Characterization and modification of cell membrane derived vesicles

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From liposomes to exosomes

Vesicles may be one of the most important pharmaceutical delivery systems on the expanding field of nanomedicines. The stability and enhanced circulation time of liposomal drug products in vivo is achieved by a surface polyethylene glycol (PEG) layer. The PEG-lipid conjugated vesicles are unilamellar, approximately 100 nm large, stable, homogeneous systems. Despite their numerous positive properties, the PEG conjugated vesicles have some serious side effects in vivo. 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. Because of their natural composition and biocompatibility in the last few years cell derived vesicles became the new focus point of nanocarrier development.

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 can be 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 the first step on a 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 the 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.

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 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.

Artificial and natural vesicle-like systems exhibit a structural hierarchy, governed by the same physico-chemical principles and their molecular constituents form nearly spherical, shell-structured vesicles by self-assembly. During my work I intended to invest the collected knowledge from the characterization of natural, extracellular vesicles, into the tailoring of vesicle-like nanoerythrosomes and I arranged the tasks into two main goals.

First, I set out to isolate and characterize a wide variety of extracellular vesicles, mainly focusing on their physicochemical and compositional properties. I considered EVs as the examples to follow during nanocarrier development, therefore I aimed to collect information about their structural elements, such as their lipid and protein content. I presumed that the observed properties could be useful in nanoerythroscope development. For this purpose I isolated EVs from (Jurkat) in vitro cell culture and red blood cell suspension, and applied microscopic and spectroscopic characterization techniques.
My second goal was in connection with nanoerythrosomes. From nanoerythrosomal membrane constituents the proteins are determined, both qualitatively and quantitatively due to their role and function in the bilayer. On the other hand a partial exchange in their lipid content seems to be possible. Based on information collected from the literature, I aimed to study the role of the typical lipid component of the inner- and outer leaflets in the modification of the size and shape of the vesicles.

Dipalmitoyl-phosphatidylethanolamine (DPPE), as a typical component of the internal erythrocyte membrane leaflet (where the membrane skeleton connects to the lipid bilayer) seemed to be an appropriate lipid additive. The phosphatidylserines (PSs), as the other main lipid components of the inner leaflet, produce an “eat me” signal when exposed in the outer leaflet, consequently they were excluded as additive lipids.

Beside sphingomyelins, phosphatidylcholines (PCs) are the most abundant phospholipids in the outer leaflet of erythrocytes, therefore I chose dipalmitoyl-phosphatidylcholine (DPPC) as another promising lipid additive. Moreover, I wanted to observe the effect of the palmitoyl-lysophosphatidylcholine (LPC). LPC has the same length and chemical behaviour as DPPC, but has an extremely conical shape, therefore drastic changes in the structure of nanoerythrosomes were expected.

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 - such as proteins and their network formation on nanoerythrosomes, which I paid great attention to in my Thesis.

Examined systems and methods of characterization

Extracellular vesicles

Extracellular vesicles were isolated from red blood cell suspension and from the medium of in vitro (Jurkat T lymphocyte) cell culture, by the mean of differential centrifugation. The different EV subpopulations: apoptotic bodies, microvesicles and exosomes can be sedimented by characteristic centrifugal speeds. The resulted EV containing pellets were suspended in PBS buffer after a washing step.
**Ghost membranes and nanoerythrosomes**

The red blood cells, which were used for ghost membrane preparation, were isolated from the blood of healthy donors. The purified red blood cells were lysed in hypotonic buffer. After the lysis the plasma membrane and the intracellular components could be separated by centrifugation. Haemoglobin free ghost membranes could be achieved as a result of numerous washing steps, in a large volume of hypotonic buffer.

The sonication and extrusion of the micrometric sized ghost membranes results in vesicles with a diameter of approximately 100 nm. The created vesicles are called nanoerythrosomes. During the preparation process we have the ability to incorporate lipid additives into the nanoerythrosomal membranes. The amount of lipid additives are calculated according to the measured protein content of the ghosts. Depending on the type of the additive and the desired effect, lipids were used in $0.5\times$, $2\times$, $5\times$, $10\times$ amount compared to the ghost protein content. The dry lipid films were hydrated by ghost suspensions and were homogenised by sonication. The applied lipids for nanoerythrosomes preparation were DPPE, DPPC and LPC.

**Methods of characterization**

The first step quality control of the isolated vesicles is the determination of their size and polydispersity by dynamic light scattering measurement (DLS). The determination of the total protein content of the suspensions (by Bradford protein assay) gives and estimation for the amount of vesicles in the samples and for the amount of lipid additives in the case of nanoerythrosomes. The visual observation of the particles in the suspension is allowed by freeze fractured transmission electron microscopy (FF-TEM).

Structural analysis of the vesicles were performed mainly by spectroscopic techniques. ATR FTIR spectroscopy was used to examine structures and interactions of the components on the atomic level. The elasticity of the membranes were observed by LD and CD spectroscopy. Small angle x-ray scattering (SAXS) also served with information from the structure of the vesicles, while the phase transitions of the lipid membranes were followed by differential scanning calorimetry (DSC). The stability of the vesicles were observed by zeta potential measurements. The particles – produced by the sonication of the ghost membranes were characterized by LC-MS technique.
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 Ca\(^{2+}\) 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.

Summarization of nanoerythrosome preparation
Thesis statements

1. I isolated extracellular vesicles from Jurkat T lymphocytes and red blood cells. I characterized the size, morphology and protein secondary structure of vesicular subpopulations (exosomes, microvesicles and apoptotic bodies). I showed that the spectroscopic protein to lipid ratio is suitable for the characterization of extracellular vesicle subpopulations that I isolated. I revealed that the extracellular vesicles are deformable by rotation, whereby the degree of deformation depends on the sheer force and the composition of the vesicles as well.

2. I produced nanoerythrosomes from red blood cell ghost membranes. By the addition of tenfold DPPC to the suspension – compared to the original ghost protein content and by sonication of the samples, I produced approximately 150 nm large nanoerythrosomes. The surface of the nanoerythrosomes was covered with a continuous polygonal protein network.

3. I presented that membrane proteins were torn out from the ghost membranes due to the sonication process. I showed that the protein pattern is significantly different in the released protein-particles and the nanoerythrosomes. DPPC addition caused the incorporation of free protein-particles into the nanoerythrosomes and when the lipid additive was applied in tenfold amount, the protein-particles constituted a protein network on the nanoerythrosomes. I realised that in the presence of tenfold DPPC the free protein-particles also formed nanoerythrosomes with protein network, which was similar to the DPPC enriched ghost membranes.

4. I showed that the polygonal protein network with pentagons and hexagons was best established if the DPPC additive was applied in tenfold excess compared to the original protein mass-content of the ghosts. The nanoerythrosomal surface pattern reversibly changed when the suspensions were heated up to 50°C and reappeared when the sample was cooled back to room temperature. This morphological change was the consequence of the mutual structural alteration of the proteins and the chain melting of the DPPC molecules. The protein network resulted in an enhanced stiffness/reduced deformability of the vesicles.

5. LPC as an additive lipid induces the formation of spherical, surface pattern free nanoerythrosomes. In the case of twofold LPC additive – compared to the original protein content of the ghosts – 40 nm large vesicles formed during
sonication. Contrary, fivefold LPC spontaneously resulted in micelle-like particles, without the need of sonication. The created particles had an average size of 20 nm and a narrow size distribution.

**Articles, presentations and posters**

**Publications in connection with the Thesis**

   *Nanoerythrosomes tailoring: lipid induced protein scaffolding in ghost membrane derived vesicles*, Materials Science and Engineering: C, Available online 30 November 2019, 110428

2. R. Deák, J. Mihály, I. Cs. Szigyártó, A. Wacha, G. Lelkes, A. Bóta*  


**Additional publications**

Presentations in connection with the Thesis

1. R. Deák, J. Mihály, I. Cs. Szigyártó, A. Wacha, Z. Varga, A. Bóta

2. R. Deák, J. Mihály, A. Bóta
   Red blood cell derived nanoerythrosomes.
   Lecture in ELETTRA, Trieste 15.02.2019.

3. J. Mihály, R. Deák, I. Cs. Szigyártó, A. Bóta, Z. Varga

Posters in connection with the Thesis

1. R. Deák, I. Gyurkó, T. Kremmer, Z. Varga


   Extracellular Vesicles Studied by Polarized Light Spectroscopy
   VBST meeting, 20 – 22 May, 2016, Twente, Netherland

   A novel approach for characterization of extracellular vesicles by polarized spectroscopy Chemistry towards Biology, Biomolecules as potential drugs (CTB9), 24-27 September, 2018, Budapest, Hungary

**Storage-sensitive red blood cell derived extracellular vesicles (RBC-EVs): an FTIR spectroscopic approach**, International Conference on Advanced Vibrational Spectroscopy (ICAVS), 7-12 July, 2019, Auckland, New-Zealand