Loaded red blood cells as natural micro carriers for drug delivery

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INTRODUCTION

The development of suitable drug delivery systems offers a huge number of advantages in contrast to conventional administration of therapeutics: reduction of toxicity and adverse side effects by keeping the blood concentration within the therapeutic window, protection from premature inactivation and loss of its systemic activity (Rossi L, 2005). A number of attempts have been made to develop controlled drug transport systems using biodegradable and non-immunogenic carriers, through which therapeutic substances can be disseminated throughout the body via the circulating blood stream. Additionally, modifications of the carrier's surface allow direct targeting of specific tissues and organs. Red blood cells (RBC) represent a potential natural drug carrier system due to the ability of their membranes to be opened and resealed (Brähler M, 2006). The morphological, mechanical and biological properties make the RBC an ideal vehicle for a broad spectrum of drugs, enzymes and any other kinds of biologically active substances (DeLoach JR, 1986). The presence of a huge amount of functional groups on the cell surface is a great additional advantage giving many possibilities for attachments of specific peptides or antibodies for targeting the desired site of action (Muzykantov V, 1994). The matured cells are highly specialised for the transport of oxygen and carbon dioxide between the lung and tissues whereas they cover a distance of 250 km in the cardiovascular system. RBC are biconcave shaped cells with a mean diameter of 7 to 8 µm and a thickness of approx. 2 µm. Due to their large surface to volume ratio (140 μm^2 / 90 μm^3) they are very elastic and thus able to pass the smallest capillaries. This extraordinary flexibility, the size, shape, integrity and durability of the RBC membrane is controlled by proteins of the cytoskeleton (Anderson RA, 1981). Human erythrocytes have a definite life span of about 120 days. The RBC membrane consists of 20% phospholipids which are asymmetrically distributed across the bilayer. Whereas phosphatidylcholin and sphingomyelin are essential parts of the outer leaflet, phosphatidylethanolamin and first of all phosphatidylserin (PS) are mainly found on the inner surface. RBC damage or normal cell aging causes a translocation and presentation of PS on the outer membrane to designate the cell for phagocytosis (Bratosin D, 1998). Hence, controlled artificial aging of the erythrocyte membrane allows specific targeting of the RES whereas the circulation time of carrier RBC in most cases depends on the membrane integrity and thus on the loading procedure (Magnani M, 2002).
Here we present studies focussed on optimization of the loading conditions in order to improve a long circulation time. For this we studied the effect of different loading procedures on the integrity of the cell membrane encapsulating model substances (BSA) and nanoparticles (QD, AmphB-nanosuspension). Subsequently, surface modification by the model peptide insulin was performed in order to provoke an uptake of the surface modified and loaded RBC by RBE4 cells in cell culture.

MATERIAL AND METHODS

Human RBC were loaded with FITC-labeled bovine serum albumin (FITC-BSA), quantum dots (QD) and AmB- nanosuspension using several hypotonic dilution methods (preparations A-F). The

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loading was carried out in hypoosmotic buffer (e.g. with osmolartity at 50% rate of hemolysis, **Figure 1**) at 4°C. The general loading procedure is schematically illustrated in **Figure 2**. Freshly drawn whole blood anticoagulated with ethylenediaminetetraacetic acid (EDTA) was used to isolate the RBC by centrifugation. To produce white ghosts (preparation A) the RBC were incubated in 20mOsm/kg lysing buffer (NaH_2PO_3/Na_2HPO_3) at pH8 and washed till no hemoglobin was left in the cells. Red ghosts were only washed once (preparation B). We implemented a pre-swelling procedure for the samples C and E. RBC were incubated with a drug of interest in the hypoosmotic environment till cell swelling and pore opening has taken place. In preparations A and B the isotonicity was reconstituted in with phosphate buffered saline (PBS, pH8). For preparations C and D the cells were lysed at an osmolarity of 70mOsm/kg with PBS and 1M potassium chloride was used for resealing. In preparations E and F the resealing buffer was PIGPA-C (containing adenine, glucose, inosine and pyruvate). After reconstitution of isotonicity and resealing of the membranes, the drug has been entrapped inside. The membrane damage was quantified by Annexin V binding intensity in which loaded and control RBC were incubated with the FITC-labeled protein. Successful drug encapsulation, loading efficiency and RBC membrane integrity was investigated by means of confocal laser scanning microscopy (CLSM), freeze-fracture scanning electron microscopy (SEM) and flow cytometry (FACS).

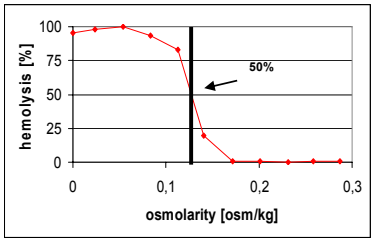

Figure 1: rate of hemolysis

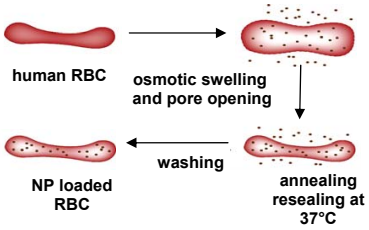

Figure 2: RBC loading procedure

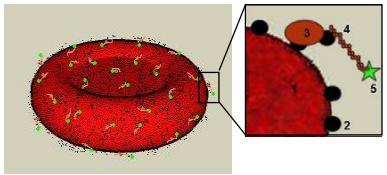

Figure 3: Surface modification of RBC

After loading, the RBC surface was doped by fluorescently labeled insulin (**Figure 3**). Therefore insulin (4) was first conjugated with hydroxysuccinimide ester of the dye PKPF-488 or -633 (5). Then the peptide was linked to sulfo-hydroxysuccinimide-biotin (2). In parallel, the amino- groups of the RBC surface were biotinylated the same way and then conjugated with avidin (3). Finally, RBC (1) and insulin were linked via the biotin-avidin bridge.

The insulin-modified und natural RBC were incubated with a rat brain endothelial cell culture (RBE4). The cells were grown in DMEM with 10% FCS and 1% penicillin/streptomycin inside of an Ibidi µ-Slide 8well chamber and incubated for 24 hours with RBC. The surface modification of erythrocytes and interaction with endothelial cells were studied by CLSM. The immune response caused by carrier-RBC was analysed using Phagotest® and Phagoburst® assays.

RESULTS AND DISCUSSION

Human RBC were loaded with BSA, NPs or phosphate buffered saline (PBS) as a control sample using different loading strategies (A-F). For examination of the membrane integrity affected by the loading procedure, an Annexin V binding test was performed and analysed by FACS. The diagram (**Figure 4**) shows the fraction of Annexin V labelled RBC in dependence on the used loading method (A: white ghosts; B: red ghosts; C, D: KCl-resealing; E, F: PIGPA-C-resealing). As illustrated the red and white ghost preparations evoke the most intense binding of Annexin V due to the strongest disturbance of the cell's integrity during loading. However, white ghosts (sample A) show the highest loading efficiency of about 99,7%. Samples C and E (subjected to a pre-swelling procedure) show a higher PS translocation in comparison to the corresponding samples without pre-swelling (D, F). In contrast to preparation A, sample C only shows a loading efficiency of about 85%. Sample F demonstrates the lowest Annexin V binding rate at all with an effective amount of FITC-BSA encapsulated similar to that achieved in samples A (about 96%). Additionally, this sample arouses less than 1% phagocytosis or burst activity by granulocytes and monocytes in heparinised whole blood samples. Consequently, this drug loading procedure (F) appears as the most gentle and favourable loading procedure possibly achieved by re-energizing of the RBC with glucose and inosine of the PIGPA-C reversal buffer.

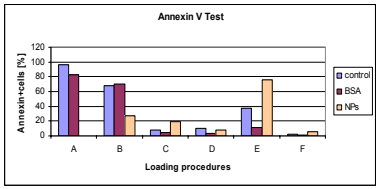

Figure 4: Annexin V test

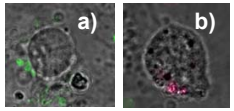

Figure 6 : cell-cell interaction

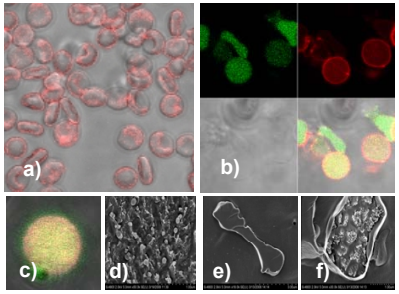

Figure 5 : loaded and modified RBC: a)-c) CLSM micrographs; d)-f) Freeze-fracture SEM

Figure 5 displays some examples of differently loaded and modified RBC: rat RBC with insulin-PKPF633 doped cell membrane (a) and with additional FITC-BSA loading (b); human RBC loaded with QD and coated with insulin-PKPF488 (c); (a-c CLSM images); freeze-fracture scanning electron microscopy of pure AmB- nano-suspension (Staedke V, 2009) (d), unloaded (e) and AmB-loaded human RBC (f;). The CLSM pictures show normally shaped RBC, where the labelling of the membrane was successful. An optimal amount of bound peptide without any deformation of the cell by linking the surface to biotin and avidin could be observed. A biotin overload together with subsequent attachment of avidin caused a strong shape transformation from discocytes to echinocytes. Under conditions of biotin excess on the membrane multivalent binding of avidin occurs. This leads to alterations and damages of the RBC membrane and finally to a deformation of the cell. Such modified RBC would be immediately eliminated by macrophages and by the

complement system in vivo and would no longer exhibit a useful carrier system for targeted drug delivery. In addition, the sterical accessibility of the residual peptide-binding sites will be lost too. The consequence would be a decrease in the insulin-binding capacity. Therefore, an optimal surface density of the biotin-avidin bonds is essential for the success of a targeted drug delivery via loaded RBC.

The peptide attached to the surface was tested concerning its ability to cause uptake of the carrier erythrocytes by endothelial cells. **Figure 6** illustrates RBE4 cells incubated with FITC-BSA loaded RBC (a) and with FITC-BSA loaded RBC additionally modified by attachment of insulin-PKPF633 (b). Whereas surface-modified erythrocytes appear to be incorporated and destructed by endothelial cells, plain FITC-BSA loaded RBC can only be found in the surrounding. Thus, surface modification with insulin seems to be an eminent tool for targeting and enhancing uptake of carrier-erythrocytes into endothelial cell.

CONCLUSIONS

RBC were successfully loaded with diverse model substances such as AmB- nanosuspension, QD and FITC-BSA. Different loading strategies were used to achieve the appropriate surface characteristics according to the desired particular application. The surface of loaded erythrocytes was additionally modified with insulin via the biotin-avidin pair. The insulin-modified surface of the carrier RBC provoked an uptake by endothelial cells confirming the possibility of specific cell targeting by this strategy.

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