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Conjugation of succinic acid to non-ionogenic amphiphilic polymers modulates their interaction with cell plasma membrane and reduces cytotoxic activity

O.V. Bondar, A.V. Sagitova, Y.V. Badeev, Y.G. Shtyrlin, T.I. Abdullin*

Kazan (Volga Region) Federal University, 18 Kremlyovskaya Street, 420008 Kazan, Russia

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ABSTRACT

Pluronic block copolymers L61 and L121 were reacted with succinic anhydride to produce, respectively, their mono- and bisderivatives with succinic acid. The critical micelle concentration of Pluronics decreased after modification. The modification of Pluronic L61 promoted its association with the plasma membrane of human cells and increased membrane damage, while the membranotropic activity of modified Pluronic L121 reduced compared to the initial copolymer. Modified Pluronics interfered with the viability, apoptosis induction and metabolism of A549 cells and skin fibroblasts to a much lesser extent presumably due to the introduction of succinic acid residue inhibited intracellular penetration of copolymers. Modified Pluronic L121 promoted the cellular uptake of doxorubicin and rhodamine 123 in A549 cells attributed to the inhibition of membrane P-glycoprotein. Our study provides an approach to assessing the mechanism of interaction of amphiphilic polymers with living cells and demonstrates that Pluronic–succinic acid conjugates can be used as safe and efficient modulators of intracellular drug delivery.

1. Introduction

To date, biomedical science has generated a vast number of active substances in the treatment of a variety of human diseases. In clinical trials the biological safety, and/or efficiency of the substances developed was often not proven [1]. One of the main reasons is that exogenous substances naturally possess a limited ability to pass through the body's barriers, resulting in their insufficient therapeutic concentration at organ-tissue/cellular levels. The use of adjuvant compounds to improve pharmacokinetics and facilitate intracellular delivery of existing therapeutics is becoming a topical approach in drug development [2,3].

Both natural and synthetic polymers provide an efficient tool in promoting the delivery of drugs to their cellular targets. Such polymers are generally designed as inert carriers that allow: increasing circulation time, a stability of administered drugs and the improvement of their biodistribution [3,4]. In particular, covalent conjugates of cytostatic agents with N-(2-hydroxypropyl) methacrylamide and polyglutamic acid have been introduced to suppress tumor growth [5]. Modification of peptide and oligonucleotide-based therapeutics with polyethylene glycol (PEG) has become a common route to improving their biological properties [6,7].

Another concept is that some synthetic polymers can be used as efficient modulators of biological response at cellular and molecular level rather than as inert drug carriers [8,9]. A promising class of such biologically active polymers is non-ionogenic amphiphilic polyethers, specifically tri-block copolymers of ethylene oxide (EO) and propylene oxide (PO) with the general formula $(EO)_x$–$(PO)_y$–$(EO)_x$ (Pluronics™). Depending on the hydrophilic–lipophilic balance (HLB) and molecular weight (MW), these polyethers exhibit different biological effects. Pluronics of low and medium HLB, e.g. Pluronics L61 and P85, were shown to substantially facilitate the accumulation of fluorophores and small drug molecules in human cells that overproduce membrane efflux transporters, e.g. multidrug resistance protein 1 (P-glycoprotein) [8–10]. High expression of P-glycoprotein in multidrug-resistant (MDR) cancer cells and brain capillary endothelial cells leads to the rapid exclusion of many xenobiotics including cytotoxic drugs taken into the cell [8,11].

Possible ways of Pluronic-medicated intracellular drug delivery include: facilitating the passive diffusion of a substance across the cell plasma membrane, the promotion of lysosomal release, and the inhibition of P-glycoprotein ATPase activity by altering the membrane fluidity and/or the suppression of adenosine-5′-triphosphate (ATP) production in mitochondria [8,9]. The inhibition of the efflux transporter, such as P-glycoprotein is believed to especially

* Corresponding author. Tel.: +7 953 4980847.
E-mail address: tabdulli@gmail.com (T.I. Abdullin).

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contribute to the enhancement of drug delivery in the presence of Pluronics [12–14]. Such an activity is of particular interest in the development of Pluronic–cytotoxic agent compositions to treat tumors that are resistant to conventional chemotherapy.

A formulation of doxorubicin with mixed micelles of Pluronics L61 and F127 (SP1049C) has recently been put forward. According to phase I and phase II studies, SP1049C was reported to increase a doxorubicin half-life and its anti-tumor activity in some patients with advanced, resistant solid tumors, whilst at the same time showing an acceptable safety profile [15,16]. In spite of this encouraging data, there is a reasonable concern about the possible adverse effects of surfactant Pluronics, including relatively toxic Pluronic L61, which could impair cellular membranes, interfere with metabolism, and hence have serious side effects.

A promising approach to improving the biological characteristics of Pluronics is modifying them with organic substances. Li et al. described a method of activating terminal hydroxyl groups in Pluronics by introducing relatively stable p-nitrophenyl groups. Activated Pluronics are readily conjugated to amino-containing molecules, including 2-pyridyldithioethylamine as a linker for thiol-containing molecules [17]. Mild oxidation of hydroxyl groups of Pluronic L121 by Dess–Martin periodinane was carried out so as to synthesize the dialdehyde derivative of polymer. Cross-linking of oxidized Pluronic with diamines was shown to promote the encapsulation of model drug into polymeric micelles and its delivery into MDR cells [18]. The activation of Pluronic P85 in the reaction with succinic anhydride by Custers et al. produced corresponding monoesters of succinic acid. The applicability of the modified Pluronic P85 to bind divalent ions was demonstrated [19]. In other reports, N,N′-disuccinimidyl carbonate-activated Pluronics F127 and F68 were coupled with 3,4-dihydroxyphenylalanine (DOPA) or its methyl ester [20], Pluronic F68 and P105 were directly labeled with fluorescein derivative [21]; Pluronic F-127 was conjugated with stearic acid to improve the encapsulation of doxorubicin into polymeric micelles [22].

While the above studies generally deal with surface chemistry or drug release application of modified Pluronics, an essential issue is how do Pluronic conjugates directly affect the activity of living cells? We hypothesized that block copolymers of EO and PO can be conjugated with succinic acid (SA) to modulate their interaction with cellular membrane interfaces. We perform a comprehensive study of the interaction of SA-modified amphiphilic polymers with plasma membrane of mammalian cells as well as their effects on basic cellular functions including transmembrane transport.

2. Materials and methods

2.1. Reagents

Two poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) block copolymers, the structural equivalents of Pluronic® L61 and Pluronic® L121 (BASF), were purchased from Aldrich. These block copolymers, further denoted as Pluronics L61 and L121, have number average molecular weight (Mn) of 2000 and 4400, respectively. The number of EO/PO units in the copolymers is 4.6/31.0 for Pluronic L61 and 10.0/68.3 for Pluronic L121 [9]. The copolymers were used without additional purification.

We used rhodamine 123, Hoechst 33342, propidium iodide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – MTT (Sigma–Aldrich); BODIPY® FL–verapamil hydrochloride conjugate (Invitrogen); Annexin V-Cy3 apoptosis probe (Biovision); doxorubicin hydrochloride and verapamil hydrochloride (Ferane, Russia). Succinic anhydride, salts and solvents were produced by Acros Organics. Cell culture reagents were purchased from Paneco (Russia).

2.2. Derivatization of Pluronics with SA

Terminal hydroxyl groups in L61 and L121 were modified in the reaction with succinic anhydride to produce SA-derivatives (Fig. S.1). Briefly, 10 g of L61 (L121) dissolved in 50 mL toluene were treated with succinic anhydride (2.9 g) for 2 h at 90 °C and then for 2 h at 100 °C. The solvent was evaporated and the residue was dissolved in water followed by pH adjustment to pH > 9 (sodium carbonate) and then to pH < 2 (diluted sulfuric acid). The product was extracted with n-butanol (2 × 180 mL). The extract was exsiccated under water-free sodium sulfate and additionally extracted into chloroform (3 × 100 mL). The resulting extract was carefully dried in vacuum.

Both synthesized products were yellow oily substances; the yield was 86% (L61) and 94% (L121). Modified L61 (M-L61) contained 1 SA residue (1 carboxylic group), whereas modified L121 (M-121) contained 2 SA residues (2 carboxylic groups) as determined by acid–base titration.

The structure of modified Pluronics was verified by nuclear magnetic resonance (NMR) spectroscopy (Varian Unity-300 NMR Spectrometer) and Fourier transform infrared spectroscopy (FT-IR) spectroscopy (Vector 22, Bruker). ^{13}C NMR ($CDCl_3$, 75 MHz) [ppm]: δ 17.25 (CH_3), 70.34 (CH_2), 73.14 (CH_2), 75.14 (CH), 172.25 (C(O)OH). FT-IR spectra of modified Pluronic were characterized by the appearance of vibrational band at 1750 cm^{-1} indicating the presence of carboxylic group (Fig. S.2).

2.3. Analysis of micelle formation

Critical micelle concentration (CMC) was determined with the use of pyrene probe as described in [23] with some modifications. Briefly, 20 μL aliquots of 1.25×10^{-5} M pyrene in methanol were cast into 96 well optical bottom plate and dried under ambient atmosphere. 200 μL aliquot of Pluronic solutions in phosphate buffered saline (PBS) with pH 7.4 at different copolymer concentrations were pipetted into the wells pre-covered with pyrene film and incubated for 1 h at 37 °C under moderate agitation to allow dissolution of pyrene and its redistribution into hydrophobic phase of polymeric micelles [24].

The emission spectra of pyrene in Pluronic solutions were registered at RT on an Infinite 200 PRO microplate analyzer (Tecan) in 365–410 nm wavelength range (λ_{ex} 339 nm) (Fig. S.3). To calculate CMC, the relationship between fluorescence intensity at λ_{max} 373 nm and the logarithm of copolymer concentration was plotted as described in [23].

Dynamic structure of Pluronic aggregates in aqueous solutions was studied with the aid of dynamic light scattering (DLS) technique on a Zetasizer Nano ZS analyzer (Malvern Instruments). The copolymers were dissolved at the concentration of 0.1–5.0 mg/mL. Hydrodynamic diameter of Pluronic associates was measured in PBS at 25 °C and in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C. Zeta potential (ζ) of the micelles was assessed in distilled water in disposable U-form cuvette. The measurements were performed in triplicates and the data were treated with Dispersion Technology Software 6.2 (Malvern Instruments). Multi-modal distribution (mean number distribution) based on non-negative least squares algorithm was utilized to evaluate DLS data assuming that the polymeric aggregates were spherical.

2.4. Cell isolation and culturing

HeLa and A549 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin under standard conditions (37 °C, 5% CO_2 atmosphere). The cells were collected from the culture flask by dissociation with