DEVELOPMENT OF ETHYLENE OXIDE AND PROPYLENE OXIDE COPOLYMER-BASED NANOPARTICLES FOR ENHANCING ANALYSIS AND INTRACELLULAR DELIVERY OF SOME BIOLOGICALLY ACTIVE SUBSTANCES

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ABSTRACT

DEVELOPMENT OF ETHYLENE OXIDE AND PROPYLENE OXIDE COPOLYMER BASED NANOPARTICLES FOR ENHANCING ANALYSIS AND INTRACELLULAR DELIVERY OF SOME BIOLOGICALLY ACTIVE SUBSTANCES

Owing to their unique physicochemical and biological properties ethylene oxide and propylene oxide block copolymers (Pluronics) are of considerable interest in the field of development of biocompatible materials and especially new drug delivery systems. Present work is dedicated to the construction of Pluronic-based nanoparticles for intracellular delivery of low molecular weight drugs and SERS analysis of biomolecules and living cells. So far, very little is known about the effect of polymer structure and introduction of chemical groups on biological properties of Pluronics. In this study Pluronics of different structure were modified with terminal carboxylic groups. According to cytotoxicity study, modified Pluronics were much less toxic for human cells than unmodified polymers. At the same time, introduction of carboxylic group did not affect the ability of Pluronic to increase cytotoxicity of anticancer drug doxorubicin towards MDR human cells. We ascertained an effect of modified Pluronics on cellular ATP level and membrane fluidity as well as localization of doxorubicin-Pluronic complexes inside the cells.

In order to fabricate SERS probes citrate-stabilized silver nanoparticles were modified with a thin layer of Pluronics of different structure. Such a modification resulted in significant amplification of SERS signal of certain fluorescent dyes and metabolites of aromatic nature probably due to the promotion of their interaction with the probe. Polymer-modified nanoparticles were applied for SERS detection rhodamine 6G released from cells into surrounding medium and certain cellular metabolites which presumably correspond to adenine nucleotides. The results obtained in this study are of particular interest for the development of informative SERS assays for analysis of drugs, fluorescent dyes and cellular metabolites.

ÖZET

BİYOLOJİK OLARAK AKTİF MADDELERİN HÜCRE İÇİ TAŞINMASI VE ANALİZİNİ GELİŞTİRMEK İÇİN ETİLEN OKSİT VE PROPİLEN OKSİT KOPOLİMER TEMELLİ NANOPARÇACIKLARIN GELİŞTİRİLMESİ

Etilen oksit ve propilen oksit blok kopolimerler(Pluronic) kendilerine özgü fizikokimyasal ve biyolojik özellikleri nedeniyle biyouyumlu malzemeler ve özellikle yeni ilaç taşıma sistemleri geliştirme alanlarında oldukça dikkat çekmektedir. Bu çalışma, düşük moleküler ağırlıklı ilaçların hücre içine taşınması; biyomoleküllerin ve canlı hücrelerin YZRS (Yüzeyde Zenginleştirilmiş Raman Saçılması) analizi için Pluronic temelli nanoparçacıkların yapımı üzerinedir. Şimdiye kadar polimer yapısının ve yeni kimyasal gruplar eklenmesinin Pluronic'lerin biyolojik aktivitesine etkisi hakkında çok az şey biliniyordu. Bu çalışmada değişik yapıdaki Pluronic'ler terminal karboksil gruplarıyla modifiye edilmiştir. Hücre toksisitesi deneylerine göre karboksil modifiyeli Pluronic'lerin insan hücrelerine toksik etkisi, modifiye olmamış polimerlere göre çok daha azdır. Aynı zamanda, karboksil grubunun eklenmesi Pluronic'in, antikanser ilacı doxorubicin'in MDR insan hücrelerine karşı toksisitesini artırması özelliğini etkilememiştir. Modifiye edilmiş Pluronic'lerin hücresel ATP seviyesini, hücre zarı akışkanlığını ve doxorubicin-Pluronic kompleksinin hücre içindeki lokalizasyonunu etkilediği gözlemlenmiştir.

YZRS probları üretmek için sitratla indirgenmiş gümüş nanoparçacıklar farklı yapılardaki Pluronic'lerle ince bir katman oluşturularak modifiye edilmiştir. Bu modifikasyon belirli florasans boyaların ve aromatik yapıdaki metabolitlerin prob ile etkileşimlerinin artması sebebiyle YZRS sinyalinde önemli ölçüde artışa neden olmuştur. Polymer ile modifiye edilmiş nanoparçacıklar, hücrelerden etraflarındaki besiyerine salınan Rhodamine 6G ve Adenin olması muhtemel belirli hücresel metabolitlerin YZRS ölçümleri için kullanılmıştır. Bu çalışmadan elde edilen sonuçlar, ilaçların, florasans boyaların ve hücresel metabolitlerin analizi için kullanılacak YZRS metodlarının geliştirilmesinde özellikle önem taşımaktadır.

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ADP	Adenosine diphosphate
AFM	Atomic force microscopy
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
AgNP-Pluronic	Silver nanoparticles Pluronic complexes
A549	Human lung adenocarcinima epithelial cells
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CLS	Classic least squares
CMC	Critical micellation concentration
DLS	Dynamic light scattering
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DPBS	Dulbecco’s modified buffer saline
Hep3B	Human hepatoma cells
HLB	Hydrophilic-lipophilic balance
IR	Infra red
MDR	Multiple drug resistance
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
LDA	Linear discriminant analysis
MW	Molecular weight
PCA	Principle component analysis
PdI	Polydispersity index
PEG	Polyethylene glycol
PEO or EO	Poly ethylene oxide
P-gp	P-glycoprotein
PLS	Partial least squares
PPO or PO	Poly propylene oxide

PSA	Penicillin streptomicyn antimicotice
RH6G	Rhodamine 6G
SEM	Scanning electron microscopy
SERS	Surface-enhanced Raman spectroscopy
Ver	Verapamil

1. INTRODUCTION

Poly(ethylene oxide)-poly-(propylene oxide)-poly(ethylene oxide) triblock copolymers, also known as Pluronics, are nonionic amphiphilic polymers with various physicochemical and biological properties. The varying hydrophilic-lipophilic balance (HLB) of Pluronics is defined by the ratio of ethylene oxide and propylene oxide units [1]. Such polymers are of considerable interest for the development of biocompatible materials, immunoadjuvants, especially, in the field of drug delivery where Pluronics are shown to promote the intracellular transport of some drugs and nucleic acids both *in vitro* and *in vivo* [2-4]. Owing to their properties, Pluronics represent a promising class of polymers for the development of novel biomedical materials.

One of the key problems of pharmacology is the development of novel modifications of Pluronics, which exhibit less toxicity and higher efficiency in drug delivery. A promising approach is to introduce a reactive chemical group into the parent polymers. In our study, we modified Pluronics of different structure with terminal carboxylic groups. The modification was verified using a variety of physicochemical methods. The association of carboxylated polymers in water solutions was analyzed by hydrodynamic diameter and zeta potential measurements using dynamic light scattering (DLS) technique.

According to cytotoxicity studies, modified Pluronics are much less toxic for human cells than unmodified polymers. At the same time, chemical modification doesn't affect the ability of Pluronics to increase the cytotoxicity of anticancer drug doxorubicin towards cancer cells in culture. We studied the effects of modified Pluronics on the levels of cellular ATP and also on membrane fluidity. On the basis of our results, putative mechanisms for the pharmacological effects of Pluronics are proposed.

Another promising application of Pluronics is the development of composite nanoparticle probes for the analysis of organic molecules and living cell. In this study we designed novel Pluronic-modified noble metal nanoparticles, which were tested as SERS probes for the detection of dyes and cellular metabolites. Modification of silver

nanoparticles with a thin film of Pluronic copolymers resulted in a considerable increase of the SERS signal of organic molecules (rhodamine 6G, doxorubicin, aminofluorescein, ATP) adsorbed on the surface of composite nanoparticles.

Pluronic-modified silver nanoparticles did not allow us the intracellular detection of rhodamine 6G (RH6G) accumulated in human cells due to strong interference with cell components. Alternatively we used these nanoparticles to probe cellular metabolites and drugs released form cells into the surrounding media. Under experimental conditions, the modified nanoparticles allowed the selective detection of trace amounts of RH6G released from cells into the extracellular solution.

We also studied a possibility of using Pluronic-modified nanoparticle for the detection of metabolites released from cells. We found that viable human cells suspended and incubated in DPBS buffer released certain metabolites into the surrounding buffer. Among released metabolites adenine nucleotides were detected by characteristic Raman band of the nucleotides at around 720 cm^{-1}. The band intensity at this wavenumber is increased with the increased cell incubation time in a buffer and this is apparently due to the intracellular level of ATP. The ATP level is of particular interest for assessment of cell metabolism, energetic status and certain pathologies.

Thus, the results obtained in the current study contribute to the development of Pluronic-based carriers for drug delivery as well as SERS analysis of cellular transport of biologically relevant compounds in model systems.

2. THEORETICAL BACKGROUND

2.1. BLOCK COPOLYMERS OF ETHYLENE OXIDE AND PROPYLENE OXIDE

2.1.1. Nomenclature

Polyoxymethylenes or polyacetales are linear or branched high-molecular weight compounds, whose maine chain is composed of simple ether bonds. Polyacetales usually can be derived from epoxides. Ethylene oxide, propylene oxide and, to a lesser degree, butylenes oxide are monomers which are components of polyacetales. In our work we will consider only water-soluble polyacetales - block copolymers of ethylene oxide and propylene oxide. Block copolymers of ethylene oxide (PEO) and propylene oxide (PPO), named under the international nomenclature as poloxamers, are products of large-capacity synthesis and are widely used as polymerous nonionic surfactants [5]. Three block copolymers with a symmetric constitution of PEO-PPO-PEO are most widely used and manufactured by the firm "BASF" under the trade mark «Pluronic», and by the firm «ICI Ltd» under the trade mark «Synperonic».

The nomenclature of commercial products is based on an alphanumeric notation. The letter means the physical condition or outward of a product (P- paste, L-liquid, F-firm), last figure means the mass fraction of ethylene oxide block expressed in tens of percent, other figures - quantity of propylene oxide blocks, divided on 5. For example, Pluronic L61 is a liquid (L), contains 1×10=10 % of ethylene oxides on mass and 6×5=30 parts of propylene oxides. Unpatented nomenclature (Poloxameres) includes number, first two figures of it means the mass of the hydrophobic block divided on 100, last figure means a mass fraction of ethylene oxide parts in a molecule. Naturally, the average values are used in nomenclature due to heterogeneity, although the heterogeneity index and composite heterogeneity of Pluronics received by anionic polymerization is insignificant [6].

2.1.2. Physicochemical Properties of Pluronics

Physicochemical properties of Pluronics are influenced by the presence in their structure of two blocks shown in Figure 2.1, different in hydrophobicity at ambient temperature. The hydrophobicity of the material is determined by contact of the hydrocarbon chains with water, which are unprofitable from the entropic factor point of view. Poly ethylene oxide contains in the main chain atoms of oxygen in sp3 hybridizations which are capable to form hydrogen bridges with water. In the absence of large substitutions, as in case of poly ethylene oxide, the polymeric macromolecule is flexible and can successfully interact with water. Thereby the solubility of the polymeric molecule is determined by the hydrophilic ethylene oxide fraction. The appearance of methyl substitutions significantly changes this property of PEO. An increase in the rigidity of the macromolecule and it geometry change leads to a significant reduction in hydrophylicity. Thereof, under usual conditions the PPO block is hydrophobic. The combination of these two chains, hydrophilic and hydrophobic, in one macromolecule leads to amphiphilic property reception by nonionic surfactant [7].

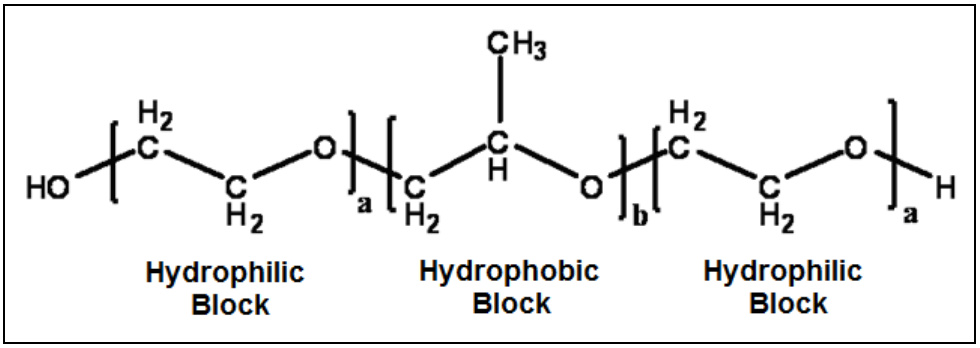

Figure 2.1. Chemical structure of PEO-PPO-PEO block copolymer

As well as the majority of other amphiphilic compounds, Pluronics organize in different associates, for example they can form micelles in water solutions seen in Figure 2.2. This phenomenon can be explained by the tendency of a system to minimize the area of PPO block contacts with water. It is known, that Pluronics possess high ability to micellation. The dependence of Pluronic critical micellation concentration (CMC) from

their composition described by the empirical correlation equation received from the work of Loh with coauthors [8]:

$$R \times T \times \ln CMC = -7.68 - 0.01 \times n - 0.15 \times m \qquad (2.1)$$

According to this equation, an increase in polymerization degree of hydrophobic (m) and hydrophilic (n) blocks promotes depressing of CMC, which is favors, the formation of micelles. At the same time, the influence of hydrophilic PEO block length on micellation is less expressed. In constant to PPO block, increase in PEO block length leads only to minor alteration of CMC. It means that, PPO block length is a determinative value for the micellation process [8].

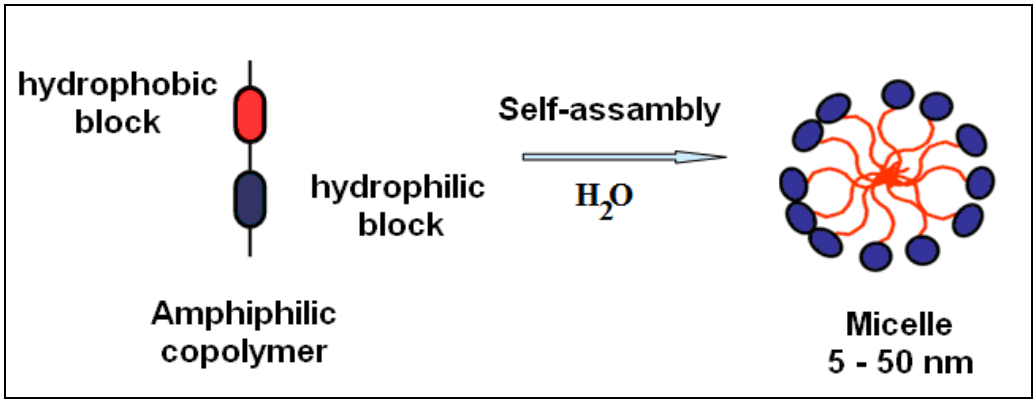

Figure 2.2. Scheme of amphiphilic block copolymer organization in micelle.

It is also shown that, at constant interrelation of EO/PO in copolymer composition, the molecular weight augmentation leads to CMC reduction. Thus, Pluronics with higher molecular weight are more favored the micellation in water solutions. An ability of block copolymers to micellation is strongly depends from the EO and PO blocks localization in a copolymer molecule [9]. The method of small angle light scattering showed that two-block copolymers are characterized by considerably smaller CMC value, than three-block copolymers composed of $EO_{n/2}PO_mEO_{n/2}$. Interestingly that copolymers of reversed constitution $PO_{m/2}EO_nPO_{m/2}$ possessed lower ability to micellation, than copolymers of $EO_{n/2}PO_mEO_{n/2}$ architecture. Most likely, this fact specifies an importance of chains location for the process of micelle formation. Thus, amphiphilic properties of Pluronics are

defined by hydrophobic PPO block and to a lesser degree by the PEO block hydrophilicity, which basically defines the solubility of the polymer in water [8, 9].

Amphiphilic properties of Pluronics cause the possibility of their interaction with biological colloidal objects such as proteins and lipids. It is possible to assume that, the degree of this interaction depends on the degree of amphiphilic properties. In turn, quantitative parameters of amphiphilic properties such as CMC are defined in the length of PEO and PPO blocks, and also their relative positioning. There are around 20 types of Pluronics that differ in their PEO and PPO block length and correspondingly in their physicochemical properties [7]. Table 2.1 assumes some important characteristics of Pluronics produced by the BASF corporation.

Table 2.1. Characteristics of commercial Pluronics produced by BASF corporation [7]

Pluronic	MW [a]	Ethylene Oxide [b]	Propylene Oxide [b]	HLB [c]	Opacity point of 1% solution , ^{o}C	CMC [d], mol/L
L35	1900	21.59	16.38	19	73	$5.3*10^{-3}$
L43	1850	12.61	22.33	12	42	$2.2*10^{-3}$
L44	2200	20.0	22.76	16	65	$3.6*10^{-3}$
L61	2000	4.55	31.03	3	24	$1.1*10^{-4}$
L62	2500	11.36	34.48	7	32	$4.0*10^{-4}$
L64	2900	26.36	30.00	15	58	$4.8*10^{-4}$
F6	8400	152.7	28.97	29	>100	$4.8*10^{-4}$
L81	2750	6.25	42.67	2	20	$2.3*10^{-5}$
P84	4200	38.18	43.45	14	74	$7.1*10^{-5}$
P85	4600	52.27	39.66	16	85	$6.5*10^{-}$
F87	7700	122.50	39.83	24	>100	$9.1*10^{-4}$
F88	11 400	207.27	39.31	28	>100	$2.5*10^{-5}$
L92	3650	16.59	50.34	6	26	$8.8*10^{-5}$
F98	13 000	236.36	44.83	28	>100	$7.7*10^{-6}$
L101	3800	8.64	58.97	1	15	$2.1*10^{-6}$
P103	4950	33.75	59.74	9	86	$6.1*10^{-6}$
P104	5900	53.64	61.03	13	81	$3.4*10^{-6}$
P105	6500	73.86	56.03	15	91	$6.2*10^{-5}$
F108	14 600	265.45	50.34	27	>100	$2.2*10^{-3}$
L121	4400	10.00	68.28	1	14	$1.0*10^{-3}$
P123	5750	39.20	69.40	8	90	$4.4*10^{-3}$
F127	12 600	200.45	65.17	22	>100	$2.8*10^{-3}$

[a] *averaged molecular weight*

[b] *averaged quantities of ethylene oxide and propylene oxide unimers*

[c] *hydrophilic-lipophilic balance (HLB)*

[d] *critical micellation concentration* (CMC)

2.2. PLURONIC INTERACTIONS WITH LIPID MEMBRANES

2.2.1. Transport of Materials Through Biological Membranes

One of the basic functions of a biological membrane is a barrier function with selective permeability between aqueous compartments divided by the membrane. Transport of materials through biological membranes can be carried out by means of three essentially different mechanisms [10]:

1. nonspecific diffusion through lipid bilayer (passive permeability);
2. pecific transport with participation of "transmitting agents";
3. mechanisms which are provided by significant changes of membrane architecture, for example, pinocytosis and transport of biopolymers.

Transport of low-molecular weight compounds which aren’t capable of interacting with specific cannels or transmitting agents is routinely described by the model considering dissolution of yielded material in a membrane and its diffusion through the bilayer. According to this model, the limiting stage is diffusion of a molecule in the lipid bilayer, the energy barrier on the phase boundary is usually neglected. To cross the bilayer a molecule should: (1) be adsorbed on the membrane, then overcome a surface interraction and a free energy barrier on the membrane border; (2) diffuse through bilayer; (3) leave from membrane to opposite side, again having to overcome an energy barrier on the phase boundary. Thus, transmembrane permeability for non-electrolytes is defined by two factors: a distribution coefficient of this material between the aqueous solution and membrane and the velocity of its diffusion through a membrane [10].

Studying of weak electrolytes transport, among of them many biologically active compounds, is of particular interest of researchers. Weak electrolytes are maintained in water solutions in a balance between the charged and uncharged forms. It is supposed, that transport of weak electrolytes through a membrane is defined by the transport of their not charged form on the mechanism of dissolution-diffusion [10, 11].

At the same time more and more experimental acknowledgement finds the alternative model of parallel channels based on the assumption that both charged and uncharged forms diffuses through the bilayer, and the transport of these two forms is differ significantly [10]. The dissolution-diffusion model and pore formation model differ in dependence of membrane permeability from it thickness. For the dissolution-diffusion model of uncharged molecules the weak correlation between these parameters was observed. The exponential interrelation between these parameters was predicted for channel model. Experimental data about the correlation between membrane permeability and it thickness had been collected for protons, potassium ions, water, urea and glycerine [6, 12].

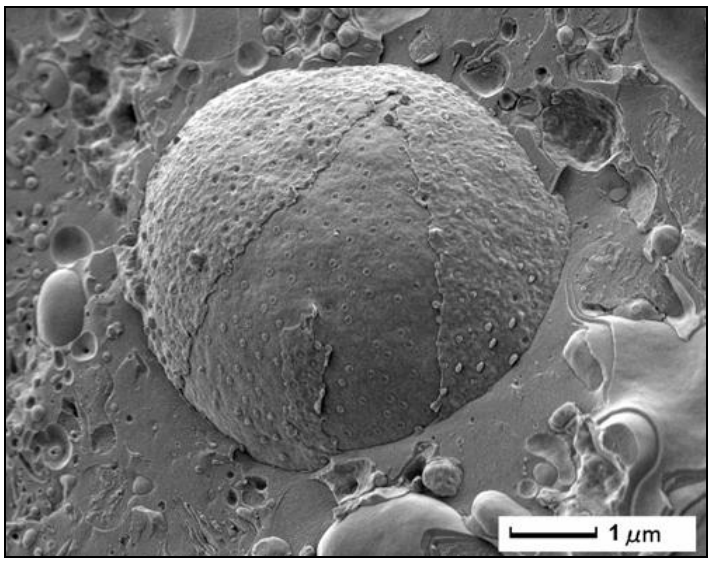

Figure 2.3. Numerous nuclear pores on the surface of the cell in a collecting duct of a uriniferous tubule, SEM image [13]

In case of water and uncharged molecules weak correlation between these parameters was observed, which means that the process can be described by dissolution-diffusion model. In contrast, membrane permeability for protons and potassium ions decreases for two orders of magnitude when the length of lipid radicals increased from 14 carbons to 18, further augmentation of radicals didn't influence the permeability. Thus, when membrane thickness is small the ion transport yields the model of pore formation [12].

The phase state affects the membrane permeability. Therefore, phase rearrangements leads to different «pore» and «defect» formation in a bilayer, which increases the membrane permeability for the water soluble ions [14]. Thus, the passive permeability of lipid bilayer is defined by its physical state. Study of cellular membrane permeability is extremely challenging.

It is known that different parallel processes overlap with the passive transport process. Among them endocytosis and exocytosis, active transport and transport through channels or pores controlled by environmental factors [14, 15]. Besides them, the transported material can be destructed as a result of metabolic processes in living cells [16]. Thus, the pristine data on passive lipid bilayer permeability can be received only by means of model systems. For transport processes studying different models are usually used, such as flat bilayer or lipid vesicles – liposomes.

Flat lipid membranes allow receiving the unique information about the conductive properties of the bilayer, superficial potential, boundary potentials and dipole potential [17-19]. At the same time, methodical difficulties of the flat membranes reception and it low stability restricts the model usage in research [20]. Other widely used modeling system is liposomes. They represent the colloidal formations consisting of small volume of an aqueous phase, isolated from solution volume by the lipid bilayer. Such "blisters" can be considered as the elementary model of a living cell. The possibility of liposome separation from surrounded solution by means of a chromatography or a dialysis is one of the advantages of the model. If pristinely put the material in surrounded solution, and then observe changes of its concentration outside and inside of liposomes, it is possible to define the lipid bilayer permeability. The cause of a passive transport is a difference of chemical potentials of material on the different sides of the membrane border. To create such difference of chemical potentials we should build the high concentration in surrounded solution, which is technically more convenient [21].

The lipid membrane permeability problem is intimately connected to pharmacology: in some cases pharmacological action of medicines is defined by their ability to penetrate through cellular membranes [22]. Therefore lipid membrane permeability in relation to medicines especially to antineoplastic drugs is in detail shined in the literature.

Interaction of daunomycin, doxorubicin and others antibiotics with lipid membrane leads to drugs embedding into the bilayer, which can be registered by changes of EPR-spectra of doxorubicin semiquinone, or by resonance energy transfer from diphenylhexatriene, piren to doxorubicine [23-26]. Doxorubicin localisation in the lipid bilayer has been studied by means of Lengmjurovsky monomolecular layers, and it has appeared that it antracyclic nuclei is dipped in phosphalipid phase, but the charged amino group is localized in the area of the glyceric residual [21]. It was revealed that doxorubicin distribution coefficient between lipid membrane and water solution strongly depends on membrane composition: introduction into the bilayer cholesterol molecule depresses the doxorubicin distribution in the membrane. Oppositely, anionic lipids introduction leads to substantial increase of doxorubicin adsorption on a membrane [26].

As a weak base doxorubicin can penetrate through bilayer in uncharged form like the majority of weak acids and bases do. Works of Garnier-Suillerot, Frezard and de Kruijff [27, 28] showed that doxorubicin is capable to penetrate through liposomes filled with DNA solution. It is known that DNA is capable to bind doxorubicin with a high constant, and as a result of this linkage antibiotic fluorescence is partially suppresed. This fact has been used for the doxorubicin transport through the membrane study. Using these model researchers showed that doxorubicin transport was carried out in the uncharged form, instead of salt, because decrease in pH leads to the significant rejection of doxorubicin infiltration [27].

Other experimental system has been offered in works of Cullis with coworkers [29]. They have considered an accumulation of some cationic medicines in the liposomes filled with an acidic buffer solution, whereas extralyposomic solution was filled with the pH 7.0 buffer. By means of this approach based on pH-induced accumulation of cationic antibiotic in liposomes, authors managed to receive liposomes containing antibiotics at high concentration. This research represents a great interest for prolonged therapy of cancer diseases.

Summing up, we will established that, for lipid bilayer permeability investigation, the usage of liposomes is methodically most simple, hence, this method is helpful for

observation of changes in lipid membrane under the influence of external factors, for example, under the influence of Pluronics.

2.2.2. Pluronics Interaction with Components of Biological Membranes

Pluronic interaction with serum albumin, α-himotripsin and phytohemagglutinin has been studied by means of gel chromatography, ultracentrifugation and spectroscopy. For this proteins and variety of Pluronics in the big range of concentration and temperature interval 20-40°C was shown, that simple mixing of components didn't formed complexes, and polymers didn't affected the structure of proteins [30].

However in other works has been shown that stable complexes of α-himotripsin with two-block copolymers (proxanols) nevertheless can be received by heating of components in water solutions [31], and also at action of high pressure [32]. Stable and catalytic active complexes himotripsin-proxanol has been received under the temperature schedule corresponding to initial state of protein denaturation (40-60°C) [31]. Products represented not covalent complexes of himotripsin and proksanol, gradually dissociating in water on initial components. Not covalent complexes conserving catalytic activity also have been received under the influence of high pressure [32].

Thermo induced complexes possess higher stability, than received under the influence of high pressure. According to authors, the mechanism of a thermo induced complex formation assumes more active penetration of PPO blocks in the protein globule, in comparison with complexes formed under high pressure. Most likely, such complexes are stabilized by hydrophobic interactions between PPO blocks of copolymers and hydrophobic parts of the protein globule [31, 32].

On the other hand, the absence of specific interaction between Pluronics and BSA or lysozyme has been shown in water solution [33]. We can assume that any interaction between proteins and polymers has an entropic nature and caused by interaction of hydrophobic chains of polymer with the hydrophobic fragments of protein exposed in the aqueous solution. Data about direct interaction of block copolymers with membrane associated proteins is absent in the literature.

The lipid bilayer composes of amphiphilic lipids, which form two hydrophilic surfaces and a hydrophobic core. Therefore, embedding or, at least, interaction of amphiphilic copolymers with colloid systems of lipid bilayer is quite probable. A series of works are devoted to study of block copolymers interaction with membranes. Lojewska with coauthors studied the influence of Pluronic F127 on some amphiphilic molecules embedding in a membrane. They used liposomes from egg phosphatidylcholine, containing amphiphilic sensitive label (di-10-ASPPS). The label strongly contacts with a membrane, but the process precedes slowly (half-cycle needs 10 hours). They revealed that the rate of di-10-ASPPS linkage with a membrane increased in the presence of F127 proportionally to Pluronic concentration. Authors have assumed that the dye molecules formed complexes with unimers or micelles of F127 before to be embedded in a membrane [34].

In the same work the influence of F127 on di-10-ASPPS and labeled lipid (FITS-PE) penetration into HeLa cells was studied [34]. The coloring of cells was observed only at the presence of F127. Probably, water unsolvable dyes di-10-ASPPS and FITC-PE can not be dissolve completely and stay as a dispersion of aggregates. Most likely, Pluronic micelles are capable to solubilize dye molecules, therefore, it facilitates the redistribution of the dye from extracellular solution to cytoplasm.

Firestone with coauthors studied the Pluronic influence on the lipid membrane organization [35]. By means of small-angle X-ray scattering they determined that Pluronics modifies the lipid bilayer, thus PPO block is embedded into the bilayer, and PEO block is exposed in an aqueous phase. The same authors studied an interaction of two-block copolymers of ethylene and propylene oxides with lipid bilayers [36]. They have shown that the length of PPO block is an important parameter influencing this interaction. By means of X-ray scattering they revealed that, PPO block, which length comparable with acyl radicals of lipids, was embedded into the membrane and formed the regular lamellar structure. Polymers with smaller length of PPO block didn’t formed the regular lamellas in the membrane. The degree of orderliness of received structures increased with PPO block augmentation and augmentation of polymer concentration.

Melik-Nubarov et al. provided future studies of tri-block and two-block copolymers interactions with lipid membrane and their influence of membrane permeability for small

solutes including doxorubicin [37]. They possessed that; the size of PPO block embedded into the bilayer for tri-block and two-block copolymers is differing significantly. Since two hydrophilic blocks tailoring the macromolecule of tri-block copolymer are exposed in the aqueous phase, PPO block is less conformational mobile and disturb the membrane more than PPO block of two-block copolymer. The schematic representation is shown in Figure 2.4. In case of two-block copolymer, it PPO block is fixed in the membrane only in one spot, which leads to lose in entropy energy and also in it disturbing influence on lipid membrane fluidity. As a result, Pluronic L61 affects the doxorubicin transport in cells more than two-block copolymer REP [37].

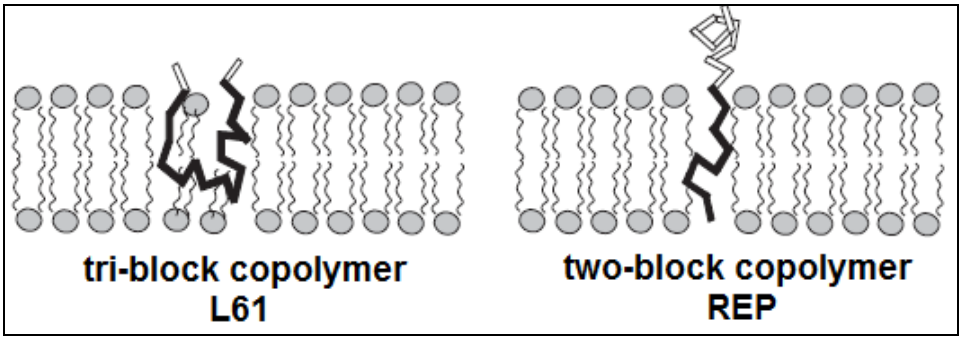

Figure 2.4. Schematic representation of tri-block and two-block copolymers interactions with lipid membrane [37]

The study of quantitative parameters of Pluronic interaction with lipid membranes is impossible without determination of the polymer quantity, which adsorbed on the surface of membrane. As Pluronics has no characteristic bands in UV-spectrum, and only two trailer functional groups can be chemically modified, the measurement of polymer quantity connected to the membrane represents a serious problem. One of the possible approaches has been offered in works of Kostarelos and Tadros with coauthors [38, 39]. Researchers observed some changes in lipid vesicles size when Pluronics was added to the suspension by the help of dynamic light scattering. As a result of polymers adsorption, diameter of liposomes increased, but at some concentration of Pluronic left on the plateau. Researchers assume that this plateau corresponds to liposome surface saturation by polymers. Authors counted the quantity of polymers adsorbed on the membrane using an assumption that PPO block was completely embedded into the bilayer, and the liposome size increment was exclusively invoked by the shell of PEO blocks. Authors have defined that in saturation

state polymers binds to the membrane in number of 1 macromolecule on 10-20 molecules of lipid [39].

Nevertheless, data cited above demonstrated an ability of Pluronics to be built in a membrane, an ability of Pluronics to strong interactions with lipid bilayer is challenged in the work of Schillen with coauthors [40]. In this work the phase diagram of the ternary system of soybean lecithin-water-Pluronic L121 has been studied. Pluronic L121 forms a lamellar phase in the aqueous medium, which indicates the presence of bilayer vesicle of L121 in a thin water solution. Researchers revealed that even at high Pluronic concentration the mixed phase Pluronic-lipid wasn't formed. Lipid vesicles and Pluronic vesicles were in symbioses, polymers didn't influenced the lipid lamellar phase.

In the same work even more indicative experiment has been made: liquid PPO was mixed with hexane, therefore the biphasic system was formed. After an establishment of the equilibriums, phases were divided and their NMR spectra were registered. It has appeared that PPO phase contained about 5 % of hexane, but the hexane phase at all didn't contained the appreciable quantities of PPO. This fact testifies that aliphatic hydrocarbons are bad solvents for PPO. The methods used in the yielded work (NMR and X-rays scattering) did not allow researches to see small amounts of Pluronics, bounded to bilayer or hexane phase. However these results revealed that we shouldn't expect the strong interaction between lipid bilayer and hydrophobic block PPO of Pluronics [40].

Nevertheless some interaction of Pluronics with lipid membranes exists, which confirmed by Johnsson's and coauthors data [41] received by the method of translucent electronic microscopy. It has been shown that Pluronics F127, F108, F87 with a long PEO chains adsorbs on the small mono lamellar liposomes, which leads to metamorphosis of spherical vesicles to disk bilayers. More hydrophobic Pluronics P105 and P85 invoked the reduction of liposome diameter without influencing their form. Thus, Pluronics interaction with lipid bilayer strongly depends from the polymer structure. The membrane composition also made an essential impact on this interaction: an addition of cholesterol inhibits the Pluronic interaction with a membrane.

Thus, in spite the fact that variety of works has been dedicated to study of Pluronics interactions with lipid membranes, the question on its quantitative assessment remains obscure to the present time. The influence of Pluronics on lipid membrane permeability is an important question of pharmacology. Pohl and Krylova studied the influence of Pluronic L61 on lipid membrane permeability for ions [42]. An addition of the polymer to the flat lipid bilayer, consisting from difitanoil-phosphatidylcholine, leads to significant increase of membrane conductivity. On this background sharp short-term splashes in conductivity was registered from time to time, which indicates the discovering-closings events of the canals. Quantitative assessments shown, that we can diminish the channel mechanism of conductivity, because of it small contribution to the general conductivity of a membrane in the presence of Pluronic. The basic mechanism, on which Pluronics facilitate transport of small ions through the bilayer, is similar to action of transmitting agents.

To investigate, does Pluronics have some selectivity to cations or anions, transmembrane gradient of potassium chloride was created and transmembrane current dependence from the enclosed potential was measured. It has observed that at zero potential Pluronic invoked a cation current of potassium chloride, which showed an ideal cationic selectivity of polymer. It is possible to assume, that Pluronics acts as transmembrane transmitting agents for metallic cations thanks to their ability to form coordination complexes with ions of metals. Formation of such complexes in a membrane should significantly depress a free energy barrier of cation transfer through lipid bilayer, which promotes the ion transport on a gradient of electrochemical potential [43]. Also it has been shown that Pluronics possesses the wide cationic selectivity: besides potassium ions, Pluronics facilitated as well conduction of calcium ions and even organic cations such as N-methyl-glucozamine.

The dependence of transmembrane ion transport invoked by Pluronics from polymer concentration was well described by square-law function of the polymer concentration [42]. Authors have assumed that at least two macromolecules of polymer form the complex with each cation. This assumption has received unexpected acknowledgement in recent work [44], in which the possibility of a phase separation of Pluronic molecules from lipids on the water-air border was shown. The monomolecular layers analysis by means of X-

rays reflectance has shown the presence of two different regions in the layer: the one are enriched by Pluronics, other by lipids, which means the phase separation. This data is also well coordinated with earlier results of Schillen and coworkers, in which the low thermodynamic compatibility of Pluronics and lipids was demonstrated [40].

Thus, summarising all listed facts, Pluronics being embedded into the membrane, aspires to precipitate out in a separate phase. Such enriched by Pluronic islands can serve as "conductors", which promotes the ion transport cross lipid bilayer, without contacts with it hydrophobic area shown in Figure 2.5. Whether the formation of such islands in a membrane influences on larger organic compounds infiltrations remains not clear.

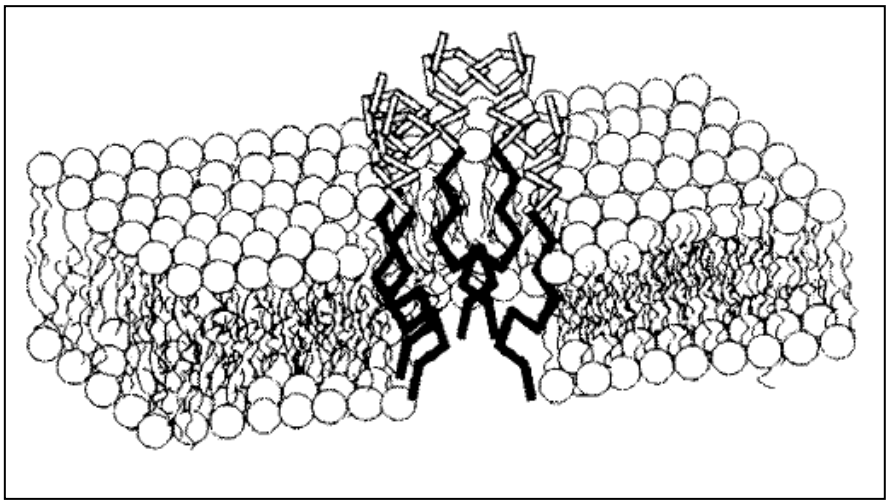

Figure 2.5. Pluronic island in the membrane, which can serve as a transducer for cations [45]

Jamshaid [46], and later Kostarelos [39] shown that as a result of Pluronic linkage to liposomes, the flow of hydrophilic dye karboksifluorestsein, incorporated into the intrinsic lumen of liposomes, appeared on the screen. Nevertheless, Pluronics were possessed as a steric stabilizer of liposome instead of PEO modified lipids in variety of works [47]. In spite the fact that, medicine included in such liposomes can be released quicker; their bioavailability is higher than the bioavailability of drugs incorporated in usual not stabilized vesicles.

Hui and coauthors are offered to use Pluronics, which conformation depends from the temperature, for the stabilization of liposomes. Incorporation of F127 into the lipid membrane leads to release not only low molecular weight dye 6-karboksifluorestsein, but also fluorescent labeled albumine from liposomes [48].

The reverse effect was rendered by Pluronics embedded into the liposome constructed from dioleoyl-phosphatidylethanolamine. Edwards and coauthors have been shown that Pluronics do not invoke the dye flow out from such liposome's when pH was ~ 9.5. At the buffer with high pH phosphatidilethanolamine underwents phase change to hexagonal phase owing to protonation of amino groups, which leads to decrease of electrostatic repultion between lipid molecules. It was accompanied by increase in lipid membrane permeability and the dye flow out of the liposomes. It was revealed that incorporation of Pluronics into the lipid membrane led to significant inhibition of this process. The effect of polymer depends from it concentration and the length of PEO block. The longer chains of PEO block were entered into its composition the bigger was effect: Pluronics F127 and F108 rendered much more expressed stabilizing influence, than Pluronics F87 and P85 [49].

Thus, the data about the Pluronic influence on lipid membrane permeability and, hence, on their physical condition are discordant enough. This question demands more regular research.

2.3. PLURONICS FOR MEDICAL PURPOSE

Previously we demonstrated that Pluronics possess the successful combination of physicochemical characteristics which allow them softly interact with biological macromolecules and lipid bilayers. The various effects invoked by Pluronics in biological systems were listed in the recent review of Mogimi and Hunter [50]. Pluronics are compounds with low toxicity. Toxicity of these polymers for living organisms correlates with their hydrophobicity. Reduction of hydrophilic (PEO) blocks of polymer molecule leads to increase of polymer toxicity. However even the most hydrophobic Pluronics are characterized by extremely low toxicity in comparison with other low molecular weight surfactants.

It defines the high interest displayed by doctors and pharmacologists to Pluronics. At the same time with a big confidence we can assume that, the basic effects invoked by Pluronics in the biological systems are caused: by amphiphilic properties of polymers and their ability to interact with biological membranes; by their surface-active properties, and also by the ability to coordinate cations of metals. In particular, Pluronics L121 and L101 are applied basically as immunoadjuvants, Pluronic L81, besides this property, is capable to inhibit synthesis of lipoproteins of low density. Pluronic L61 was offered for treatment of tumors which are displaying stability to the polyvalent therapies. More hydrophilic Pluronics P85 and P105 also possess this properties, besides it, they find application for hydrophilization of medical surfaces and polymeric nanoparticles, which allow to depress an adsorption of plasma proteins on these surfaces [50].

We will notice also, that the high ability of these polymers to form micelles is widely used in pharmacology: in a series of works it was offered to use Pluronic micelles as nano size carriers for the delivery of medicines into living cells. An ability of Pluronics to form micelles also been used for solubilization of some hydrophobic medicines in micelles of these polymers. At last, hydrophilic Pluronic F68 is widely used in a building of new medicinal forms, for stabilization of emulsions and also for production of artificial blood substitutes. Hydrophilic Pluronic F127 is capable to form heat-sensitive gels, which has found the application for the prolongation of action and controllable release of medicines. Besides it, it has been shown that some Pluronics F and P series promotes the genes delivery into the living cells [50].

2.3.1. Multiple Drug Resistance and Pluronics

Important property of hydrophobic Pluronics is their ability to overcome multiple drug resistance of tumors, which appears as a result of intensive chemotherapy. The sensitivity of cells to action of medicines is defined as quantity opposite to the concentration of a medicine necessary for the destruction of half cellular populations. Multiple drug resistance (MDR) of tumors is a phenomenon of tumor cells sensitivity decrease to wide series of structurally and functionally different cytotoxic agents, which were earlier successfully applied for tumor treatment [51]. MDR is a major problem of cancer chemotherapy.

In a variety of researches it had been shown, that MDR is determined by the hyperexpression of a set varies of genes [52], which lead to protective enzymatic cascades activation counteracting medicines influence at all stages of it interactions with cells. Firstly, the special conditions preventing the drug uptake are created by MDR cells. It is a consequence of composition changes of plasma membrane, which leads to inhibitions of specific pathways in which the drug infiltrates into the cell. This accomplished by the reduction of drug concentration gradient as a result of cytoplasm acidity changes.

Secondly, MDR cells activate an active efflux of the drug by means of membranous protein-carriers activation. Thirdly, MDR cells promote the linkage and subsequent destruction of a drug in the sour of cytoplasmic vesicles. At last, MDR cells educe an ability to repair targets, which were damaged by a medicine (for example, DNA) also inhibit the processes awaked by the target damage, including apoptosis. It is known that the relationships between these mechanisms not equal for various populations of tumors [52].

Among mechanisms of resistance the important role belongs to ATP-dependent transmitting agents (so-called ABC transporters) - the membrane associated proteins which are accomplish an active efflux of the drug out from the living cells. Normally, transporters belonging to this family express in the tissues, which are carrying out the barrier function – endothelium of pots, of a blood-brain barrier, of placenta and small intestines epithelium.

However, under the action of chemotherapeutic agents (antracyclic antibiotics and others) the genes of resistance activate. One of most widespread genotype of resistant cells is activated gene of P-170 glycoprotein (P-glycoprotein), which specifies on a wide range of cationic and hydrophobic substrates [53]. After MDR phenomenon discovery many researchers started to search for compounds, which are capable to overcome this phenomenon. It was founded that substrates of P-170, which are less toxic than anticancer drugs, are most effective inhibitors of P-170. Verapamil is the most widely used example of P-170 inhibitors. Besides verapamil, well-known immunosuppressant cyclosporine A and the estrogen antagonist tamocksifen also possess an inhibiting effect [54]. The majority of P-glycoprotein inhibitors directly contacts with P-170 inhibits their activity, or acts on cellular regulation mechanisms [55, 56]. Despite certain achievements in MDR-

effect overcoming, the usage of P-170 inhibitors in chemotherapeutic practice reduced by their high toxicity. Therefore a problem of MDR-effect overcoming is rather actual.

Pluronics P85 and L61 are successfully applied as an anticancer drugs enhancing therapeutics. The influence of several Pluronics (F108, P85, L61) on anticancer activity of a series of antracyclic antibiotics (doxorubicin, epirubicin and daunomycin) has been recently studied [57]. Experiments on mice with imparted mielom SP2/0 showed that an addition of Pluronics to antibiotic solution strengthened their effect in comparison with pure drug solution injections. Effect of the polymer increased with its hydrophobicity: among F108 <P85 <L61. Also it is revealed that an introduction of Pluronics to antibiotics decreased the overall cytotoxicity of the drug-composition, correspondingly, the lifetime of experimental animals increased. One more effect of Pluronics is an accumulation of polymers in a tumor, which cases the antibiotic redistribution from a liver to tumor.

Rapoport with coauthors showed that [58, 59], the usage of Pluronic P105-doxorubicin compositions in a combination with focused tumor cells irradiation by ultrasound has allowed to raise anticancer activity of an antibiotic considerably. It was shown that Pluronics together with doxorubicin were incorporated into the nuclei of tumor cells. Thus, incorporated in the nuclei doxorubicin and ultrasound reacted in symbiosis and promoted the DNA destruction.

In variety of works the assumption, that exactly Pluronic micelles are responsible for doxorubicin transport through the cellular membrane is prevailing. However, Kabanov with coauthors [60-62] revealed that the action of Pluronics is presumably determined by unimers of copolymer molecules. They also founded, that antibiotic incorporation in micelles even reduces it anticancer activity, because the antibiotics incorporated in micelles can penetrate through a membrane only by means of liquid-phase endocytosis, which characterized by considerably low rate than passive diffusion. Rapoport with coauthors [63] confirmed this data; they demonstrated that an efficiency of doxorubicin delivery into the cells by Pluronic P105 micelles under the influence of ultrasonic sound decreased when the Pluronic concentration was amplified. These data also specifies that drug association in micelles is rather negative, than positive factor influencing transmembrane delivery of a medicine in cells.

Kabanov et al. have shown that Pluronics are capable to reverse resistance of the cancer cells containing P-glycoprotein and other representative of ABC-transporters - MRP. By the measuring of the drug efflux rate and the activity of ABC-transporters in the presence of Pluronics, authors demonstrated that the action of polymers on resistant cells is determined by their inhibiting effect on ABC-transporters. Also, P-glycoprotein was more affected by Pluronics, than MRP-transporter [62]. Earlier it was reported that some other PEO-containing surfactants (Cremophor EL, Tween-80, Triton X-100, Solutol HS-15) also posses the ability to sensitize tumor cells [64, 65], however their action was less effective than Pluronic effect.

As P-glycoprotein (P-gp) constitutes the hemato-encephalic barrier (GEB) in a vascular endothelium, Pluronics influence on drug delivery in a brain also can be explained by P-gps activity inhibition. In work of Batracova with coauthors [66] was shown that Pluronic P85 significantly inhibited an efflux of P-gp modeling substrate rodamin-123 from the monolayer of renal pork epithelium transfected by the P-glycoprotein gene - mdr1. These data is confirmed by experiments on mice hoes mdr1 gene contained the deletion. Results showed that, GEB permeability of P-170 positive animals in the presence of Pluronic P85 was comparable to GEB permeability of mutant animals (mdr1 deletion) in the absence of Pluronic. On a model of BBMEC cells evolved from a bull brain, and on cells from intestinal epithelium - Caco-2 was shown that, the greatest activity on P-gps possess Pluronics with a median extent of PPO blocks (30-60 parts) and HLB<20 [60].

The important result explaining the Pluronic influence on P-glycoprotein activity consists from the found ability of Pluronics to reduce the endocellular concentration of ATP the energy supplier of P-glycoproteins. Probably, the reduction of endocellular ATP concentration is important factor, which can explain Pluronic influence on P-gp activity. Other important factor explaining the mechanism of Pluronics effect is their influence on cellular membrane microviscosity. The greatest activity on microviscisity possessed hydrophobic Pluronics [67].

To sum up, in last decade researches showed that Pluronics are capable to considerably reduce an activity of P-glycoproteins, which explains Pluronic influence on multiple drug resistance and blood-brain barrier permeability. Besides, it was shown that Pluronic P85

influences other mechanisms of resistance. In particular, the expression of some genes responsible for apoptotic pathways [68] and P-glycoprotein synthesis was reduced by the Pluronic P85 [69]. In work [70] it is shown that an addition of Pluronic L61 to doxorubicin leads to redistribution of this antibiotic from cytoplasmic vesicles to the nuclei. These data allow us to conclude, that Pluronic effect possesses the complex character.

Thus, in variety of works it is shown that Pluronics possess the expressed ability to reduce the sensitivity of MDR-cells to antibiotic therapy, and the degree of this influence depends on polymeric structure and composition. Nowadays the medicine SP1049C based on Pluronics is passing the clinical tests in the Great Britain and Canada [71]. Composition of this drug has been selected experimentally. It has been positioned that Pluronics contacts with cells and influences variety of cellular processes. Nevertheless, the molecular mechanisms of this influence and the dependence of mechanism of action on polymer composition till now remain unclear.

In conclusion, Kabanov and Batracova [72] summarized all known effects of Pluronics on MDR cells, which can help them successfully penetrate multiple resistances to anthracycline antibiotics. They revealed that, Pluronic affects several drug resistance mechanisms including inhibition of drug efflux transporters, promoting drug escaping from acidic vesicles and also inhibiting the glutathione / glutathione S-transferase detoxification system. All these mechanisms of drug resistance are energy-dependent and therefore ATP depletion induced by Pluronics in MDR cells is considered as one potential reason for chemo sensitization of these cells. The schematic representation of Pluronic block copolymers is shown Figure 2.6. Furthermore, Pluronics affects the gene expression in MDR cells and promotes the pro apoptotic agents synthesis. Although, the Pluronic influence on apoptotic pathways are not sufficiently studied till now.

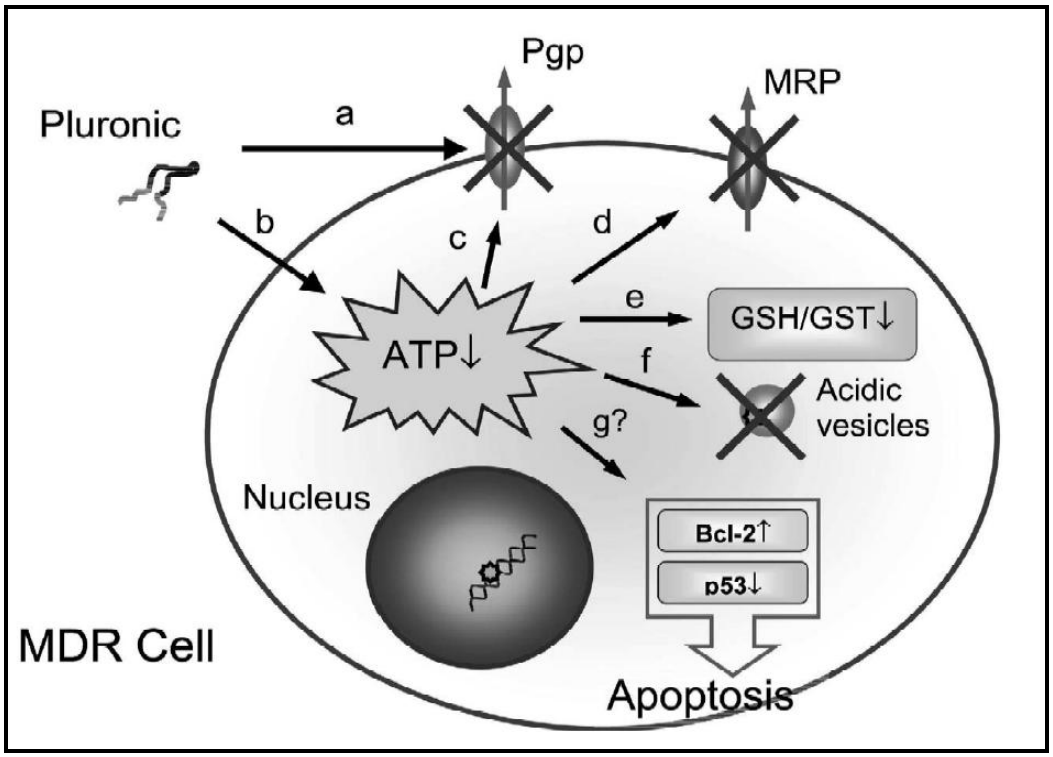

Figure 2.6. Schematic representation of Pluronic block copolymers multiple effects on MDR cell: (a) decrease in membrane viscosity; (b) inhibition of ATP synthesis; (c, d) inhibition of drug efflux transporters; (e) reduction in GSH/GST detoxification activity; (f) drug release from acidic vesicles in the cell; (g) effects on apoptosis (are not sufficiently studied) [72].

2.4. AMPHIPHILIC BLOCK COPOLYMER STABILIZED METAL NANOPARTICLES

2.4.1. Stabilization of Metal Nanoparticles by Ethylene Oxide and Propylene Oxide Copolymers

In recent years, the development of metal and semiconductor based nanomaterials are of particular interest of researchers due to their unique optical properties, nanometer-size structures, which appropriate for interaction with biomacromolecules (proteins, DNA) and intracellular environments probing [73]. For biomedical applications nanoparticles are usually covered with organic molecules providing solubility, long term colloidal stability and functionalization. Two main coating strategies have been developed, one involved

chemical binding of ligands to the surface of the nanoparticle [74, 75], and another based on physical adsorption of macromolecules [76] or surfactants [77].

Usually to stabilize nanoparticles researches use polymeric compounds, which are more capable to adsorb on the surface than molecules of low molecular weight due to the lower entropy loss during adsorption. The polymer shell around the nanoparticles reduces the approach distance between particles and correspondingly the tendency of aggregation, which provides colloidal stability of nanoparticles [78].

The nonionic polyethylene glycols (PEGs) is commonly used as dispersants for various industrial applications, but nowadays many research works are devoted to stabilization of nanoparticles by PEGs for biological application. Coordinating diblock or triblock copolymers such as polystyrene-b-poly (4-vinylpyridine) can be also used to stabilize many colloidal metal nanoparticles [79].

There are two main approaches to stabilize nanoparticles by block copolymers, one is based on the polymeric micelles and unimers usage for nanoparticles stabilization by means of self assembly and adsorption [80, 81]. Gaoet et al. used so named ABC tri-block copolymer with a hydrophobic–hydrophobic–hydrophilic structure to encapsulate and solubilize trioctylphosphine oxide - capped quantum dots by means of self-assembly [82]. Alternatively, amphiphilic comblike polymer can be used to solubilize nanoparticles by means of encapsulation [83].

For instance, stable noble metal nanoparticles were covered by micelles of poly(styrene-b-4-vinylpyridine) [84] and poly(2-ethyl-2-oxazoline)-b-poly(e-caprolactone) [85]. Many examples involve micelles of nonionic poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) EOnPOmEOn triblock copolymers [86], which are commercially available under the trade name Pluronics (BASF). Gold nanoparticles were stabilized by Pluronics micelles, which are forming in solution and/or on the surface of nanoparticles shown in Figure 2.7. Pluronics also stabilize nanoparticles in the absence of micelles. Pluronics solutions at concentrations below the critical micelle concentration (CMC) were used to control the growth of Pt nanoparticles [87].

Another approach for nanoparticles stabilization includes the direct nanoparticle synthesis in the presence of nonionic amphiphilic copolymers. For instance, Sakai and Alexandridis performed single-step synthesis and stabilization of gold nanoparticles in aqueous Pluronics solutions [88]. Nanoparticle forms by the reduction of $AuCl_4$, accompanied by oxidation of EO blocks [89].

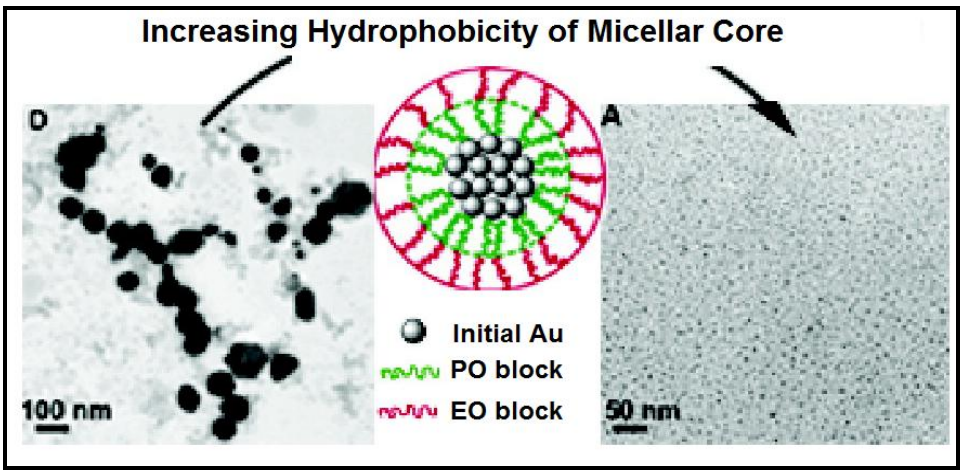

Figure 2.7. Gold nanopartocles stabilization by micelles of triblock copolymer increased it colloidal stability. With good stabilization, gold particles were spherical and uniform in size with a diameter of 5-10 nm (TEM images) [90]

2.4.2. Nanoparticle Synthesis in Aqueous Solutions of Amphiphilic Block Copolymer

Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) block copolymers also known as templates for direct synthesis of metal nanoparticles [91, 92]. PEO-PPO-PEO block copolymers are capable to self-assembly in water solutions. Researchers recently founded that PEO-PPO-PEO block copolymers can act as very efficient reductants and also stabilizers in the single-step synthesis and stabilization of gold nanoparticles in aqueous solutions at ambient temperature in the absence of additional reductant or energy input. This synthesis proceeds in less than 2 hours in room temperature, which helps safe energy and resources, since only water and nontoxic commercially available polymers are needed for synthesis [88, 89].

Synthesized in this way gold nanoparticles remain highly stable for several months. In contrast, when the PEO homopolymer with a same molecular weight was used instead of PEO-PPO-PEO block copolymers, the nanoparticle synthesis proceeds slowly (more than 2 days are needed), additionaly, produced colloids were unstable [88, 89]. The nanoparticles synthesis and their properties, such as size and colloidal stability, depends on block copolymer characteristics, such as molar concentration, PEO and PPO content, critical micelle concentration (CMC).

Toshio Sakai and Paschalis Alexandridis provided schematic mechanism of $AuCl_4$ reduction and nanoparticle synthesis in the presence of Pluronics. They reported that gold nanoparticles synthesis by Pluronics consists of three main steps: (1) reduction of metal ions by block copolymers in solution, (2) absorption of block copolymers on gold clusters and reduction of metal ions on the surface of the clusters, (3) growth of gold particles and their stabilization by block copolymers. The schematic mechanism is shown in Figure 2.8 [89].

The final product was examined by absorption spectra analysis. They compared an ability of various PEO-PPO-PEO block copolymers and PEO homopolymer to form and stabilized composite gold nanoparticles. They revealed that both PEO and PPO blocks contributes to $AuCl_4$ reduction and particle formation. PEO is more dominant in initial stages of reduction, while PPO facilitates block copolymer adsorption on gold clusters and reduction of ions on the surface of these gold clusters. The difference in particle formation observed between PEO-PPO-PEO block copolymers and PEO homopolymer is attributed to the block copolymer adsorption and $AuCl_4$ reduction processes on the surface of gold particles [89].

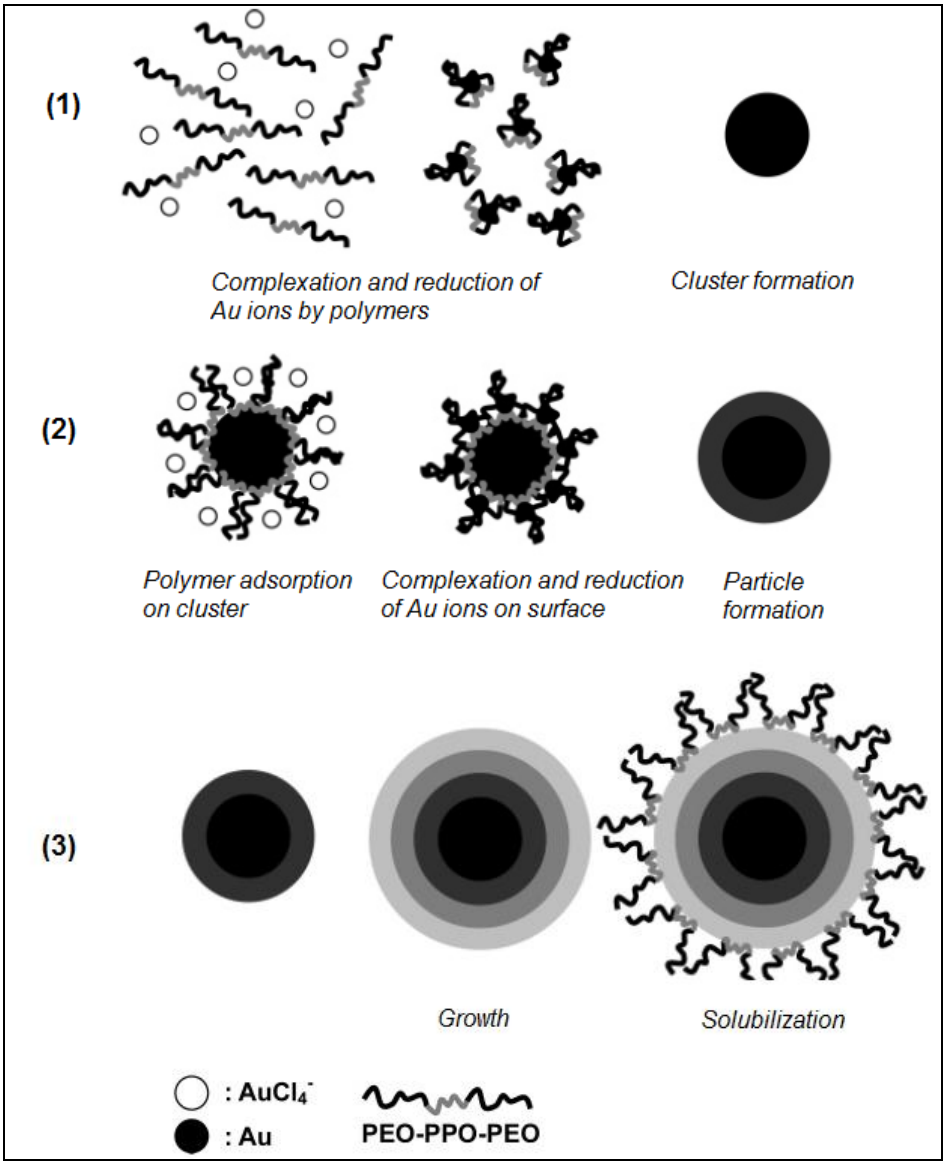

Figure 2.8. Schematic mechanism of $AuCl_4^-$ reduction by Pluronics and gold particle formation [89]

2.5. OPTICAL PROBING OF LIVE CELLS USING NOBLE METAL SERS LABELS

The modern cell biology is interested in developing of new advanced optical methods and tools for living cells probing and intacellular processes monitoring. Optical studies of living cells can be based on fluorescence, reflectance, and elastic and inelastic scattering. Many studies are based on the usage of fluorescence labels composed of organic dyes and quantum dots, which are providing high sensitivity bat can't get the information about

chemical composition or molecular structure of the targeted object [93, 94]. Moreover, majority of fluorescence labels exhibit photobleaching and may be toxic for living objects. Therefore, the main task of biotechnology is improving stability, biocompatibility, sensitivity, specificity and information content provided by optical labels.

Surface-enhanced Raman scattering SERS is a powerful spectroscopic technique that combines high sensitivity with opportunity to get some structural information about the object. The SERS phenomena originated from localized surface plasmon resonances of nanometer sized metal structures. This phenomenon leads to large enhancement of usually low Raman scattering. Using SERS phenomena it is possible to detect Raman signal even from single molecule [93]. SERS mechanism is originated from the coupling of electromagnetic fields of optically excited surface plasmons in metals and Raman scattering of the organic molecule. Nanostructures work as resonant nano-antennas and enhance Raman emission of organic molecules. During recent years, it has been demonstrated that SERS opens new capabilities for living cells probing [94-96]. SERS labels have been widely used for DNA strands and proteins labeling [97] and also for *in vivo* imaging in mice [98].

SERS labels usually consist from reporter molecules attached to gold or silver nanoparticles [99]. Compared to fluorescence, SERS labels can provide several advantages for live cell probing: (1) in comparison with a broad and relatively nonspecific fluorescence signal, the Raman spectrum of the label consists from several bands an optical signature of a fluorescent dye; (2) Raman spectrum is highly specific to the molecule of interest, spectral overlap between different labels isn't common, even when their chemical structures are similar [100].

Additionally, SERS labels are photo stable, because they don't undergo electronic excitation. SERS analysis doesn't cause photodecomposition of the probed object and also do not case sample autofluorescence. The biggest advantage of SERS labels comes from their ability to enhance Raman signals of intracellular molecules, which adsorbs on the surface of gold or silver particles. Therefore, besides detection and imaging based on the specific SERS of the label, nanoparticles can also act as tiny Raman sensors, which provide structural information about cellular environment. Figure 2.9 illustrates the basic

concept of SERS labels. In this study researchers used two SERS hybrid labels made from gold nanoparticles with either rose bengal or crystal violet as reporter molecules [100].

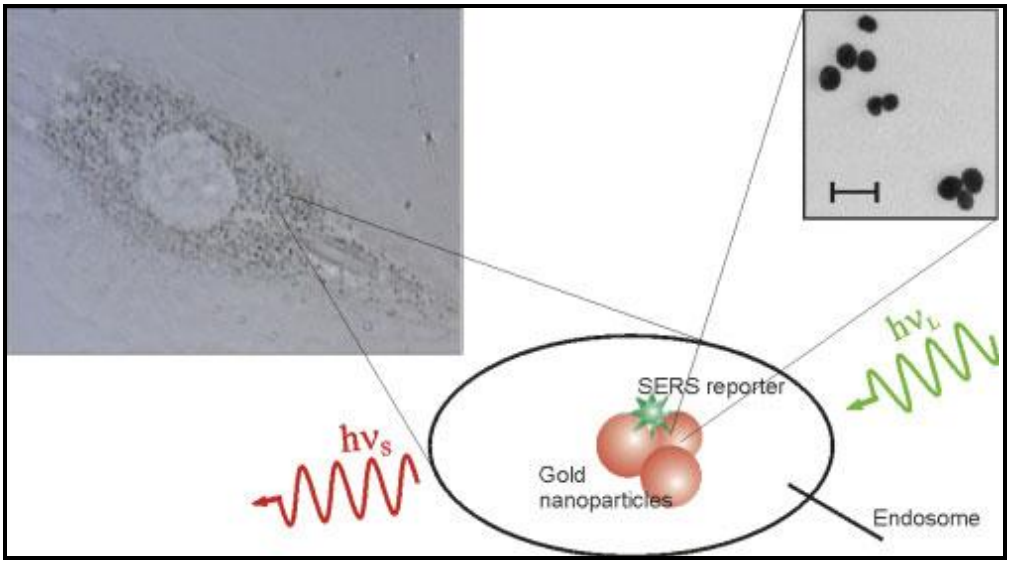

Figure 2.9. SERS labels applications in live cells. The bright-field image shows large nanoparticle aggregates incorporated into 3T3 cell. The electron microscopy image shows typical gold nanoparticles used as SERS labels. Raman scattering signals of the dye indicate the position of the gold particles probes and the chemistry of intracellular compartments [100]

SERS is extremely sensitive to chemical composition of living cells, and this information can be obtained in relatively short times without staining or fixing procedure. The cell culture media produce low Raman signal, which makes possible *in vitro* experiments of living cells in culture media [96].

Intracellular delivery of nanoparticles and cellular compartments targeting can be achieved in various ways, depending on the cell nature and physicochemical parameters of nanoparticle, such as size, shape, and surface functionalization [101, 102]. Kneipp et al. developed Raman-based optical sensors to probe live cells employing the fact that many cells absorb nanostructures by themselves without induction [103]. Nonphagocytic cells can internalize structures of less than 1 μm in size, with endocytosis and transport into endosomes. The highest efficiency for intracellular nanoparticles uptake was shown for

nanoparticles several tens of nanometers in size. This size requirement is in agreement with optimum size conditions for SERS enhancement.

Internalized gold nanostructures enable surface-enhanced Raman spectra measuring and sensitive chemical probing of the surrounding nanoparticle intracellular environment. For SERS analysis the time requirements are also short, less than 1 s per spectrum and also low laser power is needed ($\leq$2 mW/1 μm spot). Such short acquisition times are impossible in normal Raman experiments and are on the order of the time scales of intracellular processes [104]. Typical SERS spectra from a living cell show features of cellular components, such as proteins (1245 cm^{-1}, 1267 cm^{-1} amide III, side chains Phe 1002 cm^{-1}, Tyr 825 cm^{-1}) and various nucleic acid constituents (e.g. 1580, 1575, 1098 cm^{-1}) shown in Figure 2.10.

It is also possible to attach a reporter molecule, for example, a dye, to the gold nanostuctures. Such probes can be localized inside single cells and the characteristic SERS spectrum of the reporter molecule can be detected. Nanoparticle also can deliver SERS contributions to intracellular content [95]. The transfer of reporter molecules together with nanostructures enables the ultra sensitive probing of the particle environments. Further, an observation of transferred molecules may be useful for a question of intracellular drug delivery.

The formation of nanoaggregates and clusters are extremely important for electromagnetic enhancement and correspondingly for further development of SERS probes for cell analysis. Results showed that nanoparticles, after immersing them into culture medium, form stable nanoaggregates. We can conclude that the controlled formation of nanoaggregates will be one the major tools which helps to employ the large enhancement factors of nanoparticles in live biosystems [103].

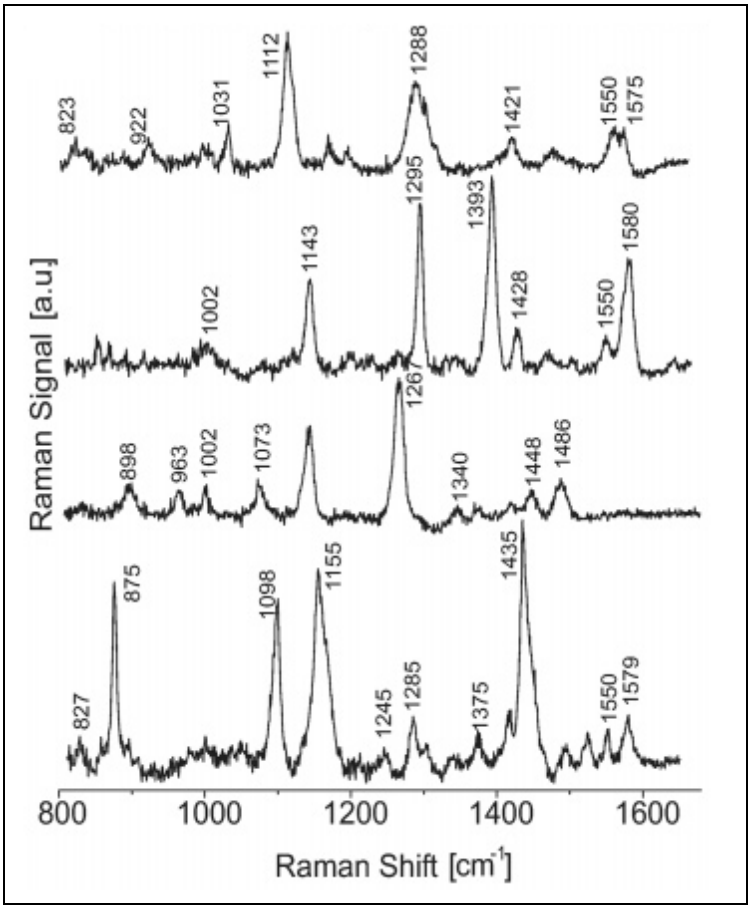

Figure 2.10. Examples of SERS spectra of NIH/3T3 cells after 3 h incubation with gold nanostructures, excitation wavelength 830 nm, 1 s collection time [103]

The major problem of live cell SERS analysis is complex composition of cells, which leads to complex Raman spectra with overlapped peaks originated from molecular vibrations in various cellular components. Such complex Raman spectra are difficult for analysis, therefore useful information about intracellular processes is difficult to extract. Notingher et al. demonstrated the possibilities of multivariate analysis for living cells Raman spectra differentiation. In contrast with univariate methods based on calculation of intensity or area of individual peaks, multivariate analysis is a statistically based analytical methods allows simultaneous multiple peaks or whole Raman spectra analysis [105].

Most common multivariate technique is Principal Component Analysis (PCA), which finds combinations of variables that describe the major trend in the data [106]. PCA reduces the insignificant variable components of the measurement matrix and represents

the data using a smaller number of significant principal components (PCs). Each principal component has a score calculated as a projection of the Raman spectra on the directions defined by the PC factors. PCs describe the most significant variance between the spectra, while the less significant PCs, describing mostly random noise are diminished.

However, PCA is luck of the machinery which helps to separate the matrix data on sample group. Linear Discriminant Analysis (LDA) is a more powerful method for the discrimination data on sample groups. LDA maximize the variance between groups and minimise the variance within groups according to Fisher's criterion [107]. The main disadvantage of LDA is that the number of variables used as input has to be smaller than the total sample number. But the number of wavelength in Raman spectra usually larger than the number of analyzed cells, so the initial Raman spectrum cannot be analysed by LDA directly [105].

To overcome this disadvantage the scores of the most significant principal components (PCs) of the each Raman spectra are used as input to a LDA model [108]. Combining LDA and PCA methods allow to increase the application of Raman spectroscopy to cancer detection, tissues imaging and identification of micro-organisms [105].

Another multivariate method for the analysis of Raman spectra of biological samples is Classical Least Squares (CLS) and Partial Least Squares (PLS). CLS and PLS have been successfully applied for determination of the biochemical composition of various tissues, such as skin [109], breast tissue [110], artery walls [111] and cells [112]. In these methods, the measured Raman spectrum of a complex matrix is decomposed into a linear combination of Raman spectra of each component of the sample. The problem is that, the prior information of the chemical composition of the sample is not available, or, if available, the measurement of the Raman spectra for all components is not feasible. In this case morphological models can be used where the Raman spectra of the major morphological features of the sample should be taken *in situ* by the help of high spatial resolution spectrometers [112]. If morphological models cannot be built, the Raman spectra of commercially available pure chemical compounds should be taken as models of

the species presented in the sample and expected to undergo the most significant changes during the experiments.

Notingher and coworkers showed how the combination of PCA and LDA can be successfully applied in Raman spectra analysis of individual living cells and tissue analysis. They presented results on LDA–PCA analysis of healthy and cancerous cells discrimination. CLS model and commercially available biopolymers also been applied in cellular differentiation studying [105].

3. MATERIALS

We used commercial poly(ethylene oxide)-poly(propylene oxide) block copolymers (Pluronics) L61, L121, F127, and F68 (Sigma Aldrich). Pluronics L61 and L121 were modified with terminal carboxyl groups in the reaction with succinic anhydride in the Department of Applied Chemistry of Kazan (Volga Region) Federal University and kindly provided by Dr. Yuriy Shtyrlin.

Silver nitrate ($AgNO_3$) was purchased from Fluka, rhodamine 6G, aminofluorescein, doxorubicin hydrochloride and verapamil were obtained from Sigma-Aldrich. All solutions were prepared using deionized water and salts of analytical quality.

For the cell culture experiments we used DMEM media supplemented with 10% fetal calf serum, antibiotics (penicillin 100 units/ml + streptomycin 100 μ/ml), and L-glutamine 2 mM. For cell culture experiments we used DPBS solution (0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8 g/L NaCl, 1,15 g/L NaH_2PO_4). Hep3B human hepatoma cells, A549 human lung adenocarcinoma epithelial cells and HeLa cells were kindly provided by Dr. Albert Rizvanov. For adhered cell trypsinization we used trypsin-EDTA solution (Gibco).

4. METHODS

4.1. MODIFIED PLURONICS CHARACTERISATION

4.1.1. Characterization of Pluronic Micelles Using Dynamic Light Scattering

Pluronics L61, L121 and their carboxylated derivatives denoted as L61-COOH and L121-$(COOH)_2$ form micelles in water. The hydrodynamic diameter of the micelles and their zeta-potential were measured using dynamic light scattering (DLS) technique on Nano-ZS analyzer (Malvern Instruments, UK). The measurement of hydrodynamic diameter of Pluronics in water solution was performed at a polymer concentration above the CMC (200 μg/ml) and at 25ºC. For zeta-potential measurements, Pluronic solutions were prepared at the concentration of 1 mg/ml.

4.1.2. Cytotoxicity Study of Modified Pluronics (MTS-Test)

Cytotoxicity of carboxilated Pluronics L61 and L121 was studied by means of proliferative MTS-test (Promega). For this purpose, HeLa and A549 cells were cultured in DMEM in 5% CO_2 atmosphere at 37ºC. Cells were seeded onto 96-well plate. After 24-h culturing, fresh media was added with Pluronic solution (1/10 volume of the media) at different concentrations of copolymers.

The studied concentration range was 125…1,05 μg/ml for Pluronics L61 and L61-COOH and 1500…23,44 μg/ml for Pluronics L121 and L121-$(COOH)_2$. Pluronics were incubated with cells for 72 hours, and then the medium was replaced with fresh one supplemented with MTS-reagent (tetrazolium salt) according to Promega protocol [113]. MTS reagent was incubated 1 hour in 5% CO_2 atmosphere at 37º C with living cells, which reduced it to a colored product (formazan) registered on a photometer (Stat Fax 2100) at 490 nm.

The percent of viable cells for each concentration of Pluronics was calculated relative to the control cells cultured without polymers. In Figure 4.1, we plotted

relationship between Pluronic concentration and cellular viability using OriginLab-8 software to find polymer concentration (µg/ml), which caused 50% inhibition of cell growth in culture (LD_{50}).

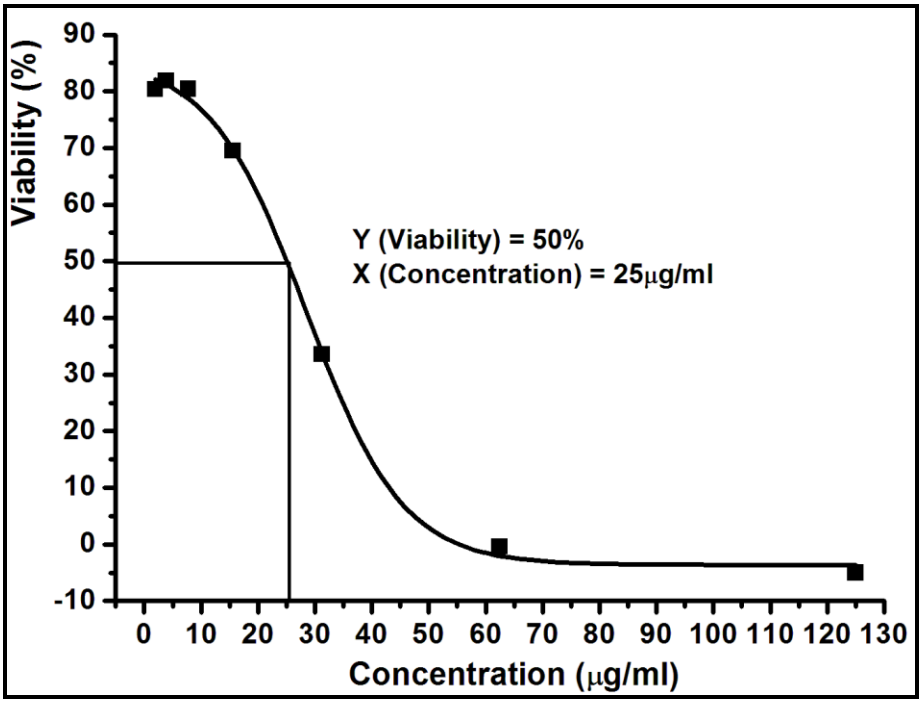

Figure 4.1. Determination of LD_{50} of Pluronic L61-COOH on HeLa cells by plotting relationship between Pluronic concentration (µg/ml) and cellular viability (%)

4.1.3. Study of an Effect of Modified Pluronics on ATP Synthesis in Cells

In order to perform the experiment, HeLa cells were cultured in 12-well plate in DMEM in 5% CO_2 atmosphere at 37ºC until they formed a monolayer. Next, Pluronics L61, L121, L61-COOH, and L121-$(COOH)_2$ were added into fresh DMEM at concentration of 200µg/ml (this concentration was found to be non toxic to cells during 4 hours of incubation and produce highest effect on cytotoxicity of doxorubicin) and incubated with cells for 4 hours. The treated cells were transferred into the suspension and subjected to a lysis procedure.

ATP content in cellular lysates was determined by a bioluminescent assay. In this assay, luminescence of D-luciferin, which is catalyzed by firefly luciferase, is detected on a

chemiluminometer (CHEMILUM 12, Russia). A 0.05 ml of ATP reagent (Lumtek, Russia) containing lyciferase enzyme and D-luciferin was added into a cuvette, and then blank signal (I blank) was recorded. Next, 0.05 ml of ATP control was added in the same cuvette and the control signal (I1) was measured. The ATP standard signal was calculated as a difference between I1 and I blank.

In order to measure the ATP concentration in the samples, 0.05 ml of cellular lysates were added to the cuvette instead of ATP control and the sample signal (I2) were registered as a difference between I2 and I blank. All measurements were performed in triplicates; the average values of the signals were presented. The ATP concentration in each sample was calculated using a formula:

$$[ATP, mol/L] = 10^8 \times (Isample/Icontrol) \quad (4.1)$$

4.1.4. Assessment of an Effect of Modified Pluronics on Cytotoxicity of Doxorubicin

To study an effect of Pluronics L61, L121 and their carboxylated modifications L61-COOH, L121-$(COOH)_2$ on doxorubicin cytotoxicity we used MTS-test (Promega). Briefly, A549 cells were seeded in 96-well plate and cultured in DMEM at 37ºC in 5 % CO_2 atmosphere. After 24-hour culturing, equimolar complexes of doxorubicin with Pluronics (200, 100, and 50 μM) were added in fresh DMEM. In order to reveal how Pluronics affect an activity of drug reverse transporters we additionally added verapamil (60 μM) as competitive inhibitor of P-glycoproteins alone and with doxorubicin and doxorubicin-Pluronic mixture.

Preliminary experiments showed that, 6 hours of incubation was enough to observe changes between samples. Therefore, all samples were incubated with cells for 6 hours in DMEM, then, cultural medium was replaced with the fresh one supplemented with MTS-reagent (Promega) and incubated with cells for 1 hour followed by the registration of colored product as described above.

4.1.5. Study of an Effect of Pluronics on Doxorubicin Accumulation in Cells

HeLa cells were cultured in 6-well plate on the surface of cover slips under standard conditions. After 24-h culturing, the medium was replaced with fresh one supplemented with doxorubicin (10 μM) and its complexes with Pluronics (100 μg/ml). Doxorubicin-Pluronic complexes were incubated with cells during 2 hours, then washed and fixed in 4 % p-formaldehyde. Intracellular localization of doxorubicin and its complexes with Pluronics was investigated by doxorubicin fluorescence at 550 nm (λ excitation 485 nm) on a fluorescence microscope WHICH (Carl Zeiss) and laser scanning confocal microscope WHICH TCS (Leica). Fluorescence intensity of doxorubicin inside the cells was estimated with the use of LAS AF Lit software (Leica).

4.1.6. Preparation of Liposomes

Granulated lecithin (300 mg) was dispersed in 10 ml chloroform in round-bottom flask followed by the solvent evaporation on a vacuum evaporator at 120 rpm and 30ºC for 10-15 min until dry lecithin film was formed. Lecithin film was dispersed in DPBS solution under intensive shaking. The resulting emulsion was repeatedly freezed and thawed and then subjected to sonication (4 impulses per 1 min). According to dynamic light scattering study, hydrodynamic diameter of liposomes was about 50 nm and zeta potential was – 30 mV. According to our results obtained liposomes can be corresponded to small unilamellar liposomes (SUL).

4.1.7. Determination of Liposome Microviscosity

Lipid bilayer microviscosity was determined by fluorescence anisotropy measuring of membrane probe diphenylhexatriene (DPHT). The chemical composition was shown in Figure 4.2.

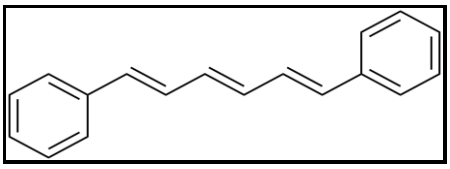

Figure 4.2. Chemical composition of diphenylhexatriene (DPHT)

DPHT is a hydrophobic probe, which is accumulated in lipid membrane phase leading to a significant enhancement of its fluorescence. As a model membrane, we used liposomes composed of natural admixture of phosphatidylcholines from egg lecithin (Acros Organics). The lipid membrane microviscosity was determined by fluorescence anisotropy of DPHT as described in [114]. DPHT was added to liposome suspension (0.5 mg/ml) to final concentration of 100 nM and incubated 1 hour under ambient conditions. Next, Pluronics were added to liposome suspension at a concentration of 1, 10 and 100 μg/ml, and incubated for 30 min. DPHT fluorescence intensity (λ emission 433 nm) was measured on a spectrophotometer FluoroLog-3 (HORIBA Jobin Yvon SAS, France) at λ excitation of 366 nm at the parallel and crossed positions of polarizers. Anisotropy of fluorescence r was calculated using the formula:

$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}} \tag{4.2}$$

Where I_{II} и $I_{\perp}$ are intensities of vertically (II) and horizontally ($\perp$) polarized fluorescence when the sample is excited by vertically polarized light. Using anisotropy of fluorescence r we can calculate the microviscosity η with the aid of Perren equation describing depolarization of fluorescence by rotational diffusion:

$$\frac{r_0}{r} = 1 + C(r)\frac{T \times \tau}{\eta} \tag{4.3}$$

Where r_o and r are limiting and measured fluorescence anisotropy respectively, T - absolute temperature, τ - life time of excited state. C (r) is a structural parameter which can be defined under the standard relationship of measured intensities of fluorescence anisotropy from a solvent of known viscosity.

4.2. DEVELOPMENT OF AGNP-PLURONIC SERS PROBE

4.2.1. Synthesis of Silver Nanoparticles

Ag nanoparticles were synthesized in water by means of standard reduction of AgNO3 in the presence of sodium citrate as a reductant under heating. Briefly, sodium citrate was added to a boiling solution of 1.0×10^{-3} M AgNO3 under vigorous stirring to obtain final concentrations of the reductant of 0.9×10^{-3}. Resulting solution was boiled for 1 h to produce spherically shaped nanoparticles stabilized with citrate ion. The final concentration of synthesized AgNPs was 180 μg/ml.

4.2.2. Adsorption of Pluronics and Organic Molecules on AgNPs

Synthesized AgNPs were modified with Pluronic block copolymers (L121, F68, or F127) by the method commonly used to prepare colloidal gold-proteins conjugates. Briefly, the suspension of AgNPs was placed in a 96-well plate and mixed with an aliquot of Pluronic solution to obtain final concentration of the copolymers 0.1…500 μg/ml. The mixture was incubated for 1 h at room temperature allowing the copolymer to be adsorbed on the surface of the nanoparticles. The procedure resulted in the modification of AgNPs and protection them against salt aggregation visually observing the disappearance the color of nanoparticle suspension. Thus, we ascertained minimal concentrations of Pluronics required for the formation of stable modified nanoparticles, which were resistant to the aggregation in 0.04 M sodium chloride solution. Pluronic-modified nanoparticles, which contained minimal amount of unbound polymers, were further used for analysis without a separation. AgNPs pre-modified with Pluronics were mixed with rhodamine 6G dye or other analytes in water. The resultant mixture was incubated for 1 h at room temperature to produce Pluronic-nanoparticle-dye complexes, which were analyzed using SERS.

4.2.3. Study of Rhodamine 6G and Metabolites Realease from Cells using Pluronic-Modified Probe

Hep3B and A549 cell lines were cultured under standard conditions in 6-well plates until reaching a monolayer confluence. The cells were washed three times with DPBS and

pre-incubated with 3-6 μM rhodamine 6G for 30 min to accumulate the dye inside the cells. The cells with accumulated rhodamine 6G were washed from adsorbed dye molecules and transferred into DPBS to study rhodamine release from cells into surrounding solution. After incubation of the cells for determined time (5, 15, 30, 60, 90, 120, 180 min, and 17 h) 20 μl aliquot of the surrounding solution was taken and carefully mixed with composite silver nanoparticle-Pluronic F127 probe (180 μl). A 5 μl of the mixture was placed on calcium fluoride slide and air-dried. Upon drying the nanoparticles formed aggregates of about 1 μm in diameter on the surface of the slide, which were further analyzed using SERS.

4.2.4. SERS Study of Intracellular Rhodamine 6G

For detection of intracellularly accumulated rhodamine 6G, A549 and Hep3B cells were cultured on cover slips at the density of 5×10^5 cells per slip. The cells were pre-incubated with 100 μM of rhodamine 6G for 30 min, washed three times with DPBS and then treated with Pluronic F127-modified AgNPs at final concentration of nanoparticles of 18 μg/ml for 2 hours. After washing the cells were fixed in 2% p-formaldehyde and enclosed in mounting medium (Sigma-Aldrich) prior to SERS analysis.

4.2.5. Surface-Enhanced Raman Spectroscopy

SERS spectra were obtained by an automated InVia microRaman system (Renishaw) pre-calibrated against monocrystalline silicon wafer peak (520 cm^{-1}). An aliquot of bare or Pluronic-modified AgNPs with adsorbed fluorescent dye or biomolecule(s) were cast on a calcium fluoride substrate and air-dried to obtain uniformly distributed nanoparticle aggregates on the substrate. The spectra were acquired in the range 400-1700 Raman shift (cm^{-1}) using an objective 50× and a diode laser 830 nm (power 30 mW; exposure time 10 s). For the quantification of rhodamine 6G adsorbed on the surface of modified nanoparticles, the intensity of characteristic SERS peaks from 10 aggregates of similar size were measured in each sample. To study fixed cells we focused on the nanoparticle aggregates inside the cells. SERS spectra were collected and averaged from ten different cells in the sample.

5. RESULTS AND DISCUSSION

5.1. CHEMICALLY MODIFIED PLURONICS FOR DRUG DELIVERY

5.1.1. Verification of the Modified Copolymers Structure Using Physicochemical Methods

Pluronics L61 and L121 modified with carboxylic groups were synthesized and kindly provided by Dr. Yuriy Shtyrlin, Department of Applied chemistry of A. Butlerov Chemistry Institute (Kazan, Russia). Pluronic L61 was offered for treatment of tumors which are displaying stability to the polyvalent therapies [50], which determines the chose of Pluronic L61 for our study. Pluronic L121 with higher molecular weight and lower HLB was chosen as a reference polymer. Some physicochemical characteristics of Pluronics L61 and L121 are presented in Table 5.1. The chemical composition of modified polymers, which is presented below in Figure 5.1, was verified with the use of physicochemical methods (IR and PMR spectroscopy) along with acid-base titration. According to acid base titration, the modified Pluronic L61-COOH contains one carboxylic group per polymer molecule while Pluronic L121-$(COOH)_2$ contains two carboxylic groups. The molecular weights of L61-COOH and L121-$(COOH)_2$ were found to be 2100 g/mol and 4600 g/mol, respectively.

Table 5.1. Physicochemical characteristics of commercial Pluronics L61 and L121

Pluronic	MW (g/mol)	Ethylene oxide	Propylene oxide	HLB	CMC (mol/L)
L61	2000	4.55	31.03	3	$1.1*10^{-4}$
L121	4400	10.00	68.28	1	$1.0*10^{-6}$

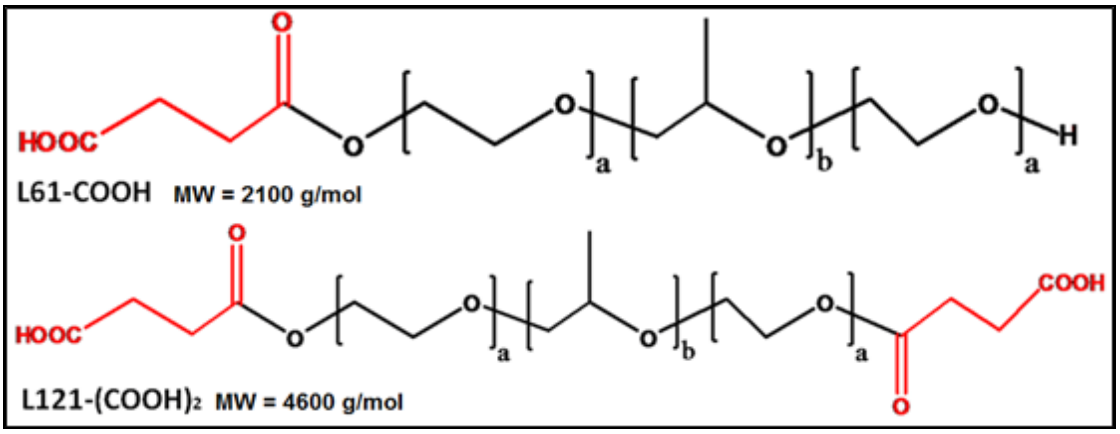

Figure 5.1. Chemical composition of modified Pluronics L61-COOH and L121-$(COOH)_2$

As an example, IR spectrum of initial and modified Pluronic L61 is presented in Figure 5.2. On IR spectrum of modified L61-COOH, we can observe the presence of vibrational band at around 1750 sm^{-1}, which is characteristic for carboxylic groups. Similarly, the NMR ^{13}C spectra of L61-COOH contain common for carboxylic groups characteristic signal at around 172 p.p.m. (data not shown).

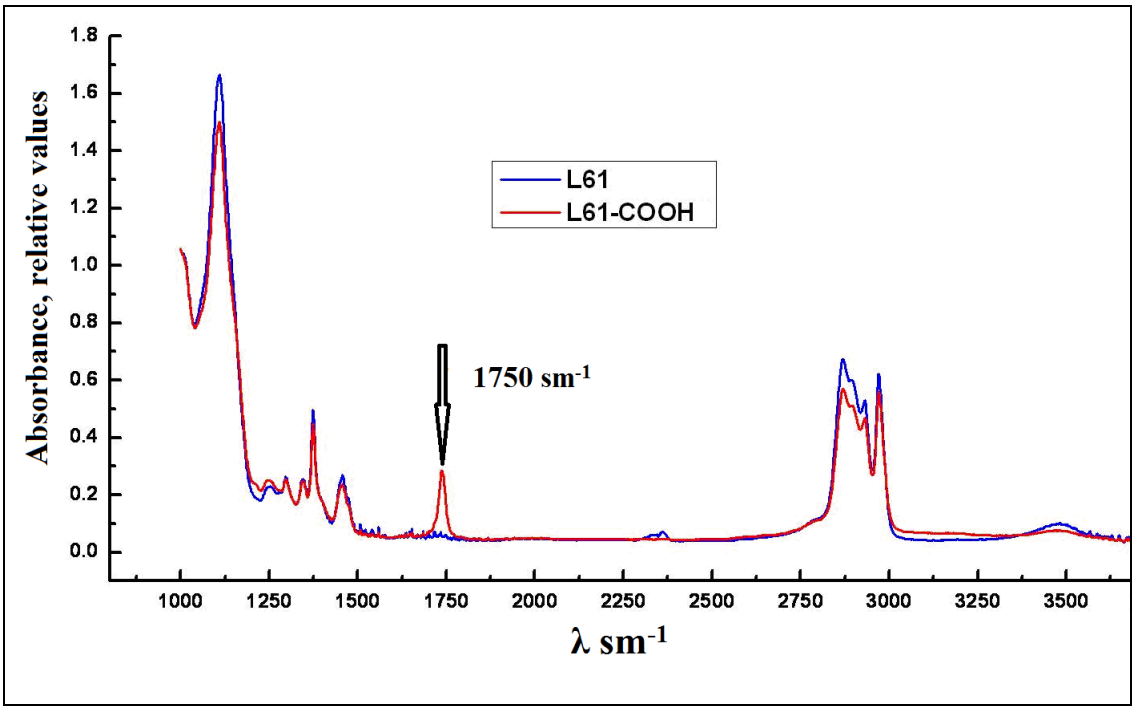

Figure 5.2. IR spectrum of initial and modified Pluronic L61.

5.1.2. Studies of Modified Pluronics Micellation Using Dynamic Light Scattering

In aqueous solutions, Pluronics self-assemble into micelles. The association of modified Pluronics into micelles was analyzed by micelle size and zeta potential measurements using ZetaSizer Nano-ZS analyzer. Based on the dynamic light scattering data, the modification introduces a high negative charge to both L61-COOH and L121-$(COOH)_2$ copolymer micelles shown in Table 5.2. Negative charge introduction to the polymeric structure made Pluronics more hydrophilic, which might influence their biological properties.

After modification with carboxylic group, the size of modified L61 increases significantly (8.3 times), but the size of modified L121-$(COOH)_2$ decreases by 10%. This can be explained by the influence of carboxylic group on intermolecular hydrogen bond formation and copolymer conformation. Pluronics L61 and L121 are differing significantly in HLB. Hydrophobic L121 (HLB = 1) forms dense micelles, thus intermolecular hydrogen bonds formation is unfavoured, and even carboxylic group introduction into the polymeric molecule does not promote the hydrogen bond formation. In case of less hydrophobic L61 (HLB = 3), the micelle conformation is more relaxed, thus, carboxylation promotes intermolecular hydrogen bonds formation between –OH and –COOH groups of molecules, which leads to micelle size augmentation.

Table 5.2. Hydrodynamic diameters (D) and zeta potentials (ζ) of initial and modified Pluronics

Pluronic	**D (nm)**	**ζ (mV)**
L61	**28,4** ± 7,5	**-6,3** ± 0,1
L61-COOH	**234,6** ± 3,4	**-59,8** ± 3,2
L121	**100,7** ± 16,1	**-7,5** ± 1,4
L121-$(COOH)_2$	**89,4** ± 10,2	**-68,6** ± 0,4

5.1.3. Cytotoxicity Studies of Modified Copolymers

The cytotoxicity of modified copolymers was tested on HeLa and A549 cell lines using MTS-assay. Cytotoxicity test revealed that modified copolymer L61-COOH was 2.2 and 1.8 times less toxic to HeLa and A549 cells than initial copolymer, respectively. L121-$(COOH)_2$ was 14 and 2.2 times less toxic to HeLa and A549 cells than initial polymer, respectively seen in Table 5.3. It is known that hydrophilic block copolymers are less toxic to cells than hydrophobic ones due to their disability to penetrate nonspecifically into the cell. Introduction of the negatively charged carboxylic group into the polymeric structure made copolymers more hydrophilic and consequently less toxic to cells than initial copolymers. Our study demonstrates that modified copolymers are more promising materials for applications in medicine and biotechnology than the commercially available Pluronics. These results demonstrate the importance of prospective studies for better understanding the relationship between chemical composition of the polymer and its biological impact.

Table 5.3. LD_{50} (μg/ml) of Pluronics for HeLa and A549 cells

Pluronic	HeLa	A549
L61	**11,43** ± 0,59	**22,55** ± 1,51
L61-COOH	**25,06** ± 3,02	**40,34** ± 1,23
L121	**65,26** ± 5,04	**82,06** ± 25,99
L121-$(COOH)_2$	**919,19** ± 163,53	**179,08**± 61,06

5.1.4. Modified Pluronics Influence Cytotoxicity of Doxorubicin

New chemically modified Pluronics were tested as carriers of common anticancer drug – doxorubicin for the purpose of delivery into human cells. The impact of Pluronics on doxorubicin cytotoxicity was tested on multiple drug resistant A549 cell line. Equimolar compositions of doxorubicin with Pluronics were added to the cells and incubated for 6 hours, then, the cytotoxicity was determined using MTS-test. The results showed that compositions of doxorubicin with Pluronics are 1.5 times more toxic to cells than doxorubicin alone shown in Figure 5.3. In the case of L61-COOH, the modification

with carboxylic groups has a little negative effect on the ability of Pluronic to sensitize MDR cells to doxorubicin. For L121-$(COOH)_2$, chemical modification does not have any noticeable effect. Further studies should be performed to reveal how a chemical composition affects the mechanism of action of Pluronics.

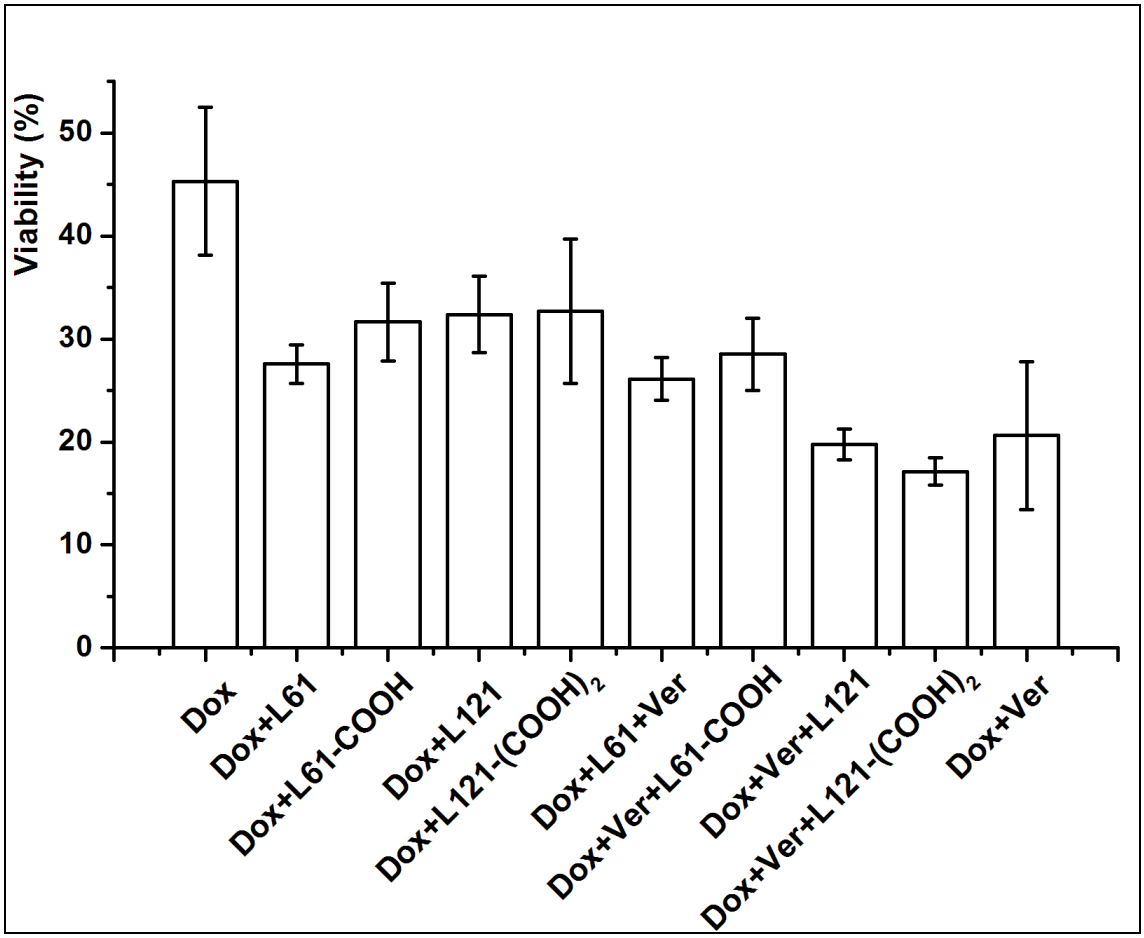

Figure 5.3. A549 cells viability (%) in the presence of doxorubicin (Dox) and its compositions with Pluronics L61, L121, L61-COOH, L121-$(COOH)_2$ and Verapamil (Ver)

Kabanov and coworkers showed that Pluronics can sensitize MDR cells to antibiotic therapy due to their ability to incorporate drugs into the micelles and increase drug endocytosis; alter cytoplasm viscosity and correspondingly increase passive drug transport; inhibit ATP synthesis and also ATP-dependent reverse drug transporters [60-62, 67]. In order to reveal how modified Pluronics inhibit reverse drug transporters P-glycoproteins, we applied the method of additions. Verepamil – a substrate and a

competitive inhibitor of the P-glycoproteins was added to doxorubicin and its compositions with Pluronics, after which the cytotoxicity of compositions was analyzed using MTS-test.

As a result of MTS-test, we showed that Verapamil addition increased the cytotoxicity of doxorubicin to A549 cells. This is due to enhancement of doxorubicin uptake caused by reverse transport blocking by Verapamil. We also showed that Verapamil doesn't influence the cytotoxicity of doxorubicin complexes with Pluronics L61 and L61-COOH. This means that, most probably, Verapamil, Pluronic L61 and L61-COOH are acting on the same target – P-glycoproteins. In contrast, the cytotoxicity of Pluronics L121 and L121-$(COOH)_2$ complexes with doxorubicin increased significantly in the presence of Verapamil. For Pluronics L121 and L121-$(COOH)_2$, we propose prevailing of other than P-gps blocking mechanism of doxorubicin cytotoxicity enhancement.

5.1.5. Study of Modified Copolymers Influence on ATP Synthesis in Living Cells.

ATP is an important biochemical marker, which determines the energetic metabolism intensity and overall cell viability. Kabanov and coworkers showed that Pluronics inhibit the ATP synthesis in mitochondria by altering the mitochondrial membrane fluidity [62, 67]. The ATP-starvation causes inhibition of ATP-dependent drug reverse transporters, which leads to drug accumulation in cells and, finally, to MDR cells sensitization to anticancer therapy. We studied the influence of modified Pluronics on ATP synthesis in HeLa cells using bioluminescent kit (Lumtek, Russia). Bioluminiscent kit contains the luciferase enzyme, which converts uncoloured luciferin into luminescent oxiluciferin in the presence of energy supplier ATP. HeLa cells were treated with both modified and initial Pluronics, then osmotically lysated and analyzed using bioluminescent method.

The analysis showed that, both L61 and L121 Pluronics decrease the intracellular levels of ATP in HeLa cells. Modified Pluronic L61-COOH decreased the ATP concentration to lower extent than unmodified L61. In contrast, modified L121-$(COOH)_2$ stimulated the ATP synthesis. In Figure 5.4, results showed that membranes and enzymes, participating in ATP synthesis, were less altered by the modified copolymers than by initial ones. This proves that modified copolymers are less toxic and more promising materials for biomedical applications than the initial copolymers. On the other hand, less inhibited

ATP synthesis can decrease the ability of modified Pluronics to sensitize MDR cells to anticancer therapy. Fortunately, the cytotoxicity studies revealed that modification didn't have a negative impact on the ability of Pluronics to increase the cytotoxicity of doxorubicin.

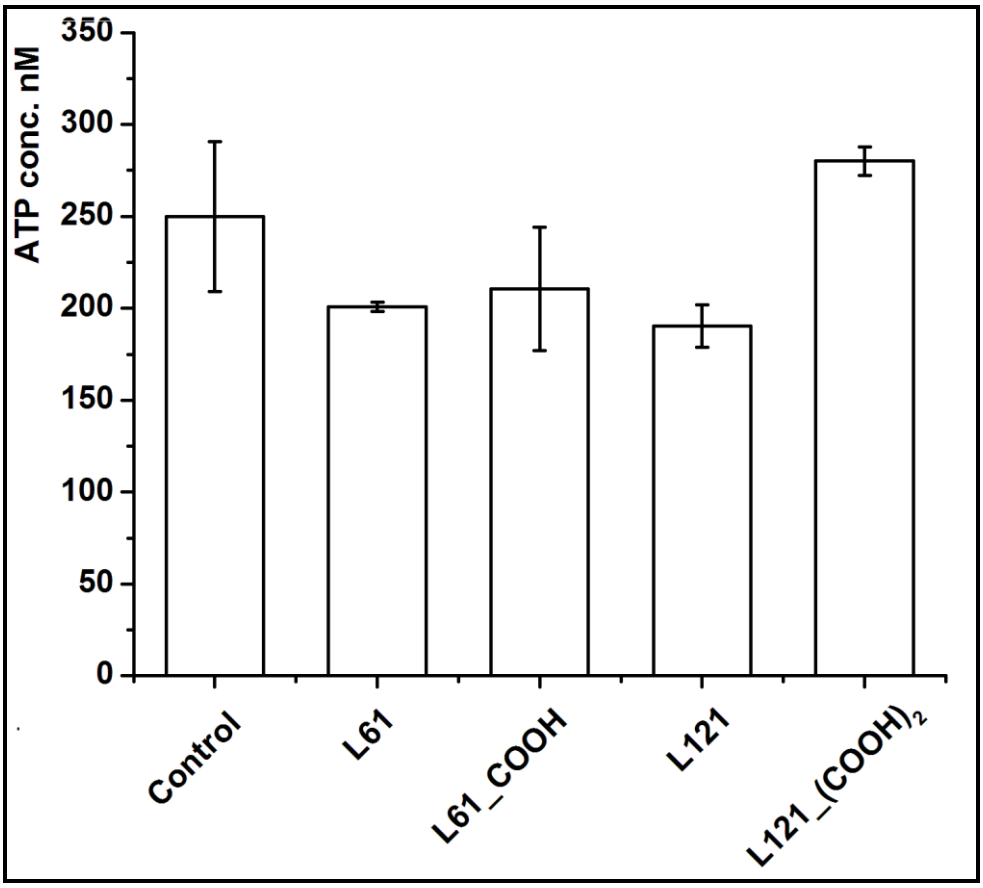

Figure. 5.4. ATP concentration (nM) determined using bioluminiscent method on HeLa cells treated with Pluronics L61, L121, L61-COOH, L121-$(COOH)_2$

5.1.6. Modified Pluronics Influence the Intracellular Accumulation of Doxorubicin

Along with cytotoxicity studies we also analyzed an effect of Pluronics on doxorubicin uptake and distribution in A549 cells using confocal and fluorescent microscopy. With the help of confocal microscopy, we demonstrated that Pluronics don't influence the intracellular distribution of doxorubicine, which gets localized in the nuclei of the cells alone or in the form of complexes with Pluronics shown in Figure 5.5.

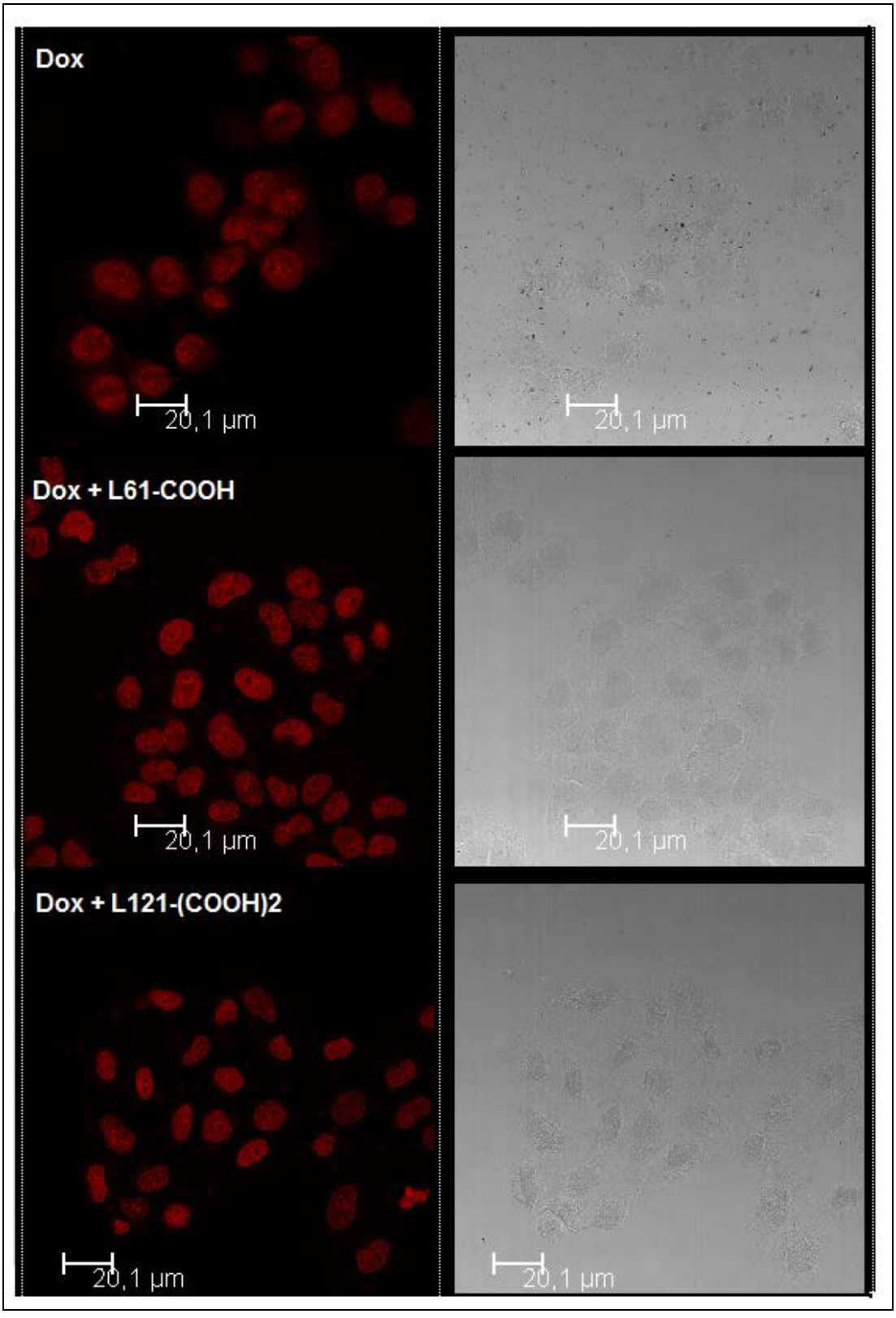

Figure 5.5. Fluorescent and light photography of A549 cells treated with doxorubicin (Dox) and it compositions with Pluronics L61-COOH, L121-$(COOH)_2$

Although, confocal microscopy showed that Pluronics increased the intracellular accumulation of doxorubicin, which correlates with cytotoxicity data shown in Figure 5.6. We showed that both initial and modified Pluronics promotes intracellular delivery of doxorubicin into the nuclei, which leads to enhancement of doxorubicin cytotoxicity to MDR cells. It has also been shown that modification slightly improved an ability of Pluronics to deliver doxorubicin into the nuclei.

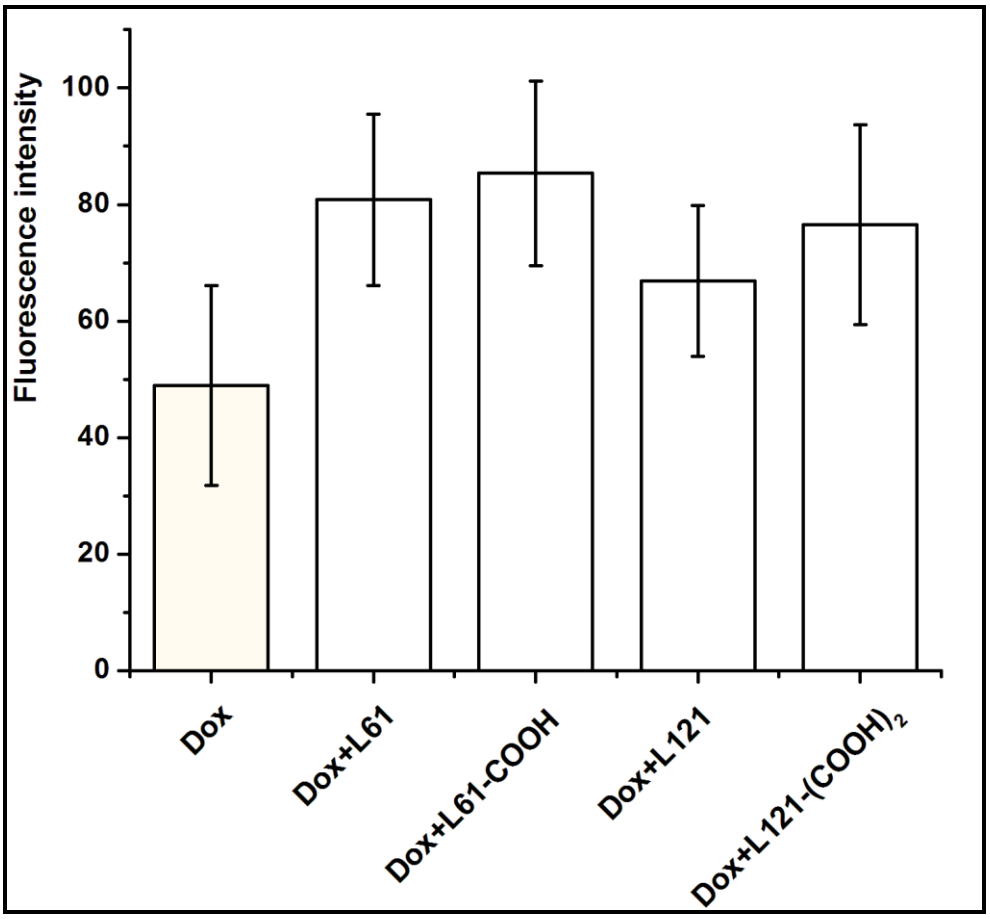

Figure 5.6. Fluorescence intensities of doxorubicin (Dox) and it compositions with Pluronics in the nuclei of A549 cells

In the literature, it has been shown that Pluronics are able to form coordinative complexes with a wide range of cations and promote their transport through the membrane [43]. Modification by carboxylic group can significantly improve an ability of Pluronics to form coordinative complexes, which explains the enhanced delivery of doxorubicin into the nuclei by means of modified Pluronics.

It is possible to assume, that Pluronics, especially modified by carboxylic group, act as transmembrane transmitting agents, due to their ability to form coordination complexes with weak base doxorubicin. Formation of such complexes in a membrane should significantly depress a free energy barrier of doxorubicin transfer through lipid bilayer, which promotes transport on a gradient of electrochemical potential.

5.1.7. Modified Pluronics Influence the Lipid Membrane Microviscosity

Microviscosity is one of the most important characteristics of the cytoplasm. Cytoplasm microviscosity depends on the lipid mobility and determines the functioning of membrane-incorporated enzymes. Particularly, cytoplasm microviscosity determines the activity of ATP-dependent drug reverse transporters, such as P-glycoproteins. P-glycoproteins play one of the key roles in multiple drug resistance (MDR) development in cancer cells. Kabanov and coworkers showed that Pluronics could significantly affect the membrane fluidity and as a result inhibit P-glycoproteins [67]. This is one of the most probable mechanisms of MDR cells sensitization to anticancer therapy in the presence of Pluronics.

Cellular membrane is a complex matrix made from lipids, proteins and carbohydrates, and thus is difficult for analysis. Considering this fact we studied the influence of initial and modified Pluronics on liposome membrane composed of the main component of the native cytoplasm phosphatidilcholine. The liposome microviscosity was determined using anisotropy of fluorescent probe – difenylhexatriene as described in materials and methods.

We determined that Pluronics at a concentration of 10μg/ml didn't affect the liposome viscosity, but at concentration of 100μg/ml produced significant effect. We showed that Pluronics decreased the liposome viscosity: Pluronic L61 up to 4.2 %, Pluronic L121 up to 8.6 %. This data shown in Figure 5.7 correlates with the HLB of Pluronics, more hydrophobic L121 (HLB = 1) disturbs liposome membrane more than L61 (HLB = 3). In comparison with initial Pluronic L61 modified L61-COOH decreased the liposome membrane fluidity more significantly (up to 15.8%), but modified L121-$(COOH)_2$ acts similarly to initial L121.

Results showed that Pluronic L61-COOH affects the lipid membrane fluidity more strongly than its precursor. It can be explained by the influence of introduced carboxylic group on polymer conformation. The reciprocal repulsion of the negatively charged carboxyls of the modified Pluronic introduced into the lipid bilayer can cause significant disruption of membrane fluidity. In case of Pluronic L121 with a low HLB, the modification didn't alter the conformation of the polymeric molecules sufficiently to change their influence on membrane fluidity. This data explains the mechanism of intracellular accumulation of doxorubicin in the presence of initial and modified Pluronics, which resulted in sensitization of MDR cells to chemotherapy.

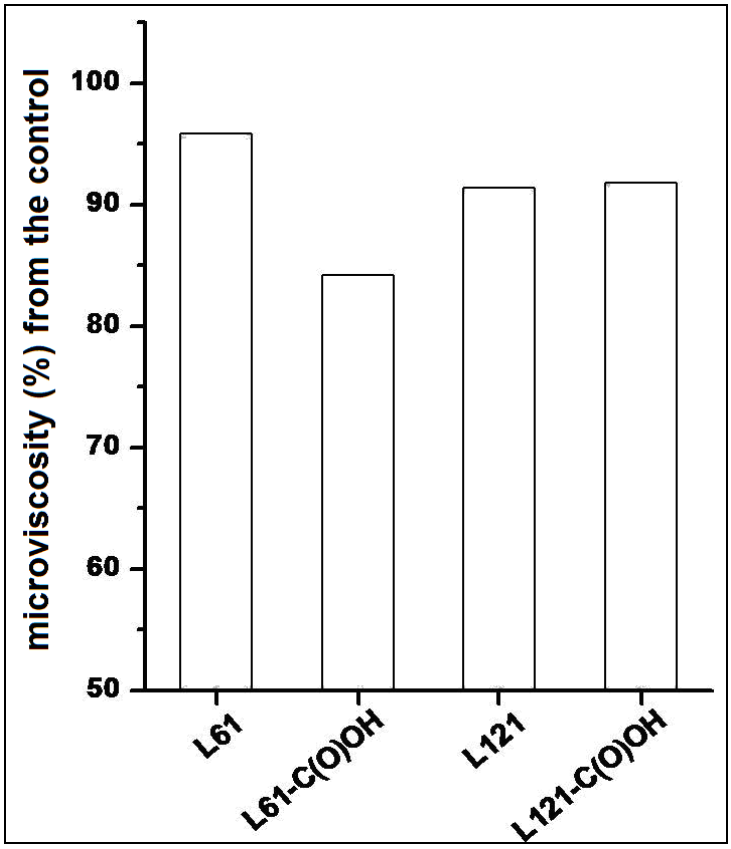

Figure 5.7. Alteration of liposome microviscosity in the presence of Pluronics at concentration of 100 μg/ml

5.2. PLURONIC-MODIFIED SILVER COLLOIDS AS OPTICAL PROBE FOR CELL ANALYSIS

5.2.1. Modification of Silver Nanoparticles with Pluronics and their Analysis Using UV-Visible Spectroscopy, Atomic Force Microscopy, Dynamic Light Scattering

The synthesized silver nanoparticles (AgNPs) were modified with Pluronics by means of hydrophobic association. Specifically, metal NPs were incubated with Pluronics at different concentrations to produce modified nanoparticles, which were more stable in salt solutions than the unmodified ones. As a result of incubation amphiphilic copolymers were adsorbed on the surface of nanoparticles to form AgNP-Pluronic complexes.

The addition of sodium chloride induced irreversible aggregation and disappearance of color of the prepared AgNP suspension due to masking of negative charge of citrate-capped nanoparticles by sodium ions. By detecting decolouration of the suspension of AgNPs, we ascertained minimal stabilizing concentrations (MSC) of Pluronics required for stable modification of the nanoparticles. The MSC was found to be 1.5µg/ml for all Pluronics, indicating generally unspecific character of their interaction with colloidal AgNPs The fact that Pluronics improve colloidal stability of AgNPs upon salt addition assumes that the amphiphilic copolymers are bound to the surface of AgNPs to form polymer-nanoparticle complexes. The binding presumably caused by hydrophobic interactions between colloidal silver and poly-(propylene oxide) block of the copolymers.

We also compared optical spectra of AgNPs and Pluronic-modified AgNPs in sodium chloride solution in order to evaluate stabilizing effect of the copolymers. The adsorption of Pluronics on AgNPs prevented the disappearance of the optical spectra of nanoparticles upon addition of the salt. The UV/Vis absorbance peak at about 410 nm was considerably decreased at different extents with the addition of NaCl solution to AgNP colloidal suspensions depending on Pluronics used for the modification. Figure 5.8 shows the affect of addition of NaCl solution to different AgNP suspsnsions. In particular, the optical absorbance of AgNPs-Pluronic complexes decreased distinctly in the order of Pluronics F68, F127, and L121. Results showed that copolymers themselves didn’t affect the optical

properties of AgNPs. Although, we demonstrated that Pluronics enable to protect nanoparticles against aggregation in salt solution and they are differ in this ability.

The more hydrophilic Pluronic F68 exhibited the highest stabilizing effect on AgNPs, whereas hydrophobic L121 showed the lowest stabilization. These results indicate that Pluronics of higher HLB provide better stabilization of the colloidal AgNPs than Pluronics of lower HLB. Specifically, after the modification with Pluronic L121 (HLB = 1) AgNPs are more prone to aggregate in NaCl solution apparently due to L121 promoting hydrophobic interactions between modified nanoparticles. In comparison to Pluronic L121, Pluronic F127 (HLB = 22) and especially Pluronic F68 (HLB = 29), possessing relatively long PEO blocks, impart higher hydrophilicity to AgNPs reducing their nonspecific interactions.

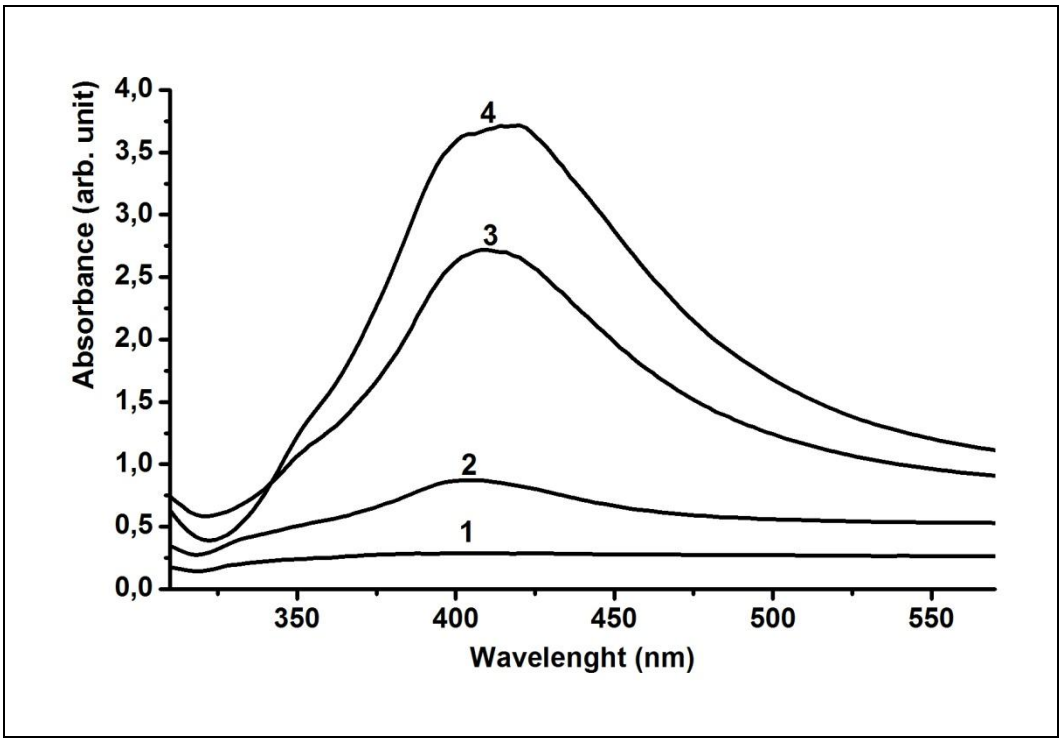

Figure 5.8. UV/vis absorption spectra of AgNP-Pluronic copolymer complexes in 0.04 M sodium chloride solution. (1) unmodified AgNPs; (2) AgNP-L121; (3) AgNP-F127; (4) AgNP-F68

The structures of metal NP-Pluronic complexes were analyzed by means of atomic force microscopy (AFM) as well as dynamic light scattering (DLS). Figure 5.9 shows representative AFM images of bare AgNPs and Pluronic-modified AgNPs spread on a mica substrate. In Figure 5.9A, unmodified AgNPs appeared on the substrate in a form of aggregated complexes composed of three or more spherical particles. Two types of AgNPs were generally observed on AFM images: smaller nanoparticles 13.1±4.7 nm and larger ones 32.7±7.7 nm (size was measured by the height of nanoparticles).

Modification of AgNPs with Pluronics F127 or F68 resulted in a partial decrease of their aggregation. The resulting AgNP-F127 and AgNP-F68 complexes represented well-defined aggregates consisting mainly of 2/3 particles shown in Figure 5.9B and C. No significant changes in the size of AgNPs were noticed after their modification with Pluronics F127 and F68. In Figure 5.9D, unlike these complexes, AgNPs modified with Pluronic L121 formed amorphous and relatively massive aggregates with an average particle size of 16.6±4.5 and 37.4±6.6 nm for smaller and larger fractions, respectively. This can be explained by hydrophobic nature of Pluronic L121 which promotes the aggregation of AgNP-L121 complexes upon sample preparation.

The DLS analysis revealed that light scattering profile of the nanoparticles is composed of two distinct peaks with a maximum at about 84.9 and 11.5 nm indicating that AgNPs consisted of two particle types. Hydrodynamic diameter of the larger fraction was almost three-times bigger than the size of large AgNPs measured by AFM. This probably arises from certain heterogeneity of AgNPs synthesized by citrate reduction and also from reversible interparticle association in the suspension. We found that hydrodynamic diameter of AgNPs remained almost unchanged after their modification with Pluronic copolymers seen in Table 5.4.

Based on DLS data, the mean zeta potential of unmodified AgNPs was -42.2 mV (pH $\geq$ 7) as synthesized. The AgNPs forms relatively stable negatively charged colloidal system. After the association with Pluronics, the zeta potential of AgNPs reduced to -35 - 37 mV indicating that Pluronics affect the surface properties of AgNPs to some extent.

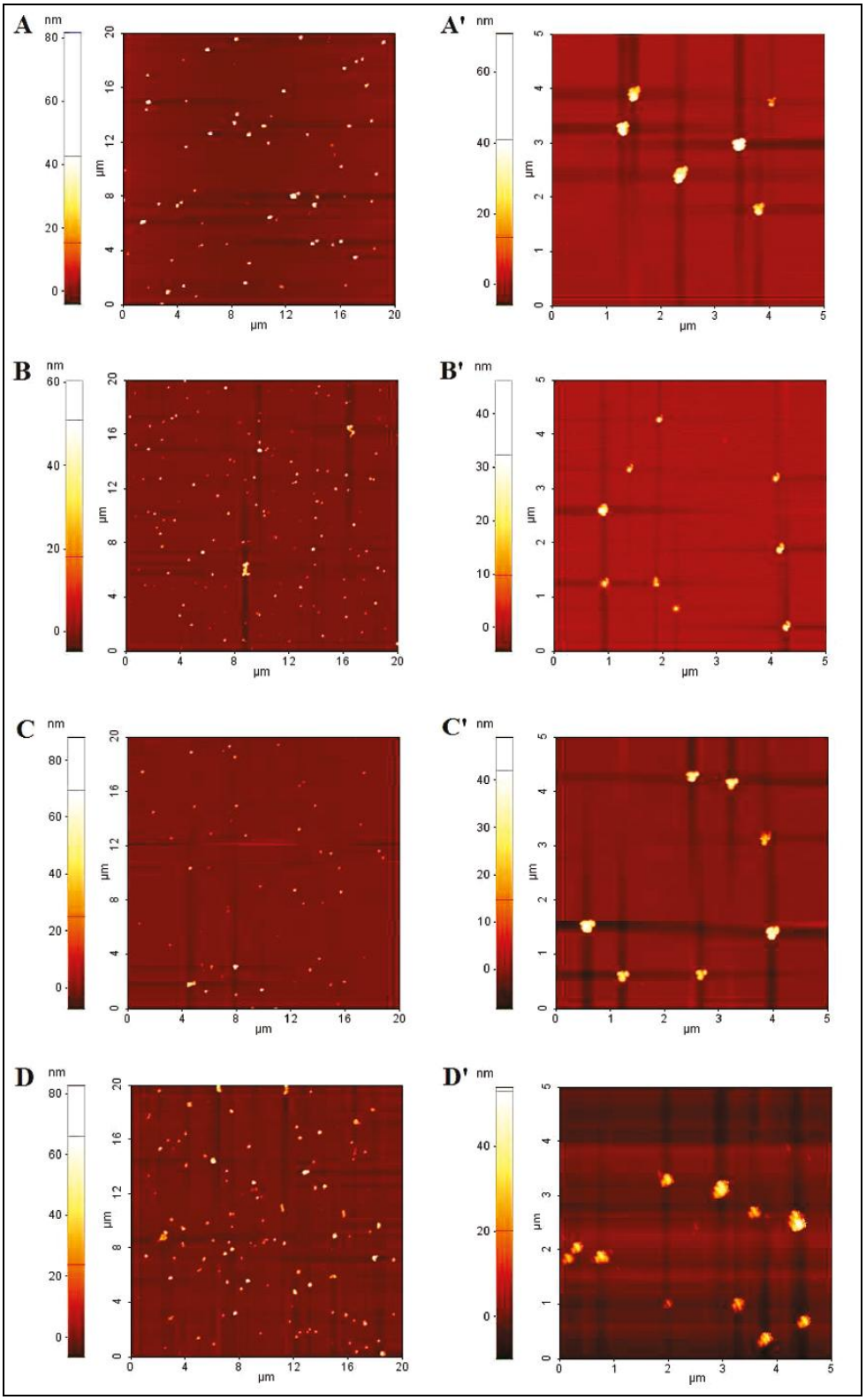

Figure 5.9. Topographic AFM-images of AgNPs and their complexes with Pluronic copolymers on mica substrate: (A) unmodified AgNPs; (B) AgNP-F127; (C) AgNP-F68; (D) AgNP-L121; (A`-D`) magnified sections of the images

Table 5.4. Dynamic light scattering data and zeta potential of metal nanoparticle–Pluronic copolymer complexes in water

Nanoparticle	Z-average (nm)	PdI	Peak 1 (nm)	Peak 2 (nm)	Zeta potential (mV)
AgNPs	54.26	0.307	83.86 (91.0*)	11.47 (9.0*)	–42.2
AgNP–L121	54.77	0.307	84.80 (90.5)	11.83 (9.4)	–36.8
AgNP–F68	54.44	0.306	83.39 (90.5)	11.65 (9.5)	–35.4
AgNP–F127	54.70	0.306	83.83 (91.7)	11.07 (8.3)	–37.2
* Peak relative intensity (%)					

Overall, the results allow us to propose the structural model of AgNP-Pluronic complexes, which are composed of colloidal core surrounded by a very thin polymeric shell. The shell is presumably formed as a result of the interaction between hydrophobic PPO blocks of Pluronic monomers with the surface of AgNPs, whereas hydrophilic PEO blocks are expected to be oriented towards the water phase.

5.2.2. Interaction of Organic Compounds with Silver Nanoparticle–Pluronic Complexes

Our results demonstrated that the association of AgNPs with Pluronic copolymers at MSCs led to the formation of composite nanoparticles. These nanoparticles exhibited reproducible optical properties, which were dependent on physicochemical characteristics of modified copolymers. Next, we applied surface-enhanced Raman spectroscopy (SERS) in order to ascertain how the copolymer components affect the adsorption of organic species on modified AgNPs.

SERS activity of polymer coated AgNPs was tested on xanthene dye - rhodamine 6G (RH6G) chosen as a model compound commonly used in spectroscopy; another dye aminofluorescein; anticancer drug – doxorubicin and ATP/ADP as biological molecules.

For RH6G we calculated the enhancement factor using bulk Raman spectrum of Rh6G and SERS spectra obtained from AgNPs-Pluronic complexes. Results showed significant increase in SERS-signal of RH6G on AgNP-polymer complexes in comparison with bulk AgNPs. Using the formula provided in [115], the enhancement factor was calculated as 9.04×10^6 for AgNP-F127, 2.78×10^6 for AgNP-L121, 1.05×10^6 for AgNP-F68, and 1.12×10^5 for AgNPs shown in Figure 5.10.

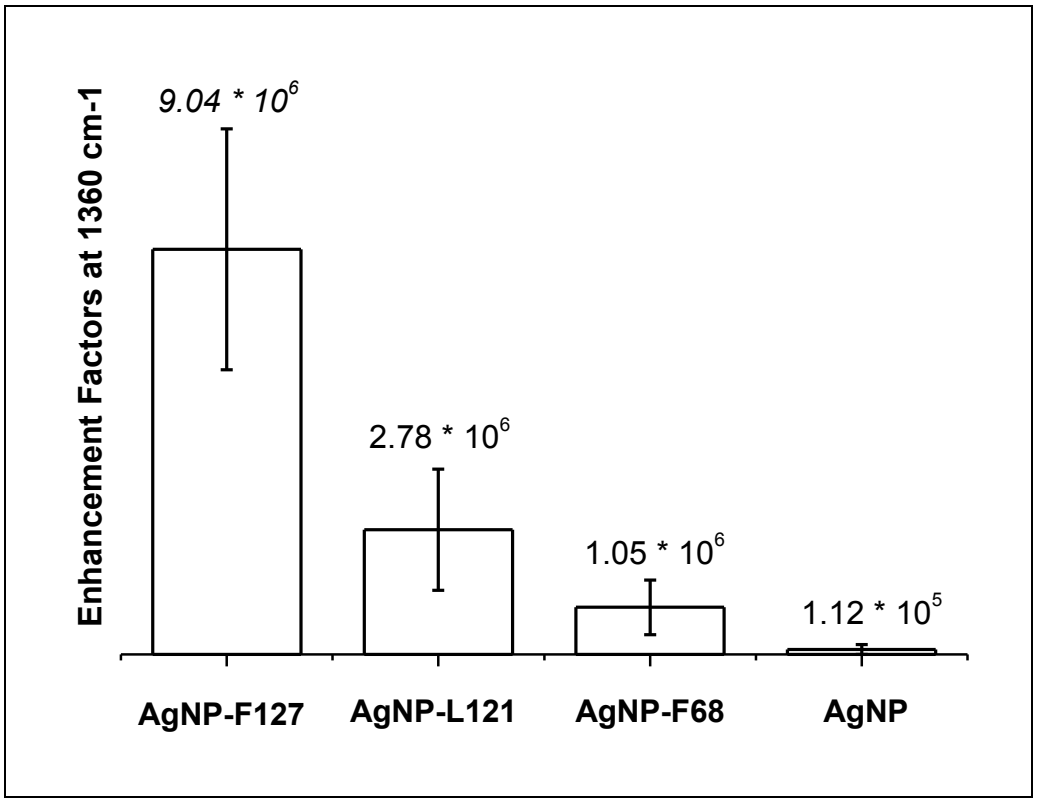

Figure 5.10. Enhancement factors for rhodamine 6G at Ag nanoparticles and AgNP-Pluronic complexes

Modification by Pluronics promotes hydrophobic association of RH6G on the surface of AgNPs, which leads to SERS signal amplification. The enhancement of RH6G signal, demonstrated for Pluronic modified AgNPs, depends on the hydrophilic lipophilic balance (HLB) of the copolymers. Pluronics L121 and F127 rich in hydrophobic polypropylene oxide blocks, which favors the adsorption of aromatic dye, showed higher SERS signal amplification of Rh6G compared to Pluronic F68. Pluronic F127 was found to enhance the signal of Rh6G more than three times higher than Pluronic L121 seen in

Figure 5.10. This indicates that polyethylene blocks richly presented in Pluronic F127 are also important for nanoparticle-dye interactions, probably they help to stabilize the probe against undesirable aggregation.

The results revealed that Pluronic F127 possessed optimal HLB for SERS-signal amplification of aromatic compounds such as RH6G. In subsequent experiments we used AgNPs modified by Pluronic F127. Additionally we demonstrated that AgNP-F127 complexes possessed the high SERS enhancement for another dye - aminofluorescein and anticancer drug - doxorubicin in comparison with bulk AgNPs. Figure 5.11 represents typical SERS-spectra of AgNPs and AgNP-F127 with adsorbed aminofluorescein and doxorubicin.

At a concentration of 1μM on AgNP-F127 complexes both aminofluorescein and doxorubicin produced well defined SERS spectra with characteristic Raman bands, but on bulk AgNPs the same concentration of the dye didn't result in defined spectra. The enhancement factors were 4.15×10^4 and 5.45×10^5 for aminofluorescein and doxorubicin repetitively. The same results were obtained for ATP/ADP, and uncharged aromatic amino acids (data not shown).

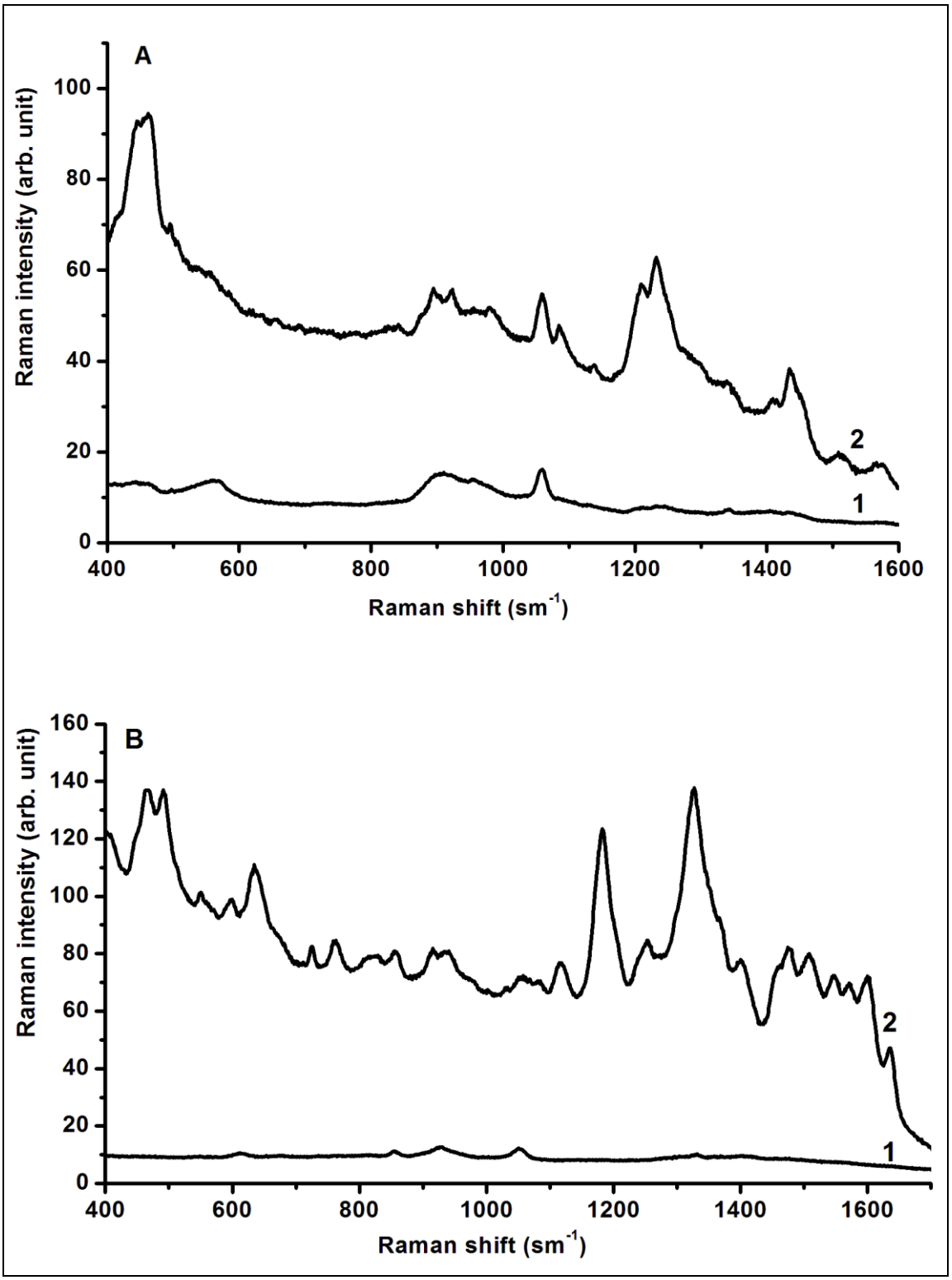

Figure 5.11. (A) SERS spectra of 1 μM doxorubicin adsorbed on AgNPs (1) and AgNP–Pluronic F127 complex (2). (B) SERS spectra of 1 μM aminofluorescein adsorbed on AgNPs (1) and AgNP–Pluronic F127 complex (2). Each spectrum is the average of the ten SERS spectra

5.2.3. Rhodamin 6G and Cellular Metabolites Efflux Studies Using Composite Nanoparticles as Extracellular Probes

The developed composite nanoparticles were applied for direct intracellular probing of the model drug compound RH6G, a strong SERS scatterer. For this experiment human hepatoma Hep3B and human lung adenocarcinoma epithelial A549 cells were grown on cover slips, preincubated with 100μM of RH6G and then washed and coincubated with developed composite probes. After the treatment cells were fixed and analyzed by means of confocal microscopy and SERS.

Based on fluorescent and confocal microscopy data both RH6G and modified nanoparticles successfully penetrated the cytoplasmic membrane and localized in the cytoplasm (data not shown). In order to take spectra, 40x objective was focused at dark spots inside the cells produced by the captured aggregates of modified nanoparticles. In spite of the fact that the developed probe showed high sensitivity to RH6G (10 nM of RH6G was enough to observe well defined SERS spectrum using modified probe), internalized nanoparticles did not allow us detection of accumulated RH6G by registering it characteristic Raman bands. Intracellular SERS probing of RH6G using modified nanoparticles was found to be difficult, probably because of the interfering influence of the cellular component, particularly proteins. The cellular periplasmic space and growing media both contain proteins at high concentrations. It was shown by us and other researchers that proteins easily adsorb on the surface of metal nanoparticles by means of hydrophobic and ionic interactions [116]. The layer of proteins on the surface of nanoparticles prevents adsorption of RH6G, which makes the RH6G SERS detection problematic.

Considering the fact that metabolites and drugs can be transported out of different cell types, we tested nanoparticles as extracellular probes. The proposed method consists of several steps: the first step assumes triple washing of adhered cells by the buffer solution; second step includes the coincubation of the cells with compound of interest. The third step implies transferring cells to a fresh buffer solution, incubation and collection of the matrix buffer containing the drugs and metabolites released from the cells. The aliquots of the matrix buffer are then mixed with composite AgNPs, dried on the CaF_2 slides and analysed

by SERS. Human cells which were held in DPBS buffer solution for several hours during the analysis remained viable, which was shown by acridine orange/ethidium bromide double staining assay (data not shown).

RH6G was chosen as a model compound for drug efflux studies in living cells using SERS and developed AgNP-Pluronic F127 probe. RH6G efflux was studied on A549 and Hep3B cell lines, which correspond to MDR cells with a high expression of drug efflux transporter – P-glycoprotein (P-gp). Little is known about intracellular accumulation of RH6G and its P-gp mediated efflux. Normally, in P-gp kinetic experiments researchers measure the fluorescence of the RH6G, which was taken up by the isolated cells or tissues. In the present study we attempted to develop an alternative SERS analysis of P-gp mediated RH6G efflux. The proposed method based on the detection of the RH6G released from the cells into the surrounding media. For these experiments we used the AgNP-Pluronic F127 composite as a SERS probe.

Adhered Hep3B cells were pre-incubated with 6 μM of RH6G, washed three times and placed in a fresh DPBS solution allowing accumulated RH6G to release from the living cells. The RH6G efflux process is a sum of passive diffusion and active transport with a participation of membrane pumps such as P-gps. Figure 5.12A represents SERS spectra of RH6G released from the kidney cells during prolongated of incubation and the kinetic representation of RH6G-signal versus time. Under these experimental conditions, the modified nanoparticles allowed selective SERS detection of trace amounts of RH6G released from cells to extracellular solution presumably via active transport. In Figure 5.12B The kinetic curve of RH6G release is sigmoidal, during the first 30 min the drug efflux proceeds quickly and reaches the maximal RH6G concentration, then from 30 min to 120 min of incubation the process slows down and levels off.

We noticed that after 120-180 min of incubation SERS-signal of RH6G slowed down, even further, after 17 hours of incubation completely disappeared seen in Figure 5.12A. This can be caused by the strong interference between RH6G and intracellular components, especially proteins, which are also being released from the cells and compete with RH6G for the adsorption spots on the surface of AgNP.

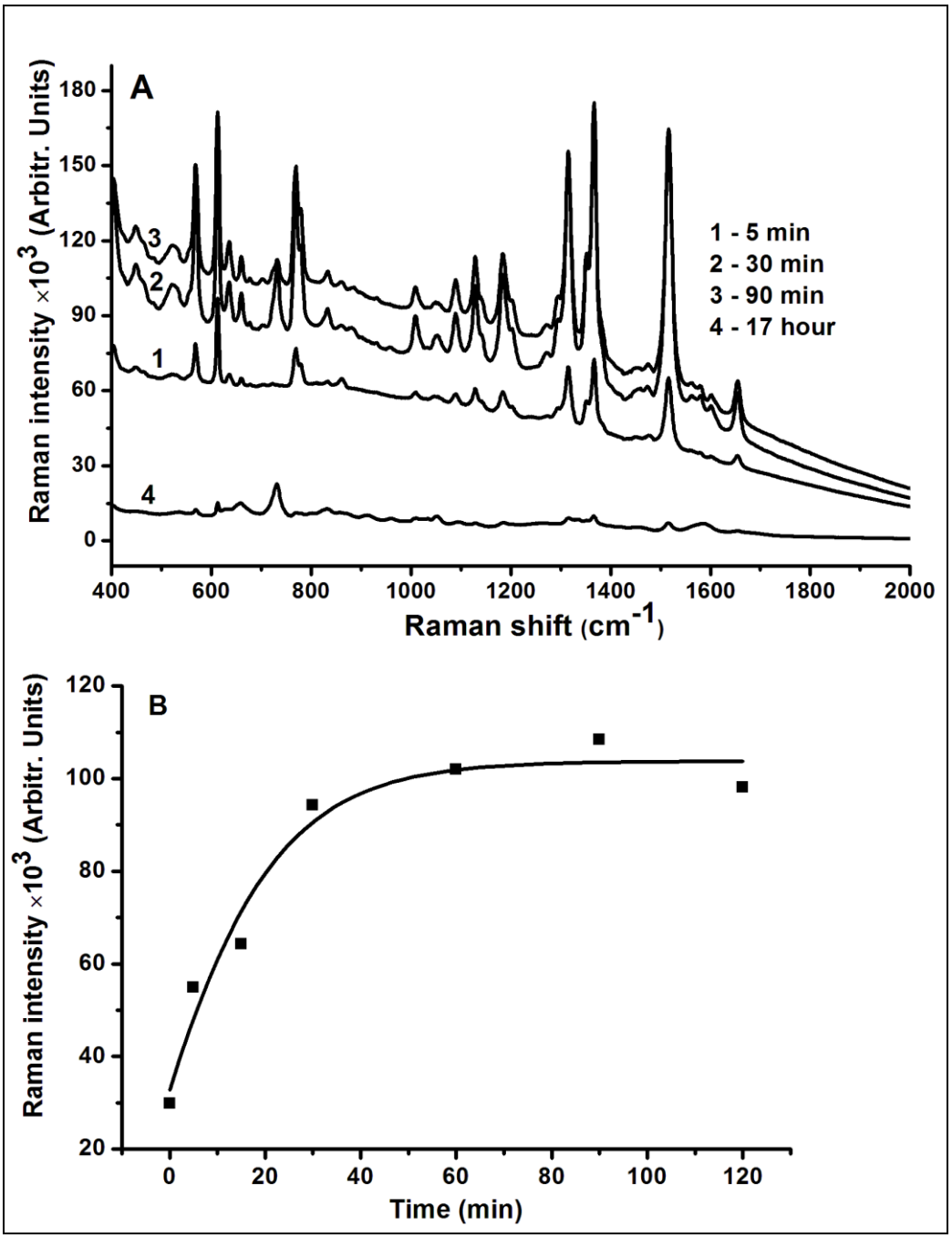

Figure 5.12. A) SERS spectra of RH6G released from the Hep3B cells versus time obtained using developed probe and excitation at 830 nm; B) RH6G signals versus time (the peak height around 1360 cm^{-1} of the spectrum was used to calculate the signal). Initial RH6G concentration added to the cells was 6 μM. Each spectrum is the average of 10 spectra obtained from the different areas of the dried colloids

The RH6G reverse transport in A549 cells happens in a similar way to kidney cells, but Raman intensity of the RH6G signal is more than 10 times lower than for Hep3B cells. The data can be explained by the higher uptake capacity of Hep3B cells for RH6G in comparison with A549 cells and also by their higher metabolic activity.

In present work silver nanoparticles modified by Pluronic F127 were also used for SERS probing of adenine nucleotides excreted from the Hep3B and A549 cell lines. We found that A549 and Hep3B cells in buffer (DPBS) solution retained their viability and secreted some metabolites to the surrounding buffer during incubation. Figure 5.13 (1) represents typical SERS spectrum of released metabolites in comparison with the control buffer solution (3) and cultural media spectra (2). The major bands of the spectrum are numbered.

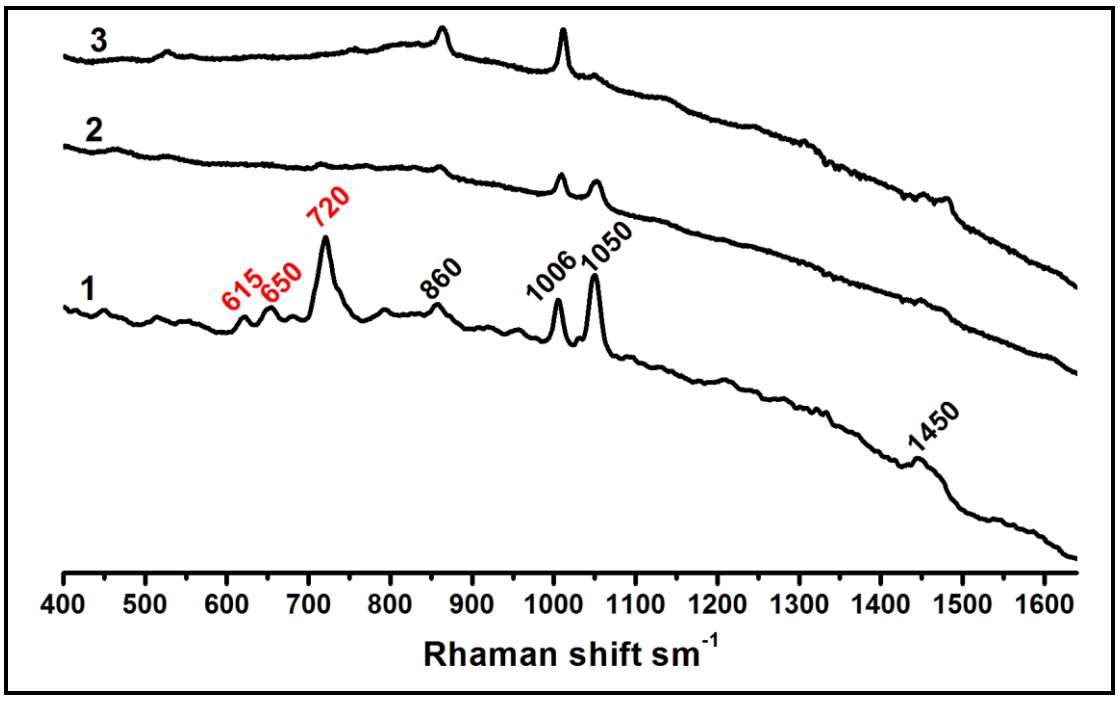

Figure 5.13. 1) Typical SERS spectrum of Hep3B cellular metabolites released to the extracellular media during 17 hours of incubation in DPBS solution 2) SERS spectrum of culture medium DMEM supplemented with 10% BSA and 1% PSA (Sigma-Aldrich); 3) SERS spectrum of buffer solution DPBS (Sigma-Aldrich). All spectra acquired with the developed AgNP-F127 probe at 830 nm excitation. Each spectrum is the average of 10 spectra obtained from the different area of the dried colloids

The majority of the bands (1450, 1050, 1006, 860 cm^{-1}) were observed both in DPBS, DMEM and in cellular metabolite spectra. We could not attribute them to any cellular components which made them of little use for metabolite efflux analysis. However, Raman bands around 615, 650, 720 cm^{-1} probably originated from the released cellular components that couldn't be produced by growing in media or DPBS solution shown in Figure 5.13.

It was necessary to identify the origin of the 720 cm^{-1} Raman band of the cellular metabolite spectrum. We made an assumption that the 720 cm^{-1} band originated from low molecular weight aromatic molecules such as adenine nucleotides, which adsorption is favored on AgNPs.

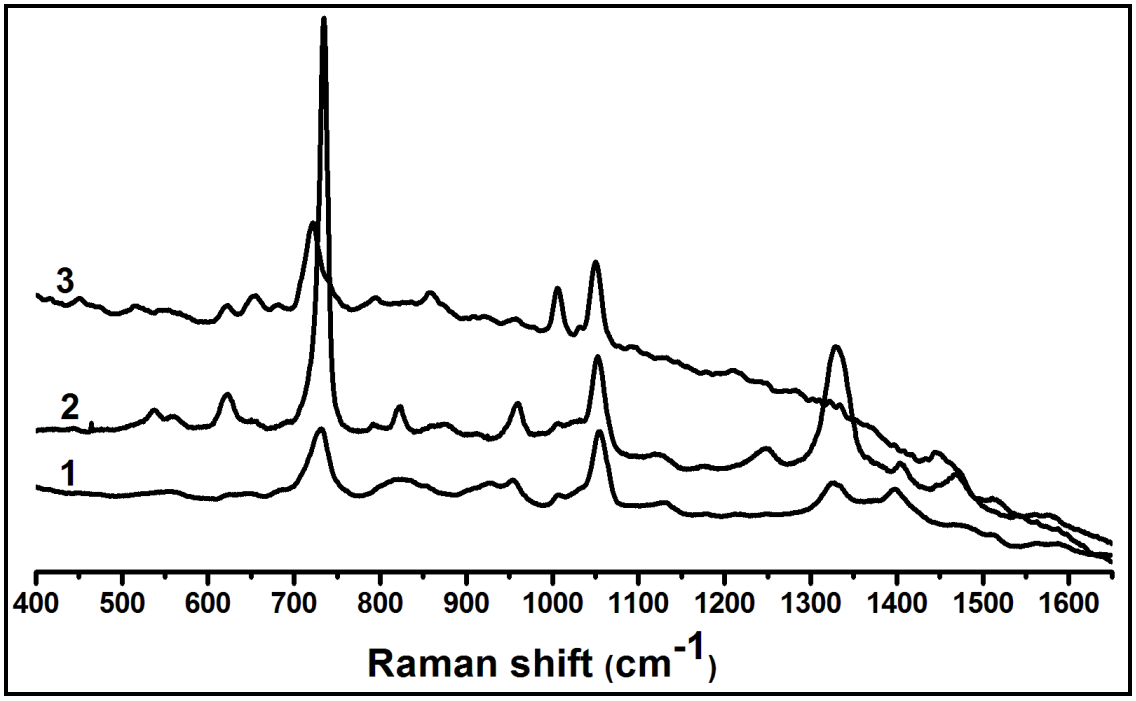

Figure 5.14. SERS spectra of (1) ATP (10^{-3}M); (2) ADP (10^{-3} M); (3) Hep3B cellular metabolites released to the extracellular media during 17 hours incubation in DPBS solution, obtained with the developed AgNP-F127 probe at 830 nm excitation. Each spectrum is the average of 10 spectra obtained from the different areas of the dried colloids

In order to confirm that hypothesis, SERS spectra of ATP and ADP were acquired using developed probe and compared with cellular metabolites spectra which was shown

Figure 5.14. Our results showed that peaks at 615, 650, 720 cm^{-1} of the cellular metabolites spectrum might indeed correspond to ADP and/or ATP. Moreover, the intensity of 720 cm^{-1} signal increased with the prolongation of cell incubation in buffer solution, which additionally indicates that this signal might belong to adenine nucleotides.

Cellular ATP content is of particular interest for assessment of cell metabolism, energetic status and certain pathologies. The results obtained in our studies might be of particular interest for the development of new SERS-based techniques and methods for informative analysis of cellular drug transport, as well as for imaging agents and metabolites identification.

6. CONCLUSION and RECOMMENDATIONS

6.1. CONCLUSIONS

The current study is dedicated to the development of Pluronic based nanoparticles for intracellular delivery and SERS analysis of some biologically active substances. In order to develop novel modifications of Pluronics, which exhibit less toxicity and higher efficiency in drug delivery, we modified Pluronics of different structure with terminal carboxylic groups. The modification was verified using a variety of physicochemical methods. The association of carboxylated polymers in water solutions was analyzed by dynamic light scattering. It was demonstrated that introduction of the carboxylic group into polymeric structure imparts strong negative charge to the Pluronic micelles and also affects the size of micelles.

According to cytotoxicity study, modified Pluronics were much less toxic to human cells than unmodified polymers. Additionally, modified Pluronics exert less negative impact on the intracellular ATP synthesis than initial Pluronics. At the same time, introduction of carboxylic group did not affect Pluronic ability to increase cytotoxicity of anticancer drug doxorubicin towards MDR cells. Both modified and initial Pluronics increase the lipid membrane fluidity, which promotes transport of molecules such us doxorubicin through the bilayer and correspondingly enhance doxorubicin toxicity to MDR cells. On the basis of our results, some probable mechanisms of pharmacological effects of Pluronics were proposed.

For the development of Pluronic based SERS probe we modified citrate-stabilized silver nanoparticles with a thin layer of Pluronics with different structure. Pluronics get adsorbed on the surface of silver nanoparticles by means of hydrophobic interactions. We demonstrated that Polymeric shell promotes the adsorption of some aromatic compounds on the surface of modified nanoparticles, which results in significant enhancement of SERS signals observed for rhodamine 6G, doxorubicin, and purine bases. Developed composite AgNP-Pluronic complexes can be used for extracellular probing of adenine

nucleotides and accumulated rhodamin 6G which are excreted from the living cells by means of active and passive transport.

The results obtained in our studies might be of particular interest for the development of new SERS-based techniques and methods for informative analysis of cellular drug transport, as well as for imaging agents and metabolites identification.

6.2. RECOMMENDATIONS

In this study we used Pluronics modified with terminal carboxylic group. The modification allows us to attaché some fluorescent dyes to polymeric molecule using carbodiimide reaction. Such composite nanoparticle we can apply as intracellular imaging agent and also study intracellular accumulation and distribution of Pluronics in cells, because this question still remains unclear.

As a future work we are planning to modify Pluronic structure with other chemical groups, such as amino or sulfhydryl group. Pluronics modified with different groups will be of particular interest in the development of new drug and gene carriers, and also in design of composite nanoparticles for SERS analysis of living cells.

Results of present research, or more exactly Pluronics modified with carboxylic groups, will be patented in the nearest time as promising materials for biomedical applications, especially in the field of drug and gene delivery.

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