Design and Characterization of Anticancer Engineered Resealed Erythrocytes

Department of Pharmaceutics, K.L.E’S College of Pharmacy, JNMC Campus, Nehru Nagar, Belgaum - 590 010, Karnataka, India.

ABSTRACT: A number of investigators have been focusing their attention on the encapsulation of antineoplastic drugs within erythrocytes to diminish their side effects. In this study, human erythrocytes have been loaded by methotrexate (MTX) as a model drug using hypotonic hemolysis method for targeted delivery of this drug. A series of in vitro tests have been carried out to characterize the carrier cells in vitro, including loading parameters, hemoglobin release kinetics, particle size distribution, SEM analysis, osmotic and turbulence fragilities. Carrier erythrocytes having acceptable loading parameters, released their drug content according to zero-order kinetics. Mean corpuscular hemoglobin content values of the cells decreased, the apparent cell sizes measured using dynamic 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 MTX-loaded cells were remarkably more fragile compared to the normal cells. Drug loaded erythrocytes showed preferential drug targeting to liver followed by lungs, kidney and spleen. Totally, MTX-loaded erythrocytes seem to be a promising delivery system for targeting the drug to reticuloendothelial system (RES).

KEYWORDS: Carrier Erythrocytes; Methotrexate; Dimethyl sulphoxide; Gluteraldehyde; Hypotonic hemolysis.

Introduction

MTX is an antifolate that competitively binds to dihydrofolate reductase (DHFR) which inhibits precursors of DNA and RNA and inhibits cell replication. It is an important drug in the treatment of acute lymphoblastic leukaemia (ALL), choriocarcinoma, related trophoblastic tumors and psoriasis. However, it has a short distribution half-life (1.5–3.5 h) and its tumor exposure time is considered short. Consequently, its therapeutic efficacy is impaired by its short in vivo half-life, and its low tumor accumulation produces adverse accumulation in healthy tissue. These processes substantially contribute to its severe and sometimes fatal life threatening toxicities of bone marrow depression, ulcerative colitis, hepatotoxicity, and nephrotoxicity (Chabner BA 2001).

Recently, a number of investigators have been focusing their attention on the encapsulation of antineoplastic drugs within erythrocytes or nanoerythrosomes (Clyde M et al., 2006) Carrier erythrocytes circulate well in vivo, can be targeted to specific organs (liver and spleen), and can carry large quantities of encapsulated drugs. Because, the drugs which are diffuse rapidly from the cells, to prevent the drug efflux, erythrocytes are treated with cross-linking reagents such as gluteraldehyde (Matsumoto S et al., 1986). Gluteraldehyde is a bifunctional reagent, which can link methotrexate to the erythrocyte protein.

Previous experiments demonstrated that subsequent treatment of drug-loaded erythrocytes with glutaraldehyde increased specific targeting of the cells to the liver and decreased the metabolism of the drug inside the erythrocytes (Maternhe CM et al., 1994). That carrier erythrocytes, treated with glutaraldehyde, might be useful in targeting antitumor agents, is supported by promising results reported from preliminary clinical studies of these carriers, administered to patients with massive hepatic metastases and to dogs with lymphosarcoma (Tonetti M et al., 1992).

In this study, the human intact erythrocytes have been loaded by methotrexate using hypotonic hemolysis method with two different crosslinking agents, namely dimethyl sulphoxide (DMSO) and gluteraldehyde, series of in vitro tests have been carried out to characterize the carrier cells in vitro, including loading parameters, hemoglobin release kinetics, particle size distribution, SEM analysis, osmotic fragility, turbulence fragility and in vivo targeting efficiency in rats.

Materials and Methods

Materials

Methotrexate (MTX) was received as a gift sample from Biochem Industries, Mumbai India; Sodium phosphate...
dibasic; potassium dihydrogen orthophosphate; sodium chloride; gluteraldehyde; dimethyl sulphoxide (DMSO); (Loba Chemie, Mumbai, India); acetonitrile; (Ranbaxy Lab, Delhi, India); and Heparin sodium (Biological Evans, Mumbai). All other chemicals were of the best quality commercially available.

**Preparation of erythrocyte suspensions**

Human peripheral blood from healthy donors in Hanks-PBS solution was centrifuged (800×g, 10 min., 4° C) and plasma as well as buffy coat was subsequently removed. RBCs were washed three times in isotonic Hanks phosphate-buffered saline (Hanks-PBS, 136.8 mmol/l NaCl, 5.3 mmol/l KCl, 0.43 mmol/l Na2HPO4, 0.43 mmol/l KH2PO4, 1.5 mmol/l MgCl2, 0.83 mmol/l MgSO4 and 6 mmol/l glucose, pH 7.4). After the last wash, the cell suspension was centrifuged to obtain packed RBCs (50% hematocrit).

**Encapsulation of methotrexate in erythrocytes**

(Marczak A et al., 2006; Dhir SS et al., 1995)

Erythrocytes were used immediately after isolation. Drug was encapsulated within the RBCs by hypotonic hemolysis according to the procedure of Shyamala et al., 1994. Different concentration of two crosslinking agents, 2ml of 0.005% v/v (F1), 0.01% v/v (F2), 0.015% v/v (F3) of DMSO solution and 2ml of 0.005% v/v (F4), 0.01% v/v (F5) and 0.015% v/v (F6) of Glutaraldehyde solution were added. The resealed cells were collected by centrifugation at (800×g, 10 min., 4°C). The collected RBCs were washed with Hanks phosphate-buffered saline and finally lyophilized.

**The content of drug in the extracellular medium**

The content of methotrexate in extra cellular medium after incubation of drug pretreated erythrocytes membrane with dimethyl sulphoxide and gluteraldehyde was determined by UV spectrophotometrically at 304 nm (Shimadzu UV/Vis spectrophotometer, Japan) using Hanks phosphate-buffered saline as blank ((Marczak A et al., 2006).

**Scanning Electron Microscopy (SEM)**

(Milian CG et al., 2003; Carmen L et al., 2001)

MTX loaded erythrocytes were prepared for scanning electron microscopy (SEM) as described by Carmen et al., (2004). Gold coating was performed by sputter coater (Polaron SEM coating system). Finally, cell samples were observed under the scanning electron microscopy (JSM-T330A, JEOL) at a beam voltage of 15 kV.

**Vesicle size and size distribution**

The mean diameter and vesicle size distribution were determined using dynamic light scattering (DLS) technique with a Zetasizer Nano ZS (Malvern instruments, U.K). This technique measures the time dependent fluctuations in the intensity of scattered light, which occurs because the particles are under Brownian motion. Analysis of these intensity fluctuations enables the determination of the diffusion coefficient of the particles, which are converted into a size distribution. This system is equipped with a 4 mW Helium/Neon laser at 633 wavelengths and measures the erythrocyte sample with the noninvasive backscatter technology at a detection angle of 173°. The software used is DTS Nano, version 4.20 supplied by the manufacturer (Malvern instruments, U.K.). Erythrocytes were diluted with PBS pH 7.4 (40% hematocrit) and all measurements were carried out at 25°C.

**Percent hemoglobin release**

To exploit the release kinetics of hemoglobin from carrier erythrocytes, 0.5 ml of packed drug loaded cells was diluted to 10 ml of PBS (pH 7.4), and the suspension was mixed thoroughly by several gentle inversions. Then, the mixture was divided in to 10 1ml portions in 2ml eppendorf safe lock vessels (Eppendorf Nethler Hinz GmbH, Germany). Which is centrifuged at 500 g for 5 min, the supernatant was separated and the absorbance of the same was determined at 540 nm using UV spectrophotometer (Shimadzu UV/Vis, 1201 Japan) to monitor the hemoglobin release (Gothoskar AV et al., 2004)

Percent hemoglobin content was calculated using the formula.

\[
\text{Percent hemoglobin content} = \frac{(A - B) \times 100}{C}
\]

A = Absorbance of sample of 540 nm
B = Absorbance of background at 540 nm
C = Absorbance of 100% hemoglobin released cells

**Osmotic Fragility**

The osmotic fragility of drug loaded resealed erythrocytes was measured by resuspending the cells (45% hematocrit) in a series of hypertonic saline solutions with increasing
osmolality (0 – 300 mOsm/kg) at room temperature over 30 min. The suspensions were centrifuged (800 g for 5 min) and the absorbance (A) of each supernatant was measured at 540 nm. The hemolysis percentage was calculated as the ratio between each A540 value and that of the supernatant at 0 mOsm/kg (Tajerzadeh H et al., 2001).

**Turbulence Fragility**

The mechanical strength of the erythrocytes membranes was determined by using 0.5ml sample of packed erythrocytes were suspended in 10ml of PBS (pH 7.4), and were shaken vigorously using a high-speed multiple test tube shaker (Spinix., Mumbai, India) at 800 g for 2 hr. To determine the time course of hemoglobin release, 0.5ml portions of each of the suspensions were withdrawn in 0, 0.5, 1, 2, 3 and 4 hr and after centrifuging at 500 g for 5 min, the absorbance of the supernatant was determined spectrophotometrically at 540 nm. The percent of release of haemoglobin was determined in reference to a completely lysed cell suspension with the same cell fraction (i.e., 0.5 ml of packed cells to 10 ml of distilled water).

**in vitro drug release studies**

The *in vitro* release of methotrexate from the ressealed erythrocytes formulation was determined using dialysis cassettes (Slide-A-Lyzer®3.5K, Pierce, U.S.A). Briefly, 4 ml of erythrocytes suspension containing known amount of drug was placed in a dialysis cassettes (previously soaked in distilled water for 24 hrs). It was immersed in 50 ml of PBS (pH 7.4), maintained at 37 ± 1°C and stirred with the help of a magnetic stirrer. Aliquots were withdrawn at specific time intervals 1, 2, 4, 8, 12, 24 and 48 hrs from the receptor compartment and the sample was replaced with fresh PBS (pH 7.4) and the hemogonate was centrifuged at 900×g for 30 min. The supernatant was collected and filtered through 0.45 µ filters (Minisart, Germany.) and analyzed spectrophotometrically after suitable dilution with sterile PBS at 304 nm.

**Results and Discussion**

The spectrophotometric method was used to estimate the content of methotrexate in supernatants, after incubation of erythrocyte membrane with different concentrations of DMSO and gluteraldehyde. Analysis of the obtained data indicates that erythrocyte membranes incubated with drug linked only 18% of methotrexate. Content of drug in the medium decreases after treatment of the cell membranes with gluteraldehyde. Noticeably, a decrease of drug concentration in supernatant is observed even with the smallest concentration of DMSO and gluteraldehyde. However, the results are statistically significant when its concentration is higher than 0.005%. The application of DMSO in concentration of 0.010% and 0.015% increases the content by 11% and 16%, whereas gluteraldehyde increases the content by 13% and 21%, respectively in comparison with the erythrocytes incubated with methotrexate only.

 Nine healthy adult wistar rats weighing 200 ± 20 g were selected. The rats were kept on a 12 hour light-dark schedule and they were fasted for 12 hrs. The animals were divided into 3 groups, each containing 3 rats. Group-I received ressealed erythrocytes equivalent to 360µg of methotrexate intravenously in the tail vein after redispersing them in sterile phosphate buffer saline solution. Resealed erythrocytes (gluteraldehyde treated) were selected for the study. Group-II rats received 360µg of pure methotrexate intravenously. Group-III rats were treated as solvent control and were injected intravenously with sterile phosphate buffer saline solution.

After 4 hrs, the rats were sacrificed and their liver, lungs, spleen and kidneys were isolated. The organs were rinsed thoroughly in sterile PBS to remove adhering debris and dried with tissue paper. The individual organs of each rat were homogenized separately by using a micro tissue homogenizer (Remi motors Ltd., Mumbai, India) with 5 ml sterile PBS (pH 7.4) and the homogenate was centrifuged at 900×g for 30 min. The supernatant was collected and analyzed spectrophotometrically after suitable dilution with sterile PBS at 304 nm.

**in vivo tissue distribution studies**

(Carmen L et al., 2001 Stehle G et al., 1997; Mishra PR et al., 2002)

This study was carried out to compare the targeting efficiency of drug loaded ressealed erythrocytes with that of free drug in terms of percentage increase in targeting to various organs of reticuloendothelial system like liver, lungs, spleen and kidneys. Experiments were performed on rats of 200 ± 20 gm weight. All the experiments were carried out in accordance with the protocols approved by the Institutional animal ethics committee (K.L.E.S College, Belgaum, India).

Analysis of the obtained data indicates that erythrocyte membranes incubated with drug linked only 18% of methotrexate. Content of drug in the medium decreases after treatment of the cell membranes with gluteraldehyde. Noticeably, a decrease of drug concentration in supernatant is observed even with the smallest concentration of DMSO and gluteraldehyde. However, the results are statistically significant when its concentration is higher than 0.005%. The application of DMSO in concentration of 0.010% and 0.015% increases the content of linked methotrexate by 11% and 16%, whereas gluteraldehyde increases the content by 13% and 21%, respectively in comparison with the erythrocytes incubated with methotrexate only.
Scanning Electron Microscopy (SEM)

To investigate the possible morphological changes of erythrocytes on loading process, samples of resealed erythrocytes were observed under the scanning electron microscope. Figure 1 (A, B and C) shows the images from scanning electron microscopy at different magnifications (x1500 and x2000). As illustrated, the loading process with drug and different crosslinking agents resulted in the formation of cup-form erythrocytes with very disperse sizes. Native human erythrocytes (Fig. 1 A) show the expected discocytes (biconcave) morphology under SEM. After drug loading and treatment with gluteraldehyde (Fig. 1 B), a slight change in their shape (biconcave) were observed with the same magnification (x1500), and were replaced by spherostomatocytes (uniconcave) coexisting with spherocytes. After drug loading and treatment with DMSO (Fig. 1 C), several membrane invaginations or endovesicles could be observed in this population and some discocytes were coexisting with spherostomatocytes and isolated spherocytes. This shows that, erythrocytes undergo considerable morphological changes during the loading process, which is confirmed by particle size analysis. Furthermore, from these micrographs we can conclude that the inclusion of methotrexate itself has no observable effect on the morphology of the carrier cells, and the observed changes are due to the encapsulation process and the crosslinking agents.

Vesicle size and size distribution

As expected, swelling of erythrocytes during the loading procedure increased both the mean and modal diameters as well as uniformity of the size of erythrocytes. From the data obtained by this test, it is obvious that the loading process, both alone and in combination with drug, increases the mean and modal diameters of erythrocytes, which is higher in the case of drug-loaded erythrocytes. Furthermore, the size uniformity of the erythrocyte population decreased dramatically, particularly in drug loading. This is possibly due to the changes in the natural structure of erythrocyte membrane that resulted from the intensive osmotic shock during the loading process that finally leads to heterogeneous cells. However, it seems that the drug itself have some unique effects on the cell structure such as cell enlargement and more size dispersion of the carrier erythrocytes population. The mean vesicle size was found to be 6.43, 6.96, 7.28, 6.94, 7.10 & 7.42 for formulation F1, F2, F3, F4, F5 and F6 respectively. The possible effects of these changes may affect the in vivo life span of the natural drug delivery system.

Percent hemoglobin release

The release profile of hemoglobin and methotrexate from carrier erythrocytes at 37°C are shown in Figure 2. As seen, the efflux of hemoglobin from carrier cells almost follows zero order release kinetics during the entire experimental period. In addition, the release profile of methotrexate has also influenced by the release of hemoglobin and the rate of drug release is considerably higher than that of hemoglobin. The result also shows that the amount of hemoglobin in drug loaded resealed erythrocytes were less as compared to normal erythrocytes. This may due to loss of hemoglobin from the erythrocytes during the drug loading process and loading of drug in to the cell by replacing the hemoglobin and other cellular constituents.

Osmotic Fragility

It is an indicator of the possible changes in cell membrane integrity and resistance of these cells to osmotic pressure of the suspension medium. This test is carried out by suspending cells in media of varying sodium chloride concentration and determining the hemoglobin released. The results obtained are depicted in Figure 3. In most cases, osmotic fragility of resealed cells is higher than that of the normal cells because of increased intracellular osmotic pressure. In other words, the lower resistance of the carrier cells to osmotic changes is a measure of loss of integrity of the erythrocyte membrane and its natural behaviour. In addition, the change in the shape of the osmotic fragility curve from S-shaped to a relatively linear trend, indicating the presence of more heterogeneous cell population, can confirm a hypo-osmotic hemolysis process.

![Fig 1. SEM photographs of (A) unloaded, (B) gluteraldehyde treated and (C) DMSO treated erythrocytes.](image-url)
Fig 2. The release profiles of methotrexate and hemoglobin from carrier erythrocytes at 37°C. Data represents the mean ± S.E. (n=3). Methotrexate (■ - ■) and Hemoglobin (▲ - ▲).

Fig 3. Osmotic fragility curves of unloaded and methotrexate-loaded erythrocytes. Data represents the mean ± S.E. (n=3). Methotrexate-loaded gluteraldehyde-treated erythrocytes (■ - ■) and Unloaded erythrocytes (▲ - ▲).

Turbulence Fragility

The results of turbulence fragility on two types of erythrocytes are shown in Figure 4. According to these results, the resistance of the erythrocytes against the vigorous turbulent flow shows a decreasing trend from unloaded cells to methotrexate loaded erythrocytes. These indicate that, more erythrocytes that are fragile formed during the loading process alone and together with methotrexate encapsulation. This trend also seen during the osmotic fragility test and this similarity is probably the result of some deviation of the carrier erythrocytes from their normal state, an effect that became more pronounced in the presence of methotrexate during the loading process.
In vitro drug release studies

The release profile of an entrapped drug predicts how a delivery system might function and gives valuable insight into its in vivo behaviour. The two formulations (DMSO and gluteraldehyde treated) of resealed erythrocytes and pure drug methotrexate were subjected to in vitro release studies. These studies were carried out using dialysis cassettes (Slide-A-Lyzer®3.5K, Pierce, U.S.A) in phosphate buffer saline pH 7.4.

The release data obtained for both formulations and pure drug were shown in Figure 5, shows the plots of cumulative percent drug release as a function of time for different formulations and for pure drug. Cumulative percent drug release of pure drug was found to be 98.12% at 12 hrs, where as, cumulative percent drug release for DMSO treated drug loaded erythrocytes were 99.48% and gluteraldehyde treated were 93.31% at 48 hrs. It was observed that drug release from the formulation (DMSO) increased as compared to formulation treated with gluteraldehyde. It also indicates that, when compared with pure drug, the in vitro release of methotrexate from erythrocytes is prolonged over a period of 36 hrs. The in vitro release of both batches of resealed erythrocytes showed an interesting diffusion controlled release throughout the study period, ensure the better entrapment of drug within the erythrocyte membrane.

In vivo drug targeting studies

In vivo drug targeting studies were carried out for formulation treated with gluteraldehyde and with optimal particle size, high entrapment efficiency and satisfactory in vitro release. The comparison between the amount of drug targeted from resealed erythrocytes and free drug in various organs is presented in Figure 6. The average targeting efficiency of drug loaded erythrocytes was found to be 26.5% of the injected dose in liver, 18.51% in lungs, 10.15% in kidney and 10.08% in spleen whereas accumulation of pure drug in liver was 21.52%, in lungs it was 12.96%, in kidney it was 15.73% and spleen 09.00% of the injected dose. These results revealed that the drug loaded erythrocytes showed preferential drug targeting to liver followed by lungs, kidney and spleen. It was also revealed that, as compared to pure drug, higher concentration of drug was targeted to the organs like liver and lungs after administering the dose in the form of resealed erythrocytes, higher drug targeting in liver, as compared to lungs, kidney and spleen may be attributed to uptake of the drug loaded erythrocytes by reticuloendothelial system and large size of liver as compared to other organs.
Fig 5. In vitro release profile of pure drug MTX and resealed erythrocytes treated with two different crosslinking agents. Data represents the mean ± S.E. (n=3). Methotrexate (♦), DMSO-treated erythrocytes (■), Glutaraldehyde-treated erythrocytes (▲).
Acknowledgements

The authors are thankful to Shri S. K. Krishnan, Analytical research (INDAL), Belgaum, India, for providing the facility of SEM. We also thank Biochem Pharma industries Ltd, Mumbai, India, for providing the gift sample of methotrexate.

References


