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Cellular internalization of PCL_{20}-*b*-PEO_{44} block copolymer micelles

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Abstract

The cellular internalization of polycaprolactone-*b*-poly(ethylene oxide) (PCL_{20}-*b*-PEO_{44}) copolymer micelles were investigated in PC12 cells cultures. The micelles were found to be internalized into PC12 cells when followed over the 4-h incubation period. Also, the internalization process was found to fulfill the basic criteria for endocytotic uptake in that it was time, temperature, pH and energy dependent. In addition, the use of other pharmacological manipulations (hypertonic treatment, Brefeldin A) provide further evidence that the mode of cellular internalization is in fact endocytotic.

Keywords: Block copolymer micelles; Endocytosis; Drug delivery

1. Introduction

In recent years, there has been much interest in the use of block copolymer micelles as delivery vehicles for hydrophobic drugs [1–8]. The micelles are formed from amphiphilic di- or triblock copolymers. In an aqueous solution, the copolymers aggregate to form micelles which contain a hydrophobic core and a hydrophilic corona. The core of the micelle serves as cargo space for hydrophobic drugs while the corona acts as an interface between the hydrophobic core and the aqueous medium.

At present little is known of the mechanism or pathway of cellular internalization of block copolymer micelles or micelle-incorporated probes. Kabanov's group found that the cellular internalization (MDCK and Jurkat cells) of fluorescein containing poly(ethylene oxide)-*b*-polypropylene oxide-*b*-poly(ethylene oxide) (pluronic) micelles is inhibited at 4°C [9]. In a later study they also found that the cellular (bovine brain microvessel endothelial cells) accumulation of [^{3}H]pluronic micelles is decreased in the presence of deoxyglucose (40 mM) [10]. Together this evidence suggests that the mechanism of cellular uptake of the pluronic micelles may proceed by an endocytotic mechanism. We are now interested in determining if the cellular uptake of polycaprolactone-*b*-poly(ethylene oxide) (PCL_{20}-*b*-PEO_{44}) micelles proceeds by an endocytotic mechanism in PC12 [11] cell cultures.

The different mechanisms of endocytosis may be divided into two broad categories, namely phagocytosis ('cellular eating') and pinocytosis ('cellular drinking') [12]. Pinocytosis includes both fluid phase pinocytosis and adsorptive or receptor-mediated pinocytosis. The many pathways of pinocytosis which

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have been identified include macropinosome, clathrin-coated vesicle, non-coated vesicle and caveolae. Cellular uptake by an endocytotic mechanism is known to be characterized by certain basic criteria including time, temperature, pH and energy dependence [12–15]. Many pharmacological manipulations which enable one to selectively inhibit or stimulate the individual pinocytic pathways have been identified (e.g., hypertonic treatment inhibits clathrin mediated endocytosis) [13]. It is by the exploitation of these pharmacological manipulations that the mechanism of cellular uptake of a particular macromolecule or ligand may be identified [16].

The present work describes the effect of various pharmacological manipulations on the uptake of PCL_{20}-*b*-PEO_{44} micelles. The time, temperature, pH and energy dependence of the uptake are first investigated to determine if the basic criteria for endocytotic uptake are fulfilled. The effect of hypertonic treatment and brefeldin A on the uptake process are also studied. These studies are of importance owing to the growing interest in block copolymer micelles as drug carriers. The lack of information available on micelle–cell interactions, the cellular internalization of micelles and their intracellular fate will prevent them from being fully exploited as drug delivery vehicles.

2. Materials and methods

2.1. Materials

The PCL_{20}-*b*-PEO_{44} (where subscripts refer to the number of monomer units in each block) block copolymer was synthesized by anionic polymerization. The description of the copolymer is provided elsewhere [8]. All chemicals for the synthesis were purchased from Aldrich Chemical Co. The [^{3}H]FK506 was purchased from Mandel Scientific and the fluorescent probe CM-DiI was purchased from Molecular Probes Co. The tissue culture reagents were all purchased from Gibco BRL.

2.2. Micelle preparation

Ten mg of PCL-*b*-PEO block copolymer was dissolved in up to 0.2 g of dimethylformamide and stirred for 4 h. Micellization was induced by dropwise addition of 0.8 g of water at a rate of approximately one drop every 10 s. The micelles were placed in a dialysis bag and dialyzed against MilliQ distilled water. The water was changed every hour for the first 4 h and then every 3 h for the next 12 h.

2.3. Preparation of micelle-incorporated probes (DiI, [^{3}H]FK506)

2.3.1. Micelle-incorporated [^{3}H]FK506

A 100-μl aliquot (amount equal to 3.7×10^6 Bq, i.e., 37 MBq/ml) of a [^{3}H]FK506 solution in ethanol was added to an empty vial and the ethanol was allowed to evaporate. The amount of [^{3}H]FK506 added to the vial was 1.19 nmol since the specific activity of [^{3}H]FK506 is 3108 GBq/mmol. 0.01 g of the PCL_{20}-*b*-PEO_{44} copolymer was then added to the vial along with up to 0.2 g of DMF. The solution was stirred at room temperature for 4 h at which point 0.8 g of water was added slowly to the vial to make a 1% (w/w) copolymer solution. The micelle solution was then left to stir overnight and then dialyzed against MilliQ water. The loading efficiency of the micelle solution was found to be 28%; in this way, the final concentration of the micelle-incorporated [^{3}H]FK506 solution was 333 nM.

2.3.2. Micelle-incorporated CM-DiI

An aliquot of CM-DiI (λ_{ex} = 553 nm) in DMF was initially added to an empty vial such that the final concentration of CM-DiI was 1×10^{-8} mol/g. Ten mg of PCL_{20}-*b*-PEO_{44} copolymer was then added to the vial followed by the addition of DMF up to 0.2 g. The solution was then allowed to stir for 4 h. Micellization was achieved by the dropwise addition of water (0.8 g) and then after stirring overnight the solutions were dialyzed against MilliQ water in the dark.

2.4. Electron microscopic analysis

Several different preparative techniques were tried in order to determine the optimal method for the electron microscopic analysis of this system. However, the freeze-fracture/freeze-etch technique was found to enable the morphology of the individual aggregates to be seen most clearly.

2.4.1. Freeze-fracture/freeze-etch electron microscopy

A small drop of a 1% (wt.) PCL-*b*-PEO micelle solution was placed in the center of a sample holder and dipped into liquid nitrogen-cooled liquid propane (−140°C). After approximately 5 s the sample holder was removed and placed into a liquid nitrogen bath. The sample holder was then placed in the freeze-fracture apparatus (Balzers 300) wherein the temperature ranged between −100 to −120°C. Sample fracture was performed by using a liquid nitrogen-cooled knife. Following freeze-fracture and freeze-etch the fracture plane was shadowed with platinum/carbon (95:5) at a 30° angle and then coated with carbon at a 90° angle. The Pt/C replica was floated off the holder using distilled water and placed onto uncoated copper grids. The sample grids were analyzed by transmission electron microscopy using a JEOL microscope.

2.5. Cell uptake experiments

2.5.1. Cell cultures

PC12 cells [11] were grown in flasks containing RPMI supplemented with 5% fetal bovine serum, 500 μg/ml penicillin and 500 μg/ml streptomycin. The cells were seeded into 24-well plates containing 500 μl per well such that there are approximately 2×10^5 cells/ml. Following a 24-h incubation period the medium was removed and replaced by fresh medium, and treatments were performed as described below.

2.5.2. Study of time-, temperature- and pH dependence

Time dependence: The medium was removed and 245 μl of fresh RPMI 1640 medium was added to each well in the culture plate. A 5-μl aliquot of micelle-incorporated [^{3}H]FK506, micelle-incorporated DiI or [^{3}H]FK506 alone was added to each well at specific time points. The 5-μl aliquot of the micelle-incorporated [^{3}H]FK506 or [^{3}H]FK506 alone corresponds to 6.6 nM [^{3}H]FK506 per well. Following the specific incubation period the supernatant and cells were treated as described below depending on the micelle-incorporated probe used.

Temperature dependence: The cells were incubated at 4°C for 15 min prior to the start of the experiment.

pH Dependence: The RPMI (pH 5) was prepared by the mixture of a specific ratio of RPMI medium and an acetic acid/Na acetate buffer solution.

2.5.2.1. Cells treated with micelle-incorporated [^{3}H]FK506 or [^{3}H]FK506 alone. Following the incubation period, 200 μl of the medium was removed and placed in scintillation vials containing 4 ml of Scinti-Safe Plus 50% scintillation cocktail. The rest of the medium was removed and the cells were washed twice with 200 μl PBS. One hundred μl of trypsin was then added to each well and incubated at 37°C for 15 min. Each well was then scraped, 100 μl of PBS was added and the contents were removed and placed in a scintillation vial containing 4 ml of Scinti-Safe scintillation cocktail. The scintillation vials were counted and the CPM value for each was recorded. A calibration curve was used to change CPM to μCi.

2.5.2.2. Cells treated with micelle-incorporated DiI. Following the incubation period the supernatant was removed and the cells were washed twice with 250 μl PBS. The cells were then analyzed under a fluorescence microscope (Olympus BH-2).

2.5.3. Study of the effect of hypertonic treatment

The cells were preincubated with sucrose (450 mM) containing medium for 30 min or 1 h prior to the addition of 5 μl of micelle-incorporated [^{3}H]FK506. Following a 1-h incubation period the cells and supernatant were treated as above.

2.5.4. Study of the effect of Naz/DOG or brefeldin A

The cells were preincubated with DOG (25 mM)/Naz (10 mM) for 15 min, 30 min or 1 h while pretreatment with Brefeldin A (5 μM) was for 1 h. The addition of 5 μl micelle-incorporated [^{3}H]FK506 was followed by a 1-h incubation period after which the cells were treated as described above.

3. Results and discussion

3.1. Characteristics of the PCL-b-PEO micelles employed in the cellular internalization studies

Fig. 1 (left) is a schematic representation of the block copolymer micelles formed from the PCL_{20}-*b*-

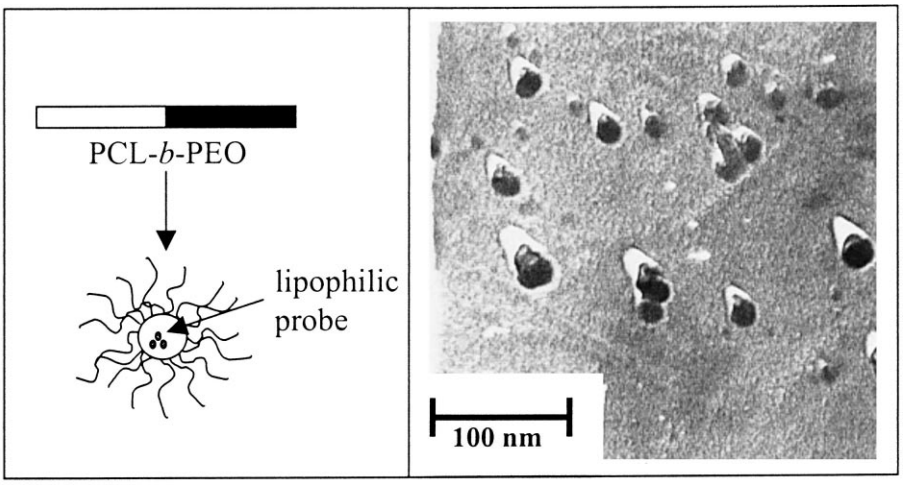

Fig. 1. (Left) A schematic representation of the PCL_{20}-*b*-PEO_{44} copolymer micelle system. (Right) The freeze-fracture/freeze-etch electron micrograph of the PCL_{20}-*b*-PEO_{44} copolymer micelle system.

PEO_{44} copolymers. The block copolymer micelles in these studies are spherical in shape and approximately 25 nm in diameter as shown in the electron micrograph in Fig. 1 (right). The stability of the micelles in RPMI medium and their biocompatibility in PC12 cell cultures were previously confirmed as described elsewhere [8].

The ideal tool for these studies would be labeled block copolymer micelles; however, this was not available and for this reason we chose to use micelles containing highly lipophilic fluorescent or radioactive probes [8]. The high degree of lipophilicity of the probes ensured a high partition coefficient for the probe between the micelles and the external medium. In addition, the kinetics of the release of the selected probes from the micelles is very slow with respect to the duration of the cell uptake experiment (maximum 4 h). In this way, the micelle-incorporated probes may be used to follow the internalization process of the micelles.

3.2. Cellular uptake studies

3.2.1. Time, temperature and pH dependence

This study demonstrates that the micelles are internalized into PC12 cell cultures. Furthermore, these studies demonstrate that the uptake is time-, temperature- and pH-dependent. The cellular uptake of both micelle-incorporated [^{3}H]FK506 and micelle-incorporated DiI were found to be time dependent as the uptake increased progressively over the 4-h incubation period as seen in Fig. 2A,B. The uptake profile of the micelles was similar to that seen for the uptake of other delivery vehicle incorporated probes, such as liposomes containing carboxyfluorescein and microparticles containing 6-coumarin [17,18].

As seen in Fig. 2A,B, a decrease in temperature from 37°C to 4°C, known to be an effective non-invasive means of inhibiting endocytosis, severely inhibits the uptake of the micelles [19]. However, there was a slight progressive increase in the uptake at 4°C over the 4-h period, which is similar to the results obtained for the cellular uptake of fluorescein labeled biodegradable nanoparticles in bovine arterial smooth muscle cells over time at 4°C [20].

The pH dependence of the internalization was also confirmed (Fig. 2A,B). The acidification of the cytoplasm has been shown to inhibit receptor-mediated endocytosis by preventing the budding of coated pits to from coated vesicles [21–25]. Several different methods may be used to induce cytosol acidification; these include prepulsing with NH_4Cl, use of weak acids (e.g., acetic acid) which are able to penetrate the cell membrane, and incubation with nigericin in isotonic KCl [12]. In this case, we chose to induce cytosol acidification by incubating the cells with medium containing acetic acid (pH 5). The uptake of the micelles was not as great at low pH as it was at 4°C.

In addition, the time dependence of the uptake of the micelle-incorporated [^{3}H]FK506 was compared to the uptake of the free drug. As shown in Fig. 3,

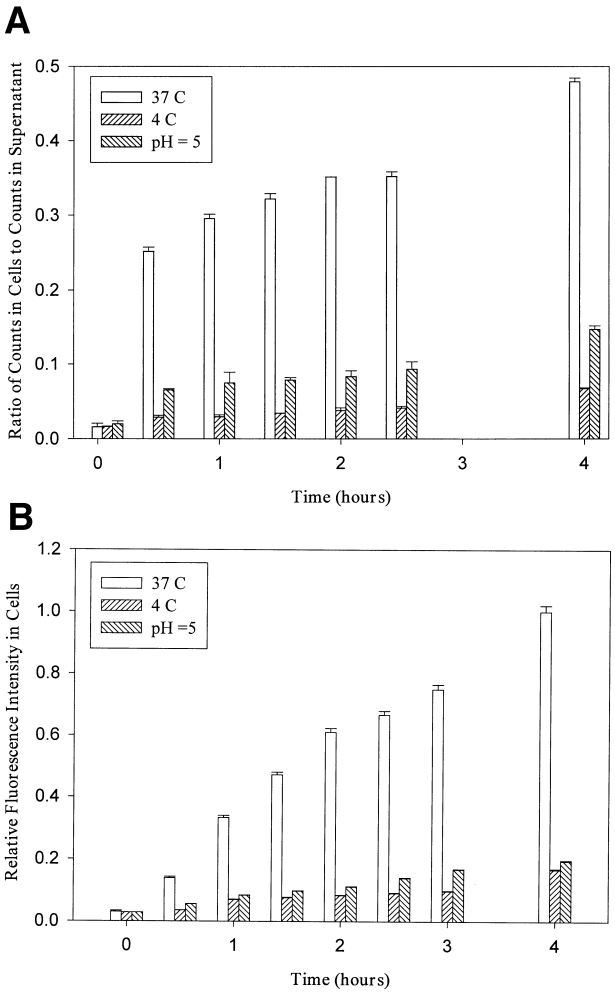

Fig. 2. (A) Temperature and pH dependence of the cellular internalization of PCL_{20}-*b*-PEO_{44} micelle-incorporated [^{3}H]FK506 in PC12 cell cultures. Each bar represents the mean of four samples ± S.D. (B) Temperature and pH dependence of the cellular internalization of PCL_{20}-*b*-PEO_{44} micelle-incorporated DiI in PC12 cell cultures. Each bar represents the mean of four samples ± S.D.

the kinetics of the cellular entry of the drug alone is much faster than that of the micelle-incorporated drug. Also, it appears that much less uptake of the micelle-incorporated drug, compared to the free drug, has occurred over the 4-h period.

3.2.2. *Summary of the effect of the various pharmacological manipulations*

Fig. 4 summarizes the effect of various pharmacological manipulations on the cellular uptake of the micelle incorporated [^{3}H]FK506 in PC12 cell cultures. The energy dependence of the uptake was studied by use of the metabolic poisons sodium azide (NaAz) and 2-deoxyglucose (DOG). The preincubation of the cells with deoxyglucose (DOG) (inhibitor of glycogenolysis) and sodium azide (NaAz) (inhibitor of cellular respiration) for 1 h decreased the uptake to 11%. Preincubation of the cells with DOG/NaAz for 15 or 30 min also decreased the degree of cellular internalization of the micelle-incorporated [^{3}H]FK506 but not to the same extent (results not shown). The inhibition of the uptake by the met-

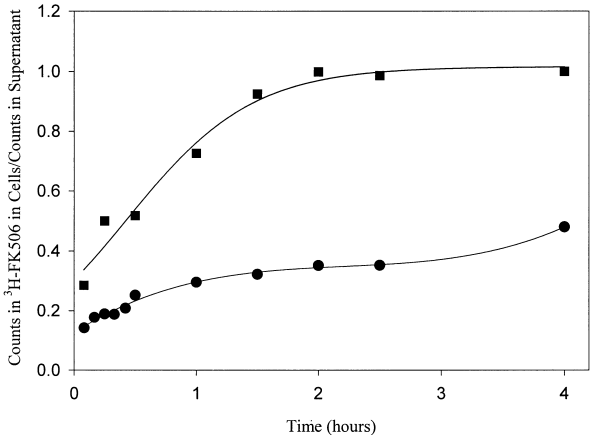

Fig. 3. Time dependence of the uptake of [^{3}H]FK506 alone (■) and PCL_{20}-*b*-PEO_{44} micelle-incorporated [^{3}H]FK506 (●) in PC12 cell cultures over a 4-h period. Each point represents the mean of four samples ± S.D.

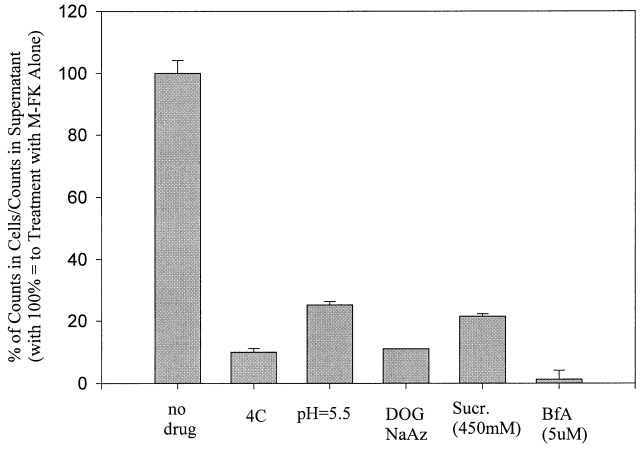

Fig. 4. The effect of various pharmacological manipulations on the cellular internalization of PCL_{20}-*b*-PEO_{44} micelle-incorporated [^{3}H]FK506 in PC12 cell cultures. Each bar represents the mean of four samples ± S.D.

abolic inhibitors confirms that the internalization process is energy dependent.

Hypertonic treatment by preincubation of the cells with 0.45 M sucrose was also found to decrease the uptake. The inhibition of receptor-mediated endocytosis by exposure of the cells to hypertonic media was first demonstrated by Daukas and Zigmond [26]. They found that receptor-mediated endocytosis of chemotactic peptides was inhibited in polymorphonuclear leukocytes in the presence of media containing 0.43 M sucrose. The specific means by which hypertonic treatment inhibits receptor-mediated endocytosis was later established by Heuser et al. [21]. They discovered that hypertonic treatment provokes inhibition in the same manner as does potassium depletion; it reduces the number of clathrin coated pits present at the cell surface. Also, the clathrin-coated pits present were much flatter and smaller than normal and clathrin was found to be accumulated in 'microcages' [22]. The fact that the hypertonic treatment did not completely abolish the uptake of the micelles may be attributed to the use of only 0.45 osmolar medium. Daukas and Zigmond [26] had found that complete inhibition of receptor-mediated endocytosis of chemotactic peptides only occurred in 0.7 osmolar medium while partial inhibition occurred in the presence of 0.45 and 0.6 osmolar medium.

In addition, the pretreatment of the cells with the macrocyclic antibiotic Brefeldin A also inhibited the internalization of the block copolymer micelles. Specifically, Brefeldin A is known to morphologically alter the endosomes by inducing the formation of tubules [15]. The inhibition of cellular uptake of the micelles in the presence of Brefeldin A is indicative that the internalization may proceed by an endocytotic mechanism.

Together, this evidence strongly suggests that the PCL_{20}-*b*-PEO_{44} micelles are internalized into PC12 cells via an endocytotic mechanism. However, in order to support this finding further studies must be done using labeled micelles visible by either confocal or electron microscopy. Also, it is of interest to study the internalization of the micelles at the molecular level in order to determine which proteins are involved in the process.

The question of whether or not the block copolymer micelles are internalized into PC12 cells via an endocytotic mechanism is only the first in a series of questions that remains to be answered including: (1) what is the specific mechanism of endocytotic uptake of the micelles; (2) what intracellular trafficking pathway do they follow upon cell entry; (3) what is their final subcellular distribution or intracellular fate; and (4) are the micelles stable within the cell? The answers to these questions will be of necessity in furthering the use of block copolymer micelles in drug delivery. For instance, the knowledge of the subcellular distribution of the micelles will enable the appropriate drug to be delivered to a particular subcellular organelle, enabling the most efficient use of the micelles as drug carriers.

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References

[1] V.Y. Alakhov, E.Y. Moskaleva, E.V. Batrakova, A.V. Kabanov, Hypersensitization of multidrug resistant human ovarian carcinoma cells by pluronic P85 block copolymer, Bioconj. Chem. 7 (1996) 209–216.

[2] M. Yokoyama, M. Miyauchi, N. Yamada, T. Okano, Y. Sakurai, K. Kataoka, Characterization and anticancer activity of the micelle-forming polymeric anticancer drug adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer, Cancer Res. 50 (1990) 1693–1700.

[3] M. Yokoyama, T. Okano, K. Kataoka, Improved synthesis of adriamycin-conjugated poly(ethylene oxide)-poly(aspartic acid) block copolymer and formation of unimodal micellar structure with controlled amount of physically entrapped adriamycin, J. Control. Release 32 (1994) 269–277.

[4] A. Harada, K. Kataoka, Novel polyion complex micelles entrapping enzyme molecules in the core-preparation of narrowly-distributed micelles from lysozyme and poly(ethylene glycol)-poly(aspartic acid) block copolymer in aqueous medium, Macromolecules 31 (1998) 288–294.

[5] S.A. Hagan, A.G.A. Coombes, M.C. Garnett, S.E. Dunn, M.C. Davies, L. Illum, S.S. Davis, Polylactide-poly(ethylene glycol) copolymers as drug delivery systems. 1. Character-

ization of water dispersible micelle forming systems, Langmuir 12 (1996) 2153–2161.
[6] S. Cammas, T. Matsumoto, T. Okano, Y. Sakurai, K. Kataoka, Design of functional polymeric micelles as site-specific drug vehicles based on poly(α-hydroxy ethylene oxide-co-β-benzyl-L-aspartate) block copolymers, Mat. Sci. Eng. C4 (1997) 241–247.
[7] S. Yeon Kim, I.L. Gyun Shin, Y. Moo Lee, Methoxy poly-(ethylene glycol) and ε-caprolactone amphiphilic block copolymeric micelle containing indomethacin. II. Micelle formation and drug release behaviours, J. Control. Release 51 (1998) 13–23.
[8] C. Allen, Y. Yu, D. Maysinger, A. Eisenberg, Polycaprolactone-*b*-poly(ethylene oxide) block copolymer micelles as a novel drug delivery vehicle for neurotrophic agents FK506 and L-685,818, Bioconj. Chem. 9 (1998) 564–570.
[9] A.V. Kabanov, V.I. Slepnev, L.E. Kuznetsova, E.V. Batrakova, V.Y. Alakhov, N.S. Melik-Nubarov, P.G. Sveshnikov, V.A. Kabanov, Pluronic micelles as a tool for low-molecular compound vector delivery into a cell: effect of *Staphylococcus aureus* enterotoxin B on cell loading with micelle incorporated fluorescent dye, Biochem. Int. 26 (1992) 1035–1042.
[10] D.W. Miller, E.V. Batrakova, T.O. Waltner, V.Y. Alkhov, A.V. Kabanov, Interactions of pluronic block copolymers with brain microvessel endothelial cells: evidence or two potential pathways for drug absorption, Bioconj. Chem. 8 (1997) 649–657.
[11] L.A. Greene, A.S. Tischler, Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor, Proc. Natl. Acad. Sci. USA 73 (1976) 2424–2428.
[12] S. Mukherjee, R.N. Ghosh, F.R. Maxfield, Endocytosis, Physiol. Rev. 77 (1997) 759–803.
[13] C. Lamaze, S.L. Schmid, The emergence of clathrin-independent pinocytic pathways, Curr. Biol. 7 (1995) 573–580.
[14] Z. Mamdouh, M.C. Giocondi, R. Laprade, C. Le Grimellec, Temperature dependence of endocytosis in renal epithelial cells in culture, Biochim. Biophys. Acta 1282 (1996) 171–173.
[15] I. Mellman, Endocytosis and molecular sorting, Annu. Rev. Cell Dev. Biol. 12 (1996) 575–625.
[16] J. Oehlke, E. Krause, B. Wiesner, M. Beyermann, M. Bienert, Extensive cellular uptake into endothelial cells of an amphipathic β-sheet forming peptide, FEBS Lett. 415 (1997) 196–199.
[17] R. Bajoria, S.R. Sooranna, S.F. Contractor, Endocytotic uptake of small unilamellar liposomes by human trophoblast cells in culture, Hum. Reprod. 12 (1997) 1343–1348.
[18] M.P. Desai, V. Labhasetwar, E. Walter, R.J. Levy, G.L. Amidon, The mechanism of uptake of biodegradable microparticles in caco-2 cells is size dependent, Pharm. Res. 14 (1997) 1568–1573.
[19] R.M. Steinman, I.S. Mellman, W.A. Muller, Z.A. Cohn, Endocytosis and the recycling of plasma membrane, J. Cell Biol. 96 (1983) 1–27.
[20] H. Suh, B. Jeong, F. Liu, S. Wan Kim, Cellular uptake study of biodegradable nanoparticles in vascular smooth muscle cells, Pharm. Res. 15 (1998) 1495–1498.
[21] J.E. Heuser, R.G.W. Anderson, Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin coated pit formation, J. Cell Biol. 108 (1989) 389–400.
[22] S.H. Hansen, K. Sandvig, B. van Deurs, Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium and cytosol acidification, J. Cell Biol. 121 (1993) 61–72.
[23] J.E. Heuser, Effects of cytoplasmic acidification on clathrin lattice morphology, J. Cell Biol. 108 (1989) 401–411.
[24] J. Davoust, J. Gruenberg, J.E. Howell, Two threshold values of low pH block endocytosis at different stages, EMBO J. 6 (1987) 3601–3609.
[25] K. Sandvig, S. Olsnes, O.W. Petersen, B. van Deurs, Acidification of the cytosol inhibits endocytosis form coated pits, J. Cell Biol. 105 (1987) 679–689.
[26] G. Daukas, S.H. Zigmond, Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leukocytes, J. Cell Biol. 101 (1985) 1673–1679.