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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; Hypotonic preswelling

1. 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 (e.g., Banker and Rhodes, 2002; Rossi et al., 2005; Hamidi and Tajerzadeh, 2003). 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 (Gutierrez Millan et al., 2004a,b; Hamidi and Tajerzadeh, 2003; Magnani et al., 2002).

Carrier erythrocytes can be utilized, for prolonged periods of time, as circulating drug reservoirs within the circulation (Jain and Jain, 1997; Garin et al., 1996; Jain et al., 1997); they can also function as circulating bioreactors, especially for enzymes (Jaitely et al., 1996; Alpar and Lewis, 1985; Magnani et al., 1989). Also, as aging erythrocytes are normally phagocytized by cells of the reticuloendothelial system, thus, these cells could serve as a natural target for delivery of their payload to these organs. Potential clinical indications for "RES targeting" include iron over-storage diseases (Jaitely et al., 1996), parasitic diseases (Summers, 1983; Talwar and Jain, 1992a,b), hepatic tumors (Al-Achi and Boroujerdi, 1990), and lysosomal storage diseases (Jaitely et al., 1996).

In recent decades, a major goal of researches in vaccine design and formulation has been the development of sustained-release or pulsed-release delivery systems capable of eliminating the requirement for a multiple dosing schedule inherent to the administration of conventional vaccines (Storni et al., 2005; Coombes et al., 1996). This would result in the decreased number of vaccination periods and, therefore, reduced problems with

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patient non-adherence and population coverage (Powell, 1996; Zhao and Leong, 1996). Aside from this, new generation of vaccines are poorly immunogenic and need potent adjuvants to induce an effective protective immunity (Zhou et al., 2003; O'Hagan et al., 1998). Therefore, development of more efficient and safe adjuvant/vaccine delivery systems to obtain high immune responses is of primary importance. Most of the vaccine delivery systems, especially those based on polymeric vehicles, have high immunogenic potency, but are potentially toxic and immunogenic at the same time. Therefore, they may cause non-specific stimulation of host immune system (Giudice et al., 2002; Babiuk et al., 2000; Gupta and Siber, 1995).

Erythrocytes, as the autologous cells of the host body have been regarded as a safe adjuvant/vaccine delivery systems (Murray et al., 2006). Beside the general advantages of erythrocytes as an ideal delivery system, they have some remarkable characteristics for being used as a vaccine delivery system: (i) they can be used for controlled release of vaccines with an aim to reduce the number of doses for primary immunization or to develop single dose vaccines; (ii) they can act as a vehicle to target antigen to antigen-presenting cells (APCs), for instance macrophages and dendritic cells.

Depending on the 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.

Bovine serum albumin (BSA) is a protein generally used in protein/vaccine delivery studies as a model protein (Qiu et al., 2003; Chia et al., 2001; Kyselova et al., 2003) or antigen (Hoshi et al., 1998; Sprott et al., 1997; Ramaldes et al., 1996), owing to its ease of assay, well-known immunogenicity pattern, well-known physicochemical properties, low cost, and general availability (Hoshi et al., 1998). The possibility of BSA encapsulation in erythrocyte ghosts has been evaluated (Prausnitz, 1994). In this study, human erythrocytes freshly prepared from normal volunteers have been loaded by BSA, intended for vaccine delivery, and some important in vitro characteristics of the carrier cells was evaluated.

2. Materials and methods

2.1. Materials

BSA (Sigma, St. Louis, USA; Art. No. A2153) was purchased locally. Other chemicals and solvents were from chemical lab or HPLC purity grades, as needed, and were purchased locally.

2.2. 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).

2.3. Encapsulation of BSA in human erythrocytes

A modified hypotonic preswelling method described by Tajerzadeh and Hamidi (2000) 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.

2.4. 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 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.

2.5. 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.

2.6. Protein and hemoglobin release

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 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.

2.7. Hematological indices

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).

2.8. 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.

2.9. Scanning electron microscopy (SEM)

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.

2.10. 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.

2.11. Turbulence fragility

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 $1000 \times g$ 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).

2.12. Osmotic competency

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.

2.13. 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.

3. Results and discussion

3.1. Loading parameters

The loaded amount of BSA was 1979.25 ± 9.45 μg with entrapment efficiency of $30.06 \pm 0.20\%$. This amount is notable in comparison to those values reported in the literature for a variety of proteins (Gutierrez Millan et al., 2004b; Ihler et al., 1973; Garin et al., 1996; Bird et al., 1982; Updike and Wakamiya, 1983). This can ensure sufficient entry of BSA into the body upon reinjection of fairly low volumes of the packed cells. A cell recovery of about 87.53%, being practically appreciable, is comparable to recovery result of other drugs and proteins reported by others (Magnani et al., 2002; Rossi et al., 2005).

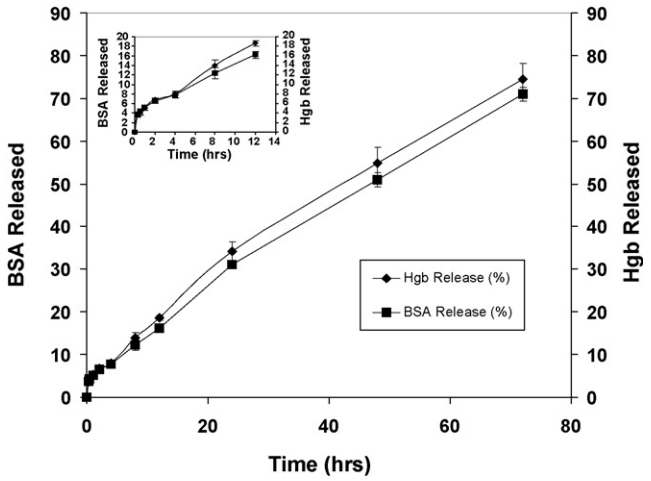

Fig. 1. The release profiles of BSA and hemoglobin (Hgb) from carrier erythrocytes, prepared by hypotonic preswelling method, at 37 °C ($n = 3$). Inset: the expanded release profiles for the early time interval of 0–12 h.

3.2. Protein and hemoglobin release

The kinetic behavior of BSA efflux from the carrier erythrocytes is an important factor affecting the plasma concentration profile of the protein upon re-injection of these microspheres to the host body. As seen in Fig. 1, the efflux of BSA from carrier cells follows zero-order kinetics during the entire experimental period ($r^2 = 0.9924$). In addition, the release profile of BSA has a remarkable consistency to that of hemoglobin. These findings are in agreement with the results published for other polar drugs such as heparin (Hamidi and Tajerzadeh, 2003), gentamicin (Erchler et al., 1986) and enalaprilat (Hamidi et al., 2001), enzymes such as asparginase (Updike and Wakamiya, 1983; Alpar and Lewis, 1985; Kravtzoff et al., 1990) and peptides such as L-lysine-L-phenyl alanine and urogastrone (Hamidi and Tajerzadeh, 2003), and support the theory that polar drugs can leave the intact erythrocytes only when the cells lose their hemoglobin content as a result of hemolysis. Thus, carrier erythrocytes may be a good candidate for targeted antigen and protein delivery to RES. In fact, it is possible to retain the protein inside the carrier erythrocytes for prolonged time periods, until the carriers are trapped in RES. Therefore, in order to obtain a suitable RES-targeting delivery system using this model antigen, controlling the life-span of carrier erythrocytes can be an effective method to achieve the desired profile of delivery.

Table 1
Hematological indices of three types of erythrocytes prepared by hypotonic preswelling method ($n = 3$)

Hematological index	Unloaded erythrocytes	Sham-encapsulated erythrocytes	BSA-loaded erythrocytes
MCV (fl)	92.7 (1.34)	94.6 (1.42)	95.66 (0.94)
MCH (pg)	32.7 (1.56)	18.55 (1.64)[a]	23.83 (2.02)[a]
MCHC (g/dl)	35.3 (1.70)	19.65 (1.78)[a]	24.86 (1.61)[a]

[a] Different from unloaded erythrocytes at a significance level of 0.05.

3.3. Hematological indices

The major hematological indices of three types of erythrocytes have been shown in Table 1. These parameters, being a part of the routing clinical hematology tests, may provide some useful estimates of the biological state of these cells. From the data in Table 1, no significant changes are evident with the entrapment process, both alone or together with BSA loading, on the volume of erythrocytes, as indicated by MCV values ($P > 0.05$). However, both MCH and MCHC decrease following exposure of the erythrocytes to loading procedure with or without BSA, with the extent of decrease being higher in sham-encapsulated cells ($P < 0.05$). This latter observation can be explained by some protective effect of BSA on hemoglobin loss from carrier erythrocytes. The overall hemoglobin loss from the erythrocytes upon loading procedure is quite expected since the procedure is destructive in nature, as the hemoglobin loss in three final washings supports this finding.

In other studies (Ihler et al., 1973; Kravtzoff et al., 1990; Garin et al., 1996), all these parameters have shown decrease in carrier erythrocytes compared to the normal unloaded cells. However, our results show that the loading procedure did not have any significant effect on mean volume. This finding is in agreement with the results of Hamidi et al. (2001). These different findings may be explained by the different measurement techniques and/or data uncertainty because of the indirect nature of all these measurements.

3.4. Laser-assisted particle size analysis

It is obvious from the data in Tables 2 and 3 that the particles are unidisperse (unimodal curves) and both the mean and modal diameters remain without any significant changes, after the loading process ($P > 0.05$). Furthermore, the size uniformity of the erythrocytes population is about the same in three types of erythrocytes (see span values in Tables 2 and 3). This means that the loading procedure has no significant effect on particle size distribution of the erythrocytes. This observation, being consistent with our measured MCV values, is in contrast with the previous report which has claimed that swelling of erythrocytes during the loading procedure causes cell enlargement with higher dispersity of the cell diameters (Hamidi et al., 2001). This observation seems to be technique-dependent, since the sizes of three groups of erythrocytes illustrated in SEM micrographs (see later sections) are apparently different. Since the basis of this technique is laser diffraction by cells and, therefore, an apparent size considering some shape approximations is measured, there may be some deviations from the reality in this method outputs.

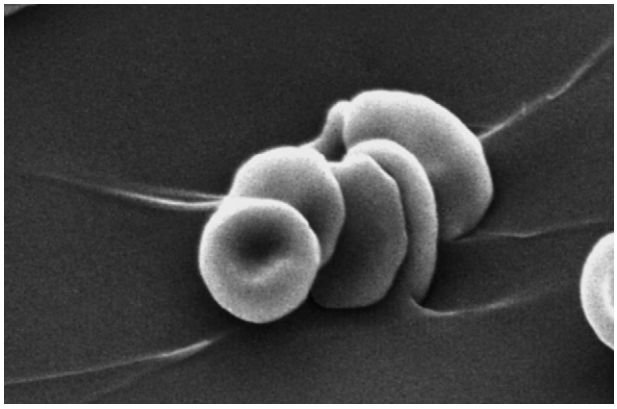

Fig. 2. SEM photographs of unloaded erythrocytes (magnification of 5000).

3.5. Scanning electron microscopy (SEM)

The morphology of the erythrocytes plays a crucial role in their life-span in circulation. As illustrated in Figs. 2–4, the loading process with and without the BSA encapsulation resulted in the formation of cup-form erythrocytes with very disperse sizes. In addition, different stages of biconcave, cup-form, stomatocyte (a particular form of spherocytes with an invagination in one point), spherocyte (spherical erythrocytes), echinocyte and irregular shapes are evident in samples. These findings show that erythrocytes undergo considerable morphological changes during the loading process, a finding which is predictable considering the destructive nature of the loading method. Furthermore, from these micrographs one can conclude that inclusion of BSA itself has no observable additional effect on the morphology of the carrier cells, and that the observed changes are due to the entrapment process.

Table 2
Statistical parameters of the number-based diameter distribution of the erythrocytes prepared by hypotonic preswelling method ($n = 3$)

Statistical parameters	Unloaded erythrocyte	Sham-encapsulated erythrocytes	BSA-loaded erythrocyte
Mean (μm)	5.890 (0.12)[a]	5.266 (0.22)	5.712 (0.14)
Mean S.D.	0.075 (0.001)	0.076 (0.004)	0.074 (0.002)
Median (μm)	5.855 (0.17)	5.239 (0.22)	5.684 (0.19)
Mode (μm)	5.623 (0.09)	5.237 (0.14)	5.623 (0.16)
Span[b]	5.103×10^{-1} (0.03)	5.277×10^{-1} (0.02)	5.280×10^{-1} (0.05)

[a] Mean (S.D.).
[b] A method parameter indicating the dispersity of data and defined as d(N, 0.9)–d(N, 0.1)/d(N, 0.5).

Table 3
Statistical parameters of the volume-based diameter distribution of the erythrocytes prepared by hypotonic preswelling method ($n = 3$)

Statistical parameter	Unloaded erythrocytes	Sham-encapsulated erythrocytes	BSA-loaded erythrocytes
Mean (μm)	6.444 (0.14)[a]	5.781 (0.22)	6.270 (0.17)
Mean S.D.	0.076 (0.002)	0.075 (0.005)	0.075 (0.003)
Median (μm)	6.478 (0.18)	5.799 (0.23)	6.296 (0.13)
Mode (μm)	6.078 (0.11)	5.697 (0.17)	6.078 (0.24)
Span[b]	4.514×10^{-1} (0.01)	4.816×10^{-1} (0.03)	4.752×10^{-1} (0.05)

[a] Mean (S.D.).
[b] A method parameter indicating the dispersity of data and defined as d(V, 0.9)–d(V, 0.1)/d(V, 0.5).

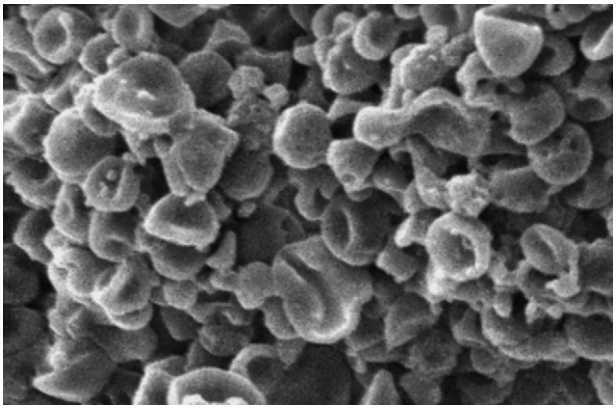

Fig. 3. SEM photographs of sham-encapsulated erythrocytes prepared by hypotonic preswelling method (magnification of 3500).

Other studies have reported the cases similar to our observations. SEM study by Garin et al. (1996) has revealed that the majority of these cells maintain their normal biconcave discoid shapes after exposure to the loading procedure with some spherocytes and a few stomatocytes and, in some cases, a few microcytic erythrocytes (erythrocytes with smaller sizes than the normal cells). Recently, Hamidi et al. (2001) reported the presence of cup-formed cells with different sizes as well as stomatocytes in the samples of carrier erythrocytes analyzed by SEM technique. The extent of irreversible shape changes occurred in carrier erythrocytes compared to normal cells, is a function of the loading method used which, in turn, exert different changes in erythrocytes shape and surface properties.

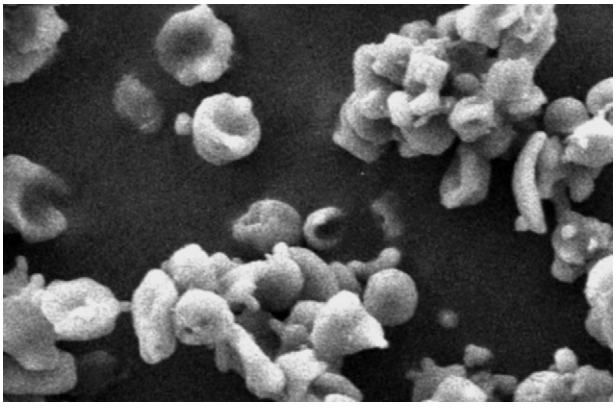

Fig. 4. SEM photographs of BSA-loaded erythrocytes prepared by hypotonic preswelling method (magnification of 3500).

The SEM results in this study indicate some size reduction in regenerated erythrocytes upon the loading procedure. This observation is inconsistent with the results of MCV and laser-assisted particle size determination, both of which are based on the assumption of some regular 'theoretical sphere' shapes for the cells under test. Since as it has been illustrated in SEM micrographs, the BSA-loaded as well as sham-encapsulated cells acquire highly irregular shapes upon loading procedure, the discrepancy between the data obtained from SEM test and hematological indices as well as laser-based particle size analysis can be attributed to the non-spherical shapes of the cells which, in turn, leads to positive deviations in 'calculated sizes' of the cells compared to their real sizes.

3.6. Osmotic fragility

The osmotic fragility curves of the tested erythrocytes are shown in Fig. 5. This parameter is an indicator of the possible changes in the integrity of the cell membrane upon loading procedure and measures the resistance of these cells against the changes in the osmotic pressure of the surrounding media. The osmotic fragility indices (OFIs) of unloaded, sham-encapsulated, and BSA-loaded erythrocytes, being 147, 175 and 202, respectively, indicate that the loading process with and without protein entrapment, results in more fragile cells against the osmotic pressure changes. In addition, incorporation of BSA in cells increases the osmotic fragility of the cells sig-

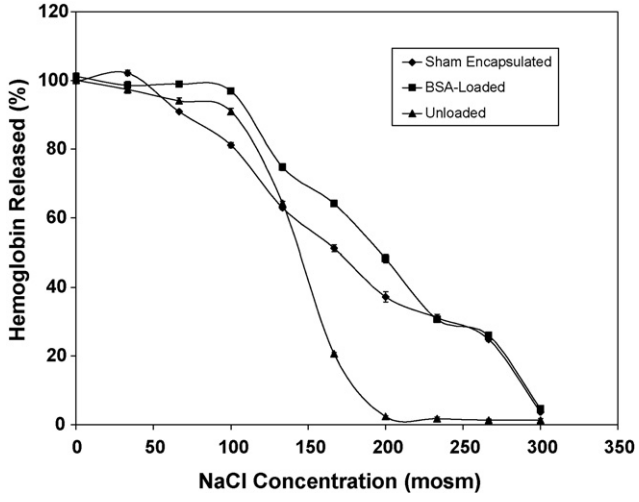

Fig. 5. Osmotic fragility curves of unloaded, sham-encapsulated, and BSA-loaded erythrocytes prepared by hypotonic preswelling method ($n = 3$).

nificantly, a result with no explanation within the scope of this study. As it has also been reported by Hamidi et al. (2001), the trend of osmotic fragility curves, changes from sigmoidal in the case of unloaded cells to linear in the case of both drug-loaded and sham-encapsulated erythrocytes. This is indicative of more heterogeneous cell population in terms of the cell membrane resistance against the extracellular osmotic pressure in the cases of BSA-loaded and sham-encapsulated cells, compared to the normal unloaded erythrocytes.

In most of the studies testing this parameter, osmotic fragility of the carrier erythrocytes was higher than the normal unloaded cells along with a change in the trend of fragility curves from sigmoidal in the cases of normal cells to some linear curves similar to our findings (Kinosita and Tsong, 1978; Jain et al., 1995; Hamidi et al., 2001; Talwar and Jain, 1992b; Garin et al., 1996; Jain et al., 1997). Interestingly, in some cases a decrease in osmotic fragility has been reported which has been explained by a reduction in the average cell volume upon loading procedure, leading to a decrease in the intracellular osmotic pressure (Kravtzoff et al., 1990).

3.7. Turbulence fragility

The turbulence fragility of the carrier erythrocytes is mainly determined by passing the isotonic erythrocytes suspensions through needles with a very narrow internal diameter, e.g., 30G (Talwar and Jain, 1992a,b) or shaking the cell suspensions vigorously (Hamidi et al., 2001) and, in both cases, measurement of the hemoglobin released at different times. The latter method was used in this study. The turbulence fragility of the carrier erythrocytes is a measure of the changes in integrity of the erythrocyte membrane upon loading procedure (similar to the osmotic fragility) and reflects the resistance of the protein-loaded cells against the hemolysis resulting from the turbulent flow within the circulation.

From turbulence fragility curves shown in Fig. 6, the values of TFI for unloaded, sham-encapsulated and BSA-loaded erythrocytes are 3.68, 2.26, and 1.42, respectively. According to these results, the resistance of the erythrocytes against the vigorous turbulent flow shows a decreasing trend from unloaded to BSA-loaded erythrocytes ($P < 0.05$). This test is indicative of production of more fragile erythrocytes upon loading process alone and together with BSA-entrapment. The results of other studies have also shown that the turbulence fragility of the carrier erythrocytes increases significantly in comparison to the normal unloaded cells (Talwar and Jain, 1992a; Jain et al., 1995; Jain et al., 1997).

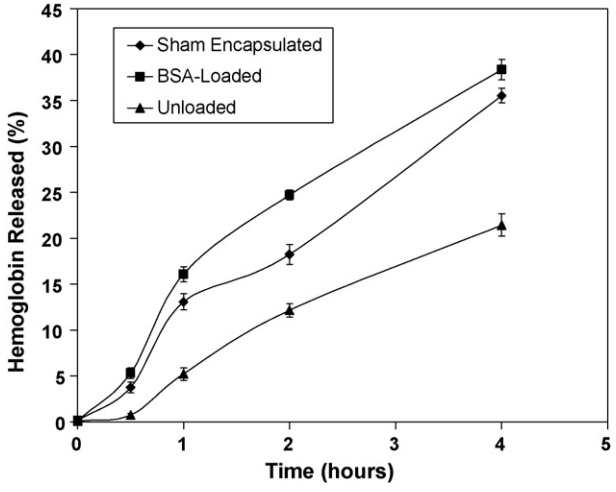

Fig. 6. Turbulence fragility curves of unloaded, sham-encapsulated, and BSA-loaded erythrocytes prepared by hypotonic preswelling method ($n = 3$).

3.8. Osmotic competency

Deformability (shape change) of the carrier erythrocytes is another parameter directly related to the life-span of the cells within the circulation because of the critical role of the deformability in cell survival when passing through the narrow pathways in circulation as well as the RES organs. Furthermore, this parameter is one of the main determinants of the rheologic behavior of the whole blood as a fluid. The deformability of the erythrocytes depends on the viscoelasticity of the cell membrane, viscosity of the cell contents, and the cell surface-to-volume ratio (Jrade et al., 1987).

Different methods have been used for evaluation of the deformability of erythrocytes, including physically-based methods (e.g., passing through filters with a definite pore size or transit time between two points in a capillary with a diameter of about 4 μm) (Jrade et al., 1987) and chemically based methods (e.g., shape changes induced by different concentrations of chlorpromazine HCl on erythrocytes) (Hamidi et al., 2001). In this study, as a result of problems inherent to each of the previous methods, which lead to some unreal cell deformability indices, a physical method, based on the continuous changes of the particle size of erythrocytes as a result of the progressively decreasing extracellular medium osmolarity, was developed and used to evaluate the cell membrane flexibility without using any mechanical or chemical stresses.

The results of the deformability test on three types of erythrocytes are shown in Fig. 7 as the changes in mean particle diameter in response to progressively decreasing osmolarity of the surrounding media. The results of standard deviations between the starting maximal, intermediate minimal and the final maximal diameter of the cells, i.e., osmotic deformability index, in each case, are shown in Table 4. The ODIs of unloaded, sham-encapsulated and BSA-loaded erythrocytes are 0.363, 0.187,

Table 4
The standard deviations between the starting maximal, intermediate minimal and second maximal diameters of erythrocytes prepared by hypotonic preswelling method ($n = 3$)

Sample no.	SD (μm)		
	Unloaded	Sham-encapsulated	BSA-encapsulated
1	0.376	0.168	0.120
2	0.334	0.200	0.125
3	0.371	0.193	0.253
Mean (ODI)	0.363	0.187	0.212
S.D.	0.017	0.016	0.035

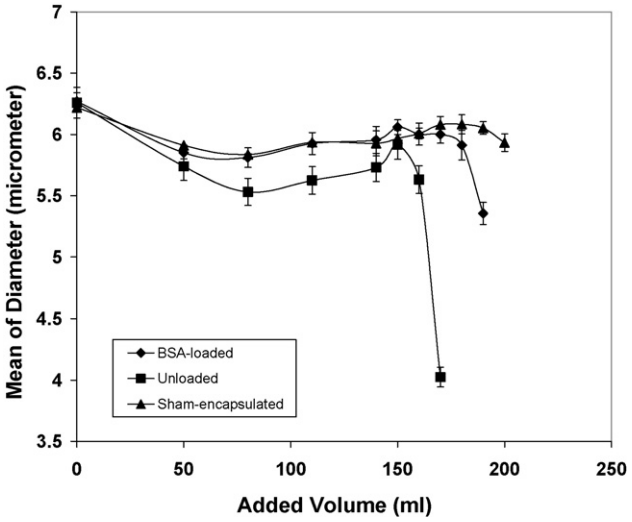

Fig. 7. Osmotic competency curves of unloaded, sham-encapsulated, and BSA-loaded erythrocytes prepared by hypotonic preswelling method ($n = 3$).

and 0.212, respectively. According to the ODI values found and the trends of the curves, entrapment of BSA in human erythrocytes has a decreasing effect on the deformability of erythrocytes exposed to different osmolarities ($P < 0.05$). However, the difference between the ODI values of the sham-encapsulated and BSA-loaded cells is not significant ($P > 0.05$).

Different reports have shown that entrapment procedure results in a significant decrease in deformability of the erythrocytes (Pitt et al., 1983; Field et al., 1989; Hamidi et al., 2001). In other words, the unloaded normal erythrocytes can acquire continuously decreased or increased sizes, because of flexibility of their membrane. However, this flexibility decreases dramatically upon protein loading, causing a lower degree of size changes both in the case of BSA-loaded and sham-encapsulated cells. This finding is of great value in terms of the entrapment of carrier cells in RES organs, a parameter with high importance considering the aim of this study.

4. 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 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.

In general, our study indicated that the erythrocytes undergo some irreversible changes upon the loading process, which, in some cases, are more extensive in the presence of the protein. Collectively, these changes are favorable for a delivery system intended for RES-targeting. However, the relative impacts of different in vitro findings on the overall in vivo antigen delivery efficacy of these cellular carriers remain to be evaluated during the prospective in vivo studies.

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