JPP Journal of Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY

JPP Journal of Pharmacy and Pharmacology

JPP 2011, 63: 322–332

Received April 20, 2010
Accepted September 21, 2010
DOI
10.1111/j.2042-7158.2010.01207.x
ISSN 0022-3573

Research Paper

Preparation and in-vitro characterization of tramadol-loaded carrier erythrocytes for long-term intravenous delivery

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Abstract

Objectives The hypo-osmotic dialysis method was used for preparation of tramadol-loaded human intact erythrocytes. In response to rapid drug escape from the erythrocytes, a membrane cross-linker, glutaraldehyde, was used successfully.

Methods The resulting carrier cells were validated in terms of the accuracy and precision of the whole drug loading procedure.

Key findings The average loaded amount, entrapment efficiency and cell recovery were 1.9041 mg, 95.98% and 85.13%, respectively. The effects of different drug concentrations on loading parameters were studied with the concentration of 10 mg/ml selected as optimal. A series of in-vitro characteristics of carrier erythrocytes, including tramadol release behaviour, haematological indices, particle size distribution, scanning electron microscopy, and osmotic/turbulence fragilities were determined compared with the sham-entrapped and unloaded cells. The results of these in-vitro tests indicated that the erythrocytes did not undergo remarkable irreversible size and shape/topology changes, but the fragility of the membranes of the processed cells were increased.

Conclusions The collective results of this study showed that the optimized method of entrapment was suitable for the encapsulation of tramadol in erythrocytes with the final carrier cells ready to enter the in-vivo animal studies as a promising long-circulating carrier for tramadol.

Keywords carrier erythrocytes; hypotonic dialysis method; long-circulating drug delivery; systemic drug delivery; tramadol

Introduction

Erythrocytes, the most abundant and available cells in the human body, have gained a high degree of interest as a promising particulate drug carrier intended for slow drug release in circulation and site-targeted drug delivery.[1] In 1959, Marsden and Ostling[2] reported the entrapment of dextrans with molecular weights of 10–250 kDa in erythrocyte ghosts. Fourteen years later, the first reports on loading the erythrocyte ghosts by therapeutic agents for delivery purposes were published separately by Ihler *et al*.[3] and Zimmerman.[4] The term 'carrier erythrocyte' was used for the first time in 1979 to describe the drug-loaded erythrocytes.[1]

The normal life-span of an erythrocyte in the systemic circulation is approximately 120 days.[5] As an advantage, in the optimum conditions of the loading procedure (using more gentle methods for loading), the life-span of the resulting carrier cells may be comparable with that of normal erythrocytes.[1,6,7] Erythrocytes have been used as circulating intravenous slow-release carriers for the delivery of antineoplasm agents, antiparasitic drugs, antiretroviral agents, vitamins, steroids, antibiotics, and cardiovascular drugs among others.[1,6–34]

The advantages and drawbacks of using erythrocytes as particulate drug delivery systems have been summarized in Table 1.[1,35–38]

It has been suggested that the treatment of the carrier erythrocytes with certain substances gives rise to alterations in the properties of the loaded erythrocytes. The treatment of loaded erythrocytes with glutaraldehyde enhances their properties as a carrier system, given that it has been observed that erythrocytes treated in this way were more stable, which increased

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Table 1 Advantages and drawbacks of carrier erythrocytes[1,35–38]

Advantages
1. Biocompatibility; particularly in the use of autologous cells;
2. Biodegradability; the lack of toxic biodegradation product(s);
3. No undesired immune responses against the entrapped drug;
4. Considerable protection of the organism against the toxic effects of the entrapped drug e.g. antineoplasms;
5. Remarkably longer life-span of the carrier erythrocytes in circulation in comparison with the synthetic particulate carriers and even comparable with normal cells;
6. Controllable life-span within a wide range from minutes to months;
7. Desirable size range and the considerably uniform size and shape;
8. Protection of the loaded compound from unwanted degradation within the host body inactivation by the endogenous factors;
9. Possibility of targeted drug delivery to the reticuloendothelial system organs;
10. Relatively inert intracellular environment;
11. Availability of the knowledge, techniques, and facilities for handing, transfusion, and work with erythrocytes;
12. Possibility of an ideal zero-order kinetics of drug release;
13. Wide variety of compounds with the capability of being entrapped within the erythrocytes;
14. Possibility of loading a relatively high amount of drug in a small volume of erythrocytes, which in turn assures the dose sufficiency in clinical as well as animal studies using a limited volume of erythrocyte samples;
15. Modification of the pharmacokinetic and pharmacodynamic parameters of the drug;
16. Remarkable decrease in concentration fluctuations in steady state in comparison with the conventional drug-administration methods;
17. Considerable increase in drug dosing intervals with drug concentration in the safe and effective level for a relatively long time;
18. Possibility of decreasing the drug side effects;
19. Due to their natural roles, they are ideal carriers for intravascular drug delivery;
20. Possibility of using synthetic erythrocyte counterparts (artificial erythrocytes);
21. Possibility of using special apparatus for loading (i.e. red cell loader) and thus completely controllable loading procedure;
22. Possibility of loading without extracting the erythrocytes out the patient body (in-vivo loading of circulating erythrocytes).
Drawbacks
1. Being biodegradable, they are removed in-vivo by the reticuloendothelial system. This, although expands its capability to drug targeting, seriously limits their useful life as long-circulating drug carriers and in some cases may pose toxicological problems;
2. The rapid leakage of certain entrapped substances from the loaded erythrocytes;
3. Several molecules may alter the physiology of the erythrocyte;
4. Being from biological origin, entrapped erythrocytes may present variability and lesser standardization in their preparation, compared with other carrier systems;
5. Inaccessibility of many important therapeutic targets like solid tumours, extravascular tissue components, and central nervous system;
6. Safety and technical concerns related to the storage of the loaded erythrocytes;
7. Liability to biological contamination due to the origin of the blood, the equipment and the environment, such as air. Rigorous controls are required accordingly for the collection and handling of the erythrocytes.

their osmotic resistance as well as their resistance to turbulence. It also meant that the output of the encapsulated substance from these erythrocytes into the circulatory flow was reduced.[39]

To the best of our knowledge, inspired by those first reports of 1973 on the loading of erythrocytes by therapeutic agents for delivery purposes, red blood cells have been used as the delivery system for numerous therapeutic agents, both small and large molecules.[3,4] They have experienced most of the clinical applications among cell-based drug delivery systems.[40] In 1977, Beutler *et al*.[41] used placental glucocerebrosidase entrapped into erythrocytes for a selective delivery to macrophages for the first time. Adenosine deaminase, L-asparaginase, desferrioxamine, thymidine phosphorylase, and dexamethasone are among other drugs entrapped in erythrocytes and tested in human studies.[27–29,42–45] Those findings, in particular recent clinical trials and orphan drug designation of dexamethasone-loaded erythrocytes for the treatment of cystic fibrosis (Orphan Drug Designation EMEA/OD/039/04EU/3/04/230), are the basis for great hope that erythrocytes have considerable potential in the field of drug delivery.

Two major approaches have been in use to associate therapeutic agents and erythrocyte carriers. Carrier erythrocytes can be obtained either through the most widely used encapsulation methods or by reversible or irreversible attachment of the drug to erythrocyte membrane.[1] Several methods have been reported for encapsulation of drugs or other bioactive agents in erythrocytes. Preparation of carrier erythrocytes using osmosis-based methods is the usual way of encapsulating substances in erythrocytes. In the most commonly used method of this type, hypotonic dialysis, the erythrocytes are exposed to a hypotonic buffer solution while being kept in a dialysis sac for a relatively long time period, followed by immersion of the sacs in two successive isotonic and hypertonic buffers to restore the openings that have occurred in the cell membranes. The drug intended to be loaded is incorporated usually in hypertonic and/or isotonic media. With hypotonic dialysis, erythrocytes best retain their biochemical and physiological characteristics, which, in turn, results in long-circulating apparently normal shaped carrier cells.[46]

Tramadol is a centrally acting opioid-like analgesic widely used in different pain states used for chronic drug manage-

ment. Tramadol hydrochloride is a white, bitter, crystalline and odourless powder. It is readily soluble in water and ethanol and has a pK_a value of 9.41, molecular weight of 299.8 and partition coefficient (logP) of 1.35 at pH 7.[47] In this study, tramadol was selected as a model for chronic systemic delivery using long circulating carrier erythrocytes prepared by a simple hypotonic dialysis method. The loading parameters were measured and validated and the resulting optimum carrier cells were characterized *in vitro* extensively.

Materials and Methods

Materials

Tramadol hydrochloride was kindly donated by Alborz Darou Pharmaceutical Co. (Ghazvin, Iran). The dialysis tubing (molecular weight cut-off 12000) and glutaraldehyde solution (25% aq.) were from Sigma, St. Louis, MO, USA. All other chemicals, solvents and reagents were from Merck, Darmstadt, Germany with HPLC purity grades, whenever needed.

Methods

Preparation of human erythrocytes

Blood samples were withdrawn by venipuncture from healthy volunteers aged 20–35 years using 19-G hypodermic syringes and transferred to preheparinized polypropylene test tubes. After centrifuging at 3000*g* for 10 min, the plasma and buffy coat were separated by aspiration, and the remaining packed erythrocytes were washed three times using phosphate buffer saline (PBS: NaCl 150 mmol/l, K_2HPO_4 5 mmol/l; pH = 7.4).

Drug assay

A reversed-phase HPLC method with UV detection was developed and used throughout the study to quantitate the tramadol concentrations in samples of loaded erythrocytes. The chromatographic system consisted of a C_{18} column (150×46 mm, 5 μm; Teknokroma, Barcelona, Spain) with a precolumn guard with the same packing and a binary mixture of phosphate buffer (0.01 M, pH 5.9) and acetonitrile (70 : 30) as stationary and mobile phases, respectively. A pump-controller unit (Smartline, model 1000, Knauer, Berlin, Germany) and a Rheodyne injection device (Rheodyne, Model 71251, IDEX health & Science, Rohnert Park, CA, USA) equipped with a 100 μl loop were used for solvent delivery (flow rate 0.75 ml/min) and sample injection, respectively. The analyte detection was made by a UV-detector (model 2500, Knauer, Berlin, Germany) at a wavelength of 218 nm. The chromatograms were processed using compatible software (EuroChrom, Knauer, Berlin, Germany).

Encapsulation of tramadol in human erythrocytes

An optimized hypotonic dialysis method using a very simple set up developed in-house was used for loading the human erythrocytes with tramadol. For this purpose, 0.75 ml washed packed erythrocytes was suspended and mixed gently in 0.25 ml isotonic PBS within a dialysis sac (molecular mass cut-off 12000, 1.5×6 cm), which was then closed and sealed at both ends with plastic clips. Each dialysis bag was placed in a 150-ml glass beaker and supported firmly by wedging the dialysis clips against the base. Dialysis was performed against 100 ml hypo-osmotic phosphate buffer (KH_2 PO_4 5 mmol/l, K_2HPO_4 5 mmol/l, pH = 7.4) with osmolarity of 100 mOsm at 4°C while kept in an ice bath for 30 min, with very gentle stirring (less than 10 rev/min) at all stages. The erythrocytes were annealed by transferring the dialysis bag into 100 ml PBS (20 min, 37°C), containing 10 mg/ml tramadol, except for the experiments evaluating different drug concentrations, followed by transferring them to 100 ml hypertonic buffer (prepared as described for PBS except for the addition of two times the NaCl concentration) containing the same tramadol concentration for 30 min at 37°C. Finally, the carrier erythrocytes obtained by this manner were washed three times using 10-ml PBS by centrifugation to wash out the unentrapped tramadol and the released haemoglobin and other cell constituents during the loading process.

In some experiments sham-encapsulated erythrocytes (i.e. the erythrocytes under complete loading procedure with no drugs added) were needed. These were prepared as described except that the tramadol aqueous solution was replaced by blank (drug-free) buffer solution during the procedure.

Loading parameters

To evaluate the drug loading in the final erythrocyte carriers, three indices were defined as loading parameters. These were: loaded amount (the amount of tramadol entrapped in 1 ml of the final packed erythrocytes); efficiency of entrapment (the percentage ratio of the loaded amount of tramadol, to the amount expected if the drug entered the cells freely in the encapsulation stage and then was completely retained within the cells); and cell recovery (the percentage ratio of the haematocrit value (% volume of packed erythrocytes to total volume of supernatant and pack cells after centrifugation) of the final loaded cells to that of the initial packed cells, both measured using equal suspension volumes).

Effect of concentration of tramadol solution

The encapsulation procedure was performed, as described, using tramadol concentrations of 5, 10, 20, 50 and 100 mg/ml in the isotonic and hypertonic dialysis stages, with the loading parameters determined in each case.

Glutaraldehyde effect

To improve the loaded amount of tramadol in final carrier erythrocytes, different volumes of glutaraldehyde, a membrane cross-linking agent, were added to the hypertonic medium from the beginning of the third dialysis stage to reach the final concentrations of 0.005, 0.01, 0.015, and 0.02% (v/v), with the loading parameters being determined in each case.

In-vitro characterization of human intact erythrocytes loaded by tramadol

Drug and haemoglobin release

To exploit the release behaviour of tramadol as well as haemoglobin (an indicator of cell lysis) from carrier erythrocytes, 0.5 ml packed tramadol-loaded cells was diluted to 5 ml using Ringer solution containing 0.01% sodium azide (NaN_3), an antimicrobial preservative for erythrocytes. The suspension was mixed thoroughly by several gentle inversions. The

mixture was divided into ten 0.5 ml portions in 1.5-ml polypropylene microtubes. The samples were shaken vertically (15 rev/min) while incubated in 37°C using a vertically shaking incubator designed and assembled in-house. At the beginning of the test and after 0.25, 0.5, 1, 2, 4, 10, 20, 48, 72 and 120 h, and then at one-, two- and three-week intervals, one of the samples was harvested and after centrifuging at 1000*g* for 2 min, 100 μl of the supernatant was used for tramadol assay. In addition, the absorbance of a 0.3-ml portion of the supernatant was determined at 540 nm using a UV/visible spectrophotometer (model Series 9000, Cecil, Cambridge, UK) to monitor the haemoglobin release. These experiments were carried out in triplicate and the percent of tramadol and haemoglobin release were 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. To determine the possible effect of NaN_3 on cell survival, a series of release test were performed with Ringer solution with and without NaN_3, and, again, the % haemoglobin release was determined in reference to a completely lysed sample.

Haematological indices

The haematological indices of three types of erythrocytes i.e. tramadol-loaded, unloaded and sham-encapsulated, obtained from the same subject were determined using a coulter counter-based instrument (Model MS9, Hematology, Stockholm, Sweden). The tested parameters consisted of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin content (MCHC).

Laser-assisted particle size analysis

To investigate the effect of the loading process on the particle size distribution of the erythrocyte population, a laser-based particle size analysis (model SALD-2101, Shimadzu, Kyoto, Japan) was used. For this purpose, 20 μl of tramadol-loaded, unloaded and sham-encapsulated erythrocytes were analysed while suspended in saline in a dilution according to instrument operation conditions (approximately 1 : 200).

Scanning electron microscopy (SEM)

To investigate the possible morphological changes which occurred on erythrocytes during the loading process, samples of the three types of erythrocytes were analysed using an electron microscope (SEM 360, Cambridge, UK) after dehydration and coating with gold particles by a Sputter Coater (model 7640, Fisons, Sussex, UK) in 18 mA for 40 s.

Osmotic fragility

To evaluate the resistance of erythrocyte membranes against the osmotic pressure changes of their surrounding media, 0.1 ml portion of the packed samples of each type of erythrocyte (tramadol-loaded, unloaded and sham-encapsulated) were suspended in 1.5 ml NaCl aqueous solution, having the osmolarity of 0–300 mOsm/l. After gentle vertically shaking at 37°C for 15 min, the suspensions were centrifuged at 1000*g* for 5 min and the absorbance of the supernatants were determined spectrophotometrically at 540 nm. The released haemoglobin was expressed as % absorbance of each sample to a completely lysed sample prepared by diluting 0.1 ml packed cells of each type with 1.5 ml 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% haemoglobin release.

Turbulence fragility

To exploit the mechanical strength of the erythrocyte membranes, 0.5 ml packed erythrocytes of each of three types were suspended in 10 ml PBS. The samples were shaken vigorously using a multiple test tube orbital shaker (model VIBRAX VXR basic, IKA, Staufen, Germany) at 2000 rev/min for 16 h. To determine the time course of haemoglobin release, 0.5 ml portions of each suspension were withdrawn at 0, 0.5, 1, 2, 4, 8, 12 and 16 h. After centrifuging at 1000*g* for 5 min, absorbances of the supernatants were determined spectrophotometrically at 540 nm. The % release of haemoglobin 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 distilled water). To compare the turbulence fragility of the different types of erythrocytes, a turbulence fragility index (TFI) was defined as the shaking time producing 20% release of haemoglobin from the erythrocytes.

Data analysis and statistics

The results are presented as mean ± standard deviation (SD). Statistical comparisons were performed using a nonparametric multiple comparison Kruskal–Wallis test, or the Mann–Whitney *U*-test, whenever applicable, using SPSS 12 for Windows. The level of significance was taken as 0.05 in all cases. A Dunn's post-hoc test was carried out to analyse the individual differences between the variables, whenever applicable.

Results

Drug assay

The HPLC method produced linear responses in erythrocyte lysate matrix throughout the wide tramadol concentration range of 5–1000 μg/ml with *r*, slope and y-intercept values of 0.9998 ± 0.0001, 0.6799 ± 0.0106 and 0.9122 ± 0.7247, respectively. Limits of detection (LOD) and quantitation (LOQ) of the method were 2 and 5 μg/ml, respectively. Within- and between-run variations of the method throughout the linear range were all less than 10% with corresponding accuracies of more than 90% in all cases. The average drug recovery was determined as 96.25% ± 2.65.

Encapsulation of tramadol in human erythrocytes

Loading parameters

The loaded amount, efficacy of drug entrapment and cell recovery of the method were determined as 1.904 ± 0.004 mg/ml, 95.98 ± 1.39% and 85.13 ± 0.34%, respectively.

Effect of initial drug concentration

The effect of different tramadol concentrations on cell recovery, loaded amount and efficiency of entrapment is shown in Figure 1. Tramadol was added at both isotonic and hypertonic stages, according to Figure 2.

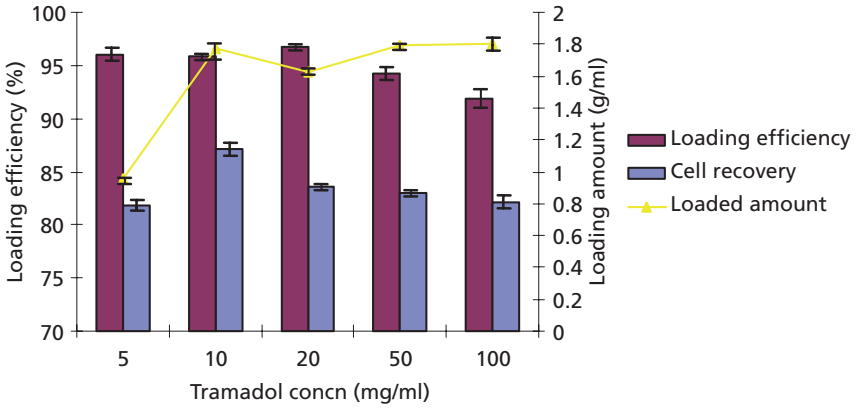

Figure 1 Effect of tramadol concentration on cell recovery, loading amount and efficiency of entrapment of tramadol. $n = 3$.

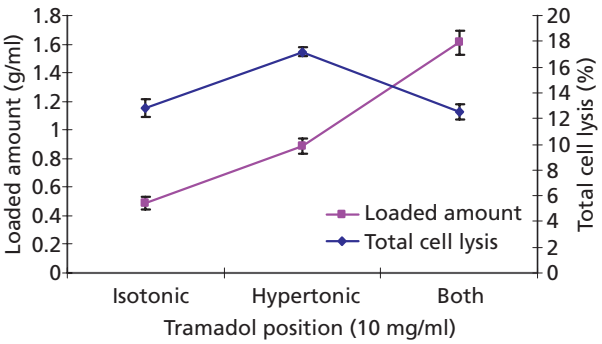

Figure 2 Effect of tramadol position in procedure on loaded amount and total cell lysis $n = 3$.

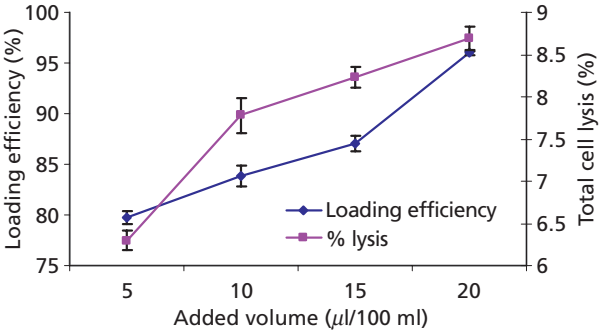

Figure 3 The effect of glutaraldehyde added volume on loading efficiency and total cell lysis $n = 3$.

Based on the data from the different drug concentrations added in different stages, the tramadol concentration selected as optimal was 10 mg/ml. This was added in both isotonic and hypertonic stages of the loading procedure.

Glutaraldehyde effect

The optimized glutaraldehyde concentration was selected as 5 μl/100 ml (0.05 μl/ml) using a glutaraldehyde aqueous solution of 5% (v/v).according to Figure 3. The effect of this amount of glutaraldehyde on the loading efficiency and the loaded amount is shown in Table 2.

Table 2 Loading amount and efficiency with and without addition of glutaraldehyde

Glutaraldehyde (μl/100 ml)	Loaded amount (g/ml)	Loading efficiency (%)
Without glutaraldehyde	0.0439 ± 0.0032	2.38% ± 0.9801
With 5 μl/100 ml glutaraldehyde	1.7741 ± 0.0054	95.98% ± 0.1300

In-vitro characterization of human intact erythrocytes loaded by tramadol

Drug and haemoglobin release

The release profiles of tramadol and haemoglobin from carrier erythrocytes while being shaken at 37°C are shown in Figure 4. The released haemoglobin profile in Ringer solution was the same with and without NaN_3, as is shown in Table 3.

Haematological indices

The haematological indices of the tested human erythrocytes are shown in Table 4.

Laser-assisted particle size analysis

The typical number-based particle diameter distribution curves of the three types of erythrocytes are shown in Figure 5. The derived statistical measures are shown in Table 5.

Scanning electron microscopy

In Figure 6 the scanning electron micrographs of unloaded, sham-encapsulated, and tramadol-loaded erythrocytes are shown.

Osmotic fragility

The osmotic fragility curves of unloaded, sham-encapsulated, and tramadol-loaded erythrocytes are shown in Figure 7. The osmotic fragility indices of unloaded (as native control), sham-encapsulated and tramadol-loaded erythrocytes were 152 ± 1.009, 135 ± 1.101 and 118 ± 0.008, respectively.

Turbulence fragility

The results of the turbulence fragility test on unloaded, sham-encapsulated, and tramadol-loaded erythrocytes are

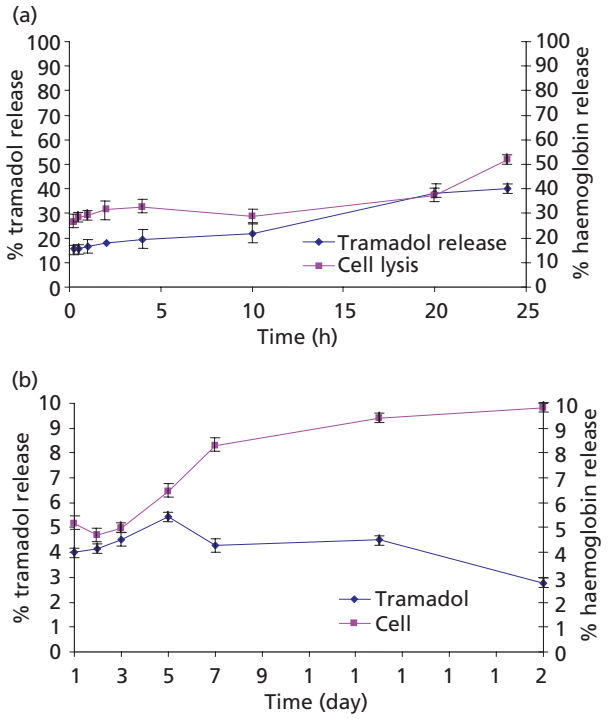

Figure 4 The release profiles of tramadol and haemoglobin from carrier erythrocytes (a) The first 24 h. (b) From day 1 up to day 21. $n = 3$.

Table 3 The percent of released haemoglobin from carrier erythrocytes in Ringer solution with and without NaN_3

Time (h)	Released haemoglobin with NaN_3 (%)	Released haemoglobin without NaN_3 (%)
0.25	2.94 (0.36)[a]	3.22 (0.49)
0.5	4.32 (0.62)	4.12 (0.54)
1	5.71 (0.41)	5.29 (0.71)
2	7.18 (0.68)	6.69 (0.64)
4	7.46 (0.53)	7.94 (0.44)
8	13.09 (0.62)	12.13 (0.85)
12	16.86 (1.14)	17.32 (0.98)
24	31.76 (0.92)	32.29 (1.89)
48	50.84 (1.73)	49.77 (2.23)

[a]Mean (SD). $n = 3$.

shown in Figure 8. From these curves, the values of turbulence fragility indices (TFI) for unloaded (as native control), sham-entrapped and tramadol-loaded erythrocytes were 15.2 ± 0.067, 12.8 ± 0.129 and 7.8 ± 0.004, respectively.

Discussion

Encapsulation of tramadol in human erythrocytes

The loaded amount of tramadol in carrier erythrocytes achieved in this study could ensure sufficient dosing of the drug upon re-injection of a fairly low volume of packed cells into the host body. We reached 95% entrapment efficiency for tramadol in our laboratory. This was remarkable compared with the results published for amphotericin B (15–18%), methotrexate (60%), prednisolone (85.4%) and, obviously, for large molecular weight drugs such as bovine serum albumin (59.8%), β-glucocerebrosidase (40–50%), and insulin (5%), upon loading in erythrocytes using the same method.[13,48–52] The use of glutaraldehyde in this study played a crucial role in improvement of drug loading parameters, from a nonsignificant final drug loading to almost complete drug retention within the cells. This effect could be clearly attributed to the well-established membrane stabilizing/cross-linking effect of glutaraldehyde, which in turn resulted in the decreased permeability of cell membrane to the drug during the final washing steps.

Considering the loaded amount (approximately 1.9 mg/ml) on one hand, and the predicted slow and continuous release of tramadol in the bloodstream on the other hand, it seemed that the acquired loaded amount was appropriate for designing the next step, especially for in-vivo studies.

A cell recovery of approximately 85% (see Results) was practically appreciable and was comparable with values in other published studies of erythrocyte loading with various drugs and proteins.[8,53]

It was reasonable to conclude that the loaded amount of tramadol was related to the tramadol concentration used in the loading procedure. Throughout the concentration range of 5–100 mg/ml, an increase was seen in the loaded amount up to a drug concentration of 10 mg/ml, beyond which the loaded amount remained unchanged. The efficiency of entrapment showed no significant change up to the tramadol concentration of 20 mg/ml, with a slight decreasing trend beyond this range. The cell recovery showed its highest value at the drug concentration of 10 mg/ml, which could be attributed to the harmful effect of the drug on cell viability beyond this limit. Based on these three trends, the concentration of 10 mg/ml tramadol was selected as optimal.

Tramadol was used in both isotonic and hypertonic stages of the loading procedure based on the data obtained, considering the minimal cell lysis along with the maximal loaded drug amount. According to Figure 1, it was clear that the loaded amount of tramadol was somehow related to the concentration of tramadol solution used. Throughout the concentration range of 5–100 mg/ml, we could see an obvious increase when we changed the concentration from 5 to 10 mg/ml, but beyond that, we could see only slight changes. Although the maximum loaded amount was reached using 100 mg/ml, the efficiency of entrapment was better in concentrations lower than 50 mg/ml, and the best cell recovery was seen at the concentration of 10 mg/ml. Due to the acceptable loading amount, entrapment efficiency and the best cell recovery, and also economic considerations in the amount of tramadol which was going to be used, the concentration of 10 mg/ml was selected for use during the process. A suitable amount of loaded tramadol in unit volume of packed carrier cells at the concentration of 10 mg/ml could be completely acceptable for dose adjustment during in-vivo studies on this delivery system.

Table 4 Haematological indices of three types of erythrocytes

Haematological index	Unloaded erythrocytes	Sham-encapsulated erythrocytes	Tramadol-loaded erythrocytes
Mean corpuscular volume (MCV; fl)	92.7 (1.00)[a]	90.1 (1.98)	93.0 (1.79)
Mean corpuscular haemoglobin (MCH; pg)	32.70 (1.56)	27.25 (1.70)	30.12 (0.31)
Mean corpuscular haemoglobin content (MCHC; g/dl)	33.30 (1.58)	30.22 (0.17)	32.40 (0.91)

fl, femtolitre; g/dl, gram per decilitre; pg, picogram. [a]Mean (SD). $n = 3$.

Table 5 Statistical parameters of the number-based distribution of the diameters of erythrocytes

Statistical parameters		Unloaded erythrocytes	Sham-encapsulated erythrocytes	Tramadol-loaded erythrocytes
Mean	Mean (μm)	5.398 (0.0298)	5.283 (0.0277)	5.356 (0.0890)
	SD	0.072 (0.0005)	0.072 (0.0005)	0.073 (0.0017)
Median (μm)		5.376 (0.0375)	5.234 (0.0338)	5.328 (0.2531)
Mode (μm)		5.623 (0.0000)	5.623 (0.0000)	5.623 (0.0000)
Span[a]		0.5070 (0.0038)	0.4941 (0.0025)	0.5062 (0.0008)

Values are mean (SD). $n = 3$. [a]A method parameter indicating the dispersity of data, defined as d(N, 0.9)–d(N, 0.1)/d (N. 0.5).

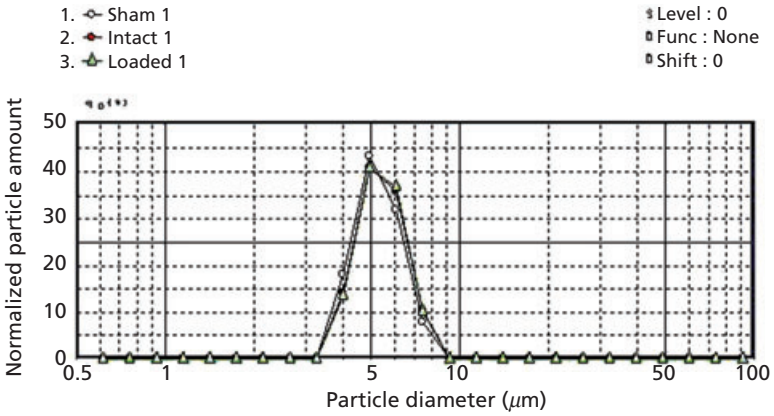

Figure 5 Comparison of number-based diameter distribution of unloaded, sham-encapsulated and tramadol-loaded erythrocytes.

Tramadol was added at both isotonic and hypertonic stages, according to Figure 2, because of the minimal cell lysis, besides the maximal loaded amount. The latter effect may be explained by the higher amount of tramadol in the surrounding media of cells and also the long time that tramadol needed to reach equilibrium, according to a separate study performed in our laboratory (unpublished data).

The optimum glutaraldehyde concentration was selected as 5 μl/100 ml using 5% glutaraldehyde stock aqueous solution according to Figure 3. As can be seen in Figure 3, while the addition of further cross-linker amounts resulted in approximately a 15% improvement in drug loading, the increased cell lysis and the problem of higher amounts of glutaraldehyde residual in the final drug-loaded cells led us to limit the use of glutaraldehyde to 5 μl/100 ml in the surrounding media. In addition, since the purpose of this study was preparation of long-circulating stable and normal carrier erythrocytes and, on the other hand, the effect glutaraldehyde has on the integrity and flexibility of these cells, the cells became more susceptible to detection by the reticuloendothelial system.[39] Therefore, we selected the minimal volume of glutaraldehyde which was able to provide us with acceptable entrapment efficiency. The effect of adding this amount of glutaraldehyde on the loading efficiency and the loaded amount is shown in Table 2. According to Table 2, this glutaraldehyde amount was able to improve the loading efficiency from only 2.38% up to 95.98%, while keeping the cells in their near normal, functional as well as morphological condition.

In-vitro characterization of human intact erythrocytes loaded by tramadol

Tramadol and haemoglobin release

From the drug and haemoglobin release profiles shown in Figure 4, it could be concluded that firstly, tramadol was a slow-release process which obeyed cell lysis kinetics, represented by haemoglobin release, especially during the first hours of the process. In other words, the drug could exit the

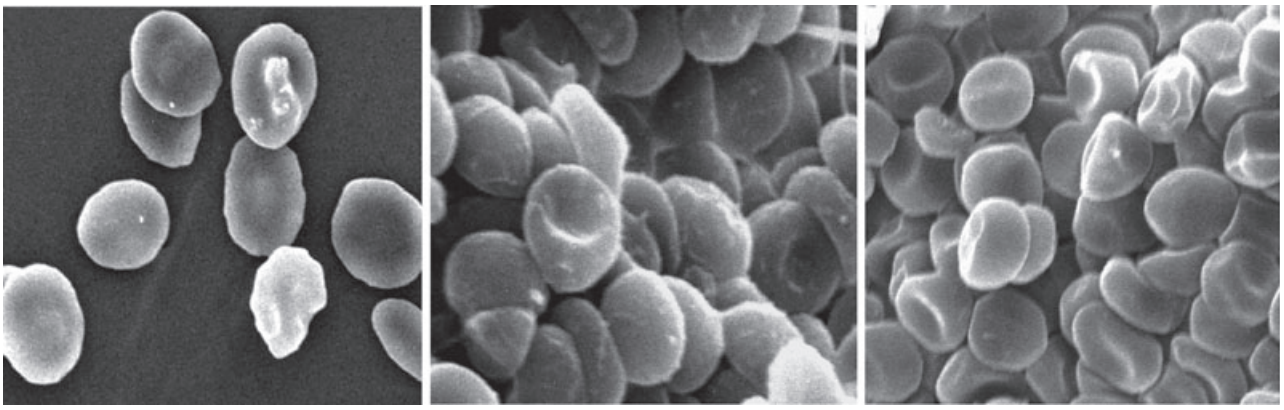

Figure 6 Scanning electron photomicrographs of loaded and unloaded erythrocytes. (a) Unloaded erythrocytes ×3500; (b) sham-encapsulated erythrocytes ×3500; and (c) tramadol-loaded erythrocytes ×5000.

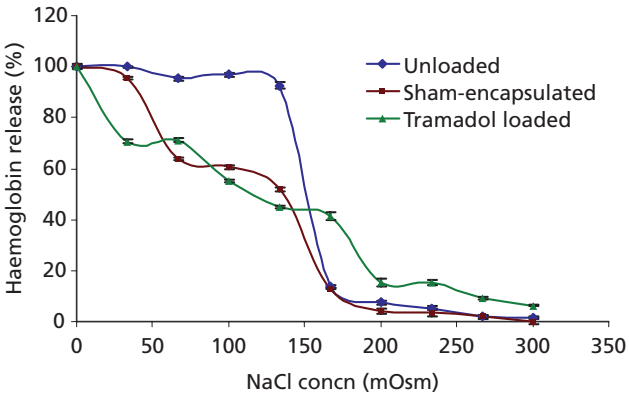

Figure 7 Osmotic fragility curves of unloaded, sham-encapsulated and tramadol-loaded erythrocytes $n = 3$.

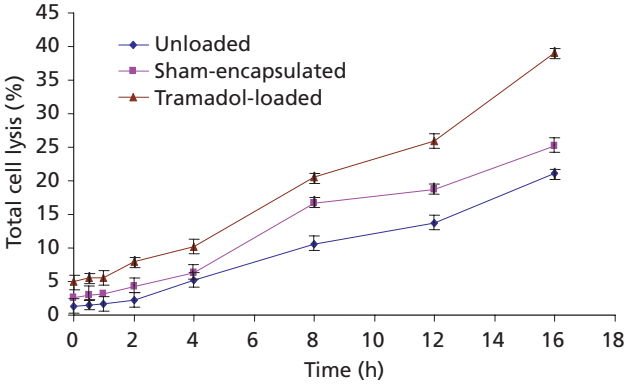

Figure 8 Turbulence fragility curves of unloaded, sham-encapsulated and tramadol-loaded erythrocytes $n = 3$.

cells only when the cell lost its natural state and integrity. This finding was favourable in terms of achieving a long-circulating drug carrier. Secondly, that drug release approached approximately 55% on day 5, and then decreased, probably due to drug stability issues, as opposed to the increasing trend of haemoglobin release up to almost 100% in three weeks. The declining trend of released tramadol from five days onwards could be explained by the presence of destabilizing chemical reactions for tramadol in the experimental condition used for the release test, an effect which was covered early on by drug release from cells, but became significant by progressing the time to days and weeks. Finally, it could be concluded that considering the relatively slow drug release (leak out) from carrier cells, it seemed that there was no need for any further release-controlling factors other than glutaraldehyde to achieve practically feasible rate-controlled tramadol release using this system.

The addition of NaN_3, as antimicrobial preservative, showed no significant effect on the extent of released haemoglobin ($P > 0.05$; Table 3) when compared point-to-point. Therefore, NaN_3 could be used as a suitable preservative in this type of study.

Haematological indices

From the data in Table 4 and according to statistical analysis, no significant changes were evident with the entrapment process alone or together with tramadol loading on all haematological indices tested ($P > 0.01$). This observation was predictable, considering the gentle process condition of the method used for preparation of the carrier erythrocytes. In other studies all these parameters decreased in carrier erythrocytes compared with the normal unloaded cells.[54–57]

Laser-assisted particle size analysis

It was obvious from the data in Table 5 and Figure 5 that the particles were unidispersed (unimodal curves) and all the mean, medium, and modal diameters remained without any significant changes after the loading process ($P > 0.05$). Furthermore, the size dispersity of the erythrocytes population was about the same for the three types of erythrocytes. This meant that the loading procedure had no significant effect on particle size and size distribution of the erythrocytes. This effect was in contrast with previous reports claiming that

some kind of enlargement occurred in erythrocytes during the loading procedure, along with wider dispersity of the cell diameters.[58]

Scanning electron microscopy

As illustrated in Figure 6, the loading process, with and without tramadol entrapment, resulted in the formation of unidispersed erythrocytes with normal sizes and shapes. The presence of some spherocytes (spherical erythrocytes) between processed erythrocytes indicated that when the dialysis sacs were transferred into the hypertonic (i.e. third) stage, their membrane became stabilized immediately, thus leaving no time for their gradual restoring in a complete form. Nonetheless, the majority of the cells regained their apparent normal shape as shown in the SEM micrographs. Furthermore, from these data, we could conclude that inclusion of tramadol itself had no observable additional effect on the morphology of the carrier cells.

Other studies have reported similar findings. An SEM study by Leung *et al*.[59] revealed that the morphological characteristics of most cells were restored to the 'normal' morphological appearance after completion of the dialysis cycle. By contrast, Hamidi *et al*.[33] reported the occurrence of extensive morphological changes as a result of the destructive hypotonic preswelling method used for preparation of the carrier erythrocytes. It was obvious from those studies and the current data that the extent of irreversible shape changes occurred in carrier erythrocytes compared with normal cells, and was a function of the loading method used which, in turn, exerted different degrees of changes in erythrocyte shape and surface properties.

The unchanged erythrocyte shape and topology evidenced in this study, being one of the main determinants in erythrocyte disappearance kinetics in circulation, could be potentially beneficial in terms of a successful long-circulating carrier preparation.

Osmotic fragility

The osmotic fragility indices of unloaded, sham-encapsulated and tramadol-loaded erythrocytes (152 ± 1.009, 135 ± 1.101 and 118 ± 0.008, respectively) indicated that, first, the loading process with and without tramadol entrapment resulted in more fragile cells compared with the normal ones ($P < 0.05$). This was quite predictable considering the use of the membrane fixing agent glutaraldehyde in the encapsulation method. In other words, the processed cells could not respond to the osmotic changes of the surrounding media as shape change and therefore broke up on these occasions. Secondly, the incorporation of the drug itself made the cells more fragile compared with the sham-encapsulated cells. In most of the studies testing this parameter, the osmotic fragility curves (OFC) indicated that dialysed/resealed red blood cells were more resistant to hypotonic haemolysis than normal red blood cells.[40,43]

Turbulence fragility

From turbulence fragility curves, the values of TFI for unloaded, sham-encapsulated and tramadol-loaded erythrocytes were 15.2 ± 0.067, 12.8 ± 0.129 and 7.8 ± 0.004, respectively. These data, being significantly different with unloaded cells ($P < 0.01$), were in very good agreement with the findings of the osmotic fragility test and, thus, confirmed the explanation set front. The results of other studies have shown that turbulence fragility of the carrier erythrocytes increased significantly in comparison with the normal unloaded cells.[1,33]

Conclusions

In early stages of this study, after an extensive evaluation of the hypo-osmotic dialysis method for loading human erythrocytes with drugs, which resulted in the preliminary optimization of method variables in terms of maximal drug loading and minimal final cell lysis, the human erythrocytes were loaded successfully with tramadol, with practically acceptable loading parameters. The loaded cells were evaluated with respect to their in-vitro drug delivery characteristics, including drug and haemoglobin release behaviour, haematological indices, particle size distribution, shape and surface properties, and osmotic and turbulence fragilities. The results of these tests, collectively, were indicative of no notable changes in cell size and morphology, but significant increases in cell membrane fragility. The relative impact of each side in the ultimate success of long-circulating tramadol delivery in systemic circulation using the prepared carrier erythrocytes remains to be investigated by in-vivo studies.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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