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Laser Interferometric Determination of Liposomes Diffusion Through Artificial Membranes

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1. Introduction

Cellulose membranes have been used in the medical field as wound dressing and artificial skin material for the treatment of skin wounds such as burns, ulcers, and grafts and as an adjuvant in dermal abrasions (Cheng et al., 2009; Fontana et al., 1990). Using liposomes as drug-delivery strategies in connection with a cellulose-based wound dressing might have important impact on increasing the efficacy of wound healing. The concentration of liposomes used in this therapy is crucial. The amount of liposomes might permit increasing the efficacy of their diffusion through the cellulose membrane. In the present study we propose a laser interferometric method as a novel technique to investigate liposome diffusion through membranes and cellulose-based wound dressing to optimize drug delivery.

1.1 Analysis of diffusion by laser interferometry

Laser interferometric methods are widely used for studying diffusion through membranes or in polymeric media (Dworecki et al., 2003; Dworecki et al., 2006; Wąsik et al., 2010). Substance transport through any membrane or release from gel structure are connected with the formation of concentration boundary layers (CBLs). The analysis of thickness and hydrodynamic properties of the CBLs play an important role in determining parameters of substance transport. These parameters might be analyzed by laser interferometry, for example, determination of antibiotics transfer through cellulose biomembrane in the presence of native and O-deacylated (lack of ester-bound fatty acids) forms of lipopolysaccharide (Arabski et al., 2007). Lipopolysaccharides (LPSs), a constituent of the outer membrane of gram-negative bacterial cell walls, are one of the important factors of pathogenicity of bacteria. LPS characterized amphiphilic biopolymeric compounds combining, in a single molecule, hydrophilic (O-specific chains, core oligosaccharide, etc.) and hydrophobic (lipid A) entities. The interferometric technique combined with digital image analysis of the antibiotic CBLs showed that the amount of colistin transported through the cellulose membrane was not influenced by the presence of native LPS, in

contrast to presence of O-deacylated form. This effect might be associated with the phenomenon of micelles formed by native LPSs (Arabski et al., 2007).

The modification of laser interferometric technique by immobilising the tested molecules in agarose gel and measuring the amount of released substances allowed investigating interactions of partially insoluble mixtures as lipopolisaccharide with chitosan (Arabski et al., 2009a). The results of studies with laser interferometry indicated that chitosan's binding to LPS and this interaction is weaker than that of colistin. It might be associated with presence of fatty acid residues as colistin components. Chitosan reduced the speed of colistin diffusion from the gel measured by laser interferometry. These results were confirmed with electron microscopy and precipitation assay (Arabski et al., 2009a)

Additionally saponins enhance the interaction of colistin with the S and Re types of *P. mirabilis* LPSs. Analysis by laser interferometry method shows that less colistin diffused from the S1959 and R45 LPSs aggregates pre-incubated with saponins. This effect might be associated with disaggregation of LPSs micelles by saponin and facilitation of colistin binding to the lipid A part of the LPSs. These results were confirmed in a whole bacterial cell experiment (Arabski et al., 2009b).

Because this method was successfully used in our previously studies we decided to apply interferometry to examine the physical properties of PC:Chol:DOTAP (3:4:3) liposomes.

1.2 Liposomes as antibiotic carriers

Liposomes are spherical vesicles consisting of one or more phospholipid bilayers surrounding a water space. The diameter of the liposome varies from 0.02 to 10 μm. Vesicle formulations are usually based on natural and synthetic phospholipids and cholesterol. The structure may also possess lipoproteins (Urlich, 2002). The base property of these compounds is to form bilayer structure in water environment. The physicochemical properties of liposomes can be modified by changing: the types of lipids, the composition and proportions of lipids in the liposomal formulation, the size of the liposome, the charge of the liposomal surface, pH and temperature sensitivity and the fluidity of the liposomal membrane.

Regarding the variety of liposomal formulations, the vesicles are universal carriers for both hydrophilic and hydrophobic compounds. Hydrophilic elements are dissolved in the water space inside the vesicles. The most useful for this are LUVs (*Large Unilamellar Vesicles*) because the volume of encapsulated water is relatively high (Gregoriadis, 1995; Sharma & Sharma, 1997). Hydrophobic compounds are located in the lipid bilayer, and MLVs (*Multilamellar Vesicles*) or SUVs (*Small Unilamellar Vesicles*) may be applied. Charged drugs can be associated to the lipid surface (Gregoriadis, 1995; Sharma & Sharma, 1997). The size of the liposomal vesicles significantly influences drug distribution. Large (>1 μm) MLV formulations are usually not used as drug carriers, but SUVs of ~100 nm exhibited high efficacy in the eradication of bacterial pathogens (Krieger et al., 1999; Drulis & Dorotkiewicz, 2010). Encapsulation of the drugs in lipid vesicles is a good solution for designing the required pharmacokinetic and pharmacodynamic properties (Allen, 1998; Bakker-Woudenberg 1993, 1994, 2002; Swenson et al., 1988). There are many advantages of liposomes as antibiotic carriers: improved pharmacokinetics and biodistribution, decreased

toxicity, enhanced activity against intracellular pathogens, target selectivity and enhanced activity against extracellular pathogens, in particular to overcome bacterial drug resistance.

The variety of liposomal formulations allows the design of effective antibiotic forms and subsequent therapeutic success (Abeylath & Turos, 2008; Schiffelers et al., 2001d; Sharma & Sharma 1997; Yimei et al., 2008). There is much evidence of the benefits of liposomes as antibiotic delivery systems. The advantage of liposomal carriers is the possibility of a gradual and sustained release of antibiotics during drug circulation in the body. This allows maintaining the proper drug concentration for a relatively long term. In comparison, administration of the free antibiotic exhibits a quick and short effect and requires several doses per day (Hamidi et al., 2006). Drug encapsulation in liposomal vesicles improves the pharmacokinetics and also protects drug against the hydrolytic activity of enzymes and chemical and immunological deactivation (Allen, 1998; Omri & Ravaoarinoro 1996a,b; Schiffelers et al., 2001). Conventional liposomes applied by intravenous administration are recognized as foreign antigens by the immunological system and are opsonised. This activates nonspecific defence mechanisms and the liposomes are taken up by the mononuclear phagocyte system (MPS), which leads to lower blood circulation time and fast blood clearance. Liposomes accumulate in the liver, spleen, lung, and kidneys (Bakker-Woudenberg, 1994, 2002; Schiffelers et al., 2001). This phenomenon (phagocytosis of liposomes) is desirable for intracellular pathogen eradication, but unfavourable for other kinds of infection (Lasic, 1998; Voinea & Simionescu, 2002). The MPS uptake rate depends on several liposomal properties, such as size, charge, and fluidity. The blood clearance of small vesicles (~100 nm) rises to several hours, in comparison with several minutes for MLV formulations. Rigid and uncharged vesicles circulate longer than fluid and charged ones (Beaulac et al., 1997; Scherphof et al., 1997). The plasma circulation time of antibiotics can be improved by encapsulation in polyethylene glycol-coated ("pegylated") (STEALTH) liposomes. The hydrophilic layer composed of PEG protects the vesicles from the MPS and allows a long liposome circulation in the blood system. Sterically stabilised STEALTH liposomes exhibit sustained release of drug and are able to accumulate selectively at sites of infection (Bakker-Woudenberg et al., 1993; Ceh et al., 1997). Liposomes are currently in common use as universal drug carriers in the cosmetic and pharmaceutical industries. In healthcare there are antitumor anthracyclines such doxorubicin and antifungal amphotericin B liposomal formulations available (Allen & Martin, 2004; Bakker-Woudenberg et al., 1994). Intensive research is focused on antibiotics entrapped in liposomes to enhance their antibacterial activity and pharmacokinetic properties. Lipid vesicles as drug carriers significantly influence drug distribution and reduce toxic side effects during antibiotic therapy (Allen 1998; Bakker-Woudenberg 1993, 1994, 2002; Drulis et al., 2006ab, 2009; Gubernator et al., 2007; Sapra & Allen 2003).

The present study was designed to examine the physical properties of PC:Chol:DOTAP (3:4:3) liposomes, especially the diffusive transport of the liposome through different membranes, as a model of a liposomal drug system for local applications. Interferomentric description of diffusive transport of the liposome through different membranes would be useful, as a model of a liposomal drug system for local applications.

2. Laser interferometric method

The laser interferometry method for the investigations of substance transport was presented previously (Arabski et al., 2007). A sketch of the measuring system is presented in Fig. 1.

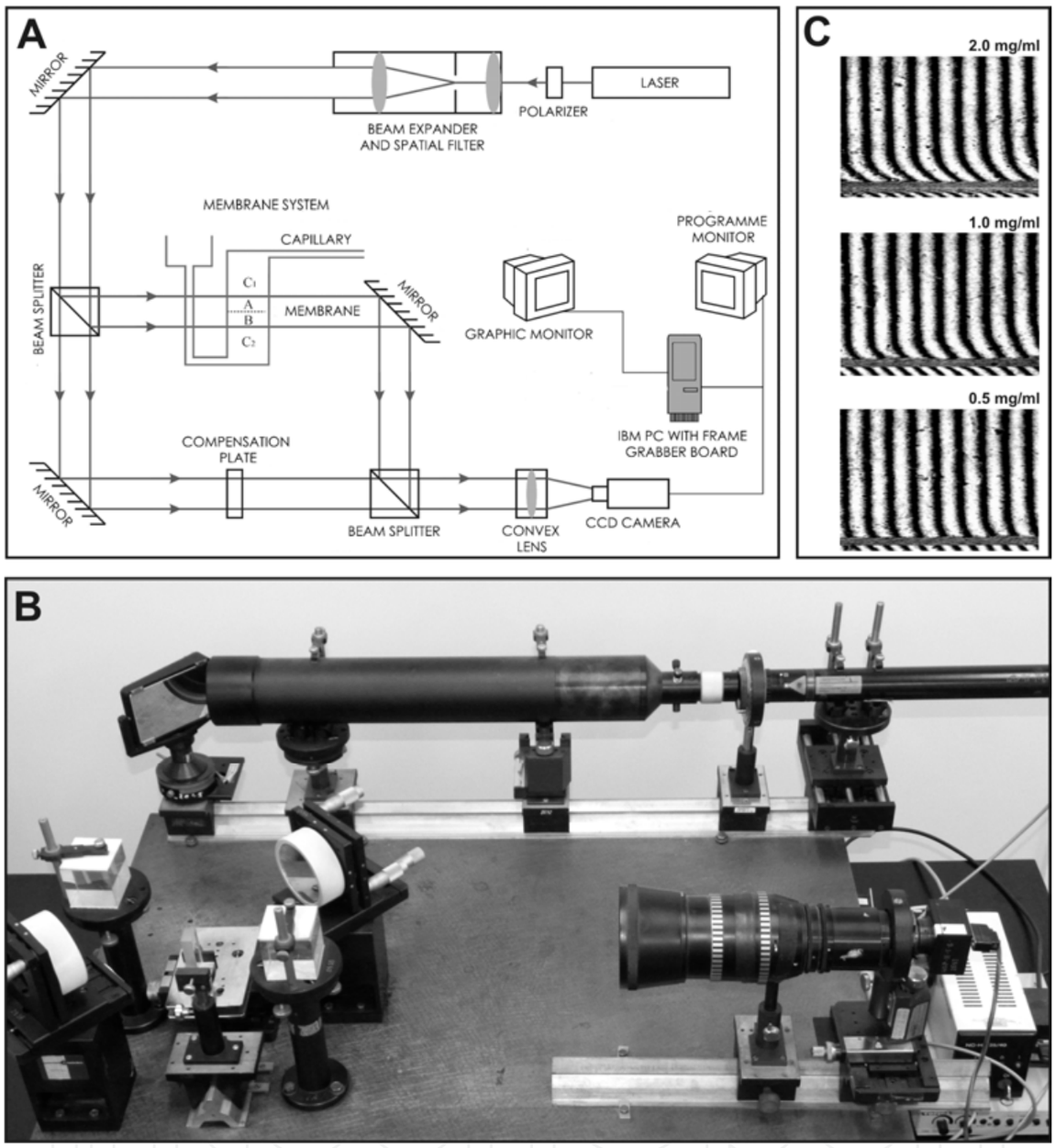

Fig. 1. Experimental set-up (A) of the interferometric investigations of the transport processes in a membrane system and its photo (B). Examples of interferograms (C) obtained for PC:Chol:DOTAP 3:4:3 liposomes at 0.5, 1, and 2 mg/ml initial concentrations transported through BioFill membrane after 10 min. C_1 denotes the initial solution concentration in the upper cuvette (water C_1=0), C_2 is the initial solution concentration in the lower cuvette (aqueous liposome solution at 0.5, 1 or 2 mg/ml). B→A- direction of liposomes diffusion (A).

It consist of a Mach-Zehnder interferometer with a He-Ne laser, a membrane system, a TV-CCD camera and a computer with a system for the acquisition and processing of interference images. The membrane system under study consists of two glass cuvettes

(internal dimensions: 70 mm high × 10 mm wide, optical path length: 7 mm) separated by the horizontally located membrane. The cuvettes are made of optical glass of high uniformity. In our experiments the upper cuvette held pure water while the lower was filled with aqueous liposome solution (0.5, 1 or 2 mg/ml).

The laser light (Fig.1A,B) is spatially filtered and is transformed by the beam expander into a parallel beam ca. 80 mm wide and then split into two beams. The first beam goes through the investigated membrane system parallel to the membrane surface, while the second goes directly through the compensation plate to the light detection system. As a consequence of the superimposition of these beams, respective interference images are generated (Fig.1C). The images depend on the refraction coefficient of the solute, which in turn depends on the substance concentration. When the solute is uniform the interference fringes are straight, and they bend when a concentration gradient appears. In this system, water and dissolved liposomes diffusing through the membrane will lead to the formation of concentration boundary layers (CBLs). The applied computer program to analyse these images allows, among other things, to find the concentration profiles and the CBL thicknesses. The concentration profile $C(x,t)$ is determined by the deviation of the fringes from their straight line run $d(x,t)$. Since the concentration C and the refraction coefficient are assumed to be linear (Robinson & Reid, 1993), we have:

$$C(x,t) = C_0 + a\frac{\lambda d(x,t)}{hf} \tag{1}$$

where C_0 is the initial liposomes concentration, a the proportionality constant between the concentration and the refraction index, λ the wavelength of the laser light, h the distance between the fringes in the field where they are straight lines, and f the thickness of the solution layer in the measurement cuvette. The CBL thickness δ was defined according the following criterion. δ is the distance from the membrane to the point at which the concentration C_δ is a certain part (k) of concentration on the membrane surface C_m:

$$k = \frac{C_\delta}{C_m} \tag{2}$$

In our investigations we took arbitrary k=0.08. The flux J_s of the solute which flows through the membrane is given by:

$$J_S = \frac{N(t)}{St} \tag{3}$$

where $N(t)$ is the amount of liposomes which diffuses after time t through a membrane of area S from one compartment of the membrane system to the other. $N(t)$ at any time t was calculated by integrating the concentration profile according to:

$$N(t) = S\int_0^\delta C_1(x,t)dx \tag{4}$$

and $N(\Delta t)$ in the given time interval Δt was calculated by integrating the concentration profile according to:

$$N(\Delta t) = S\int_0^{\delta'} C_1(x, t+\Delta t)dx - S\int_0^{\delta} C_1(x,t)dx \tag{5}$$

On the basis of (3) and (5) we obtain:

$$J_S = \frac{\int_0^{\delta'} C_1(x, t+\Delta t)dx - \int_0^{\delta} C_1(x,t)dx}{\Delta t} \tag{6}$$

where $C_1(x,t+\Delta t)$, δ' and $C_1(x,t)$, δ denote the concentration profiles and the thickness of CBL for times $t+\Delta t$ and t, respectively.

The membrane permeability coefficient was determined on the basis of Fick's first law. In the case of diffusive transport of binary solutions, this law can be written as:

$$J_S = RT\omega_m\left[C_2(x=0,t) - C_1(x=0,t)\right] \tag{7}$$

where J_s is the solute flux and $C_1(x=0,t)$ and $C_2(x=0,t)$ the concentrations of the solutions on the membrane surfaces at time t. On the basis of Eqs. (6) and (7) we obtain the formula for the membrane permeability coefficient:

$$\omega_m = \frac{\int_0^{\delta'} C_1(x, t+\Delta t)dx - \int_0^{\delta} C_1(x,t)dx}{RT\left[C_2(x=0,t) - C_1(x=0,t)\right]\Delta t} \tag{8}$$

We present method of diffusion coefficient calculations based time evolution of CBLs measured by laser interferometry. The diffusion coefficient D is determined by the CBLs thickness criterion mentioned above and the theoretical concentration profile (Dworecki et al., 2000):

$$C_1(x,t) = C_2\frac{1-\sigma}{2}erfc\left(\frac{x}{2\sqrt{Dt}}\right) \tag{9}$$

where σ denotes the membrane selectivity coefficient, C_2 is the initial solution concentration in the lower cuvette, *erfc* is complementary error function defined as:

$$erfc(x) = \frac{2}{\sqrt{\Pi}}\int_x^{\infty} e^{-\eta 2}d\eta \tag{10}$$

On the basis of the Eqs. (2) and (9) we obtain the formula for the diffusion coefficient D(t) after time t for the membrane sytems in whose one compartment held pure solvent.

$$D(t) = \frac{\delta^2(t)}{4t\left(erfc^{-1}(k)\right)^2} \tag{11}$$

where $erfc^{-1}$ is the inverse function to *erfc*. The formula of diffusion coefficient $D(\Delta t)$ in the time interval Δt is given:

$$D(\Delta t) = \frac{\delta_2^2(t_2) - \delta_1^2(t_1)}{4\Delta t(erfc^{-1}(k))^2} \tag{12}$$

The interferograms were recorded from 120 to 2400 s with a time interval of Δt = 120 s and the profiles for a given initial liposome concentration (0.5, 1 or 2 mg/ml) were reconstructed. Such profiles were used to calculate the time dependencies $N(t)$, $N(\Delta t)$, $J_s(t)$, D(t), D(Δt) and ω_m. All parameters were measured on the basic of interferograms by *KALPROST* application descript by Mr. Grześkiewicz using *VISUAL C++ 2005 EXPRESS EDITION*. All experiments were performed at a temperature of 37°C.

A cationic liposomal formulation (PC:Chol:DOTAP 3:4:3) was prepared using a thin lipid film method. Appropriate amounts of lipids dissolved in chloroform (10 mg/ml) were mixed in a 100-ml round-bottom flask. Then the chloroform was evaporated under vacuum on a rotary evaporator and the resulting thin lipid film was hydrated by agitation with 1 ml of PBS at room temperature. The MLV liposomes were then extruded 10 times through a 100-nm-pore filter using a 10-ml extruder. The size of the liposomes was determined with a ZetaSizer Nano-ZS (Malvern Instruments, UK) and was usually in the range of 105-110 nm. The stability of liposomal formulation was previously determined by carboxyfluorescein release profile. The liposome leakage reached approximately 6, 8, 14, and 24% after 1, 3, 12, and 24 h, respectively. In the experiments of vesicles stability Sephadex columns were used and no lipid exchange was observed (Drulis-Kawa, 2006b). 1,2-dioleoyloxy-3-trimethylammonium-propane (DOTAP) and phosphatidylcholine (PC) were purchased from Northern Lipids Inc. (Vancouver, BC, Canada). Cholesterol (Chol) was obtained from E. Merck (Darmstadt, Germany). HPLC solvents were supplied by J. T. Baker (Deventer, the Netherlands).

The liposome diffusion efficiency was examined using commercial membranes such as Biofill and Nephrophane. The bacterial cellulose membrane (BioFill) is a transduced film with a gram-meter of 9 gm^{-2} to 20 gm^{-2}, pH between 6.0 and 7.0, obtained through biosynthesis from bacteria of the genus *Acetobacter* (Klemm et al. , 2001). Its thickness varies between 20 μm and 52 μm in dehydrated and in hydrated conditions, respectively. Bacterial cellulose has a microfibrous structure and it is a hypoallergic, nontoxic, non-irritant, biodegradable, non-pyrogenic, highly hydrophilic, and biocompatible material (Pitanguy et al., 1988; Kucharzewski et al., 2003). A cellulose acetate membrane Nephrophane is a hydrophilic membrane and its thickness varies from 25 μm in the dehydrated state to 200 μm under hydrated conditions (Kargol, 2001). Nucleopores, a polymeric nuclear track membranes, with different pore diameters (0.092-0.1 μm, 0.2 μm, 0.9 μm, and 1.27-1.35 μm) were purchased from Joint Institute for Nuclear Research in Dubna, Russia.

3. Results

The results of liposome diffusion at three concentrations (0.5, 1, and 2 mg/ml) through nucleopore membranes of different pore diameter, bacterial cellulose (BioFill), and cellulose acetate (Nephrophane) membranes are shown in Table 1.

Membrane	Liposome concentration		
	2 mg/ml	1 mg/ml	0.5 mg/ml
Nucleopore 0.092-0.1 μm	no diffusion observed	no diffusion observed	no diffusion observed
Nucleopore 0.2 μm	0.031 mg of liposomes pass membrane, blocked after 2 min of diffusion	0.030 mg of liposomes pass membrane, blocked after 8 min of diffusion	0.035 mg of liposomes pass membrane, blocked after 12 min of diffusion
Nucleopore 0.9 μm	diffusion of 0.069 mg of liposomes after 40 min. (Fig. 2A●)	diffusion of 0.069 mg of liposomes after 40 min. (Fig. 2A●)	diffusion of 0.036 mg of liposomes after 40 min. Fig. 2A○)
Nucleopore 1.27-1.35 μm	filtration	filtration	filtration
Biofill	diffusion of 0.187 mg of liposomes after 40 min. (Fig. 2B●)	diffusion of 0.082 mg of liposomes after 40 min. (Fig. 2B●)	diffusion of 0.034 mg of liposomes after 40 min. (Fig. 2B○)
Nephrophane	diffusion of 0.144 mg of liposomes after 40 min. (Fig. 2C●)	diffusion of 0.088 mg of liposomes after 40 min. (Fig. 2C●)	diffusion of 0.028 mg of liposomes after 40 min. (Fig. 2C○)

Table 1. Diffusion of liposomes through different membranes.

Filtration of the liposome vesicles was noted for nucleopore membrane of pore diameters 1.27-1.35 μm. In the case of 0.2 μm pores, diffusion was blocked after 2, 8, and 12 min. for the 0.5, 1, and 2 mg/ml concentrations of lipids, respectively, although the vesicle sizes were lower than the pore diameters. No diffusion through 0.092-0.1 μm pores was observed although fluid formulation of liposome was tested and the plastic deformation of vesicles thus the passing across smaller pores would be possible. Mathematical analysis of the time-dependent concentration profiles indicates that the amount of liposomes (0.069 mg) transported through the nucleopore membrane of 0.9 μm pore diameter at the initial concentration of 2 mg/ml was the same as for 1 mg/ml after 40 min in distance of 0.995 mm from membrane (Fig. 2A).

In the first phase of diffusion we observed throw of liposomes through nucleopore membrane of 0.9 μm pore diameter and then (after ~10 min.) diffusion was relatively constant (Fig. 3).

The same kinetics of liposomes transport across nucleopore 0.9 μm after analysis of flux J_s through a membrane area was observed (Fig. 4).

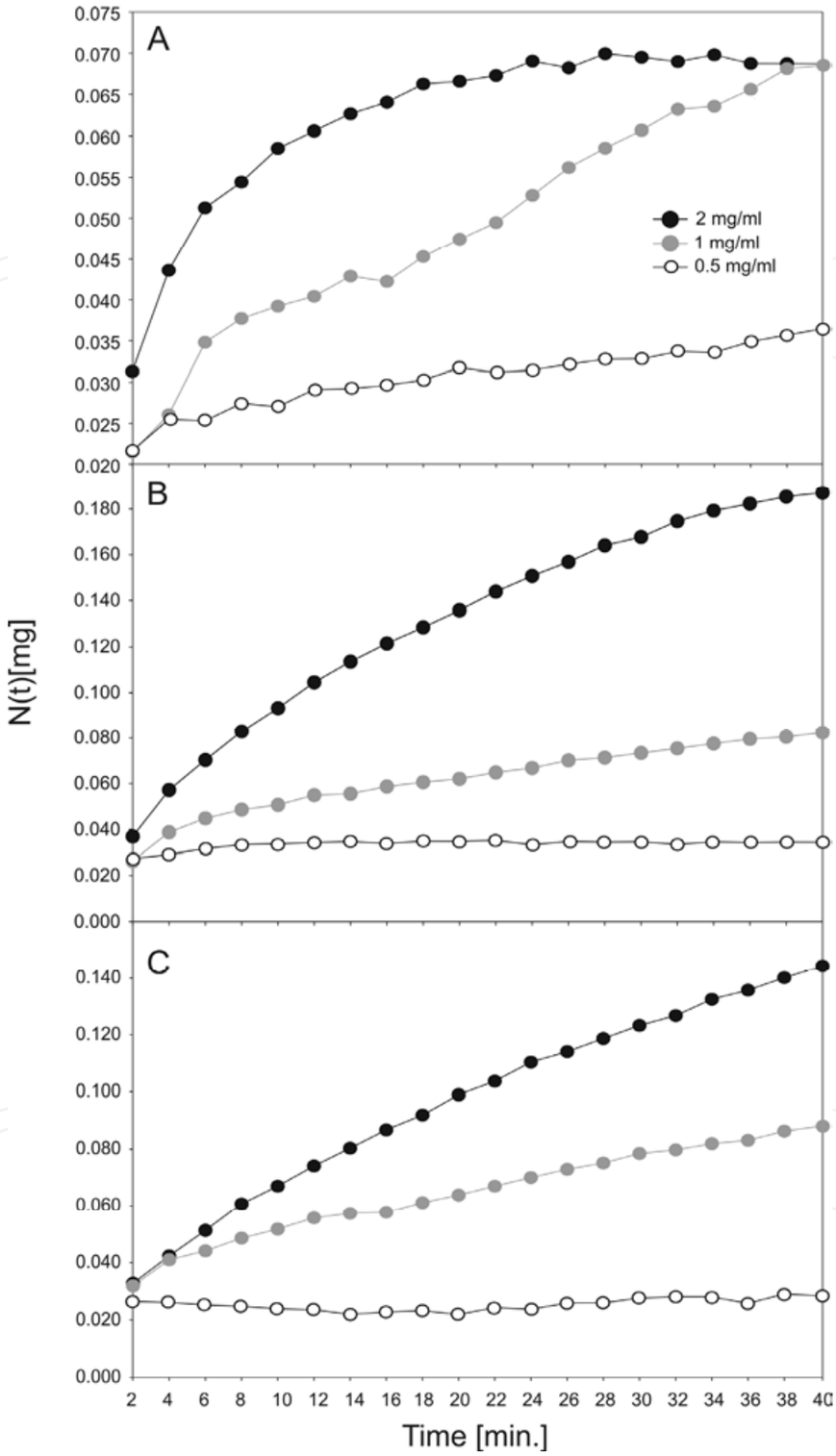

Fig. 2. The amount *N(t)* of PC:Chol:DOTAP 3:4:3 liposomes transported through nucleopore membrane of pore diameter 0.9 μm (A), BioFill membrane (B) and Nephrophane membrane (C) in 40 min at 37ºC measured by the laser interferometric system.

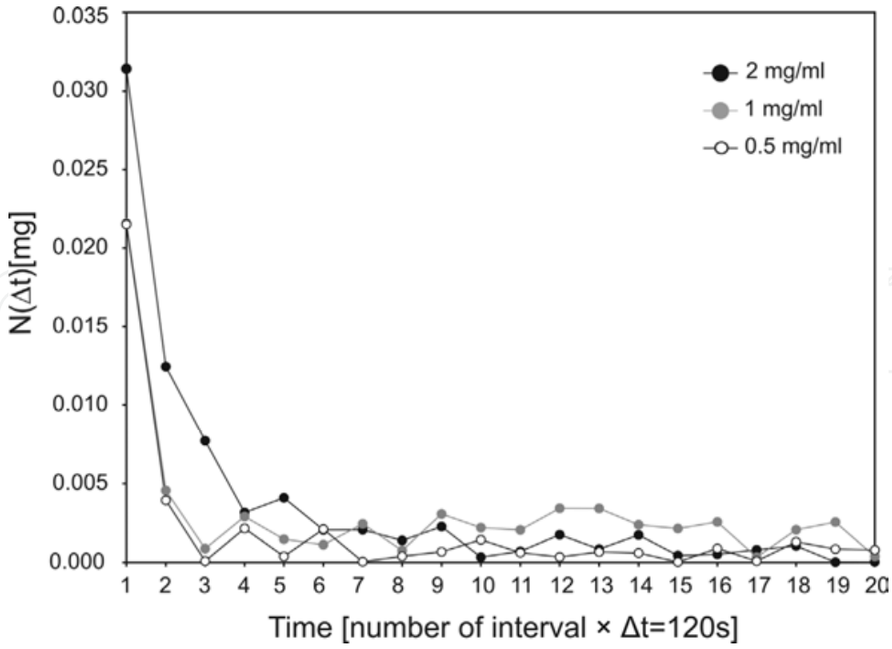

Fig. 3. The amount *N(Δt)* of PC:Chol:DOTAP 3:4:3 liposomes transported through the nucleopore membrane of 0.9 μm pore diameter measured by the laser interferometric system.

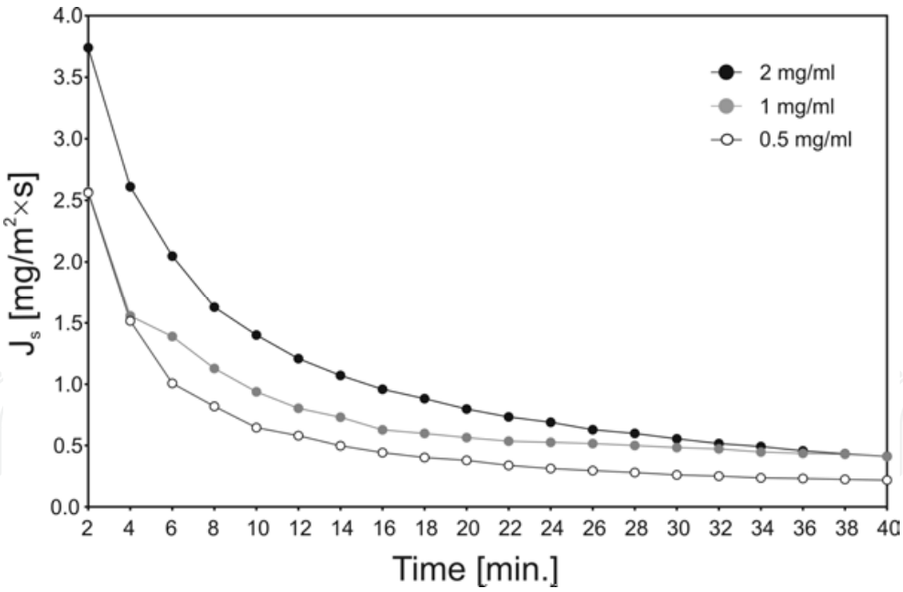

Fig. 4. The flux J_s of the PC:Chol:DOTAP 3:4:3 liposomes which cross the nucleopore membrane of 0.9 μm pore diameter measured by the laser interferometric system.

More efficient diffusion of liposomes was noticed in regards to BioFill (Fig. 2B) and Nephrophane (Fig. 2C) membranes than to 0.9 μm nucleopore (Fig. 2A). For the highest liposome concentration, 0.187 and 0.144 mg of lipids were transported across BioFill and Nephrophane, respectively.

The next step of our mathematical analysis was calculations of the average membrane permeability coefficient for aqueous solution of liposomes. The greater amounts of liposomes transported through BioFill, Nephrophane in contrast to nucleopore membrane was associated with the different values of this coefficients for above membranes (1.21×10^{-10}, 1.06×10^{-10}, and 4.31×10^{-11} mol $N^{-1}s^{-1}$, respectively). We observed different kinetics of liposomes diffusion through both cellulose membranes. The amount of transported substances was positively correlated with the initial concentration of liposomes in dose-dependent manner and increase was regular for 40 min.

Additionally, analysis of the membrane permeability coefficient for different liposomes concentrations shown the lower value of this coefficient for 2 mg/ml than for 1 and 0.5 mg/ml.

The same conclusions are based on analysis of diffusion coefficient D values for liposomes solutions measured both after time t and in the given time interval Δt. Analysis of diffusion coefficient for liposomes solution in the time interval Δt (Fig. 6) was more precise than after time t (Fig. 5). Therefore, we could noted that throw of liposomes from initial concentration 2 mg/ml took place in the first ~10 minutes of diffusion (Fig. 6)

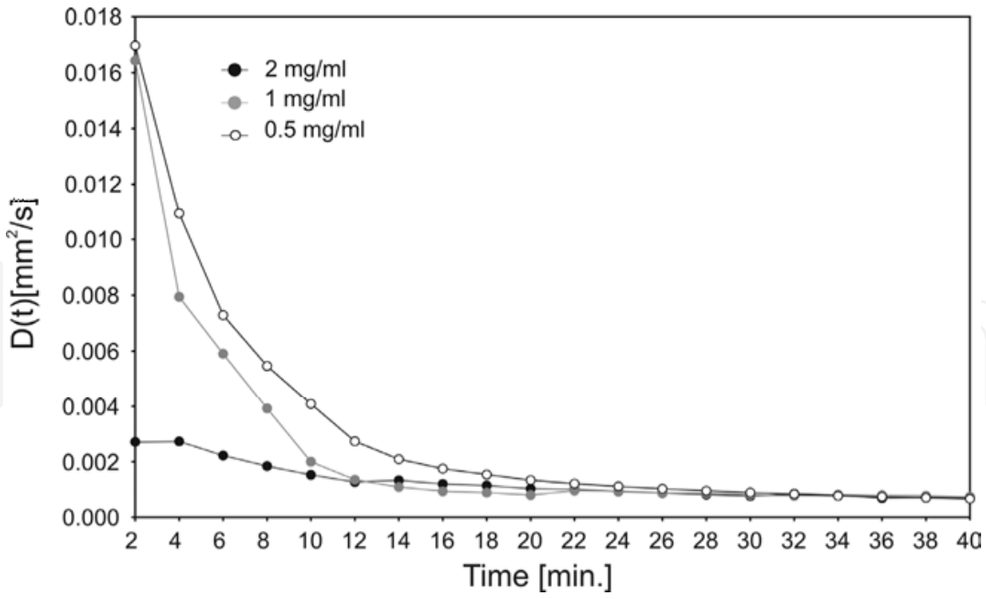

Fig. 5. The diffusion coefficient in water solution of liposomes measured in time *t* by the laser interferometric system.

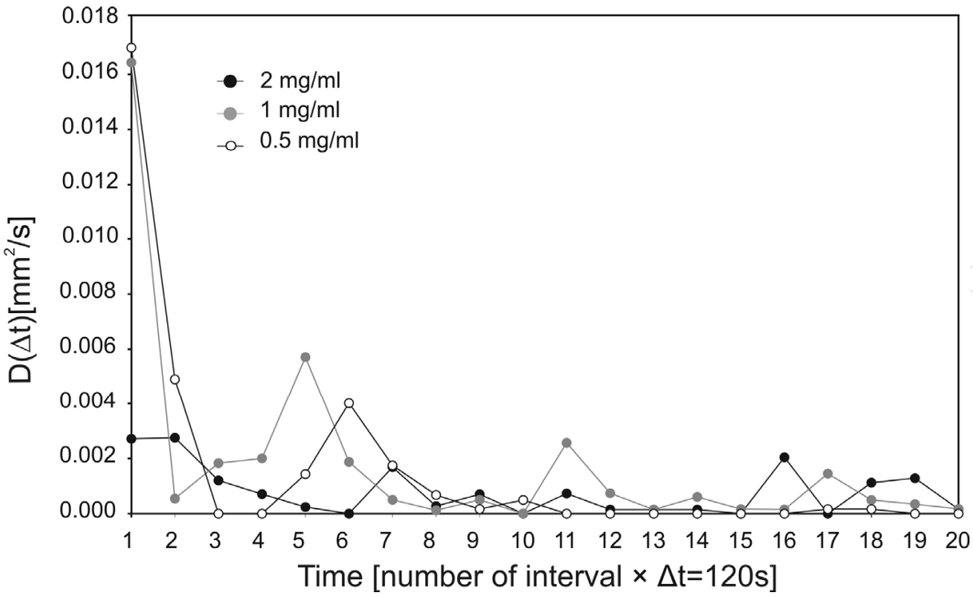

Fig. 6. The diffusion coefficient in water solution of liposomes measured in the given time interval Δt by the laser interferometric system.

4. Conclusions

Liposome diffusion through the membrane separating solutions of lipid concentration leaded to build-up of the concentration boundary layers (CBLs) on both membrane sides measured by laser interferometry. The CBL dependents on the distance from the membrane, the physical properties of the membrane, and the type of solutes. These factors leaded to concentration polarization on both sides of the membrane (Grzegorczyn & Ślęzak, 2007).

In this study the measurements of the concentration profiles and liposome fluxes were done using laser interferometry. The analysis of liposome diffusion was performed for 40 min and in the given time interval Δt. Analysis of diffusion of liposomes through nucleopore and cellulose membranes showed different kinetics of diffusion process which determined amount of transported substance (Fig.7).

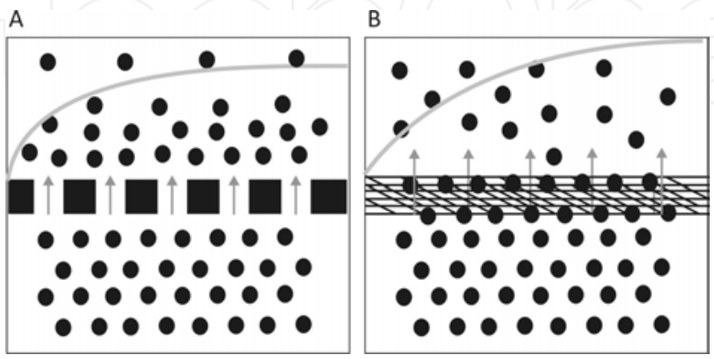

Fig. 7. Schematic presentation of liposomes diffusion through nucleopore (A) and cellulose (B) membranes proposed by the authors. Grey arrow denotes direction of diffusion. Grey curves denote layout of substance concentration.

In the first phase of diffusion across nucleopore membrane of 0.9 μm pore diameter throw of liposomes was observed and then diffusion started to be constant (Fig. 7A and Fig. 2A). This effect might be associated with different polarizations of the liposome concentrations on both sides of the nucleopore membrane. The difference of substance concentrations influenced on physical properties of diffusion process, and amount of transported particles. The lower concentration (1 mg/ml) might promote constant transport of liposomes through the membrane. At the greater difference in liposomes concentration (2 mg/ml) on the two membrane sides, reciprocal inhibition of liposome diffusion was observed. The bacterial cellulose membrane transfered greater amounts of liposomal vesicles in a gradual manner and no inhibition of diffusion was detected (Fig. 7B and Fig. 2BC). In conclusion, in the first step of diffusion process cellulose membranes were saturated by liposomes and thus releasing of substance to aqueous solution in upper side of cuvette was observed. Both commercial membranes exhibited efficient diffusion and no filtration effect.

In conclusion, the determination of dependence between the concentration of the liposome vesicles and membrane diffusion efficiency by laser interferometry method might be important from a clinical point of view. The effect of liposome concentration polarization on diffusion process through a wound dressing and artificial skin materials might be useful in the optimization of therapy using liposome-encapsulated drugs. The wound dressing based on cellulose membrane might promote more effective transport of liposomes as antibiotics carriers in comparison to standard nucleopore membranes. Additionally, laser interferometry is a very precise and quick method for determining of liposomes diffusion.

5. Acknowledgment

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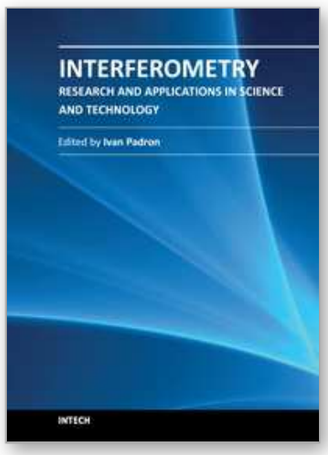

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