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PharmDr. et Bc. Josef Mašek, Ph.D.

EDUCATION

He graduated from Pharmacy at the Veterinary and Pharmaceutical University of Brno, Faculty of Pharmacy in 2006, and from Biology at the Masaryk University Brno, Faculty of Science in 2009. For his post-gradual studies, he was working on the project of Metallochelating nanoliposomes for the construction of recombinant vaccines. Josef Mašek defended his dissertation in 2013 at the Masaryk University Brno, Faculty of Science.

PROFESSIONAL ASSIGNMENTS

Josef Mašek is currently the head of the Department of Pharmacology and Toxicology at the Veterinary Research Institute, Brno. He is interested in the research and development of nanoparticle-based drug and vaccine delivery systems, oromucosal drug delivery, microscopic techniques, enhancement of drug bioavailability, mucosal and dermal barrier functions, modern drug and vaccine dosage forms, and the research in the field of pharmacology. He was awarded the Sanofi-prix Award in 2013 and the Discovery Award in 2017 for his work in the field of drug and vaccine delivery. He is currently the author or co-author of more than 30 impacted publications, 5 book chapters, and 3 international patents. He is a member of the Scientific Board of the Faculty of Pharmacy, Masaryk University Brno.

PREPARATION AND TRANSFORMATION OF LIPOSOMES

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WORKFLOW

– Preparation and transformation of the liposomes with different morphologies

– Analysis of the liposomes: DLS (dynamic light scattering), TEM (Transmission electron microscopy)

MOTIVATION

Liposomes are a very common delivery system in medicine because of their biocompatibility, non-toxicity, encapsulation of hydrophilic and hydrophobic compounds (e.g. drugs) and specific transport to the target region/area. The composition and surface of liposomes can be modified for specific targeting, long circulation in the body or better stability. These modifications are a great tool for designing liposomes according to current needs.

THEORETICAL BACKGROUND

Liposomes

Liposomal spontaneous formation in the aqueous environment is controlled mainly by the hydrophobic effect. Minimizing the interaction between the acyl chain of the phospholipid and the surrounding aqueous phase leads to the organization of phospholipid molecules into a bilayer and subsequent vesiculation [1]. Their rigidity, structure and size are determined by both the lipid composition and the method of preparation. In general, liposomes can be categorized by size into:

- SUVs (small unilamellar vesicles)
- LUVs (large unilamellar vesicles)
- GUVs (giant unilamellar vesicles)

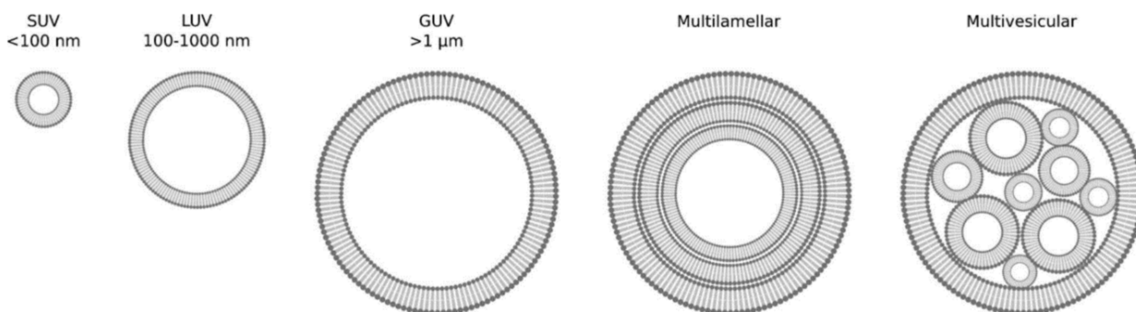

Figure 1 – Schematic representation of different types of vesicles [2].

according to the number of lamellae as unilamellar, multilamellar, multivesicular, multivesicular multicentric particles (see Figure 1) and according to the charge on neutral, anionic or cationic. An important parameter is the phase transition temperature (the temperature of the transition from an ordered, rigid state to a fluid membrane). This temperature depends on the degree of saturation and the length of the acyl chain and can range from −20 °C for unsaturated dioleoyl to about 55 °C for saturated chains for distearoylphosphatidylcholine [1].

The classic methods of preparation of liposomes in bulk (batch methods) include in particular: The hydration of the lipid film where the given phospholipid composition is dissolved in an organic solvent, most often chloroform or a mixture of chloroform/methanol. The solvent is then evaporated under reduced pressure on a rotary evaporator. Evaporation produces a thin film of the individual phospholipid bilayers on the wall of the glass flask. The film is then hydrated with a suitable buffer with vigorous stirring at a given temperature, according to the highest transition temperature of the phospholipid. The vesicles thus formed are characterized by a large polydispersity and a different number of lamellae (MLV). The MLV can be modified by freezing and thawing the suspension, which produces unilamellar liposomes with a larger volume of the encapsulated aqueous phase. To achieve the targeted size and polydispersity/homogeneity of vesicles, other secondary methods are used, especially extrusion, in which the liposome suspension is repeatedly pushed through uniform cylindrical pores of the polycarbonate membrane, reducing their size according to pore size [3].

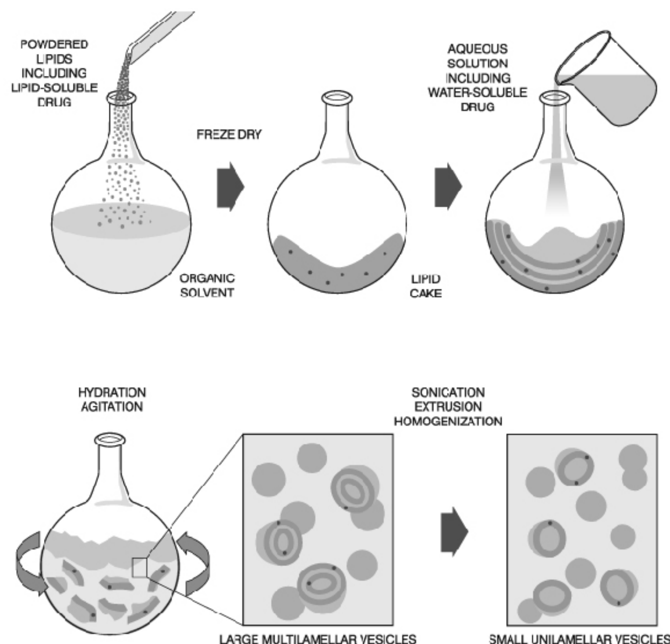

Figure 2 – Scheme of preparation of liposomes by the method of hydration of lipid film with secondary treatment (Avanti® Polar Lipids).

Another possibility for the preparation of liposomes is the **Microfluidic** method, which results in lipid nanoparticles (LNPs) with a precisely designed structure and a high-level dose of encapsulated substance. The principle is based on laminar mixing of organic (lipid) and aqueous (RNA) phases in a capillary of constant flow and under a specific angle. Laminar or turbulent flow is given by the Reynolds number according to the equation

$$Re = \frac{\rho V D}{\mu} \quad (1)$$

where ρ - fluid density; V - fluid velocity; D - capillary diameter; μ - dynamic viscosity of the fluid. For Re <2100 values, this is a laminar flow that ensures homogeneous mixing with a high level of reproducibility.

Upon mixing, the liposome is gradually assembled from the inside, thanks to the change in polarity of the environment. The lower pH causes the ionizable lipid to become cationic, initiating the first electrostatic interactions with the anionic RNA to form the core of the particle. Other lipids assemble around the core. An important factor in determining the encapsulation efficiency (EE) and biological activity of LNP formulations is the N / P ratio. This is the molar ratio between amines (N, which become cationic at low pH) found on the ionizable lipids and phosphates (P, negative) found on the RNA backbone. The N / P ratio varies depending on the formulation, so it is not possible to determine one optimal ratio for all formulations. A lower N / P ratio leads to a larger volume of an encapsulated substance, however, it should never be 1:1, due to a positive charge would not be sufficient to protect the RNA.

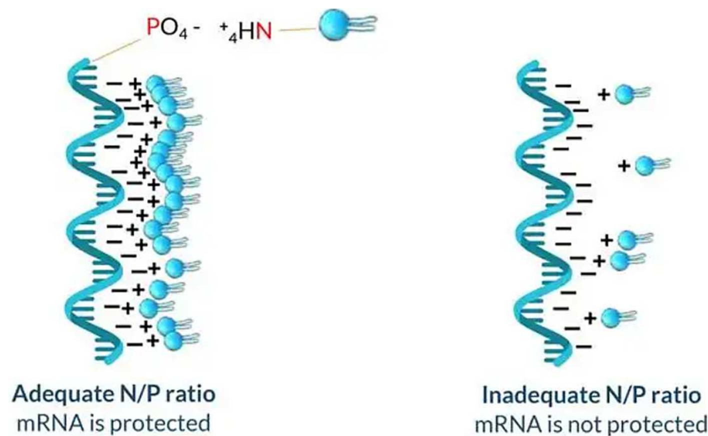

Figure 3 – Schema of the RNA protection principle (®2020 Precision nanosystems inc.)

Among other factors, mixing parameters may influence characteristics such as particle size and PDI. The Total Flow Rate (TFR) and the Flow Rate Ratio (FRR) are the most influential mixing parameters to tune for achieving optimal particles for a given application. In general, higher TFR and FRR result in smaller particles.

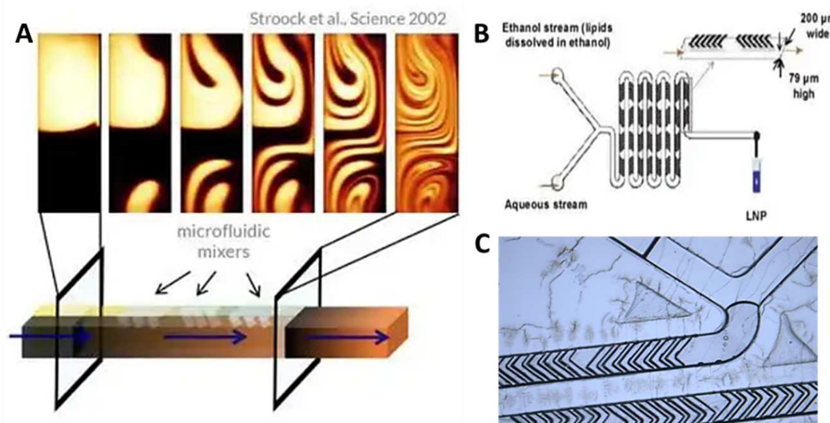

Figure 4 – Principle of microfluidic mixing in herringbone channel (A, B); (®2020 Precision nanosystems inc.); Detail of the microfluidic mixing channel (C).

Characterization of the liposomes

Dynamic light scattering

The DLS method correlates Brownian motion with particle size. Scattered light (red laser, 633 nm) shows a phase shift at different times by different particles and the individual waves interact with each other. The interaction can be positive or negative, depending on the relative position of the particles. Over a given time, the particle changes its position precisely due to Brown's motion, small particles being more affected by this motion than the large particles. As the relative position of the particles changes, the phase shift and thus the total intensity of the scattered light fluctuates. The intensity fluctuation correlates with particle velocity. For the small particles moving fast, there will be a higher fluctuation of the intensity of the scattered light than in the case of large, slower-moving particles. Measurement of such an intensity changes-fluctuation is expressed by the autocorrelation function. The device, the correlator, assesses the degree of similarity between two signals over a short period [4, 5].

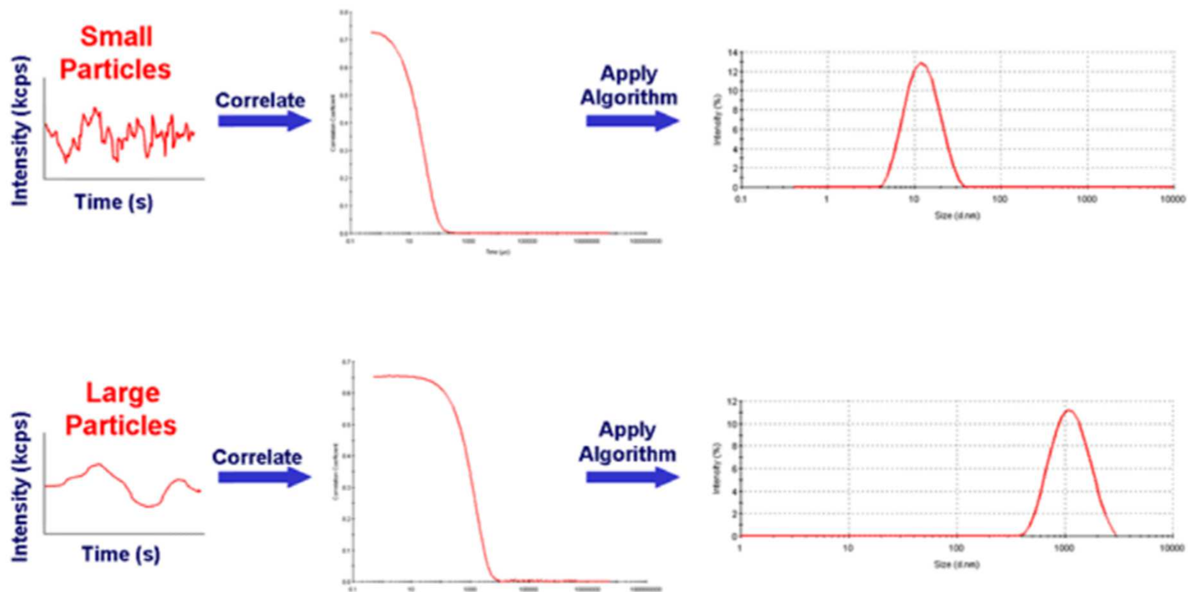

Figure 5 – Schematic representation of the fluctuation of the intensity of scattered radiation as a function of time [4].

The initial intensity of the scattered light is compared with the intensity over time $t + \mathrm{d}t$, $t + 2\mathrm{d}t$, $t + 3\mathrm{d}t$, and $t = \infty$. Time slots (dt) are in the order of nanoseconds/microseconds. The individual intensities deviate in time; time t = ∞ mean time in the order of tens of milliseconds, when the correlation no longer occurs and the correlation factor equals zero (for the ideal correlation, the correlation factor equals one). The graph of correlation function versus time is called a correlogram. From the correlogram, the translation diffusion coefficient is obtained. The particle size is then calculated from the translational diffusion coefficient using the Stokes-Einstein equation:

$$R_h = \frac{kT}{6\pi\eta D} \qquad (2)$$

where R_h is the hydrodynamic radius, D is the diffusion coefficient, k is the Boltzman constant, T is the temperature and η is the viscosity of the medium [4].

Transmission electron microscopy

The TEM is a microscopic method in which a specimen (a thin section about 100 nm thick or a suspension of particles mounted on a grid) is irradiated with an electron beam. The primary electrons are emitted from a cathode, a thin tungsten fibre (about 0.1 mm in diameter) shaped into a "V". The cathode is heated to the limit temperature (approx. 2800 K) when this temperature is exceeded, electron emission occurs. In addition to thermo-emission, we also distinguish auto-emission, where a high-voltage electrode is placed opposite the cold tip-shaped cathode. The generated strong electric field releases electrons from the tip surface. A vacuum in the range of 10^{-3} to 10^{-5} Pa is required for the thermo-emission principle, and a vacuum of 10^{-7} to 10^{-8} Pa is required for the auto-emission principle. The cathode fibre is cantered in the hole of the so-called Wehnelt cylinder. The primary electrons are "sucked" from the Wehnelt cylinder bore to the circular anode. Electrons having the right direction are accelerated further into the tube to the lenses. Electromagnetic lenses focus electrons into a thin beam and maintain their trajectory, so the focused primary electrons interact with the sample. The interaction results in elastic and inelastic scattering. During elastic scattering, the primary electrons interact with the nuclei of the atom, due to the interaction the electrons deviate from the original trajectory. In the case of inelastic scattering, the primary electrons interact with the electrons in the electron shell, thereby temporarily exciting these electrons. This primary excitation process is used in Electron Energy Loss Spectroscopy (EELS). Different types of deexcitation, for example, X-ray emission, Auger electron emission, cathodoluminescence, make it possible to combine the structural information of a sample with information about its composition or electronic characteristics [6].

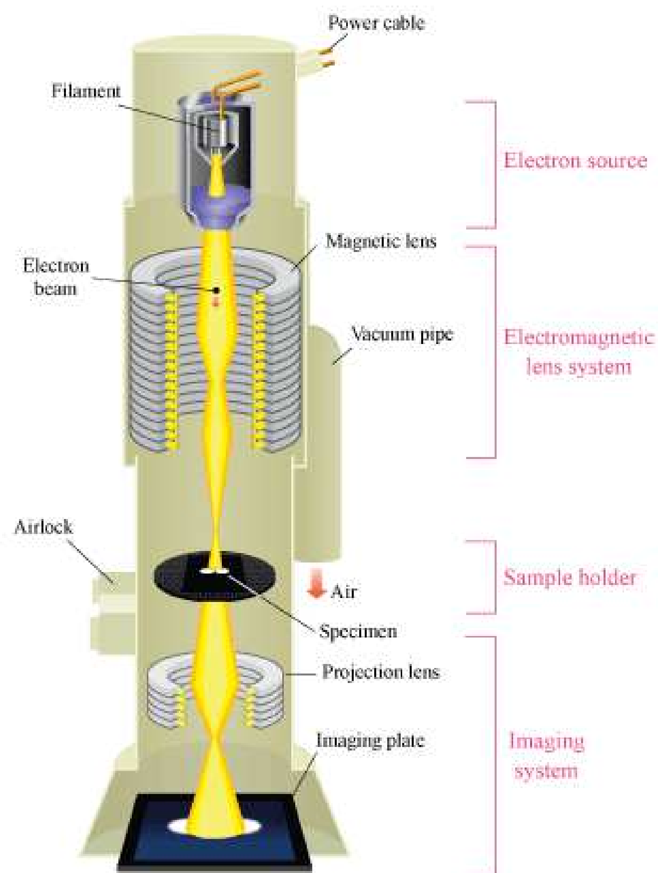

Figure 6 - Schematic representation of the principle of transmission electron microscopy [6].

To display the electrons passed through the sample, it is necessary to convert the electrons to the visible light region. For this purpose, a screen (covered with ZnS) is used, which, depending on the intensity of the incident electrons, converts the signal into visible light (most often around 550 nm). The images are then acquired using a CCD camera [6].

DESIGN OF EXPERIMENT

Solutions and reagents:

1. PBS buffer: 10 mM Na_2HPO_4 1.78 g; 1.8 mM KH_2PO_4 0.245 g; 2.7 mM KCl 0.20 g; 137 mM NaCl 8.01 g in 1 liter Milli-Q H_2O.

2. BSA: 10 mg protein to 1 ml PBS

3. Lipid: EPC (1,2-dioleoyl-sn-glycero-3-ethylphosphocholine) and Cholesterol

Preparation of liposomes using vacuum evaporator:

1. A given amount of individual lipids according to the composition EPC (1,2-dioleoyl-sn-glycero-3-ethylphosphocholine) / Cholesterol in a molar ratio of 75/25 will be weighed into two individual ground-glass flask flasks, which will then be dissolved in chloroform so that the final concentration in each flask will be 1 mg/ml of a total lipid.

2. Using a vacuum evaporator, evaporate the solvent to form a lipid film with constant rotation and elevated temperature

3. This creates four lipid films, which will then be hydrated:

   a. 1 ml of PBS buffer will be pipetted into the second flask which results in the releasing the lipid film and forming of the MLV.

   b. 1 ml of a 10 mg/ml solution of bovine albumin in PBS will be pipetted into the second flask, and after releasing the lipid film, the mixture will be repeatedly frozen and thawed in liquid nitrogen for 5 cycles which results in the formation of unilamellar vesicles.

4. Individual samples will be further extruded through a polycarbonate membrane (200 nm).

Preparation of liposomes using the microfluidic method:

1. A given amount of individual lipids according to the composition will be dissolved in ethanol to obtain the final concentration of 4 mg/ml of total lipid. This solution will be used as the "organic phase". As a "water phase" the ultrapure Milli-Q water will be used.
2. Individual solutions will be placed into the plastic syringe and mounted to the microfluidic cartridge.
3. The process parameters will be following: TFR 7 ml/min, FRR 3:1

Characterization using DLS:

1. Pipette approximately 70 µl of liposome suspension into a clean quartz cuvette

2. The samples will be measured successively at a constant temperature of 25 °C in duplicate.

Characterization using TEM:

1. A drop of liposome suspension will be placed on the grid

2. After about one minute, the remaining solution will be removed from the grid using filter paper

3. The sample will be stained with 2% phosphotungstic acid solution or ammonium molybdate.

4. Excess acid will be dried with filter paper.

5. The samples will be observed at a magnification of 7 500 × and an accelerating voltage of 80 kV.

LITERATURE

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