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PREPARATION AND IN VITRO CHARACTERIZATION OF CARRIER ERYTHROCYTES FOR VACCINE DELIVERY

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ABSTRACT

Erythrocytes as the most readily available and abundant cells within the body, have been studied extensively for their potential application as drug delivery carriers. In this study, human erythrocytes have been loaded by bovine serum albumin (BSA) as a model antigen/protein using hypotonic preswelling method for targeted delivery of this antigen to antigen-presenting cells (APCs). A series of in vitro tests have been carried out to characterize the carrier cells in vitro, including loading parameters, BSA and hemoglobin release kinetics, hematological indices, particle size distribution, SEM analysis, osmotic and turbulence fragility, and osmotic competency. BSA was loaded in erythrocytes with a loaded amount of 1.98 ± 0.009 mg with antigen release from carrier cells showing a zero-order kinetic consistent to that of the cell lysis. The apparent cell sizes, measured using laser scattering, were not significantly different from normal erythrocytes, but the real sizes, measured using SEM, and surface topologies were quite different between loaded and unloaded cells. The BSA- loaded cells were remarkably more fragile and less deformable compared to the normal cells. Totally, BSA- loaded erythrocytes seem to be a promising delivery system for reticuloendothelial system (RES) targeting of the antigens.

KEYWORDS

Cellular carriers, Carrier erythrocytes, Vaccine delivery, Bovine serum albumin

INTRODUCTION

Cellular carriers, including erythrocytes, leukocytes, platelets, islets, hepatocytes, and fibroblasts all have been exploited as potential carriers for drugs and other bioactive substances in recent decades In this context, erythrocytes offer many advantages over the other cellular carriers in selective and effective delivery of the bioactive agents to any organs, where the modified erythrocytes destruction occurs, like liver, spleen and lymph nodes, generally referred to as reticuloendothelial system (RES). Moreover, the possibility of targeting carrier erythrocytes to non-RES organs has been exploited in recent years, e.g., using homing devices such as IgG or IgM. Also these cells are non-immunogenic and biodegradable; they freely circulate throughout the body and offer ease of preparation; they have the capacity to carry large amounts of drug; and can behave as a slow-release, long-acting system. Depending on the

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extent of changes occurring in cell physiology and/or morphology during antigen loading procedure, one can prepare erythrocytes loaded with antigens, capable of serving as controlled antigen release and/or antigen targeting vehicles. In addition, the possibility of the attachment of different antigenic groups/molecules on the erythrocyte surface with the ultimate goal of qualitative as well as quantitative improvements in vaccination endpoint can be regarded as potential advantages of this delivery system.

Active Substances Encapsulated Into Red Blood Cells: A number of active substances have been encapsulated into RBCs with the specific aim of using carrier RBCs as a slow delivery system. In this case the drug is encapsulated as a non-diffusible pro- drug that is converted into a diffusible drug by RBC resident enzymes and released in circulation. Alternatively the RBCs are used as a drug carrier system. In this case the drug is maintained into the RBCs until these are targeted to and phagocytised by macrophages where their content is released. RBCs have been also used as circulating bioreactors for the degradation of metabolites or xenobiotics. In this case an enzyme is encapsulated into RBCs where it remains catalytically active as long as the cell circulates. These modified RBCs are able to perform as circulating bioreactors when a metabolite, and/or a xenobiotic able to cross the RBC membrane reach the enzyme within the cell. A list of representative constructs is shown[3]

Coupled molecules to RBC membranes: For several years RBCs have been used as diagnostic tools for *in vitro* agglutination studies taking advantage from the presence of external bound molecules. More recently several methods have been developed to couple molecules of interest to RBCs for their *in vivo* use. Selected examples include the biotinylation of RBCs: this was useful for the evaluation of RBC survival or blood volume determination [93], [94] and [95] but has been also extensively used for the coupling of other biotinylated molecules by way of an avidin bridge. The coupling of fibrinolytic agents, such as clinically applicable tissue-type plasminogen activator (tPA) and urokinase (uPA), could maintaining high fibrinolytic activity without compromising the biocompatibility of the carrier. These were tested *in vivo* in an animal model in which it was demonstrated that blood level and tissue distribution of RBCs carrying tPA or urokinase were similar to those of control RBCs [96]. Furthermore, when compared to plasminogen activators, RBC-coupled enzymes showed a dramatically prolonged life-time and a higher bioavailability in the blood stream of intact animals [96] and [97]. As further examples of plasminogen activators coupled to RBCs, streptokinase retains its ability to convert its substrate, plasminogen, into plasmin *in vitro*, while RBCs carrying both collagen antibody and streptokinase were even able to bind immobilized collagen and degrade fibrin clots formed over the collagen target [98].

Encapsulation of drugs and contrasting agents[1]: The methods and procedures described above could in some cases be adapted to co-encapsulate within RBCs different entities comprising drugs and contrasting agents. Although these developments are limited, it is envisaged that they will increase in the near future due to the possibility of visualizing the *in vivo* behaviour of said erythrocytes and at the same time, reached the goal of delivering proper active substances [99]. The drugs to be encapsulated could be the same discussed above but could also be new chemical entities. Envisaged drugs that could benefit from the procedure of co-

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encapsulation are those that could interact with the contrasting agent when said compound is for example a nanoparticle with a mesoporous surface that facilitates the encapsulation of hydrophobic drugs or poorly soluble drugs. The contrasting agents that could be encapsulated include fluorescent agents with excitation and emission spectra in the infra red region of the spectra and/or superparamagnetic nanoparticles. Said nanoparticles could also be surface optimized to interact preferentially with the drug of interest [100].

Preparation of human erythrocytes: Blood samples were withdrawn by venipuncture from healthy volunteers aged 25–30 years, using 19-G hypodermic needles connected to disposable polypropylene syringes, into pre-heparinized polypropylene tubes. After centrifuging at 600 × *g* for 10 min, the plasma and buffy coat were separated by aspiration, and the remaining packed erythrocytes were washed three times with phosphate-buffered saline (PBS; 150 mM NaCl and 5 mM K_2HPO_4; pH 7.4).

Encapsulation of BSA in human erythrocytes[2]: A modified hypotonic preswelling method described by was used for loading the human erythrocytes by BSA. For this purpose, 1 ml of washed packed erythrocytes was transferred gently to a polypropylene test tube, 4 ml of a hypotonic PBS with osmolarity of 0.67 times that of the eutonic solution was added, and the resulting cell suspension was mixed gently by 10 times inversion. The swollen cells produced were separated by centrifugation at 600 × *g* for 10 min and the supernatant was discarded. A 200 µl aliquot of a hemolysate, prepared by diluting another portion of erythrocytes with distilled water (1:1), was, then, added gently onto the remaining swollen cells. It is assumed that this hemolysate layer plays an important role as an osmotic shock barrier and also as a reservoir of cell constituents for underlying cells and thus prevents them from substantial loss of cellular components near the lysis point. Then, 250 µl of an aqueous solution of BSA (8 mg/ml) was added gently to the cell suspension, and the resulting mixture was inverted gently several times and centrifuged at 600 × *g* for 5 min. Addition of protein solution, mixing, and centrifuging were repeated three more times to achieve the lysis point of the cells. This point, defined as the tonicity value where the cell starts to be irreversibly damaged with the loss of cell contents, was detectable by a sudden increase in transparency of the cell suspension and the disappearance of the distinct boundary between cells and supernatant on centrifuging. At this point, the drug/ protein to be loaded, enters the cell through the orifices occurred upon hemolysis. The erythrocytes were, then, resealed by the rapid addition of 100 µl of hypertonic PBS with an osmolarity of 10 times the eutonic solution, followed by gentle mixing of the suspension by several inversions. Finally, the resulting mixture was incubated at 37 °C for 30 min to reanneal the resealed cells. The carrier erythrocytes obtained by this manner were washed three times using 10 ml aliquots of PBS to wash out the unentrapped BSA and the released hemoglobin as well as other cell constituents during the loading process. In some experiments there was a need for sham-encapsulated cells, which was prepared as described except for replacing BSA aqueous solution with distilled water.

BSA assay: A reversed-phase HPLC method was developed and used throughout this study for BSA assay. The method consisted of a gradient system of 0.1% trifluoroacetic acid (TFA) in water (A) and 0.08% TFA in acetonitril (B) with

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initial A/ B ratio of 70/30 which changed linearly to the final ratio of 35/65 (A/ B) within 20 min. The reversal to the initial condition was, then, occurred within 2 min and finally, the system was re-equilibrated over 8 min (total run time of 30 min). The flow rate was 1 ml/min all over the gradient steps. The analyte separation was carried out using a wide-pore Symmetry 300® C_4 protein analysis column (50 mm × 4.6 mm; particle size 5 µm; pore size 300 Å ; Waters, MA, USA) operated at 40 °C and equipped by the corresponding guard column (Waters). The solvent delivery system used was a double-reciprocating pump (Waters, model 600) and a UV-detector (Waters, model 746), with a wavelength of 280 nm was used for analyte detection with the outputs processed and recorded by a compatible integrator (Waters, model 486). Sample injection was made by a loop injector (Rheodyne®; Cotati, CA, USA) equipped by a 50 µl loop.

To determine the amount of loaded BSA, 0.1 ml of final washed erythrocytes was diluted with 0.1 ml of distilled water to completely lyse the cells. Then, the suspension was centrifuged at 10,000 × *g* for 20 min and the supernatant was filtered through a 0.45 µm syringe filter (Teknokroma, Spain, Prod. No. TR-200507). Finally, 50 µl of the filtrate was injected to the chromatograph.

Loading parameters: To evaluate the effect of any changes in encapsulation method variables on the loading efficiency, three indices were defined as loading parameters:

• Loaded amount, the total amount of BSA encapsulated in the final packed erythrocytes;

• Efficiency of entrapment, the percentage ratio of the loaded amount of BSA to the amount added during the entire loading process;

• cell recovery, the percentage ratio of the hematocrit value of the final loaded cells to that of the initial packed cells, measured on equal volumes of two suspensions.

Protein and hemoglobin release: To investigate the effect of the loading process on the particle size distribution of the erythrocytes population, a laser-based particle size analyzer (Shimadzu, model SALD-2101, Japan) was used. For this purpose, BSA- loaded, unloaded and sham-encapsulated erythrocytes were analyzed while suspended in saline in a dilution according to the instrument operation conditions.

Haematological indices[5]: To exploit the release kinetics of BSA as well as hemoglobin (an indicator of cell lysis) from carrier erythrocytes, 0.5 ml of packed BSA- loaded cells was diluted to 5 ml using ringer solution (calcium 4.5 meq/l, chloride 156 meq/l, sodium 147.5 meq/l, and potassium 4 meq/l) containing 0.01% sodium azide (NaN_3), as an antimicrobial preservative. The suspension was mixed thoroughly by several gentle inversions and, then, was divided into ten 0.5-ml portions in 1.5 ml polypropylene microtubes. The samples were rotated vertically (15 rpm) while kept in 37 °C, using a vertically shaking incubator designed and assembled in-house. At the beginning time of the test and also at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72 h elapsed, one of the aliquots was harvested and after centrifuging at 1000 × *g* for 5 min, 50 µl of the supernatants were injected directly to chromatograph for BSA assay. In addition, the absorbance of another 0.3 ml portion of the supernatant was determined at 540 nm using an UV/visible spectrophotometer (Cecil, model

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9000, UK) to monitor the hemoglobin release. Our tests on the possible interference of the cell matrix used in this study with hemoglobin absorbance at 540 nm showed that there is no significant interference in this context and, also, BSA or other cell constituents released has no significant effect on the absorption pattern of hemoglobin in the visible wavelength range. These experiments were carried out in triplicate and the percent of BSA and hemoglobin release was determined in reference to a completely lysed sample (100% release) which was prepared by adding distilled water instead of ringer solution to one replicate of the above mentioned samples. The hematological indices of three types of erythrocytes, i.e., BSA- loaded, unloaded and sham-encapsulated, obtained from the same volunteer were determined using a coulter counter-based instrument (Hematology, model MS9, Sweden). The parameters determined consisted of mean corpuscular volume (MCV; the estimated average cell volume), mean corpuscular hemoglobin (MCH; the estimated average hemoglobin content per each cell) and mean corpuscular hemoglobin content (MCHC; the estimated hemoglobin content per 100 ml of cell volume).

Laser-assisted particle size analysis: To investigate the effect of the loading process on the particle size distribution of the erythrocytes population, a laser-based particle size analyzer (Shimadzu, model SALD-2101, Japan) was used. For this purpose, BSA- loaded, unloaded and sham-encapsulated erythrocytes were analyzed while suspended in saline in a dilution according to the instrument operation conditions.

Scanning electron microscopy (SEM)[6]: To investigate the possible morphological changes of erythrocytes upon loading process, samples from three types of erythrocytes were prepared by, briefly, fixation in glutaraldehyde (4%), followed by osmium tetroxide (1%) treatment, dehydration using a concentration gradient of ethanol from 35 to 100% and, finally, drying using pure hexamethyldisilazane. The prepared samples were, then, analyzed using an electron microscope (model Cambridge, SEM 360, UK) after being coated with gold particles by a Sputter Coater (Fisons, model 7640, UK) in 18 mA for 40 s.

Osmotic fragility: To evaluate the resistance of erythrocytes membranes against the osmotic pressure changes of their surrounding media, 0.1 ml aliquots of the packed samples of each type of erythrocytes, i.e., BSA- loaded, unloaded and sham-encapsulated, were suspended in 1.5 ml of NaCl aqueous solutions having osmolarities of 0–300 mosm/l. After gentle vertically shaking at 37 °C for 15 min, the suspensions were centrifuged at 1000 × *g* for 10 min, and the absorbance of the supernatants were determined spectrophotometrically at 540 nm. The released hemoglobin was expressed as percentage absorbance of each sample to a completely lysed sample prepared by diluting 0.1 ml of packed cells of each type with 1.5 ml of distilled water instead of NaCl solutions. For comparative purposes, an osmotic fragility index (OFI) was defined in each case as the NaCl concentration producing 50% hemoglobin release.

Turbulence fragility[8]: To exploit the mechanical strength of the erythrocytes membranes, 0.5 ml samples of packed erythrocytes of three types (see above) were suspended in 10 ml of PBS in polypropylene test tubes and were shaken vigorously using a multiple test tubes orbital shaker (IKA, model VIBRAX VXR basic, Germany) at 2000 rpm for 4 h. To determine the time course of hemoglobin release, 0.5 ml portions of each suspension were withdrawn at 0, 0.5, 1, 2,

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and 4 h elapsed, and after centrifuging at 1000 × *g* for 10 min, the absorbance of the supernatants were determined spectrophotometrically at 540 nm. The percent of hemoglobin release was determined in reference to a completely lysed cell suspension with the same cell fraction (i.e., 0.5 ml packed cells added to 10 ml of distilled water). To compare the turbulence fragilities of the different types of erythrocytes, a turbulence fragility index (TFI) was defined as the shaking time producing 20% hemoglobin release from erythrocytes (arbitrary value selected based on the usual cell lysis extent occurred in these studies).

Osmotic competency[7]**:** To exploit the mechanical strength of the erythrocytes membranes, 0.5 ml samples of packed erythrocytes of three types (see above) were suspended in 10 ml of PBS in polypropylene test tubes and were shaken vigorously using a multiple test tubes orbital shaker (IKA, model VIBRAX VXR basic, Germany) at 2000 rpm for 4 h. To determine the time course of hemoglobin release, 0.5 ml portions of each suspension were withdrawn at 0, 0.5, 1, 2, and 4 h elapsed, and after centrifuging at 1000 × *g* for 10 min, the absorbance of the supernatants were determined spectrophotometrically at 540 nm. The percent of hemoglobin release was determined in reference to a completely lysed cell suspension with the same cell fraction (i.e., 0.5 ml packed cells added to 10 ml of distilled water). To compare the turbulence fragilities of the different types of erythrocytes, a turbulence fragility index (TFI) was defined as the shaking time producing 20% hemoglobin release from erythrocytes (arbitrary value selected based on the usual cell lysis extent occurred in these studies). The basis for this method is the evaluation of the shape changes induced by changing osmolarities of the erythrocyte surrounding media on unloaded, sham-encapsulated and BSA- loaded cells, using laser-based particle size analysis (Shimadzu, model SALD 2101) as an indicator of cell membrane deformability. For this purpose, 20 µl of each type of packed erythrocytes was added to the sample compartment of the particle size analyzer filled by saline. Progressing amounts of filtered double distilled water were, then, added to the suspensions under continuous stirring until achievement of the lysis point, determined by sudden disappearance of the particle size distribution curves, and upon addition of each portion, the particle size distribution was recorded. For comparative purpose, an osmotic deformability index (ODI) was defined in each case as the average of standard deviations between the starting maximal, intermediate minimal and final maximal diameters of the cell samples.

STATISTICAL ANALYSIS

All the experiments were carried out in triplicate ($n = 3$) and the differences between the results were judged using ANOVA parametric test at a significance level of 0.05.

CONCLUSION

A number of in vitro tests were performed on an erythrocyte -based antigen delivery system consisting of human intact erythrocyte loaded by BSA, because of their potential impact on the practical applicability of this system. The results of our study showed that the carrier erythrocytes have practically acceptable loading parameters, and release their protein content with a zero-order kinetic consistent to that of the cell lysis. The hemoglobin content of the carrier erythrocytes showed significant decrease compared with unloaded cells. Statistical analysis of the diameters of carrier erythrocytes using a laser-based technique showed that the mean diameter of red

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blood cells as well as the dispersion of the diameters around the mean value remains without any significant changes as a result of the loading process and the protein entrapment. The SEM analysis of carrier erythrocytes showed a biconcave to cup-form transformation with a broad variation in erythrocytes shapes and sizes. Both the osmotic and turbulence fragilities of the carrier erythrocytes increased significantly. Deformability test of the carriers showed that encapsulation of BSA in erythrocytes has a decreasing effect on the deformability of erythrocytes.

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