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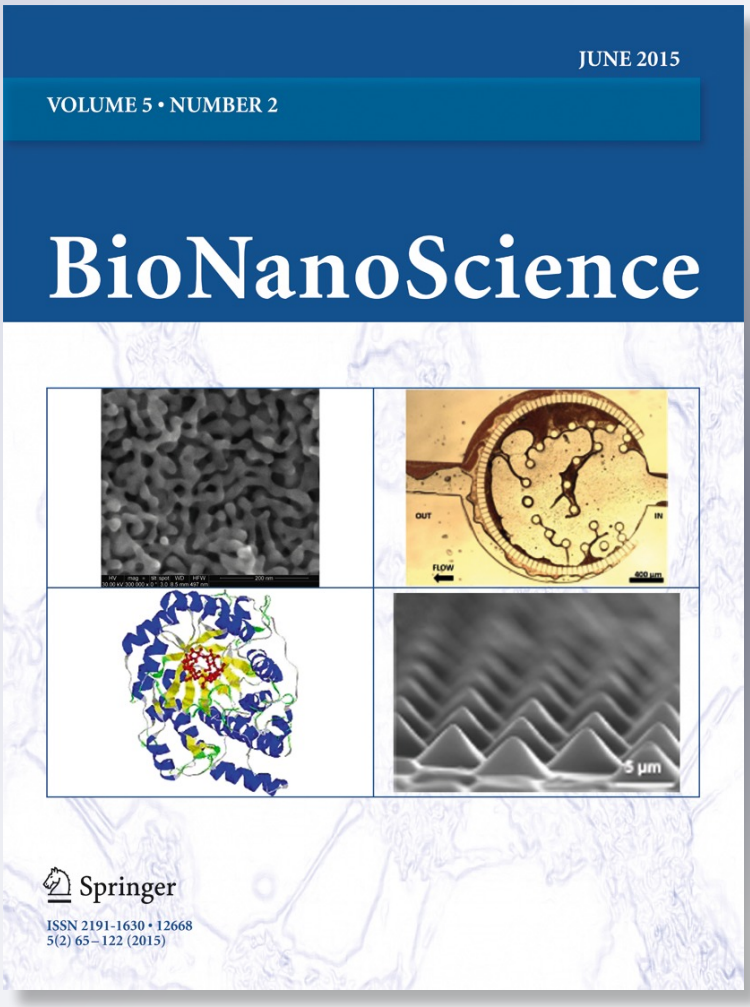

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Evaluation of Cell Membrane-Modulating Properties of Non-Ionic Surfactants with the use of Atomic Force Spectroscopy

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Abstract Analytical possibilities of atomic force spectroscopy (AFS) in liquid were studied upon interaction of membranotropic polymers with the plasma membrane of human cells. Topographical visualization of tightly adherent dermal fibroblasts, but not relatively soft prostate cancer (PC-3) cells, was achieved using a conventional triangular cantilever. A microsphere-based probe has been developed and applied for AFS analysis of micromechanical properties of PC-3 cells. Non-ionic block copolymers of ethylene oxide and propylene oxide, bi-functional Pluronic® L61, and glycerol-based trifunctional copolymer (TFC) were studied as potential modulators of cellular membranes and drug delivery systems as reported by Bondar et al. (Int. J. Pharm. *461*(97), 104, 2014). As indicated by dynamic light scattering and fluorescent techniques, Pluronic® L61 and TFC were adsorbed onto the cell surface and inserted into the plasma membrane in different extent. Analysis of AFS curves for surfactant-treated PC-3 cells showed that both Pluronic® L61 and TFC decreased the Young's modulus of cellular surface by almost 1.6 and 2 times, respectively. This is in accordance with the ability of amphiphilic polymers of decreasing the microviscosity of cellular membrane and promoting intracellular drug uptake as shown previously by Bondar et al. (Int. J. Pharm. *461*(97), 104, 2014). Our results are of particular interest for the characterization of interaction of living cells with amphiphilic polymer-based nanocarriers and drug formulations using AFS and other surface-sensitive techniques.

Keywords Cellular membranes · Elastic properties · Cancer cells · Atomic force spectroscopy · Non-ionic surfactants · Block copolymers of ethylene oxide and propylene oxide · Membrane-modulating properties · Microviscosity · Nanocarriers

1 Introduction

In the last decade, many physicochemical methods, initially designed to analyze materials and nanostructures, are widely used in biological investigations. Atomic force microscopy (AFM) and AFM-derived atomic force spectroscopy (AFS) are promising techniques for the visualization and characterization of different biological objects at cellular and molecular levels [1]. While AFM has been used for nanoscale visualization, AFS allows for measuring the surface deformation of the sample, contacting with the probe, and registering the modulus of elasticity (Young's modulus). The high spatial resolution of the AFS and its ability of operating in liquid environment makes it a very informative tool for biological, medical, and pharmaceutical applications [2, 3].

With the aid of AFM, the probing of the surface and interior of living prokaryotic [4, 5] and eukaryotic cells [6, 7] as well as model membranes [8] was carried out. Along with the visualization of ultrastructure of cell membrane, including pores in lipid bilayer [8], transmembrane sensing of intracellular substructures and organelles, e.g., nucleus and cytoskeletal elements [9–11], was achieved. The force of the interaction

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of cell membrane receptors with biological ligands was directly measured by the AFM probe with the ligand attached to its tip [12, 13].

Studying the interactions of membrane-targeted drugs and polymers with membranes of living cells and organelles using AFM and AFS is of particular fundamental and practical significance [9, 14]. In particular, AFS was used to assess the membrane-damaging effect of various polymers, e.g., polyethyleneimine, poly-L-lysine, PEG, and polyvinyl alcohol, designed as nanocarriers to deliver small drugs and non-viral genes into human cells [14]. Among these polymers, block copolymers of ethylene oxide (EO) and propylene oxide (PO), particularly, copolymers with $(EO)_x$–$(PO)_y$–$(EO)_x$ structure known as Pluronics®, are a promising drug delivery system [15].

Kabanov et al. showed that Pluronics exhibit distinct biological effects on mammalian cells, mainly due to their ability of altering physicochemical properties of cellular membranes [16, 17]. Membrane-modulating properties of Pluronics, in turn, allow for the increase of intracellular accumulation of cytostatic agents and inhibition of multidrug resistance of cancer cells. A drug composition on the basis of Pluronic L61 and doxorubicin has been recently developed to treat solid tumors [18]. The screening of new polymeric systems, targeted at the plasma membrane and other membrane structures of cancer cells, is an important problem in polymer-mediated therapy. Earlier, we proposed glycerol-based tri-functional block copolymers of EO and PO as promising analogs of Pluronics [19]. In this study, we evaluate analytical possibilities of the AFS to reveal membranotropic and membrane-modulating effects of non-ionic surfactants, Pluronic L61, and tri-functional block copolymer of EO and PO Laprol® 6003 (TFC).

2 Materials and Methods

2.1 Reagents

Bi-functional block copolymer of EO and PO (Pluronic® L61) was purchased from Sigma–Aldrich. Tri-functional block copolymer of EO and PO (Laprol® 6003), the structural analog of Voranol®6008 (Dow Chemical), was produced by JSC Nizhnekamskneftekhim (Russia). Cell culture reagents were purchased from PAA Laboratories. BODIPY FL C_{11} fluorescent probe was purchased from Invitrogen. Inorganic salts and solvents were produced by Acros Organics.

2.2 Cell Isolation and Culturing

PC-3 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 % CO2). Human dermal fibroblasts were isolated from skin explants according to a conventional protocol. Primary fibroblasts were expanded in α-MEM and used at early passages. Adherent cells were detached by trypsin–EDTA solution and collected by centrifugation at 200×*g* in PBS. Cell viability was verified by trypan blue staining.

2.3 Fabrication of Silicon Microsphere Based AFS Cantilever

Microspherical silicon probes were fabricated with the use of atomic force microscope Solver-Bio (NT-MDT) as described earlier [20]. Briefly, individual silicon microballs were deposited from water solution onto the glass substrate. Epoxy resin drops were applied onto the same substrate apart from microballs, and a tipless cantilever NSG 11 (NT-MDT), established in the AFM holder as a precision 3-D manipulator, was sequentially brought into contact with the adhesive drop and the silicon microballs. Finally, the microsphere-modified cantilever was removed from the holder and allowed to harden. Fabricated cantilevers were verified using the needle-shaped substrate TGT01 which appeared as hemispherical structures on the AFM image due to the convolution effect indicating the interaction between tip and the substrate.

Commercially available cantilevers with a spring constant of 5.5 N/m (Bruker) were used, while softer cantilevers strongly adhered to the cell surface complicating force curve measurements. The spring constant was additionally verified using the thermal tune technique as a part of standard Bruker software.

2.4 Calculation of the Young's Modulus

In order to calculate an absolute value of the Young's modulus from force curves, the Hertz model was used [21], which describes the interaction of a rigid hemisphere (an AFM tip) with the infinite plane (a cellular surface) (Fig. 2b). The interaction force (F) of the probe depends on the depth of its penetration into the plane and is defined by the expression:

$$F = \frac{4\sqrt{R}}{3} E^* \Delta h^{3/2} \quad (1)$$

where R is the radius of probe curve; Δh is the depth of probe penetration; and E^* is the effective Young's modulus for tip sample system.

2.5 AFM of Living Adherent Human Cells in Liquid

Surface morphology and micromechanical properties of living cells in liquid were studied on an atomic force microscope Dimension FastScan (Bruker) in the AFS mode which allows

for elastic properties (Young's modulus) measurements. To calculate the Young's modulus from force curves recorded, the theoretical Hertz model of the interaction of a rigid hemisphere and an infinite plane (Fig. 2b) [21] and NanoScope Analysis (Bruker) software were used. PC-3 cells and dermal fibroblasts (300,000 cells) were seeded on a cover glass and cultured 2 days under standard conditions. Prior analysis cells were washed with PBS and then transferred into PBS buffer (pH 7.4) supplemented with an amphiphilic polymer (0.1 mg/mL) and incubated for 15 min. AFS scanning was carried out in the same solution. At least five individual cells were analyzed and ten force curves were registered for each sample.

2.6 Zeta Potential Measurements of Cells

Zeta potential of viable suspended PC-3 cells (0.5×10^6 cells/mL) was detected by dynamic light scattering technique on Zetasizer Nano ZS analyzer (Malvern Instruments) [22]. Cells were pre-incubated with amphiphilic polymers at concentrations 0.05–0.2 mg/mL for 10 min. Measurements were performed in U-capillary cuvette in 50 mM HEPES buffer (pH 7.4) at 25 and 37 °C. Light scattering data were analyzed with the use of Dispersion Technology Software.

2.7 Detection of Displacement of Membrane-Bound Fluorescent Probe

Membrane-specific BODIPY FL C_{11} fluorescent probe which incorporates into lipid bilayer of cell plasma membrane was used. PC-3 cells were collected and suspended in PBS at a density of 5×10^5 cells/mL. Cells were pre-labeled with 1 μM BODIPY FL C_{11} and washed with PBS by means of centrifugation. Pluronic L61 or Laprol 6003 was added to the cell suspension at the concentration of 0.2 mg/mL. The mixture was incubated for 40 min under gentle agitation, and cell fluorescence was analyzed on a flow cytometer (Guava easyCyte 8HT, Millipore) in green FL channel.

3 Results and Discussion

3.1 AFM Visualization of Adherent Human Cells

A conventional AFM triangular cantilever with the curvature radius of the tip ~10 nm was used to visualize single primary human skin fibroblasts grown on a glass slide. Figure 1 shows representative AFM image of the part of adherent and spread fibroblast which was acquired with the aid of a PeakForce QNM® (Quantitative nanomechanical property mapping) method.

In addition to surface topography (Fig. 1a) and peak force error (Fig. 1b), the adhesion map (Fig. 1c) and elasticity map (Fig. 1d) were also obtained using corresponding scanning modes. All modes generate similar pictures of a region of adherent and well-spread single cell with defined boundaries and surface microstructures. Adhesion and elasticity mappings show that the cell surface possesses higher adhesive and lower elastic properties compared to the substrate.

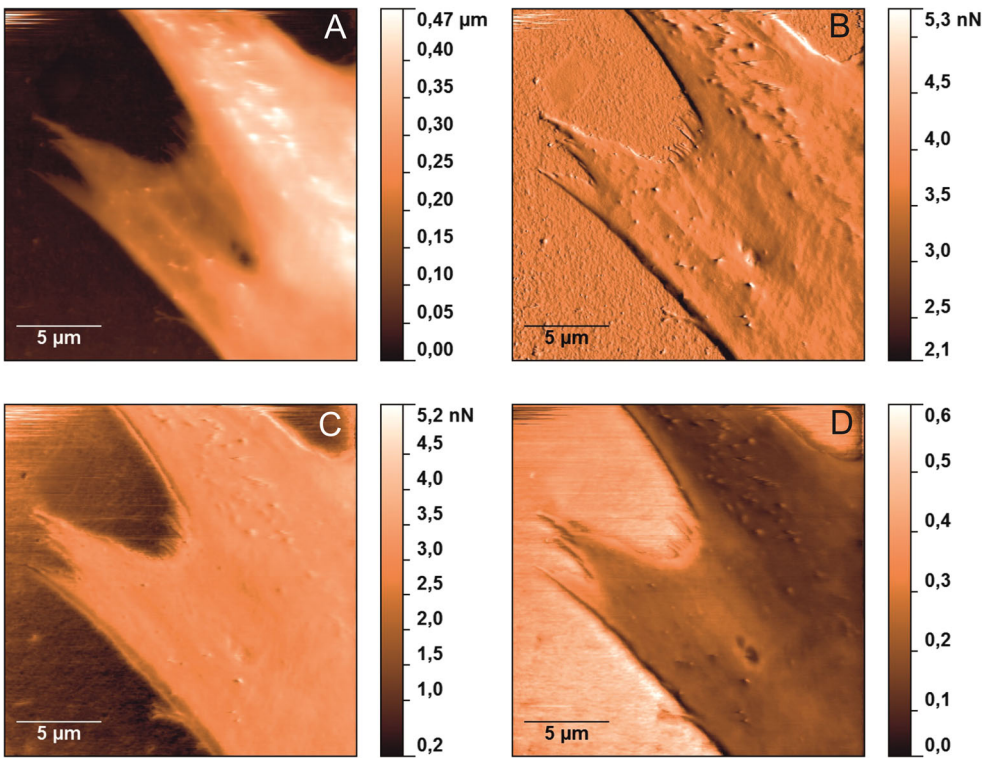

Fig. 1 Representative AFM images of adherent human skin fibroblast on a cover glass in different scanning modes (PeakForce QNM®). **a** Surface topography, **b** peak force error, **c** adhesion map, and **d** elasticity map (log of Young's modulus)

In the same conditions, scanning of adherent PC-3 cancer cells was accompanied by their damage, indicating their higher softness in comparison with skin fibroblasts, which were characterized by a tight contact with the glass substrate. This is apparently due to the small curvature radius of the conventional AFM probe which pierces soft cells, resulting in their detachment from the substrate even at a relatively weak pressure, which is in agreement with existing data [23]. Therefore, in order to determine viscoelastic properties of the plasma membrane of PC-3 cells, we applied earlier developed AFM probe with the microsphere-based tip [20].

Figure 2a shows SEM image of this AFM probe with attached silicon microsphere of 5 μm in diameter. Owing to its large contact area, the probe exerts lower pressure on the surface and allows for the analysis of biological objects using AFS technique. Moreover, better controlled geometry of this probe increases the accuracy of the determination of Young's modulus according to the Hertz model (Fig. 2b).

3.2 Evaluation of Cell Surface Adsorption of Non-Ionic Surfactants

Figure 3 shows the chemical structure of non-ionic surfactants: bi-functional block copolymer of EO and PO (Pluronic L61) and glycerol-based tri-functional block copolymer (Laprol 6003, TFC) which have been studied as modulators of the plasma membrane of human cells [16, 19]. These copolymers contain different amount of EO and PO units and have different supramolecular structure, but, at the same time, they are characterized by similar hydrophilic-lipophilic balance (HLB). HLB values, calculated theoretically by the Davies method, were 5.8 for Pluronic L61 and 6.7 for TFC, indicating the predominance of hydrophobic properties in these polymers.

Adsorption of Pluronic L61 and TFC on the surface of suspended PC-3 cells was investigated with the use of previously proposed method which is based on the registration of the zeta potential of mammalian cells [22]. PC-3 cells suspended in HEPES buffer generate negative zeta potential value of about −23 mV. It was found that Pluronic L61 at 25 °C slightly alters cell surface charge, while TFC at the same

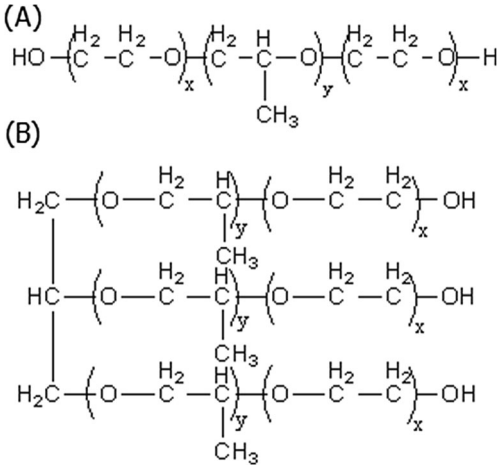

Fig. 3 Chemical structure of **a** Pluronic L61 (y=31; x=2.3; MW 2000) and **b** tri-functional block copolymer Laprol 6003 (y=27.8; x=8; MW 6000)

conditions induces significant decrease of negative zeta potential of cells to almost −10 mV value (Fig. 4). Observed zeta potential changes indicate an interaction of non-ionic polymers with the cell surface, and therefore, an alteration of interface properties between the plasma membrane and surrounding solution, presumably, as a result of the adsorption of hydrophobic polypropylene oxide block of amphiphilic polymers at the lipid bilayer.

More obvious decrease in zeta potential of cells in the presence of TFC than Pluronic L61 suggests the more efficient adsorption of the former polymer on the cell surface due to its higher affinity lipid-like structure. The effect of TFC on cell zeta potential is becoming more obvious upon temperature increase up to 37 °C as a result of the promotion of hydrophobic properties of polymeric molecules due to heat-induced dehydration of their blocks. Furthermore, the increase in hydrophobic properties of the amphiphilic polymer promotes its adsorptive interactions with the plasma membrane of cells.

To further characterize the binding of non-ionic surfactants to the plasma membrane of PC-3 cells, a new approach has been applied which is based on the detection of displacement of membrane-bound fluorescent probe. This probe contains C_{11} alkyl tail which stably integrates into the membrane lipid bilayer. Figure 5 shows the distribution of fluorescence of

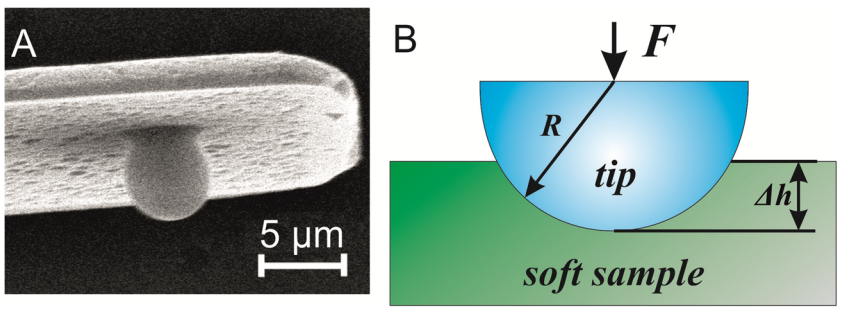

Fig. 2 **a** SEM image of the AFM probe tip with the silicon microsphere [20]; **b** scheme of the interaction of the probe hemisphere with the infinite plane according to Hertz's model [21]

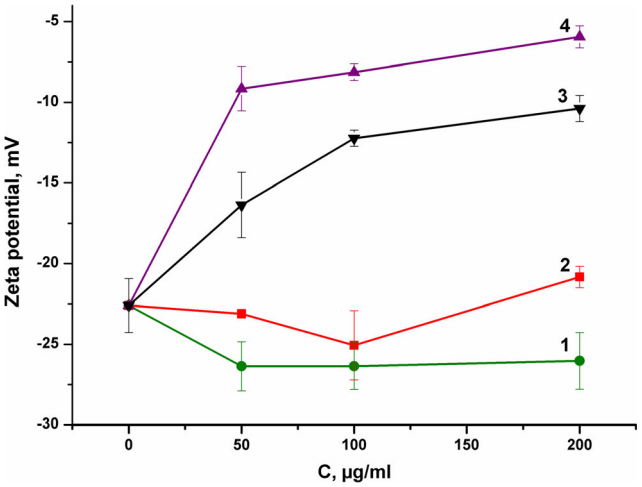

Fig. 4 Alteration of zeta potential of PC-3 cells in the presence of Pluronic L61 and TFC. Cell suspension 0.5×10^6 cells/mL in HEPES buffer (pH 7.4). Incubation time 10 min. (*1*) Pluronic L61, T 25 °C; (*2*) Pluronic L61, T 37 °C; (*3*) TFC, T 25 °C; (*4*) TFC, T 37 °C

lipophilic probe-labeled PC-3 cells after incubation with amphiphilic polymers according to flow cytometry analysis.

Pluronic L61 did not induce significant decrease in the cell fluorescence, indicating its low ability of integration into the cellular membrane and displacement of the lipophilic probe. Unlike Pluronic L61, TFC resulted in almost 1.7-fold decrease in the average fluorescence intensity of labeled cells, which indicates the displacement of lipophilic probe from the cellular membrane, apparently, due to partial insertion of this polymer into the membrane lipid bilayer.

These results are consistent with the ability of amphiphilic polymers to alter zeta potential of suspended cells (Fig. 4), as well as with the literature data that hydrophobic Pluronics incorporate into the lipid bilayer with the participation of their polypropylene oxide block to change membrane microviscosity and permeability for low molecular weight substances [24].

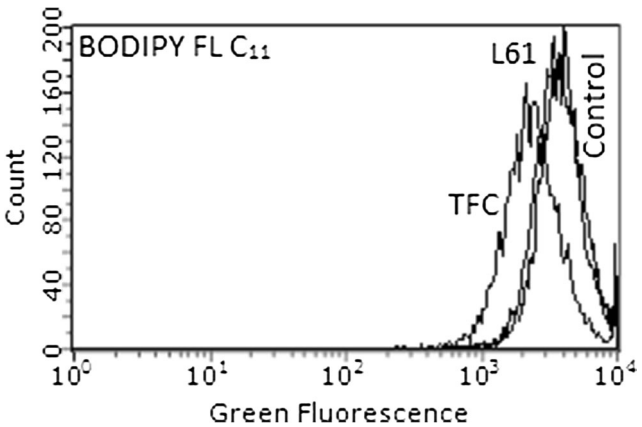

Fig. 5 Distribution of fluorescence intensity of PC-3 cells labeled with BODIPY FL C11 membrane probe after incubation with Pluronic L61 and TFC. Polymer concentration 0.2 mg/mL, incubation time 40 min

3.3 AFS Study of an Effect of Non-Ionic Surfactants on Elastic Properties of Plasma Membrane

Elastic properties of the plasma membrane of single PC-3 cells pre-grown on the glass slide were assessed by AFS with the aid of the microsphere-based probe (Fig. 2). The probe was brought into the contact with cell surface, and force curves upon the probe deviation as well as Young's modulus were measured as described earlier [20]. Figure 6 shows average AFS force curves for the glass substrate, control PC-3 cells, and the cells treated with amphiphilic polymers. As seen in the figure, the surface of glass substrate was characterized by an almost steep curve which is typical for solid materials, while adherent cells generated a curve with a smaller tilt angle. Under the same conditions, amphiphilic polymer-treated PC-3 cells generated force curve with even smaller tilt angle in comparison with intact cells, which showed that samples became softer (Fig. 6). In case of the tri-functional polymer, a relatively noisy curve was registered under experimental conditions. This can be explained by a dynamic membrane-fluidizing effect of this polymer which results in the modulation of interaction of AFM tip with the plasma membrane of living cells.

These results correlated with mean values of Young's modulus for analyzed cell surfaces which were 0.156 MPa for untreated cells, 0.096 and 0.079 MPa for cells treated with Pluronic L61 and TFC, respectively. The standard error for Young's modulus values did not exceed 20 %, indicating a good reproducibility of the analysis [25]. The increase of force curve slope and decrease in Young's modulus observed by AFS study indicate the lowering of elasticity of the plasma membrane of human cells in the presence of ampiphilic polymers. Similar effects on micromechanical properties were established earlier for F-actin targeted drugs [9].

We believe that alterations in AFS signal of PC-3 cells observed in the presence of amphiphilic polymers mainly result from their effect on the plasma membrane rather than the cell interior. Copolymers of EO and PO are known to interact

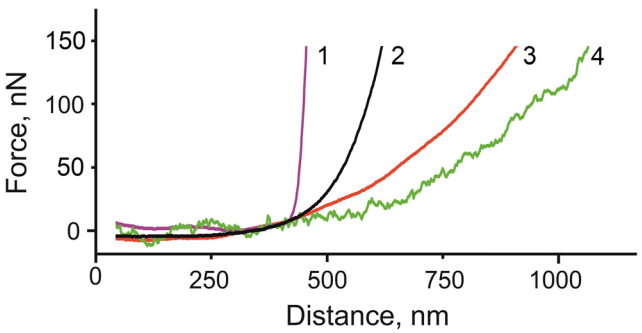

Fig. 6 Average force curves acquired from the surface of adhered PC-3 cells with the aid of microsphere based probe. (*1*) Glass slide; (*2*) untreated cells; (*3*) Pluronic L61 treated cells; (*4*) TFC-treated cells. Polymer concentration 0.1 mg/mL, incubation time 15 min at room temperature

with cellular membranes as a primary 'target' and modulate their physicochemical characteristics, e.g., microviscosity [17, 19, 26]. The cells were briefly exposed to polymers for 15 min in buffer solution. Such conditions do not imply the significant penetration of polymer molecules into cytosol.

Altogether, ASF data show that amphiphilic polymers induce softening and reduce elasticity of cellular membranes as a result of their interaction. Such an effect was more pronounced for lipid-like TFC compared to bi-functional Pluronic L61 and also correlated with an ability of surfactants to change cell zeta potential (Fig. 4) and displacement of membrane-bound fluorescent probe (Fig. 5). Our results obtained in this study are also in agreement with the previous report [19] that TFC decreases microviscosity of cell plasma membrane in higher extent than Pluronic L61 presumably due to the better adsorption of the former polymer onto the lipid bilayer and its destabilizing effect on lipid packing. Such an effect of TFC is responsible for its marked ability of promoting intracellular delivery of fluorescent dyes and drugs into cancer cells [19] as well as plasmid DNA-polycation complexes into human dermal fibroblasts [27].

Our study shows that AFS with the microsphere-based probe can be used to investigate and compare membrane-modulating properties of non-ionic surfactants as candidates for drug delivery systems. Together with other surface-sensitive techniques, AFS is a useful tool for pharmaceutical screening of existing and newly synthesized membranotropic polymers.

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Compliance with Ethical Standards O.V. Bondar, D.V. Lebedev, V.D. Shevchenko, A.A. Bukharaev, Y.N. Osin, Y.G. Shtyrlin, and T.I. Abdullin state that there are no conflicts of interest. This article does not contain any studies with human or animal subjects. All procedures followed were in accordance with the ethical standards of the Local Ethical Committee of Institute of Fundamental Medicine and Biology of Kazan Federal University.

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