Univerza v Ljubljani
Fakulteta za matematiko in fiziko

Seminar IV

Interaction of liposomes on endothelial cells

Author: Rok Podlipec
Mentor: doc. dr. Janez Štrancar

November 18, 2010

Abstract

Liposomes are small vesicles (∼100 nm) composed of various of lipid molecules which build their membrane bilayers. Different forces acting on and among lipid molecules in liposome membrane influence their dynamics and interactions with other particles (e.g. cells). In the case of liposome interacting with endothelial cells (e.g. blood-brain barrier) various of transport mechanisms have to be considered (e.g. receptor-mediated transcytosis).
Liposome-cell interaction which is exchange of lipids, absorbtion of liposomes on cell membranes and specially fusion or endocytosis, can be described with proper equations of mass action. Furthermore, experimental measurements of liposome-cell interaction on MDCK cells with similar charachteristics compare to actual endothelial cells in BBB were made. The measurements showed the correlation of specific membrane properties with the rate of interaction or transcytosis, which could lead to an optimal liposomal conformation to effectively traverse BBB.

Contents

1 Introduction

Liposomes have rapidly spread into various sciences and applications and well over ten thousand research articles have jet been published. Their mechanical and surface properties are defined by their composition, size and lamellarity, where stability also plays an important role, which depends additional on external factors, such as temperature, pressure, physical stresses, chemical and biological agents, and the presence of various cell types [1].
Liposomes are spherical, self-closed structures composed of curved lipid bilayers which encapsulate part of the solvent, in which they float. They are unilamelar (consist of one concentric membrane) or multilamelar (consist of several concentric membranes). The size vary from 20 nm to several μm and the thickness of the membrane is around 4 nm. They consist mainly of amphiphilic molecules, which have a hydrophilic (water-soluble) and a hydrophobic (water-insoluble) part (figure 1 above and left). The lipid composition of the membrane defines liposomal fluidity, permeability and surface charachteristics, which directly affect the rate of their interaction with cells. Liposomal composition and structure can also be used as a cell model in the study od membrane processes [1].
Endothelial cells form barriers in the vascular system which seperate blood from tissues. The cells are bond with tight junctions which prevent transport of drugs, and therefore acts as an obstacle for the systematic delivery (figure 1 right). In the brain, blood-brain barrier, which consists of endothelial cells, separates brain capilares (apical side of barrier) from brain tissue or central nervous system (basolateral side of barrier). Blood-brain barrier is selectively permeable to nutrients necessary for healthy brain function through diverse collection of molecular transport systems. One of them is receptor-mediated transcytosis[1] (RTM), which involves the vesicular traffickng system of the brain endothelium, where circulating ligand interacts with specific receptor at the apical plasma membrane[2] of the endothelial cell (figure 4 a) [2]. Drug loaded liposomes coupled with particular ligands or targeting vectors on the external surface could be used to increase drug transcytosis through the barrier and consequently more effectively cure brain disfunctions [3].

2 History

First observations of liposomes, meaning *fat bodies* in Greek, date back in late 19. century where lecithin colloidal solutions were studied. In the early twentieth Century Otto Lehmann observed, what he called

[1] Transcellular transport.
[2] Composed of phospholipid bilayer where intrinsic glycoproteins and glycolipids are embedded.

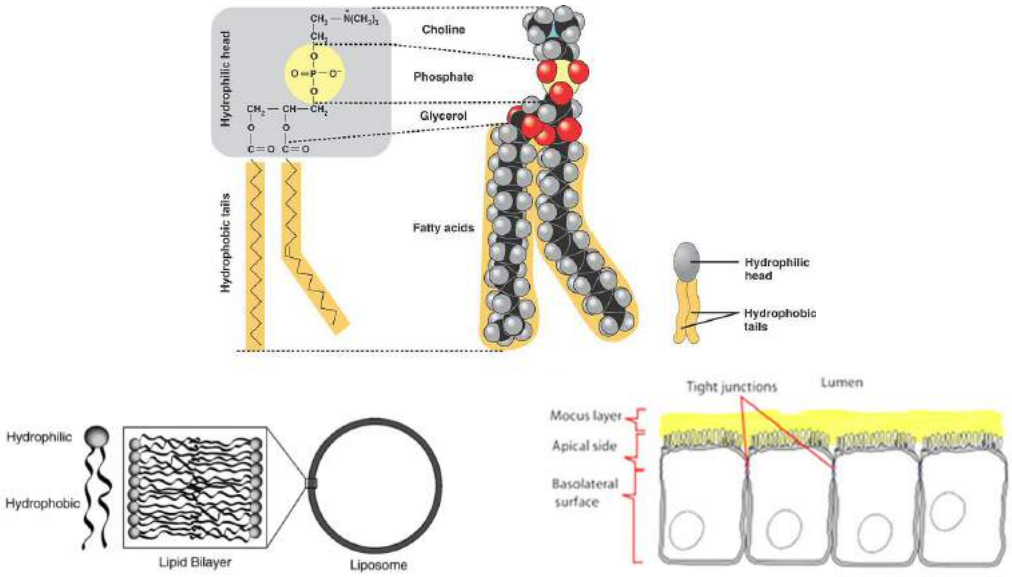

Figure 1 – Above: phospholipid molecule with two hydrophobic fatty acid tails [4]. Hydrophilic head is composed of glycerol, phosphate and choline molecules. Left: schematic representation of liposome composed of phospholipid molecules [5]. Right: cellular tight junction; M. Ruiz (2006).

artificial cells, which are today rather called multilamelar liposomes. Just as other scientist at that time, he did not precisely understand the system. In the mid 60's Alec Bangham and his colleauges recognized the unique properties of liposomes which are an encapsulation of the part of the solvent into their interior during agitation of the of thin lipid films in water. After this discovery the field of liposome research grew rapidly, ranging from pysics to medicine and has continued ever since. After first decades liposomes were found very useful in the basic research of properties of biomembranes, comformation and function of proteins, mechanism of various physical, chemical or biological interactions etc. In the 80's several liposome-oriented pharmaceutical companies were developed in aspiration for the medical aplications of liposomes, but due to unforeseen difficulties, like unstableness of the liposome formulations, faded away quickly [1].
The potential of liposome-mediated drug delivery to cells was recognized in the 1970s but the mechanisms of liposome-cell interaction have not been elucidated jet completely [6]. Over the last 15 years, many drug targeting and delivery strategies to enter the brain tissue have been explored, e.g. protein drug covalently linked to a transport vector (Pardridge, 1998) or drug targeting to the transferrin receptor (Visser et al., 2004) [7].

3 Liposome dynamics

Important parameter which can be used to characterize lipid structure in dinamical changes is order parameter S. In general it means different ordering of molekules in different directions and is second rank tensor. Long-axis rotational motion of the lipid molecules in membrane bilayer has very anisotropic components and the chains are strongly ordered in an orienting potential (membrane normal). Deeper as we go into the membrane bilayer (fatty acid tails of lipid molecules), lower is the order parameter (minor contribution of van der Waals forces among fatty acid tails, fluidity increases).
In simplest model which describes the motion of a long molecular axis (in case of biological membranes) is wobbling model, where the direction of molecular axis is changing randomly within the maximal angle γ (the angle between membrane normal and molecular axis).
For the description of molecular ordering in liquid crystals, 3×3 matrix of order parameters was introduced

(Saupe, 1964). Matrix element are

$$S_{ij} = \frac{1}{2} \langle 3 l_{Ni} l_{Nj} - \delta_{ij} \rangle , \tag{3.1}$$

where l represents $\cos\theta$ between reference axis of the sample or molecule (e.g. lipid bilayer normal) and axis of the coordinate system of the molecule. The order parameter is gained by time averaging of the molecular motion if it is fast compared to the time scale of the measurement method. In lamellar or axial symetry, molekules rotate along their long axes, parallel to the bilayer normal, where only one component is non-zero [1].

$$S_{33} = \frac{1}{2} \big(3 \left\langle \cos^2 \theta \right\rangle - 1 \big), \tag{3.2}$$

where θ is the angle between the molecular long axis and the lipid bilayer normal. In perfect alignment ($\theta = 0$), we obtain $S_{33} = 1$. In complete disorder, time averaged $\cos^2\theta$ is $\left\langle \cos^2 \theta \right\rangle = 1/3$, so $S_{33} = 0$. We can measure temperature and composition dependent S_{33} parameter directly by NMR, EPR or fluorescence methods which have different time scales and therefore give different time averaging.
Lipid molecules within a lipid bilayer exhibit also lateral difusion and process of transfer between the two monolayers (flip-flop). First varies from $\sim 10^{-10}$ to $\sim 10^{-8}$ cm^2/s, while flip-flop may take weeks.
In solution, liposomes exhibit Brownian motion which is directly proportional to the temperature and inversly to the liposome size. Translational motion can be, in a first approximation, estimated by the Stokes-Einstein relation [1].

$$D = \frac{kT}{6\pi r \eta}, \tag{3.3}$$

where D is diffusion coefficient, k Boltzmann constant and η viscosity of the medium. The approximation is valid for diluted solutions, where there is practically no attractive or repulsive force. From this hydrodynamic analysis we can also derive the rotational frequency or rotational correlation time[3] of liposomes [1]:

$$t_r = \frac{4\pi r^3 \eta}{3kT} \tag{3.4}$$

The exchange of lipid molecules with other liposomes or cells is in case of phospholipids, because of very low concentration of monomers, a rather slow process. Concentration of free phospholipid molecules in solution in the presence of vesicles (liposomes) is normally bellow 10^{-7}M. Aggregation of almost all phospholipids is the consequence of intermembrane forces which tend to minimizate the free energy of the system and is dependent on the lipid concentration (critical micellar concentration, which denotes the critical concentration of all self-assembled structures) [8]. Explained with thermodynamical chemical potencial, the necessary condition for the formation of large stable aggregates is $\mu_N^0 < \mu_1^0$, where μ_N^0 is the mean interaction free energy *per molecule* in an aggregate state and μ_1^0 in isolated state. For 1D, 2D and 3D (spheres) aggregates, μ_N^0 in general decreases with N. Interaction free energy of the molecules can be expressed as [8]:

$$\mu_N^0 = \mu_\infty^0 + \frac{\alpha kT}{N^p}, \tag{3.5}$$

where α is a posotive constant dependent on the strength of intermolecular interactions and p is a number that depends on the shape of the aggregates.
For one particular phospholipid (DMPC) the rate constant transfer between liposomes was measured to be approximately 10^{-4} s^{-1} [1]. This transfer however in some cases cannot be neglected, specially when we have lipids with higher water solubility. When liposomes interacting with endothelial cells, it is important to obtain the conditions to increase rate constant transfer.
Very important in liposome dynamics is also the leakage of encapsulated substances. It briefly depends on the temperature, chemical composition of bilayer, ionic strength od the medium and characteristics of the encapsulated substance [1].
Permeability of the liposomal membranes shows strong dependence on the dynamics of the membrane. It is different for anions, cations and neutral particles. In general it is increasing with temperature. The simplest model for permeation is the Fick's first law of flux [mol/m^{2}s] across the membrane [1]:

$$J = -D \frac{\partial C}{\partial X}, \tag{3.6}$$

[3]The time it takes the particle to rotate by one radian.

where D is diffusion constant and C concentration gradient. In the case of the membrane-water system we can define permeability coefficient as $P = K_d D/\delta = J/\Delta C$, which is diffusion coeficient over thickness δ, corrected with the distribution coefficient K_d. This model correlates well for the permeation of substances soluble in the bilayer. In general, charged particle (anions, cations) are less permeable than neutral ones. The reason is in reduced solubility in the nonpolar phase. Furthermore, anions are in general 50-200-fold more permeable than simple kations which is due to formation of short lived complexes which can help to shuttle them across the bilayer or due to internal membrane potential of +240 mV (oriented dipoles on its surface). Pointed in numbers, water permeability coefficient through lecithin[4] is of the order of $10^{-3} - 10^{-4}$ cm/s, which means that 3000-4000 water molecules cross the lipid bilayer per second per area of polar head (0.7 nm^2) while it takes about 70 h for one sodium atom to cross the membrane. In general, permeability is difficult to understand, specially for charged particles. Most of these measurements were performed in lecithin bilayers [1].

3.1 Forces between lipid molecules

Lipid molecules aggregate into various structures to minimize the free energy, δG, of the whole system by trying to maximize hydrophilic interaction and minimize the hydrophobic one. Hydrocarbon chains bind with London-van der Waals forces, which lower their free energy gained while transferred from water to the nonpolar phase, whereas simultaneously, the polar head groud hydration also greatly reduces δG. Total interfacial energy for molecules in an aggregate can be written in the first order as:

$$\mu_N^o = \gamma a + \frac{K_r}{a}, \tag{3.7}$$

where γa is the hydrophobic contribution of interface with a being the surface area and γ free energy per unit area. The second term presents repulsion of polar heads via empiric constant K_r. Intramembrane and intermembrane forces which are responsible for the intra-bilayer and interbilayer interactions consist of hydrophobic forces (attractive, between hydrocarbon molecules in water), van der Waals/London dispersion forces (attractive, between neutral atoms or molecules, in case of nonpolar molecules we have London dispersion), electrostatic forces (repulsive, systems with nonzero surface charge), hydration forces (repulsive, when the polar heads are hydrated) and steric forces (repulsive, thermal undulations of the bilayer, specially polar heads), which can all operate simultaneously [1, 7, 8].
Numerous theories and models have been proposed to explain these forces. They are responsible for the mechanical and thermodynamic behavior of membranes. Characteristics of aggregates is dependent on thermal.. There are also weak attractive depletion forces which act among polymers and surfaces (colloids) in close proximity. However in our case these forces are irrelevant, because only phospholipid molecules fill the solution. Casimir forces which are physical forces between two uncharged metallic plates in a vacuum also do not contribute to the forces among lipid molecules.

Van der Waals forces

These are forces between neutral atoms and molecules, where the interacting molecules are polar with permanent dipole moments or both, polar and nonpolar where polar molecules induce dipole moment in nonpolar ones which causes the attraction. London dispersion forces are responsible for the attraction of nonpolar molecules, where fluctuating dipole moment of molecules derives from statiscally distribution of electrons in time. Free energy for nonretarded van der Waals attraction between two spheres with radii R_1 and R_2 is [1]

$$V_{vdW} = -\frac{A_H}{6d}\frac{R_1 R_2}{R_1 + R_2}, \tag{3.8}$$

where A_H is the Hamaker constant and is proportional to the atom density and polarizability of the media, $A_H = \pi^2 C \zeta_1 \zeta_2$. The van der Waals force can be obtained by differentiation of equation xx.
VdW forces between amphiphilic structures are generally small. The reasons are more, that Hamaker constant is not constant above separation 3 nm, that in the presence of electrolyte there is an additional

[4] A class of phosphatides containing glycerol, phosphate, choline, and fatty acids. It is essential as a component of all cell membranes, for fat metabolism, etc.

reuction of A_H due to ionic *screening*, etc. VdW force between bilayers and membranes has therefore no effect beyond separation of 15 nm [8].

Electrostatic forces

These interactions depend on the ionic strength (concentration of electrolyte present), surface charge density or potential, pH and dielectric properties of the medium [1, 8]. They can be calculated by the Poisson-Boltzmann equation

$$\nabla^2\psi(x) = -\frac{4\pi\rho(x)}{\varepsilon}, \tag{3.9}$$

where $\psi(x)$ represents electrostatic potential, $\rho(x)$ charge density and ε dielectric constant. We can solve the equation with the expression of Laplace operator in spherical coordinates (in case of spherical particles, e.g. liposomes or cells) and appropriate boundary conditions [1].

Hydration forces

These repulsive forces arise whenever water molecules bind strongly to hydrophilic surface groups. Water molecules bound prevent the close approach of two surfaces (e.g. hydrated liposomes) and high energies are needed to dehydrate the surfaces. The range of these forces is ¡ 4 nm and can be empirically writen as [1]

$$F_n(d) = K_h e^{-\frac{d}{\lambda}}, \tag{3.10}$$

where λ is the characteristic decay length ($\sim$ 0.2-0.3 nm - dimension of water molecules). These forces often dominate over vdW or electrostatic forces at small separations (they are responsible for the lack of strong adhesion of bilayers composed of uncharged lipids such as lecithin). The impact of hydration forces at small separations of interacting bilayers is also dependent on the salt concentration in the solution (binding of hydrated Na^+, higher the concentration is strongly the repulsion is) [8]. The energy nedeed to dehydrate the polar head decreases with increasing temperature.

Steric forces

They originate from thermal undulations of the bilayers and are important specially in the systems with larger polar heads. There are four different types of repulsive steric forces (undulation, peristaltic, protrusion and overlap force) where undulation force, which is the only one expected to have an indefinitely long range, can be described with the equation [8]

$$F \approx \frac{(kT)^2}{2k_b D^3}, \tag{3.11}$$

where D is the distance and k_b membrane's bending modulus, which is important parameter in bending energy, $E_b = k_b/2R^2$ [8].

Hydrophobic forces

Attractive hydrophobic interaction between hydrocarbon molecules in water is of long range and much stronger than van der Waals attraction at small separations. Hydrophobic interaction between hydrocarbon groups is in case of free bilayers effectively shielded by hydrophilic headgroups. In case of bilayer expansion due to a stretchin forces, more of hydrophobic area is exposed to the aqueous phase and hydrophobic interaction can emerge. The enhanced hydrophobic interaction caused by either electric field applied, osmotic swelling or particular ion binding to stress the bilayers may be the primary causes leading to the fusion of bilayers and membranes. These forces can also be enhanced by increasing the temperature [8].

4 Liposome-cell interactions

Understanding of liposome interactions with cells (e.g. endothelial cells) is beside improvements in drug delivery important also for understanding the cell-cell interactions. There are four different mechanisms of interaction [1]:

- exchange of lipids or proteins with cell membranes,
- adsorption or binding of liposomes to cells,
- internalisation of liposomes by endocytosis or phagocytosis,[5]
- fusion of bound liposome bilayer with the cell membrane

shown in figure 2. All four interactions are dependent on lipid composition, type of cell, presence of specific receptors and targeting vectors etc.
Lipid transfer occurs by two separate processes, either by associated transfer proteins or upon events during collisions. Due to the similarity of liposomes with the phospholipid monolayer of lipoproteins[6], lipid transfer proteins in the cell membrane also recognize liposomes, which consequently causes lipid exchange [1].
In case of endocytosis, after adsorbing on the cell surface, liposomes are engulfed into endosomes[7] which transport them to the lysosomes (enzymatic activity where proton pumps in the membrane lower pH value) which in general digest the lipids and encapsulated molecules are therefore released into surrounding cytoplasm, where they are reduced by oxy-redoxy systems inside cell (figure 2) [1].
The remaining mechanism of interaction is fusion, where liposomal content is directly delivered to the cytoplasm. In general, fusion of liposomes with cells occur rarely, where the process is controlled by membrane proteins. The fusion can be enhanced by adding fusogenic lipids in the liposome membranes [1]. Fusion of lipid billayers is explained physicaly by their intermixing, driven by concentration and surface presure gradients, i.e. lateral diffusion and Marangoni effect[8]. To fuse, it is required to overcome the energy barrier, which is due to the internal bilayer interactions. The lack of direct experimental measurements of the process of lipid bilayer fusion, because of very short life-times of the intermediate structures, hinders the understanding of this molecular mechanism, while in case of lipid bilayer interaction with cells (higher heterogeneity and complexity), the mechanisms are even more complex [9].

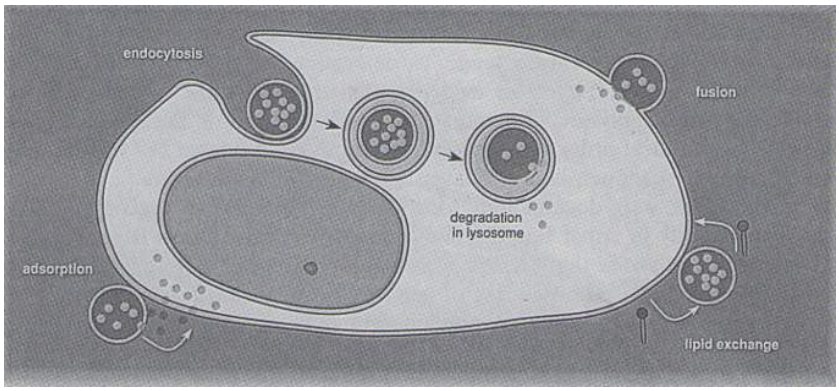

Figure 2 – Liposome-cell interaction. The first process is liposomal adsorbtion to the cell membrane (lower left). Some of the contents may be released into the extracellular fluid and some fraction may pass through the membrane. The second possible process is the uptake of liposomes by endocytosis (upper left), which then degrade in organelles called lysosomes and release the content into the cytoplasm. The third possible process is fusion, where the liposome's content enters the cytoplas directly (upper right). The last mechanism is the exchange of lipids (lower right) [1].

[5]The cellular process of engulfing solid particles by the cell membrane.
[6]Water-soluble carrier particles compounded of lipids and proteins, the way encapsulated lipids (cholesterol) travel in the blood, where protein molecules allow tissues to recognize and take up the particle.
[7]A membrane-bound vesicle found in a cell by an inward folding of the cell membrane to hold foreign matter taken into the cell by endocytosis.
[8]The mass transfer along an interface between two fluids due to surface tension gradient.

5 Drug encapsulation

Spherical phospolipid-based liposomes can be used to carry large amount of small water-soluble molekules (therapeutic drugs) in their aqueous core or lipophilic ones in their lipid bilayer membrane through covalent, non-covalent or avidin-biotin binding (figure 3). The effective delivery of therapeutics to targeted sites (disease sites) of the body is largely dependent on sufficient local concentration of a therapeutic agent, resistance to the rapid clearance by the reticuloendothelial system[9], amount of polyethylene glycol (PEG) moiety[10] incorporated into the liposome bilayer and adequate amount of transport agents when crossing the endothelial barriers through various mechanisms (e.g. the transport via receptor-mediaton) [2, 5].
PEG molekules, which are used as a spacer (figure 3), results in a better flexibility of the targeting vector bound. That particular binding increases the efficiency of targeting to specific receptors or in general transport of drugs into targeting tissues [10].
The size of liposomes has also been shown to be important factor in the efficient delivery of therapeutic drugs to the disease sites (e.g. antitumor drugs to a tumor site) [11]. Size affects their circulation half-life ($T_{1/2}$). As the size of liposome increases, the $T_{1/2}$ decreases, where optimum size was found to be 114.3 nm. Smaller it is, easier and more effectively can penetrate to the tumorous sites.

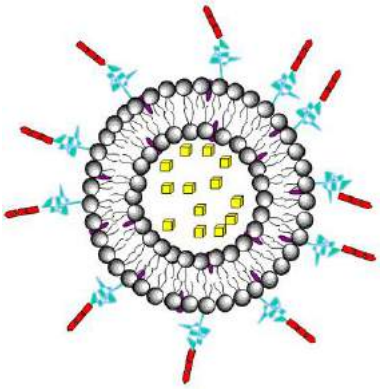

Figure 3 – Shematic representation of drug encapsulation in liposomes. Hydrophilic drugs (yellow) are encapsulated in the interrior of the liposome, while hydrophobic ones (violet) are bound in the interior of phospholipid membrane. Vesicles (red), bound on PEG molecules (blue) represent targeting vectors which bind to a specific cell receptors [12].

6 Blood-brain barrier

Because of tight junctions, low vesicular transport and high metabolic activity, nearly 100% of large-molecule drugs (e.g. proteins, peptides, and nucleic acids) do not cross the BBB endothelial cells. Only small molecule drugs (molecular weight < 600 Da[11], e.g. histamine[12]) can pass the BBB either paracellularly (through tight junctions) or transcellularly depending on their lipophilicity (if the lipid solubility of the molecules is higher, more efficient is the transport) [13, 14].
BBB is approximately 200 nm thick, separating more than 100 billion capillaries from brain tissue, neurons. The surface area of capillares is $\sim$20 m^2 [13].
The figure 4 shows shematic representation of BBB and the processes involved. Endothelial cells are sealed together with tight junctions and surrounded by astrocytes[13] that contribute to the selective pas-

[9] Class of cells that occur in widely separated parts of the human body, that take up particular substances and are part of the body's defense mechanisms as they can engulf and destroy bacteria, viruses, and other foreign substances.
[10] Flexible, water-soluble polymer which is in particular case of binding to a liposome membrane bilayer important for stabilization and ability to effectively bind targeting vectors.
[11] Dalton (symbol: Da), unified atomic mass unit (symbol: u).
[12] Organic nitrogen compound involved in local immune responses acting as a neurotransmitter.
[13] Characteristic star-shaped glial cells in the central nervous system.

sage of substances across BBB. Substances like alcohol, caffeine, nicotine most anasthetics etc. (small molecules soluble in fat, which is lipophilic) and glucose (active transport with specific proteins makes an exception of otherwise incapability of the transport, due to large, water-soluble nature) pass the barrier rapidly, whereas proteins, ions and most antibiotics (nonlipophilic nature) cross the barrier slowly.

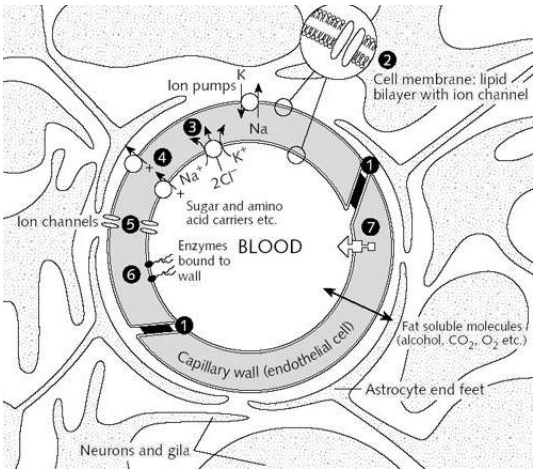

Figure 4 – Blood-brain barrier. (1) tight junction between endothelial cells; (7) the efflux pumps which extrude fat-soluble molecules that have been transported into the cells [15].

6.1 Noninvasive blood-brain barrier transport via receptor-mediation

Nutrition transport of ions, amino acids, vitamins and proteins through the brain endothelium is induced by several molecular transport systems in the plasma membrane: carrier-mediated transport (CMT, molecular carriers at both sides of barrier, selective transport of small molecules), absorptive-mediated transcytosis (AMT, relies on nonspecific charge-based interactions, e.g. initiated by polycationic molecules binding to negative charges on the plasma membrane, lack of specific targeting) and receptor-mediated transcytosis (RMT, vesicular trafficking system, where specific ligand bound to therapeutic interacts with a specific receptor at the apical side of endothelial cell) (figure 5) [2]. I will focus on the latter, vesicle-based receptor-mediated transcytosis, which allows the passage of larger molecules, including liposomes.

For successful transport, therapeutic carriers (e.g. liposomes) must be conjugated to a molecule that has the capability of targeting an RTM system. These molekules can couple to therapeutics or therapeutic carriers through chemical linkage (e.g. covalent conjugation through a stable thioether bond) or non-covalent streptavidin-biotin linkage[14] (biotinylated therapeutic-streptavidinylated targeting vector, high binding affinity - $K_d \sim 10^{-15}$, where $K_d = [P][L]/[C]$ and where variables in brackets represent molar concentrations of the protein, ligand and complex, respectively). Due to necessity to retain therapeutic activity and sterical stabilization (in case of liposomes as therapeutical carriers), polyethylene glycol (PEG) is used. In case of non-covalent linkage PEG is used to better separate the therapeutic and targeting moiety[15], while its incorporation into liposome bilayer serves, beside stabilization, to prevent rapid clearance by the reticuloendothelial system and as a bond to thiolated targeting molecule [2].

The most studied receptor known to undergo RTM is the transferrin receptor (TfR), which is known to mediate iron delivery to the brain. In human, TfR is transmembrane glyco-protein. Table 1 represents *in vivo* RTM systems for liposomal brain drug delivery [2].

[14]Streptavidin - 52800 Da tetrameric protein which has an extraordinarily high affinity for biotin. Biotin - water-soluble B-complex vitamin

[15]Part or functional group of a molecule.

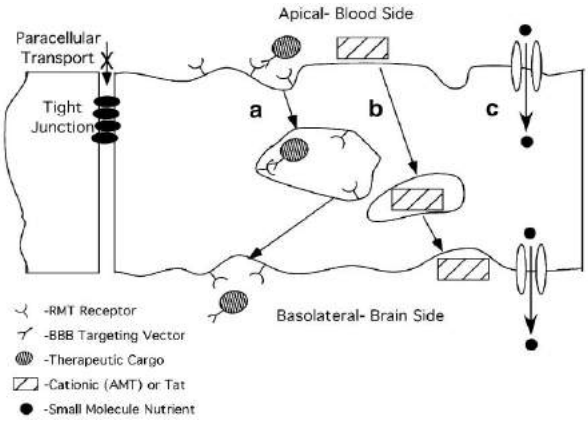

Figure 5 – Shematic presentation of transport across the BBB. Because of tight junctions between adjacent endothelial cells, therapeutics must either difuse through cell membranes or be transported by one of the mechanisms in order to successfully reach brain tissue. **a.** Receptor-mediated transcytosis. **b.** Absorptive-mediated transcytosis. **c.** Carrier-mediated transport [2].

Table 1 – *In vivo* Studies of RTM Systems for Brain Drug Delivery [2]

BBB Receptor	BBB Targeting Vector	Linkage	Species used	Secondary Target	Payload
Rat TfR	OX26 MAb[a]	PEGylated liposome	Rat	Neurons	Expression plasmid encoding β-Galactosidase (model enzyme)
Mouse TfR	8D3 MAb[b]	PEGylated liposome	Mouse	U87 human glial tumors	Expression plasmid encoding antisense mRNA[c] directed to human EGFR[d]
Human IR[e]	83-14 MAb[f]	PEGylated liposome	Monkey	Neurons	Expression plasmid encoding β-Galactosidase or luciferase[g]
Human IR	83-14 MAb	PEGylated liposome	Monkey	Ocular cells	Expression plasmid encoding β-Galactosidase or luciferase

[a] The mouse monoclonal antibody (made by identical immune cells that specifically bind to the substance) against the rat TfR.

[b] The mouse monoclonal antibody against the mouse TfR.

[c] Messenger RNA. Its sequence of nucleotides causes a gene production (protein).

[d] Epidermal growth factor receptor which exists on the cell surface and is activated by binding of its specific ligands.

[e] Human insulin receptor.

[f] The mouse monoclonal antibody against the human TfR.

[g] Class of oxidative enzymes.

6.2 Model of blood-brain barrier transport

Madin-Darby canine kidney (MDCK) cells have been successfully used as a model of endothelial barrier in the study of transcellular transport. This model was used due to the similarity to endothelial cells concerning the different transport mechanisms, different degrees of permeability and specially because of their physiochemical properties and the transport of selected pharmaceutical compounds. Because of high reproducibility they are furthermore convenient for extensive studies [16].
For the study of liposome-cell interaction, confocal laser scanning microscopy was used, where the liposomes were labelled with fluorescence dyes (Orthmann et.al., 2010). Figure 6 shows cellular localisation of various liposomes in MDCK cell line through the fluorescence dyes.

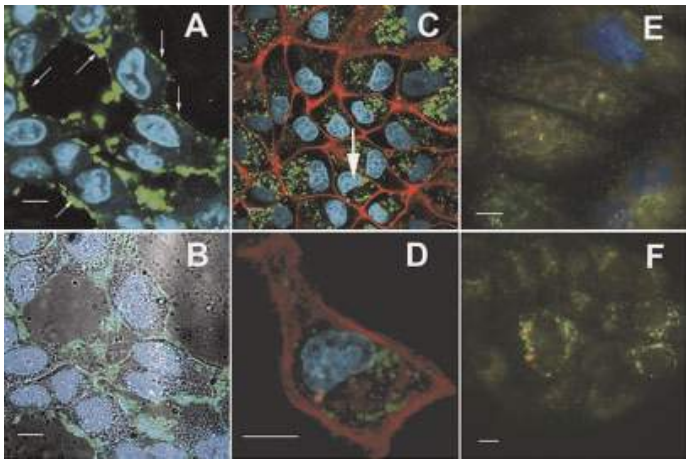

Figure 6 – Confocal laser scanning microscopy images of fluorescence dye calcein labelled liposomes in MDCK cells after 24 h of incubation at 37°C. Liposomes, nucleus and cell membrane are indicated by green (calcein), blue (Hoecht reagent) and red (trypan blue) dye, respectively. **(A,B)** Incubation of positively charged liposomes $L2^{16}$. Green areas represent location of calcein loaded liposomes, mainly at the membrane sites. **(C).**. Incubation of cells with negatively charged liposomes L4. Trypan blue dye was used to better visualise the cell membrane. Liposomes (green dye) are mainly distributed in the cytosol. Picture **(D)** shows the magnification of particular cell pointed with the arrow on previous picture C. Incubation of negatively charged, less fluid L3 and fluid L4 liposomes is shown on pictures **(E)** and **(F)** respectively. From these results we do not know if the calcein marker is free or still encapsulated in liposomes [16].

Impact of membrane properties on trascytosis of liposomes across endothelial barrier model

Only a few studies have investigated the impact of liposomal composition on cellular transcytosis by endothelial cells. In the study [16] it was shown that the membrane properties affect essentially the mechanisms of transport. They discovered that the charge and the fluidity of the liposomes plays an important role in their transport efficiency into and through a tight cellular barrier. Membrane structure and fluidity of selected liposomes were characterised by electron paramagnetic resonance (EPR) technique with lipophilic spin probes, while mechanism of the transport was observed by fluid-phase hydrophilic fluorescence marker calcein (figure 6) encapsulated in liposomes [16].
The composition of liposomes (table 2) was modified to differ mainly in charge and fluidity. PC-phosphatydilcholin and CH-cholesterol are basic lipids (structural formulas are shown in figure 7), DCP molecule carries negatively charge, while DDAB molecule positively charge, DOPE and OPP are helper lipids, first for nonviral gene transfection and second for ability to disturb membrane organisation and to promote membrane-membrane interaction, and PEG-PE sterical stabiliser, already mentioned previously in seminar.
Transcytosis activity was quantified after 24 h of incubation of calcein loaded liposomes with tight MDCK cell monolayer by determination of the calcein flourescence in the basal compartment of the transwell system (table 2, shematic presentation of the process shown in figure 8). The highest transcytosis was determined after incubating the cells with L4 liposomes, which have the highest molar ratio of membrane helper lipids among liposomes and are the most fluid and are negatively charged. On the other hand, the highest uptake of the negatively charged MDCK cells was shown in case of L2, positively charged liposomes where strong charge-charge attraction forces play important role, hence prevent the calcein to cross the barries, which is indicated in low transytosis (table 2) [16].
The measurements of the stability of liposomes through encapsulated calcein leakage over particular time interval (7 h) have shown the correlation with corresponding transcytosis values (figure 9 left). It has been proposed that during the transport process, calcein molecules are released from endosomes in the cell and that the release rate is proportional to the leakage of the liposomes. Calcein as a drug model is then transcytosed into the basolateral site of the membrane by the cell proteins.
Different values of calcein transcellular transport for various compositions of liposomes gives an assumption of different mechanisms of cell-liposome interaction. To clarify this hypothesis the reduction

measurements by EPR spectroscopy of hydrophilic spin probe tempocholine (ASL, figure 7) encapsulated in liposomes L3 and L4 added on tight set of MDCK cells were made (figure 9 right). ASL is reduced by oxy-redoxy systems inside the cells which reflecs in an EPR spectra intensity decrease [17]. From the reduction curves for both liposomes we have made the conclusion that the mechanism of the liposomal transport is the same (likely by the endocytosis).
To fit the ASL reduction curve we must write the model of liposome-cell interaction (page 6.3). From the fitted curve we can in general get the charachteristics of interaction.

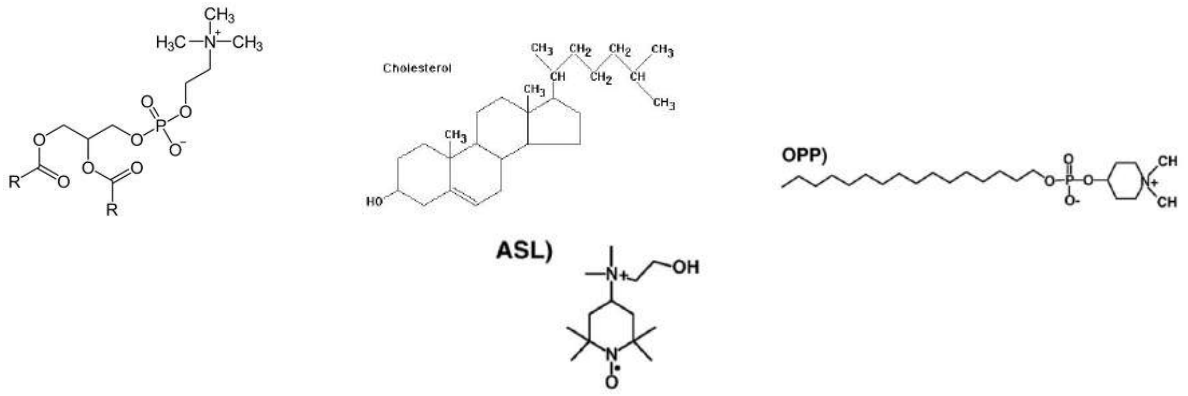

Figure 7 – Structural formulas of: PC) phosphatidylcholin; CH) cholesterol; OPP) octadecyl-1,1-dimethylpiperidin-1-ium-4-yl phosphate; ASL) charged hydrophilic spin-probe.

Table 2 – Composition of Selected Liposomes (the numbers in first five columns present molar ratios) and relative Amounts of Calcein in different Compartments in Comparison to the total Amount of Calcein used for Incubation [16].

						Calcein [%]		
Liposome	PC	CH	DCP/DDAB	DOPE/OPP	PEG-PE	Basal Media	Cells	Ratio (basall/Cell)
L1	70	30	10/-	-	-	11.5±2.3	2.9±0.4	4.0
L2	70	30	-/10	-	-	11.5±4.8	5.3±2.1	2.2
L3	40	30	10/-	30/-	-	4.4±0.7	3.1±0.8	1.4
L4	50	30	10/-	20/20	-	28.1±1.0	2.8 ±0.3	10.1
L5	70	30	10/-	-	5	4.9 ±0.9	2.2±0.2	2.2

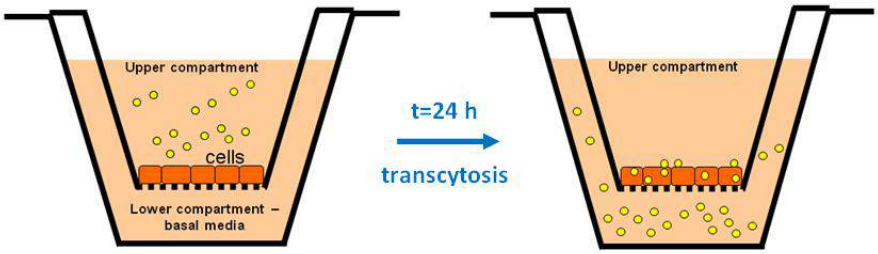

Figure 8 – Shematic presentation of the transcytosis process across tight MDCK monolayer.

Detailed studies of the influence of liposomal membrane properties on endothelial cells should in the future improve efficient liposomal formulations to traverse cellular barriers (e.g. blood-brain barierr) and hence improve the drug delivery, which is important for a treatment of metastases and primary tumours in the brain [16].

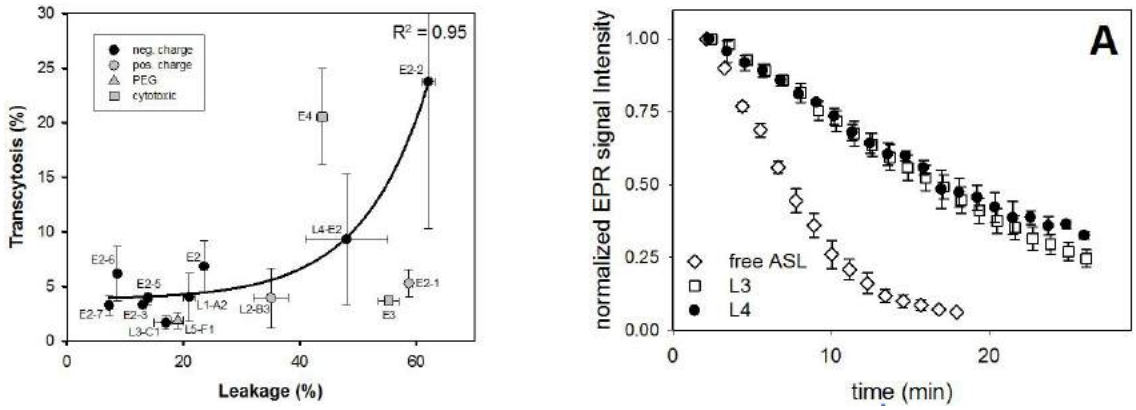

Figure 9 – Left graph: Transctosis of calcein encapsulated in liposomes as a function of liposome stability. Leakage was measured after 7 h, while for the transcytosis liposomes were incubated with cells for 24 h at 37°. Right graph: reduction of liposomal ASL (L3-average of 5 measurements, L4-average of 3 measurements) and free ASL by MDCK cells.

6.3 Kinetics of interaction

A lot of studies of liposome-cell interaction have already been made. Different lipid compositions and concentrations of prepared liposomes have shown different rates of cell interaction, where two main mechanisms have been observed: fusion and endocytosis. Kinetic analysis can elucidate rate constants for endocytosis or fusion, the number of various surface receptors and the corresponding binding constants [6, 18]. In some cases it has been shown that charged liposomes induce higher uptake by various of cells in comparison to uncharged ones [6].

Model

To study the liposome-endothelial cell interaction we mixed dense solution of MDCK cells and liposome solution with encapsulated spin probe ASL. By EPR spectroscopy we observed the signal decrease due to reduction of ASL molecules (with one unpaired electron which absorbes the EM field at resonance and produces the EPR signal) by oxy-redoxy systems in cell cytoplasm. By making a very dense solution, we forced the liposomes to fuse or endocytose with cells. To fit the reduction curve (figure 9 right), namely obtain the time change of non-reduced ASL concentration in cells (dc_{ASL}/dt), we had to use proper dynamic equations (law of mass action equations and diffusion equations which base on a concentration gradient of particular substances, in our case ASL molecules).
Due to the heterogenic structure of plasma membrane, we have to assume that the mechanism of the transport is not the same over the whole cell surface. Some of the liposomes will fuse (N_f), some of them will be transported by endocytosis (N_{end}) and some will stay associated at the cell surface [18]. Due to the time change of the plasma membrane, we presume the transport ability of the liposomes would consequently be reduced. The number of cells which are unable to transport the liposomes or have no receptors left are denoted as N_{fnr} for fusion and N_{endnr} for endocytosis, respectively. Furthermore, we suppose that the spin probe ASL concentration c_l in liposomes, that have not associated jet remain constant, because they are unable to pass the liposome membrane easily. Reduction of ASL in cell cytoplasm is dependent on reductant concentration c_r. This parameter is constant in case $N_{ASL} << N_r$, where N_r is the number of reductant molecules and N_{ASL} the number of ASL released in cytoplasm. Shematic representation of mechanism is shown on figure 10.

Mass action equations for this interaction are [18]:

$$\frac{dN_f}{dt} = -k_f N_f(t)\big(N_c - N_{fnr}(t)\big) \tag{6.1}$$

$$\frac{dN_{end}}{dt} = -k_{end} N_{end}(t)\big(N_c - N_{endnr}(t)\big) \tag{6.2}$$

$$\frac{dN_{fnr}}{dt} = \frac{k_f}{\alpha} N_f(t)\big(N_c - N_{fnr}(t)\big); \qquad N_{fnr}(t=0) = 0 \tag{6.3}$$

$$\frac{dN_{endnr}}{dt} = \frac{k_{end}}{\beta} N_{end}(t)\big(N_c - N_{endnr}(t)\big); \qquad N_{endnr}(t=0) = 0 \tag{6.4}$$

$$\tag{6.5}$$

where N_c is the number of all cells, while α and β represent the ratio of rate constant of cell transport saturation and rate constant of cell transport mechanism. If we do not do any assumption like $c_r >> c_{ASL}$ (the reductant concentration is much higher than released ASL concentration) or $k_r >> k_f, k_{end}$ (the rate constant of reduction is much higher than rate constant of transport mechanisms) which simplify the differential equations, time changes of concentrations are written with the next differential equations [18]:

$$\frac{dc_{ASL}}{dt} = k_P\big(c_{le} - c_{ASL}\big) - k_r c_{ASL} c_r + k_f N_f(t)\big(N_c - N_{fnr}(t)\big) c_l \frac{v_l}{v_c} \tag{6.6}$$

$$\frac{dc_{le}}{dt} = -k_P\big(c_{le} - c_{ASL}\big) + k_{end} N_{end}(t)\big(N_c - N_{endnr}(t)\big) c_l \tag{6.7}$$

$$\frac{dc_r}{dt} = -k_r c_{ASL} c_r \tag{6.8}$$

$$\tag{6.9}$$

where c_l is the concentration of spin probe in liposomes and v_l/v_c volume ratio of liposomes and cells. The first term on the right side of the equation 6.6 (describes the reduction of ASL or EPR signal decrease) is diffusive term derived from Fick equation, the second term derives from the law of mass action[17].

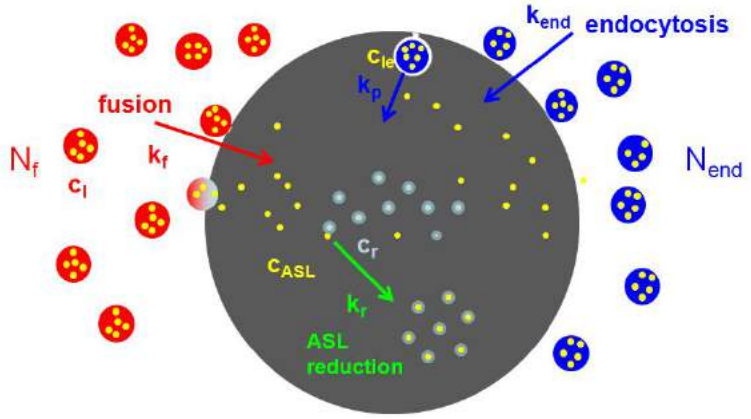

Figure 10 – General description of the cell-liposome interaction. N_f: the number of liposomes that fuse with cells; N_{end}: the number of liposomes that pass the plasma membrane by endocytosis; c_l: concentration of spin probe in liposomes that have not interacted jet; c_{le}: concentration of spin probe in endosomes; c_r: concentration of reductant in cell cytoplasm; c_{ASL}: concentration of spin probe in cytoplasm; k_f: rate constant of fusion; k_{end}: rate constant of endocytosis; k_p: endosome membrane permeability for spin probe; k_r: rate constant for spin probe reduction in cytoplasm.

[17]Mathematical model that explains and predicts behaviors of solutions in dynamic equilibrium. It can describe kinetics concerning the rate equations or elementary reactions.

7 Conclusion

The liposomes are used as drug carriers with numerous potential applications in medicine. One of them is the transport across endothelial cells into tumorous tissues. Efficient transport of drugs across the endothelial blood-brain barrier is still a big issue (today there is still no BBB drug-targeting technology program), which could be improved with the use of optimal liposomal formulations (long circulation time, bound with efficient BBB targeting vectors, etc.). Furthermore, the study of liposomal membrane properties (e.g. fluidity, charge, polarity, etc.), could essentially contribute to the effeciveness of the transport.

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