

Nanoengineered Erythrovesicles: Camouflaged Capecitabine as a Biomimetic Delivery Platform

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Abstract

Aim: Circulation half-life has become one of the major design considerations in nanoparticulate drug delivery systems. By taking cues for designing long circulating carriers from natural entities such as red blood cells (RBCs) has been explored for many years. Among all the cellular carriers including leukocytes, fibroblast, islets, and hepatocytes, RBCs offer several distinctive features. Nanovesicles (NVs) represent a novel transporter for cell signals to modify functions of the target cell. Therefore, NVs play many roles in both physiological and pathological process. **Materials and Methods:** This report highlights biogenesis, composition, and biological rules of erythrocytes derived NVs (EDNVs). Furthermore, we address utilization of EDNVs as novel drug delivery cargo as well as therapeutic target. **Result and Discussion:** EDNVs are biocompatible, biodegradable, efficient drug loading target specificity, and prolonged biological half-life. It is also rich in phospholipids, proteins, lipid raft, and hemoglobin. In this study, nanosize lipoprotein membrane vesicles (EDNVs) bearing capecitabine were prepared by sonication method. **Conclusion:** Developed capecitabine nano erythrocytes conjugate formulation were preliminary optimized on the basis of vesicle morphology, size and size distribution, loaded drug concentration, and *in vitro* release studies.

Key words: Capecitabine, erythrocytes derived nanovesicles, red blood cells, sonication

INTRODUCTION

Capecitabine is a prodrug that is converted to fluorouracil in the body tissue following the oral as well as parental administration. It is widely used in the treatment of metastatic colorectal cancer and breast cancer. The adverse effects associated with capecitabine including bone marrow depression, cardiotoxicity, diarrhea, nausea and vomiting, and dermatitis. Hence, formulating capecitabine as a controlled release dosage form would provide greater or longer *in vitro* and *in vivo* antitumor activity, thereby reducing its toxic side effects. Moreover, its adverse effects are enhanced because the drug must be frequently administered at high doses, due to its limited oral bioavailability. To reduce capecitabine side effects and increase its therapeutic efficacy, several studies have suggested different formulations for capecitabine administration such as transdermal systems, nanoparticles microparticles, and capecitabine loaded erythrocytes.^[1] Pharmacokinetic studies revealed that these formulations achieved sustained release, dose reduction, and improved

stability erythrocytes are preferred as a drug delivery system because they are biocompatible and biodegradable, have a long half-life, and can be loaded with a variety of drugs. Anticancer, antiviral, and antiparasitic drugs are examples of therapeutic agents that have been loaded into nano erythrocytes. Nano erythrocytes (NERS) were used either as a carrier for sustained release of the drugs or to accomplish targeted delivery of the drugs to infected organs.

The safety and utilization of nano erythrocytes as carrier systems have been potentially explored.^[2-4] The major problem encountered in the use of biodegradable natural cells as drug carriers is that they are removed *in vivo* by the reticuloendothelial system (RES) as a result of modifications

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that occur during the loading procedure in cells. Although this expands the capability of erythrocytes to target the RES, it seriously limits their lifespan as long-circulating drug carriers in circulation and, in some cases, may pose toxicological.^[5]

Utilization of erythrocytes as a drug carrier in humans also has the inherited problems of transfusion of blood from one to another, possible contamination due to the origin of the blood, the equipment used, and the loading environment.^[5] Therefore, screenings of these carriers for the absence of diseases as well as rigorous controls are required for the collection and handling of NERs to eliminate any risk of contamination.^[6] Furthermore, erythrocytes as a drug carrier raises other potential concerns due to the changes in their biochemical nature. Drugs or other bioactive agents can be loaded in nano erythrocytes either by physical methods or by chemical methods.^[7] For successful entrapment into the erythrocytes, the drug should have a degree of water solubility and resistance to degradation within erythrocytes. Certain drugs have been entrapped in erythrocytes by endocytosis, including vinblastine, chlorpromazine, hydrocortisone, propranolol, tetracaine, retinol, and capecitabine.^[8] Anchoring the drugs to red blood cell (RBC) membranes with the help of nanotechnology is a powerful method to load the drug that shows binding affinity toward the membrane protein and remains effective during the long circulation lifetime of the carrier. RBCs can be engineered to load specific drugs to facilitate the localized delivery of chemotherapeutic drugs in target-specific organs, which can prolong period of time in circulation and to overcome systemic toxicity. The conjugation of the drug on cell membrane surface enhances their survival and avoids drug leaching during the transit time. Biodegradable lipoproteosomes are small vesicles that are produced from RBCs by hypotonic lysis method to produce erythrocyte ghosts by removing their hemoglobin content.^[9,10] Subsequently, these erythrocyte ghosts were extruded to form small vesicles having a mean diameter of about 100 nm.^[10]

This study aims to improve the physicochemical properties and anticancer activity of capecitabine. The capecitabine was conjugated on the carrier membrane for sustained and targeted drug delivery to avoid drug leakage, to improve the circulatory time of carrier, to increase the stability, and to reduce cost and toxicities of capecitabine therapy which would present a significant advantage over many conventional systemically administered formulations. The study focuses on development and optimization of NERs-based formulation of capecitabine by probe sonication method and subsequently drug loading with the help of hypotonic preswelling method.^[11,12]

MATERIALS AND METHODS

Materials

Analytical grade materials were used for the study. Capecitabine (Reliance Life Science, India) was received

as gift sample. Acetonitrile, chloroform, glutaraldehyde, distilled water, and phosphate buffer pH (7.4) were also used throughout the study. All other chemicals and reagent were of analytical grade and were used without further purification. Male albino mice weighing 250 ± 25 g was obtained from Animal House of Tatyasaheb Kore College of Pharmacy, Warananagar. The protocol for animal use and care were approved by the Institutional Animal Ethics Committee (IAEC), New Delhi, India (1090/PO/ac/07/CPCSEA; Date 29/6/2016).

Isolation of ghost

The whole blood obtained from registered blood bank (Galaxy Hospital, Maharashtra, India) was centrifuged at 3000 rpm for 5 min at $4 \pm 1^\circ\text{C}$ in cooling centrifuge (Remi Corp, Mumbai). The serum and buffy coats were removed by washing 3 times with normal saline. The washed erythrocytes were hemolyzed with distilled water and centrifuged at 2000 rpm for 15 min and stored at 4°C until further use.^[11] Erythrocytes were hemolyzed by incubating them sequentially in 50 and 30 mOsm hypotonic solutions, prepared from isotonic normal saline solution (~ 300 mOsm). The hemoglobin in the supernatant was removed after centrifugation and cream-colored pellet was resuspended in hypotonic solutions and subjected to centrifugation again. The colorless ghosts thus obtained were incubated in hypertonic solution for 60 min at 37°C for resealing.^[12] The resulting sealed ghosts were washed 3 times with isotonic Normal saline and stored at 4°C until further use.

Drug loading

Capecitabine loaded into erythrocyteghosts using Preswell dilutional hemolysis method. For drug loading into resealed ghosts, we first closed cell membrane pores by incubating the cells in hypertonic solution for 45 min at 37°C .^[13] Then, drug solutions containing varying concentrations of capecitabine (5-30 mg/ml) were incubated with an aliquot of sealed cells. For loading drug before resealing, drug solution was incubated with the cells recovered from the hypotonic solution. The drug loading was assessed lysing the drug loaded ghosts (20 μl) with methanol (980 μl). The drug was finally separated from cellular carriers by sonication and centrifugation. The amount of capecitabine in supernatant was measured at 245 nm using an ultraviolet (UV) spectrophotometer (Cary 60, UV/Vis Spectrophotometer, Agilent Corp.), and the percent drug loading was calculated using the following equation:

$$\text{Percent drug loading} = \frac{\text{Amount of drug loaded}}{\text{Amount of drug added}} \times 100$$

Preparation of NERs

NERs were prepared by reducing the size of erythrocyte ghosts containing capecitabine. Two size reduction methods

were used to prepare nanosized erythrovesicles: Bath sonication (Sonic, Unique Biologicals, Kolhapur, India) and probe sonication (Remi 210, Remi Corp, Mumbai). Sonication was performed for 15 min at 25°C and the drug loading in membrane ghost was done using hypotonic preswell dilutional method [Figure 1]. Glutaldehyde solution was used as cross-linking agent, resealed using hypertonic solution and incubated for 15 min, under refrigerated condition at $4 \pm 1^\circ\text{C}$, sonication process was done for 25 min.^[14,15]

Lyophilization of prepared NERs

Lyophilization of the NERs was carried out using Labconco freeze drying system (Free Zone 4.5, USA, Model: 7948030). Capecitabine-loaded NERs preparation was dispensed in glass vials and frozen at -40°C for 8 h, then subjected to freeze drying maintaining condenser temperature at -50°C and vacuum of approximately 0.5 bar.^[16]

Characterization of NERs

Drug content and entrapment efficiency

Drug content was determined by deproteinized loaded cells done using acetonitrile followed by centrifugation, NERs were added in acetonitrile (10 ml) and centrifuged.^[17] The supernatant was collected, and the drug content was estimated using UV-visible (Cary 60, 2100, Agilent technology, Germany) spectrophotometric method at 245 nm, using following equations.

$$\% \text{ Drug content} = \frac{\text{Amount of drug loaded}}{\text{Amount of drug added}} \times 100$$

$$\% \text{ Entrapment efficiency} = \frac{\text{Amount of drug added} - \text{amount of drug loaded}}{\text{Amount of drug added}} \times 100$$

Hemolysis

The tendency of NERs to hemolyze blood was studied by incubating NERs with the whole blood, and subsequently, measuring the degree of hemolysis. NERs containing capecitabine were added to whole blood at either 1:1 or 2:1 ratio and incubated at 37°C for 30 min in the dark followed by centrifugation for 10 min at 5000 rpm. The amount of hemoglobin released was determined in the supernatant by measuring the absorbance at 245 nm using a UV/visible spectrophotometer.^[18] A completely lysed blood sample (prepared by adding distilled water to whole blood) was used as a control. The percent hemoglobin release was calculated using the following equation:

$$\text{Percent hemolysed} = \frac{(\text{A540 of sample} - \text{A540 of background})}{(\text{A540 of 100\% hemoglobin})} \times 100$$

A540 is the absorbance at 540 nm.

Hematological indices

Control erythrocytes, sham-loaded erythrocytes, and capecitabine loaded NER's were hematologically

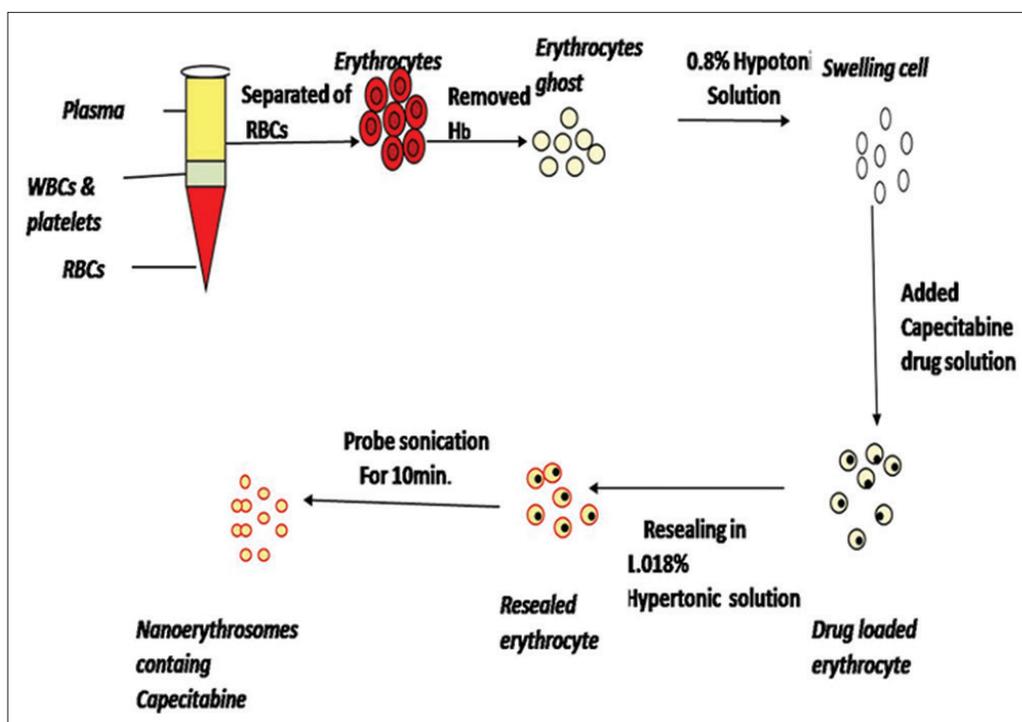


Figure 1: Preparation of capecitabine loaded nano erythrovesicles

characterized. The mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), the MCH concentration (MCHC), and the hematocrit were measured using a hematology analyzer.^[19] To estimate the morphological variation between normal and capecitabine-loaded NER's, both normal and capecitabine-loaded erythrocyte samples were examined using a scanning electron microscope (SEM). NER's were diluted 1:10 ml using autologous plasma and then mixed by several gentle inversions. The mixture was aliquoted into Eppendorfs tubes. The samples were incubated at 37°C and rotated vertically. Samples were removed at 0.5, 1, 2, 4, 8, 12, 24 and 48 h and then centrifuged at 3000 rpm for 5 min. 100 µl of the supernatant was separated for capecitabine assays, and the remaining portion was centrifuged for 5 min. The supernatant was used for hemoglobin analysis using measuring the absorbance at 540 nm. The results are expressed as percentages of the absorbance of a completely hemolyzed sample.^[20]

Osmotic shock

Capecitabine contained NERs were incubated with distilled water for 15 min followed by centrifugation at 3000 rpm for 10 min, may cause the release of drug and it was estimated using UV-visible spectrophotometer at 245 nm.^[21]

Osmotic fragility

The osmotic fragility test was used to assess the ability of erythrocyte membranes to resist lysis caused by exposure to solutions of NaCl ranging from 0.0 to 0.9 g%. A 25 µl NERs sample was added to each of a series of 2.5 ml saline solutions containing 0.1-0.9 g% NaCl. After gentle mixing and standing for 15 min at room temperature, the NERs suspensions were centrifuged at 5000 rpm for 5 min. The absorbance of the supernatant was measured at 540 nm. The released hemoglobin was expressed as percentage absorbance of each sample in reference to a completely lysed sample prepared by diluting packed cells of each type with 1.5 ml of distilled water.^[22]

Turbulence fragility

Aliquots of 0.5 ml of packed erythrocytes of each of the three types were suspended in 10 ml of phosphate buffer saline (PBS) in polypropylene test tubes and shaken vigorously using a multiple test tube orbital shaker at 2000 rpm for 4 h. To evaluate the time course of hemoglobin release, 0.5 ml portions of each suspension were withdrawn at 0, 1, 2, 3, 4 and 5 h. The samples were centrifuged at 1000 g for 10 min, and the absorbance of each supernatant was determined spectrophotometrically at 245 nm. The percent hemoglobin release was determined relative to that of a completely lysed 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, the turbulence fragility index (TFI) was used.^[23] This value is calculated as the shaking time required to produce 20% hemoglobin release from erythrocytes.

In vitro drug release

Release of capecitabine from NERs was studied using the dialysis (Slide-A-Lyzer, 3500 MWCO, Thermo-Scientific) method at 37 ± 2°C and was compared with the pure drug solution. Briefly, the dialysis bags were first hydrated for 30-60 min with PBS (pH 7.4) and NERs (500 µl) were loaded carefully using a syringe without puncturing the dialysis membrane. Then, the tubes were immersed in 100 ml of release medium PBS (pH 7.4). While stirred the release medium using the magnetic stirrer at 150 rpm/min, 1 ml samples were withdrawn at predetermined time intervals. From the release medium and the same volume was replaced with fresh medium.^[24,25] The sample was analyzed at 245 nm using UV-Visible spectrophotometer (Shimadzu Corporation, Japan).

Shape and surface morphology

Particle size and shape analysis was performed using photomicrograph using Brightfield optical microscope. A small amount of NER's suspension was placed on a clean slide; pictures of resealed erythrocytes were taken by random scanning of the slide. Finally, diameter of about 10-20 resealed erythrocytes was manually measured from photomicrographs of each batch.^[26] A JEOL-JSM-6360 SEM, equipped with a digital camera, at a 20 kV accelerating voltage was used to evaluate morphological differences between normal and capecitabine loaded NER's. Both normal and 8 mg/mL capecitabine loaded erythrocyte samples were processed as follows. After the samples were fixed in buffered glutaraldehyde, the aldehyde medium was drained off. The cells were rinsed 3 times for 5 min in phosphate buffer and post-fixed in osmium tetroxide for 1 h. The samples were then rinsed with distilled water and dehydrated using a graded ethanol series: 25%, 50%, 75%, 100%, and another 100%, each for 10 min. The samples were rinsed in water, removed, mounted on studs, sputter coated with gold, and then viewed using SEM.^[27]

Particle size and zeta potential

Vesicle size and polydispersity index (PI) of drug-loaded NERs were measured using Dynamic light scattering method using Zetasizer, ZEM5002 (Malvern Instruments Ltd., Worcestershire, UK) at a fixed angle of 90°.

Evaluation of stability profiles

Vesicle size

To evaluate the stability of capecitabine -loaded NERs, an aliquot of the particles (500 µl) was stored at 4°C, 25°C and 37°C for 21 days. Samples were withdrawn each day (0, 7, 14 and 21) for determination of vesicle size and drug content as described above. Capecitabine loaded NERs suspended in PBS were placed in the syringe attached with the aerosolizer and then sprayed ten times. The fine droplets thus generated were collected in a small tube for assessing vesicle size, PI and entrapment efficiency as discussed above.^[20]

Centrifugal stress

The centrifugal stability was assessed to determine the effect of centrifugation speeds on capecitabine leakage from NERs. NERs containing capecitabine (500 µl) were centrifuged at various speeds ranging from 1000 to 5000 rpm for 10 min at 4°C and the amount of drug released in the supernatant was quantitated.^[28]

Drug leakage

The drug leakage that may occur due to turbulence during handling and administration to animals was also evaluated. NERs containing capecitabine were passed through a 271/2 gauge needle at a flow rate of 10 ml/min, comparable with the blood flow rate *in-vivo*. The number of passes was varied (5-20 times) to change the intensity of turbulence that may result due to repeated injections. The formulations were then centrifuged for quantitation of capecitabine as described above.^[28]

In vivo tissue distribution

The study was conducted for distribution of drug loaded nano erythrocytes to various organs of RES such as liver, lungs, kidney, and spleen. Nine healthy adult Wistar rats weighing 200-240 g were taken from Animal House, TKCP, Warananagar, India. All procedures with animal were reviewed and approved by the IAEC, TKCP, Warananagar, India. A constant day and night cycle was maintained, and they were fasted for overnight. The animals were divided into three groups, in which two groups each containing four rats and one group containing one rat. Group I rats received developed NERs equivalent to 250 µg of capecitabine intravenously (IV) in the tail vein after pre-dispersing them in sterile PBS pH 7.4 solution. Group II rats received 250 µg of pure capecitabine IV. Group III rats were treated as solvent control and injected IV with 1.0 ml of sterile PBS pH 7.4 solution. After 24 h, the rats were scarified and their liver, lungs, spleen, and kidneys were isolated. The organs were rinsed thoroughly in sterile PBS to remove and dried with tissue paper the organ of each rat were homogenized and added the 5 ml phosphate buffer solution pH 7.4, and the homogenate was centrifuged at 1000 rpm for 30 min in this process extraction was done and protein remove, the supernatant collected and filter through 0.45 µ filter and analyzed UV-Visible spectrophotometrically after suitable dilution with PBS solution at 245 nm.^[20]

In vivo drug release

The study was carried out for distribution of drug loaded nano erythrocytes to release drug from NERs. The albino rats were allowed to eat commercial food pellets and drink water except during the first 5 h of each test.^[20] All procedure with animal were reviewed and approved by the IAEC, TKCP, India. The animals of the first group received IV injection

of capecitabine drug solution equivalent to 250 µg of capecitabine. The animals of the second group were treated with capecitabine NERs formulation equivalent to 250 µg of capecitabine concentration administered through caudal vein. The blood samples were collected at different time intervals from 0, 1, 2, 3, 4, 5, 6, 7 and 8 h from retro-orbital plexus using heparinized syringe. Blood sample was centrifuged in refrigerated centrifuge at 2000 rpm for 30 min, and then plasma was separated, treated with acetonitrile, and kept in a shaker incubator. Capecitabine extracted in acetonitrile was filtered through a 0.22 µm filter and then analyzed UV-Visible spectrophotometrically after suitable dilution with its PBS solution at 245 nm.^[20]

Data analysis and statistics

The data are presented as mean ± standard deviation and were analyzed by one-way ANOVA analysis using GraphPad Prism 5.0 software (GraphPad Software, SanDiego, CA, USA). $P \leq 0.05$ was considered as statistically significant.^[29]

RESULTS AND DISCUSSION

In the present investigation, we sought to develop and explore the potential of NERs for delivery of an anti-cancer drug, capecitabine. Thus, we have performed a series of *in-vitro* and *in-vivo* studies to optimize drug loading and stability of capecitabine encapsulated NERs.

Isolation of ghost

To prepare cells with surface pores, we incubated erythrocytes in hypotonic solutions of varying strengths. By controlling the osmolarity, cells with surface pores between 10 and 500 nm can be prepared. When cells were incubated in 30 mOsm solution, we obtained cell ghosts completely devoid of hemoglobin and intracellular organelles; hemoglobin depleted cells had numerous pores on the surface. The pores on ghost cell membranes were then closed by incubating in hypertonic solution (PBS ×10) at 37°C that resulted in spherical ghost cells. After optimization of preparation parameters, we examined the morphological features of resealed ghosts under a microscope. Hypertonic solution mediated resealing was efficient in retaining spherical morphology of the NER's.

Drug loading

The loaded amount, the entrapment efficiency, and the percent cell recovery were determined. The UV method was used to estimate the capecitabine content of the supernatants after the incubation of erythrocytes with capecitabine. The obtained data indicate that 70 µg of capecitabine was loaded with an entrapment efficiency of 29.3%. This amount is notable in

comparison to those values reported in the literature for a variety of drugs. The observed cell recovery of approximately 85.94% is comparable to the recovery results for various drugs reported in other studies.

Preparation of NERs

First, the drug was loaded into resealed erythrocyte ghosts (erythroosomes) by simple incubation with drug solution. However, drug loading by this process was minimal. When capecitabine was incubated at a concentration of 7.07 $\mu\text{g/ml}$ with ghosts, only $7.21 \pm 1.18\%$ drug was loaded. Reduced drug loading was perhaps because erythrocyte membrane contained no pores for drug molecule to enter the cells. Alternatively, cell membranes might have lost all transporters during exhaustive hypotonic lysis. Further, no increase in drug loading was observed when incubation duration was extended. This data are consistent with the previous assumption that the drug did not move in and out of the cells in the absence of pores. However, incubation of drug with the ghost cells before resealing resulted in a major increase in drug loading: $7.21 \pm 1.18\%$ drug was loaded. Drug loading increased with the increase in concentration but amount of loading went down when the drug concentration was increased, which is attributed to saturation of the ghost core. Hence, capecitabine solution at a concentration of 7.07 $\mu\text{g/ml}$ was found to be optimum for maximum drug loading. Drug solution incubated with varying volumes of cell suspension suggests that drug loading was maximal when drug solution-to-ghost cell volume ratio was 1:1. Thus, drug loading was maximal when 70 μl capecitabine solution was added into 500 μl cell suspension.

Characterization of NERs

Hemolysis

An increase in erythrocyte hemolysis destabilizes the heme structure in hemoglobin molecules, leading to a release of free iron ions that generate more free radicals. Moreover, the presence of capecitabine in the media surrounding the erythrocytes promotes the production of reactive oxygen species (ROS). Furthermore, several studies have reported that capecitabine stimulates ROS production. The *in vitro* hemolysis study was performed at different time interval of standard ghost sample and capecitabine loaded NERs. The % HR observed [Figure 2] maximum hemolysis was observed at 4 h from this conclude that these results, the does not resistance of the nano erythrocytes against the loss of cell integrity it showed if increase their time of shaking and observed the also increase their hemolysis. This study carried out at 4 h and amount of percent hemolysis was found to be 33% at 4 h.

Hematological indices

The major hematological indices of the control, ghost cell, and capecitabine-loaded NER's are shown in Table 1.

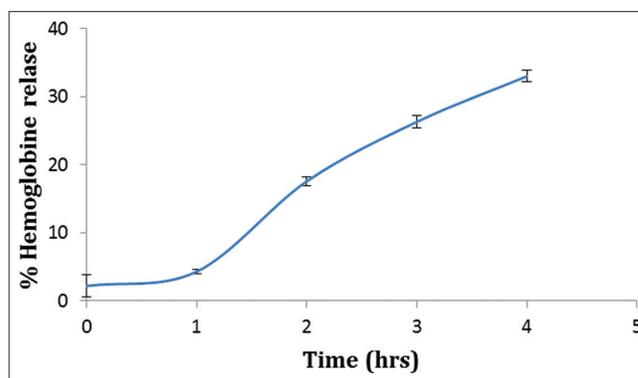


Figure 2: Hemolysis profile of nano erythroosomes loaded with capecitabine. Data represent the mean \pm standard deviation ($n = 3$)

Table 1: Hematological parameters of control erythrocytes, ghost erythrocytes and capecitabine loaded NER's

Test	Ghost	Capecitabine loaded NER's	Control
MCV (fL)	67.0 \pm 3	69.1 \pm 9.9 fL	90.9 \pm 1.2
MCH (pg)	25.3 \pm 0.5	29 \pm 1.1 pg	31.6 \pm 2.5
MCHC (g/dL)	33.4 \pm 1.3	31.1 \pm 4.6 g/dL	33.2 \pm 1.1

MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, NER: Nano erythroosomes

These parameters, which are measured as part of routine clinical hematology tests, may provide some useful estimates of the biological state of the erythrocytes. The results of this study showed that significant changes in erythrocyte volume were caused by the entrapment process in ghost and capecitabine loading, as indicated by the MCV values. However, both the MCH and the MCHC decreased following the exposure of the erythrocytes to the loading procedure, in ghost and capecitabine loaded erythrocytes. The overall loss of hemoglobin from the erythrocytes upon loading procedure was expected because the procedure is destructive in nature. In similar studies, all of these parameters were found to be lower in carrier erythrocytes than in normal unloaded cells.

The cholesterol and protein level in nano erythrocytes was estimated using UV-Visible spectrophotometer. The cholesterol levels of nano erythrocytes membrane were found to be 102.53 mg/dl and protein level was found to be 14.80 g/dl. Protein value which was found to be normal range (Not more than 13.24 ± 1.82 mg/dL) and it may conclude that protein was present in the NER's; the cholesterol level was significantly less than the normal value (Not more than 128.17 ± 14.2 mg/dL) from this concluded that the formation of erythrocyte membrane during this process also removed the fatty substance from the cell.

Osmotic shock

The capecitabine content of NERs was determined using UV-visible spectroscopy method at 245 nm. The changes in osmotic condition of cell resembles to change their integrity. When drug loaded NERs were incubated with distilled water the cell were completely ruptured and there was complete release of drug from the cell. This indicates that there was complete lysis of the cell when formulation was incubated with water for osmotic shock study. Hence, change their osmotic condition then drug released was increased at 90% in distilled water as compare to isotonic condition [Figure 3]. Therefore, the storage of carrier erythrocytes in isotonic suspensions may be more suitable in lower temperatures.

Osmotic fragility

In the developed osmotic lysis method for encapsulation of capecitabine, osmolality of the buffer used is crucial. Developed formulation of NERs in that drug was encapsulated by using preswelling method. From this concluded that drug loaded NERs are swelled at higher concentration of hypotonic solution hence it was minimum drug entrapped in the cell and hence maximum drug retained in the solution [Figure 4], however it clear that low concentration of sodium chloride solution indicated greater drug release in the cell to through formulation, 0.2% sodium chloride have entrapment efficiency decreased in the resealed erythrocyte. A hypotonic

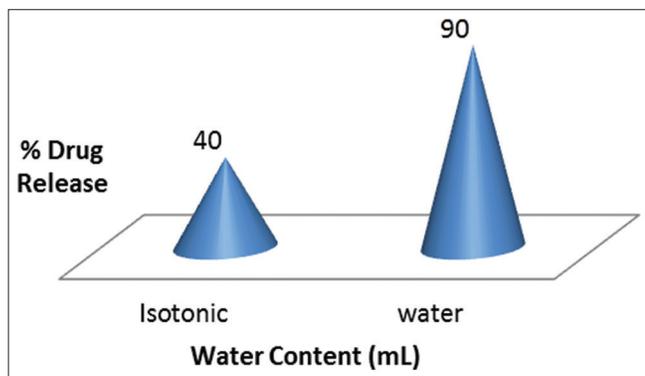


Figure 3: Osmotic shock studies of capecitabine loaded nano erythrovesicles

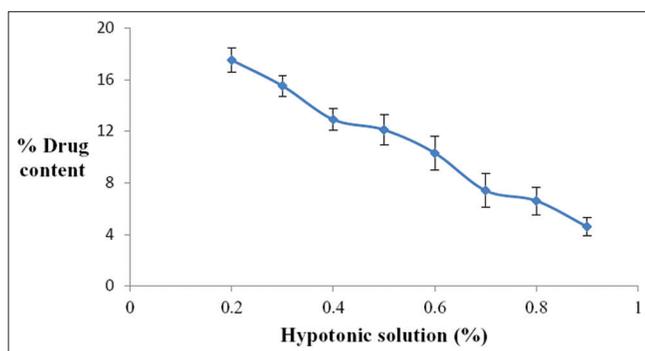


Figure 4: Effect of osmotic fragility on drug content of capecitabine loaded nano erythrovesicles

solution of concentration 0.3% w/v induced cell swelling and the formation of pores that allowed the drug to penetrate the erythrocyte [Figure 5]. However, hemolysis of erythrocytes was greater with low concentration of sodium chloride. The results obtained are depicted in Table 2. It indicates that at 0.2% NaCl there was less resistance of cells to hemolysis as compared to other concentrations of sodium chloride used for the study.

Turbulence fragility

The turbulence fragility test is used to exploit the mechanical strength of the erythrocyte membranes. In this study, this test was mainly carried out by shaking the cell suspensions vigorously. The hemoglobin released was measured at different times. The results indicated that the turbulence fragility of the capecitabine-loaded NER's was greater than that of the control erythrocytes. The TFI values for unloaded, control and capecitabine-loaded erythrocytes were 3, 2 and 1 h, respectively. Similarly, other studies have shown that the turbulence fragility of the erythrocytes as drug vehicles increases significantly relative to that of normal control cells. These results indicate that the resistance of the erythrocytes to vigorous turbulent flow shows a decreasing trend from control cells to capecitabine-loaded erythrocytes. These results indicate that erythrocytes become more fragile during the loading process and that this fragility is enhanced by capecitabine encapsulation.

In vitro drug release

The *in vitro* drug release from capecitabine solution, optimized capecitabine loaded NER's were studied at $37^{\circ}\text{C} \pm 2$ in PBS buffer [Figure 6]. When the release of plain capecitabine was evaluated using dialysis bag as barriers, ~100% drug was available in the receiver chamber only after 1 h, suggesting that bags were not controlling the passage of drug molecules from donor to receiver chambers. No degradation in the release media is expected since capecitabine remained stable over an extended period of time (~24 weeks) at various storage conditions. However, a slower release may stem from

Table 2: Effect of osmotic fragility on % drug retention and % entrapment efficiency of capecitabine loaded NER's

Concentration of NaCl (% w/v)	Drug content (%)	Entrapment efficiency (%)
0.9	4.6 \pm 0.7	95.4 \pm 0.9
0.8	6.6 \pm 0.65	93.4 \pm 0.65
0.7	7.4 \pm 0.92	92.6 \pm 0.87
0.6	10.3 \pm 1.08	89.7 \pm 1.02
0.5	12.1 \pm 0.95	87.7 \pm 0.79
0.4	12.9 \pm 1.02	87.1 \pm 0.84
0.3	15.5 \pm 0.75	84.5 \pm 0.91
0.2	17.5 \pm 0.62	82.5 \pm 0.73

NER: Nano erythrovesicles

the compact structure of nano erythroosomal membrane that is composed of natural lipids, cholesterol, and surface proteins. The percent cumulative drug release from the capecitabine solution was found 71.19% to be after 8 h, capecitabine loaded NERs to be 48.49% after 8 h. The release was better controlled from capecitabine loaded NER's compared to standard drug solution. From the above results, it is very clear that the drug loaded NER's would show very slow release of capecitabine in the blood circulation, targets more to tumor tissues and therefore meets the requirements for an effective drug delivery system.

Shape and surface morphology

To investigate the possible morphology changes of nano erythrocytes on loading process, samples of resealed nano erythrocytes were observed under the SEM [Figures 7 and 8]. As from the concluded that the loading process with drug and different crosslinking agent resulted in the formation of cup-form nano erythrocytes very disperse sizes. Native human nano erythrocytes show the expected biconcave morphology under SEM. After drug loading and treatment with gluteraldehyde, a slight change in their shape was observed with the same magnification $\times 1500$. This shows that nano erythrocytes undergo considerable morphology change during the loading process, which is confirmed by particle size. From this find out the no observed effect on the morphology of the carrier cells, and the changes of the drug loading encapsulation cell. The main morphological change in capecitabine-loaded NER's, as revealed by scanning electron microscopy [Figure 8], was the transformation of

loaded cells from biconcave (normal) to near spherocytes. Attainment of a spherical shape due to drug loading makes the erythrocytes more fragile. The fragile cells may be destroyed and rapidly cleared from the circulation by macrophages. Further studies are required to elucidate and demonstrate erythrophagocytosis of capecitabine-loaded NER's.

Vesicle size

We used different sizing methods to assess their influence on the homogeneity and entrapment efficiency of the formulations. Sizing with sonication produced polydispersed particles (PI > 0.5) and drastically reduced the entrapment efficiency. The particle size of capecitabine loaded NER's were found to be 2.96 nm and PI was found to be 0.097 [Figure 9]. The PI of capecitabine loaded NERs was less than one and concludes that NER's formed are monodispersed or of uniform size. The sizes of NER's were found in nanometer; therefore, we could expect better accumulation at tumor by enhanced permeability and retention effect. Further, sonication had minimal effect on entrapment efficiency, suggesting little or no disruption

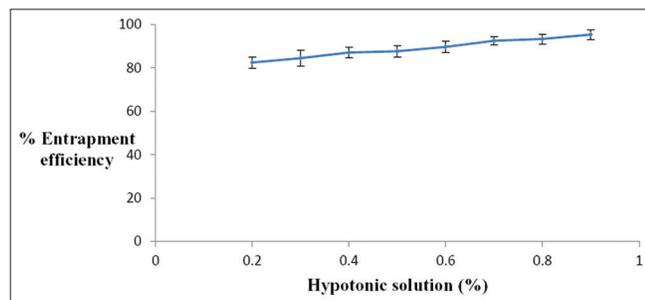


Figure 5: Effect of osmotic fragility on entrapment efficiency of capecitabine loaded nano erythrocytes

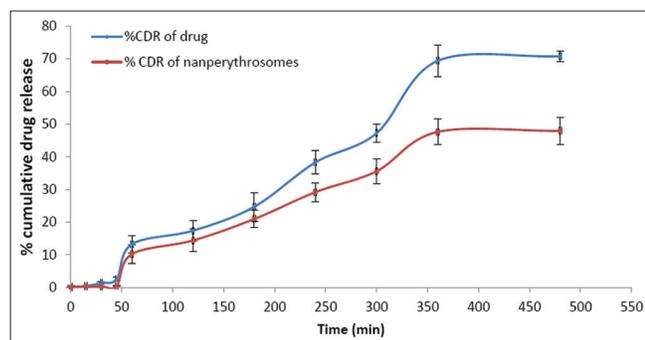


Figure 6: Percent cumulative drug release of capecitabine and capecitabine loaded nano erythrocytes

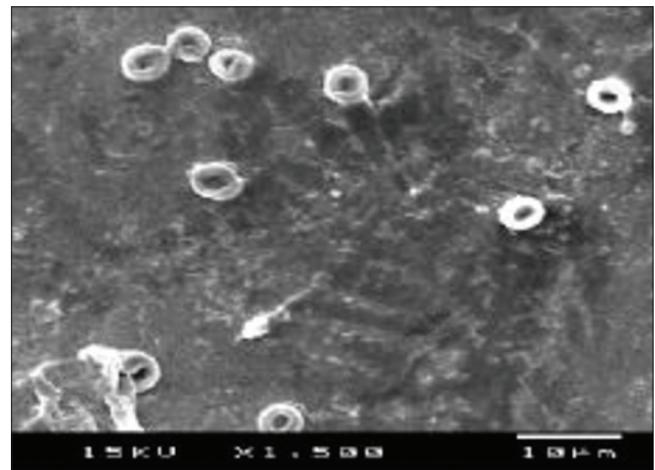


Figure 7: Photomicrograph image (scanning electron microscope) of unloaded/ghost nano erythrocytes

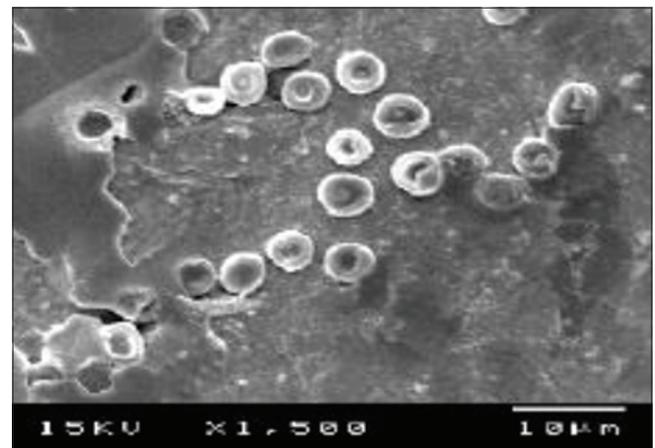


Figure 8: Photomicrograph image (scanning electron microscope) of capecitabine loaded nano erythrocytes

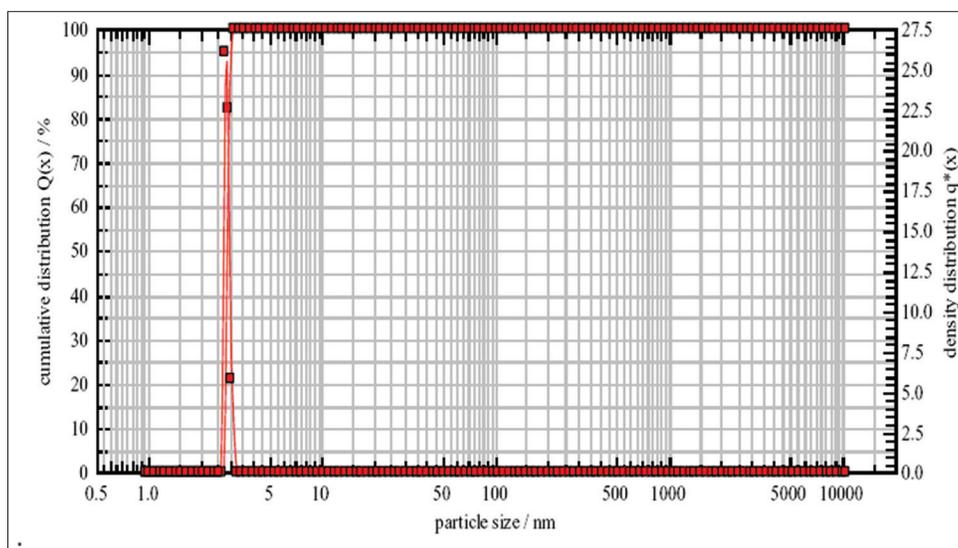


Figure 9: Vesicle size distribution of capecitabine loaded nano erythrovesicles

of cells. This is consistent with the flexible structure of erythrocytes that continually travel through narrow capillaries and slits of sinusoids in the physiological system. In fact, structural flexibility is a very important feature of erythrocytes that determines the fate of cells and a slight deviation in terms of shape or rigidity can lead to clearance of cells by macrophages. Thus, NERs are expected to be optimal for *in-vivo* efficacy and are likely to avoid clearance by alveolar macrophages.

Evaluation of stability profiles

Stability studies of the prepared NERs were carried out by storing all the formulations at 4°C, and at RT for 2 weeks [Table 3]^[30,31]. Parameters such as percentage drug content, osmotic shock, and *in vitro* release studies of the formulation were carried out. The results revealed that 4°C is the ideal storage condition for capecitabine loaded NERs.

The capecitabine loaded NER's were kept at room temperature and at 4°C for 15 days. After specified time, the formulations were evaluated for % drug content using osmotic shock. This study would reveal that NER's are not able to retain or prevent drug leakage during storage at different conditions. The preparation stored initially at 4°C and room temperature showed a large amount of changes in % EE, % drug release [Figure 10]. This indicates complete retention of loaded capecitabine in NERs during storage.

Vesicle size

The primary morphological change in capecitabine-loaded NER's revealed by SEM was the transformation of loaded cells from biconcave to spherocyte shape. The spherical shape of loaded cells makes them more fragile, and fragile cells are destroyed and rapidly cleared from the circulation by macrophages.

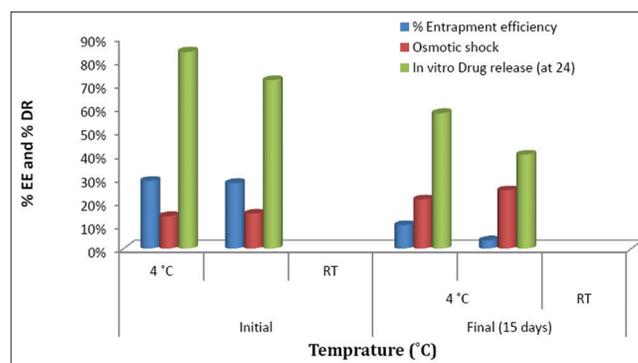


Figure 10: Stability assessment of capecitabine loaded nano erythrovesicles

Table 3: Stability of NERs at various temperatures

Test	Initial		Final (15 days)	
	4°C	RT	4°C	RT
% Entrapment efficiency	29%	28%	10.20%	3.36%
Osmotic shock	14%	15%	21%	25%
<i>In vitro</i> drug release (at 24)	84%	72%	58%	40%

NER: Nano erythrovesicles

Centrifugal stress

In Figure 11 showed entrapment efficiency was essentially unaltered when formulations were centrifuged at 1000 rpm. With the increase in centrifugal force to 5000 rpm, NERs underwent disruption and released ~60% of the encapsulated drug.

Centrifugal stress study was determined using UV spectroscopy method at 245 nm. As we increase, the centrifugal force to 5000 rpm drug retention was decreased, means fragility was

not maintained in at high centrifugal force. After disruption of capecitabine loaded NER's maximum drug retention was 74% at 1000 rpm and minimum was 3.17% at 5000 rpm [Figure 12]. It indicates that high centrifugal force on NER's does not maintain cellular integrity.

In vivo tissue distribution

In vivo drug targeting studies were carried out for formulation treated with glutaraldehyde and with optimal particle size, high entrapment efficiency, and satisfactory *in vitro* release. The comparison between the amount of drug targeted from resealed NERs and free drug in various organs is presented in Figure 13. The average targeting efficiency of drug loaded nano erythrocyte was found to be 26.5% of 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. It can be assumed that drug loaded NER's 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 distributed to the organs after administering the dose in the form of NERs and the order of drug distribution was found to liver > lungs > kidney > spleen. Large amounts of

distribution in the liver may be attributed to uptake of the drug loaded NERs by RES and large size of the liver as compared to other organs.

In-vivo drug release

The blood serum concentration of drug solution and formulation after IV administration has been showed at Figure 14. After administration of pure drug solution 58.19 ± 1.1 $\mu\text{g/ml}$ of capecitabine was found in the blood at 8 h while it was found to be 43.89 ± 0.5 $\mu\text{g/ml}$ for capecitabine loaded NER's. The results revealed that capecitabine loaded NER's is capable of controlling the release of the drug for an extended time period. The release of drug was for an extended period at a slow rate as compared to pure drug.

CONCLUSION

NERs is a suitable carrier for the preparation of capecitabine loaded NERs prepared by sonication methods. Prepared carriers were optimized for many formulation variables such as morphology of vesicles, size and size distribution, polydispersity, integrity of membrane, loaded drug concentration, release rate, and *in vivo* bioavailability. The

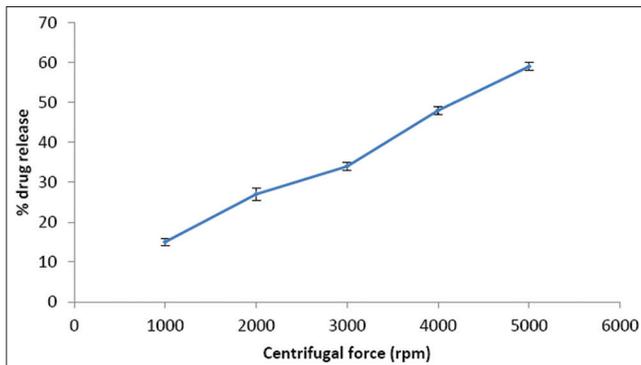


Figure 11: Effect of centrifugation force on drug release of capecitabine loaded nano erythrocytes

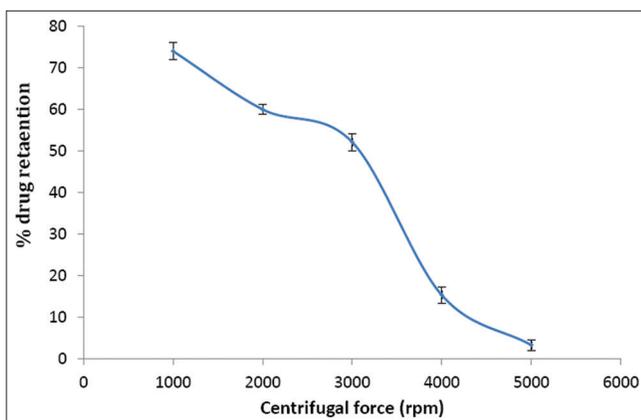


Figure 12: Effect of centrifugal force on % drug retention of capecitabine loaded nano erythrocytes

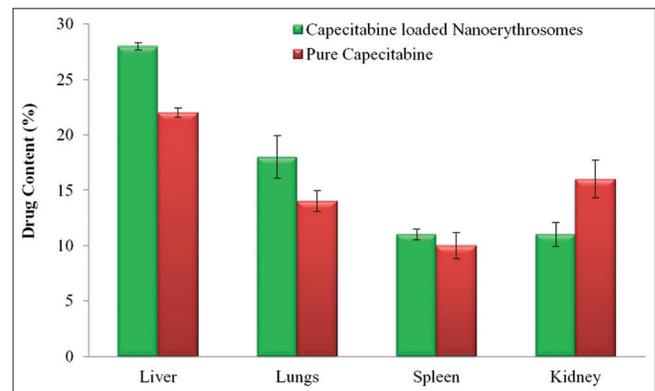


Figure 13: Tissue distribution of capecitabine drug solution and nano erythrocytes

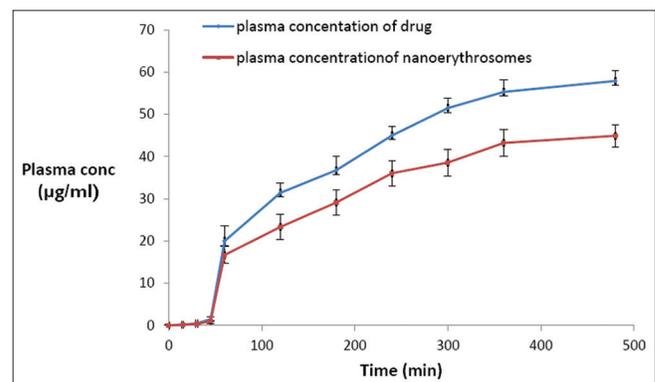


Figure 14: *In vivo* drug release of pure capecitabine solution and capecitabine loaded nano erythrocytes

developed CAPECITABINE – NER's carrier revealed nonaggregated, polydispersed vesicles with smooth surfaces. Preparations containing glutaraldehyde as cross-linking agent showed maximum drug entrapment efficiency. Capecitabine-NER's formulation was best fit for zero-order kinetics and capable of controlled release of drug for 8 h. Capecitabine-NER's formulation showed good re-dispersibility with normal saline which is desirable for parenteral administration. The Capecitabine-NER's formulation was successfully administered IV and showed higher plasma concentration compared to free drug signifying controlled release rate *in vivo*. Targeting efficiency of drug-loaded erythrocytes over free drug is higher, which may provide increased therapeutic index and drug targeting to various organs. It may help in the reduction of dose required for the therapy and thereby dose-related systemic side effects could also be minimized.

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