PhD Thesis

Characterization and modification of cell membrane derived vesicles

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Abbreviations

AB – apoptotic body
ATR – attenuated total reflection
BSA – bovine serum albumin
CD – circular dichroism
DLS – dynamic light scattering
DOPC - 1,2-dioleoyl-sn-glycero-3-phosphocholine
DPPC - 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPPE - 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
DSC – differential scanning calorimetry
ED – electron density
EMP – extra-membrane particle
EV – extracellular vesicle
EXO - exosome
FBS – fetal bovine serum
FF – freeze fracture
FTIR - Fourier transform infrared spectroscopy
LC MS - liquid chromatography–mass spectrometry
LD – linear dichroism
LPC - lysophosphatidylcholine
MLV – multilamellar vesicle
MVA – multivesicular aggregate
P/L – protein to lipid ratio
PBS – phosphate buffered saline
PC - phosphatidylcholine
PE - phosphatidylethanolamine
PEG - polyethylene-glycol
PI - phosphatidylinositol
PS - phosphatidylserine
RBC – red blood cell
REV – red blood cell derived extracellular vesicle
RNA – ribonucleic acid
SAXS – small angle x-ray scattering
SM - sphingomyelin
TDM – transition dipole moment
TEM – transmission electron microscopy
UCF - ultracentrifugation
ULV – unilamellar vesicle
US - ultrasonication
WAXS – wide angle x-ray scattering
1. Introduction

Vesicles may be one of the most important pharmaceutical delivery systems on the expanding field of nanomedicines. Intravenously injectable liposomes contain PEGylated phospholipids (polyethylene glycol covalently coupled with phosphoethanolamine, DSPE-PEG 2000 Da). The PEG chains cover the surface of the vesicles and result in the steric stabilisation of the objects, whereby a long circulation time in the vascular system is assured. Although the PEGylated surface of the vesicles may induce a reduced response from the immune system of the patient, a complement pseudo-allergy frequently occurs, which can lead to lethal cases in the clinical praxis. To avoid the toxic effects, an intense worldwide research is in progress to replace the PEGylated lipids and find alternative constituents for steric stabilisation. Different biomacromolecules (for example: hyaluronan, chitosan, poly-malic-acid, poly-lactic-acid) have already been used for the manufacturing of vesicles. Recently the presence of hyaluronan coated extracellular vesicles was reported in human synovial fluid, which directs our attention on natural vesicles, where the stabilization is assured by the automatized mechanisms of biological evolution.

Natural vesicles are complex, versatile relatives of the liposomes, produced by all kinds of living cells, from the smallest prokaryotes to the most complex organisms. These vesicles are derived from the plasma membrane and secreted into the extracellular space, earning their name: extracellular vesicles (EVs) based on this process. Extracellular vesicles have an extremely complex composition, which grants them properties best fit for their biological role. As EVs are the tools of transport and communication between cells, they are small (certain types have an average size of 30 nm), stable, targeted and loaded with numerous functional biomolecules (e.g. proteins and RNAs).

The connection between artificial and natural vesicles is represented by nanoerythrosomes. Nanoerythrosomes are derived from erythrocyte ghosts. The produced vesicles reportedly keep their natural composition and achieve stable, approximately 150 nm size. Due to their natural origin, these particles are biocompatible and could possibly be further modified by synthetic additives as well. Beside their useful properties, nanoerythrosomes can be produced in abundance, as red blood cells are arguably one of the best and most frequent source of cell membrane material.
Nanoerythrosomes can bypass the blood-brain barrier and have been used as additives to camouflage nanoparticles - for example gold nanoparticles in biological imaging. They could also be used as reference materials for extracellular vesicles, due to their similar biological membrane. As a fully biomimetic system, nanoerythrosomes are promising tools for personalized medicines. The elaboration of a nanoerythrosomal drug delivery system may start from the patient’s own blood, considerably reducing the risk of immunogenicity. I hope that my work represents perspectives on the path leading in this direction.

The studying of both the artificial and natural vesicle-like systems require a number of techniques, not only to determine different features (e.g. structural, morphological, calorimetric, optical, mechanical) in static and dynamic manners, but also to reveal quantities on a wide size range extending from the atomic scale to the micrometres. In the Thesis, after the presentation of the vesicle-like systems I briefly describe the applied methods, focusing on parts, which may be important for the interpretation of the results. The results are mainly concerning the novel types of nanoerythrosomes, produced by me first, therefore I intend to give a detailed description on the preparation and measurement protocols, to assure the reproduction of the systems by potential successors.
2. Background and Literature

2.1. Vesicles; their formation and main characteristics

Vesicles, or with other word liposomes, are lipid based, nearly spherical, typically multilamellar objects, which are composed from alternating lipid bilayers and water shells (1). The „vesicle” terminology was originally referring to small compartments of different natural, biological systems used in cytology, while “liposome” was a biochemical denomination. Recently both names are widespread. Vesicles, as artificial creations are formed via self-assembly in a two-component liquid phase system consisting of amphipathic lipids and water (Figure 1) (2) (3). The self-assembly results in different formations depending on the ratio of the two components as well as the size, shape and chemical behaviour of the lipid at a given temperature (4). The rigorous classification is used in physics and the systems of hydrated lipids are referred to as lyotropic systems (5) (6).

The vesicles could be considered as model systems of the biological membranes because their characteristic structural units, the lipid bilayers are highly similar (7). For bilayer formation the shape of the molecules is an important attribute: majority of the phospholipids have to be cylindrical, or slightly conical. Cylindrical lipids support the horizontal growth of the membrane, because the head group and the chain regions of the molecules occupy approximately the same lateral space and they do not induce curvature when densely packed together. As huge planar bilayers are not favoured thermodynamically, the large membranes eventually turn into spherical vesicles, which minimalize their surface energy (8).

Vesicles are either produced from one (or more) types of artificial lipids, or from natural lipids extracted from different organisms (bacteria, plants, mammals) or tissues (brain etc.). In the latter case more realistic and fully bio-mimic membranes could be obtained, but these vesicles exhibit complex, hardly adjustable physico-chemical features (9) (10).

Hydrated phospholipids typically result in large stacks of concentric bilayers, called multilamellar vesicles (MLVs) – or even vesicles inside another: multivesicular aggregates (Figure 1) (11). In this case the correlation between the multilayers is strongly disrupted and “vesicles in vesicle” can occur. Single bilayer containing unilamellar vesicles (ULVs) could also be prepared but these forms are not stable without additives.
When the number of lamellae is a reduced number, typically between 2 and 6, the objects are called oligolamellar vesicles (12) (13).

Figure 1. Phospholipid molecules and their formations in aqueous buffers. The effect of lipid geometries [e.g. phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and phosphatidylethanolamine (PE)] on the membrane curvature. Typical bilayer formations: unilamellar- (ULV) and multilamellar vesicles (MLV) and multivesicular aggregates (MVA).

The spontaneous arrangement of phospholipids and the aggregation of membrane particles make vesicular suspensions highly polydisperse. To produce stable, homogeneous, unilamellar vesicles, typically additives - such as cholesterol, or special phospholipids (conical or conjugated ones) are required. The preparation process almost always include some kind of sonication or extrusion, in which the applied intensity and pore size are used to adjust the size of the particles, respectively (14).

Furthermore, membrane-mimicking vesicles are not static systems. Their molecular constituents make intensive fluctuations and rotations, they move laterally in the membrane with high frequency and rarely even change their place between the inner and outer leaflet of the bilayer. This fluid character of the membranes and the interactions between the vesicles induce the aggregation and fusion of the particles with time (15).

As lipid molecules are the smallest essential packing elements of the membrane, their character and composition have a significant impact on the resulting vesicles. Lipids
represent a diverse group of organic compounds including fats, oils, hormones and other small, organic, membrane constituents, which are not water soluble. The three principal classes of lipids that can construct bilayers are glycerophospholipids (shortly: phospholipids), sphingolipids, and sterols (principally cholesterol) (16). In the Thesis the observed physicochemical effects are in connection with phospholipids, therefore the term “lipid” generally refers to “phospholipids”. The basic structure of the bilayer is created by phospholipids. The length and saturation of the lipids acyl chains define the hydrophobic region of the bilayer. By the modification of the lipid chains, the thickness and fluidity of the membrane can be altered. Incorporation of guest molecules, such as cholesterol, into the hydrophobic region also has a significant effect on the dynamics and thermotropic attributes of the membranes (17).

![Figure 2. Modification of the liposome membrane (18)](image)

The phosphate head group of the lipid molecules and its alternation is responsible for the surface character of the vesicles (Figure 2) (19) (20). The phosphate groups could be conjugated with functional molecules, which might either help in signalling and targeting (e.g. functional proteins), or grant steric stabilization (polyethylene-glycol - PEG) for the vesicle (21). The head group is also responsible for the charge of the lipid: positive, negative or zwitterionic lipids are all available. Finally, the size of the head group compared to occupied space of fatty acid chains (defined by their number, length
and saturation), determines the geometrical properties of the phospholipids. The shape of the molecule created the structure of the established membranes, resulting in bilayers, or different types of micelles, by cylindrical or conical lipids, respectively (Figure 1) (16).

2.2. Extracellular vesicles (EVs)

2.2.1. Genesis and biological function of EVs

Extracellular vesicles are natural vesicles, created by living cells and secreted into the extracellular space (22) (23) Figure 3. They serve as tools of intercellular communication and transport processes (24) (25). The EVs are derived from cellular membranes and have similar composition to their parent cells (26). They carry a wide variety of molecules, depending on the type of the vesicle as well as the physiological state of the emitter cell. Typical transported molecules are functional proteins and nucleic acids – especially RNAs, like messenger- (mRNA), micro- (miRNA) or small interfering RNA (siRNA) (27) (28) (29) (30). The composition and concentration of the released vesicles alter in healthy and in sick patients, therefore their detailed analysis is important for diagnostic and therapeutic purposes (31) (32) (33) (34).

![Extracellular vesicle classification](image)

Figure 3. Extracellular vesicle classification according to production pathway and characteristic size

EVs are a diverse group of vesicles that can be classified easiest by the way of production. According to their origin, the common classes of EVs are microvesicles (MVs), exosomes (EXOs) and apoptotic bodies (AB) (35). Strictly speaking the former
two, MVs and EXOs form a smaller group of EVs as they are created by an internal desire of the cell to affect on its environment. Microvesicles are derived from the plasma membrane by a shedding process, while exosomes are built up in an intracellular organell, called the multivesicular body (MVB). The MVB is responsible for the loading and functionalization of the exosomes and by its fusion with the cell membrane the vesicles are secreted into the extracellular matrix. In contrast, apoptotic bodies are the products of the programmed cell death (apoptosis), where the cell is disrupted in a controlled process, resulting in giant vesicles of residual cellular material (36) (37).

Due to their different production pathways, the subpopulations of extracellular vesicles have significantly different characteristics. Apoptotic bodies are the most heterogeneous vesicles, as far as their size and composition is concerned (25). They are the largest of all the EVs, usually above one micrometre, often comparable to the size of the cells. According to their function ABs may be viewed as degraded cellular packages, containing partially decomposed organelles and inflammation molecules (38). Their in vivo clearance from the extracellular matrix is quick, consequently an increased quantity of ABs can be a sign of cell damaging agents (39). Microvesicles are approximately 100-300 nm large particles produced by a shedding process from the plasma membrane (40). As these particles are the direct derivatives of the outer membrane, they carry significant information about its biomolecular pattern (41). Examination of microvesicles may be important in processes that are related to the plasma membrane, or in the case of certain cell types, which have simplified intracellular composition (such as red blood cells). Exosomes are the smallest and most specific group of EVs. They are approximately 30-100 nm large particles with the ability to transport signalling molecules to great distances (42) (43). This function requires a unique composition that grants the vesicle stability and targeting ability as well as the possibility to fuse with the membrane of the recipient cell. These special characteristics put the exosomes in the spotlight of scientific experiments in order to develop new and effective diagnostic, therapeutic and carrier nanostructures (44) (45) (46). The exosomes represent the most advanced vesicles that the model lipid systems aim to mimic in the future.

2.2.2. Characterization of EVs

The EV containing extracellular matrix is a complex suspension of cells, submicron particles and biological molecules. The cells, cellular debris and other
nanoparticles make the isolation and characterization of EVs complicated. Furthermore, the detection and studying of EVs from a single cell type could be challenging due to their low concentration, especially in vivo (47) (48). Consequently, for modelling purposes and fundamental research in-vitro cell cultures or purified suspensions of a single cell type are used. In either case the general methods of isolation are the same. Namely: separation by centrifugation (differential or gradient ultracentrifugation), gel filtration (size exclusion chromatography) or using an immunoassay that isolates the vesicles according to their specific surface markers (e.g. exosomal marker proteins: CD9, CD63 or CD81) (49).

The purity and concentration are critical quality attributes of isolated EV suspensions, but also limiting parameters for their characterization techniques. Techniques that are used to examine unilamellar model vesicles could be applied for extracellular vesicles as well. The structure and composition of small unilamellar vesicles could be used for modelling exosomes and microvesicles with a few simplification - such as the lack of membrane proteins and the lower diversity of lipid components (50). Techniques that have limitation for complex membranes – such as DSC – cannot be used for EV characterization.

The first step quality control of EV suspensions is the determination of their particles size distribution (51). For this purpose generally DLS is used. DLS gives information about the EV type and the purity of the EV population, by measuring its size distribution and polydispersity. Compositional analysis is also important to typify the vesicular suspensions, by the identification of EV marker molecules. There are a number of techniques available for compositional analysis, ranging from simple electrophoretic or colorimetric experiments to the complete proteomics and lipidomics of the vesicles (52) (30). The previous ones have the disadvantage of being fairly unreliable, while the latter ones are time and sample consuming and also not commonly accessible.

2.3. Artificial vesicles with natural origin

On the field of organic nanoparticle development, the ultimate goal could be the reproduction of extracellular vesicles. EVs, especially exosomes and microvesicles are small (approx. 100-200 nm) unilamellar vesicles that are stabilized by their unique biomolecular - mostly lipid and membrane protein - composition (53). This special composition grants them natural targeting, enhanced membrane permeability- and
therapeutic potential (46). EVs are the products of the cells, they are fully biocompatible and degradable. Although the listed positive properties make them ideal nanocarrier candidates, their direct application is complicated because of their low concentration and difficult purification from the biological milieu.

On the other hand model vesicles are accessible in large quantities and are easily tuneable by different additives. They are prepared from synthetic lipids in a laboratory or industrial environment (54). By their modification many positive properties can be achieved, such as enhanced stability, stealth character or even targeting ability (55) (56). However, the stability of the vesicles can be increased by additives in vitro as well as in vivo, their clearance from the organism is generally fast and the applied surface modifications could easily lead to strong immune responses (57).

In the development of artificial vesicles one step towards the EVs is the combination of natural membranes, or membrane components, with synthetic vesicles (58) (59). By this process “hybrid” vesicles can be created that combine the biological properties of the cellular membranes with the variability of model vesicles. These systems are trying to reproduce extracellular vesicles, while remaining their more controlled models, hoping to combine the positive properties of the two.

There are two practical approaches for the preparation of “hybrid” vesicles. The first is to prepare the model vesicles in the desired amount and composition and then trying to incorporate the extracted cell membrane components into them. Since in this case the incorporated membrane particles are typically functional proteins, the created vesicles are called proteoliposomes (59). Another method is when the cellular membrane is isolated and purified in high concentration and then transformed into vesicles by physicochemical processes (usually by extrusion and sonication) (60) (61). These vesicles can be further supplemented with additives – typically with phospholipids, as their protein content is already high. The nomenclature of these vesicles is based on their cellular origin, for example vesicles prepared from red blood cell (erythrocyte) membrane are called erythrosomes, or nanoerythrosomes – reflecting their small size (62).

Red blood cells (RBCs) are the most abundant cellular components of the blood, giving approximately 40% of its total volume. In their matured form RBCs lose their nucleus and most of their cellular organelles, to maximize space for haemoglobin molecules. Haemoglobin makes up approximately 96% (w/w %) of the dry material content of the cell and it is responsible for the binding and transport of oxygen and carbon-dioxide. Red blood cells are ideal sources of membrane material as their plasma
membrane is widely characterized and can be isolated in high purity, without intracellular membrane contamination (63).

Figure 4. Schematic representation of red blood cell membrane structure (64)

The RBC membrane has a unique composition that grants the cell high deformability and flexibility (Figure 4) (65) (66). Their membrane is composed of three layers: a glycocalyx (rich in carbohydrates) on the exterior, a lipid bilayer (containing numerous transmembrane proteins) and a membrane skeleton, which is connected to the inner side of the bilayer (67). The RBC membrane is rich in proteins, in human RBCs the protein content is approximately half of the total membrane mass. According to their function, the proteins can be transport proteins for molecules and ions (e.g.: Band 3 proteins, Na⁺/K⁺ transporters), adhesive molecules (e.g.: ICAM-4) or structural proteins (e.g.: Ankyrin, Adducin or Spectrin). The lipid composition is also characteristic and widely detailed for human RBCs (Figure 5). Generally speaking, the bilayer is composed of phospholipids and cholesterol in 1:1 mass ratio. The phospholipid content has a special distribution: the outer monolayer is mainly built up from phosphatidylcholine (PC) and sphingomyelin (SM), while the inner monolayer contains phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). This asymmetric phospholipid distribution is crucial for the cell integrity and functions and it is maintained by a series of lipid transporter molecules (scramblases, flippases and floppases) (68).
Red blood cells tolerate the extremities of the environmental parameters very well. Their structural properties allow them to go through the smallest capillaries (approx. 2 μm, while the diameter of RBCs is approximately 7-8 μm) and to be swollen or shrunken in hypotonic or hypertonic solutions, respectively. If the cell is put in a hypotonic medium, its volume is increased until a point when the integrity of the plasma membrane is broken and the cytoplasm flows out, through the newly formed holes. This process is often induced artificially and used for RBC membrane preparation. The empty residue membranes are called ghosts (70). For membrane studies and nanoerythrosome preparation typically RBC ghosts are used (71).

Nanoerythrosomes are approximately 100-200 nm large vesicles, produced from RBC ghost membrane by extrusion or sonication (72) (62). The vesicles are direct derivatives of the original plasma membrane, having its compositional heritage. With lipid additives further modification of the nanoerythrosomes is possible. The character of the lipid additive has a significant impact on the physicochemical properties of the produced vesicles, whereby the homogeneity of the suspension could also be increased. Their natural origin and tailoring ability make nanoerythrosomes ideal tools for personalised therapeutics in the future (73) (74).

2.4. Characterization methods of vesicles and membrane systems

The relationship between function and structure is a fundamental importance for understanding membrane mechanisms. The exploration of this relationship is frequently effortful and in the case of complex membrane systems not trivial. In case of novel, vesicle-like nanosystems - such as nanoerythrosomes, we put emphases on the complex structural characterization.
The self-assembly of lipid molecules generally result in large multilamellar vesicles with a diameter of several micrometres. Combined sonication and extrusion processes as well as the application of additives produce different, characteristic size-ranges, which have an impact on the function and applicability of the products. For example the laterally extended (7-8 µm) ghost membranes can be transformed into vesicle-like nanoerythrosomes with a characteristic diameter of approx. 150 nm. Further additives can cause the formation of small vesicles with a size of only 30 – 40 nm. The formation of nanoerythrosomes goes with the appearance of different surface patterns, composed from a variety of membrane particles. Therefore the size characterization should be supported with picturesque information as well. Nowadays, the cryo-electron microscopy provides excellent possibilities in the description of three-dimensional shapes even in sub-nanometre range (75), but freeze-fracture combined transmission electron microscopy has an advantage over cryo-EM by showing all local forms of surface patterns and domain formations in the vesicles (76). Beside the size, shape and morphological characteristics of vesicles and vesicle-like nanoerythrosomes, one of the main concerns is their stability over time. Few of the pure lipid-water systems are considered to be stable on their own, most of the time this property is achieved by additives and surface modification, like the conjugation of polyethylene-glycol chains, or incorporation of sterane molecules (e.g. cholesterol or ursolic acid).

When vesicles with a net charge on their surface are dispersed in liquids, a layer from counter-ions gather around them. This oppositely charged layer of ions is known as the Stern layer. The Stern layer induces the formation of a second, outer layer, which unlike the Stern layer only loosely connects to the vesicle. This outer layer is called a diffusive layer and the two layers together form the electrical double layer. When the vesicle moves in the liquid there is a boundary between the ions of the diffusive layer that move with the particle and one that remains in the bulk dispersant. The electrostatic potential in this moving plane boundary is called the zeta potential, its measurements is also required for the characterization of the colloidal stability (77).

2.4.1 Size determination

Dynamic Light Scattering (DLS) is a routinely used technique to determine the size distribution of a vesicle suspensions (51). The technique is based on the Brownian motion of the particles (78). This means, that the solvent molecules are in permanent movement by their thermal energy and continuously collide with the suspended particles...
These collisions induce a movement of the particles, which is called the Brownian motion. The speed of the Brownian motion can be directly measured from the scattered light pattern of the moving vesicle. The relation between the speed of a particle’s Brownian motion and its hydrodynamic diameter is described by the Einstein-Stokes equation:

\[
D = \frac{kT}{3\pi\eta D_H}
\]

Where \( D \) is the diffusion speed; \( k \) is the Boltzmann’s constant; \( T \) is the absolute temperature; \( \eta \) is viscosity and \( D_H \) is the hydrodynamic diameter of the particle.

According to the equation, the hydrodynamic diameter of the vesicles can be calculated from the measured diffusion speed, if the temperature and viscosity of the suspension is known and kept constant. DLS is suitable for the examination of nanoparticles in a size ranging from 1 nm to micrometers, in concentration between 0.1 and 0.01 % (w/w), typically. Biggest limitation of the technique is its sensitivity for the monodispersity of sample. Furthermore, sedimentation- or multiple light scattering of the particles could also be limitation parameters of the method.

Measurement of zeta potential gives an indirect information about the net charge on the particles surface. This charge is important, because it influences the stability of the vesicle as well as its interactions with other particles in biological systems. Zeta potential values can also predict the aggregation tendency of vesicles in the aqueous medium (79).

During zeta potential measurement an electrical field is applied, which induces the movement of the charged particles in the suspension. The ratio between the vesicles velocity and the applied external field – called electrophoretic mobility (\( \mu_e \)) is then measured and converted into the zeta potential (\( \zeta \)) using the Henry equation:

\[
\mu_e = \frac{2\varepsilon zf(ka)}{3\eta}
\]

Where \( \varepsilon \) is the dielectric constant, \( \eta \) is the absolute zero sheer viscosity of the medium and \( f(ka) \) is the Henry function. In details \( a \) is the radius of the vesicle and \( k \) is the Debye parameter that represents the electrical double layer, depending on the ionic strength of the medium and on the temperature of the medium being \( 1/k \):
Here, $k_b$ is the Boltzmann constant, $T$ is the temperature of the sample, $e$ is the charge of electron, $N$ the Avogadro number and $I$ is the ionic strength of the medium.

For particles in polar medium (water) the $f(k\alpha)$ is approximately at its maximum value: 1.5, for particles in non-polar medium the $f(k\alpha)$ can be approximated by its minimal value: 1. In water based buffers, which are relevant for biological conditions, the commonly used value is 1.5. During a zeta potential measurement, the electrophoretic mobility is quantified by measuring the small frequency shift of the light of a coherent laser source, scattered by the charged NPs during their movement in the external electric field.

2.4.2 Morphology

The self-assembly of phospholipid molecules can lead to a wide variety of membrane structures in aqueous solutions. Although the thermodynamically favoured particles are typically spherical objects like micelles or vesicles, depending on the compositional and environmental parameters destroyed and non-lamellar (hexagonal, or cubic) formations could also be possible.

The fine details of vesicular systems are in the size range of only a few nanometres. The visual observation of these small elements need a very high resolution microscopic technique, which could only be achieved by transmission electron microscopy (TEM) (80). The problem with conventional TEM measurement is that it is carried out in high vacuum, which could damage the biological samples irreversibly by the evaporation of their water content. The high energy of the irradiating electron beam could also disrupt the sensitive membrane particles, making their observation impossible. Freeze fracture (FF) sample preparation offers a solution for both problems (76). During the freeze fracturing process the sample is in frozen form, which is used for the creation of a fractured surface, followed by the preparation of a replica from the surface. This replica could then be easily observed by TEM and also stored for a very long time.
Figure 6. The process of freeze fracturing in the case of membrane bilayers. The fracturing frequently opens up the two membrane leaflets, resulting in a cytoplasmic- and an extracellular half, which faces are called P face and E face, respectively.

There are some key parameters for the freeze fracturing of vesicle samples. First of all, the suspension has to be rapidly frozen to avoid the formation of large ice crystals. During rapid freezing a cooling-rate of approximately $10^4 - 10^5 \degree C/s$ is applied. This speed is achieved by a secondary cryogenic liquid, such as freon, cooled by liquid nitrogen. As the sample is rapidly cooled to approximately -196°C (the boiling point of nitrogen, at 1 bar), the water is frozen in an amorphous form. Amorphous ice consists of very small crystals, consequently it is less harmful to the membranes (81).

The frozen sample is then put into a cooled vacuum chamber (incubated at -110°C, instead of -196 °C, to increase the sublimation of ice), where a cooled knife produce fractured surface of it. During the fracturing process the sample is “broken” in its weakest plane. Covalent, hydrogen and ionic bonds are strong, while hydrophobic interactions are the weakest. When the plane of fracturing encounters the vesicles, it either breaks off the ice from them, breaks the vesicles out of the icy sample, or brakes the membrane bilayer into two half. As a result of this process the outer surface of the vesicles as well as the structure of the bilayer could be observed. The two characteristic sides of the fractured bilayer are called E (extracellular)-face or P (protoplasmic)-face, referring to the membranes position in the living cells (Figure 6) (82) (83).

In the final step of freeze-fractured procedure, platinum and carbon are vaporised on the fractured surface, creating and shading the final replica, respectively. The resolution of the FF-TEM images is approx. 2±1 nm, due to the average size of the platinum particles formed during the sputtering process. The secondly vaporized carbon
forms a continuous covering film, whereby the features of the fractured surface are conserved. After the removal of the sample residues, the replica can be stored and observed for an unlimited amount of time.

Freeze fracturing provides an excellent tool to visualize the individual artificial or natural vesicles and nanoerythrosomes embedded in the aqueous matrix. In electronmicrographs three kinds of fracturing could be observed. Vesicles with convex fractured outer surfaces, frequently protruding from their local flat surrounding, and concave imprints of other vesicles broken out entirely from the medium are visible. The third type of characteristic fractured surface represents vesicles that are broken through entirely, leaving half of them in the medium. Instead of being sharp, the contours of these fractures are rather wide, corresponding to the wall-thickness of the vesicles.

It must be noted, that the electron microscopic study can serve information about the individual particles and their details therefore it does not render high statistical reliability. Relatively long and laborious procedure is required to obtain numerical data and to achieve a high statistical relevance.

2.4.3 Thermal behaviour

Thermal analysis of lipid membranes is generally carried out by Differential Scanning Calorimetry (DSC). DSC is capable of accurately determining the phase transition temperatures and the associated enthalpies of pure lipid systems. The impact of guest molecules and different mediums can also be studied via the changes of the thermal behaviour.

The principle of the measurement is fairly simple. The DSC device has a sample and a reference cell. Both cells are heated, timely following the exact same temperature profile. In case a thermal event – such as a phase transition happens, the device measures a heat flow between the sample and the reference cell. The informational output of the DSC is a differential heat flow as the function of temperature, in which the intensity of the signal is proportional to the scanning rate (dT/dt).

On the thermogram of the vesicles one or more peaks could be seen. In pure lipid systems the peaks correspond to the phase transitions. The peak position \( T_m \), is the temperature value of the melting point and the peak area under the DSC trace is a measure of the change in enthalpy, corresponding to phase transition, \( \Delta H \) [kJ/mol].
2.4.4 Structure in the nanometre range

It was already mentioned, that the vesicles and nanoerythrosomes are objects with a typical diameter of few hundred nanometre. Their main attribute is the double-layered lipid sheets extending up to 5 – 11 nm in thickness. This size-range can be characterized by scattering of X-ray or neutron beams in the frame of the coherent scattering, while the wavelength of the incoming and scattered beams remains the same. From a practical point of view, x-ray scattering is one of the most commonly used for the examination of vesicular systems (84) (85) (86) (87). This scattering technique is dissected into two types; the small angle x-ray scattering (SAXS) and the wide angle x-ray scattering (WAXS) corresponding to the angle of the scattered x-ray wave. SAXS gives information about the shells of the vesicles, the location of guest molecules, the lamellarity of the vesicles and in the case of unilamellar vesicles it serves with an estimation for the size of the vesicles as well (88) (89). The WAXS holds information about finer details about the bilayer structure, for example, about the distance between the neighbouring lipids, the geometrical parameters of subcells constituted by strongly correlated alkyl chains of lipids (90).

When a monochromatic x-ray radiation passes through a matter, part of the x-rays are scattered by electrons and by nuclei of targeted atoms. As the intensity of scattering is inversely proportional to the quadrate of the mass of the particles, the scattering of x-rays on the atomic nuclei can be neglected. Therefore the scattering pattern provides information on the time averaged electron density profile of the sample. The electrons are given by atoms, molecules, and finally, by the associates or aggregates of all molecules of the sample. The chemical content of the sample provides the actual electron density. The goal of the scattering measurement is inverse, namely by means of scattering phenomenon we intend to determine the electron density, whereby the local chemical content, in the end the structure can be determined. The amplitude and phase of the scattered wave depends on the position of each scattering centres (e.g. electrons) in the matter. As the position of electrons change permanently by thermal motions, the position of scattering electrons is given by the average special electron density (ED). Due to the reciprocal relation between the distances in matter (real length) and scattering angle (reciprocal length), information about the atomic scale are provided at high angles, while scattering at low angles corresponds to structures of the nanometre scale. The principles of SAXS and WAXS measurement are the same, with the differences being only practical
and technical details; SAXS appears in the small angle scattering regime (between 0 – 10°, in the vicinity of the transparent beam), while WAXS is detectable in the wide angle regime, between 10 – approx. 130. WAXS (or WAXD wide-angle X-ray diffraction in a case of a more regular lattice-type arrangement) provides information in the range of several 0.1 nm. The SAXS describe the whole vesicle with its bilayer structure. As it was mentioned, for the incoming X-ray beam the vesicles represent complex shell-shaped centrosymmetrical electron clouds created by the electrons of constituent lipids, proteins and other constituent molecules with a diameter, which corresponds to vesicle size, approx. several hundred nm. The electron density of this electron cloud shell along the radial direction represents the thickness of the bilayer in a range of 5 – 11 nm.

SAXS pattern, i.e. the one-dimensional scattering intensity, is usually presented as a function of q [nm⁻¹] (scattering variable) instead of the scattering angle (2Θ), its absolute value defined as

\[ q = \frac{4\pi \sin \Theta}{\lambda} \]

where, \( \lambda \) is the wavelength of X-ray (for example \( \lambda=0.1542 \) nm for Cu Kα radiation, typically used).

The introduction of “q” variable is very practical because the scattering curves measured by different wavelengths, can be compared with each other. The small angle X-ray scattering (SAXS) curves of the vesicles and vesicle-like systems are similar. The scattering intensity of multilamellar vesicles is given by the multiplication of two terms, when the correlation between the vesicles is neglected (This assumption is fulfilled in case of diluted system.)

\[ I(q) = |F(q)|^2 \cdot S(q)/q^2 \]

where \( S(q) \) is the structure factor for the one-dimensional layer arrangement and \( F(q) \) is the form factor of each double-layered unit (it is the Fourier transformed electron density of double-layered unit), \( 1/q^2 \) is the geometrical transformation factor (from sheet to sphere).

In case of unilamellar vesicles, the expression of the intensity is simplified to the next form:

\[ I(q) \propto |F(q)|^2 \]
The scattered intensity is proportional to the number of the vesicles embedded in the aqueous sample randomly and to the quadrate of the form factor of bilayer. This factor is the Fourier transformed electron density of the centrosymmetrical shell of ULVs (shown in Figure 1). The quadrate of the form factor of bilayer generally appears in every SAXS curve of unilamellar systems as a broad peak ("hump"), centered about at the q=1 (nm\(^{-1}\)).

When the systems contain an increased amount of membrane proteins, scattering curves exhibit a reduced sign of lipid bilayers (91). In the next paragraphs, I summarize general information about the SAXS curves of regular unilamellar vesicles, which will be useful in the interpretation of other curves obtained on complex nanoerythrosomes (92).

Figure 7. Typical SAXS curve of a unilamellar vesicle

The entire SAXS curves (extending from very small q up to high q, corresponding to the scattering angle regime of 0.007 – 20°, it can be obtained only at some synchrotron station) make possible the description of both the overall size of the unilamellar vesicles and the fine structure of their bilayers and yields complete structural and morphological information. The bend in the scattering curve at the very small q-range (marked with A in the Figure 7) characterizes the overall mean size of vesicles through the Guinier approximation. Near q=0.1 1/nm a local maximum appears (B) which is a consequence of a relatively narrow size distribution. After a local minimum in the scattering curve a wide scattering peak (C) with two smaller ones (D and E) follows which carry information on the fine structure of the bilayer. A thorough inspection can reveal that the main peak (C) is in fact composed of two or more double-layer components (marked with F and G).
These features can be comprehensively interpreted by modelling the scattering curve using different mathematical models. The basis of the calculation is that the different parts of the bilayer (two head groups and one carbon chain region) can be described by different, characteristic electron-densities given by the stoichiometry of their respective chemical constituents. Although step-like radial electron density functions would give adequate description for each region, Gaussian functions are more convenient to respect the thermal fluctuations of the constituents and the lack of sharp boundaries, which are not present in real systems. Taking into account the size distribution of vesicles and modelling the electron density (ED) profile along the lipid bilayer normal with sum of Gaussian functions corresponding to the different regions, the experimental SAXS curves can be fitted in the least-squares sense.

When the system contains periodical structure (nanostructures, the periodicity falls into the nanometre range), beside the monotonically decaying SAXS curve, diffraction appear periodically. Bragg’s law describes these periodicities as:

\[ 2d \sin \Theta = n\lambda \]

Where \( d \) is the space between the periodic layers (which are bilayers in the case of vesicles), \( \Theta \) is the half of scattering angle, \( n \) is an integer and \( \lambda \) is the wavelength of the incident ray.

In the case of multilamellar vesicles, x-ray scattering is used to reveal the structural features of membranes giving information about their “lamellarity”. In a suspension of multilamellar vesicles Bragg peaks appear on the scattering curve due to the well-ordered structure of their multilayers. According to the equation: \( q_0 = n2\pi/d \), the periodic distance \( d \) is equal to the sum of bilayer thickness and the inter bilayer water shell thickness. The presence and the character of the equidistant Bragg peaks on the scattering curve holds information about the ordering of the bilayers. This effect can be best observed during lipid phase transitions. The intensive and sharp peaks of gel-phase are decreased and broadened in rippled gel phase, but narrow and grow again in liquid crystalline phase. This alternation of the diffraction peaks shows the changes in the bilayer structure during the rising temperature Figure 8.
Figure 8. Characteristic scattering curves of multilamellar vesicles (MLV) of DPPC-water system at typical phase temperatures. In the biologically relevant temperature range the lipids can either be in gel, rippled gel or in liquid crystalline phase (46 and 55°C are presented), whereby the number and sharpness of the diffraction peaks on the SAXS curves indicate different kind of regularity of the membrane system.

Considering the Bragg’s law, we can see that the periodicity of sub-nanometre range results in a wide angle diffraction (discrete scattering in certain and regular angular positions). When the periodicity is smoothed instead of wide angle X-ray diffraction the wide angle X-ray scattering is the correct terminology. The distance between the acyl chains of lipids in the layer falls in the length-scale of several Angstrom. Consequently, the vesicles, especially the multilamellar vesicles in concentrated form (typically more than 10 w/w% water), exhibit a very characteristic diffraction peak around 2Θ= 24°, corresponding to approx. 0.5 nm chain – chain distance. It means that the rod-shaped acyl chains of lipid are in parallel positions with each other and the distance between the neighbouring lipids is 0.5 nm. This value and the character of diffraction peak is altered during the phase transition, and provide information about the lateral arrangement of lipids during the thermal processes. The arrangement of lipids is strongly perturbed in the presence of other additives, especially in the presence of different macromolecules, proteins, therefore this signal is vanished in the complex systems, for example in cases of extracellular vesicles, nanoerythrosomes.

2.4.5 Structure in the atomic scale

a) Infrared spectroscopy

Infrared (IR) spectroscopy is based on the interactions between IR light and matter. The energy of the absorbed (or reflected) IR light is equal to an energy difference
between two energy levels of vibrations of atom pairs, group of atoms in the molecules (93). Different functional groups absorb characteristic frequencies of the IR radiation, therefore the detected IR spectrum (the intensity as a function of wavenumber [cm\(^{-1}\)]) is a fingerprint of the molecule (94). A vibrational mode of the sample is IR active only if it can be associated with a change in dipole moment. Since molecular vibrations reflect chemical features of a molecule, such as, an arrangement of the nuclei and chemical bonds within the molecule, infrared spectroscopy contributes not only to the identification of the molecule, but also to the investigation of the molecular structure. In the case of biological samples, despite the complexity of the spectrum characteristic and well-defined IR vibrational modes of nucleic acids, proteins, lipids and carbohydrates could be found (Figure 9).

The IR spectrum of a biological membrane serves with information about the conformation and dynamics of its molecules. A molecular vibration is defined by its absorption maximum and peak frequency in the spectrum. It is characteristic for the atoms of the chemical bond and reflects their conformation, which is also slightly affected by its environment.

The basic constituents of biological membranes are phospholipids and membrane proteins. In the case of extracellular vesicles the nucleic acid content can also be a subject of examination.

Figure 9. FT-IR spectrum of biological samples, with typical molecular vibrations corresponding to biomolecules highlighted (95)
As far as phospholipids are concerned, their acyl chains could be identified by the symmetric and asymmetric stretching vibrations of CH₂ and CH₃ groups. These vibrations are found in the 3050–2800 cm⁻¹ spectral region. The =CH moiety of unsaturated chains shows a stretching vibration at around 3010 cm⁻¹, furthermore the C=O ester carbonyl stretching vibration is centred around 1734 cm⁻¹. The frequencies of the CH₂ and CH₃ bands are conformation sensitive and respond to the trans/gauche ratio in the lipid’s alkyl chains. In the densely packed acyl chains of the phospholipid bilayer, the CH₂ vibrations have a characteristic wavenumber. If a perturbation happens in this region, the interactions between the acyl chains weaken, consequently the CH₂ vibrations shift in the direction of higher wavenumbers. This phenomenon makes it possible to observe the phase transition of the lipids, because during the main transition the ratio of the gauche conformers is significantly increased, resulting in a 2-3 cm⁻¹ shift of the stretching bands to higher wavenumbers.

Regarding proteins, there are two characteristic bands at 1650 cm⁻¹ (Amide I) and 1540 cm⁻¹ (Amide II), corresponding to the vibrations of peptide bonds (C=O and C–N stretching, and of N–H bending modes). These bands are sensitive to the secondary and tertiary structure of proteins. Particularly, the Amide I band (composed of C=O stretching in approx. 80%) is used to estimate protein secondary structure: the broad envelope of the Amide I can be resolved to individual band components after its second derivation (or by Fourier self-decomposition). The individual band components can be used to characterise the α–helical (~1653 cm⁻¹), β–sheet (~1635 cm⁻¹) and unordered (~1640 cm⁻¹) content of the given proteins (96).

Further peaks of the spectra in connection with the observed biomolecules:

- Peaks at approximately 1460 cm⁻¹ and 1400 cm⁻¹ are a result of the bending modes of CH₂/CH₃ groups present both in amino acid side chains and in fatty acids.
- In the region of 1300–900 cm⁻¹, absorptions derived from carbohydrates and phosphates can be detected, however, the relative weak Amide III band (C-N stretching coupled with N-H bending, C-H and N-H deformations) of proteins appears in this region.
- At wavenumbers of 1241 cm⁻¹ and 1085 cm⁻¹ the asymmetric and symmetric phosphate/phosphodiester vibrations of nucleic acids and that of phospholipids could be found. As far as nucleic acids are concerned, RNA shows absorption peaks at approximately 1120 cm⁻¹ (derived from the ribose C–O stretching) and around 998
cm⁻¹ (due to uracil ring stretching), on the other hand DNA peaks could be found at 1020 cm⁻¹ (as deoxyribose C–O stretching) and at 964 cm⁻¹ which corresponds to the motions of DNA backbone.

- The C–OH vibrational mode of carbohydrates is found at around 1050 cm⁻¹.

The presence of water could complicate the IR measurement. Its absorption peaks at around 3285 cm⁻¹, 2100 cm⁻¹ and 1640 cm⁻¹ are overlapping with the bands of other components. The application of attenuated total reflection (ATR) serves with a possible improvement. In a single reflection ATR unit a droplet (3-5 µl) of the sample is placed on an internal reflection element (diamond, germanium or ZnSe). During the measurement the infrared light strikes the sample and enters the ATR element. Due to internal reflection, the beam is reflected within the crystal, creating an evanescent wave that extends beyond the ATR element. Because the sample is in close contact with the ATR element, this evanescent wave loses energy at frequencies identical to the sample’s absorbance. The resultant beam is used to generate the absorption spectrum of the sample. The sample and the ATR element are in full contact, which reduces the effective path length of IR light inside the sample. Consequently, absorbance of water bands does not saturate the signal received by the detector. Other possibilities for reducing the water signals during the measurement may be the subtraction of a water background (spectrum) and/or measurement of dried film samples (Figure 10).

Figure 10. FTIR spectra of DPPC/water suspension in different measurement conditions: A: DPPC/water suspension (20m/m%); B: after subtraction of water background; C: dry-film spectrum
b) Dichroism spectroscopy

In dichroism spectroscopy the incidental light is either linearly (LD) or circularly (CD) polarized. When the polarized light interacts with the optically active matter, its differently polarized components are absorbed in different amounts (97) (98).

Linearly polarized light consists of light waves, which electric (and magnetic) field vectors point in the same direction, forming a classic sinusoidal wave. At a single point of space, the vector oscillates with a given frequency, which can be imagined as a series of parallel arrows. Linear dichroism is the difference between the absorption of polarized light parallel and perpendicular to an orientation axis.

\[ LD = A_\parallel - A_\perp \]

LD spectroscopy is applied for the characterization of systems that are either intrinsically oriented, or can be oriented by external forces. Vesicles are spherical objects that can be distorted to a more ellipsoid shape by a sheer force (generated artificially in a Couette flow cell, coupled to the LD instrument, (Figure 11)). The degree of macroscopic orientation is derived from the orientation of membrane probes inserted, or associated to the vesicle membrane. These probe molecules have a well-defined transition dipole moment (TDMs). Typical probe molecules for LD measurement of vesicle suspensions are retinoic acid, curcumin and pyrene. For a particular TDM of a membrane probe oriented in a lipid bilayer the calculated macroscopic orientation factor (S) has a relationship with the measured LD signal:

\[ LD_r = \frac{LD}{A_{iso}} = \frac{3}{4}S(1 - 3\cos^2 \alpha) \]

Where \( LD_r \) is the reduced LD signal, \( A_{iso} \) is the isotropic absorption and \( \alpha \) is the angle of TDM relative to the membrane normal. As a result LD serves information from membrane insertion, orientation angles and structure of associated molecules.

A wave of light is circularly polarized if the electric field vector rotates about its propagation direction with a constant magnitude. At a single point of space, the vector trace out a circle through a period of a wave frequency. A circularly polarized light can be resolved into two characteristic components: a right- and a left circularly polarized part. In some circumstances, right circularly polarized light is absorbed in different amounts than left circularly polarized light. This differential in absorption of circular polarized light is the principle of circular dichroism spectroscopy.
Figure 11. Deformation of vesicle-like objects and probe molecule (pyrene) orientation in LD flow cell (99)

CD spectroscopy is often used to study structural properties of the membrane associated proteins. The far-UV CD signals, recorded below $\lambda=250$ nm originate from peptide n-$\pi^*$ and $\pi^*$-$\pi^*$ transitions and represent structural elements, such as $\alpha$-helix and $\beta$-sheets and turns.
3. Aims of the work

The aim of my PhD work was to elaborate a biocompatible, stable, vesicle-like nanocarrier system, by using red blood cell ghost membranes as starting material. Although, red blood cell ghosts had already been used for the preparation of vesicle-like nanoerythrosomes in the past, either by extrusion, or by ultrasound treatment, until now, nanoerythrosomes were only considered as relatively simple, lipid based vesicular derivatives of the original cell membrane. Their protein content and especially its structural role had not been paid attention to. I presumed that ghosts contain the proper macromolecules for vesicle stabilisation and they could be a promising source material for the tailoring of nanocarrier systems.

The artificial and natural vesicle-like systems exhibit similar structural hierarchy, governed by identic physico-chemical principles. Their molecular constituents form nearly spherical, shell-structured vesicles by self-assembly, which membrane curvature and size are defined by certain – mostly lipid – constituents. The protein constituents of natural membranes are determined, both qualitatively and quantitatively, due to their role and function in the bilayer. I assumed that a partial exchange in their lipid content could be effective for shaping the laterally extended ghosts into vesicle-like objects. The complete knowledge about both sides in this “guest - host” relation – both the incorporated lipid and the enriched natural membrane – is important. On one hand, the lipid additives as guest molecules are thoroughly studied and described in the literature, on the other hand the characterization of the natural vesicles, as potential hosts, is in its infancy. Consequently, I planned to isolate and characterize a wide variety of extracellular vesicles focusing on their physicochemical and compositional properties. I aimed to collect information about their structural elements, such as their lipid and protein content. I planned to study the effect of the releasing cell type on the characteristics of secreted EVs. For this reason, I isolated EVs from (Jurkat) in vitro cell culture and red blood cell suspension.

As far as tailoring of the nanoerythrosomes were concerned, I decided to use artificial forms of representative red blood cell membrane lipids. I chose typical lipid components of the inner and outer membrane leaflets. Beside the typical constituents of both leaflets, I also intended to study the effect of the molecular shape on the nanoerythrosome formation. Therefore I chose the cylindrical dipalmitoyl-
phosphatidylcholine (DPPC), the conical-shaped dipalmitoyl-phosphatidylethanolamine (DPPE) and the inversely conical lyso- phosphatidylcholine (LPC) as additive lipids.

Finally, I have to mention that the Biological Nanochemistry Research-group offered a unique research infrastructure for my experiments. Beside the large laboratorial equipments, freeze-fractured electronmicroscopy provided an outstanding possibility to observe the nanostructural formations that I paid great attention to in my Thesis.
4. Materials and Methods

4.1. Chemicals and reagents

Lipids:
- DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and
- DOPC (1,2-Dioleoyl-sn-glycero-3-phosphocholine) were purchased from NOF America Corporation.
- DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine) and
- LPC (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids.
- Cholesterol was purchased from Sigma Aldrich.
- Brain Total Lipid Extract (Cholesterol:DPPC:DOPC:DPPE:DPPE = 5:1:1:2:1) was purchased from NOF America Corporation.

Bovine Serum Albumin (BSA):
- Used for reference sample preparation in ATR-FTIR spectroscopy, was purchased from Sigma Aldrich.
- Used as protein standard, for Bradford protein assay was purchased from Bio-Rad Hungary Ltd.

Reagents and buffers:
- Physiological salt solution (9 g NaCl in 991 ml Millipore water),
- Red blood cell lysis buffer (7.5 mM TRIS/HCl, pH: 7.6),
- Phosphate buffered saline (PBS),
- Bradford reagent for protein concentration determination assay were purchased from Sigma Aldrich

Medium and chemicals for cell culture:
- Cell culture media (RPMI-1640),
- Fetal bovine serum (FBS),
- Glutamine and penicillin/streptomycin were purchased from PAN Biotech (Germany).
4.2. Preparation protocols

4.2.1. Preparation of reference – model – vesicles

a. Preparation of BSA loaded DOPC vesicles for reference material of ATR-FTIR

DOPC and BSA were mixed in 1:1 weight ratio and were solved in PBS buffer in a final concentration of 1.3 mg/ml. To achieve homogenous mixture and maximal BSA-content, repeated heating-cooling cycles (between 37 °C and −196 °C) were applied. The resulted liposome suspension was used as a stock solution, from which 1 – 1 ml was extruded through 600, 200 and 80 nm pore sized membranes, respectively, using a LIPEX extruder (Northern Lipids Inc., Canada). The produced unilamellar vesicles were purified from soluble BSA by Sepharose CL-4B (GE Healthcare) gelfiltration.

b. Preparation of DOPC liposomes for reference material of LD/CD spectroscopy

DOPC liposomes were prepared according to the lipid thin film hydration technique, in a final concentration of 1021.3 µg/ml (100). First DOPC was dissolved in a mixture of chloroform:methanol 2:1, then the solvents were evaporated. The resulting lipid film was kept in vacuum chamber for 8 hours to remove the traces of residual solvents. The dry lipid film was hydrated with PBS buffer. To achieve a homogenous mixture of liposomes, repeated heating-cooling cycles were applied (between 37 °C and −196 °C). The solution was extruded through polycarbonate filters with 200 nm pore size (at least 10 times) using a LIPEX extruder.

4.2.2. Extracellular vesicle (EV) isolation protocols

a) Isolation of Jurkat cell derived extracellular vesicles for spectroscopic P/L ratio determination

EV subpopulations (apoptotic bodies, microvesicles and exosomes) were isolated from the cell culture of Jurkat T-cell line. RPMI-1640 medium containing 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin was used as growing medium. The collection of vesicles started when approximately 10⁷ cell number was reached, then the growing medium was replaced with FBS free medium to avoid bovine serum vesicle contamination. Jurkat cells were incubated in FBS free “EV collecting medium” for 24 hours. Collecting medium was mixed with 1 µM doxorubicin to induce apoptosis (101).
Prior the vesicle isolation cells and cellular debris were removed from the collecting medium in two consecutive sedimentations 300×g, 5 min (Nüve NF800R centrifuge). EV isolation was performed by differential (ultra)centrifugation. In the first step the cell free collecting medium was submitted to a 3000×g centrifugation for 30 min to sediment the apoptotic bodies. The supernatant was further centrifuged at 20000×g for 30 min to obtain microvesicles (Thermo Sorwall WX ultra centrifuge, with Thermo Sorwall 1270 fixed angle titanium rotor). Finally, the isolation of exosomes was carried out by 110000×g ultracentrifugation for 1 hour. All pellets were washed with isotonic PBS buffer and the final pellets were suspended in 200 μl PBS. For ATR-FTIR experiments four independent isolations (JK1-JK4) were performed.

b) Isolation of red blood cell derived extracellular vesicles (REVs) for spectroscopic P/L ratio determination

Red blood cell derived EVs were isolated from just outdated cell concentrate (from Hungarian National Blood Transfusion Service). The cell concentrate was diluted to double volume with isotonic PBS buffer, the cells were sedimented (300×g, 10 min, Nüve NF800R centrifuge) and their supernatant was used for microvesicle isolation. After two consecutive sedimentations the cell free supernatant was submitted to a 20000×g centrifugation for 30 min (Thermo Sorwall WX ultra centrifuge, with Thermo Sorwall 1270 fixed angle titanium rotor). The pellets were washed with isotonic PBS buffer and the final pellet was suspended in 200 μl PBS.

c) Isolation of red blood cell derived extracellular vesicles (REVs) for vesicle elasticity experiments

Red blood cells were isolated from the blood of healthy volunteers, collected at the Central Laboratory of the National Institute of Rheumatology and Physiotherapy, Hungary. The use of human blood samples was approved by the Scientific Ethics Committee of the Hungarian Health Scientific Council (ETT TUKEB 6449-2/2015). To obtain EVs from human red blood cells (RBCs), 15 ml blood was collected from healthy adult donors in tripotassium ethylenediamine-tetraacetic acid containing tubes (K₃EDTA, VWR Hungary).

Briefly, the erythrocytes were isolated via centrifugation at 2500×g for 15 min at 4 °C (Nüve NF 800R, swing out rotor). RBCs were washed with physiological salt solution until the platelets and the buffy coat was completely removed. Buffy coat free
erythrocytes were suspended in PBS, and were kept at 4 °C for vesicle production. After 7 days the erythrocyte suspension was used for REV isolation. The cells and the cellular debris were removed by two centrifugation steps at 2500×g and 3000×g for 15 min at room temperature. The supernatant containing erythrocyte EV was collected and further ultracentrifugated at 138000×g for 60 min at 4 °C (Thermo Sorwall WX Ultra centrifuge, T-1270 fixed angle rotor). The REV pellets were suspended in PBS, and stored at 4 °C until further use.

For spectroscopic experiments, the REV samples were purified from contaminants, i.e. soluble proteins and protein aggregates, on a Sepharose CL-2B column to perform size-exclusion chromatography (SEC) according to a modified protocol published by (102). The column with a diameter of 1.6 cm, height of 5.3 cm and total volume of 10.6 ml was washed 4 times with PBS buffer and then was loaded with platelet-free supernatant of erythrocyte (2.5 ml), followed by elution with PBS. After the sample passed the filter top, buffer was added. The first 2.5 ml of eluate was discarded and the next 2 ml eluate which contained REV was collected in 2 fractions of 1 ml.

4.2.3. Preparation of haemoglobin free ghosts and nanoerythroso
a) Ghost membrane isolation protocols (1-2.)

Freshly collected anticoagulated blood was donated by four healthy volunteers, 18 ml from one donor at a time. Although the preparations were addressed to each donors, we did not observed any differences in the characteristics of nanoerythroso described. For blood collection 6 ml K₃EDTA tubes were used (Vacuette, Greiner Bio-One, Austria).

Ghost membrane isolation was carried out at 4°C, according to a modified version of the protocol of Dodge et al. (70) briefly described in the following section.

Cellular components were sedimented from whole blood by centrifugation, 2480×g, 10 min in swing out rotor (Nüve NF 800R centrifuge). Plasma and the white blood cell containing buffy coat were removed and the erythrocyte pellet was suspended in physiological NaCl solution for three times washing. After the last sedimentation the red blood cells were lysed in hypotonic TRIS buffer (lysis buffer, 7.5 mM TRIS/HCl), stirred in 40× buffer volume. The erythrocyte ghost membranes were sedimented with an Avanti J26XP centrifuge in JA-14 fixed angle rotor with an average of 13900×g for 1 hour at 4°C. The final ghost pellet was suspended in isotonic PBS buffer. As a first step quality attribute of the ghost membrane stock solution, its total dry material was determined. 10
µl of homogenized, sonicated ghost suspension was pipetted on a silicate plate. After complete drying its weight was measured with analytical balance. (This protocol is referred to as Ghost isolation protocol 1. in the corresponding parts of the thesis.)

The original isolation protocol (Ghost isolation protocol 1.) was improved in the later stages of the work, in order to achieve higher membrane yields and more precisely characterized ghost suspensions. (The improved protocol is referred to as Ghost isolation protocol 2. in the corresponding parts of the thesis.)

In the part of the preparation process, where haemoglobin free ghosts were achieved, the membrane pellets were suspended in PBS and washed two times in ultracentrifuge (Thermo Sorwall WX ultracentrifuge, T-1270 fixed angle rotor), average 22200×g, 30 min, 4°C. The final ghost membrane pellet was suspended in PBS, its protein content was determined by Bradford protein assay and was used as a stock solution for nanoerythrosome preparation. The ghost membrane aliquots were rapidly frozen and stored in liquid nitrogen until further use.

b) Nanoerythrosome preparation protocols (1-2.)

In the initial nanoerythrosome preparation protocol (Protocol 1.) ghost membrane suspension was diluted with PBS and treated with a Biologics INC ultrasonic homogenizer (5 minutes, 10% power, 50% pulsar) to achieve a homogeneous stock solution. Nanoerythrosomes were made from the stock solution by consecutive sonication and extrusion through a 200 nm pore size polycarbonate membrane, using an Avanti Mini-Extruder.

For samples without any additive the sonication and extrusion resulted in the final nanoerythrosome structure and composition. In the case of samples with additional phospholipids (DPPC, DPPE or LPC), approximately fivefold, tenfold or half of the original lipid amount of the ghosts, were mixed to the stock solution. The amount of lipid additives were calculated – according to literature ratios (65) –, from weight measurements via analytical balance. For nanoerythrosomes with extra lipid an additional sonication step was applied after the mixing of the ghosts and lipids.

In the case of improved nanoerythrosome preparation protocol (Protocol 2.), the ghost membrane suspension was put in a bath sonicator (Elmasonic S10, 30W) in glass vial, either alone or with additional lipids. Sonication lasted 10 min, with pre-set power level and continuous operation (37 kHz, in 1.5 ml glass vial, sample volume 200 µl). This
resulted in a gentler and less invasive mixing of the components compared to the process used in Protocol 1.

The amount of the lipid additive was determined by Bradford protein assay, assuming the lipid-protein ratios found in literature (65). As far as additional lipid (5×, 10× phospholipid additive + ghost) containing samples are concerned, the lipid powders were first solved in chloroform:methanol 2:1 mixture, followed by the complete removal of the solvents in vacuum. Finally, the dry lipid film was hydrated with the ghost membrane suspension.

Ultracentrifugal separation of sonicated ghost membrane components (vesicles and EMPs) was carried out, using a Beckman TL-100 ultracentrifuge with TLA 100.1 fixed angle rotor, average 96400×g, 30 min.

4.3. Instruments and parameters of measurements

a) Transmission electron microscopy combined with freeze fracture (FF-TEM)

Approximately 1 µl droplets of the samples, previously incubated at the desired temperatures, were used for freeze fracturing. The samples were pipetted onto a golden sample holder and rapidly frozen in liquid freon, then put into liquid nitrogen. Fracturing was performed at -100°C in a Balzers freeze-fracture device (Balzers BAF 400D, Balzers AG, Liechtenstein). A replica was made from the fractured surface with vaporized carbon-platinum. The replica was washed with surfactant solution and distilled water, and it was transferred to 200 mesh copper grid for transmission electron microscopic (MORGAGNI 268D, FEI, The Netherlands) examination.

b) Dynamic light scattering (DLS)

A W130i dynamic light scattering apparatus (AvidNano, United Kingdom) was used for measuring the average size and size distribution of the samples. Because of the small sample volume, a 50 µl microcuvette was applied. The analysis of the measurement data was performed with the i-Size software, supplied with the apparatus. In the original concentration of the samples yielded by the procedure, or diluted with MQ, buffer systems.
c) Zeta potential measurement

Stability of nanoerythrosome samples was assessed by zeta potential measurements using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom) instrument. The samples, originally in PBS, were diluted to 50× volume with ultrapure (MilliQ) water, in order to make the surface characteristics of the vesicles measurable.

d) Differential scanning calorimetry (DSC)

A μDSC 3 EVO (Setaram, France) apparatus was used for calorimetric experiments. Approximately 10 mg samples were loaded per measurement and an empty sample holder was used for reference. The heating protocol consisted of three cycles, between 20 and 60°C, with two different scan rates, first a faster 1°C/min and then a slower 0.2°C/min for better resolution. The results are presented from the latter.

e) Bradford protein assay (UV-Vis)

The total protein content of ghost vesicle samples was determined using the Bradford assay that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. Bovine Serum Albumin (BioRad) was used as standard, from which eight consecutive dilution steps were carried out in three parallel lines. To 5 μl of each standard (0-25 μg/ml) and unknown sample solution, 250 μl dye reagent was added and the mixtures were vortexed for 15 sec. The samples were incubated at room temperature for at least 5 min and the absorbance was measured at 595 nm with BioTek Synergy 2 plate reader on 96 well plates.

f) Protein identification using LC-MS(MS)

Proteins were extracted from sonicated ghost membrane by repeated freeze-thaw cycles. 10 μg protein/sample was digested in solution as previously described and following tryptic digestion desalted using PierceTM C18 spin columns (Thermo Fisher Scientific, Waltham, MA, United States). Peptides were analysed using a Dionex Ultimate 3000 RSLCnano LC (Dionex, Sunnyvale, CA) coupled to a high resolution Bruker Maxis II Q-TOF mass spectrometer (Bruker, Bremen, Germany) equipped with CaptiveSpray nanoBooster ionization source. Peptides were separated using gradient elution on a 25 cm Waters Peptide BEH C18 nanoACQUITY 1.7 μm particle size UPLC column (Waters, Milford, MA).
Data were processed using ProteinScape 3.0 software (Bruker Daltonik GmbH, Bremen, Germany). Proteins were identified using Mascot (version Mascot 2.5; Matrix Science, London, UK) search engine against the Swissprot Homo sapiens database (2017_11). The following search parameters were applied: trypsin enzyme, 10 ppm peptide mass tolerance, 0.15 Da fragment mass tolerance, 2 missed cleavages. Carbamidomethylation was set as fixed modification, while deamidation (NQ) and oxidation (M) as variable modifications. Proteins with a minimum of two identified, unique peptides were accepted.

The measurements were carried out by the researchers of the MS Proteomics Research Group, Institute of Organic Chemistry, Hungarian Academy of Sciences (in courtesy of Dr. László Drahos and Dr. Lilla Turiák).

g) Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopic measurements were carried using a Varian 2000 (Scimitar Series, United States) FT-IR spectrometer, fitted with a diamond attenuated total reflection cell (‘Golden Gate’ single reflection ATR unit with active area of 600 x 600 μm², Specac, United Kingdom).

Approximately 5 μl of the sample was spread onto the diamond ATR surface and a thin dry film was obtained by slowly evaporating the solvent under ambient conditions (approx. 5 min). For temperature controlled measurements, a custom-made liquid cell was applied; the temperature was equilibrated for 5 min before collection of each spectrum. Typically, 64 scans were collected at a nominal resolution of 2 cm⁻¹. After each data acquisition, ATR correction (and water background spectral subtraction) was performed. The actual frequencies of complex IR bands were determined by fitting their peaks with Lorentzian curves. For all spectral manipulation the Grams/32 software package (Galactic Inc., USA) was used.

h) Absorption and Polarized light spectroscopy (LD)

Linear (LD) dichroism spectra were recorded using a JASCO-1500 spectrometer equipped with a Couette flow cell system (CFC-573 Couette cell holder). For recording LD spectra, the vesicles were oriented under a shear gradient of 2270 s⁻¹ with a total path length of 0.5 mm. The LD spectra were collected between 195 and 500 nm in 1 nm increments at a scan speed of 100 nm/min. For LD, baselines at zero shear gradient were measured and subtracted from all spectra.
UV-Vis absorption spectra of all samples were measured at 25°C using a Hewlett-Packard 8453 diode array spectrophotometer thermostated with Grant LTD 6G circulating water bath. Spectra were recorded in 1 nm increments between 190 and 450 nm in a quartz cuvette with a 1 mm optical path.

i) Small angle X-ray scattering (SAXS)

Small-angle X-ray scattering measurements were performed using CREDO, an in-house transmission geometry set-up. Samples were filled into thin-walled quartz capillaries of 1.2 mm average outer diameter. After proper sealing, these were placed in a temperature controlled aluminium block, which was inserted into the vacuum space of the sample chamber. Measurements were done using monochromatized and collimated Cu Kα radiation (0.1542 nm wavelength), and the scattering pattern was recorded in the range of 0.23-1.03 nm⁻¹ in terms of the scattering variable, q (q=4πsinθ / λ, where 2θ is the scattering angle and λ is the X-ray wavelength). The total measurement time was 7.5 hours for each sample. In order to be able to assess sample and instrument stability during the experiment, the exposures were made in 5 minute units, with frequent sample change and reference measurements. These individual exposures were corrected for beam flux, geometric effects, sample self-absorption and instrumental background, as well as calibrated into physical units of momentum transfer (q, nm⁻¹) and differential scattering cross-section (absolute intensity, cm⁻¹×sr⁻¹). The average of the corrected and calibrated 5 minute scattering patterns was azimuthally averaged to yield a single one-dimensional scattering curve for each sample.
5. Results and Discussion

5.1. Physicochemical characterization of extracellular vesicles

The physicochemical characterization of extracellular vesicles was performed on EVs isolated from in vitro cell culture (Jurkat T lymphocyte) and suspension of red blood cells. Corresponding to the actual guidelines, determination of the size is an important qualitative parameter to classify these membrane particles (103).

The size analysis of the vesicles was performed by dynamic light scattering (DLS). The EV preparation process generally resulted in small vesicle concentrations, which were convenient for this method. Here I present the size characteristics of extracellular vesicles derived from Jurkat T lymphocytes. These vesicles exhibit three, strictly different particle subpopulations, corresponding to exosomes (EXO), microvesicles (MV) and apoptotic bodies (AB). The size-distribution curves are plotted in Figure 12. The measured average particle sizes are 100 nm, 200 nm and 1500 nm, corresponding to EXOs, MVs and ABs, respectively. The separated vesicle suspensions are fairly polydisperse, indicated by their polydispersity indexes (P.d.). Presumably, this characteristic is in connection with their origin – exosomes and microvesicles are groups of small, intentionally created vesicles with high homogeneity and similarity to each other, while apoptotic bodies are large, heterogeneous, encapsulated cellular “debris”.

![Size distribution of Jurkat cell derived extracellular vesicles](image)

Figure 12. Size distribution of Jurkat cell derived extracellular vesicles [exosomes (EXO), microvesicles (MV) and apoptotic bodies (AB)], measured by DLS
Despite the separation of the EV subpopulations, we can observe some overlapping between their size intervals, which can be the result of the separation method. The appearance of smaller particles – such as exosomes, lipoproteins or protein aggregates-, in the suspensions of MVs and ABs could happen by co-sedimentation, which may explain the observed overlap.

Beside size-analysis, morphological studies represent another characterization possibility for EVs. Due to freeze-fracture procedure, we were able to observe fine structural features of extracellular vesicles. Figure 13 represents typical FF-TEM images of the Jurkat T lymphocyte derived EV subpopulations.

Because of the similar physicochemical properties of microvesicles and exosomes, their visual appearance is also comparable. There is, however a difference in their size range, as exosomes can be significantly smaller than 100 nm, while MVs are approximately 200 nm large, which is in full agreement with our DLS measurements. The similarities of their physicochemical properties often result in their co-sedimentation, resulting in increased polydispersity (P.d. indexes) observed by both TEM and DLS.

The outer surface of MVs and EXOs contain numerous membrane particles – similarly to the membrane of ABs, but in their case, the particles frequently form associates, or dense groups, separated from each other by smooth lipid surfaces.

Apoptotic bodies (ABs) have an average diameter of 700-1000 nm. Their surface is densely packed with 1-5 nm large membrane particles, sometimes standing as single particles, other times clearly forming associates of one or two, similarly to membrane protein complexes. The outer surface of ABs is more particulated, which resembles to the cellular membranes, where most of the protein content can be found on the outer surface as well. The membrane particle covering is significantly different on the surface of apoptotic bodies, which can be due to the heterogeneous encapsulation of the cellular material during apoptosis.

Beside the convex fractured outer surfaces of the EVs, we can also observe vesicles broken through entirely, whereby the observation of the inner leaflet of the bilayer becomes possible. This phenomenon can be observed in the case of EXOs, in Figure 13, where the unilamellar structure of the vesicles is clearly seen.
5.2. Spectroscopic characterization of extracellular vesicles

5.2.1. FTIR analysis of Jurkat cell derived EV suspensions

In the following section, I show the spectroscopic results whereby the size- and morphological measurements are supplemented with an inner atomic fingerprint information of the EVs. The typical ATR-FTIR spectra of Jurkat derived apoptotic bodies, microvesicles and exosomes are presented in (Figure 14), supplemented with the spectra of their originator cell. These spectra were collected from dried films of an approximately 4 µl droplet of the suspension. The drying was applied to decrease the signals from overlapping water bands. The spectra are typically normalized to the intensity of the phosphate stretching vibrations, which are mostly derived from the signal of PBS buffer. This way the approximate dry matter content of the EV suspensions could be compared to each other. The concentration of the suspension is the highest in the case of apoptotic bodies and decrease in microvesicles and exosomes, respectively. This suggests that in Jurkat cell medium the vesicle population mainly consists of apoptotic bodies with significantly fewer microvesicles and a small portion of exosomes.

The FTIR spectra of extracellular vesicles provide structural and conformational information about their molecular constituents. The main features of the spectra are common for all biological samples (94) (95), but with detailed analysis special characteristics of the vesicular samples could be unravelled (104). These characteristics concern the protein content of the membranes most of all. The absorption bands in connection with the vesicular proteins: amide I and amide II, could be found at around 1651 cm\(^{-1}\) and 1540 cm\(^{-1}\), respectively. Another absorption band originated from the protein content of the vesicles is amide A, which gives a strong, but highly masked signal at 3285 cm\(^{-1}\) wavenumbers. This peak could hardly be seen in the case of diluted
suspensions (MV and exosomes), due to the overlapping $\pi$-OH stretching vibration of water bands.

Figure 14. Representative ATR-FTIR spectra of EVs isolated from Jurkat cell line: exosomes (EXO), microvesicles (MV) and apoptotic bodies (AB) (104)

Absorption peaks from the vesicular lipids appear around 1738 cm$^{-1}$, 2924 cm$^{-1}$ and 2850 cm$^{-1}$. The peak at 1738 cm$^{-1}$ originates from the ester groups of phospholipids, triglycerides and cholesterol esters, while the latter two are the dominant antisymmetric and symmetric stretching vibrations of the lipid acyl CH$_2$ groups.

The spectral region of 1200–950 cm$^{-1}$ of vesicular suspensions is attributed to the broad phosphate vibration bands of the isotonic PBS buffer. However, this spectral region contains the stretching vibrations of the phosphodiester groups of phospholipids and the C=O-C stretching vibrations of phospholipids, triglycerides and cholesterol esters, in our case these bands are completely masked by the broad and intensive phosphate bands of the PBS. The reason for this phenomenon is the diluted nature of the EV suspensions. As Jurkat cell cultures produce low amount of vesicles, which are further diluted during the centrifugation and purification steps of the isolation process, the final sample protein concentration (which is a way to estimate the particles in the suspension) rarely exceeds 0.05 mg/ml (Table 1). Due to the very low sample concentration, the IR absorption band intensities of the buffer molecules are comparable with the molecules of the vesicles. Consequently, the subtraction of a pure PBS dry film spectra is applied prior to further analysis of EV samples.
<table>
<thead>
<tr>
<th>EXO</th>
<th>MV</th>
<th>AB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein concentration (±SD) [mg/ml]</td>
<td>0.051±0.02</td>
<td>0.053±0.03</td>
</tr>
</tbody>
</table>

Table 1. Average protein concentration of Jurkat EV fractions measured by Bradford protein assay

Different EV fractions (exosomes, microvesicles and apoptotic bodies) have distinct physicochemical and compositional characteristics, even when isolated from the same cell type (102). FTIR spectra in the range of 1750-1450 cm⁻¹ corresponds to the lipid (C=O stretching vibration of glycerol backbone) and protein (amide I, C=O stretching of peptide backbone) content (Figure 15/A).

The amide I band at approximately 1700–1600 cm⁻¹ contains information about the secondary structure of the proteins (105). By the second derivative of the amide I band, its board envelope could be resolved into individual band components. These components provide information from the α-helical, β-sheet, β-turn and unordered content of the proteins (Figure 15/B). As far as EVs are concerned, the different subpopulations have an expense in β-sheet conformations, favouring β-turns (band component at 1676 cm⁻¹) and unordered protein motifs (1640 cm⁻¹) when going from apoptotic bodies to exosomes. In the spectra of microvesicles and exosomes, the arising band component around 1627 cm⁻¹, is characteristic for non-native intermolecular β-sheets and suggests the appearance of aggregated proteins or apolipoproteins. Another new band component at 1660 cm⁻¹ is related to triple-helix structure, associated with the presence of immune complexes or nucleic acid (RNA) content of the EVs.
Figure 15. Representative ATR-FTIR spectra of EVs after PBS buffer subtraction in the 1800-1350 cm\(^{-1}\) wavenumber region: C=O stretching from lipid esters, amide I and amide II bands of proteins (A). Second derivative IR spectra of the amide I region for protein secondary structure assessing (B). Each minima in the second derivative spectra correspond to a component band peak. (104)

In microvesicles (MV) the relative intensity of the carbonyl stretching bands (1737 cm\(^{-1}\)) is higher, compared to other fractions. This indicates higher phospholipid content which could be the consequence of their plasma membrane origin. On the other hand in exosomes, at around 1737 cm\(^{-1}\) the C=O stretching band intensity is suppressed. This could be explained by their composition, as exosomes are significantly enriched in cholesterol, sphingomyelin, and ceramide for the expense of phosphatidylcholine and phosphatidylethanolamine. Furthermore, the similar spectral features of Jurkat cells and
apoptotic bodies is due to their similar composition reflecting on their direct relation to each other.

5.2.2. Spectroscopic assessment of protein and lipid components of biological vesicles

*Spectroscopic examination of Jurkat cell derived EVs*

The IR spectra of an EV suspension simultaneously contain the absorption bands related to all of its constituent molecules. Consequently in a single spectra, between the different absorption band intensities stand a quasi-quantitative relation. This means that the ratio of an absorption band intensity characteristic for one constituent and for another represents concentration ratios for the constituents as well. Accordingly a spectroscopic protein to lipid ratio (P/L ratio) could be determined by dividing the relative intensities of the protein amide I band, with the intensities of the lipid associated –CH₂/CH₃ alkyl bands (104).

During the utilization of amide I band as a parameter of the P/L ratio, the presence of non-vesicular materials, like aggregated proteins or amino acids could cause difficulties. To overcome this problem the integrated amide I intensity is determined by the curve fitting by Lorentz-function of the 1653 cm⁻¹ band component (Figure 16/A). Due to this method, the band components derived from amino acids (1600 cm⁻¹) and protein aggregates or apolipoproteins (1622 cm⁻¹) are not used in the calculation. Although concerning the lipid content the first approach was to use of lipid related C=O bonds (around 1735 cm⁻¹) (105), its intensity in the diluted EV samples (MV, EXO) is very low. This would result in inaccurate and unreliable P/L determination. Therefore, instead of the carbonyl, the alkyl related bands were used. The intensity of the alkyl bands is generally higher in EV suspensions than the carbonyl, especially after the subtraction of the PBS buffer background, which would otherwise overlap with its high intensity –OH stretching vibration. Beside the –CH₂/CH₃ bands the olefinic =CH stretching bands (~3010 cm⁻¹) of unsaturated lipid acyl chains are also included into the calculation (Figure 16/B).

By the usage of the C-H band region instead of the C=O as the lipid component of the P/L ratio, the sterols – like cholesterol might also have a contribution. This could be important in the case of exosomes, which membrane is reportedly rich in cholesterol and cholesterol-ester.
Figure 16. Selected wavenumber regions of Jurkat cell derived exosomes used for P/L determination protocol: amide I and amide II wavenumber region (1770-1470 cm⁻¹) (A) deconvoluted by curve fitting with Lorentz-function (band denoted by dotted lines), C-H stretching region (3040-2700 cm⁻¹) acting for lipid components (B). (104)

Calculated spectroscopic P/L values of Jurkat cell derived EVs are presented in Table 2. The different subpopulations of Jurkat derived vesicles have significantly different P/L values. The highest ratio belongs to the apoptotic bodies. In their case the average P/L value is above 1 (approx. 1.2), which means that the apoptotic bodies have relatively high protein content, compared to their lipid concentration. This could be a result of their origin, as in the process of apoptosis, a high amount of intracellular debris is packed into the ABs. This cytoplasmic contamination could give an intensive protein
signal. This idea is strongly supported by the standard deviation values, which are also the highest for ABs, reflecting their heterogeneous composition. Microvesicles and exosomes have more similar composition and as a result more similar P/L values. From these two types of EVs, the MVs have higher lipid content, while the exosomes are richer in proteins, according to the results.

<table>
<thead>
<tr>
<th></th>
<th>1.Isolation</th>
<th>2.Isolation</th>
<th>3.Isolation</th>
<th>4.Isolation</th>
<th>Average±(SD)</th>
</tr>
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</tr>
<tr>
<td>AB</td>
<td>1.08</td>
<td>1.36</td>
<td>1.19</td>
<td>1.18</td>
<td>1.20 ± 0.12</td>
</tr>
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</table>

Table 2. Spectroscopic protein:lipid ratios of Jurkat cell derived extracellular vesicles

The compositional reliability of the method was examined by measuring the P/L ratios of artificially produced BSA-lipid vesicles. The lipids were either a mixture of Cholesterol:DPPC:DOPC:DPPE:DPPS (5:1:1:2:1), or a pre-purchased Total Brain Lipid Extract. The protein (BSA) to lipid ratios of the vesicles were 0.2; 0.5; 1; 2 and 4. The spectroscopically determined P/L values were plotted against the known, real ratios of the suspensions. In both cases (either when lipid mixture or Brain Total Lipid Extract was used), the obtained plots could be best fitted with a liner regression (Figure 17). The correlation coefficients for the fitting were 0.98675 and 0.99084 for the lipid mixture and the Brain Total Lipid Extract, respectively.

![Figure 17. Linear regression diagram of BSA-Lipomix (104)](image-url)
Beside the vesicle composition, the effect of vesicle size was also investigated on the robustness of the method. Vesicles from DOPC and BSA were prepared and extruded on different pore sized membranes. From the same composition, particles with a mean diameter of 600, 200 and 80 nm were created, which resemble the size of AB, MV and EXO populations, respectively. The calculated spectroscopic P/L ratios of the DOPC-BSA vesicles showed no significant difference by the alternation of their size, neither if they were extruded, nor when sonicated during the preparation process (Figure 18). Consequently we were able to rule out the possibility of interference due to size and protein encapsulation of the vesicles during the ATR-FTIR measurement.

**Spectroscopic examination of ghost and red blood cell EV**

The technique was also applied for EVs of different sources (Figure 19). Red blood cell derived extracellular vesicles, show a high diversity in their composition, depending on the isolation protocol as well as the aging of the cells (106). The RBC EVs derived from outdated cell concentrate, had a P/L ratio of approximately 0.6. This value correlates well with the ratios measured for Jurkat microvesicles. Reproduction of the EV suspensions, from the same cell concentrate had similar P/L ratios, which means that the approximately 0.6 value is characteristic for the EVs found in outdated blood packs (Table 3). When the same cells (RBCs) were isolated from freshly collected blood and were kept in PBS buffer for 5-7 days, the secreted vesicles had a significantly different 2.95 P/L ratio, while their other parameters (such as size distribution) did not change. This could be due to the different environmental parameters as well as the relatively young age of the cells. Because RBCs use their microvesicles to remove unnecessary
cellular material, the compositional change should be in correlation with the changed cellular functions. By this phenomenon the characterization of RBC EV microvesicles and RBC products (e.g. blood packs) with ATR-FTIR technique could be possible. Concerning the microvesicular properties of RBC EVs, their composition should be similar to the plasma membrane of the originator cells. To compare the two, RBC ghost membrane was isolated. The detailed analysis of IR spectra of RBC ghosts and EVs revealed not only differences in their P/L ratios, but also in their protein content (Table 3). The second derivative of the amide I region showed dominantly α helical protein structures in the RBC EVs (Figure 19/B). Furthermore, a slight shift in the main amide I component peak suggests a change in the MV protein conformation compared to the cellular membrane of ghosts.

![Figure 19](image.png)

Figure 19. Representative ATR-FTIR spectrum of RBC derived EV (blue line) compared with RBC ghost membrane spectrum (red line) (A). Second derivative IR spectra of the amide I region for protein secondary structure assessing (B). (104)
<table>
<thead>
<tr>
<th></th>
<th>1.Isolation</th>
<th>2.Isolation</th>
<th>3.Isolation</th>
<th>Average</th>
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<tr>
<td><strong>Ghost membrane</strong></td>
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<td>0.88</td>
<td>0.91</td>
<td><strong>0.90 ± 0.02</strong></td>
</tr>
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</table>

Table 3. Spectroscopic protein:lipid ratios of red blood cell ghosts and extracellular vesicles

5.2.3. Structural analysis of EVs by polarized light spectroscopy

RBC EV is an ideal subject for chiroptical examination, as it is homogeneous in size and composition and it can be produced in high concentration, repeatedly. In the case of membrane particles, such as extracellular vesicles, LD spectra serves with information about constituents that are either intrinsically oriented or could be oriented by the externally applied sheer force of a Couette flow cell (99). In the LD spectra of freshly isolated RBC EVs (Figure 20/B, LD, black line) a characteristic absorption peak appears at approximately 421 nm wavelength. This band matches with the Soret band of heme proteins perfectly. As red blood cells carry an enormous amount of haemoglobin and in addition their intracellular composition lacks other heme containing membrane associated proteins, this band is a strong signal of vesicular haemoglobin. Its peak on the LD spectrum suggests that the haemoglobin is somehow oriented in the system, probably due to membrane association. Contrary to LD spectra, which Soret region only shows the membrane associated (consequently oriented) portion of the haemoglobin, the standard UV-Vis spectroscopy provide information from the total haemoglobin content of the vesicles. The UV-Vis spectrum of the freshly collected RBC EVs had a maximal absorbance at $\lambda= 415$ nm (Figure 20/B, UV, black line), derived from the haemoglobin content of the vesicles. The broad absorption band could be resolved into two components by Gaussian function based deconvolution. One at $\lambda= 412$ nm (oxygen bound haemoglobin) and one at $\lambda= 428$ nm (deoxygenated haemoglobin), suggesting that the RBC EVs contain both oxy- and deoxy-haemoglobin. The CD spectra of freshly isolated RBC EVs (Figure 20/C, black line) revealed interesting structural characteristics of the vesicular proteins. On the far-UV CD curve two negative bands could be seen: a strong, broad one with $n-\pi^*$ origin and another weaker $\pi-\pi^*$ excitation CD component, centred at $\lambda= 225$ nm and $\lambda= 210$ nm wavelengths, respectively. In contrast, CD spectrum of free, dissolved
haemoglobin has a typical, α helical pattern with two approximately equal band amplitudes, above λ= 200 nm. The character of the freshly collected RBC EV CD curve suggests that despite its high α-helical content additional structural elements such as β-sheets and turns could be present in the vesicles. These results support the previous ATR-FTIR conclusions on vesicular protein structures well.

Figure 20. Polarized light spectroscopy of red blood cell EVs. LD spectra (A, upper) and absorption spectra (A, bottom) of EVs from 0 (black) and 28 (red) days old vesicles. Far-UV CD curves of 0 (black) and 28 (red) days old vesicles next to the CD spectrum of free haemoglobin (blue) (B). (99)

By the applied spectroscopic methods, a significant structural alternation could be detected in stored RBC EVs. The LD spectra of 28 days old EVs showed the disappearance of the λ= 421 nm peak (Figure 20/B, LD, red line). Because of the
unchanged intensity of the Soret bands in UV-Vis spectroscopy, the disappearance of the haemoglobin signal could only be associated with the detachment of the molecules from the lipid bilayer. Furthermore a shift in its UV-Vis absorption peak from λ= 415 nm, to λ= 405 nm showed the conversion of haemoglobin from oxy- form to methaemoglobin. CD spectral changes also occur in the case of 28 day old samples: the zero crossover point of the fresh curve moves from λ= 210 nm, to λ= 203 nm, which is the crossover point measured also for the free haemoglobin. Moreover the band intensities and shape above λ= 200 nm becomes similar to the free haemoglobin. The observed detachment and transformation of haemoglobin in the RBC EVs could further support the previously detailed FTIR results, when different P/L ratios were calculated for fresh and aged RBC EVs.

The membrane rigidity can also be studied by LD spectroscopy. The vesicle deformation during external mechanical stress can be directly described by the factor S (detailed in Introduction). To establish a more confirmed macroscopic orientation factor, by which the deformation of the vesicles could be characterized, pyrene was employed. The characteristic absorption bands of pyrene could be seen on the LD spectra at low wavenumbers λ< 350 nm (Figure 20/A, LD). The intensities of the pyrene peaks are significantly higher in the 28 day sample than in the fresh suspension of RBC EVs. This difference can be numerically expressed by the S values of the two samples: S₀= 0.007-0.011 and S₂₈= 0.045-0.067. As a conclusion, the results show that the originally rigid membrane of the RBC EVs during the ageing process becomes more deformable, which is potentially a consequence of the haemoglobin detachment till the end of the 28th day.

5.3. From ghost membranes to nanoerythrosomes

5.3.1. The utilization of cell membranes in vesicle preparation

Preparation and characterization of RBC ghosts

The RBC ghost membranes express large, laterally extended membrane sheets as it can be observed in their FF-TEM images (Figure 21/A,B). The empty membranes keep numerous properties of their cellular form, like the micrometric size and the high concentration of membrane particles.
Figure 21. FF-TEM images of RBC ghost membrane suspensions (A-D), with typical representatives of P- and E-faces of the fractured bilayers, (C), (D), respectively. The P-face (inner cytoplasmic half membrane) is densely packed with membrane proteins, while in the E-face (outer extracellular half membrane) contains loosely packed proteins and protein associates.

These observations suggest that the ghosts preserve the main attributes of the cell membranes, in spite of the fact of destruction caused by hypotonic treatment. The membrane sheets are single formations, they are not attached to each other rather well dispersed in the medium. Unfortunately, the resolution of the freeze-fracture technique does not allow the measurement of the thickness of the membrane bilayers. The method, however, provides possibility to distinguish the two leaflets (inner and outer) of membrane and serves with visual information from the interior of the bilayers as well (107). Namely, the procedure can open the ghost membrane bilayer, whereby the morphological features of the separated cytoplasmic and extracellular neighbouring leaflets can be observed. The inner leaflet, also called the cytoplasmic half membrane, is called the P half and the extracellular half is the E half. Their surfaces facing towards each other are the P face and the E face, respectively (82). If the subjects of the fracturing are vesicles, the two sides could be easily distinguished by the convex or concave character of the replica. In the case of large membrane sheets – such as ghosts, their particle density is a more reliable attribute for the identification of the different surfaces.
The P face (Figure 21/C) of the ghost membranes is more densely packed with particles than the E face (Figure 21/D), because membrane proteins remain in the cytoplasmic half in abundance. The detachment of the bilayer leaflets is more common in the case of biological membranes than in artificial vesicles because the large peripheral macromolecules (such as glycoproteins) extend relatively far into the aqueous matrix, consequently, fracturing close to membrane surface is not favourable.

The mechanical stability and the shape of RBCs is assured by the membrane skeleton located underneath the inner membrane leaflet. This, a quasi-hexagonal cytoskeletal network, constituted from long and flexible (α,β)-spectrin tetramers interconnecting short actin filament juctional complexes (108) (109). During the hypotonic process, the connections between the complexes (ankyrin complex, band 3, actin juctional complex) and the cytoplasmic side cease to exist and these characteristic skeletal elements mostly turn into the medium. Presumably, this is the explanation why we cannot observe (not even partially) morphological features in ghosts, resembling to the skeletal system of the RBCs.

Sonication of the ghost membrane: the formation of vesicles and extra-membrane particles

Corresponding to the widespread laboratory practice, a gentle sonication was used to transform the large, empty ghost membranes of red blood cells into smaller, vesicle-like nanoerythromes (110) (111). Sonication is a general method for small-sized unilamellar vesicle preparation as far as synthetic liposomes are concerned. In the case of nanoerythrosome preparation, the parameters of sonication proved to be key factors of the ghost membrane transformation. Generally, I used the shortest sonication time and lowest energy, whereby nanoerythromes could be obtained. The transformation of ghosts to nanoerythromes is accompanied by drastic morphological changes, shown in Figure 22 and Figure 23. A significant decrease in the particles size clearly appear in the DLS measurements, also represented by Figure 22 and Figure 23. The ghost suspension contains large sheets and the presented form exhibits an “E”-face with inhomogeneous surface location of membrane proteins (inset of Figure 22). Although, the DLS indicates the presence of large scattering objects, the calculated size values cannot be realistic, because of the presence of anisotrophic scattering particles. The sonication results in the formation of uniform and spherical-like nanoerythromes. These creations are the dominant formations of the suspensions, seemingly being the main successors of the
ghost membranes. They have an average diameter of approximately 150 nm in the FF-TEM images. On the outer surface of the nanoerythrosomes numerous embedded particles could be observed, these objects are presumably aggregated membrane particles, originating from the membrane proteins of the RBCs. The size and distribution of these surface particles resemble to the membrane proteins of the ghosts, embedded in their E and P faces, demonstrated in Figure 21 and Figure 22.

Figure 22. Surface morphology of ghost membrane (A). The FF-TEM images shows a characteristic “E” face with loose surface protein aggregates. The DLS indicates large scattering objects (B).

Figure 23. Surface morphology of nanoerythrosomes formed during the sonication of ghost membranes (A). Size (mass) distribution, measured by DLS (B), shows the presence of smaller objects too.

A reduced magnification allows the overview observation of the occurring objects, formed in larger amount during the sonication. Figure 24 reveals morphological
characteristics of the sonicated ghosts. Here, two types of particles can be found; larger ones that were highlighted and described in Figure 23, and smaller, frequently aggregated creations, marked by white circles and arrows in Figure 24. These particles are nominated as extra-membrane particles (EMPs). The EMPs are originated from the ghost membrane and presumably torn out by sonication. Consequently, these out-of-membrane particles have similar size characteristics to the ghost membrane proteins. Such small, dispersed particles, however, were absent in the aqueous media of the precursor ghosts. Thus, EMPs were identified as the product of the sonication process. The exploration of their role in the nanoerythrosome formation required the separation of the sonicated ghosts components and the analytical-structural examination of the fractions.

![Figure 24 Nanoerythrosomes and free membrane protein particles (marked with circles) (A). Surface of nanoerythrosomes and free membrane protein particles (EMPs) under higher magnification (B).](image)

**Separation and characterization of nanoerythrosomes and EMPs**

In order to separate the nanoerythrosomes and the extra-membrane particles from the sonicated ghost suspension, ultracentrifugation (UCF) was used (described in the section of *Materials and Methods*). The size distribution of the obtained supernatant and pellet was measured and the characteristic data is summarized in Table 4. Compared to the approximately 150 nm large particles in the sonicated ghost suspension, The UCF pellet had a dominant fraction (98 mass%) with an average size of 74 nm, while the particles, which remained in the supernatant, had only a diameter of 30 nm on average. Beside these fractions, both the supernatant and the pellet contained a small portion of larger components as well. In the case of supernatant this part (with an approx. 100 nm extension) could be the un-sedimented nanoerythrosomes, while as far as the pellet is
concerned the larger (~310 nm) components could be the result of the ineffective resuspension of the nanoerythrosomes or their aggregates. The measured polydispersity supports the effectiveness of the separation, as in the case of UCF supernatant the standard deviation values are only ±9 nm for 90% of the particles, while in the pellet the same attribute is ±36 nm. These values suggest an effective separation of the particles into two distinct fractions (UCF supernatant and UCF pellet) prior to their characterization.

<table>
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<th>Mean diameter [nm]</th>
<th>Standard dev. [nm]</th>
<th>Mass distribution [%]</th>
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<tr>
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Table 4. Size distribution of sonicated ghost and its ultracentrifuge-separated fractions

In accordance with the DLS results, the FF-TEM morphological studies also indicate significant differences in the sonicated ghost and its UCF fractions (Figure 25). The sonicated ghost and its pellet fraction contain a high amount of nanoerythrosomes, while in the UCF supernatant the detectable particles are almost always small, extra-membrane particles (EMPs) (Figure 25/A,B and C, respectively). The TEM images reveal that the sedimented nanoerythrosomes are highly particulated and form loose association (Figure 25/B). This attribute can be the result of the centrifugal force that attached the particles in the UCF pellet together. Next to the pelleted nanoerythrosomes, a high number of extra-membrane particles could also be seen, frequently associated with the nanoerythrosomes (see the inset in Figure 25/B). According to DLS and TEM measurements, the supernatant of the ultracentrifugation could be viewed as a suspension of membrane particles torn out of the vesicles by the sonication process, while the pellet contains mainly the vesicles and those particles that remained embedded or associated to their membranes.
Figure 25. Nanoerythrosomes and extra-membrane particles (EMPs) in sonicated ghost suspension (A). Vesicle rich pellet- (B) and mostly EMP containing supernatant (C) of ultracentrifugation. (111)

FTIR spectroscopic analysis provides compositional and structural information from the particles found in the fractions of UCF separation. The absorption bands with the highest intensities are the lipid derived –CH₂/CH₃ stretching vibrations (3020-2800 cm⁻¹) and C=O stretching vibration (1800-1700 cm⁻¹) as well as the protein derived amide A (3290 cm⁻¹), amide I (1700-1600 cm⁻¹) and amide II (1600-1500 cm⁻¹) bands. The FTIR spectra reveal a significant compositional difference between the supernatant and the pellet of the ultracentrifugation. Namely, the particles of the UCF supernatant are enriched in proteins according to their high intensity amide- and surpassed C-H and C=O bands (Figure 26).
Figure 26. FTIR spectra of ghost, sonicated ghost and its UCF fractions. The whole spectra (A) and the part containing amid I, amid II and C=O vibrations [1350-1800 cm\(^{-1}\)] (B). (111)

This compositional difference is reflected in the spectroscopic P/L ratio, which as far as nanoerythrosomes are concerned is based on the integrated band intensities of the amide I and C=O bands – due the higher C=O band intensity, compared to EVs. The calculated spectroscopic P/L ratio (Table 5) for UCF supernatant is approximately 35.9, which compared to the 17.2 P/L ratio of the UCF pellet indicates significantly higher protein content. If we compare these values to the P/L ratio of the ghost starting material: 31.9 (or the slightly lowered value of the sonicated ghost: 28.2) we can conclude, that the particles released from the ghosts by the sonication are proteins – together with their nearest membrane-lipid surrounding. After the centrifugation, the protein particles...
remained in the supernatant, due to their small size, while the nanoerythrosomes with lower protein content were sedimented into the pellet.

<table>
<thead>
<tr>
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<th>UCF sup.</th>
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Table 5. Spectroscopic P/L ratios of ghost, sonicated ghost and its UCF fractions

Beside the different protein- and lipid content of the UCF supernatant and pellet, the structure of the proteins shows alteration in the two fractions too. By the analysis of amide I region of the spectra (Figure 26/B), the bands in connection with α-helix (1654 cm\(^{-1}\)) and β-sheet (1635 cm\(^{-1}\)) secondary structures are revealed. The deconvolution of the envelope curve shows that most of the proteins has α helical structure in each fraction. The calculated α-helix/β-sheet ratios - according to the ratio of the integrated area of band component at 1654 cm\(^{-1}\) and that at 1635 cm\(^{-1}\), are generally 60/40, slightly varying due to the sonication and sedimentation of the vesicles (Table 6). A significant difference could be identified in the UCF supernatant, where the β-sheet seemed to be absent. The symmetricity of the envelope curve of amide I indicates that the band is constituted from one peak (at approx. 1654 cm\(^{-1}\)), which suggests that the absorption band contains essentially α-helix or unordered structures (which have absorption around the same 1656-1640 cm\(^{-1}\) wavenumber).

<table>
<thead>
<tr>
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<th>Ghost US</th>
<th>UCF pellet</th>
<th>UCF sup.</th>
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<td>60.2/39.8</td>
<td>60.5/39.5</td>
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</tr>
</tbody>
</table>

Table 6. α-helix/β-sheet ratios of ghost suspension and its UCF fractions calculated from ATR-FTIR spectra. In the UCF supernatant practically only α-helical structures could be observed.

In order to obtain a closer information about the kinds of embedded and secessive proteins, mass spectrometric (MS) protein analysis of the pellet and supernatant was carried out. The proteomic data correlated well with the findings of FTIR spectroscopy (protein enrichment in UCF supernatant).
The proteomic analysis showed qualitative and quantitative differences in the proteins of the ultracentrifugally separated vesicle-like nanoroerythrosomes and extra-membrane particles (EMPs). According to MS analysis, the preparation procedure of nanoerythrosomes resulted in significantly different protein content for the vesicles compared to the separated “free” membrane particles (EMPs), however the four most frequent proteins in both fractions were the same. MS also showed higher protein diversity in the EMP suspension, compared to the pelleted vesicles. The number of identified proteins was nearly twice as much in the supernatant (148) than in the pellet (79). Furthermore, not just the total number of identified proteins, but the ones that are specific for one fraction or the other were excess in the supernatant. In the supernatant of UCF 79 unique proteins were found and only 10 as far as the pellet was concerned. Surprisingly, the four most frequent proteins identified in both fractions were the same: Spectrin α and β chains, Ankyrin and Band 3 protein. For each of these proteins significantly more peptides were identified in the supernatant than in the pellet. The most common protein contained exclusively by the pellet was the Actin cytoplasmic 2 protein.

To summarize the structural and morphological examinations, a gentle sonication turned the large, micrometric ghost membranes into spherical nanoerythrosomes, with a 150 nm average size and characteristic membrane protein content. Beside the vesicles a high concentration of small particles (10-30 nm) were also identified by FF-TEM and nominated as EMPs. Ultracentrifugation was applied to separate the components into a mainly nanoerythrosomal pellet and extra membrane particle containing supernatant. The examination of the different fractions by ATR-FTIR and MS concluded that the nanoerythrosomes had lower protein to lipid ratio, turning out to be the lipid enriched derivatives of ghost membranes. On the other hand the EMPs were mainly consisted of proteins with only a little amount of their original lipid surroundings. These findings raised the idea of phospholipid addition to the suspension, to achieve homogeneous nanoerythrosomes. With lipid additives the re-incorporation of EMPs into the newly formed nanoerythrosomes would also be possible, based on the analogy of proteoliposomes (112).

5.3.2. The tailoring of nanoerythrosomes with phospholipid additives

As it was presented previously, the sonication of ghosts resulted in the formation of a complex system consisting of nanoerythrosomes and EMPs. The (UCF) separation
and analysis of the particles, implied the need of extra lipid during the vesicularisation process. When deciding on the types of lipid additives the composition of natural membranes served with examples to follow. The smallest structural elements of all membranes are the phospholipids, consequently the phospholipid components of the natural membranes were taken into consideration. As far as red blood cells are concerned, the most abundant phospholipids of their membrane are phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (113) (114). The first one is a typical constituent of the inner half of the bilayer, while the latter can mainly be found in the outer monolayer. Building on their properties, these two lipids were chosen to be the synthetic lipid additives of nanoerythrosome preparation. The extra lipid was applied in high excess in order to significantly express its effect on the vesicle-like formation. Its amount was based on the measured protein concentration of the ghost membranes. According to the detailed information about ghost membranes in the literature, their lipid and protein content is generally 1:1 in mass ratio. Therefore the measured total protein concentration of ghosts by Bradford protein assay, serves with an estimation on their total lipid concentration as well. Compared to the original lipid content of the ghosts, generally five- (5x) or ten (10x) times more phospholipid (calculated in molar ratio) was added to the suspensions prior to the sonication. Significant differences were in the case of lysophosphatidylcholine (LPC) due to its extreme character and strong impact on the vesicles.

a) Non-vesicular formations due to DPPE addition

The addition of dipalmitoyl-phosphatidylethanolamine (DPPE) to red blood cell ghosts, resulted in highly polydisperse membrane suspensions. The samples remained cloudy despite to any amount of sonication. Because of the visible polydispersity and rapid sedimentation of the suspensions DLS measurements couldn’t be carried out. To still get an insight on the morphology of the membranes, FF-TEM was applied. The freeze fractured images of the ghost suspensions sonicated together with tenfold of DPPE (ghost total lipid:DPPE = 1:10; w/w) revealed large, planar stacks of multilayers (Figure 27). Beside the multilayers EMPs (free membrane proteins) could not be observed. This result is not surprising when considering the physicochemical properties of the DPPE molecule. DPPE is a phospholipid with long (C16) acyl chains and small ethanolamine headgroup. The resulted geometrical shape of the molecule is truncated conical, which does not support positive curvature radii, necessary for the formation of vesicles. The dominantly peripheral membrane proteins complement the conical-shaped form of DPPE molecules.
and constitute a parallel-shaped form practically, making possible the formation of laterally extended sheets. This finding is not conflicting with our previous information, as the peripheral proteins in the erythrocytes mainly connect to the inner leaflet rich in DPPE. Moreover, the membrane of the erythrocytes can be considered as extended, more planar than nano-spherical. As our aim was to create nanoerythrosomes, changing the shape (type) of the lipid additive seemed to be reasonable.

Figure 27. FF-TEM images of multilayers in 10×DPPE containing ghost membrane suspension. The top-view of a laterally extended stacks, consisting of parallel-arranged sheets (left). A side-view of a laterally extended stack, containing multilayers (right). In the top view (left) image, some gaps are visible where the larger proteins, protein aggregates are located in the membrane. (111)

b) Nanoerythrosomes with protein scaffolding

*DPPC enriched nanoerythrosomes*

Dipalmitoyl-phosphatidylcholine (DPPC) is a cylindrical shaped phospholipid, which is – compared to DPPE –, more capable of bilayer formation in its pure form (115). The amount of DPPC additive was calculated to be five (5×DPPC) and ten (10×DPPC) times more than the total lipid content of the ghost membrane suspensions. The characteristics of sonicated ghost (nanoerythrosomes and EMPs), ghost+5×DPPC (nanoerythrosomes with five times of their original lipid content) and ghost+10×DPPC (nanoerythrosomes with ten times of their original lipid content) were compared to each other, regarding their homogeneity, morphology and structure of their molecular constituents.
The size-distribution measurement revealed the crucial role of DPPC in the formation of homogeneous nanoparticles (Table 7). The sonication of ghost membranes resulted in the formation of vesicle-like objects with an average diameter of 155 nm. The system did not contain particles larger than approximately 300 nm, but its polydispersity was quite high (standard deviation: ±89 nm). When an additional fivefold of DPPC was mixed to the suspension, the sample had a bimodal size distribution till the end of the sonication. A dominant fraction (89 w/w %) of the particles had an average diameter of 54 nm, but a smaller portion of the particles (11 w/w %) had an average size of 420 nm. However, in the case of such a wide bimodal distribution, due to the model fitting of light scattering the calculated mean diameters are not precise, the presence of a small and a large particle fraction indicates an imperfect mixing of the molecules. The risen amount of DPPC (10×DPPC) was able to take up (and likely incorporate) the protein particles (EMPs) into the nanoerythrosomes more effectively. In the 10×DPPC containing suspension the average size of the vesicles was 134 nm, with a standard deviation value of only ±36 nm.

<table>
<thead>
<tr>
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<th>Mean diameter [nm]</th>
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<td><strong>Sonicated ghost</strong></td>
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<tr>
<td></td>
<td>54</td>
<td>13</td>
<td>89</td>
</tr>
<tr>
<td><strong>Ghost+5×DPPC</strong></td>
<td>420</td>
<td>335</td>
<td>11</td>
</tr>
<tr>
<td><strong>Ghost+10×DPPC</strong></td>
<td>134</td>
<td>36</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 7. Size distribution of sonicated ghosts prior and after (5× and 10×) DPPC addition

The zeta potential values served with an estimation on the stability of the vesicular suspensions and showed good correlation with the amount of DPPC additive (Figure 28). The sonicated ghost suspension without extra lipid had the largest negative zeta potential. In this case, the nanoerythrosomes contained the highest concentration of membrane proteins, and they preserved their original lipid composition as well. These two attributes together resulted in a high net charge of the particles. The measured zeta potential decreased with the risen amount of DPPC additive, but in the nanoerythrosomes with
10×DPPC concentration its value was still significantly negative, suggesting that nanoerythrosome suspensions even with the most lipid additive have a decent stability.

![Graphs of Zeta Potential Distribution](image)

Figure 28. Zeta potential of sonicated ghosts prior and after (5× and 10×) DPPC addition (62)

The morphological and structural studies definitely proved that the newly formed DPPC containing nano-objects were nanoerythrosomes (Figure 29). In the case of 5×DPPC additive, nanoerythrosomes with approximately 50-80 nm size, and slightly particulated surface were created (Figure 29/A,B). The amount of EMPs in the aqueous medium was also hardly observable, suggesting a possible uptake of the protein particles by the extra lipid. When the amount of DPPC was ten times compared to the ghost’s original lipid content, the resulting nanoerythrosomes had an unexpected morphology. The objects were roughly still spherical, but a peculiar scaffolding appeared on their surface (Figure 29/C,D). This network as a surface pattern could only be observed by the application of FF-TEM, as the freezing process conserved the fine structural details of the
nanoerythrosomes in its original, hydrated state. The nanoerythrosomal scaffolding usually consisted of hexagons and pentagons. This surface pattern was similar to fullerenes, but only in the geometrical sense. The contact points of the polygons fell on to the regular surface of spherical balls. The side of the polygons seemed to be characteristic, it extended to approx. 25 nm. The sides, as edges, protruded from the spherical surface. They were not homogeneous creations, but they were constituted from small particles closely packed to each other. The contour of the network could be detected on the inner side of the bilayer as well. On the nanoerythrosomes, which were broken through entirely, shells of the network could be seen. These cases are shown in Figure 29/D. In this vesicle, the contour of the broken shell is not cyclic, but consists of linear sections. These linear sections exhibit the same length as the sides of the hexagons and pentagons on the outer side of nanoerythrosomes (these hexagons and pentagons can be observed on surface of the neighbouring nanoerythrosomes also in Figure 29/D). Furthermore, the scaffolding was continuous on the broken edge of the vesicles in every observed case, indicating it is an extended formation, which covers the whole of the nanoerythrosomes and not a local association of the constituents.

Figure 29. FF-TEM images of nanoerythrosomes with 5×DPPC (A,B) and 10×DPPC (C,D) additive. Particles (white arrow) inside the nanoerythrome and the characteristic length of the polygonal network (red arrow) can be seen on the nanoerythrome surface and also on the linear section of the shell of the broken nanoerythrome (D).
From the electron micrographs it is clear, that the addition of 10×DPPC caused a structural change in the nanoerythrosomes. The originally smooth surface of the nanoerythrosomes without DPPC additive expressed an angular formation, which indicates the association and possibly also the segregation of certain components. The appearance of the scaffolding, in combination with the observable disappearance of the protein particles (EMPs) from the extravesicular space could mean, that the extra lipid induced the uptake of the proteins and their association in the DPPC enriched membranes. Consequently, the nanoerythrosomal scaffolding could be a protein network that surrounds DPPC rich lipid rafts.

Small angle x-ray scattering (SAXS) provides further information on the shell structure of the nanoerythrosomes. The ghosts (suspended in PBS, in the same condition as it was obtained after the sonication treatment, without additional lipids), and its DPPC modified forms, the nanoerythrosomes with 5× and 10×DPPC ratios were measured. These samples were identical with the ones used for FF-TEM studies. The scattering patterns were recorded in the q-range between 2·10⁻¹ and 6 nm⁻¹ in our Credo laboratory apparatus which are shown in Figure 30. This measurement covers significantly narrower interval compared to the one that was demonstrated in Figure 7. The small q-range is missing, therefore I could not receive information about the whole nanoerythrosomes, namely about their shape, size and size-distribution. The observed q-interval carries information about the layer-structure of the objects as the quadrature of the bilayer formfactor, centred at approximately q=1 nm⁻¹ appears (116). In Figure 30 we can see that the shape of all the three curves are strictly alter from the regular unilamellar vesicles, taken into consideration the same relevant q-range (2·10⁻¹ – 6 nm⁻¹), shown in Figure 7. The SAXS curve exhibits a strongly decreasing exponential form. This scattering attribute originates from the proteins and protein-aggregates, which are abundantly present in the samples. The FF-TEM images (Figure 24) show the existence of single, smaller or larger proteins and their aggregates that can cause this exponential scattering pattern. It has to be noted that the scattering of proteins is always present independently even if the proteins or their associates are associated to the nanoerythrosomes or dispersed in the medium. Beside the dispersed proteins, their membrane embedded forms are also present in the suspensions, therefore the scattering of the lipid bilayers should be very weak. That is the explanation for shape of the SAXS curve of nanoerythrosomes without lipid additive (Figure 30, blue line), where only a “shoulder” appears. Shortly summarized; the scattering of proteins suppresses the scattering of lipids. When extra-added 5×DPPC is
present and consequently the amount of lipid bilayers is increased, instead of the shoulder, a significant intensity of the quadrature of the bilayer formfactor is observable. This part will be referred in the followings as “the peak of bilayer”. After adding 10×DPPC to the ghosts, the intensity of the bilayer derived peak became double, compared to the case of 5×DPPC. The intensity of the peak (at q=1) correlates with the increased lipid concentration in the system very well. This strengthening of the unilamellar character by the additional lipid indicates that the DPPC molecules were incorporated into the nanoerythrosomal membrane and did not suffer any segregation, by creating multilamellar structures. However a very weak sign of oligolamellarity (the formation of a few concentric bilayers) appears in the 10×DPPC containing nanoerythrosomes (Figure 30, green line), as a small and narrow diffraction peak is observable at q=1 value. This extremely small peak corresponds to a very small amount of oligolamellas, therefore the nanoerythrosomes could still be considered as unilamellar vesicles, with heterodisperse domains consisting of lipids and proteins.

![Small diffraction peak (first Bragg reflection) of oligolamellar hydrated DPPC domains, d=2π/λ=6.3 nm is the periodicity](image)

Figure 30. SAXS curves of ghost membrane and nanoerythrosomes with DPPC additives. The enhancement of scattering peak, corresponding to the bilayer form (black circle) is due to the rise of incorporated DPPC. (111)

Considering the visual information obtained by the freeze-fracture method we can suppose that the scattering objects are nearly spherical unilamellar nanoerythrosomes. Although the shell of the DPPC enriched nanoerythrosomes is not homogeneous, a
spherical shell structure, as a crude model can quantify the shape of their scattering curve. In other words, a structural analogy between nanoerythrosomes and sterically stabilized unilamellar vesicles is assumed. (The sterically stabilized vesicles contain additional shell-layers on both sides of the bilayer, which can be analogous with the proteins of the nanoerythrosomes.) By employing a shell model of spherical symmetry, an approximate description of the layer structure becomes feasible. Neglecting the interactions between individual nanoerythrosomes and assuming their spherical symmetry, the scattering intensity can be calculated by the symmetric ‘2 Gaussian’ (89). Here one pair of Gaussian functions represent the polar head group regions of the lipid bilayers, while another pair the protein molecules located in the bilayer and on its both sides (surfaces?). It must be noted that a third, single Gaussian contribution is also used to describe the hydrocarbon chain region of lipid double layers. To reduce the number of independent parameters in the model and to support its convergence to a physically relevant result, the characteristic values for the Gaussians of the lipid head groups and the chain region of a pure DPPC bilayer were used in the first attempt and only the Gaussians for protein localization were fitted. Even though this model might be overly simplistic, the best fit of 5×DPPC containing nanoerythrosomes indicates an asymmetric protein localization in the nanoerythrosomes. It shows that the membrane proteins are mainly located in the bilayer and on its outer leaflet. The layer thickness, approximated to be about 11 nm, is significantly larger than that of the hydrated pure DPPC bilayer as it is demonstrated in Figure 31.

Figure 31. Model description of nanoerythrosome containing 5×DPPC. The visual observation on a nanoerythrosome by FF-TEM (left). Concept of the nano-structure of the object (middle). Shell model for reconstruction of the SAXS curve of the nanoerythrosomes (right). (62)
After the nanostructural characterization, calorimical studies were executed in order to obtain thermal features of the lipid matrix and its molecular composition in nanoerythrosomes. The thermal behaviour of the vesicles membrane highly depend on their composition. The bilayers composed of different lipid molecules can exhibit characteristic phase transitions according to their components (117). The composition of biological membranes is extremely diverse. The phospholipids are varied on a large scale, the cholesterol content is high (approx. 1:1 in molar ratio compared to the phospholipid content) and the bilayer is constantly disturbed by proteins associated to- or embedded into the membrane (118). Consequently, the biological membranes are highly perturbed, and do not express a characteristic phase transition. The same could be observed on the thermograms of nanoerythrosomes (Figure 32).

![Figure 32](image)

**Figure 32.** The appearing minor phase transition in DPPC enriched nanoerythrosomes: 5×DPPC - blue, 10×DPPC – green –, values scaled up twenty times compared to a typical DPPC/water system. (62)

Nanoerythrosomes even with 5×DPPC additive were free from characteristic phase transitions (blue line). An extremely small sign of chain melting occurred only in the 10×DPPC containing vesicles (green line). The value of its corresponding change in enthalpy was approximately 0.4 kJ/mol(DPPC) which is only 1% of the same value of a pure hydrated DPPC system (ΔH= 33.7 kJ/mol(DPPC)) (Table 8). Also the temperature of the phase transition was slightly shifted in nanoerythrosomes, compared to DPPC liposomes, from 41.5°C to 39.6°C. The results suggest that the additional DPPC was fully mixed in the system, if applied in fivefold amount compared to the amount of ghost lipid.
Although the higher amount of extra lipid (10×DPPC) showed some thermal feature, its intensity and position was far from the typical DPPC membranes.

To summarize: the thermal characteristics of the nanoerythrosome suspensions revealed similar attributes to the ones obtained by SAXS. The DPPC, even in its highest concentration incorporated into the vesicles’ membrane. Due to its enrichment in the 10×DPPC containing vesicles, the additive lipid formed DPPC rich domains. These domains were capable of expressing a small and shifted change in enthalpy, observed by DSC.

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<tr>
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Table 8. Phase transition temperature (T) and change in enthalpy (ΔH) values of nanoerythrosomes containing 5×DPPC and 10×DPPC additives and DPPC liposomes

The structural consequences of the nanoerythrosome formation on the atomic size-range were revealed by ATR-FTIR spectroscopy. The IR spectra of the nanoerythrosomal suspensions show that the intensities of the lipid related C-H and C=O absorption bands are significantly getting higher with the rising amount of DPPC (Figure 33). Additionally the peak positions of CH$_2$ symmetrical and asymmetrical stretching vibrations are shifted towards the characteristic values of the pure DPPC ($\nu_s$ 2918 cm$^{-1}$; $\nu_{as}$ 2850 cm$^{-1}$) (Figure 33/A). Further changes could be seen in the amide bands of the nanoerythrosomes (Figure 33/B). The protein related amide I peak is slightly shifted, from 1651 cm$^{-1}$ to 1655 cm$^{-1}$ due to the DPPC addition, furthermore the small satellite peak at approx. 1630 cm$^{-1}$ also shows decreasing tendency. These two processes indicate a change in the secondary structure of the proteins, from a generally β-sheet character to a more α-helical form.
Figure 33. The effect of additional DPPC on the structure of nanoerythrosomes. The changes of lipid –CH$_2$ (A) and protein – amide I (B) region (111)

The deconvolution of the amide I band - achieved by the help of spectroscopist colleagues - shows significantly higher α-helix components in the 10×DPPC containing nanoerythrosomes than in the vesicles without lipid additive (Figure 34). The structural changes of the proteins could be the consequence of their enriched DPPC environment.
The formation of lipid rafts, enriched in DPPC is supported by the thermotropic response of the symmetric stretching vibration of $-\text{CH}_2$ groups (Figure 35) (119). For this measurement the vesicles were prepared according to preparation protocol I (detailed in Materials and Methods), consequently the effect of lipid addition is only analysed as general tendencies. The sharp increase of the $\nu_s\text{CH}_2$ is caused by the change from trans- to gauche conformers of the lipid acyl chains during the main transition. In the pure hydrated DPPC systems, liposomes exhibit an intensive transition from gel to liquid-crystalline phase, with a $T_m$ around 41.5°C. This phenomenon also occurred in nanoerythrosomes, after DPPC addition, however the change of enthalpy during the phase transition is reduced and the transition is extended in a wider temperature interval. With the increased amount of DPPC the shape and characteristics of the DSC curve resembles...
significantly to the curve of the pure hydrated DPPC system, indicating the formation and enhancement of lipid domains with the risen amount of DPPC additive. This result shows an interesting consequence of the compounding. In spite of the fact that the lipid (DPPC) is present in significant amount, its phase transitional enthalpy-change disappears but its structural change is in progress. In this case the phase transition character is changed; the first order type of phase transition changed to second order type. This change is also accompanied with further alterations; the system became sensitive against any perturbation, the correlation length became also long, whereby the range of interaction between the molecules became also extreme long. Presumably, these attributes play important roles in the formation of protein scaffolding of nanoerythrosomes.

Figure 35. Changes in lipid acyl chain from trans- to gauche conformers during phase transition of DPPC enriched nanoerythrosomes and synthetic DPPC liposomes (62)

In order to examine the contribution of the scaffolding to the mechanical properties of the nanoerythrosomes, LD measurements were performed. The nanoerythrosomes were oriented in linear flow cells – similarly to the extracellular vesicles -, in the presence of pyrene probe molecules. Due to the sheer force applied in the flow cell, the spherical vesicles could be deformed according to their flexibility, resulting in the orientation of their membrane associated probe molecules. The absorption and LD spectra of membrane embedded pyrene probe molecules are shown in Figure 36. The values of the orientation factors (S, its connection to LD signal is shown in page 31) indicate a decreasing tendency starting from the UCF pellet of sonicated ghost (S=0.016), followed by the 5×DPPC (S=0.011) and 10×DPPC (0.005) containing nanoerythosome
suspensions. The decreasing tendency in the orientation factor shows, that the formation of the protein scaffolding increased the rigidity of the nanoerythrosemes and resulted in its lower deformability.

Figure 36. The decreasing elasticity (which is roughly proportional with the magnitude of LD signal) of nanoerythrosomes – measured by LD spectroscopy - shows correlation with the formation of the surface scaffolding in 10×DPPC containing vesicles. (111)

**Addition of DPPC to purified EMP suspension**

The appearance of the protein scaffolding on nanoerythrosomes seemed to be the consequence of either the lipid addition or the EMP reincorporation, but most probably both. The importance of the DPPC had been pointed out clearly by a set of measurements so far. Therefore, to examine the role of the EMPS, the supernatant of ultracentrifugation (a purified EMP suspension) was mixed with tenfold DPPC - compared to its measured protein concentration. In the final step of preparation a gentle sonication was applied to mix the components and induce the particle formation.

Electron microscopic examination of the DPPC enriched EMPS revealed extremely angular structures. The originally 4-10 nm sized individual particles and their 2-3 fold larger associates (Figure 37/A) disappeared from the suspension and were replaced by a heterodisperse system. Angular membrane sheets extended into 200-300 nm range (Figure 37/B) and nanoerythrosome-like particles with polyhedral surface character, with a size up to 100 nm (Figure 37/C) were visible in the electronmicrographs of DPPC-EMP containing system. These formations are not typical for the pure DPPC systems, consequently their presence in the suspension had to be the result of an
interaction between DPPC and EMPS. Because of the high polydispersity and heterogeneity of the formations, the suspension seemed to be in an intermediate state between a DPPC and EMP containing mixture and nanoerythrosomes with partially expressed polygonal surface pattern. To enhance the reorganization of the system and to help the formation of the thermodynamically stable formations, a widely used thermal homogenization procedure was applied. This process is based on the perturbation of the lipid molecules due to their increased mobility and altered geometrical shape above the phase transition temperature. The suspension was heated to 50°C and it was incubated there for 30 minutes. After cooling back to room temperature its morphology was once more observed by FF-TEM (Figure 37/D,E). The microscopic images show an increased structural homogeneity, the single EMPS, dispersed in the medium, could not be seen at all. However, nanoerythrosomes with two typical surface pattern could still be distinguished. On one hand a wide variety of large (generally 200-600 nm) objects were formed, these objects had angular shape and a surface covered with long, densely packed protrusions (Figure 37/D). On the other hand there were spherical objects with a polygonal surface pattern (Figure 37/E), which resembled to the previously created nanoerythrosomes (Figure 29).

The FF-TEM images of the DPPC enriched EMP suspension demonstrate, that the dispersed protein particles of the aqueous medium (EMPS) are not only just capable for incorporation into the nanoerythrosomes, but they also have an essential role in the formation of the protein network on the vesicles’ surface.

![image](https://example.com/image)

Figure 37. FF-TEM images of separated EMPS - UCF supernatant (A), EMPS mixed with 10×DPPC (B,C) and the lipid enriched EMPS after a heating cycle (D,E) (111)
Temperature induced changes in the established nanoerythrosomal scaffolding

Electron microscopic images revealed that the sample incubation at 50°C had a significant effect on the surface pattern of nanoerythrosomes, formed by DPPC and EMPs. The alternation of the structure from planar sheets (Figure 37/B) to spherical objects (Figure 37/D,E) and the formation of the network elements suggested a new kind of association between the EMPs. As this transformation was induced by the heating of the suspension, the effect of temperature change was examined on nanoerythrosomes with an already established scaffolding as well – prepared directly from ghosts with 10×DPPC additive.

To examine the effect of consecutive heating and cooling treatments, freeze fracture was carried out from three parallel batches of nanoerythrosome suspensions. The first fracturing was made from freshly prepared nanoerythrosomes with 10×DPPC additive, according to the general procedure (rapidly frozen in liquid Freon, approx. T= -180°C). The second batch was heated to 50°C and was incubated there for a few minutes, to reach the desired temperature in its whole volume. The heated suspension was then quenched and rapidly frozen. Because of the rapidness (approx. 2×10^5 °C/s) of the freezing, the molecular interactions were conserved, which is represented on their replicas. The third sample was also incubated at 50°C for the same amount of time, but then it was cooled back to room temperature (approx. 25°C) and quenched prior to the freeze fracturing process. This way the three observed nanoerythrosome suspensions were: one in its original (general) state (A), one in a heated state (representing the 50°C conditions) (B) and one that was heated to 50°C, then cooled back to 25°C before the fracturing process (C).

The electron microscopic examination of incubated nanoerythrosomes showed significant difference between the structure of vesicles at 50°C and 25°C (Figure 38). At room temperature, before heating the nanoerythrosomes had a characteristic 100-200 nm size and polyhedral shape (Figure 38/A). At 50°C a remarkable increase in size (often particles with an average diameter of 300-400 nm appear) and association of the vesicles could be seen. The association could result in their fusion, especially above the phase transition temperature of DPPC, which could be the source of the larger vesicles. The large vesicles seem to have an oligolamellar structure – on their fractured surface often more (typically 2-3) concentric bilayers could be identified. Another attribute of the
heated membranes is their waved surface (Figure 38/B). On the spherical nanoerythrosomes long, parallel bands could be seen, covering practically their whole surface. On the other hand, the surface of the smaller nanoerythrosomes is rough and particulated, but the original, polygonal structure is less expressed. It seems that at 50°C the angular associations like polygons (Figure 29/C,D) or folded sheets (Figure 37/B) are not typical. The closely packed, long bands, presumably the associations of the side elements of the dissected polygons, are running parallel and cover the whole of the nanoerythrosome.

Figure 38. The morphology of nanoerythrosomes at different temperatures during a heat cycle: 25°C (A), 50°C (B) and at the end of the heat cycle – 25°C (C) (111)

When the 50°C suspension is cooled back to room temperature, the morphology of the nanoerythrosomes changed in a way that resembles to their original, 25°C form (Figure 38/C). However, most of the vesicles achieved the polyhedral structure, the heritage of the high temperature could be also detected. Elongated waves are often expressed on the surface of the nanoerythrosomes, although the edges of these waves are significantly sharper than it was in the case of curved bands – found at 50°C (Figure 37/D).
These features indicate that the protein scaffolding of the nanoerythrosomes is thermo-sensitive. The protein particles in the nanoerythrosomal membranes form different types of associations on 50°C and on 25°C. This indicates a loose, rearrangeable connection between the proteins. Furthermore, the polygonal network seems to be a favoured formation at room temperature, if the system contains an appropriate amount of DPPC additive. This explanation is supported by the observation that the nanoerythrosomes express polygonal surface pattern, when cooled from 50°C back to 25°C.

Beside morphological changes, the increased temperature (up to 50°C) had structural consequences on the nanoerythrosomes as well. Structural changes on the molecular level were observed by ATR-FTIR spectroscopy. The IR spectra of nanoerythrosomes with 10×DPPC additive were collected in three consecutive heating-cooling cycles between 25°C and 50°C. The spectra were recorded at every incubation point both in the heating and in the cooling steps. From the processed spectra the wavenumber values of the lipid related C-H bands and the protein related amide I bands were retrieved. The average values of the three consecutive measurements are plotted as the function of temperature (Figure 39).

As far as the lipid related bands of the IR spectra are concerned, with the rise in the temperature, a shift in the symmetric stretching vibration of the CH_2 bands could be seen (Figure 39). The position of υ_s CH_2 at 2850 cm⁻¹ is characteristic for DPPC membranes and it represents the DPPC enriched lipid rafts as shown in Figure 33/A previously. The shift in its position and the shape of the curve is in connection with the geometrical alteration of the lipid molecule, which results in the phase transition of the bilayer (detailed previously in Figure 32). In our case, however, the lipid phase transition is extremely weak (Figure 32; Table 8), but the shift in the CH_2 peak position from approximately 2850 cm⁻¹ to 2852 cm⁻¹ wavenumbers is a significant change. This shift corresponds to the alteration of the lipid acyl chain conformation, which results in a truncated conical shape of the molecule.

The shift in the amide I peak position from approx. 1641 cm⁻¹ to 1634 cm⁻¹ is the sign for the protein conformational change (Figure 39). A shift in the direction of lower wavenumbers indicates a growing amount of β-sheet conformation in the nanoerythrosomes. This could either be the result of the increased temperature, or the alternation of the proteins’ lipid environment.
Figure 39. Temperature induced changes in the lipid (CH$_2$) and protein (Amide I) content of the 10×DPPC enriched nanoerythrosomes: correlation between the characteristic IR band shifts and morphological alterations. (111)

By the end of the thermal cycles, the characteristic peak positions for both CH$_2$ and amide I returned to its starting value, which indicates the reversibility of the conformational changes of the components. Although the shifts in the IR spectra represent significant alterations for both biomolecules, the reversibility excludes their denaturation or degradation. Compared to the observations of FF-TEM images, the shifts of IR spectra are presumably the signs of the formation and dissociation of the nanoerythrosomal scaffolding. These results serve as further indication for the coupled atomic and nanosized structural changes both in the lipid and protein constituents.

The effect of Ca$^{2+}$ ion on the protein scaffolding

The parameters with a potential impact on the vesicles stability have been widely characterized in model systems. Arguably, the most important environmental parameters are the temperature (120) and the ionic strength (121) (122). The temperature had already been shown to have a significant effect on the structure of the nanoerythrosomes by the reversible alternation of their protein scaffolding (Figure 39). In order to examine the effect of the increased ionic strength on nanoerythrosomes, the suspensions were diluted with CaCl$_2$ solution to a final 10mM Ca$^{2+}$ concentration. The applied nanoerythrosome samples contained 10×DPPC additive and expressed a polygonal surface morphology, as detailed previously (Figure 29).
In the presence of Ca ion (10mM Ca\(^{2+}\)) a homogeneous suspension of spherical nanoerythrosomes could be observed on the electron microscopic images (Figure 40). The general size of the vesicles was approximately 120 nm and the protein scaffolding was clearly visible. The nanoerythrosomes were fairly similar to ones without Ca\(^{2+}\), except in one attribute. Their surface, beside the protein network, exhibited a peculiar feature. There were small, spherical and compact particles, resembling to the EMPs shown to be dispersed in the extra-vesicular medium earlier (Figure 25/A). The association of these particles to the nanoerythrosomes did not affect the protein scaffolding, suggesting that their interaction with the membranes happened after the establishment of the scaffolding, likely due to the addition of calcium ions. Furthermore, the newly associated particles seem to emerge out from the bilayer, suggesting that they are rather surface associated-, than intramembrane proteins. Their position is also characteristic: they can be found in the middle of smooth, polygonal surface patches (marked by red arrows in Figure 40/B). As it had been pointed out earlier, the edges of the scaffolding are presumably associated protein particles of the nanoerythrosomes complemented by the EMPs that were incorporated by the additional DPPC. Consequently, the leaflets of the polygons are the lipid enriched rafts, which can be attractive to the dispersed particles if the Ca\(^{2+}\) changed the net charge of their lipids. For DPPC model vesicles this phenomenon was presented earlier (123), as the originally neutral DPPC vesicles became a positively charged in the presence of Ca\(^{2+}\) (the process was observable from 1-2 mM Ca\(^{2+}\)).

Figure 40. Particle (EMP) association onto the nanoerythrosomes surface as a result of increased Ca\(^{2+}\) concentration (111)
The Ca\textsuperscript{2+} induced interactions of nanoerythrosomal protein molecules were analysed by ATR-FTIR spectroscopy, examining the protein related amide I peak positions (approx. 1639-1633 cm\textsuperscript{-1}) in three consecutive heating-cooling cycles. At room temperature (25°C), a slight shift could be seen from approximately 1639 cm\textsuperscript{-1} to 1637 cm\textsuperscript{-1} wavenumbers, obtained for nanoerythosome suspensions without Ca\textsuperscript{2+} and with Ca\textsuperscript{2+}, respectively (Figure 41). This shift in the amide I absorption peak indicates slightly different protein associations for the vesicles with increased Ca\textsuperscript{2+} concentration. When the suspensions were heated up to 50°C (plotted only until 45°C as further increase had no effect), the amide I peaks shifted to lower wavenumbers reaching their minimum (approx. 1633 cm\textsuperscript{-1}) at around 42°C. As the characteristic phase transition temperature for DPPC membranes is approximately 41.5°C, this suggests that the Ca\textsuperscript{2+} interacts with both the proteins and the lipid bilayer of the vesicles. By heating the suspension above the phase transition temperature, the Ca\textsuperscript{2+} induced protein associations are getting weaker, presumably due to the increased mobility of the membrane components. It is also notable, that when the suspensions cooled back to room temperature, the difference between Ca\textsuperscript{2+} enriched and Ca\textsuperscript{2+} free vesicles reappeared, consequently the effect of calcium ion is reversible.

Figure 41. The effect of 10 mM Ca\textsuperscript{2+} on the nanoerythrosomal proteins according to thermotropic FTIR measurements – the average of three consecutive heating/cooling cycles

Environmental parameters such as temperature and the presence of bivalent calcium ion had significant effects on the morphology of the nanoerythrosomes. The
finding, connecting to the presence of Ca\(^{2+}\) can also indicate that Ca\(^{2+}\) ion effect certain proteins, which are not able to take part in the constitution of the scaffolding, consequently forced to be segregated in the lipid rich domains.

c) Lysophosphatidylcholine (LPC) enriched nanoerythrosomes

*Formation of small-scaled nanoerythrosomes*

According to the composition of (RBC) cell membranes, phosphotidyletanolamins and phosphotidylcholines are the most frequent lipids of the inner- and outer leaflets of the bilayer, respectively (65) (124). For vesicle formation, a high radii curvature is necessary, which could be induced only by lipids with cylindrical or conical shape. The effect of lipids with different shape was examined by the addition of DPPE (Figure 27) and DPPC (Figure 29) molecules to the ghost suspensions. As a consequence of the extra lipid, spherical vesicles were only observed in the case of DPPC additive. To further enhance the curvature radii, conical shaped LPC was applied instead of the cylindrical DPPC. By the incorporation of the LPC into the ghost membranes, a more frequent vesicle formation and particle size reduction could be expected. Furthermore, the LPC molecule is closely related to the DPPC, having the same choline head group and palmitic (C16) acyl chain. One significant difference is that its glycerol backbone is esterified only by one palmitic acid. On one hand this means, that the surface character of the LPC rich membranes should be similar to the ones consisted of DPPC. On the other hand the morphology of the nanoerythrosomes could be significantly altered compared to the DPPC enriched vesicles, due to the highly conical and reversely shaped LPC.

For LPC enriched nanoerythosome preparation lower LPC:ghost ratios were applied (compared to DPPC:ghost ratios), due to the extreme physicochemical character of this lysolipid (125). Nanoerythrosomes with 2×, and 5×LPC additives were prepared and in the case of 5×LPC, no sonication was necessary to achieve homogeneous suspension. The cloudy suspension of ghosts became transparent as the 5×LPC film dissolved from the wall of the glass vial, without any physical perturbation. In the case of 2×LPC containing nanoerythrosomes the effect of the additive lipid was not as significant as in 5× LPC case, the suspension was sonicated according to the general nanoerythrosome preparation process.
It was obvious by visual observation that the addition of LPC resulted in a highly monodisperse suspension of nanoerythrosomes. DLS measurements showed that the size of the particles decreased significantly (Table 9), even compared to the size of the conventional nanoerythrosomes (Table 4). A dominant fraction of small particles (approx. 46 nm in (2×) and 20 nm in (5×) LPC containing samples) were typical for both LPC concentrations, complemented by a lower amount of larger vesicles. The average vesicles were larger for the 2×LPC containing nanoerythrosomes. The nanoerythrosomes, which were formed spontaneously in the presence of 5×LPC additive were extremely small (the average size was 20 nm, for 95% of the particles) and only a small vesicle fraction with a mean diameter of 107 nm could be observed. The standard deviation values were also low, indicating the homogeneity of the suspensions. Considering the micelle formation tendencies in LPC suspensions, the size distribution of pure LPC reference samples were also measured by DLS. The hydrodynamic diameter of LPC micelles appeared to be significantly smaller (approx. 5 nm) compared to the smallest of the LPC containing nanoerythrosomes.

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<th>Mean diameter [nm]</th>
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Table 9. Size distribution of LPC containing nanoerythrosomes and reference LPC micelles

The LPC containing nanoerythrosomes are small, spherical objects as observed on the FF-TEM images (Figure 42/A,B). The particle distribution according to the electron micrographs show good correlation with the measured values of DLS (Figure 42/C). In the 2×LPC containing samples most of the spherical particles are smaller than 50 nm (Figure 42/A). With the significantly reduced size, the bilayer structure of the particles became a relevant question. The spherical particles on the electron micrographs of the 2×LPC containing nanoerythrosomes clearly express a bilayer character. The
particles are generally broken into half, which could only be the result of the hollow vesicles. On the edges of the spheres that were broken through entirely the thickness of the membrane could be seen. If the ratio of the LPC additive is increased from 2× to 5×, the detergent effect of the lysolipid seems to transform the vesicles. Most of the visible particles in the 5×LPC containing suspension had a size smaller than 20 nm (Figure 42/B). These small, dispersed particles do not show a lamellar structure, they are more similar to the EMPs, obtained by the ghost membrane sonication (Figure 37/A). Consequently, it is believed that the 5× amount of the LPC disrupted the ghost membrane, by extracting and encapsulating its components into small micelles. The presence of these small-scaled nanoerythrosomes suggests that by the adjustment of the LPC concentration a tailoring of the nanoerythrosomes could be possible.

Figure 42. Structural properties of small-scaled nanoerythrosomes due to LPC addition (111)

*Disruption of nanoerythrosomal scaffolding by LPC*

The effect of lysolipid on the vesicle’s structure was so significant (when used in 2× and 5× amount) that even its lower concentration resulted in a completely new type of small nanoerythrosomes (Figure 42/A). In order to only slightly perturb the nanoerythrosomal bilayer and examine the LPC’s effect on the polygonal protein scaffolding, a reduced amount of LPC was applied. For experiments with lowered LPC, 10×DPPC containing, polyhedral nanoerythrosomes were used. The polyhedral nanoerythrosomes were mixed to a suspension of LPC, in a way to achieve a final LPC:DPPC:ghost-lipid ratio of 0.5:10:1 (n/n ratios). As the LPC was mixed to the nanoerythrosomes having 10×DPPC (after the establishment of their protein scaffolding), the impact of the lysolipid on the scaffolding could be examined.

According to the freeze fractured TEM images, the shape and size of the nanoerythrosomes are not affected by the small amount of LPC additives (Figure 43/A,B),
but the protein scaffolding changed significantly. The protrusions, expressed by the 10×DPPC containing nanoerythrosomes (Figure 43/A) were abolished in the presence of 0.5×LPC (Figure 43/B). The surface of the nanoerythrosomes became homogeneous, without the presence of the angular network, but instead of being smooth they were roughly particulated. It seemed that the particles of the protein network were evenly dispersed on the surface of the nanoerythrosomes after the addition of 0.5×LPC to the suspension.

Figure 43. Morphological (A,B) and structural (C) changes in DPPC enriched nanoerythrosomes due to a low (0.5×) amount of LPC additive (111)

Structural changes in nanoerythrosomes due to lysolipid addition could be observed by SAXS measurement (Figure 43/C). On the scattering curve of the nanoerythrosomes, having 10×DPPC and 0.5×LPC, two differences could be seen, compared to the scattering of 10×DPPC containing nanoerythrosomes. The monotonously decreasing beginning section of the scattering curve corresponds to the protein particles of the suspension. This section is increased significantly when LPC was present. This is the consequence of the interaction between LPC and the proteins resulted in a destruction of the protein scaffolding producing smaller scattering units. At the same time, the characteristic form factor of the bilayers did not change and appear as a broad peak in both cases. These features of the SAXS curves indicates the LPC molecules likely interact with the protein constituents of scaffolding causing their presence in the bilayers unobservable.

To summarize the results, the addition of lysolipid had a strong impact on the structure and surface morphology of nanoerythrosomes, even in its smallest concentration (0.5×LPC) (Figure 43). Due to its conical shape, LPC enhanced the formation of
extremely small nanoerythrosomes (with an average diameter of 46 nm) when applied in (2×LPC) concentration (Figure 42/A). If the amount of the lysolipid additive was risen to fivefold, its detergent effect became dominant and the resulted particles were rather micelles or dispersed proteins than vesicles (Figure 42/B). In its smallest concentration (0.5×) the LPC had an effect on the vesicles morphology by dissociating the protein scaffolding and resulting in homogenous nanoerythrosomal surface. The observations suggest that LPC could be useful for tailoring the nanoerythrosomes on a wide structural range.
Summarization of the results

Characterization of extracellular vesicles derived from “in-vivo” systems

I carried out the characterization of extracellular vesicles, derived from Jurkat T lymphocytes and red blood cells. The characterization techniques involved freeze fracture combined transmission electron microscopy and dynamic light scattering, for the examination of the vesicles morphology and size distribution, respectively. It was concluded, that the extracellular vesicle subpopulations could be distinguished according to their characteristic size. The exosomes, isolated by me, had a size distribution between 60-150 nm, the microvesicles appeared to be larger, approximately 100-400 nm in size. The third type of extracellular vesicles, the apoptotic bodies were significantly larger with a typical diameter between 0.5-4 μm. These values are in good correlation with the literature. The mean diameters of the isolated subpopulations were 100 nm, 180 nm and 1500 nm, for exosomes, microvesicles and apoptotic bodies, respectively. The isolated vesicles typically had a spherical shape and according to FF-TEM micrographs the vesicles had a unilamellar form. The surface of all the three vesicle populations were covered by protein particles and their associated forms. The membrane particle distribution was homogeneous in the case of apoptotic bodies, but on microvesicles and exosomes the particles were associated heterogeneously.

An interesting, novel mechanical property of the extracellular vesicles was observed by linear- (LD) and circular dichroism (CD) spectroscopy. It was revealed that the red blood cell derived extracellular vesicles could be deformed into an anisotropic form in a Couette-flow cell, whereby the degree of deformation depended on the composition and storage time of the isolated vesicles.

My extracellular vesicle isolations showed higher protein content – measured by Bradford protein assay – in the case of apoptotic bodies, than for exosomes and microvesicles. FTIR spectroscopy served with further details from the secondary structure of the vesicles. The ratio of α-helixes, β-sheets, intermolecular β structures showed variance depending on the vesicle populations and their cellular origin. By the calculation of certain integrated IR band intensities, the ratio of vesicular proteins and lipids could be determined. This – so called spectroscopic protein to lipid – ratio served with a possibility to distinguish extracellular vesicle subpopulations, which is highly important from a diagnostic point of view.
Preparation of nanoerythrosomes from red blood cell ghost membrane

The micrometric sized, planar ghost membranes were transformed into spherical nanoerythrosomes by sonication. The size of the resulting nanoparticles depended on the intensity of the sonication process, the average diameter of the vesicles was between 150 and 60 nm. The surface of the nanoerythrosomes was covered by 5-10 nm large proteins and their 20-30 nm large associates in a heterogeneous distribution. In the aqueous medium similar sized particles could be found (referred to as extra membrane particles – EMPs, in the following paragraphs). By ultracentrifugation a typically nanoerythrosome containing pellet and an EMP containing supernatant could be achieved. The protein content of the two separated fractions significantly differed from each other. According to MS analysis the supernatant contained 79- and the pellet 69 different proteins. The four most frequent proteins in both fractions were the same (spectrin α, β, ankyrin and „Band-3”). The spectroscopic analysis showed no significant difference between the protein conformation of the nanoerythrosomes prior and after of the sonication process.

The phospholipid guest molecules, which were applied to structurally modify the ghost membranes (dipalmitoyl-phosphoethanolamine (DPPE), dipalmitoyl-phosphocholine (DPPC), lysophosphatidylcholine (LPC)) transformed the ghosts. The transformation changed the structure and morphology of the precursor membranes significantly. The degree of transformation depended on the type of the lipid additive as well as the ratio of the ghost and guest molecules.

The addition of DPPE resulted in laterally extended 200-800 nm wide, planar membranes, which showed highly ordered stacks of layers on the FF-TEM images.

The addition of DPPC formed typically 150 nm large, spherical nanoerythrosomes. By changing the amount of the lipid additive (calculated according to the measured protein concentration of the ghost membrane), a change in the vesicles morphology was observable. In the case of both DPPC additive ratios (5× and 10×), a homogeneous nanoerythrosome suspension was formed. When the amount of DPPC was risen to 10×, a polygonal surface pattern (consisting of pentagons and hexagons) appeared on the nanoerythrosomes. The complex physicochemical characterization of this novel type of nanoerythrosome was carried out by FF-TEM, DSC, FTIR and SAXS methods. The temperature dependence of the polygonal scaffolding was observed by temperature controlled FTIR, in consecutive heating/cooling cycles. It was presented that the lipid and protein components have a crucial role in the observed structural alternation. The
temperature induced changes appeared to be reversible. The role of the EMPs in the formation of the polygonal scaffolding was examined by the separation of the EMPs and their selective enrichment with 10×DPPC. It was presented, that the DPPC additive forms polyhedral nanoerythrosomes with the EMPs, similarly to DPPC enriched ghosts.

By the application of LD and CD spectroscopy an enhanced stiffness – and reduced deformability – of the vesicles was seen, caused by the establishment of the polygonal network.

The addition of LPC resulted in drastic transformation of the ghosts. Its effect was visually observable in the case of 5×LPC, by the alternation of refracted light through the suspension. Due to 2×LPC additive, the size of the newly formed vesicles was approximately 40 nm, while in the case of 2×LPC additive the particles were rather micelles than vesicles, with an average size of 20 nm.

A small amount of LPC (0.5×) caused the dissociation of the previously established polygonal network in DPPC enriched nanoerythrosomes. The angular structural elements of the scaffolding were dispersed on the vesicles surface by the low amount of LPC additive.

The presence of calcium ion (10 mM) slightly perturbed the polygonal network of the 10×DPPC containing nanoerythrosomes. The effect of the Ca²⁺ could be seen both by FF-TEM, and IR spectroscopy. In the TEM images associated particles appeared at the centre of the polygons. Furthermore, FTIR spectroscopy revealed the reversible dissociation of the protein scaffolding.

Figure 44. Summarization of the nanoerythrosome preparation process
Az eredmények összefoglalása

"In vivo" rendszerek extracelluláris vezikuláinak jellemzése

Jurkat T limfocitákából és vörösvértétekből izolált extracelluláris vezikulák fagyasztatöreffél kombinált transzmissziós elektronmikroszkópos (FF-TEM) morfológiai vizsgálatát-, valamint dinamikus fényszórással (DLS) történő méretanalízisét végeztem el. Megállapítható, hogy az extracelluláris vezikulák méretben elkülöníthető szubpopulációnkból állnak. Az általam izolált exoszómák (EXO) mérete 60 és 150 nm között van, míg az ennél nagyobb mikrovezikulák (MV) 100 és 400 nm közötti méretűek. A harmadik, extracelluláris vezikula forma képviselői, az apoptotikus testek (AB), ennél lényegesen nagyobb méretűek 500 nm és 4 μm közötti kiterjedésűek. Ezek az adatok megfelelnek az extracelluláris vezikulák egy ismertetett irodalmi érdeknek. A három szubpopuláció jellemző mérete, az előbbiek sorrendjében 100, 180 és 1500 nm. Az izolált vezikulák közelítőleg gömb alakúak. Az FF-TEM képen megfigyelhető, hogy az áttört vezikulák unilamellás formájúak. Mindhárom szubpopuláció felszínét, membránfehérjék egyedi vagy asszociálódott formái borítják. A nagyobb méretű apoptotikus testek felszíne egyenletesen borított, míg a kisméretű exoszómák felületén a fehérjék, és azok asszociátumainak eloszlása egyenletlen. Az extracelluláris vezikulák különleges, eddig fel nem tárt mechanikai sajátságát vizsgáltam lineáris dikroizmus (LD) és cirkuláris dikroizmus (CD) módszerek segítségével. Megállapítható volt, hogy a vörösvértest eredetű mikrovezikulák a forgó Coutte-cellában anizotrop térbeli formátvá deformálhatók, ahol a deformáció mértéke a vizsgált vezikula összetételétől és tárolási idejétől függ. Az általam izolált extracelluláris vezikulák esetén a klaszifikus fehérjemeghatározási módszer (Bradford assay) az exoszómák és mikrovezikulák fehérjetartalmára hasonló értéket mutat, míg az apoptotikus testek esetében a fehérje koncentráció lényegesen nagyobb. A vizsgálatok FTIR spektroszkópiai módszerrel történő kiterjesztése továbbá eredményeként járt: a fehérjék másodlagos szerkezetének jellemző motívumai analízálhatók. Az α-hélix, a β-redő, az intermolekuláris kölcsönhatásokon alapuló β-redő, valamint rendezetlen régiók arányai sejttípusonként és izolált vezikula szubpopulációnként is eltérnek. A spektroszkópiai vizsgálatok további igéretes alkalmazását jelzi, hogy a megfelelő rezgési sávok felhasználásával, a vezikulák fehérjéinek és lipidjeinek aránya is megállapítható. E jellemző az úgynevezett spektroszkópiai fehérje-lipid arány felhasználásával az általam izolált exoszómák és
mikrovezíkulák is megkülönböztethetők, amelynek diagnosztikai szempontból nagy jelentősége van.

**Nanoeritroszómák kialakítása vörösvértest eredetű ghostmembránból**


Az szerkezet változtatás céljából a ghost membránhoz adott foszfolipid vendégmolekulák (dipalmitoilfoszfatidiletanolamin (DPPE), dipalmitoilfoszfatidilkolin (DPPC), lizofoszfatidilkolin: palmitoilfoszfatidilkolin (LPC)) a gazdarendszert átalakítják. Az átalakulás szignifikáns morfológiai, szerkezeti változással jár, a változás mértéke a lipid fajtájától és gazda–vendég molekulák arányától függ.

DPPE hozzáadás esetén laterális irányban kiterjedt (200 – 800 nm) síkok, az FF-TEM képeken jól megfigyelhető, szabályszerű elrendeződését mutató halmazok jöttek létre.

DPPC adalék hatására tipikusan 150 nm körüli, közel gömbbalakú nanoerythroszómák formálódtak. A lipid adalék kiindulási ghost fehérje mennyiségéhez viszonyított arányát változtatva, lényeges morfológiai változások voltak megfigyelhetőek. Mind az 5×DPPC, mind a 10×DPPC lipid beágyazódását követően homogén nanoerythroszóma szuszpenzió keletkezett. Továbbá a nagyobb DPPC
koncentráció esetében a nanoeritroszómák felszínén ötszögekből és hatszögekből álló, összefüggő poligonális hálózat jelent meg. Az újszerű, eddig az irodalomban nem ismert, felületi fehérje-mintazattal rendelkező nanoeritroszómák komplex jellemzését FF-TEM, kalorimetria (DSC), FTIR és kisszögű röntgenszórás (SAXS) módszerek segítségével végeztük el. Hőmérsékletfüggő FTIR mérésekkel, több fűtési/hűtési cikluson keresztül igazoltuk, hogy a fehérje hálózat mintázata hőmérsékletfüggő és annak változásában mind maguk az asszociálódott fehérjék, mind az asszociációt segítő DPPC molekulák szerepet játszanak. A mérések alapján a fehérje hálózat mintázat-változása reverzibilis. A hálózat kialakulásához vezető fehérje-lipid kölcsönhatást, szeparáltan, az elválasztott membránon kívüli részecskék (EMP) és DPPC molekulák összekapcsolására megfigyeltük. Az EMP:DPPC (1:10 tömegarány) is hasonló felületi mintázatú nanoerythroszómákat eredményezett, mint a 10×DPPC-vel adalékolt ghost.

Az LD és CD módszerek alkalmazásával megfigyeltük, hogy a felületi fehérje hálózat jelenlétére a nanoerythroszómára merészsevő és nyírás igénybevétel esetén nem deformálható, míg a hálózat részleges kiépülése a nanoerythroszómá plasztikusságának növekedésével jár.

Az LPC ghost membránhoz történő hozzáadása drasztikus változásokkal járt. Az LPC hatását jól jelzi, hogy 5× mennyiségben a ghost membránhoz adva a keletkező részecskék fénytörési tulajdonságainak változása már szabad szemmel is követhető. 2×LPC beágyazódását követően átlagosan 40 nm-es vezikulák keletkeztek, míg 5×LPC tömegarány esetében micella-szerű, objektumok kialakulásához vezetett. Az LPC relatív kis mennyisége (0.5×), a 10×DPPC lipid jelenlétében kialakult fehérje hálózatot megbontja és a fehérjék poligonális felületi hálózata a nanoeritroszómák felületén elhelyezkedő rendezetlen hálózattal alakul.

A kalciumion (Ca²⁺) jelenléte a tanulmányozott (10 mM) koncentráció tartományban perturbálja a 10×DPPC-vel kialakult poligonális hálózatot. A Ca²⁺ ion hatása mind morfológiailag (FF-TEM), mind spektroszkópiai úton követhető. Az FF-TEM felvételek, a poligonális hálózat öt- és hatszögeinek centrumában önálló fehérje asszociátumok jelennek meg. A felületi mintázat le és felépülésével járó reverzibilis átalakulás figyelhető meg az FTIR alkalmazásával.
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