Cellular Internalization of Poly(ethylene oxide)-*b*-poly(ϵ-caprolactone) Diblock Copolymer Micelles

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Poly(ethylene oxide)-*b*-poly(ϵ-caprolactone) (PEO-*b*-PCL) block copolymers self-assemble into micelles in aqueous solution. We have examined whether these micelles can internalize into P19 cells in vitro. Fluorescently labeled PEO_{45}-*b*-PCL_{23} block copolymer was prepared by conjugating a tetramethylrhodamine molecule to the end of the hydrophobic PCL block. Dynamic light scattering (DLS) and transmission electron microscopy (TEM) studies yielded 24 ± 2 and 25 ± 2 nm, respectively, for the diameters of the micelles. The studies also showed that chemical labeling did not effect the morphology or size. When the rhodamine-labeled PEO_{45}-*b*-PCL_{23} block copolymer micelles were tested in vitro, time-, concentration-, and pH-dependence of the internalization process suggested that internalization proceeded by endocytosis. The results from these studies provide the first direct evidence for the internalization of PEO_{45}-*b*-PCL_{23} micelles. Future studies will utilize multiple labeling of these micelles, allowing questions to be addressed related to the fate of internalized micelles as drug carriers, the destination of the incorporated drugs or fluorescent probes released from micelles, and the identification of the subcellular localization of the whole drug−carrier system within cells, both in vitro and in vivo.

INTRODUCTION

Recently, the use of block copolymer micelle based delivery systems to improve the therapeutic efficiency of many drugs has received considerable attention (*1*−*3*). Polymeric drug-carrying micelles were first prepared by the group of Ringsdorf (*4*). The drug molecules were attached to the hydrophobic side chains of amphiphilic graft copolymers or to the hydrophobic block of amphiphilic diblock copolymers. Such polymers, in solution, formed nanosized polymeric micelles with hydrophilic shells and drug-modified hydrophobic cores. One system consisted of cyclophosphamide, an alkylating antitumor agent in the core of poly(ethylene oxide)-*b*-poly(L-lysine) (PEO−PLL) micelles. In those studies, cyclophosphamide was conjugated to the poly(L-lysine) block as the sulfido derivative. It was reported that the rate of hydrolysis of cyclophosphamide derivatives in the micellar system varied from minutes to several hours. Recently, the field of polymeric micelles as drug carriers has advanced greatly by the work of the groups of Kabanov and Kataoka, among others (*5*−*21*). Many studies from the group of Kabanov utilized Pluronic copolymers with the poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO−PPO−PEO) structure, which can self-assemble into micelles with PPO in the core and PEO in the corona. Such micelles, for expample, enhance the delivery of haloperidol to the brain, resulting in a significant increase in neuroleptic activity of the drug (*5*, *9*). Similarly, Kataoka's group found, for example, that polymeric micelles carrying adriamycin are more effective in vivo than free adriamycin. Micelle-incorporated adriamycin accumulates selectively in a solid tumor, very likely due to the enhanced permeability and retention effect (EPR effect) of the vascular endothelia at the tumor site (*6*, *7*, *10*, *13*, *20*).

Drugs and other agents can be incorporated into the inner core of polymeric micelles both by chemical conjugation and physical entrapment. In fact, many hydrophobic low molecular weight drugs were successfully incorporated into hydrophobic cores (*1*−*21*). Also, cisplatin and poly(ethylene oxide)-poly(α,β-aspartic acid) (PEO−PAA) block copolymers in an aqueous medium can form polymer−metal complex micelles (*22*). The concept has also been extended to polyion complex micelles, in which the block or graft polymers contain ionic and nonionic blocks, and in which a polyion or surfactant with opposite charge interacts with the ionic block to form the core of the complex micelles. Such micelles have been developed to deliver biological macromolecules such as DNA (*23*−*25*).

Polymeric micelles with poly(ethylene oxide) (PEO) as the hydrophilic block forming the corona of the micelles can avoid uptake by the reticuloendothelial system (RES) and thus stay in blood circulation for prolonged times (*1*−*3*). A wide range of polymeric materials has been utilized as the hydrophobic block or the charged block in complexes to form the core of the micelles, the choice depending on the compatibility between the polymers and the incorporated drugs (*2*). In this group, poly(ethylene oxide)-*b*-poly(ϵ-caprolactone) (PEO-*b*-PCL) diblock copolymer micelles have been explored as a drug delivery system (*16*, *17*). PCL is a well-known biodegradable polymer which has been utilized in various biomedical applications because of its excellent biocompatibility and degradability. It has been found that PEO-*b*-PCL micelles

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are an effective carrier for lipophilic drugs, such as FK506, L-685,818, and dihydrotestosterone (*16*, *17*).

An understanding of micelle internalization is needed to develop drug delivery systems, especially carriers for subcellular targeting (*26*−*37*). At present, little is known about cellular internalization of polymeric micelles (*31*−*37*). Kabanov's group reported that Pluronic copolymer micelles can be internalized by an endocytotic pathway, and Pluronic copolymers can increase drug absorption by inhibition of the P-glycoprotein (P-gp) drug efflux system in cells (*31*−*34*). Our previous studies suggested cellular internalization of PEO-*b*-PCL into PC12 cells via endocytosis (*36*, *37*). However, since the probes used in these studies were only physically incorporated into micelles, no direct evidence has been provided for the internalization of the polymer micelles themselves. Therefore, we have chemically conjugated a rhodamine molecule to the end of the PCL block, prepared micelles containing these labeled chains, and investigated the internalization of such micelles into P19 cells, a pluripotent embryonic carcinoma cell line (*38*−*40*).

EXPERIMENTAL PROCEDURES

Materials. Poly(ethylene oxide) (number average molecule weight, M_n ca. 2000, Aldrich), tetramethylrhodamine-5-carbonyl azide (T6219) (Molecular Probes), and stannous(II) octoate (SnOct) (Sigma) were used without further purification. ϵ-Caprolactone (Aldrich) was twice dried over CaH_2 at room temperature and distilled under reduced pressure. Tetrahydrofuran (THF) and toluene were distilled just before use.

Synthesis and Characterization of PEO_{45}-*b*-PCL_{23} Block Copolymers and Rhodamine-Conjugated Copolymers. PEO_{45}-*b*-PCL_{23} block copolymers were synthesized as described elsewhere (*41*). A predetermined volume (6.6 mL) of ϵ-caprolactone monomer was placed in a flask containing a preweighed amount (5 g) of PEO under a nitrogen atmosphere. Then a drop of SnOct was added. After cooling to liquid-nitrogen temperature, the flask was evacuated, sealed off, and kept at 130 °C for 24 h. The synthesized polymers were then dissolved in THF, recovered by precipitation into cold hexane, and dried under vacuum at room temperature.

The degree of the polymerization of the PCL and the polydispersity of the polymers were determined by GPC. The degree of polymerization of the PCL was also measured by ^{1}H NMR relative to the degree of polymerization of the PEO.

The rhodamine-conjugated PEO_{45}-*b*-PCL_{23} block copolymers (PEO_{45}-*b*-PCL_{23}-Rh) were synthesized by employing a modified version of an existing procedure (*42*): 20 mg of the dried PEO_{45}-*b*-PCL_{23} block copolymers and 10 mg of tetramethylrhodamine-5-carbonyl azide were dissolved in 10 mL of freshly distilled toluene under a nitrogen atmosphere. After heating at 80 °C for 5 h, the reaction mixture was placed in a dialysis bag (MW = 1000) and dialyzed against DMF for one week to remove unconjugated rhodamine. Both the dialysis bag and the DMF were changed several times. Then the solution was dialyzed against MilliQ water for 3 days to remove any organic solvents. Again, both the dialysis bag and the MilliQ water were changed until no trace of rhodamine in either the external solvent or the dialysis bag in the absence of the internal solution could be detected. The aqueous solution was freeze-dried. The rhodamine-conjugated block copolymers were obtained in 60% yield. ^{1}H NMR was used to determine the amount of rhodamine in the conjugated block copolymers.

Preparation of Unlabeled Micelles (PM). A 20 mg amount of PEO_{45}-*b*-PCL_{23} block copolymers was dissolved in 0.38 g of DMF to yield 0.4 g of solution; the solution was stirred for 4 h. A 1.6 g amount of water was added at a rate of 10 μL every 30 s to induce micellization. The aqueous solution was placed in a dialysis bag (MW 50000) and dialyzed against MilliQ water for 1 day. After dialysis, the solution was diluted to 0.67 wt % of polymer.

Preparation of Labeled Micelles (RhPM). A block copolymer mixture consisting of PEO_{45}-*b*-PCL_{23}/PEO_{45}-*b*-PCL_{23}-Rh (molar ratio: 19/1) was used to prepare the rhodamine-labeled micelles. The polymer mixture was then handled as described above. The polymer and rhodamine concentrations in the solution were 0.67 wt % and 67 μM, respectively.

Preparation of Tetramethylrhodamine-5-carbonyl Azide (T6219) Aqueous Solution. One hundred microliters of 2 mM tetramethylrhodamine-5-carbonyl azide acetone solution was placed in a vial. After acetone was removed under vacuum, 3 mL of MilliQ water was added to dissolve the tetramethylrhodamine-5-carbonyl azide. The concentration of rhodamine in the resulting solution was 67 μM.

Preparation of Nonconjugated Tetramethylrhodamine-5-carbonyl Azide Containing Polymer Micelle Solution (NconRhPM). One hundred microliters of 2 mM tetramethylrhodamine-5-carbonyl azide acetone solution was placed in a vial. After the acetone was removed under vacuum, 3 mL of 0.67 wt % PEO_{45}-*b*-PCL_{23} block copolymers micelle solution was added to dissolve the tetramethylrhodamine-5-carbonyl azide. The total concentration of rhodamine in the micelle solution was 67 μM.

Dynamic Light Scattering (DLS). A Brookhaven laser light scattering instrument with an Uniphase μBlue laser at wavelength of 532 nm was used to determine the size distributions of the micelles at 25 °C. Labeled and unlabeled PEO_{45}-*b*-PCL_{23} block copolymers micelle solutions were filtered through 200 nm filter and used at a concentration of 0.05%.

Electron Microscopy. Transmission electron microscopy (TEM) was carried out on a JEOL microscope with a CCD camera operating at an acceleration voltage of 80 kV. Copper EM grids were precoated with a thin film of Formvar and then coated with carbon. Drops of 0.05 wt % solution of the labeled or unlabeled micelles were deposited on the resulting grids. After being dried in air overnight, the samples were shadowed with palladium/platinum alloy and carbon.

Cell Cultures. P19 mouse embryonal carcinoma cells (European Collection of Cell Cultures), as described by McBurney (*38*−*40*), were cultured in bacteriological Petri dishes (Falcon) for spectrofluorometric analyses or in 16-well chamber glass slides (Nunc) for fluorescent microscopy studies. Cells were grown in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL), and serum-free medium was used in all internalization experiments.

Fluorescence Microscopy. Fluorescence microscopy studies were performed to assess the concentration and time dependent internalization of rhodamine-labeled micelles. In the concentration dependent studies, 10, 5, 2.5, or 1 μL PM, RhPM, T6219, or NconRhPM were added to well chambers containing 90 ul RPMI 1640 medium. After a 12 h incubation period, the media were removed and the P19 cells were washed with cold phosphate-buffered saline (PBS), acid solution (0.2 M acetic acid and 0.5 M NaCl aqueous solution), and PBS. In the time course studies, 10 μL of PM, RhPM, T6219, or NconRhPM

were added to chambers containing 90 μL of RMPI 1640 medium. The incubation times were 1, 4, 12, 18, and 24 h. Fluorescent images were captured using an Olympus BX60 system microscope equipped with an Olympus BX-FLA reflected light fluorescence attachment and a Dage-MTI DC-330 color camera coupled to a Compaq Pentium II computer with 128 MB RAM. Magnification was obtained using 20X or 40X objectives. Images were stored in uncompressed tagged image file format (TIFF) at a resolution of 1200 dpi using Image Pro Plus 3.0 image analysis software.

Fluorescence Spectroscopy. To obtain more quantitative data, similar studies were performed using regular fluorescence spectroscopy. For the studies of concentration dependent internalization, cells were grown in 900 μL of RPMI 1640 medium in the Petri dishes and 100, 25, 10, or 5 μL of PM, RhPM, T6219, or NconRhPM solution was added to each dish. After a 12 h incubation period, the media were removed and P19 cells were washed with cold PBS, acid solution, and PBS. Cells were lysed in 300 μL of DMSO. The DMSO solutions were then diluted with 3.7 mL of water. The resulting solutions were used for the determination of the amount of rhodamine using a FluoroMax-2 fluorescence spectrometer: $\lambda_{ex} = 545$ nm, $\lambda_{em} = 578$ nm.

For the studies of time dependent internalization, 100 μL of PM, RhPM, T6219, or NconRhPM solution was added to each dish containing 900 μL of RMPI 1640 medium. After a specific incubation period (30, 24,18, 12, 8, 4, and 1 h, respectively), the P19 cells were treated as described in the experiments of concentration dependent internalization.

Factors Affecting Internalization: Temperature, pH, Ca^{2+}, Drugs. Cells were grown in Petri dishes in the RPM1 1640 medium containing 10% serum. Four hours prior to the start of the experiments, the cells were placed in the serum free RMPI 1640 medium (900 uL) and different agents were added in the following concentrations: calcium (3mM), chlorpromazine (5 μM), FK506 (5 μM), and EGTA (5 mM) (*43*−*48*). The effects of temperature were assessed by incubating the cells at 4 °C and 37 °C (*49*). The pH effect was investigated on cells in a serum-free medium (pH 7.4) and in an acidified medium (pH 5.5) (*50*−*54*). Incubation periods varied, and the details are indicated in the legend to Figure 6.

RESULTS AND DISCUSSION

Synthesis of PEO_{45}-*b*-PCL_{23} Block Copolymers and Rhodamine-Conjugated Copolymers. PEO_{45}-*b*-PCL_{23} block copolymers and rhodamine-conjugated copolymers were synthesized as shown in Scheme 1. The number average molecular weight (M_n) and polydispersity of PEO_{45}-*b*-PCL_{23} block copolymer determined by GPC are 4500 and 1.41, respectively. The block copolymer was also characterized by ^{1}H NMR. An NMR spectrum of the labeled polymer is shown in Figure 1. The methylene protons of ethylene oxide units of the PEO correspond to the singlet at 3.7 ppm (labeled b). The methylene peaks of the caprolactone appear at 4.1, 2.3, 1.63, and 1.37 ppm (e, c, d, and d, respectively) (*41*). The molar ratio of ethylene oxide units to caprolactone units can be calculated based on the ratios of their peak areas. Therefore, the degree of polymerization of caprolactone can be obtained relative to the degree of polymerization of ethylene oxide. In our case, the calculated degree of polymerization of caprolactone is 23. The molecular weight based on the degree of polymerization of caprolactone and ethylene oxide from NMR is 4600 g/mol, which is consistent with the GPC result (4500 g/mol).

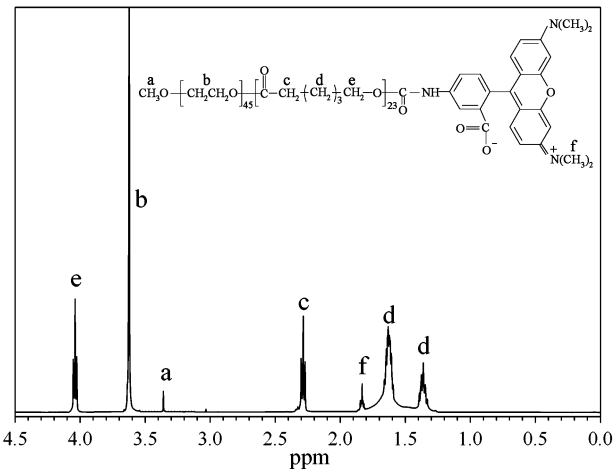
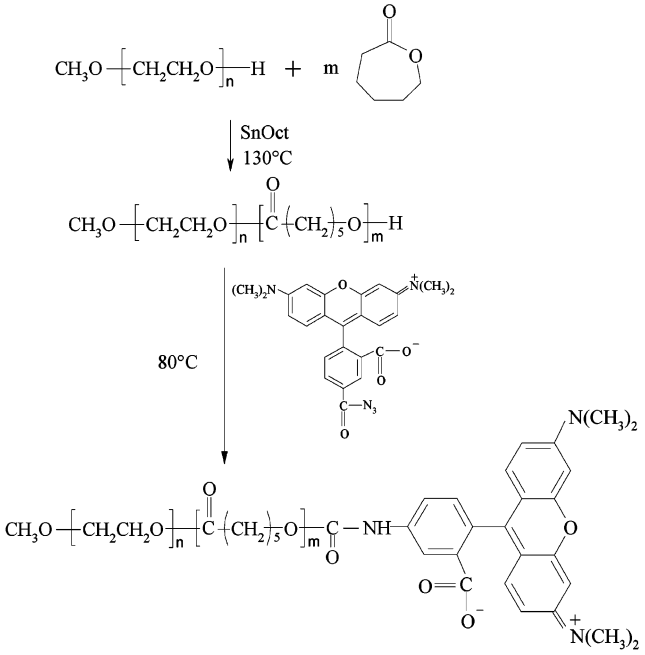


Figure 1. ^{1}H NMR spectrum of PEO45-*b*-PCL23-Rh in CDCl3.

Scheme 1. Synthesis of Poly(ethylene glycol)-*b*-poly(ϵ-caprolactone) Diblock Copolymer and Rhodamine-Conjugated Poly(ethylene glycol)-*b*-poly(ϵ-caprolactone) Diblock Copolymer



As shown in Scheme 1, the acyl azide group in tetremethylrhodamine-5-carbonyl azide is rearranged to an isocyanate on heating at 80 °C (*42*). In our PEO_{45}-*b*-PCL_{23} block copolymers, the functional group at the end of PEO chain is methoxy, while the functional group at the end of PCL chain is hydroxy. The hydroxy group can react with the freshly formed isocyanate. Therefore, tetramethylrhodamine can be attached to the end of the PCL chain. The success of the conjugation of tetramethylrhodamine to the block copolymer can be proven by ^{1}H NMR. The signal of methyl protons of tetramethylrhodamine is found at 1.84 ppm (f in Figure 1). The ratios of peak areas of the methyl group in tetramethylrhodamine to those of the methylene groups in PEO or poly(caprolactone) were used to calculate the molar ratio of tetramethylrhodamine to block copolymer chains. The average molar ratio is 0.94, suggesting that 95% of the polymer chains may be functionalized. Despite all the efforts, there may still be an undetectable trace of unconjugated rhodamine. This should lead to only a small

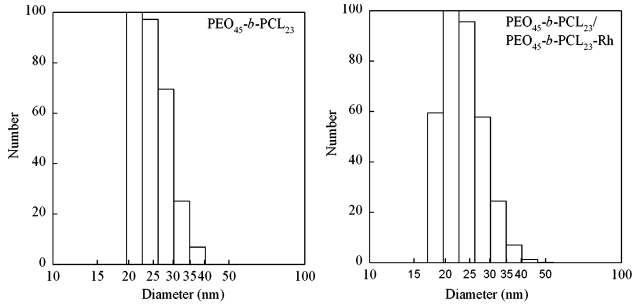


Figure 2. Size distribution of PEO_{45}-*b*-PCL_{23} and PEO_{45}-*b*-PCL_{23}/PEO_{45}-*b*-PCL_{23}-Rh (molar ratio: 19/1) micelles analyzed by DLS.

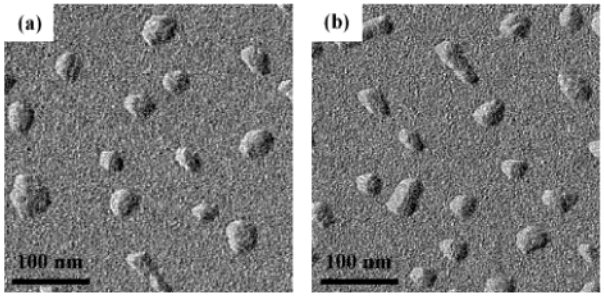


Figure 3. Transmission electron micrograph (TEM) of micelles of (a) PEO_{45}-*b*-PCL_{23} and (b) PEO_{45}-*b*-PCL_{23}/PEO_{45}-*b*-PCL_{23}-Rh (molar ratio: 19/1).

increase of size of error bar of our results, but should not affect the general conclusions.

Characterization of Labeled and Unlabeled Micelles. Rhodamine-conjugated block copolymer (5%) was mixed with the of the unlabeled block copolymer (95%) to prepare the rhodamine-labeled micelles. Both the labeled and unlabeled micelles were studied by DLS. As shown in Figure 2, the average diameters of unlabeled and labeled micelles are 25 ± 2 and 24 ± 1 nm, respectively. The results suggest that the size distributions of both labeled and unlabeled micelles are narrow, and that there is no significant change on the sizes of micelles after incorporation of rhodamine-conjugated PEO_{45}-*b*-PCL_{23} block copolymer chains.

The sizes and morphologies of labeled and unlabeled micelles were also studied by TEM. As shown in Figure 3, both labeled and unlabeled micelles are generally spheroidal. The sizes (from TEM) for both labeled and unlabeled micelles are identical (25 ± 2 nm and 25 ± 2 nm, respectively) and consistent with the DLS results. It should be noted that in the calculation of average sizes from TEM micrographs, only the spherical units are counted, since some of the elongated aggregates are probably the results of the attachment of two or more micelles during deposition.

Internalization of Micelles into P19 Cells. P19 cells are pluripotent cells, commonly used for in vitro studies, particularly those related to cell differentiation. Some drugs and also DMSO can transform P19 cells into neurons or muscle cells (*38*−*40*). We have grown the cells in the media without drug manipulations to retain them in the nondifferentiated pluripotent state.

We have investigated the rate and the extent of the internalization of rhodamine-labeled micelles, taking unlabeled block copolymer micelles (PM) as a negative control, as well as tetramethylrhodamine-5-carbonyl azide (T6219) and tetramethylrhodamine-5-carbonyl azide incorporated into block copolymer micelles (NconRhPM) as positive controls. Internalization of block copolymer

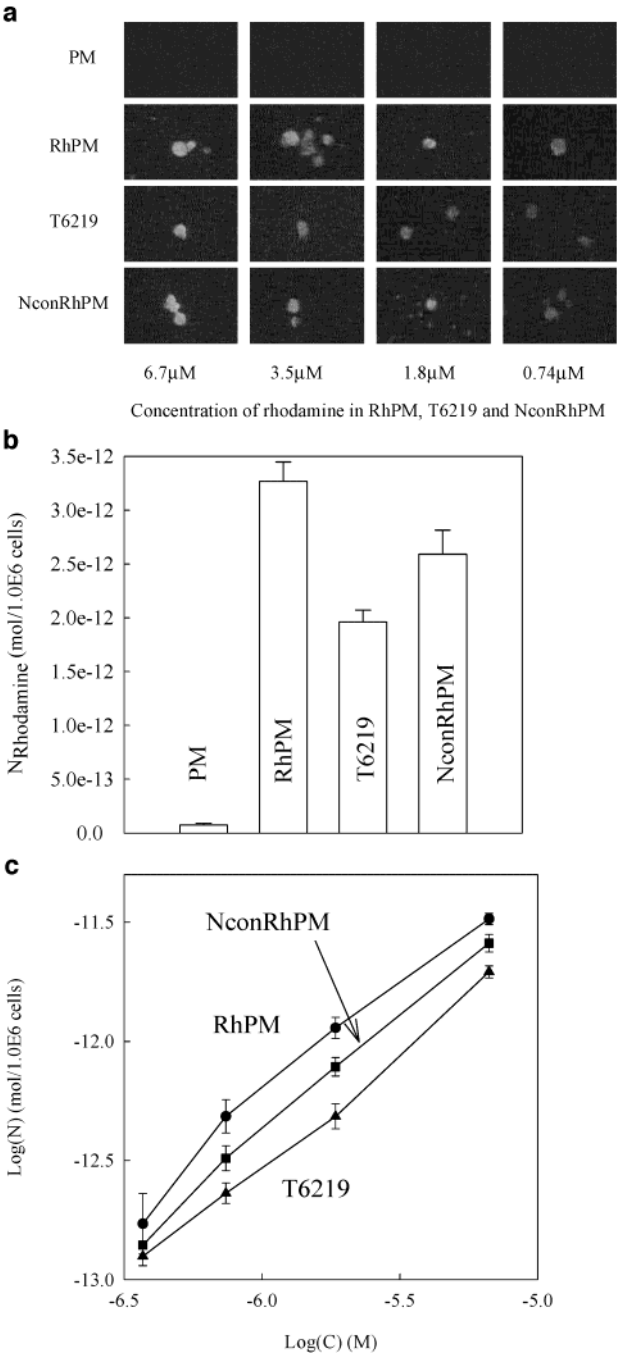


Figure 4. (a) Photographs of fluorescence intensity for rhodamine concentration-dependent internalization with P19 cells in 12 h. The polymer concentrations in RhPM, PM, and NconRhPM are 0.067%, 0.035%, 0.018%, and 0.0074%, respectively. (b) Internalization of PEO_{45}-*b*-PCL_{23} block copolymer micelles and of rhodamine T6219 into P19 cells in 12 h. The concentrations of the polymer in incubation solutions of PM, RhPM and NconRhPM are 0.067 wt %. The rhodamine concentrations of solutions of RhPM, T6219, and NconRhPM are 6.7 μM. (c) Rhodamine concentration-dependent internalization of PEO_{45}-*b*-PCL_{23} block copolymer micelles into P19 cells in 12 h. The polymer concentrations in RhPM and NconRhPM are 0.0037 wt %, 0.0074 wt %, 0.018 and 0.067 wt %, respectively.

micelles into P19 cells is dependent on both polymer concentration (Figure 4−c) and internalization time (Figure 5a,b). The fluorescence intensity of the rhodamine-labeled micelles was very low in low micromolar concentrations and at early time points (between 30 min and 6 h). Therefore, we conducted polymer concentration de-

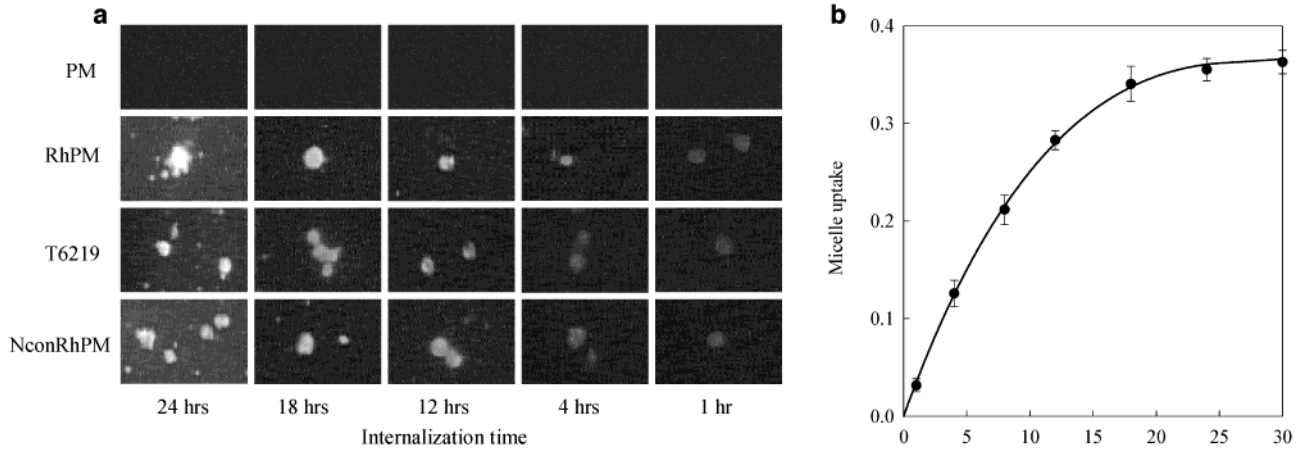


Figure 5. (a) Photographs of time-dependent internalization with P19 cells in 24 h. The polymer concentrations of PM and NconRhPM are 0.067%. The rhodamine concentrations of RhPM and T6219 are 6.7 μM. (b) Time-dependent internalization of RhPM with P19 cells as determined by spectrofluorometry. The polymer concentrations are 0.067%. The rhodamine concentrations are 6.7 μM.

pendent studies employing different concentrations of micelles at fixed 12 h incubation with P19 cells. Fluorescent microscopy studies suggest that fluorescence intensities increase in proportion to the increasing concentrations of rhodamine, and signals in RhPM-treated cells are stronger than those treated with T6219 or NconRhPM. Specrofluorometric measurements corroborate the findings from fluorescent microscopy (Figure 4b). Since the rhodamine molecules in RhPM are chemically linked to the PEO_{45}-*b*-PCL_{23} block copolymers, the results provide direct evidence for internalization of the block copolymer micelles. In addition, the amounts of rhodamine taken up by P19 cells are in the order RhPM > NconRhPM > T6219, suggesting that the block copolymer micelles enhance the accumulation of rhodamine T6219 molecules in the cells.

In most block copolymer solutions, both free unimers and micelles generally coexist. Results from studies with Pluronic P85 block copolymers suggest two different mechanisms of internalization involving free unimers and micelles, respectively (*31*−*35*). In the present case, we can show that it is the micelles which are responsible for the observed internalization. The considerations are as follows:

The polymer concentrations in the experiments described here are 0.067 wt % (150 μM), 0.035 wt % (78 μM), 0.018 wt % (40 μM), and 0.0074 wt % (16 μM), 0.0037% (8 μM), respectively. The CMC of PEO_{44}-*b*-PCL_{21} block copolymers micelles is 1.2 mg/L (unpublished observations); it is reasonable to take the CMC of PEO_{45}-*b*-PCL_{23} block copolymers micelles as also being around 1.2 mg/L, i.e., 0.00012 wt % (0.27 μM). Therefore, the PEO_{45}-*b*-PCL_{23} block copolymers in the solutions of our studies are mainly in the form of micelles, since the concentrations of polymer exceed the CMC by factors of ca. 30 to 600. Therefore, micelles are the primary form in the investigated solutions. Most importantly, free unimer concentrations of the PEO_{45}-*b*-PCL_{23} block copolymer are practically unchanged with changing polymer concentration. If the free unimers were responsible for the internalization, the internalization of RhPM should not be dependent on the total polymer concentration above the CMC. Since the internalization does depend on polymer concentration, we conclude that it is the micelles and not the free unimers of PEO_{45}-*b*-PCL_{23} that are responsible for the transport of PEO_{45}-*b*-PCL_{23} block copolymers into P19 cells.

Time dependent internalization of the block copolymers into P19 cells was confirmed by fluorescence microscopy

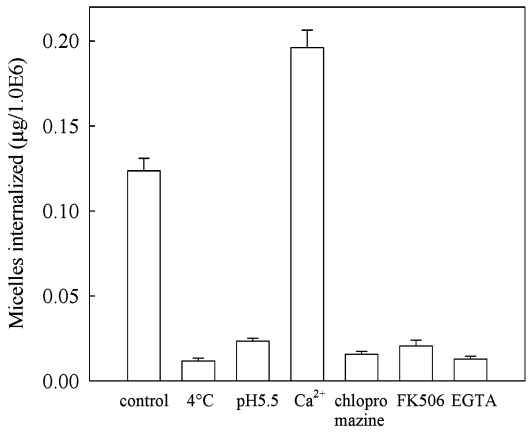


Figure 6. The effect of various pharmacological manipulations on the internalization of P19 cells with RhPM. The polymer concentration of RhPM is 0.067%. The rhodamine concentration is 6.7 μM.

(Figure 5a). As expected, there was no fluorescence in samples with empty PEO_{45}-*b*-PCL_{23} block copolymer micelles. The fluorescence intensities of P19 cells incubated with RhPM, T6219, and NconRhPM increased within 24 h. The amount of internalized PEO_{45}-*b*-PCL_{23} block copolymer micelles increased relatively rapidly initially, and the equilibrium was reached within ca. 24 h (Figure 5b).

If the micelles are internalized by endocytosis, then several basic criteria must be fulfilled (*43*−*54*). For instance, the process is time and energy dependent. Extent of internalization is reduced at low temperature (*49*) and low pH of the medium (*50*−*54*). Moreover, calcium and a number of drugs can significantly alter the extent of the internalization (*43*−*48*). In the present studies, various conditions in the medium significantly alter the extent of internalization of micelles as shown in Figure 6. For example, P19 cells treated with rhodamine-conjugated micelles and incubated at 4 °C, or in an acidified medium (pH 5.5), internalize a significantly smaller amount of micelles than the cells incubated at 37 °C or in the cell culture medium at pH 7.4 (control). Similarly, drug treatment with chlorpromazine or FK506 significantly reduces the amount of internalized micelles. These drugs are often used in internalization studies (*46*−*47*). Endocytosis is a calcium-dependent process. The ion concentrations were manipulated by EGTA (*48*). The internalization of rhodamine-labeled micelles is maximal in the presence of high (3 mM)

calcium concentration. By contrast, chelation of calcium by EGTA, significantly reduces the amount of internalized micelles.

In summary, results from the present studies show that the internalization of PEO_{45}-*b*-PCL_{23} micelles into P19 cells occurs by endocytosis. The influx of the rhodamine derivative used for labeling of the PCL chain appears to be increased when incorporated into PEO_{45}-*b*-PCL_{23} micelles. The studies also show changes in the extent of micelle internalization by pH, temperature, calcium, chlorpromazine, FK506, and ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) providing additional evidence for endocytosis (Figure 6).

Our earlier studies suggested that PEO-*b*-PCL micelles are internalized into PC12 cells and primary neuron-glia cultures via an endocytotic mechanism. However, in those early studies we did not have available fluorescent block copolymers, and the conclusions were based on studies involving noncovalently incorporated fluorescent or radiolabeled probes within the micelles (*36*, *37*). In agreement with the present findings and those by others (*31*−*37*), we now provide direct evidence for this mode of micelle internalization by employing covalently labeled block copolymers. The availability of these micelles provides a new tool for future studies in other cellular contexts in which the fate of the polymer carrier and incorporated agent can be followed at the subcellular level. Studies employing dual (micelle and probe) and multiple labels (micelle, probe, subcellular compartment) are currently underway in our laboratories to answer questions about the detailed subcellular distributions and in order to devise specific carriers to target different subcellular compartments.

CONCLUSIONS

The tetramethylrhodamine molecule was successfully conjugated to the end of the hydrophobic PCL block of PEO_{45}-*b*-PCL_{23} block copolymers. These fluorescently labeled block copolymers were used to prepare fluorescently labeled PEO_{45}-*b*-PCL_{23} block copolymer micelles. Chemical fluorescent labeling does not affect the spherical morphology or size of such micelles. Utilizing the rhodamine-labeled PEO_{45}-*b*-PCL_{23} block copolymer micelles, we have demonstrated that these micelles can be internalized into P19 cells by endocytosis. The internalization is time and concentration dependent. The results from these studies provide the first direct evidence for internalization of PCL-*b*-PEO micelles. The availability of rhodamine-labeled micelles will allow future studies involving multiple labels (drugs, drug−carrier, and subcellular compartment) to address the questions related to the fate of internalized micelles and the incorporated drugs or fluorescent probes.

ACKNOWLEDGMENT

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