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Nanocomposite biomimetic vesicles based on interfacial complexes of polyelectrolytes and colloid magnetic nanoparticles

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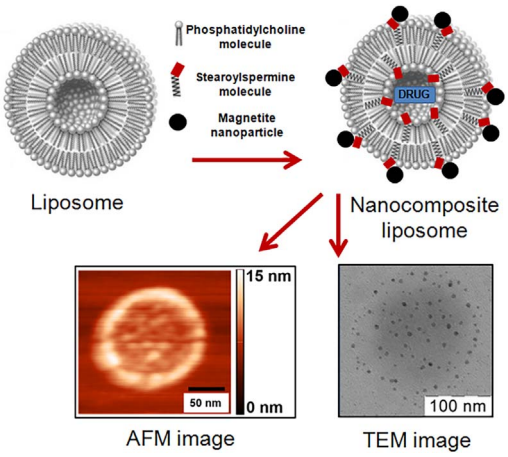
GRAPHICAL ABSTRACT



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ABSTRACT

Development and study of novel biomimetic and biocompatible functional nanofilm structures, surfaces and colloid membranous vesicles are currently important from fundamental and applied viewpoints. They can serve as model systems for insight into the basic structural-functional interconnections and physicochemical mechanisms at the nano-scale in biomembranous systems, and are useful for development of engineering solutions efficient for bio-medical applications including controlled drug delivery. We present here the results of a study of novel nanofilm composite structures (Langmuir monolayers, Langmuir-Blodgett films and liposomes) based on the interfacial complexes formed by biogenic lipid phosphatidylcholine, synthetic amphiphilic water-insoluble polyamine stearoylspermine (a derivative of biogenic polyamine spermine and stearic acid), colloid cationic ligand-free magnetite nanoparticles and polyanions (DNA, Poly(styrenesulfonate)). It was found that stearoylspermine molecules formed stable Langmuir monolayer on an aqueous subphase surface and that monolayer compression isotherm changed as a result of interactions of the stearoylspermine monolayer with aqueous subphase components (colloid cationic magnetite nanoparticles and polyanions). Monolayer Langmuir-Blodgett

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films of interfacial polycomplexes formed by stearoylspermine and magnetite nanoparticles or DNA molecules were deposited onto the mica substrate surface and the structure of polycomplex films was investigated using AFM. The data obtained using Langmuir monolayer technique were further used in formation of new composite nanofilm magnetic colloidal membranous vesicles based on the interfacial polycomplexes of phosphatidylcholine, stearoylspermine, magnetite nanoparticles and polyanions. The nanocomposite membranous vesicles were prepared successfully by sequential adsorption of colloid cationic ligand-free magnetite nanoparticles and polyanions onto the cationic surface of mixed phosphatidylcholine/stearoylspermine liposomes preliminarily formed using conventional ultrasound method. The formed vesicles were characterized by transmission electron microscopy, AFM, electron magnetic resonance technique, laser light scattering and electrophoresis techniques. The synthesized stable biocompatible nanocomposite magnetic liposomal vesicles can be useful in development of novel efficient systems for capsulation, targeted transport, controlled spatial localization and physical stimuli-addressed drug and DNA delivery.

1. Introduction

Development and study of novel biomimetic and biocompatible functional nanofilm structures, surfaces and colloid membranous vesicles are currently important from fundamental and applied viewpoints. Such nanostructures can serve as model systems for insight into the basic structural-functional interconnections and physicochemical mechanisms at the nano-scale in biological membranous systems and are useful for development of engineering solutions efficient for biomedical applications including controlled drug delivery.

For development of optimal systems for capsulation and controlled drug delivery it is necessary to find most rational solution of a series of complex interrelated biological, chemical, physical and nanotechnological tasks. Besides the problems related to the efficient drug capsulation and targeted delivery there is a number of requirements to drug carriers including the size restrictions (submicron or less scale) and the lowest possible toxicity of the carrier components and materials. Currently, various colloidal carrier systems are under development and investigation based on organic, inorganic and/or composite nanostructures formed by polymers, amphiphiles, nanoparticles, nanotubes, etc. [1–8]. Interfacial interactions and complexes play crucial role in formation and functionalization of such nanostructures. The main unresolved problems in that field are now the controlled spatial localization and efficient activation of the drug carriers by various internal or external stimuli resulting in targeted and dosed release of the encapsulated agents.

Biomimetic and biocompatible colloid membranous vesicles − liposomes − are for decades used as a model system of biological membranes in the biophysical studies and are now one of the very few systems used in real bio-medical and cosmetic practice [9–12]. The liposomal lipid bilayer membrane is similar to the biological membranes, and the size range of liposomes can be rather wide and corresponds to that of biogenic membranous systems, in particular microvesicles. Later developed membranous vesicles − polymersomes − are also formed by amphiphilic molecules, but those molecules include polar polymeric moieties as, for example, block copolymers with poly(ethylene glycol) [7]. Polymer components in the structure of membranous vesicles significantly increase their colloidal stability and blood circulation times [7,13]. New organic–inorganic hybrid lipid bilayer membranous vesicles with an inorganic silicate framework covering the lipid vesicular surface were developed for drug delivery applications and were characterised by enhanced colloid stability [14].

Membranous vesicles have attracted tremendous attention as prospective drug carriers because of their biocompatibility, tunable membrane properties and ability in encapsulating or integrating a very broad range of compounds of different nature − hydrophilic molecules and ions in the aqueous internal core and hydrophobic compounds in the bilayer membrane. Also, cationic liposomes complexed with polyanionic nucleic acid molecules are promising synthetic nonviral carriers of DNA and RNA vectors for transfection and gene therapy applications [15].

New approaches in the development of advanced liposomal compositions include the creation of functionalized liposomes capable of controlled delivery of encapsulated substances: the membrane structure of such liposomes can be changed in response to external physical and/or chemical stimuli. Examples of such liposomes are thermosensitive liposomes [16], pH-sensitive liposomes [17] and redox-responsive liposomes [18]. The sensitivity of drug carriers to external physical stimuli can be achieved via their functionalization by appropriate sensitizing components as, for example, inorganic nanoparticles which are currently widely studied as prospective candidates for biomedical applications [3,5,6,8,19–21].

A promising approach to solving the problem of controlled spatial localization of colloidal drug carriers and their targeted delivery in the body is the incorporation of magnetic nanoparticles into their structure and using an external magnetic field. The idea of targeted delivery of therapeutic agents (such as oligonucleotides, proteins, other drugs) with the use of a magnetic field has been proposed by Widder in 1978 [22]. Currently, magnetic nanoparticles of iron oxides (mainly magnetite Fe_3O_4) are the most widely used magnetic nanoparticles in biomedicine due to their low toxicity, relatively high saturation magnetization, stable structure and magnetic characteristics [3,5,8,19,23–27]. Biomedical applications of magnetic nanoparticles are mainly related now to the diagnostics (biosensors, contrast agents for magnetic resonance imaging, markers for biomolecules), to magnetic separation and targeted therapeutic effects (targeted delivery of therapeutic molecules including DNA, controlled local hyperthermia of cancer, etc.). Interesting new directions are connected with the using of functionalized magnetic nanoparticles for activation the processes at the cellular and sub-cellular level (ions channels, etc.) and in tissue engineering [8,28]. The study of organized organic-inorganic and bio-inorganic nanostructures based on the magnetic iron oxide nanoparticles is now of high interest also because biogenic magnetic nano-phase iron oxides (mainly magnetite) are found in various living systems from bacteria and plants to humans [29,30]. In humans the presence of magnetite particulate inclusions correlate with neurodegenerative diseases [31,32]. In biological systems inorganic nanostructures are formed as a result of biomineralization processes which occur under normal conditions and in which the key role is played by the composition and structural organization of the surface of bio-molecular matrix interacting with the growing inorganic nano-phase. The elucidation of the fundamental mechanisms of biomineralization and of formation of the organized bio-inorganic nanostructures is important for the development of prospective biomimetic synthetic strategies in nanotechnology. In that connection the study of interfacial nanostructure formation processes in biomimetic systems based on Langmuir monolayers and bilayer membranes with magnetite nanoparticles and polymers (in particular, polyanions including DNA), is interesting and important for understanding the mechanisms of processes with the participation of biogenic magnetite nanoparticles in living systems, and also for the development of methods for design and production of new functional biocompatible nanosystems such as composite nanofilms, magnetic

vesicles and lipoplexes for the controlled drug and DNA delivery. Magnetic membranous vesicles are known as magnetoliposomes. They are formed usually via filling the internal volume of the liposomes with magnetic fluid or magnetic particles [33–35] or by incorporation of hydrophobized magnetic nanoparticles into the lipid bilayer structure [36]. The current applications of magnetoliposomes include magnetically directed drug carriers, magnetic hyperthermia, magnetic field assisted gene transfection and contrast agents for nuclear magnetic resonance tomography.

The idea of our approach to development of magnetic field-controlled vesicular drug and DNA carriers is the formation of stable colloid liposomal structures with functionalized membrane surface capable to bind magnetic iron oxide nanoparticles and DNA molecules. For that purpose cationic amine-containing amphiphilic water-insoluble compounds should be present in the liposome membrane. It is known that polyamines can form stable complexes with magnetic iron oxide nanoparticles [37] and polyanion molecules including DNA [38,39]. In that connection biogenic polyamines are of particular interest due to their complete biocompatibility.

Biogenic polyamines (putrescine, spermidine and spermine) are intimately involved in the regulation of cellular growth and viability, they are normally found in millimolar concentrations in the cell nucleus [40,41]. Spermine molecule have maximal number of aminogroups (four) among the biogenic polyamines and can function as a free radical scavenger what is important for suppression of the lipid peroxidation in liposomal membranes [42]. Aminogroups of spermine (two primary and two secondary) can interact with various organic and inorganic ligands and can be protonated at pH values below 10 resulting in positive electrostatic charge of the spermine molecule. Spermine is soluble in water and in many organic solvents such as chloroform, ethanol, methanol, etc.

Earlier, the effect of self-assembly of highly-organized freely suspended nanofilm structures based on the complexes of colloid cationic ligand-free magnetite nanoparticles with molecules of polyamine spermine used as a linking multidentant ligand in a bulk aqueous phase in the absence of any surfaces and interfaces have been found [43]. In the present work we use the interfacial complexation effect of water-insoluble cationic amphiphile stearoylspermine (a derivative of biogenic polyamine spermine and stearic acid) with aqueous phase colloid ligand-free magnetite nanoparticles for immobilization of magnetite nanoparticles onto the surface of liposomal membrane. The presence of the amide bond in the stearoylspermine molecule makes possible its enzymatic biodegradation in living cells. Synthetic lipopolyamines, in particular stearoylspermine and related acyl spermines were prepared successfully and used in gene transfer and drug delivery research and practice [44–46].

Interfacial complexation processes of water-insoluble amphiphiles can be monitored and studied readily using Langmuir monolayer technique. Langmuir monolayers at the air/aqueous phase interface and mono- and multi-layer Langmuir–Blodgett (LB) films deposited onto the solid substrates are for many years a convenient system for studying interfacial phenomena, in particular, for modeling and study of physicochemical properties of the surfaces of biological lipid membranes, and for creating various planar nanostructures [47–51].

In this article we present results of the preparation and characterization of novel nanofilm nanocomposite systems (Langmuir monolayers, LB films and liposomes) based on the interfacial complexes and structures formed by biogenic lipid phosphatidylcholine, amphiphilic water-insoluble polyamine stearoylspermine, colloid magnetite nanoparticles and polyanion molecules (DNA, Poly(styrenesulfonate)). We have synthesized stearoylspermine and formed stable stearoylspermine Langmuir monolayer on an aqueous subphase surface. The stearoylspermine Langmuir monolayer compression isotherm changes caused by interactions of the monolayer with the aqueous subphase components (colloid ligand-free magnetite nanoparticles and polyanion molecules) have been studied. Monolayer Langmuir-Blodgett films of interfacial polycomplexes formed by stearoylspermine with magnetite nanoparticles or DNA molecules were deposited onto the mica substrate surface and characterized by scanning probe microscopy. New nanocomposite biocompatible magnetic colloidal membranous vesicles based on the polycomplexes of phosphatidylcholine, stearoylspermine and magnetite nanoparticles (magnetic liposomes) were formed successfully by sequential adsorption of colloid magnetite nanoparticles onto the mixed phosphatidylcholine/stearoylspermine liposomes preliminarily formed using conventional ultrasound method.

2. Materials and methods

Chemical reagents for our experiments were obtained from Sigma/Aldrich and were used as received without additional purification: salts $FeCl_3$, $FeCl_2$, spermine, stearic acid, phosphatidylcholine, native salmon DNA, Polystyrene sulfonate (PSS), NaOH. Milli-Q integral water purification system for ultrapure water was used to produce water with an average resistivity of 18 MΩ-cm for all experiments. The procedures of synthesis of stearoylspermine and magnetite nanoparticles used in our work, and the data of its characterization are described in Appendix A Supplementary data. Ligand-free colloid magnetic iron oxide nanoparticles (magnetite Fe_3O_4) with the mean size of about 3.7 nm were synthesized in aqueous phase according to the known Massart method [52]. The important point is the predominance of synthesized magnetite nanoparticles with diameter smaller than the lipid bilayer membrane thickness (~ 5 nm) what is important for the use of those nanoparticles in our work for formation of nanocomposite liposomes via adsorption of magnetite nanoparticles onto the outer liposomal membrane surface. The isoelectric point pI (the pH value of zero charge) of magnetic iron oxides (magnetite and maghemite) in water is about 7 [53], so at the low enough pH values in an aqueous solution the colloid ligand-free iron oxide magnetic nanoparticles possess positive electrical surface charge and can be stabilized efficiently by the interparticle electrostatic Coulomb repulsion. The electrophoretic mobility of the synthesized magnetite nanoparticles measured at pH = 3.8 corresponded to electrostatic surface ζ-potential value of about +20 mV.

Experiments with Langmuir monolayers and Langmuir–Blodgett films were carried out with the use of KSV-Nima LB Trough Medium KN 1003 device at ambient conditions (room temperature about 24 °C). Langmuir monolayer of stearoylspermine was formed by the dropwise deposition of the stearoylspermine solution in chloroform (concentration 5×10^{-4} M) onto the surface of aqueous subphase. After 10 min necessary for chloroform evaporation and uniform distribution of stearoylspermine molecules onto the aqueous subphase surface the compression isotherm (the dependence of surface pressure value on the monolayer area) was measured with the mowing barrier rate 5 mm/min. Preparation of LB films via deposition of Langmuir monolayers onto the freshly prepared mica substrate surface was carried out using conventional vertical substrate lifting method with the substrate rate 5 mm/min. The monolayer transfer ratio was high (about 1). Some details of our experiments with Langmuir monolayers and LB films can be also found in our previous related publications [50,54].

The structure of LB films and liposomes deposited onto the atomically flat freshly prepared mica substrate surfaces was studied by the method of atomic force microscopy (AFM) using Solver P47 SPM MDT device (NT-MDT, Russian Federation) in tapping AFM imaging mode in air at ambient conditions (temperature ~ 24 °C). LB film samples for AFM measurements were prepared by conventional Langmuir-Blodgett vertical lifting deposition method onto the mica substrate. Samples of liposomes for AFM study were prepared by adsorption of liposomes from the aqueous liposomal suspension onto the freshly prepared mica substrate surface with subsequent substrate washing in the ultrapure water and drying. AFM images were highly stable and reproducible.

Characterization of the samples using transmission electron microscopy (TEM) was carried out with the use of LEO 912AB, IOMEGA device (Germany). Samples for TEM measurements were prepared by

deposition of microdroplets of aqueous suspensions of colloid magnetite nanoparticles or nanocomposite liposomes onto the surface of copper grid substrate (3 mm diameter, 200 mesh) coated with an ultrathin layer of polymer (Formvar) and amorphous carbon with subsequent drying.

Measurements of the mean size of colloidal particles and their electrophoretic mobility which is proportional to the electrostatic ζ-potential value were carried out in suspensions of colloid magnetite nanoparticles and liposomes by the dynamic light scattering and electrophoresis techniques using 90 Plus device (Brookhaven instruments corporation).

Magnetic properties of the samples containing magnetite nanoparticles were studied by electron magnetic resonance (EMR) technique at room temperature with the use of X-band spectrometer Varian E-4 (USA). The microwave frequency was 9.1 GHz, magnetic field was varied in the range of 0–6 kOe. The effective g-factor value was determined by the formula $g_{eff} = g_e B_e/B_{res}$, where B_{res} – resonant magnetic field (the midpoint on the spectrum field scale between the maximum and minimum of the microwave absorption curve derivative), $g_e = 2.0023$, B_e – resonant magnetic field corresponding to g_e. Line width ΔH_{pp} was determined as the distance between the maximum and minimum of the microwave absorption curve derivative in the magnetic field units.

3. Results and discussion

3.1. Stearoylspermine Langmuir monolayer and its interfacial complexes with colloid magnetite nanoparticles and polyanion molecules

Firstly, the compression isotherm of stearoylspermine Langmuir monolayer and its changes caused by binding of aqueous phase colloid magnetite nanoparticles and DNA molecules at the stearoylspermine Langmuir monolayer/aqueous subphase interface were studied. Stearoylspermine molecules are water-insoluble due to the presence of hydrophobic hydrocarbonic portion in the molecule – stearic acid residue (see the chemical formula of stearoylspermine molecule presented on Fig. 1a). At the same time amino groups of stearyolspermine can be protonated in an aqueous phase at pH values below 10 resulting in positive electrostatic charge of the molecule. As a result, the stearoylspermine molecules have pronounced amphiphilic properties and can form stable Langmuir monolayer at the air/aqueous phase interface. The typical compression isotherm of stearoylspermine monolayer on the pure water subphase is presented on Fig. 1b (curve 1). The compression isotherms of stearoylspermine Langmuir monolayer formed on the surface of aqueous suspension of ligand-free cationic colloid magnetite nanoparticles and on the water solution of DNA molecules are shown on Fig. 1b curves 2 and 3, correspondingly.

The comparison of the compression isotherm of amphiphilic polyamine stearoylspermine Langmuir monolayer (Fig. 1b curve 1) with well-known monolayer compression isotherm of amphiphilic monoamine with the same hydrocarbon moiety (stearylamine or octadecylamine, see, for example [55,56]) shows that the monolayer area per molecule at high surface pressure values is similar (about 18 Å^2/molecule at surface pressure ~30 mN/m) but at lower surface pressure values the stearoylspermine monolayer compression isotherm is substantially expanded and shifted to the higher monolayer area values compared with the stearylamine monolayer. That difference in compression isotherms is clearly a result of different monoamine and polyamine nature of polar moieties of stearylamine and stearoylspermine molecules, respectively.

The comparison of curves 1 and 2 on Fig. 1b demonstrates the shift of stearoylspermine monolayer compression isotherm to higher values of the monolyer area per stearoylspermine molecule when the aqueous subphase contained cationic colloid ligand-free magnetite nanoparticles. That shift indicates to the adsorption of cationic magnetite nanoparticles on the stearoylspermine monolayer surface. Polyamine spermine is also cationic at those pH values and the binding of magnetite nanoparticles with stearoylspermine monolayer can be caused by high enough chemical affinity of polyamine to iron oxides. The compression isotherm of stearoylspermine monolayer formed on the aqueous solution of DNA molecules (Fig. 1b, curve 3) was also shifted to the higher monolayer area values comparatively to the compression isotherm of the monolayer formed on the pure aqueous subphase indicating to the expansion of the stearoylspermine monolayer caused by the interfacial binding of DNA molecules. Similar effect of stearoylspermine Langmuir monolayer expansion was observed when the other polyanion Polystyrene sulfonate was present in the aqueous subphase. The observed shift of the monolayer compression isotherms toward the higher values of the monolayer area per molecule means an increase in the surface pressure value and, consequently, decrease in the interfacial tension at the same values of the monolayer area as a result of the interfacial binding what complies with the Gibbs adsorption theory. Such effect of Langmuir monolayer expansion due to the binding of aqueous subphase polymeric molecules as DNA with Langmuir monolayers of oppositely charged cationic amphiphilic compounds is known [57–59] and our results are in agreement with the literature data.

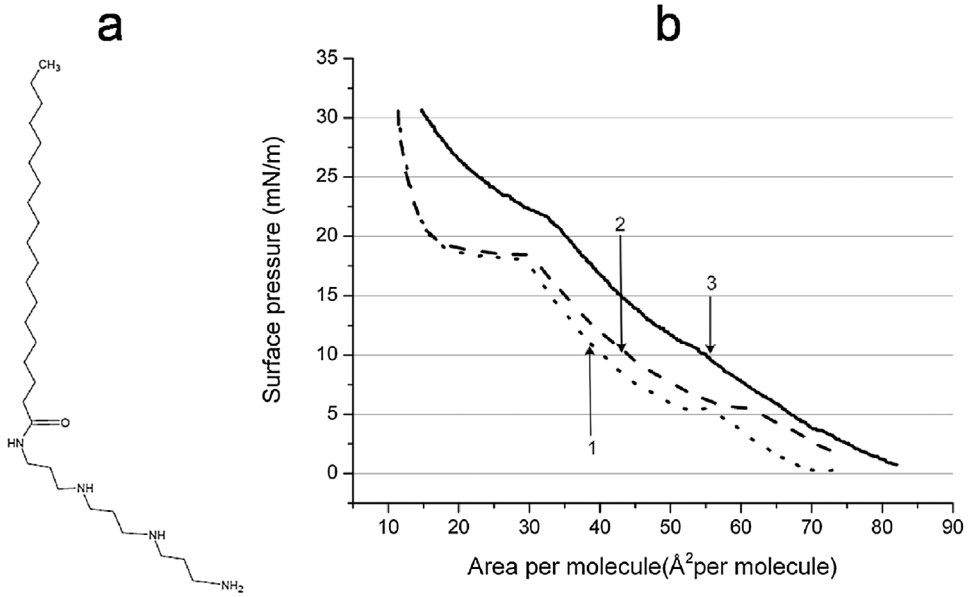

Fig. 1. (a) Chemical formula of stearoylspermine. (b) Compression isotherms of stearoylspermine Langmuir monolayer on the various aqueous subphases. Curve 1–pure aqueous subphase (pH ~ 4.0). Curve 2–aqueous subphase contains 10 mg/ml colloid cationic ligand-free magnetite nanoparticles (pH ~ 4.0). Curve 3 – aqueous subphase contains native DNA molecules (concentration 1 mg/ml, pH = 6.2).

Transfer of stearoylspermine Langmuir monolayer and corresponding interfacial stearoylspermine monolayer complexes with magnetite nanoparticles and DNA molecules from the aqueous subphase surface to the mica substrate for subsequent structural study using AFM was performed by the standard Langmuir–Blodgett method (vertical substrate lifting) at the surface pressure values about 27 mN/m corresponding to the condensed state of the monolayer shown schematically on Fig. 2a. We were interested in deposition and study of compressed monolayers because the structure and properties of compressed lipid Langmuir monolayer in its condensed state correspond most closely to the corresponding lipid matrix properties in the bilayer liposomal membrane [60]. The typical topographic image of stearoylspermine monolayer deposited onto the fresh atomically-flat mica substrate surface was obtained by tapping mode AFM technique and is presented on Fig. 2b. It is seen from the Figure that besides a few granular 3-D structures the monolayer surface is rather flat and homogeneous. The 2D/3D transition processes in monolayers can lead to the formation of such non-planar structures and are directly related to the collapse processes in compressed Langmuir monolayers [61].

The typical experimental topographic image of nanocomposite stearoylspermine Langmuir monolayer deposited onto the same mica substrate surface is presented on Fig. 2d. Cationic magnetite nanoparticles were adsorbed onto the Langmuir monolayer surface from the aqueous subphase containing colloid ligand-free magnetite nanoparticles as it is schematically shown on Fig. 2c. One can see individual and chain-like particulate nanostructures along with a few relatively

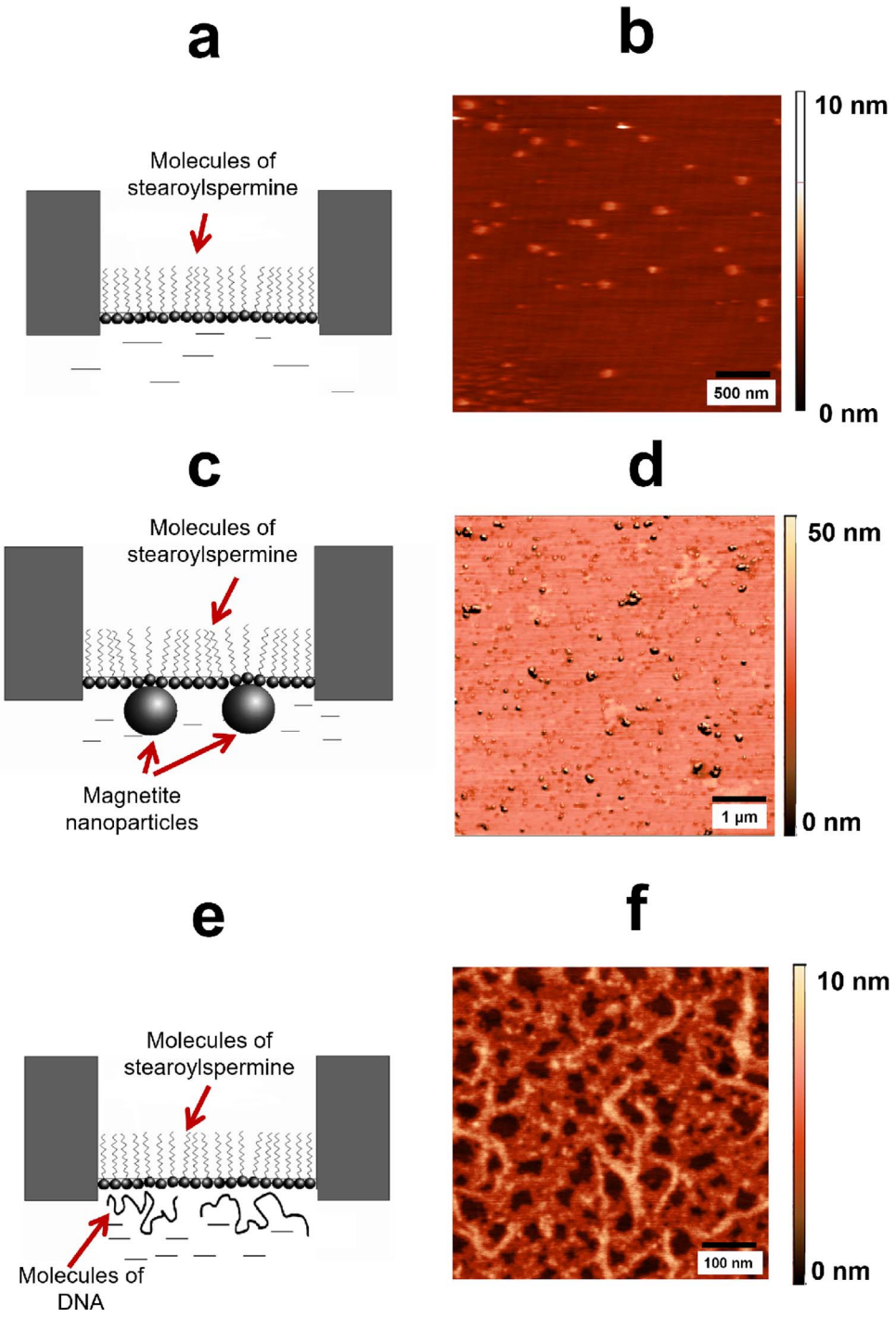

Fig. 2. (a)Scheme of condensed Langmuir monolayer. (b) Typical topographic image of the stearoylspermine Langmuir monolayer deposited onto the fresh mica substrate surface at monolayer surface pressure value 27 mN/m. (c) Scheme of formation of nanocomposite Langmuir monolayer via adsorption of colloid ligand-free magnetite nanoparticles from an aqueous subphase on the surface of stearoylspermine Langmuir monolayer. (d)Typical topographic image of nanocomposite stearoylspermine monolayer LB film containing adsorbed magnetite nanoparticles and deposited onto the mica substrate surface at monolayer surface pressure value 27 mN/m, pH = 4. (e)Scheme of formation of interfacial complex of stearoylspermine Langmuir monolayer with DNA molecules on the surface of aqueous DNA solution. (f) Typical topographic image of the monolayer LB film formed by deposition of the complex stearoylspermine Langmuir monolayer with adsorbed DNA molecules onto the mica substrate surface at monolayer surface pressure value 27 mN/m, subphase pH = 6.2. Images were obtained by AFM technique in a tapping mode of operation at ambient conditions.

large quasi-spherical aggregates. Formation of chain aggregates is a typical feature of single domain ferromagnetic particles due to their interparticle magnetic dipole–dipole interactions [43].

Formation of interfacial complex of stearoylspermine Langmuir monolayer with DNA molecules on the surface of aqueous DNA solution is shown schematically on Fig. 2e. Fig. 2f presents characteristic topographical AFM image of corresponding polycomplex monolayer LB film deposited onto the surface of mica substrate. It follows from Fig. 2f that stearoylspermine/DNA interfacial complex has a planar net-like structure with diameter of pores in the range about 100–300 nm. Similar structures were observed earlier in the interfacial complexes formed by DNA and Langmuir monolayer of amphiphilic cationic compounds [62].

The presented results of experiments with stearoylspermine Langmuir monolayer indicate to the possibility for formation of interfacial planar stearoylspermine polycomplexes with colloid cationic magnetite nanoparticles and polyanions (in particular, DNA and PSS). Such complexes can be used for creation of various practically useful functional composite nanofilm structures (vesicles, capsules, films) formed by amphiphilic polyamine, iron oxide magnetic nanoparticles and polyelectrolyte molecules via sequential layer-by-layer adsorption reactions. In the next section we describe an example of such functional structure – novel biocompatible nanocomposite magnetic colloid liposomal vesicles.

3.2. Nanocomposite membranous vesicles based on the interfacial complexes of biogenic lipid, stearoylspermine and magnetite nanoparticles

We have fabricated new nanocomposite magnetic membranous vesicles (magnetic liposomes) via sequential adsorption of colloid ligand-free magnetite nanoparticles and polyanion molecules onto the surface of mixed phosphatidylcholine/stearoylspermine liposomes preformed by the conventional ultrasound method. The scheme of transformation of an ordinary lipid bilayer liposome into the nanocomposite magnetic membranous vesicle is presented on Fig. 3.

Cationic stearoylspermine molecules do not form stable liposomes themselves and formation of stearoylspermine-containing liposomes is possible via incorporation of stearoylspermine molecules into the conventional liposomal membrane matrix. We have formed successfully the mixed liposomes consisted of biogenic electrically neutral (zwitterionic) lipid phosphatidylcholine and cationic stearoylspermine molecules with molar ratio 4:1, correspondingly. The synthesis of small mixed unilamellar liposomes composed by phosphatidylcholine and stearoylspermine molecules begins with mixing of stock solutions of phosphatidylcholine and stearoylspermine in chloroform, and the molar fraction of stearoylspermine molecules in the final mixture was 20%. The mixed solution was then dried using a rotary evaporator resulting in formation of the mixed lipid/stearoylspermine film on a wall of the evaporator flask. Further contact of the dry mixed phosphatidylcholine/stearoylspermine film with an aqueous buffer solution (pH = 4.5) resulted in the formation of multilamellar mixed liposomes. After that the aqueous multilamellar liposomes suspension was treated for 10 min by ultrasound of 22 kHz frequency in the continuous mode. Ultrasound treatment was necessary to obtain unilamellar liposomes with smaller size and a narrow dispersity. That procedure is conventional and efficient for preparation of small unilamellar liposomes [63]. The obtained liposomes were then separated from the titanium dust using centrifugation for 5 min with speed of 12000 rotations/min. Concentration of prepared liposomal suspension corresponded to 1 mg of amphiphile per ml. The typical size (hydrodynamic diameter) of the obtained mixed liposomes as determined by dynamic light scattering was 150–300 nm for different experiments, and the measured electrophoretic mobility corresponded to mean electrostatic surface ζ-potential value about +8 mV. To form nanocomposite magnetic liposomes the colloidal ligand-free magnetite nanoparticles were adsorbed onto the membrane surface of preformed mixed phosphatidylcholine/stearoylspermine liposomes. Adsorption of cationic magnetite nanoparticles onto the cationic liposomes was carried out by adding a certain volume of magnetite nanoparticles aqueous suspension to the aqueous suspension of mixed liposomes at pH = 4.5 with following incubation at ambient conditions. The resulting nanocomposite membranous vesicles were characterized by TEM, AFM, EMR and dynamic light scattering techniques. Electrophoretic mobility of nanocomposite liposomes with adsorbed magnetite nanoparticles corresponded to mean electrostatic surface ζ-potential value about +20,5 ± 0.5 mV. The increase of +12 mV in the value of surface electrostatic potential of nanocomposite vesicles compared to the initial surface potential of mixed cationic phosphatidylcholine/stearoylspermine liposomes was caused by the presence of adsorbed cationic magnetite nanoparticles on the liposomal membrane surface. In our approach to the formation of nanocomposite magnetic liposomes we used a non-standard method based on the adsorption of cationic magnetite nanoparticles onto the surface of mixed liposome membrane also having positive charge caused by protonated aminogroups of stearoylspermine. Thus, overall positive surface charge of liposomal membrane increased due to the adsorption of magnetite nanoparticles and was a strong anticoagulant factor resulting in the prevention of any aggregation of liposomes during the addition of magnetite nanoparticles in the suspension of mixed phosphatidylcholine/stearoylspermine liposomes. The binding of magnetite nanoparticles with liposomal membrane surface and formation of interfacial complexes was determined by corresponding chemical affinity of polyamine moiety of stearoylspermine and the nano-phase iron oxide

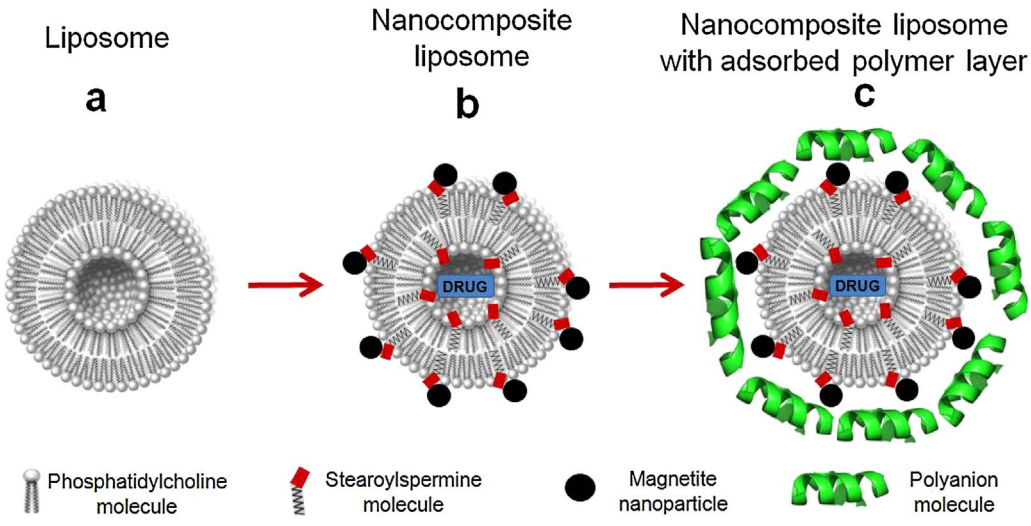

Fig. 3. Scheme of the structure of nanocomposite magnetic liposomes (b and c) and of its formation stages starting from the conventional phosphatidylcholine liposome (a). The formation procedure comprised the preparation of mixed phosphatidylcholine/stearoylspermine liposomes followed by binding of colloid ligand-free cationic magnetite nanoparticles onto the surface of preformed mixed liposome membrane (stage b) and then adsorption of a layer of polyanion molecules (stage c).

surface. Similar processes also occurred in the experiments described above on the adsorption of cationic magnetite nanoparticles onto the surface of stearoylspermine Langmuir monolayer (Figs 1 b, 2 c, d). This approach ensured an increase in the colloidal stability of the resulting nanocomposite liposomes and allowed to eliminate aggregation of liposomes in the process of formation of their interfacial complexes with colloid magnetite nanoparticles. At the same time, the permeability of nanocomposite liposomes to ions (Na^+, Cl^-) did not change noticeably in comparison with conventional phosphatidylcholine liposomes.

Figs. 4a and 4c present characteristic TEM images of nanocomposite liposomes with adsorbed magnetite nanoparticles. Fig. 4c is a detailed TEM image of such liposome. It follows from the TEM data that those liposomes have a quasi-spherical shape with a mean diameter about 200 nm corresponding well to the above mentioned liposome size data obtained by the dynamic light scattering measurements. It also follows from Figs. 4a and 4c that magnetite nanoparticles are fairly uniformly distributed over the surface of the nanocomposite liposomes with mean interparticle distance about 10 nm. Magnetite nanoparticles did not demonstrate any tendency to agglomerate and clustering when adsorbed on the liposome membranes and the liposomes also did not form aggregates due to the efficient permanent electrostatic repulsion during the binding process of cationic magnetite nanoparticles onto the cationic liposomal membrane surface. Fig. 4b represents characteristic corresponding topographic AFM image of the nanocomposite magnetic liposome localized on the mica substrate surface. To prepare the sample for AFM measurements the cationic nanocomposite liposomes were adsorbed onto the anionic fresh mica surface from the aqueous liposomal suspension and then washed with following drying. The image of the liposome on Fig. 4b indicates to its rather spherical shape and diameter corresponding to above TEM and dynamic light scattering data.

Magnetic properties of synthesized magnetite nanoparticles and nanocomposite magnetic liposomes were investigated using EMR method. EMR spectra of suspensions of magnetite nanoparticles and of nanocomposite magnetic liposomes are shown on Fig. 5. These spectra are typical for single domain colloidal magnetic iron oxide nanoparticles (magnetite, maghemite) which are superparamagnetic at room temperature [23]. The effective EMR signal width ΔH_{pp} was 620 ± 10 Oe for colloidal suspension of magnetite nanoparticles and 520 ± 10 Oe for nanocomposite liposomes with immobilized magnetite nanoparticles adsorbed onto the liposome membrane outer surface.

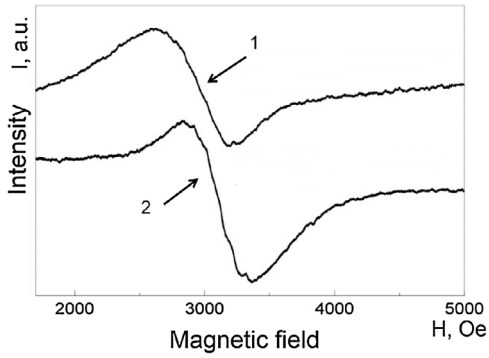

Fig. 5. EMR spectra. Curve 1–Aqueous suspension of colloid ligand-free magnetite nanoparticles. Curve 2–Aqueous suspension of nanocomposite magnetic liposomes containing magnetite nanoparticles adsorbed onto the liposome membrane surface.

Also, the resonance field value B_{res} in case of immobilized magnetite nanoparticles was increased by about 100 Oe as compared with B_{res} for free colloidal magnetite nanoparticles in the aqueous suspension. It is known that "free" colloidal magnetic nanoparticles in a suspension tend to form aggregates of various shapes due to the magnetic dipole–dipole interactions. Due to the interpartical magnetic interactions in such aggregates the efficient local magnetic field values are different from the external magnetic field value what results in a broadening of the EMR spectrum and in its shift to lower values of the magnetic field. Therefore, the observed differences in ΔH_{pp} and B_{res} values between EMR spectra of colloidal magnetite nanoparticles suspension and nanocomposite magnetic liposomes with the same magnetite nanoparticles indicate to the restricted mobility and inability to form aggregates in case of magnetite nanoparticles immobilized on the nanocomposite liposome membrane surface.

3.3. Nanocomposite membranous vesicles based on the interfacial complexes of biogenic lipid, stearoylspermine, magnetite nanoparticles and polyanion molecules

There is possibility for further functionalization and modification of the formed cationic nanocomposite magnetic liposomal vesicles via adsorption of additional components as, for example, appropriate nanoparticles or polyelectrolyte molecules. Thus, polyanions can be used

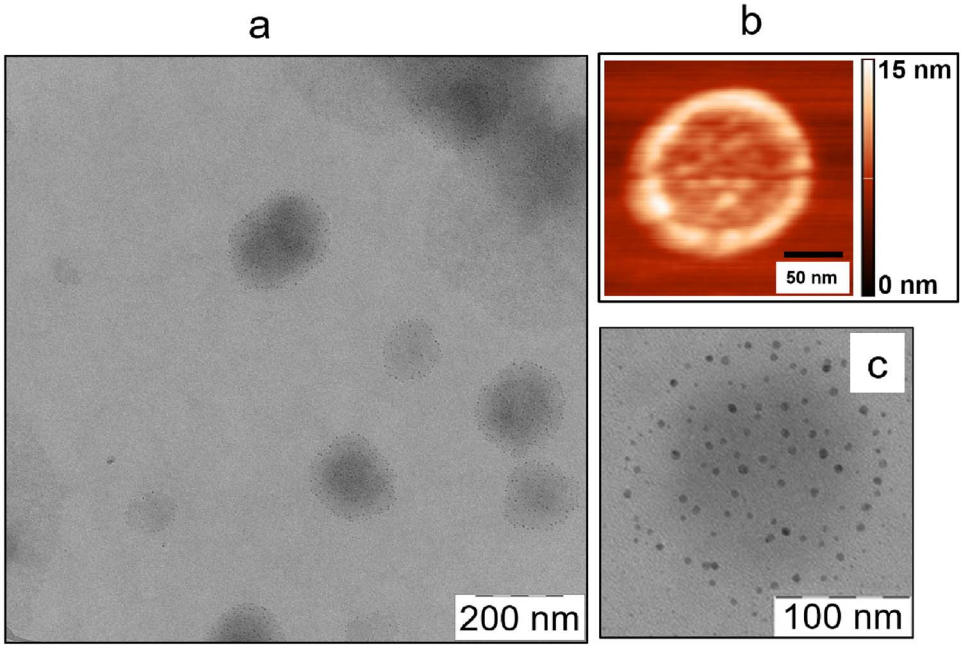

Fig. 4. (a)Transmission electron micrographs of the prepared mixed nanocomposite liposomes composed by phosphatidylcholine/stearoylspermine with 4:1 molar ratio and with magnetite nanoparticles adsorbed on the outer surface of the liposomal membrane. (b)Typical AFM topographic image of the nanocomposite liposome adsorbed on the mica substrate surface. The image was obtained using AFM technique in a tapping mode of operation at ambient conditions. (c)Enlarged TEM image of the typical nanocomposite liposome.

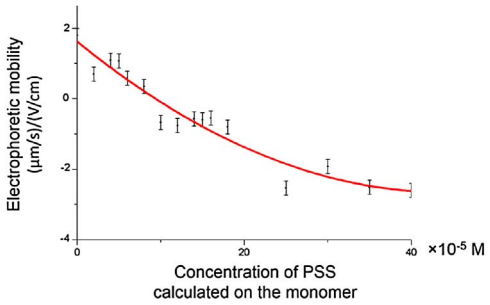

Fig. 6. The dependence of experimentally measured electrophoretic mobility value of nanocomposite liposomes on the concentration of polyanion PSS into the liposomal suspension.

to form an adsorption layer on the surface of cationic colloid vesicles resulting in the changes in surface charge sign and value [5]. We have studied interaction of polyanion DNA and PSS molecules with cationic nanocomposite membranous vesicles described in Section 3.2. The changes in electrophoretic mobility of cationic nanocomposite liposomes caused by adsorption of polyanions on their membrane surface were measured experimentally. Fig. 6 shows the dependence of electrophoretic mobility value of the nanocomposite liposomes on the concentration of polyanion PSS into the liposomal suspension. As seen in Fig. 6 the electrophoretic mobility corresponding to surface potential value of initially positively charged liposomes decreases with increasing the concentration of PSS in the liposomal suspension and the membrane surface eventually becomes negatively charged due to the binding of anionic PSS molecules. The saturation of PSS adsorption to nanocomposite liposomes corresponded to the state of maximal negative surface charge, which did not change with further increase in the concentration of PSS in the liposomal suspension as it is seen from Fig. 6.

The dependence of electrophoretic mobility value of prepared nanocomposite liposomes on the concentration of the other studied polyanion DNA in the nanocomposite liposome suspension was in the main features similar to that for PSS (Fig. 6) what implied the similar character of polyanion adsorption and formation of complex anionic layer on the surface of nanocomposite liposomes. Such polyelectrolyte layers on the surface of nanocomposite liposomes can provide their additional stabilization [63].

Fig. 7a shows characteristic TEM image of nanocomposite membranous vesicles with adsorbed DNA molecules and Fig. 7b present corresponding characteristic topographic AFM image of those vesicles localized onto the mica substrate. Adsorption of DNA on nanocomposite liposomes was carried out at saturating DNA concentration 0.2 mg/ml (about 5×10^{-4} M per monomer). Some aggregation of vesicles occurred in the process of DNA addition into the suspension of nanocomposite liposomes which manifests itself as a cellular structures in Fig. 7. The Images 7a and 7b being obtained by independent methods illustrate well the basic structural features of the individual nanocomposite vesicles present in aggregates and having a diameter in the range of 100–200 nm with noticeable dense interfacial polyelectrolyte complex layer.

For potential applications of the prepared nanocomposite liposomes as drug or DNA carriers the important point is their biocompatibility. Practically all components of such liposomes are present in living organisms – biogenic lipid phosphatidylcholine is the main component of the cell membranes, iron oxide magnetic nanoparticles are widely spread in nature and as indicated above can be found in various organisms in norm and pathology. Now some formulations containing magnetite nanoparticles have already gained approval by the Food and Drug Administration (FDA) for use in humans [64]. Synthetic stearoylspermine is composed of biogenic molecules stearic acid and spermine connected by the amide bond which can be enzymatically cleaved allowing their metabolization in a cell.

The formed nanocomposite magnetic liposomes with cationic outer membrane surface containing polycomplexes of stearoylspermine and magnetite nanoparticles represent a platform for further functionalization via sequential adsorption of additional components, in particular, polyanions including DNA. That makes them potentially useful for applications in nucleic acid transfection and magnetofection methods. It should be noted also that the suspensions of prepared colloidal nanocomposite liposomal vesicles showed reduced degradation and several times extended stability in comparison with conventional phosphatidylcholine liposomes what is important for their potential use in practice.

The other important point also is the possibility to change in a controllable way the permeability of liposomal membrane for controlled drug delivery applications. The presence of magnetic conducting magnetite nanoparticles bound on liposomal membrane gives opportunities to navigate the nanocomposite liposomes with applied magnetic fields and trigger the release of encapsulated drug or other compounds controllably in response to the appropriate external physical stimuli. Nanocomposite liposomes and polyelectrolyte capsules containing magnetite nanoparticles can be activated by electromagnetic field effects via locally heating of the nanocomposite shell or the membrane matrix [36,65,66]. Also, changes in permeability of nanocomposite magnetic liposomes can be caused by external magnetic field as a result of magnetic deformation effect present in a number of nanocomposite magnetic vesicles [67,68]. Our recent results indicate to the efficiency of ultrashort high voltage electric pulses in the activation of nanocomposite membranous vesicles containing conducting nanoparticles including magnetite nanoparticles [69]. The additional substantial advantage of nanocomposite vesicles containing magnetic iron oxide nanoparticles is their multi-functionality as potential agents for contrasting and diagnostics using magnetic resonance imaging and at the same time for therapy via hyperthermia treatment using alternating electromagnetic field. Also, precise controlled spatial localization of magnetic vesicles and capsules resulting in their targeting in the body

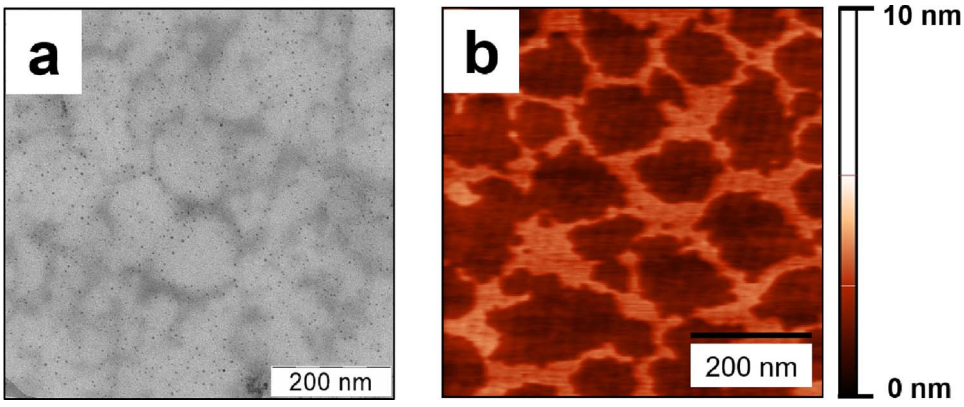

Fig. 7. Images of nanocomposite phosphatidylcholine/stearoylspermine (4/1) liposomes with bound magnetite nanoparticles and adsorbed DNA layer. (a) TEM image (b) AFM image obtained using AFM technique in a tapping mode of operation at ambient conditions.

can be realized with the use of appropriate external magnetic field gradients. As a result, the presented nanocomposite magnetic liposomes can be considered as a promising prototype of new multi-functional and multiple stimuli-responsive magnetic vesicular carrier for controlled drug and gene delivery, and for other bio-medical applications.

4. Conclusions

New nanocomposite nanofilm structures such as Langmuir monolayers, Langmuir-Blodgett films and biomimetic membranous vesicles composed of biogenic lipid phosphatidylcholine, synthetic amphiphile stearoylspermine (a derivative of biogenic polyamine spermine and stearic acid), colloid magnetite nanoparticles and polyanion molecules (PSS and DNA) have been formed and studied. The principal point of those structures was formation of quasi-planar interfacial polycomplexes which comprise water-insoluble cationic stearoylspermine molecules and aqueous phase components – cationic ligand-free colloidal magnetic iron oxide nanoparticles and polyanion molecules. Langmuir monolayer and LB film techniques have proved as an efficient tool for formation and study of such complexes. Characteristic changes of stearoylspermine Langmuir monolayer compression isotherms caused by interfacial complexation processes were observed. Stearoylspermine polycomplexes with magnetite nanoparticles and polyanion molecules formed on the outer membrane surface of liposomes provided extended colloidal stability of the nanocomposite liposomes along with their improved functionality – possibilities for further adsorption of additional aqueous phase functional components and sensitivity to magnetic and electromagnetic fields providing their controlled spatial localization and actuation by external physical stimuli. The data obtained give evidence that the prepared biocompatible nanocomposite magnetic membranous vesicles can be a base for development of new multi-functional magnetic biomimetic vesicular carriers perspective for controlled drug and gene delivery, and for other bio-medical applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfa.2017.07.035.

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