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Protein Conjugation with Amphiphilic Block Copolymers for Enhanced Cellular Delivery

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Abstract

Modification of a model protein, horseradish peroxidase (HRP), with amphiphilic block copolymer poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (Pluronic), was previously shown to enhance the transport of this protein across the blood—brain barrier *in vivo* and brain microvessel endothelial cells *in vitro*. This work develops procedures for synthesis and characterization of HRP with Pluronic copolymers, having different lengths of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) blocks. Four monoamine Pluronic derivatives (L81, P85, L121, P123) were synthesized and successfully conjugated to a model protein, HRP, *via* biodegradable or nondegradable linkers (dithiobis(succinimidyl propionate) (DSP), dimethyl 3,3′-dithiobispropionimidate (DTBP), and disuccinimidyl propionate (DSS)). The conjugation was confirmed by HRP amino group titration, matrix-assisted laser desorption/ionization-time of flight spectroscopy, and cation-exchange chromatography. HRP conjugates containing an average of one to two Pluronic moieties and retaining in most cases over 70% of the activity were synthesized. Increased cellular uptake of these conjugates was demonstrated using the Mardin-Derby canine kidney cell line and primary bovine brain microvessel endothelial cells. The optimal modifications included Pluronic L81 and P85. These copolymers have shorter PPO chains compared to Pluronic P123 and L121, which were less efficient. There was little if any dependence of the uptake on the length of the hydrophilic PEO block for the optimal modifications. The proposed modifications may be used to increase cellular uptake of other proteins.

INTRODUCTION

Use of proteins as therapeutic agents in many cases is hindered by an inability of protein molecules to cross cell membranes and physiological barriers, such as the blood—brain barrier (BBB) (1,2). To address this limitation, we have proposed to covalently modify proteins with amphiphilic groups, in particular, triblock copolymers, poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO-PPO-PEO), also known as Pluronic or poloxamers (3). Using *in vitro* and *in vivo* models, we have shown that a model protein, horseradish peroxidase (HRP), modified with Pluronic P85 (P85) *via* a degradable disulfide linker can cross the BBB

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and accumulate in the brain (4). Pluronic P85 alone is readily transported into cells *via* caveolae-mediated endocytosis (paper in preparation). Evidently, it also can serve as a synthetic transduction modulator that facilitates cell transport of a protein as large as HRP. The transport efficiency of protein—Pluronic conjugates should strongly depend on the lengths of Pluronic hydrophobic (PPO) and hydrophilic (PEO) blocks (5). Therefore, to develop optimal protein modifications one needs to establish robust procedures for (i) protein conjugation with the block copolymers with different lengths of PPO and PEO blocks and (ii) characterization of these conjugates. This work uses HRP as a model protein for conjugation with the block copolymers that differ in the (1) lengths of PPO block (L81 and P85 vs L121, and P123) and (2) overall hydrophobicity (L81 and L121 vs P85 and P123). The conjugates were synthesized by attaching one end of a Pluronic molecule to HRP amino groups via either degradable (disulfide) or nondegradable linkers. The products are characterized by amino group titration and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) spectroscopy. The uptake of the conjugates in the Mardin-Derby canine kidney (MDCK) cell line and primary bovine brain microvessel endothelial cells (BBMEC) is characterized to establish the most promising modifications. On the basis of these studies, L81 and P85 were identified as the best copolymers to enhance cell uptake of HRP.

EXPERIMENTAL PROCEDURES

Materials and Devices

HRP type VI-A, MW 43 kDa, 4-methoxyltrityl chloride (MTr-Cl), 1,1′- carbonyldiimidazole (CDI), 1,2-ethylenediamine (EDA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), ninhydrin, hydrindantin, 2-methyoxy ethanol, trifluoroacetic acid (TFA), triethylamine, anhydrous acetonitrile, anhydrous pyridine, methanol, dichloromethane, toluene, acetone, ethanol, and dimethylformamide were purchased from Sigma-Aldrich Co. (St. Louis, MO). Pluronic block copolymers, L81 (lot no. WSOO-25087), P85 (lot no. WPOP-587A), L121 (lot no. WPAC-550B), and P123 (lot no. WPIV-612B) were kindly provided by BASF Corp. (Paris-pany, NJ). Their characteristics are summarized in Table 1. Dithiobis(succinimidyl propionate) (DSP), dimethyl 3,3′-dithiobispropionimidate (DTBP), and disuccinimidyl propionate (DSS) were from Pierce Biotech Co. (Rockford, IL). Sephadex LH-20 gel and Illustra NAP-25 columns were from Amersham Biosciences (Pittsburgh, PA). Toyopearl MD-G SP column (10 mm ID × 6.8 cm) was from Tosoh Co. (Japan). Amicon ultra-15 centrifugal filter, MWCO 30K, membrane NMWL was from Millipore Co. (Billerica, MA). Spectro/Por membrane (MWCO 2000) was from Spectrum Laboratory Inc. (New Brunswick, NJ). Flexible thin-layer chromatography (TLC) plates were from Whatman Ltd. (Mobile, AL).

Monoamine P85 Derivative

To produce monoamine P85, first, the hydroxyl group at one end of the P85 chain was protected by MTr-Cl. Second, the hydroxyl group at the other end was activated using CDI and then reacted with EDA (Scheme 1) (6). The monoamine P85 was obtained after deprotecting the hydroxyl group in the presence of TFA. In detail, 3 g of P85 (MW 4600) was dried overnight *in vacuo* at 50 °C and then dissolved in 15 mL of anhydrous pyridine. One molar equivalent of MTr-Cl (200 mg) was dissolved in 10 mL of anhydrous pyridine and added to the P85 solution. The reaction was allowed to proceed for 3 h at 25 °C and then stopped by adding 1 mL of methanol. The pyridine was removed *in vacuo* by coevaporation with 3 × 15 mL of toluene. The mono-MTr-P85 was isolated from nonreacted copolymer and bis-MTr-P85 by adsorption chromatography on Silicagel column (3 × 20 cm) using stepwise gradient elution of 200 mL of dichloromethane containing 2%, 5%, and 10% methanol. Fractions containing bis-substituted MTr-Pluronic eluted first (R_f = 0.8), followed by monosubstituted MTr-Pluronic (R_f = 0.6) and free Pluronic (R_f = 0.4). The products were then analyzed by TLC in dichloromethane/MeOH, 9:1, and detected in TFA vapors (a test for MTr-group). The collected

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mono-MTr-P85 was dried by coevaporation with 2 × 15 mL of anhydrous acetonitrile *in vacuo* and then reacted with 5-fold molar excess of CDI (530 mg) in 10 mL of anhydrous acetonitrile. After incubation for 2 h at 25 °C, the reaction mixture was treated with 0.2 mL of water for 20 min to neutralize the nonreacted CDI, and then added to an excess of EDA in 20 mL of ethanol upon stirring. The mixture was kept overnight at 25 °C and then diluted by 50 mL of water and dialyzed for 18 h using a membrane with MWCO 2000 kDa against 20% aqueous ethanol (2 × 2 L) to remove small molecular mass reagents including EDA. After the dialysis, the polymer was concentrated *in vacuo*, coevaporated twice with methanol (10 mL), and redissolved in 50 mL of 2% TFA in dichloromethane. After incubation for 1 h at 25 °C, the bright yellow solution was concentrated *in vacuo* and neutralized by 5 mL of 10% triethylamine in methanol. The resulting monoamine P85 was isolated by gel filtration on a Sephadex LH-20 column (2.5 × 30 cm) and analyzed by TLC in dichloromethane/MeOH, 9:1. The monoamine P85 develops a blue color after spraying with 1% ninhydrine solution in ethanol (a test for an amino group).

Monoamine L81, L121, and P123 Derivatives

The monoamine derivatives of L81, L121, and P123 were synthesized using a modified procedure. In particular, to remove excess of EDA, modified L121 and P123 were dialyzed against 30–40% aqueous ethanol using a membrane with MWCO 2000 kDa. L81 was dialyzed against 30% aqueous ethanol using a membrane with MWCO 1000 kDa.

Amino Group Determination by Ninhydrin Assay

The stock solution of ninhydrin was prepared by adding 1.03 g of ninhydrin, 0.18 g of hydrindantin, 12.8 mL of 4 N sodium acetate buffer (pH5.5) to 38.5 mL of methylcelosolve. For the standard calibration curve, 0.5 mL of glycine (10^{-4} to 10^{-5} M) in acetate buffer (pH 5.5) was mixed with 0.25 mL the ninhydrin solution. The mixture was heated at 100 °C for 15 min and supplemented with 0.75 mL 50% aqueous ethanol. The absorbance was measured at 570 nm.

Conjugation of HRP with Monoamine Pluronic

Monoamine P85 (200 mg) in 0.5 mL of methanol was mixed with DSP (105 mg, 6-fold molar excess) solution in 0.5 mL of DMF stored over molecular sieves (4 Å). The mixture was supplemented with 0.1 mL sodium borate buffer (0.1 M, pH 8) and incubated for 30 min at 25 °C. Excess of DSP was removed by gel filtration on a Sephadex LH-20 column (2.5 × 30 cm) in methanol, and the solvent was removed *in vacuo*. Activated copolymer was dissolved in 1 mL 20% aqueous ethanol and mixed with HRP (16 mg) in 0.5 mL of 0.1 M sodium borate (pH 8). The homogeneous reaction mixture was incubated overnight at 4 °C. The activated monoamine L81, L121, or P123 (200 mg) was dissolved in 1 mL of 30–40% aqueous ethanol and reacted with HRP in 0.5 mL of 0.1 M sodium borate (pH 8). Modifications of HRP with monoamine P85 *via* DSS and DTBP were carried out using a similar procedure. DTBP was directly dissolved in 0.5 mL of sodium borate buffer (0.1 M, pH 8) and reacted with monoamine P85 in 0.5 mL of 20% aqueous ethanol.

Purification of HRP—Pluronic Conjugates

The conjugates were precipitated in cold acetone to remove the excess of nonreacted copolymers. Briefly, about 1 mL of the above reaction mixtures was added dropwise to 25 mL of cold acetone upon stirring. After incubating for 1 h at 4 °C, the brown precipitate containing HRP conjugates was collected at 3000 rpm for 10 min at 4 °C, washed by cold ethanol (2 × 10 mL), and dried *in vacuo*. Alternatively, the conjugates were washed with 20% aqueous ethanol and separated using Amicon ultra-15 centrifuge filter (MWCO 30 kDa) at 3800 rpm at 4 °C for 15 min. The extent of separation of nonreacted copolymers was assayed by TLC on silica

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gel plates in dichloromethane/methanol, 9:1. In this system, copolymers migrated (R_f = 0.4), while the conjugate was immobile. If free copolymer was detected, the purification procedure was repeated. To separate unmodified HRP, the conjugates were further purified on Toyopearl SP cation exchange column (1 cm × 6.8 cm) equilibrated with 10 mM CH_3COONa/10 mM NaCl/5% EtOH, pH 4.1, in the gradient of 0.2 M NaCl in 10 mM CH_3COONa/5% EtOH, pH 4.1. The final conjugates were desalted in Amicon ultra-15 centrifuge tube (MWCO 30 kDa) and freeze-dried.

MALDI-TOF spectra. Mass values of HRP—Pluronic conjugates were determined using a Voyager-DE Pro STR MALDITOF mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA). Samples were prepared by (1) plate coating with 0.5 μL saturated sinapinic acid in 50% acetonitrile with 0.1% TFA; (2) depositing 0.5 μL solution of HRP—Pluronic conjugates in water (10^{-5} M); and, finally, (3) coating with 0.5 μL saturated sinapinic acid in 50% acetonitrile with 0.1% TFA. External calibration was performed using bovine serum albumin (Applied Biosystems, Foster City, CA). Mass spectra were recorded with the laser intensity set between 5000 to 10 000 au Typically, three acquisitions (100 shots each) were pulled together for each sample.

Enzymatic Activity of HRP—Pluronic Conjugates

o-Phenylenediamine was used to measure the HRP enzymatic activity as described earlier (7). Briefly, 1 to 20 ng/mL HRP—Pluronic conjugates were mixed with 20 μL of a fresh *o*-phenylenediamine solution (0.05 mg/mL) in citrate buffer (0.1 M, pH 5.0) containing 0.01% Triton X-100, 0.1 g/mL BSA, and 0.2% H_2O_2The reaction mixture was added to 96-well plates to a final volume of 200 μL in citrate buffer (0.1 M, pH 5.0). After incubating at 37 °C for 5 min, the reaction was stopped by adding 20 μL of a 0.5% Na_2SO_3 solution in 2 N H_2SO_4, and the absorbance was measured at 490 nm in the microplate reader Multiskan MCC/340 (Fisher Scientific, Pittsburgh, PA).

Degree of Modification by TNBS Assay

Ten microliter aliquots of HRP—Pluronic conjugate solutions (protein concentration 0.1–0.6 mg/mL) were mixed with 10 μL of TNBS solution (1.7 mM) in 80 μL of sodium borate buffer (0.1 M, pH 9.5) and incubated at 37 °C for 2 h. The absorbance was measured at 405 nm using the microplate reader. The protein content was measured using a BCA kit from Pierce (Rockford, IL). The degree of modification (average number of amino groups modified) was calculated according to the equation

$$S\ (\text{modification degree}) = 7 \times \frac{A_{\text{native}}/C_{\text{native}} - A_{\text{modified}}/C_{\text{modified}}}{A_{\text{native}}/C_{\text{native}}}$$

where A_{native} and A_{modified} were the absorbences and C_{native} and C_{modified} the concentrations of native and modified HRP, respectively. The total number of primary amino groups including lysine residues and terminal amine group in HRP is 7.

Cells

MDCK cells (from ATCC, CCL-34) were seeded in 24-well plates at a density of 50 000 cells/well in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, California, cat no 41500–034) supplemented with 0.5% penicillin/streptomycin and 10% fetal bovine serum (Invitrogen, Carlsbad, California). The cells were cultured at 37 °C with 95% humidity and 5% CO_2, and grown for two days until 80–90% confluence. BBMEC were isolated from the gray matter of the bovine cerebral cortex by enzymatic digestion followed by subsequent centrifugations (8). Cells were seeded onto collagen-coated, fibronectin-treated 24-well plate at a density of 50 000 cells/well and grown to confluence (typically 10–12 days) in a culture

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media consisting of 45% minimum essential medium (MEM), 45% Ham's F-12 nutrient mix (Gibco, Life Tech., Grand Island, NY), 10 mM HEPES, pH 7.4, 13 mM sodium bicarbonate, heparin, gentamicin, amphotericin B (Sigma), and 10% horse serum.

Cellular Uptake

MDCK cell and BBMEC monolayers in 24-well plates were washed twice in assay buffer containing 122 mM NaCl, 25 mM $NaHCO_3$, 10 mM glucose, 3 mM KCl, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 1.4 mM $CaCl_2$, and 10 mM HEPES. Cells were exposed to unmodified or modified HRP in assay buffer for various time intervals at 37 °C, then washed with cold phosphate-buffered saline five times and lysed in 1% Triton X-100. No cellular toxicity was observed during treatment. Aliquots of cell lysates (20 μL) were taken for HRP activity determination as described above. Separate calibration curves were used for unmodified or modified HRP. The amounts of cell-associated HRP were normalized for the cell protein as determined by Pierce BCA.

RESULTS AND DISCUSSION

Synthesis of Pluronic Monoamine Derivatives

A general procedure for introduction of amino group in Pluronic is presented in Scheme 1. Good separation of monosubstituted MTr-Pluronic from bis-substituted and free Pluronic was achieved using a silicagel column for each copolymer used (L81, P85, L121, and P123). The purity of the fractions was assessed by TLC. For all polymers, the monosubstituted MTr-Pluronic exhibited R_f = 0.6, the bis-substituted MTr-Pluronic R_f = 0.8, and free Pluronic R_f = 0.4. Fractions containing the monosubstituted MTr-Pluronic were collected, and these fractions did not contain bis-substituted MTr-Pluronic or free Pluronic. Subsequent transformation of the end-protected MTr-Pluronic to the amino derivatives using CDI and EDA proceeds in mild conditions and is well-established for conjugation of poly(ethylene glycol) to amino groups of peptides (9). The reaction yields were from 70% to 80% by weight. The modification degrees (number of amino groups per copolymer molecule) were 0.96 (L81), 0.74 (P85), 0.74 (L121), and 0.81 (P123) as determined by ninhydrin assay. No trace of EDA was detected by TLC. The NMR spectra, before and after modification, show no change in -CH_2CH_2O- and -$CH_2CH(CH_3)O$- signals, suggesting that the copolymer backbone remained intact (data not shown).

Synthesis and Purification of HRP—P85 Conjugates

Three types of homobifunctional cross-linking reagents, DSP, DSS, and DTBP, were used to conjugate HRP and monoamine P85 (Scheme 2). Two reagents, DSP and DTBP, contain disulfide bond and are degradable in the reducing environments, such as intracellular glutathione (10). In contrast, DSS produces a stable nondegradable link. The water-insoluble DSP and DSS were reacted with monoamine P85 derivatives in DMF/methanol, while DTBP was reacted in water. After purification, the activated P85 derivatives were conjugated to HRP in 20% aqueous ethanol. The resulting conjugates were purified, first, by acetone precipitation or filtration and, second, by cation exchange chromatography. In the case of DSP-activated P85 derivative, the reaction yield depended on pH, with pH 8.0 being optimal (Figure 1). Similar results were obtained for DSS-activated P85 (data not shown). However, DTBP-activated P85 was more stable at higher pH and, therefore, produced high yield of the protein conjugate at pH 8.0 and 9.0.

Table 2 summarizes the characteristics of the HRP—Pluronic conjugates obtained with different reagents and modification conditions. The residual enzymatic activity of HRP—P85 conjugates was relatively higher for DSP and DSS-linked conjugates (70% to 85% of initial HRP), but somewhat lower for DTBP-linked conjugates (56%). The average modifications

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degrees of HRP-P85 as determined by TNBS titration assay were from ca. 1 to 2. However, MALDI-TOF revealed that the conjugates contained a mixture of unmodified HRP and HRP—P85 conjugates with 1, 2, and 3 P85 chains attached (Figure 2a). The presence of the free P85 in these conjugates depended on the purification procedure. No free copolymer was detected for HRP—P85 precipitated by acetone, while the filtration could not separate the free copolymer completely.

Conjugation of HRP with Monoamine L81, L121, and P123

The reaction conditions were modified for conjugation of HRP with L81, L121, and P123, which are more hydrophobic compared to P85. In these cases, the DSP-activated copolymers were conjugated to the protein in aqueous ethanol (30% to 40% v/v) mixtures to decrease hydrophobic association between the PPO chains. Furthermore, especially in the case of the most hydrophobic L121, the modification degrees considerably increased when the conjugation was carried out at lower temperature and for longer exposure time (Table 2). The PPO segment exhibits a lower critical solution temperature (LCST), and therefore, the decrease of the temperature (4 °C) favors disaggregation of Pluronic molecules (11). Notably, the excess of L81, L121, and P123 was completely removed during purification of the conjugates in cold acetone but not by the filtration (data not shown). Furthermore, even for the samples with the lower modification degrees, there was poor separation between the conjugates and unmodified HRP. However, the unmodified protein was observed along with various HRP—L81 and HRP—P123 conjugates in the mass spectra (Figure 2b,c). Obviously, separation of free HRP from the conjugates on a cation exchange column was only partial, as the mass spectra demonstrated. A possible reason was that under the separation conditions the modified HRP formed aggregates that also entrapped unmodified HRP and were eluted within the column elution volume. Increasing the ethanol content in the elution buffer from the original 5% to 20% could be a future solution to dissociate the aggregates and enhance the separation. HRP—L121 conjugates were not detectable in MALDI-TOF, possibly due to high hydrophobicity of such conjugates (data not shown in the figure). The remaining activity of HRP after conjugation was high (81% to 96% of initial HRP), except for the HRP—L121 conjugate with higher modification degree, which was purified by precipitation in acetone (65%) (Table 2). Altogether, the procedures described in most cases resulted in HPR—Pluronic conjugates with relatively high yield, purity, and enzymatic activity.

Cellular Uptake of HPR—Pluronic Conjugates

The uptake of unmodified HRP in both MDCK cells (Figure 3) and BBMEC (data not shown) increased almost linearly as the protein concentration increased up to ca. 250 μg/mL. This behavior is characteristic of fluid-phase endocytosis (12). Contrary to that, the cellular uptake of HRP—P85 was bimodal, with an initial sharp increase in uptake at 10 μg/mL followed by a slower increase up to 250 μg/mL. This suggests a different mechanism for the uptake of the modified protein and may indicate adsorption endocytosis (13). Clearly, the uptake was greatly increased after the modification, possibly due to binding of hydrophobic PPO chains with the cell membranes. (Notably, the studies presented in this work report the total uptake, which combines membrane binding and internalization components. A detailed characterization of the uptake mechanisms will be reported separately.)

Further, 50 μg/mL was selected as the concentration for the comparison of the unmodified and different conjugated HRP. In both cell models, there was a considerable increase (4- to 5-fold) in the uptake of HRP—P85 and HRP—L81 conjugates compared to the unmodified HRP (Figure 4a,b). Furthermore, the HRP—L121 conjugate also exhibited significant uptake increase, especially in the BBMEC mono-layers. The least efficient uptake was observed in the case of HRP—P123. Evidently, the length of the hydrophobic PPO block is an important factor for the membrane binding and/or cell internalization of HRP conjugates. P85 and L81

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contained about 40 propylene oxide units, whereas L121 and P123 contained about 70 propylene oxide units (Table 1). Therefore, the copolymers with shorter PPO chains overall appeared to be more efficient than those with longer PPO chains. This may be due to the anchoring of long PPO chains in the membranes that can prevent internalization of the corresponding conjugates in the cells. Notably, in the case of the free Pluronic the cellular uptake was also less efficient for the copolymers with longer PPO chains compared to the copolymers with shorter PPO chains because of strong binding of long PPO chains in the membranes (5). No aggregates were observed for all of the four conjugates in the concentration that were used in cellular experimentation when detecting in the sedimentation experiment (data not shown). Obviously, during the cellular uptake the conjugates were exposed to the membrane in the unimer form. It is also interesting that the length of the PEO chain had little if any effect on the uptake of the HRP—P85 and HRP—L81 conjugates having the same size of the PPO chains. At the same time, one may expect that the length of PEO chain could have a significant effect on the pharmacokinetics and distribution of the modified protein in the body (14). This will be examined for these conjugates in subsequent studies. Finally, in MDCK cells there was no difference in the uptake of HRP—P85 conjugates with degradable DSP and nondegradable DSS linkers (Figure 4c). However, the HRP—P85 conjugate with degradable positively charged DTBP linker was taken up much less efficiently than the conjugate with the degradable uncharged DSP linker. Since it is well-known that cationic compounds are generally transported more efficiently into cells, the reason for the decreased uptake of HRP—P85 conjugate with DTBP linker may be, for example, modification of different lysine amines in HRP compared to other conjugates. The differences in localization of the P85 chain in the HRP molecule may have a profound effect on the conjugate's transport characteristics due to the effects of the neighboring amino acids.

In summary, we have generated four monoamine Pluronic derivatives and successfully conjugated them to a model protein, HRP, *via* biodegradable or nondegradable linkers. The formation of covalent conjugates was demonstrated qualitatively by mass spectroscopy and quantitatively by TNBS assay. The purification procedures have been established. Using these methods, we obtained HRP conjugates containing an average of ca. 1 to 2 Pluronic moieties per protein molecule and retaining in most cases over 70% of the activity of the native HRP. The increased cellular uptake of the conjugates was demonstrated using MDCK cells and BBMEC monolayers. As a result of these modifications, a very hydrophilic protein, HRP, more efficiently bound with these cells than the unmodified analogue. The optimal polymers for modifications included P85 and L81. Between these two conjugates, there was little if any dependence of the uptake on the length of the hydrophilic PEO block of Pluronic, suggesting that its hydrophobic PPO block plays a decisive role in the conjugate interactions with cells. The results of this work will facilitate development of a novel method for the cellular delivery of proteins (4).

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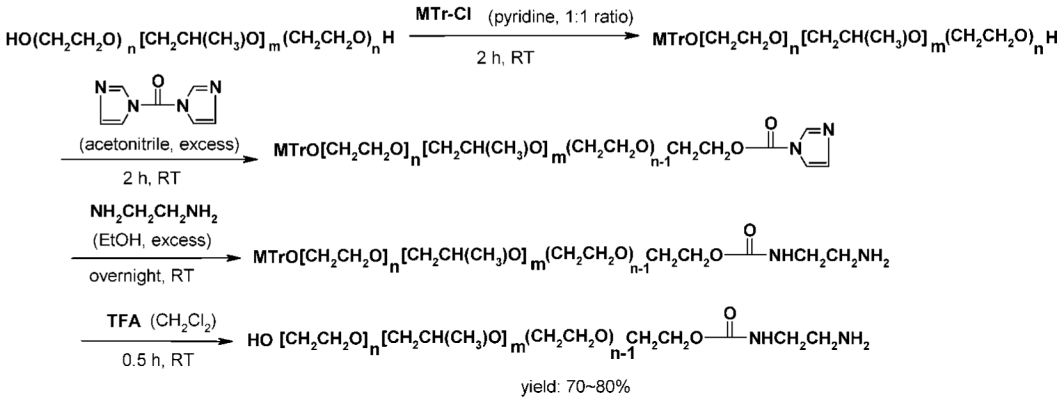

Scheme 1.
Synthesis of Monoamine Pluronic

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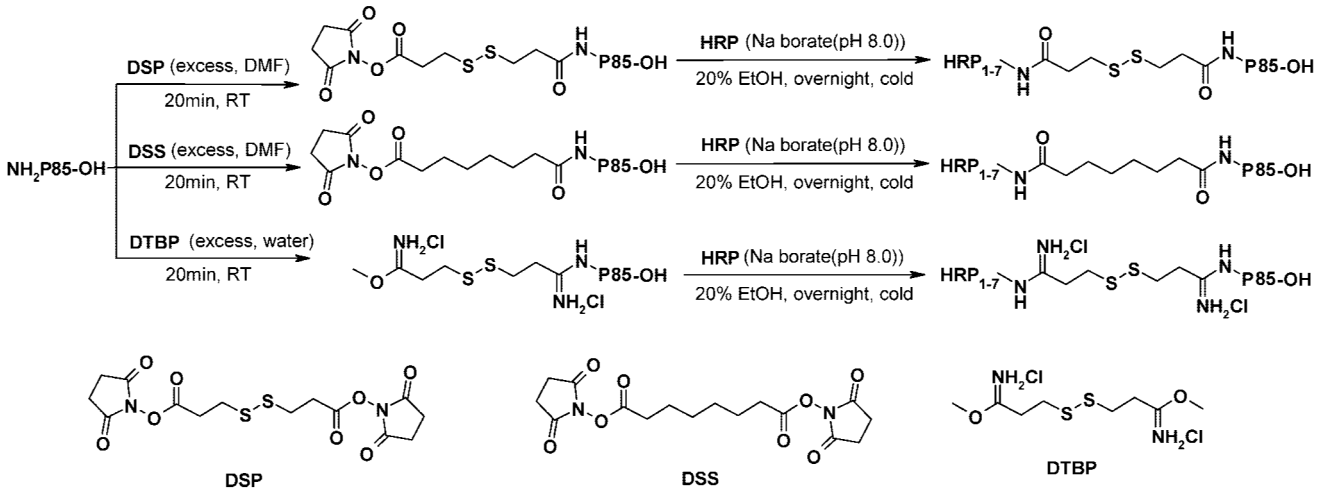

Scheme 2.

Conjugation of HRP with Monoamine P85 through Biodegradable Linkers DSP, DTBP and Nondegradable Linker DSS

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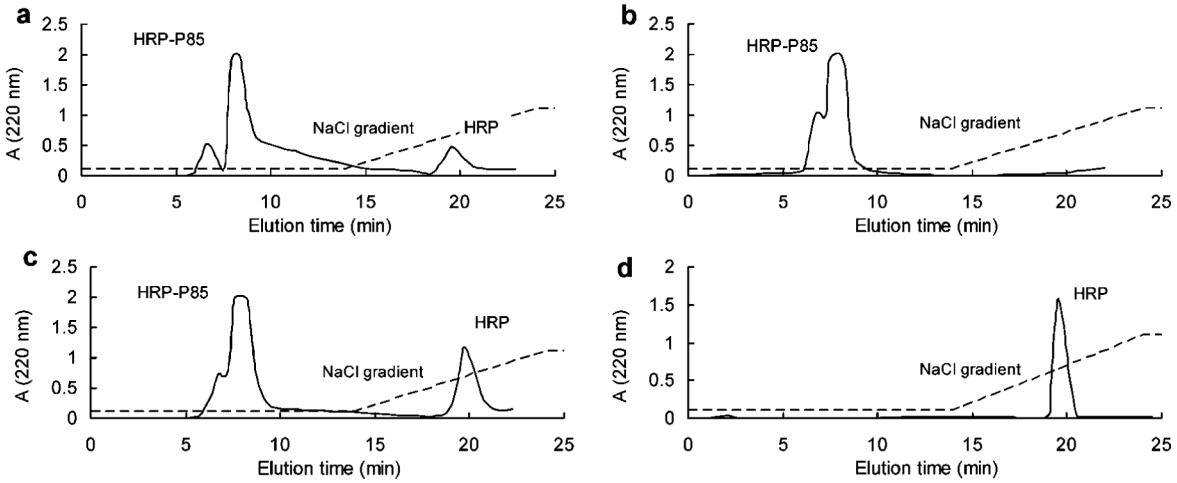

Figure 1.

Chromatographic profiles of HRP—P85 conjugates obtained using DSP at different pH: (a) 7.4, (b) 8.0, (c) 9.0, and (d) unmodified HRP. Chromatography conditions: Toyopearl MD-G SP column (10 mm i.d. × 6.8 cm) in NaCl salt gradient (0.01–0.2 M) (pH 4.3, elution rate 1 mL/min, detection at 220 nm).

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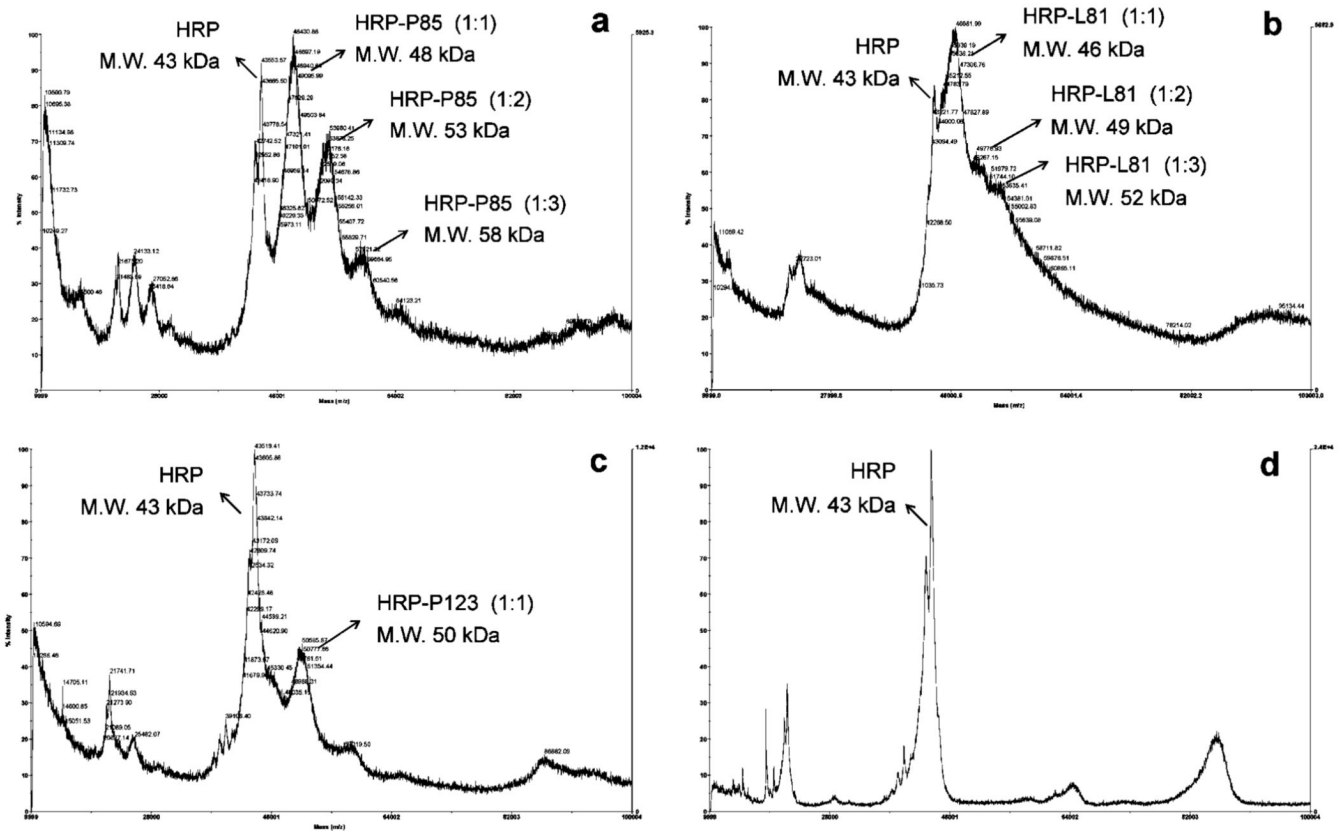

Figure 2.

Mass spectra of HRP—Pluronic conjugates obtained by MALDI-TOF: (a) HRP—P85; (b) HRP—L81; (c) HRP—P123; and (d) unmodified HRP. (a) HRP—P85 MW 48 kDa (1:1), 53 kDa (1:2), and 58 kDa (1:3); (b) HRP—P81 MW 46 kDa (1:1), 49 kDa (1:2), and 52 kDa (1:3); (c) HRP—P123 MW 50 kDa (1:1); (d) HRP MW 43 kDa.

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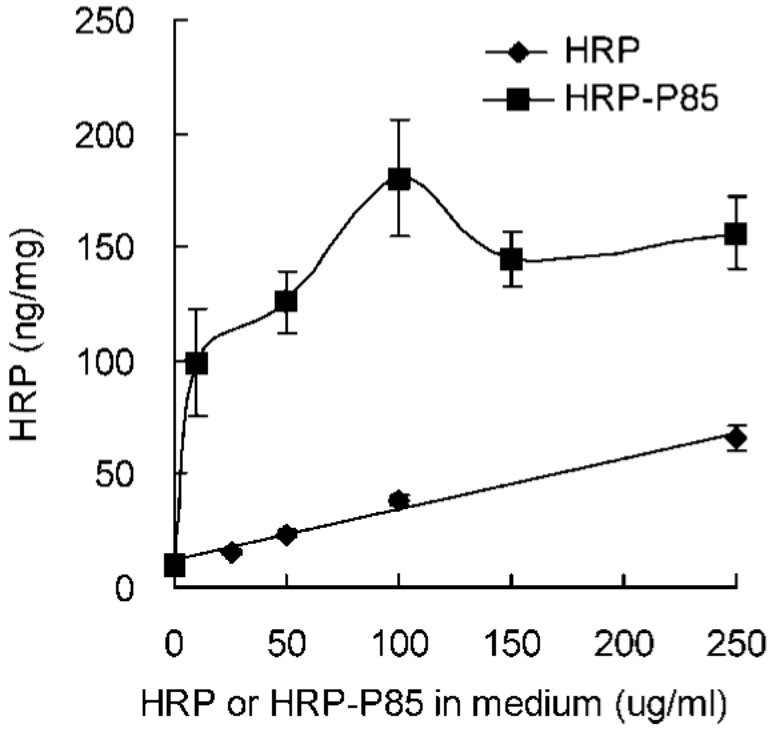

Figure 3.
Uptake of HRP and HRP—P85 conjugate with DSP link in MDCK cells at 60 min as a function of the enzyme concentration in cell culture medium. The amount of HRP is normalized for the total cellular protein (ng/mg). Data were obtained in quadruplicate and presented as means ± SEM.

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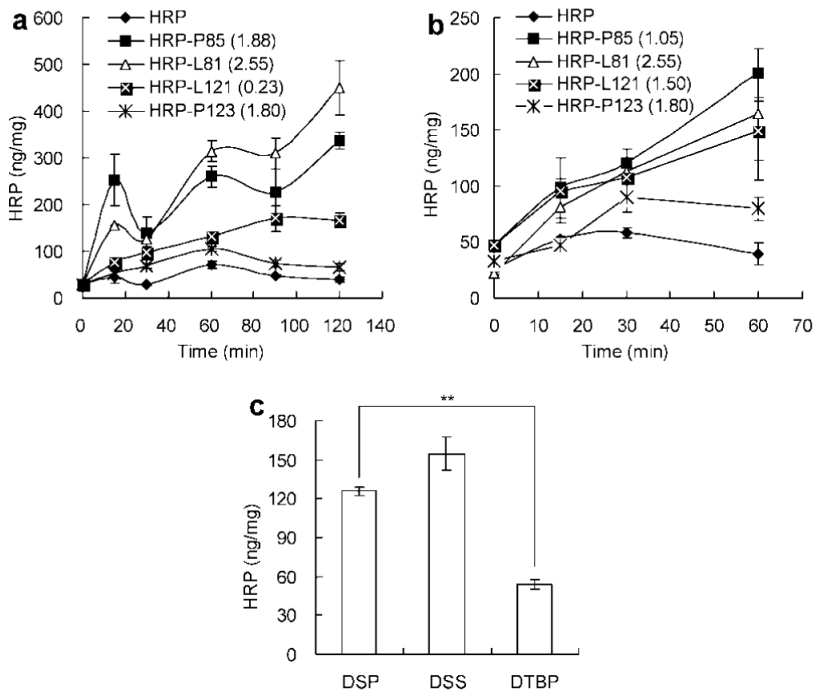

Figure 4.

Uptake of HRP—Pluronic conjugates in (a,c) MDCK cells and (b) BBMEC monolayers. The conjugates were prepared using (a,b) DSP or (c) different cross-linkers as shown in the figure. The modification degree of the conjugates was indicated in the brackets. Data were obtained in quadruplicate and presented as means ± SEM. Statistical analysis was done using the Student *t* test. The symbol ** indicates statistical significance ($p < 0.01$).

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Table 1

Structure and Properties of Pluronic Copolymers

Pluronic	structure	MW	HLB[a]	CMC (%)[b]
L81	EO_3—PO_{43}-EO_3	2750	2	0.006
P85	EO_{26}—PO_{40}-EO_{26}	4600	16	0.03
L121	EO_5—PO_{68}-EO_5	4400	1	0.0004
P123	EO_{19}—PO_{69}-EO_{19}	5750	8	0.0025

[a] Hydrophilic—lipophilic balance (HLB).

[b] Critical micelle concentration (CMC) in aqueous solution values at 37 °C as determined using pyrene probe.

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Table 2

Characteristics of HRP-Pluronic Conjugates

Pluronic	linker	Pluronic/HRP molar ratio during the reaction	reaction conditions time/ temperature/pH[a]	purification method[b]	modification degree[c]	residual activity[d]
P85	DSP	120	24 h/4 °C/pH8.0	F	1.3	85
P85	DSP	240	24 h/4 °C/pH8.0	A	1.39	ND
P85	DSP	240	36 h/4 °C/pH8.0	A	1.41	ND
P85	DSP	120	24 h/4 °C/pH8.0	A	1.03	70
P85	DTBP	120	24 h/4 °C/pH9.0	A	1.59	56
P85	DSS	120	24 h/4 °C/pH8.0	F	1.88	ND
L81	DSP	180	24 h/4 °C/pH8.0	F	2.55	96
L81	DSP	120	24 h/4 °C/pH8.0	A	0.95	81
P123	DSP	120	3 h/25 °C/pH8.0	F	1.80	85
P123	DSP	120	24 h/4 °C/pH8.0	A	1.26	ND
L121	DSP	120	3 h/25 °C/pH8.0	F	0.23	92
L121	DSP	120	24 h/4 °C/pH8.0	A	1.50	65

[a] Last stage of conjugation of HRP with activated derivative.

[b] The excess of Pluronic was removed by acetone precipitation (A) or filtration of the conjugate in the aqueous ethanol solution (F).

[c] Number of copolymer chains per HRP as determined by TNBS titration of amino groups.

[d] Residual activity was determined by *o*-phenylenediamine reaction and calculated on the basis of the percentage of the activity of the native HRP.