of surface concentration of adsorbed materials. *Langmuir 7*, 2034−2039.
(28) Li, J.-T., Caldwell, K. D., and Rapoport, N. (1994) Surface properties of Pluronic-coated polymeric colloids. *Langmuir 10*, 4475−4482.
(29) Attia, Y. A., and Rubio, J. (1975) Determination of very low concentrations of polyacrylamide and polyethyleneoxide flocculants by nephelometry. *Br. Polym. J. 7*, 135−138.
(30) Regoeczi, E. (1984) *Iodine-Labelled Plasma Proteins*, Vol. 1, CRC, Boca Raton, FL.
(31) Brocklehurst, K., Carlsson, J., Kierstan, M. P. J., and Crook, E. M. (1973) Covalent chromatography: preparation of fully active papain from dried papaya latex. *Biochem. J. 133*, 573−584.
(32) Jönsson, U., Malmqvist, M., Olofsson, G., and Rönnberg, I. (1988) Surface immobilization techniques in combination with ellipsometry. *Methods Enzymol. 137*, 381−388.
(33) Janson, J.-C., and Rydén, L. (1989) *Protein Purification: Principles, High Resolution Methods, and Applications*, VCH, New York.
(34) Rydén, L., and Carlsson, J. (1989) Covalent chromatography. *Protein Purification: Principles, High Resolution Methods, and Applications* (J.-C. Janson and L. Rydén, Eds.) pp 252−274, VCH, New York.
(35) Caldwell, K. D., Carlsson J., and Li, J.-T. (1993) Product for coating of hydrophobic surfaces to render them protein-resistant while permitting covalent attachment of specific ligands. U.S. Pat. Approved, International Publication No. WO 95/06251.
(36) Li, J.-T., Carlsson, J., Huang, S. C., and Caldwell K. D. (1996) Adsorption of poly(ethylene oxide)-containing block copolymers: a route to protein resistance. In *Hydrophilic Polymers*; *Performance with Environmental Acceptability* (J. E. Glass, Ed.) Advances in Chemistry Series 248, pp 61−78, American Chemical Society, Washington, DC.
(37) Li, J.-T. (1993) Plasma Interactions with Copolymer Stabilized Colloids, Doctoral Dissertation, University of Utah, Salt Lake City.
(38) Prisyazhnoy, V. S., Fusek, M., and Alakhov, Y. B. (1988) Synthesis of high-capacity immunoaffinity sorbents with oriented immobilized immunoglobulins or their Fab′ fragments for isolation of proteins. *J. Chromatogr. 424*, 243−253.
(39) Lin, J.-N., Chang, I. N., Andrade, J. D., Herron, J. N., and Christensen, D. A. (1991) Comparison of site-specific coupling chemistry for antibody immobilization on different solid supports. *J. Chromatogr. 542*, 41−54.

BC960048V