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CELL BIOPHYSICS

The Effect of Polyoxyethylene–Polyoxypropylene Triblock Copolymers on the Loading Degree of Poly-(Lactic-co-Glycolic Acid) Copolymer-Based Microparticles Containing Chlorin e_6 and Ethidium Bromide in Mesenchymal Stem Cells

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Abstract—The effect of different polyoxyethylene–polyoxypropylene triblock copolymers, their concentration, and mode of action on the loading of poly-(lactic-co-glycolic acid) copolymer-based microparticles containing such medicinal agents as radachlorin (chlorin e_6) or ethidium bromide in mesenchymal stem cells was studied. It has been shown that medicinal agents encapsulated inside microparticles affect the loading of these particles in the cytoplasm of mesenchymal stem cells. The number of cells that absorbed the particles with chlorin e_6 is approximately two times lower than that in the experiments with ethidium bromide. It has been shown that pretreatment of microparticles with triblock copolymers is more efficient for loading them in cells compared with simultaneous introduction of triblock copolymers and particles into the culture medium. Treatment of ethidium bromide-containing microparticles with triblock copolymers is not efficient for their loading in mesenchymal stem cells compared to the control. The exception is Pluronic 123; when particles are treated with it at concentrations of 1 and 2%, the loading of particles in cells increases compared to the control by factors of approximately 11 and 5, respectively. For particles with chlorin e_6, their pretreatment with triblock copolymers at a concentration of 4% is most efficient; the loading of the pretreated particles in cells is increased by factors of approximately 3 to 11.

Keywords: mesenchymal stem cells, poly-(lactic-co-glycolic acid) copolymer microparticles, triblock copolymers, adsorption, chlorin e_6, ethidium bromide

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Targeted drug delivery using mesenchymal stem cells (MSCs) actively migrating into pathologically altered, rapidly proliferating tissue is a promising area of research in the treatment of various diseases [1]. One of the problems of this technology is the insufficient amount of medicinal agents transported by stem cells and the creation of a high concentration of the agent in the pathological tissue to achieve a therapeutic effect. Various drug nanocontainers loaded in MSCs are used to solve this problem [2, 3].

The use of various microparticles is considered as one of the most efficient methods of drug delivery because of their pharmacokinetic properties, as well as the possibility of conjugating the drug on its surface or enclosing it in its structure followed by introduction into the MSC cytoplasm [4].

The interaction of such particles with biological systems depends on their chemical composition, size, surface charge, and physicochemical properties (hydrophilicity/hydrophobicity) of the surface. These factors determine the rate of biodegradation of particles, their ability to penetrate into the cell cytoplasm while maintaining its viability. All this will ultimately determine the therapeutic efficacy of drug delivery by stem cells to the pathological focus [5].

Abbreviations: EtBr, ethidium bromide; GFP cells, mesenchymal stem cells derived from the bone marrow of B10 mice with the green fluorescent protein gene; MSCs, mesenchymal stem cells; PEO, polyethylene oxide; PLGA, poly-(lactic-co-glycolic acid); PPO, polypropylene oxide; TBCs, triblock copolymers.

Particles based on poly-(lactic-co-glycolic acid) (PLGA) copolymers are among the most efficient for biomedical purposes. They are characterized by low toxicity and good biological compatibility with tissues of living organisms; their properties and the rate of their destruction can be regulated by the content of crystalline phase and the ratio of monomer units in PLGA [6, 7].

However, the hydrophobic properties of PLGA microparticles prevent their interaction with the cell membrane and thereby reduce the amount of pharmaceutical agents that penetrate the cell [8–10]. To change the hydrophobic properties of materials, the ethylene oxide and propylene oxide triblock copolymers (Pluronics), which are known as agents that promote the penetration of drugs through biological barriers, are widely used. Due to the successful combination of their physicochemical and physiological characteristics, Pluronics are widely used in medicine and pharmacology. In particular, they act as stabilizers of perfluorocarbon emulsions [11] and are used in the technology of low-temperature preservation of organs and tissues [12], as well as adjuvants in immunotherapy [13, 14].

In the literature it has been suggested that the effects caused by Pluronics are due, on the one hand, to their interaction with cell membranes and the possible effect of these copolymers on the structural characteristics of the membrane [15].

On the other hand, treatment of hydrophobic particles with Pluronics leads to a change in their physicochemical characteristics [16, 17]. Thus, it is known that mixing PLGA polymer with small amounts (0.5–2.0% wt/vol) of Pluronic® F-108 (PF-108) can significantly improve the surface hydrophilicity of the PLGA micro- and nanofiber networks while maintaining the bulk (thermal and mechanical) properties of PLGA [18].

The purpose of this work was to study the effect of Pluronics of various physicochemical structures (hydrophobicity–hydrophilicity) on the ability of PLGA microparticles with encapsulated medicinal substances, radachlorin, or ethidium bromide (EtBr) to penetrate the MSC cytoplasm while maintaining their viability.

MATERIALS AND METHODS

Triblock copolymers. In this paper, we used nonionic surfactants, namely, triblock copolymers (TBCs) of polyethylene oxide (PEO) and polypropylene oxide (PPO) such as Proxanol 268 (NIOPIK, Russia), Pluronic 10R5 and Pluronic 123 (Fluka Chemistry, Germany), Pluronic F-68 (Fluka Analytical, Germany), Pluronic L-31 and Pluronic L-35 (Aldrich, United States). The molecules of all these surfactants (with the exception of Pluronic 10R5) have a PEO–PPO–PEO structure, i.e., a hydrophobic block forms the central part of the molecule. Pluronic 10R5 has an "inverted" PPO–PEO–PPO structure with a hydrophilic block in the center and PPO terminal blocks.

Preparation of microparticles. Microparticles based on PLGA RESOMER PG 653H (Evonik, Germany) with a 65 : 35 block ratio and containing radachlorin (chlorin e_6, RADA-FARMA, Russia) or ethidium bromide (Serva, Germany) were obtained by the multiple emulsion method ((water in oil) in water) with the removal of the solvent [19].

Microparticles with an average diameter of 400–500 nm were used in the experiments. The calculated radachlorin content was 8 μg/mg PLGA; that of EtBr was 10 μg/mg PLGA.

Obtaining mesenchymal stem cells. We used MSCs obtained from the bone marrow of B10 mice with the green fluorescent protein (GFP) gene (Nursery of the Center of Biomedical Technology, Svetlye Gory, Krasnogorsk district, Moscow region). Cells were cultured in DMEM supplemented with 10% fetal calf serum. The ability of cells to differentiate in the chondrogenic, adipogenic, and osteogenic directions was tested using the standard method [20].

Particle treatment with Pluronics. Before adding to the cells, the particles were incubated with surfactants for 10 min and then precipitated by centrifuging (10 min, 4500 rpm). The precipitate was washed twice, resuspended in phosphate buffered saline, and added to the wells to a final concentration of 75×10^6 particles/mL per well. The particles were incubated with cells for 24 h.

Incubation of cells with particles in the presence of Pluronics. Before introducing the untreated particles into the well with the cells, TBCs were added into the culture medium to a final concentration of 0.5, 1, and 4%. A suspension of particles was then added into the wells to a final concentration of 75×10^6 particles/mL per well. The particles were incubated with the cells for 24 h. The culture medium was then removed, the well was washed five times with phosphate buffered saline (1.5 mL), and 0.5 mL of trypsin solution with 0.25% EDTA was added to the well. The resulting cell suspension was collected for the study.

Flow cytofluorometry. Due to the fluorescence of cells and particles in two different light ranges (green and red), the efficiency of the developed method was evaluated using flow cytofluorometry by the number of viable cells that "absorb" the particles. After each measurement, the location and borders of the regions in the scatter plots were corrected: we set the regions (gates) of GFP cells and a part of the PLGA in the side scatter indicator versus forward side scatter indicator graph as the regions of singlet GFP cells and the regions of cells and particles that fluoresce in the required range. As an example, Figs. 1 and 2 show the results of flow cytofluorimetry of the number of cells that "absorb" particles with chlorin e_6 and EtBr,

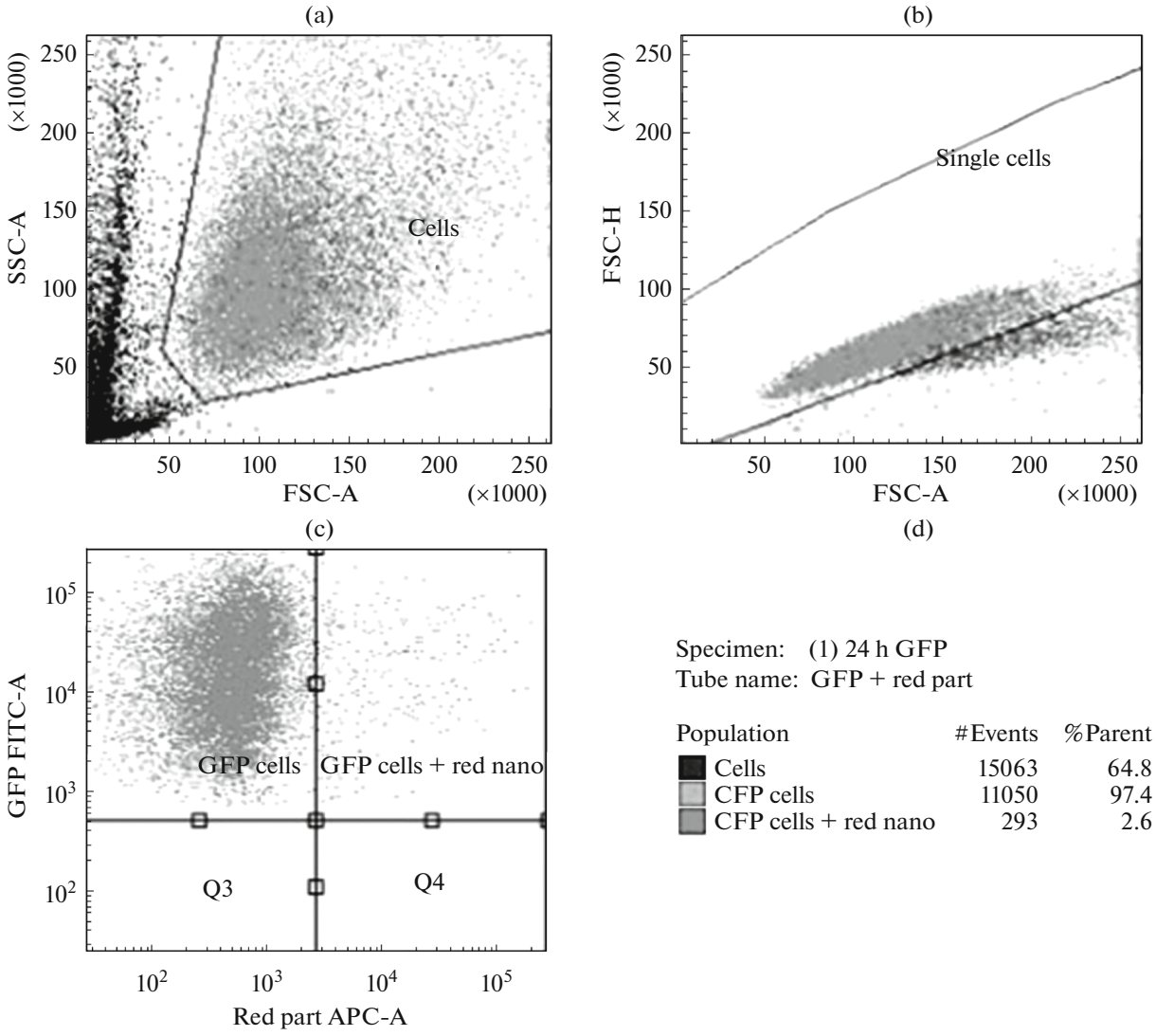

Fig. 1. The results of flow cytofluorimetry in the control group with PLGA particles containing chlorin e_6 (without surfactant): (a) population of GFP cells in forward and side light scattering; (b) gate of GFP cells; (c) gate in FITC and APC channels (upper right corner); (d) quantitative characteristics of the data.

respectively, during a 24-h incubation under control conditions (without treatment with surfactant).

Zeta potential measurement. A Zetasizer nano ZS device (Malvern, United Kingdom) was used to measure the zeta potential. The sample was measured in a DTS 1060 cuvette under the following conditions: the temperature was 25.0°C; the balancing temperature before measuring was 120 s; the number of scans to be averaged for one measurement of zeta potential was 12; the number of repetitions was at least three (in the case of obvious emissions, additional measurements were carried out); the voltage on the electrodes of the cell was 70 V; the laser power was set at its maximum (2 mW); and the wavelength was 633 nm. The Smoluchowski model was chosen for the Henry function (the Henry function was equal to 1.5); the Monomodal model was used for the analysis of the zeta potential (in the standard software).

RESULTS AND DISCUSSION

Triblock copolymers were used for the treatment of particles loaded in stem cells; the physicochemical characteristics of TBCs are presented in Table 1.

All the TBCs differ in molecular weight, hydrophobicity (the weight fraction of PPO blocks in a molecule), the PEO : PPO ratio, the critical micelle concentration, water solubility, and toxicity.

Particles containing chlorin e_6 or EtBr were incubated with cells for 4 and 24 h; we determined the percentage of cells in whose cytoplasm particles were

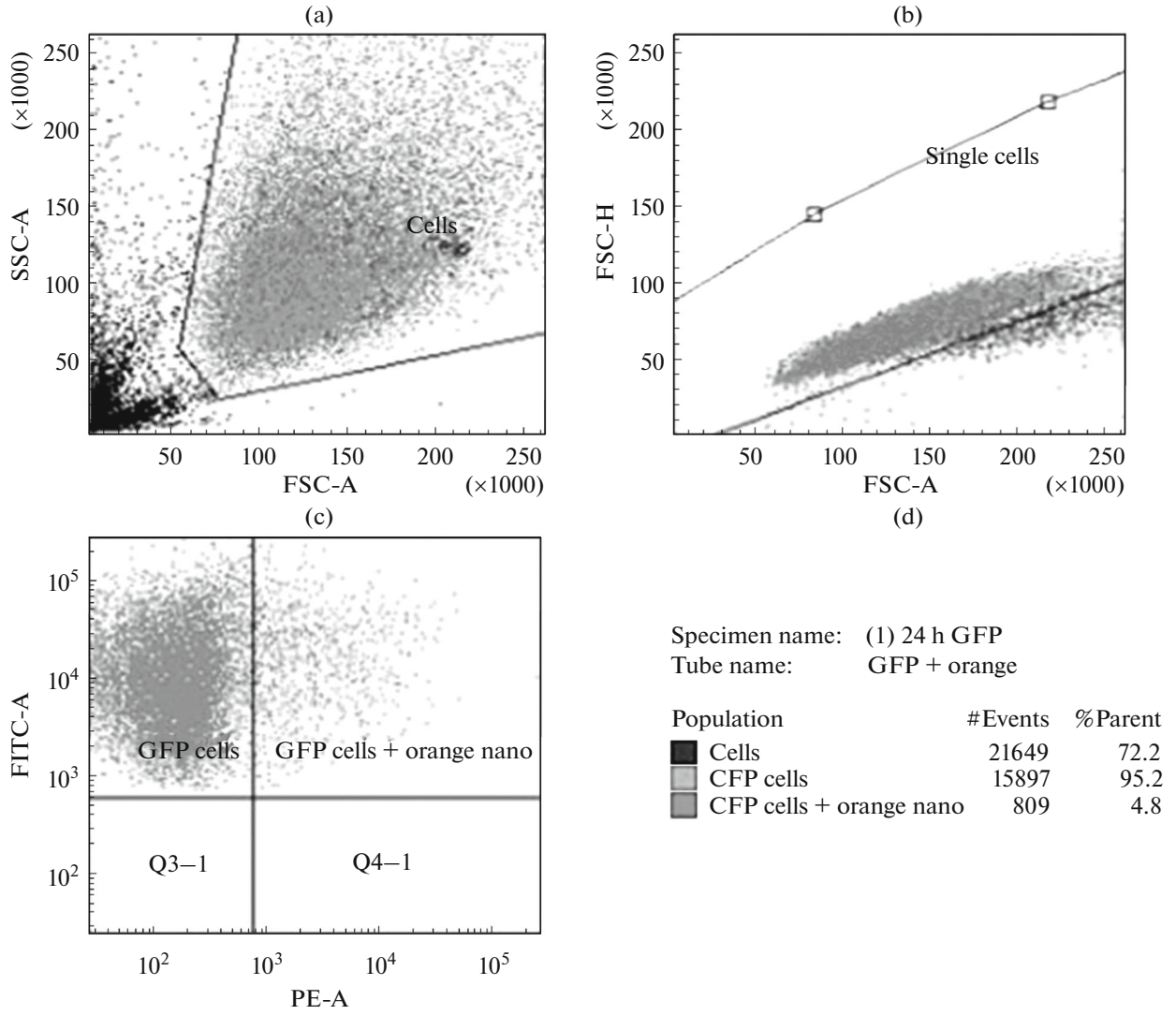

Fig. 2. The results of flow cytofluorimetry in the control group with PLGA particles containing EtBr (without surfactant): (a) population of GFP cells in forward and side light scattering; (b) gate of GFP cells; (c) gate in FITC and APC channels (upper right corner); (d) quantitative characteristics of the data.

detected (the control). The data are presented in Table 2.

It should be noted that when cells are incubated with particles containing chlorin e_6 or EtBr without TBCs for 24 h (control), the number of particle-loaded cells differs by approximately two times; for radachlorin, the loading is approximately 2.6% of the total number of cells; for EtBr, it is approximately 4.6%. Since a polymer of lactic and glycolic acids is the basis of all microparticles, medicinal substances encapsulated in particles can affect differences in the loading of microparticles into cells; they may alter the physicochemical properties of the particle (surface charge, hydrophobicity level, etc.).

To increase the load of stem cells with microparticles, the latter were subjected to treatment with the above TBCs. In the first series of experiments, we examined the loading of particles containing chlorin e_6 or EtBr into stem cells in the presence of TBCs added to the culture medium at various concentrations (0.5, 1, and 4%). The data are presented in Table 3.

During a 24-h incubation at TBC concentrations of 0.5, 1, and 4%, the number of cells containing particles with chlorin e_6 increased proportionally to the concentration. At concentrations of 0.5 and 1% for all TBCs, the number of cells containing particles was below that in the control. The block copolymer L-31 at concentrations of 0.5 and 1% inhibited the absorption of particles by the cells; at a concentration of 4% it was toxic to cells. At a TBC concentration of 4%, the number of cells containing particles was higher than the control, with the exception of Pluronic 268 and Pluronic F-68. Thus, for P-123, it was approximately 15%, which is more than six times the control level; for

Table 1. The physicochemical characteristics of triblock copolymers

TBC	Molecular weight	The number of monomer blocks in the PEO–PPO–PEO structure	Weight fraction of PPO monomer blocks in the molecule, %	Critical concentration of micelle formation at 25°C, wt/vol %	PEO : PPO block ratio in the molecule	Solubility in water at 25°C	Cytotoxicity***
Pluronic L-31	1100	2–16–2	90	–	0.25	Limited	Toxic
Pluronic 123	5800	20–70–20	70	0.03**	0.57	Good	Low toxic
Pluronic F68	8400	78–30–78	20	0.03**	5.20	Good	Nontoxic
Pluronic L-35	1900	9–14–9	50	–	1.2	Limited	Low toxic
Pluronic 268	13000	130–38–130	20	0.02**	6.84	Good	Nontoxic
Pluronic 10R5*	2000	8–22–8	50	–	0.73	Good	Nontoxic

* Structural formula PPO–PEO–PPO;
** data taken from [24];
*** measurements were made using the generally accepted method; the data were not published.

Table 2. The percentage of the cells loaded with PLGA particles with encapsulated medicinal substances

Indicators	Medicinal substances			
	chlorin e_6		EtBr	
Incubation time	4 h	24 h	4 h	24 h
% of cells containing particles	0.64 ± 0.18	2.34 ± 0.27	0.38 ± 0.16	4.62 ± 0.81

Here and in the following tables, the mean values ± standard deviations are presented.

Table 3. The percentage of cells containing particles with chlorin e_6 or EtBr after 24 h of incubation in the presence of TBCs at various concentrations

TBC	Medicinal substances					
	chlorin e_6			EtBr		
	TBC concentration					
	0.5%	1%	4%	0.5%	1%	4%
Pluronic 268	0.20 ± 0.20	0.47 ± 0.12	1.03 ± 0.25	2.90 ± 0.36	2.03 ± 0.25	1.37 ± 0.32
Pluronic 10R5	0.30 ± 0.20	0.07 ± 0.06	3.23 ± 0.25	2.87 ± 0.23	3.10 ± 0.20	0.50 ± 0.20
Pluronic L-31	0	0	Cell death	0	0.17 ± 0.29	Cell death
Pluronic L-35	0.53 ± 0.25	0.10 ± 0.10	6.57 ± 0.40	3.60 ± 1.31	2.73 ± 0.38	1.07 ± 0.21
Pluronic 123	0.93 ± 0.15	3.17 ± 0.46	15.4 ± 0.62	1.97 ± 0.35	1.73 ± 0.42	3.83 ± 0.23
Pluronic F-68	0.3 ± 0.2	0.07 ± 0.11	1.1 ± 0.2	2.43 ± 0.25	2.10 ± 0.17	1.47 ± 0.25

L-35 and 10R5 it was 6.6 and 3.2%, respectively, which is higher than the control values by factors of more than 2.8 and 1.4.

A correlation between the size of the hydrophobic polypropylene block and the number of cells containing particles is observed. Thus, at the 4% concentration of Pluronic P-123, L-35, and 10R5, with the use of which the cell loading with particles increased; the hydrophobic block in the molecule was approximately 70, 51, and 49%, respectively. When particles are treated with TBCs with a hydrophobic block constituting approximately 20% (Pluronic F-68 and Proxanol 268), the number of cells containing particles was below the control values. Thus, with this experimental design, the direct dependence of the number of particles absorbed by the cells on the TBC concentration is observed. The optimal concentration was 4%. However, when TBCs are added to the culture medium, the loading of microparticles into the cells is as a whole not efficient.

Apparently, when cells are incubated with particles in the presence of TBCs, they affect, on the one hand, the surface hydrophobicity of the particles; in this case, their chemical nature (the ratio of hydrophobic/hydrophilic units in the molecule) plays a significant role. Thus, it is known that extremely hydrophilic or hydrophobic surfaces violate the process of attaching cells to a solid surface [4] and treatment of the PLGA microparticles with TBCs can affect the surface wettability and the attachment of particles to the cell surface and thus their penetration into the cytoplasm [17].

On the other hand, TBCs can influence the state of the cell membrane, changing its microviscosity. In the performed experiment, the loading process appears to be the result of two components, namely, changes in the surface characteristics of the particles and changes in the microviscosity of the cell membrane [21].

When cells were incubated with the EtBr-containing particles in the presence of TBCs at the used concentrations for 24 h, as a rule, a decrease in the number of cells containing particles relative to the control was observed. Moreover, an inverse dependence of the number of cells containing particles on the TBC concentration was observed when the smallest number of cells containing particles is observed at a concentration of 4%, both relative to the control and relative to concentrations of 0.5 and 1%. Thus, the addition of various TBCs to the culture medium of the cells with EtBr-loaded particles in most cases inhibits the penetration of particles into the cytoplasm.

In the second series of experiments we incubated particles containing chlorin e_6 or EtBr with the above triblock copolymers (at concentrations of 1, 2, and 4%) for 10 min. After precipitation and washing the particles twice with phosphate buffer they were then incubated with the cells for 24 h. The number of cells containing the particles is presented in Table 4.

When particles containing chlorin e_6 were pretreated with triblock copolymers, we observed an

Table 4. The percentage of cells containing particles with chlorin e_6 or EtBr after preliminary treatment with TBCs followed by incubation for 24 h

TBC	Medicinal substances					
	chlorin e_6			EtBr		
	TBC concentration					
	1%	2%	4%	1%	2%	4%
Pluronic 268	2.67 ± 0.83	3.67 ± 0.31	9.53 ± 0.60	5.07 ± 0.21	5.2 ± 0.3	6.8 ± 0.2
Pluronic 10R5	1.60 ± 0.30	3.1 ± 0.17	9.87 ± 0.35	3.53 ± 0.23	5.8 ± 0.3	2.9 ± 0.2
Pluronic L-31	0.27 ± 0.25	4.37 ± 0.35	14.10 ± 4.39	6.33 ± 0.40	7.03 ± 0.25	3.37 ± 0.06
Pluronic L-35	1.87 ± 0.15	4.27 ± 0.38	11.0 ± 0.1	0.1 ± 0.1	0.33 ± 0.31	4.63 ± 0.25
Pluronic 123	4.97 ± 0.15	6.47 ± 0.45	25.07 ± 1.07	55.03 ± 4.53	23.2 ± 0.35	7.37 ± 0.31
Pluronic F-68	1.90 ± 0.10	2.9 ± 0.17	8.07 ± 0.21	7.03 ± 0.32	8.07 ± 0.35	5.87 ± 0.35

increase in the number of cells containing particles. The number of cells containing particles increases proportionally to the TBC concentration used for the treatment of particles. However, when a concentration of 1% was used, the number of cells containing particles was lower than in the control. The only exceptions were particles treated with Pluronic 123; these were loaded into the cells at level approximately two times higher than the control. The greatest number of cells containing particles was observed when the particles were treated with 4% TBC solutions. At this concentration, all TBCs increase the loading of particles into the cells above the control from approximately 3.4 to 11 times. As in the first series of experiments, Pluronic 123, L-31, L-35, and 10R5 were the most efficient; when these TBCs were used, the particle loading increased by a factor of 10.7, 6.0, 4.7, and 4.2, respectively. It should be noted that during treatment with Pluronic 268 and F68, the particle loading also significantly increased relative to the control, by factors of 4.1 and 3.4, respectively.

It was proved that when manufacturing PLGA microfiber meshes (pure and mixed with Pluronic PF-108 at concentrations of 0.5 and 2%), samples with PF-108 were more hydrophilic, which was further confirmed by analyzing the fiber surface using X-ray photoelectron spectroscopy [22]. It has been shown that ethylene oxide component of PF-108 is on the fiber surface, whereas the propylene oxide component of PF-108 remains embedded in the PLGA microfiber. At lower concentrations of PF-108, the ethylene oxide conformation on the surface of the fibers has the shape of a mushroom; at higher concentrations of PF-108, it has a rod-shaped form. Thus, depending on the TBC concentration, the structure of the surface layer on the particle will be different, which affects the hydrophobicity–hydrophilicity of the surface. This may affect the penetration of particles into the cell.

The loading of EtBr-containing particles pretreated with the indicated TBCs (Table 4) into the cells either slightly increases relative to the control or remains below control values (with the exception of Pluronic L-123). There is no correlation between the TBC concentration and the number of cells that contain particles. Thus, for Pluronic L-31, Pluronic L-123, and Pluronic F-68, the number of cells that contain particles decreases with an increasing concentration of TBC. Even in the case of Pluronic L-123, the greatest loading was observed at a concentration of 1%, approximately 12 times higher relative to the control; at a concentration of 2%, it was approximately five times higher; and at a concentration of 4%, the loading increased by a factor of only 1.7 relative to the control.

Along with the hydrophobic–hydrophilic properties of the surface, the surface charge of the particles is a very important characteristic. There are experimental data with TBC-stabilized microparticles; they consist in the fact that the greater the absolute negative value of the ζ-potential of the microparticles is, the thinner the surface layer of TBC is on the microparticle [23].

When measuring the charge (zeta potential) of the control particles, the absolute value of the negative charge of chlorin e_6-containing particles significantly exceeds the same indicator of EtBr-containing particles (see Table 5). It should be noted that when chlorin e_6-containing particles were treated with TBCs, the surface negative charge in absolute values either exceeded the charge of the untreated particles or remained at the control level. As the TBC concentration increased to 4%, the absolute values of the zeta potential decreased when all particles were treated

Table 5. The zeta potential (mV) of particles after treatment with TBCs

TBC	Chlorin e_6			EtBr		
	control	1% TBC	4% TBC	control	1% TBC	4% TBC
Pluronic 268		−38.7 ± 1.7	−22.9 ± 6.1		−19.0 ± 0.6	−16.7 ± 0.7
Pluronic 10R5		−24.7 ± 2.4	−13.0 ± 4.2		–	–
Pluronic L-31	−23.2 ± 1.0	−19.0 ± 2.5	−18.6 ± 3.0	−15.7 ± 3.2	−10.8 ± 1.0	−16.4 ± 1.0
Pluronic L-35		−28.0 ± 1.2	−18.8 ± 4.1		−17.3 ± 1.5	−16.1 ± 1.1
Pluronic 123		−32.7 ± 1.5	−33.3 ± 1.2		−18.8 ± 0.8	−16.1 ± 1.1

with TBCs, with the exception of Pluronic 123, which indirectly indicates an increase in the thickness of the adsorption layer on the particle surface. At the same time, the number of cells containing particles increased at a TBC concentration of 4%.

After treatment with TBCs at concentrations of 1 and 4%, both the surface charge of the EtBr-containing particles and the number of cells that absorbed particles were not changed and remained almost at the level of the control. Apparently, encapsulated EtBr prevents TBC adsorption on the surface of the particles, as a result of which the surface properties of the particles were not changed and no significant changes in their uptake by the cells were observed.

CONCLUSIONS

(1) Medicinal substances encapsulated inside PLGA particles affect their loading in the cell cytoplasm in the control. When the PLGA-based particles with encapsulated chlorin e_6 are loaded, the number of cells is approximately two times lower than when the particles with EtBr are loaded.

(2) Pretreatment of microparticles with TBCs and their introduction into a culture medium are more efficient for their absorption by the cells compared with the simultaneous addition of a surfactant and particles to the culture medium.

(3) Treatment of the EtBr-containing particles with TBCs at concentrations of 1, 2, and 4% does not increase the loading of particles into the cells, or this increase is insignificant relative to the control. Pluronic 123 is an exception; when particles are pretreated with it at a concentration of 1%, the loading into the cells increases by approximately 11 times.

(4) Treatment of the chlorin e_6-containing particles with TBCs at concentrations of 2 and 4% increases the number of cells containing particles. As in the case of EtBr-containing particles, Pluronic 123 is more efficient; when particles are pretreated with it at a concentration of 4%, the loading into the cells increases by approximately 11 times compared to the control.

(5) When particles are treated with TBCs, the magnitude of the charge (zeta potential) of particles with chlorin e_6 depends on the TBC concentration; at a concentration of 2%, the absolute negative value is above the control values; at a concentration of 4%, the absolute value of the charge decreases to the control values or lower, which indirectly indicates a change in the thickness of the TBC adsorption layer on particles.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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