PHARMACEUTICAL SCIENCES

UDC 546.26:615.099.092

STUDY OF IMPROVEMENT OF EFFICIENCY OF DRUG SUBSTANCE LIPOSOMAL DELIVERY SYSTEMS, AS WELL AS OF DELIVERY SYSTEMS BY MEANS OF CARBON NANOTUBES TO TARGET CELLS DUE TO CONTROLLED CHANGE OF LIPOSOME CHARGE, NANOTUBES CHARGE OR TARGET CELL MEMBRANES CHARGE BY FLUORESCENT AND SPIN PROBES METHODS.

Kartel N.T., Ivanov L.V.,
Chuiko Institute of Surface Chemistry of NAS of Ukraine, Kiev
Lyapunov A.N.,
Institute for Single Crystals of National Academy of Sciences of Ukraine, Kharkov
Okotrub A.V.,
Nikolaev Institute of Inorganic Chemistry SB RAS, Novosibirsk;
Derymedvid L.V., Vereitinova V.P.
National University of Pharmacy, Kharkov

Abstract:
In order to increase the efficiency of liposomal delivery systems (LDS) for drug substance (DS) delivery to target cells the concept of creation of LDS with two or three active DS ("self-organizing LDS") is proposed and in a number of cases experimentally confirmed, where some DS beside pharmacological activity are capable to provide structuring function - change liposome membrane charge and respectively change the affinity to liposome membranes of other oppositely charged pharmacologically active DS in liposomes; reduce membrane fluidity and respectively reduce DS efflux from liposomes; capable to charge at the final stage at fusion with target cells the membranes of these cells and facilitate oppositely charged active DS more effective penetration into target cells. New experimental data on the fact that change of target cell membrane charge by means of introduction of ionogenic surfactants substantially increases the affinity of carbon nanotubes (CNT) with opposite charge are obtained. In these cases ion-ion interactions of CNTs with oppositely charged membranes of target cells can act as the main motive force of DS delivery vector and improve efficiency of delivery systems by means of nanotubes. It is also possible to use charged CNTs carrying DS with charge opposite to target cell membrane charge.

Systems of drug substance (DS) delivery to tissues and target cells should account for and use at designing the fact that many drugs have in their molecules beside lipophilic moiety a certain charge, and target cells have charges at membrane surface. In the case of liposomal delivery systems (LDS) for drug substances, the key aspect at creation of LDS is the provision of necessary liposome charge, which is defined for every DS individually and in a certain way affects both the process of DS inclusion in liposomes and the process of directed transport of DS to target cells or target organs. Ceteris paribus, the presence in liposome membrane of charged moiety should facilitate the inclusion of DS with opposite charge. Thus, foreign researchers demonstrated that the anionic liposomes are optimal as agents of positively charged amitriptyline delivery [1], anionic inhibitor of thrombocytes is more effective when introduced to cationic liposomes [2], and isolithocholic acid derivatives facilitate DNA molecule release from polycationic liposomes [3].

In order to increase the efficiency of different systems of DS delivery to target cells we propose the concept of creation of LDS with two or three active drugs ("self-organizing LDS"), where some DS beside pharmacological activity are capable to provide structuring function - change liposome membrane charge and respectively change the affinity to liposome membranes of other oppositely charged pharmacologically active DS in liposomes; change membrane fluidity and respectively reduce DS efflux from liposomes; capable to charge at the final stage at fusion with cancer cells its membranes and facilitate oppositely charged DS (doxorubicin) more effective penetration into cancer cells. For functional carbon nanotubes (CNT) and other nanoparticles having superficial charges it is proposed to introduce into target cell membranes the ionogenic surfactants with opposite charge. In these cases ion-ion interactions of DS or CNT with oppositely charged cell membranes act as the main motive force of improvement of nanotube delivery system efficiency.

The purpose of this paper is the experimental validation of different configurations of proposed concept of improvement of DS delivery system efficiency that are related to change of liposome and erythrocyte membrane charges. The results may be used at designing of new DS delivery systems.

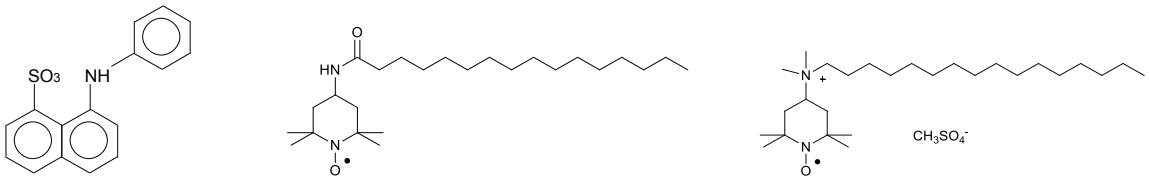
Materials and methods.

In this work we used the methods of fluorescent and spin probes, which are commonly used in molecular biology and pharmacology, biophysics, biopharmaceutics and are lately used in nanotechnological researches at study of influence of different nanoparticles on biological objects [4-10].

Liposomes were derived by means of ultrasound treatment of multilamellar vesicles from 0,05% phosphatidylcholine at frequency of 22 kHz in 0,1 M Tris buffer with pH of 7,2 by means of method described in [4]. In order to obtain the cationic liposomes the liposome suspension from phosphatidylcholine was added with cationic surfactant aethonium in an amount of 10% of phosphatidylcholine weight. Rat packed erythrocytes were obtained from the blood of male rats by

means of triple washing with physiological solution (0,9% sodium chloride) prepared on sodium phosphate buffer (5 mmol/l, pH 7,2–7,4) with further centrifugation during 20 minutes with the velocity of 3000 rpm. Human erythrocytes were obtained from fresh-preserved donated blood preserved with Glugicirum. Blood was drawn off at Kharkov regional blood transfusion station. With the purpose of object unification group A blood was used. After plasma removal the erythromass was washed three times by means of centrifugation at 1500 g during 3 minutes in tenfold volume of physiological solution (0,15 Mol/l of sodium chloride, 0,01 Mol/l of Tris buffer, pH 7,4).

In order to explore the affinity of drug substances to liposomes, as well as to evaluate the cell membrane charge, a negatively charged fluorescent probe 1- anilinonaphthalene-8- sulphonate (1,8-AHC) by "Serva"(FRG) was used. Fluorescent probes were introduced into liposomes with a concentration of $5 \cdot 10^{-6}$ mol/l by means of introduction of their aqueous solutions to liposome suspension. Fluorescence for 1,8-AHC probe was excited at 365 nm, and fluorescence changes were observed at 475 nm. Fluorescent probe fluoresces only in lipidic surrounding of liposome membranes, and in water probe fluorescence is quenched with water molecules. For exploration of influence of multiwall conventional (hydrophobic) or modified oxidized carbon nanotubes on erythrocyte membrane microviscosity were used two lipophilic spin probes on the basis of palmitic acid –charge-neutral probe **1** and positively charged by quaternary nitrogen probe **2**, synthesized according to [5].



1,8-AHC *probe **1*** *probe **2***

Introduction of probes **1** and **2** to erythrocyte water suspension was carried out by adding of concentrated probe solution to DMSO in such a way that DMSO final concentration in erythrocyte suspension was within 0,5 - 1%, and probe final concentration in erythrocytes was $2 \cdot 10^{-4}$ m/l. ESR spectra were registered with radiospectrometer *«ESR Spectrometer CMS8400»*, and fluorescence spectra were registered with spectrofluorimeter SIGNE-4M.

The evaluation of erythrocyte membrane microviscosity was carried out on the basis of processing of intensity and width of ESR spectra triplet lines for nitroxide radicals – spin probes **1** and **2** in lipidic bilayer of erythrocyte membranes [6, 7]. For calculation of correlation time for probe Brownian rotational diffusion (τ) the following spectra characteristics are used: width of central component (ΔH_0) and low-floor component ΔH_{+1}, intensity of ESR spectrum components ESR (h_0, h_{+1}, h_{-1}) with nucleus magnetic quantum number ^{14}N $(M = 0, +1, -1)$, and isotropic splitting constant ($A_{изо}$). Basic equation for evaluation of viscosity of any medium is the Stokes-Einstein equation:

$$\tau = 4\pi a^3 \cdot \eta / 3kT , \qquad (1)$$

where: τ_c is the spin probe correlation time (time during which spin probe rotates for 1 radian, 57^0), η is the medium viscosity, a is the effective radius of spin probe defined from ESR spectra [6, 7].

$$1/\tau_{c\,(+1)} = 2 \cdot 10^8 / [(h_0/h_{+1})^{1/2} - 1)]\ \Delta H_0\ sec^{-1} \quad 2\ (a)$$

$$1/\tau_{c\,(-1)} = 3{,}6 \cdot 10^9 / [(h_0/h_{-1})^{1/2} - 1)]\ \Delta H_0\ sec^{-1} 2\ (b)$$

$$\tau_{c\,(+1/-1)} = 6{,}65 \cdot 10^{-10}\ ([(h_{+1}/h_{-1})^{1/2} - 1)]\ \Delta H_{+1}\ sec^{-1} 2\ (c)$$

Hydrophobic nanotubes that were obtained at pilot installation developed by Chuiko Institute of Surface Chemistry of NAS of Ukraine and TMSpetsmash LLC (Kiev) were studied: CNT-K1 – cumulative fraction of high-purity multiwall nanotubes (content of more than 95%), obtained by means of CVD-synthesis over composite oxide Al-Fe-Mo aerosolic catalyst, nanotube inner diameter ≈ 1-2 nm, outer diameter ≈ 10-40 nm, ash content less than 0,4%; CNT-K2 – oxidized modification of CNT-K1 obtained as the result of programmable treatment with nitric acid solutions [5].

Results and discussion.

Earlier L.V. Ivanov et al. demonstrated with the method of spin and fluorescent probes that the introduction of ionogenic surfactants to liposomes from phosphatidylcholine causes rapid binding of surfactants with liposomes and notable change in fluorescence of negatively charged 1,8-AHC probe in liposomes due to charge at liposome membranes under action of surfactant [24]. Authors assumed that by changing the charge at cell membranes by means of different procedures it is possible to control the process of drug substance fixation to membranes, i.e. to influence bioavailability of DS. This idea was developed in our further research of different systems for DS delivery to cells and target tissues by means of membrane charge alternation.

For comparative study of affinity of certain charged DS to liposomes with neutral charge of membranes and with additional opposite charge at liposome membrane the diclofenac sodium was chosen as DS having in its lipophilic molecule (two residues of benzene rings) the carboxyl group, which is negatively charged at neutral pH and liposomes from phosphatidylcholine with addition of cationic surfactant aethonium. Fig. 1 presents the data on determination of affinity constant K_c for diclofenac sodium affinity to liposomes from phosphatidylcholine (neutral charge at membrane) and to cationic liposomes with addition of aethonium by graphic method in inversed coordinates. Ordinate contains a value that is inverse to ν, where ν is the fraction of DS complexes with liposomes as related to total quantity of liposomes taking part in DS fixation.

The parameter $\nu=(F_0-F)/F_0$ is calculated from fluorescence spectra of 1,8-AHC probe in liposomes in the presence of DS, where F_0 is the initial fluorescence intensity of 1,8-AHC probe in liposomes, F is the intensity of probe fluorescence under condition of diclofenac growing concentration in liposome suspension [8,11,12,23]. It is worth noting that the fixation of negatively charged diclofenac sodium in membranes of conventional liposomes caused the increase of negative charge at membrane. Herewith, the probe fluorescence in liposomes dropped due to probe displacement from membranes with diclofenac sodium molecules, as well as repulsion of negatively charged 1,8-AHC probe from liposome membranes. This effect was amplified in the presence of cationic surfactant aethonium in membrane, when probe affinity to membrane was increased due to presence of aethonium in membrane.

An abscissa intercept created with experimental line defines the value of constant Kc of diclofenac binding to conventional liposomes equal to $1\cdot10^3$ M^{-1} and of diclofenac fixation to liposomes with addition of surfactant aethonium (positively charged liposomes) equal to $3\cdot10^3$ M^{-1} [11, 12]. Herewith the last points of straight lines with coordinates (x= -3, y=0) and (x= -1, y=0) are not the experimental ones; they are extrapolated with experimental lines to abscissa for Kc determination. Thus, the introduction of positive charge to liposome membranes increases the diclofenac affinity to liposomes approximately thrice. The results show that the directed change of membrane charge allows substantial increase of LDS efficiency, at which the DS is not "hanging" inside the liposomes, but is more firmly fixed to inner surface of liposome membranes and prevents DS "efflux" at liposome diffusion through tissues and vascular barriers.

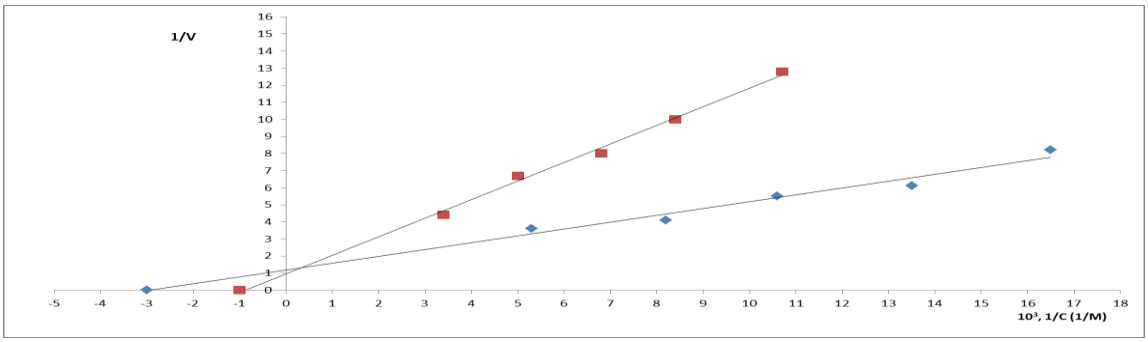
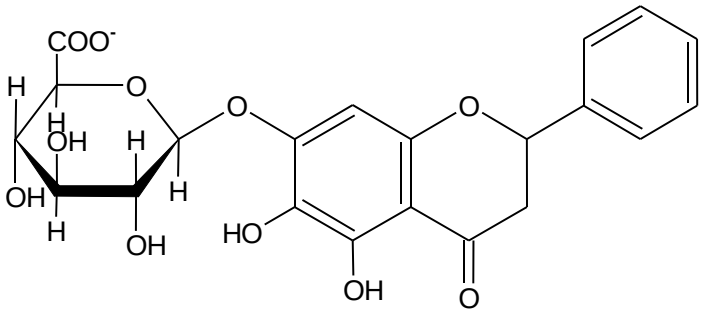


Fig. 1 Graphical determination of constant K_c of diclofenac sodium fixation to liposomes by graphical method in inverse coordinates: brown dots represent liposomes from phosphatidylcholine, blue dots represent liposomes with addition of surfactant aethonium.

Second key point at LDS creation is that at introduction in vivo the liposomes must remain maximally impenetrable for DS included in them as the result of liposomes interaction with plasma components– efflux effect [13]. This happens when membrane fluidity is high. In this case liposomes are added with cholesterine, which increases membrane viscosity [14]. We turned our attention to work of native researchers, in which by means of fluorescent probe method it is shown that the microviscosity of achromacyte membranes, reduced under inflammation conditions, is increased up to normal value (recovered) at addition to erythrocytes of a number of nonsteroidal anti-inflammatory drugs (NSAIDs) – mefenamine sodium salt, ortophenum, diclofenac and piroxicam. Herewith, the affinity constants for these NSAIDs affinity to erythrocytes under inflammation conditions are increased by 8-10 times [15]. Thus, the data obtained above (Fig. 1) evidence that the presence in liposomes of diclofenac sodium may not only charge the membrane with negative charge, but also increase microviscosity of liposome membranes preventing DS efflux from liposomes. Obviously, this opens the possibilities for improvement of DS functionality in liposomal delivery systems.

Main problem at LDS creation is the selection of dominant DS capable of forming the optimal delivery system. For doxorubicin delivery system it is required to have in liposomes a membranothropic negatively charged drug substance capable of charging the liposome membrane with negative charge and increase doxorubicin affinity to inner surface of liposomes. We offered using flavonoid baicalin obtained from Baikal skullcap as a structure forming DS for liposomal delivery systems containing doxorubicin and other positively charged DS. Flavonoid baicalin possesses a number of unique properties, which allowed creation of a separate magazine dedicated to major researches on baicalin in the USA. Baicalin possesses high antioxidant activity and reduces toxicity of chemotherapeutic agents by 2-3 times when taken simultaneously, which allows substantial increase of chemotherapeutic agent dose. Baikal skullcap flavonoids demonstrate hepatoprotective action [16,17], are capable of modulating the activity of microsomal cytochrome P 450 and oxidoreductase [18], demonstrate antimicotic and antiviral activity, reveal cytostatic activity [19-21] and antioxidant activity



[22].

Fig. 2. Baicalin structural formula

Major problem of implementation of baicalin action in an organism is the protection from fast metabolism in liver and stomach, where it is metabolized to aglycone. That is why baicalin introduction into liposomes can protect baicalin from fast metabolism and los of pharmacological effect. Using baicalin as dominant DS in LDS is primarily related to presence of negative charge in baicalin molecule and hydrophobic moiety, which can bind to lipids of liposome membranes. For experimental validation of baicalin membranothropic properties and its ability to increase negative charge of liposomes we earlier used the fluorescent probes method [12]. Introduction of flavonoids into liposome suspension causes notable drop in probe fluorescence intensity due to competition between probe and flavonoids for places of binding at membranes and probes displacement to water, where probe fluorescence is quenched. For baicalin the maximum fluorescence drop is observed, which is caused with increase of negative charge of liposomes at baicalin fixation and repulsion of negatively charged probe from membrane (fluorescence quenching with water molecules). Flavonoids affinity to liposomes drops in the row: baicalin > hyperoside > myricetin > quercetin > flakumin [12]. Baicalin molecule has high affinity to liposome membranes, which causes the increase of membrane negative charge. This fact, versatile pharmacological activity of baicalin, as well as the reduction of drugs toxicity in the presence of baicalin are convincing arguments for baicalin use as a forming DS for a system of positively charged doxorubicin delivery, as well as of other DS.

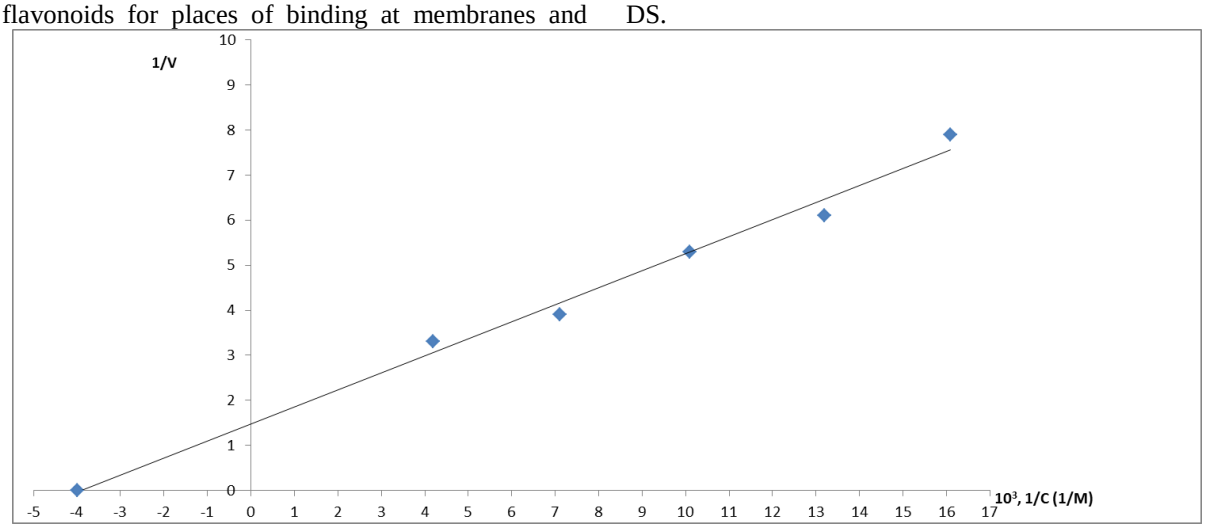


Fig. 3. Determination of binding constant Kc for baicalin fixation to liposomes from phosphatidylcholine with graphical method in inverse coordinates.

Kc of baicalin binding to liposomes (Fig. 3) determined with graphical method amounts $4 \cdot 10^3$ M^{-1}, and for diclofenac it is $1 \cdot 10^{-3}$ M^{-1} (Fig. 1), i.e. baicalin has sufficiently high affinity to liposomes and can act as an effective regulator of liposome charge in delivery systems for doxorubicin or other DS. At final stage – liposome fusion with cancer cells - membranothropic baicalin may diffuse to membranes of cancer cells and add negative charge to tumour cell membranes, which in turn increases affinity of positively charged doxorubicin to tumour tissue. It is worth noting that for pegylated liposomes there is no fusion with cancer cells, and doxorubicin is splashed out to aqueous medium between liposomes and cancer cell. Considering weak affinity of water soluble doxorubicin to membranes, it is idle to expect an effective process of doxorubicin binding to cancer cell without baicalin, and respectively effective suppression of cancer cells.

In our recent work [5] we performed a comparative analysis of impact of CNT with different structure on microviscosity of erythrocyte membranes with spin probes method using two lipophilic probes on the basis of palmitic acid – uncharged probe **1** and probe **2** having positive charge by quaternary nitrogen and being ionogenic surfactant (see Materials and methods in the present paper). Spin probes **1** and **2** were integrated in erythrocyte membrane similar to phospholipids and gave information on different areas of surface of lipidic

bilayer of erythrocyte membranes. The analysis results demonstrated that the ESR spectra of spin probe **2** were more sensitive to changes of membrane microviscosity at binding of different nanotubes to erythrocyte membrane, and the strongest effect on membranes (increase of membrane viscosity) was caused by oxidized multilayer nanotubes CNT-K2, and not the initial hydrophobic multilayer CNT-K1 (Table 1). This result is unexpected, as erythrocyte membrane is charged negatively and the affinity of oxidized (negatively charged) CNT-K2 to erythrocytes should be weak, as well as the effect in the result of repulsion of CNT-K2 from erythrocyte membranes.

Table 1 is a fragment of summary table published earlier [5].

Table 1.

Values of τ calculated from ESR spectra for probe 2 in erythrocytes in the presence of CNTs at a temperature of 25 ºC, and the evaluation of membrane viscosity changes.

System	Incubation time, h	Correlation time, sec		Viscosity, relative units
		$\tau_{+1} \cdot 10^9$	$\tau_{+1-1} \cdot 10^9$	
Probe **2**				
Erythrocytes (control)	-	9,93	7,72	1*
Erythrocytes +CNT-K1	4	8,78	7,06	0,91
	24	14,2	14,3	**1,85**
Erythrocytes +CNT-K2	4	9,65	8,02	1,04
	24	12,4	17,7	**2,29**

**Erythrocyte membrane viscosity in control experiments with probe 2 (calculation of τ_{+1-1}) is accepted as 1.*

Table 1 presents the data only on the effect of two multiwall nanotubes - initial CNT-K1 and oxidized CNT-K2 - on time of probe correlation in erythrocyte membrane proportional to microviscosity of erythrocyte membranes according to formula 1. In the work the nanotube incubation with erythrocytes was conducted during 4 hours and 24 hours in order to define the dynamics of nanotubes influence on membrane microviscosity. The results of experiments described above on change of affinity of charged DS to membranes at alternation of membrane charge allows new explanation of the greatest impact on erythrocyte membranes by oxidized nanotubes CNT-K2 in the range of nanotubes with different structure [5], including one as compared to the same nanotubes not being oxidized CNT-K1: introduction of spin-labelled cationic surfactant (probe **2)** to erythrocyte membranes increases membrane positive charge, which causes significant increase in affinity of negatively charged CNT-K2 to membranes with further slow immobilization of erythrocyte membranes during 24 hours. It is worth noting that the concentration of spin-labelled surfactant (probe **2**) in erythrocyte suspension amounted $2 \cdot 10^{-4}$ m/l, i.e. 0.01%. While using neutral lipophilic probe **1** no great effects of membrane immobilization in the presence of CNT-K1 and CNT-K2 were observed.

Thus, the directed alternation of liposome membranes charge in LDS, target cell membrane charge, as well as of nanotubes charge in DS delivery system by means of nanotubes can substantially increase the affinity of liposomes or modified nanotubes to target cells, i.e. to increase the efficiency of these DS delivery systems.

Conclusions

The increase of binding constant (K_c) for diclofenac sodium fixation to liposomes by 3 times at the expense of alternation of liposome membranes charge at introduction of cationic surfactant aethonium is shown by means of fluorescent probes method. Membranothropic properties of baicalin are studied, the constant K_c of baicalin binding to liposomes equal $4 \cdot 10^3$ M^{-1} is determined. The change of liposome membranes charge at baicalin binding to liposomes by means of fluorescent probe method is shown, which together with notable membranothropic properties of baicalin and possession of different kinds of pharmacological activity advances baicalin as dominant DS for liposomal delivery systems. The increase of affinity of modified oxidized multiwall carbon nanotubes to erythrocyte membranes as compared to the same unoxidized (hydrophobic) nanotubes is shown by spin probes method.

In order to increase the efficiency of different systems of drug substance (DS) delivery to target cells we propose the concept of creation of LDS with two or three active drugs ("self-organizing LDS"), in which some DS beside pharmacological activity are capable to provide structuring function - change liposome membrane charge and respectively change the affinity to liposome membranes of other oppositely charged DS in liposomes; change membrane fluidity and reduce DS efflux from liposomes; capable to charge at the final stage at fusion with cancer cells the membranes of cancer cells and facilitate oppositely charged DS (doxorubicin and other) more effective penetration into cancer cells.

New experimental data on the fact that change of target cell membrane charge by means of introduction of ionogenic surfactants substantially increases the affinity of carbon nanotubes (CNTs) with opposite charge, are obtained. In these cases ion-ion interactions of CNTs with oppositely charged membranes of target cells can act as the main motive force of DS delivery vector and improve efficiency of delivery systems by means of nanotubes. It is also possible to use charged CNTs carrying DS with charge opposite to target cell membrane charge.

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