RESEARCH ARTICLE

Engineered cellular carrier nanoerythrosomes as potential targeting vectors for anti-malarial drug

Jaya Agnihotri, Virendra Gajbhiye, Narendra Kumar Jain

Pharmaceutics Research Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar (MP), India

The present investigation was aimed at developing and exploring the use of nanoerythrosomes (nEs) for targeted delivery of anti-malarial drug, pyrimethamine (PMA). The formulation was prepared by the extrusion method and drug was conjugated to nEs with the help of gluteraldehyde used as a cross-linking agent. The nEs formulation was optimized for drug concentration, surface morphology, viscosity, and sedimentation volume. The drug-loaded nEs showed delayed *in vitro* release, good stability at 4±1°C, and controlled *in vivo* release. Tissue distribution studies showed higher accumulation of drug in liver (17.34±1.3 µg/ml) at 3 h in the case of nEs as compared to free drug (12.82±0.7 µg/ml). A higher amount of drug i.e. 13.14±0.9 µg/ml was found after 24 h in liver in the case of nEs as compared to free drug concentration of 9.72±0.5 µg/ml. Data showed that developed PMA-loaded nEs hold promise for targeting and controlling the release of drug and improve treatment of malaria.

Key words: *Anti-malarial, cellular carrier, nanoerythrosomes, pyrimethamine, targeting*

INTRODUCTION

Malaria is the most important disease of humans, affecting 109 endemic countries in year 2008 with a population of over 1 billion people and causing around 1 million deaths each year.[1] There has been a resurgence of this disease in many parts of the tropics not withstanding enormous control efforts. In addition, drug resistance causes increasing problems in most malaria-affected areas.[2] Malaria remains today as it has been for centuries, a major burden on tropical communities and a danger to travelers. Benign malarias (*Plasmodium vivax*, *P. ovale* and *P. malariae*) are rarely fatal and are still sensitive to chloroquine in most of the clinical situations. Malignant malaria caused by *P. falciparum*, ranks high as a cause of morbidity and mortality in tropics.[3] *P. falciparum* infection causes cerebral malaria as a complication, although complicated malaria can and does involve other tissue and organs in the body.[4] The *P. falciparum* malaria may sometimes be managed with chloroquine, although there is now an increased tendency toward the use of either pyrimethamine (PMA) with quinine or mefloquine. PMA is an anti-malarial drug which acts by inhibiting the conversion of folic acid to folinic acid.[5] It is widely used in the treatment of *P. falciparum* infection along with sulfodixine as combination therapy, but successful use of PMA is limited due to its toxic effect, particularly depression of hematopoisis. Acute over dose causes convulsion, tachycardia, respiratory depression, circulatory collapse, and death may also occur.[6] Therefore, it is necessary to target PMA directly to liver cells, where its activity is most required, using some nanometric molecular drug delivery systems.

Numerous novel carriers have been proposed for reducing toxic effects of anti-malarial drugs. However, low drug encapsulation, inadequate shelf life, and plasma instability limit their use. Among the various carriers used for targeting of drugs to various body tissues, the cellular carriers meet several criteria desirable in clinical applications, most importantly the biocompatibility of carrier and its degradation product.[7] Leucocytes, platelets, neutrophils, resealed erythrocytes, and nanoerythrosomes (nEs) have been potentially used. Among these, nEs possess great potential in drug delivery.[8] The rationale behind the use of nEs as drug carriers is based on the certainty that senescent (damaged) cells will be removed from circulation by phagocytic reticuloendothelial cells in liver, where targeting of anti-malarial drug is required. The present investigation was aimed at developing and exploring the use of nEs for targeted delivery of anti-malarial drug, PMA.

Address for correspondence:
Prof. N K Jain, Pharmaceutics Research Laboratory,
Department of Pharmaceutical Sciences,
Dr. Hari Singh Gour University, Sagar (MP) – 470 003, India.
E-mail: jnarendr@yahoo.co.in

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MATERIALS AND METHODS

Materials

PMA was obtained as benevolent gift from IPCA Laboratories Ltd (Mumbai, India). Gluteradehyde was purchased from SpectroChem (Mumbai, India). All other chemical and reagents used were of analytical grade.

Preparation of erythrocyte ghost

The hypotonic osmotic lysis method described by Deloach was used for the preparation of ghost suspension.[9] The method involves washing with a series of hypotonic saline solution, each wash being followed by centrifugal concentration of cells. One volume of red blood cells was incubated with an equal volume of hypotonic saline solution (0.7%). The cells were lysed and washed several times with this solution. After each wash, the content was centrifuged at 1000 rpm for 10 min at 4±1°C in a refrigerated centrifuge (model Z206, Hermle, Germany) and supernatant was aspirated and discarded. The ghost suspension was finally obtained when supernatant became colorless. The ghost cells was diluted with 0.9% saline to obtain 50% hematocrit and stored at 4±1°C until use.[10]

Preparation of nanoerythrosomes and drug loading

An extrusion technique reported earlier[11,12] was used with little modification for conjugation of drugs with nEs. The erythrocytes ghost suspension (50% hematocrit) was extruded through the polycarbonate membrane filter (0.4 μm pore, Nucleopore Corp., USA) attached to an adapter. nEs were obtained by 8-10 consecutive extrusions and final preparation was stored at 4±1°C in the refrigerator. PMA was conjugated to the nE membrane using gluteraldehyde as a cross linker. Two thousand five hundred microgram of drug was added to 2 ml of nEs preparation (50% hematocrit) in the presence of 0.3% gluteradehyde (in a 0.9% saline solution). The mixture was incubated at 4±1°C for 6 h and then reaction was stopped by the addition of 2 ml of 15% glycine solution in isotonic saline. Finally, the preparation was stored at 4±1°C in refrigeration until used.

Optimization of formulation

The PMA-loaded nE (DnEs)' formulation was optimized on the basis of drug concentration, surface morphology, viscosity, and sedimentation volume as follows.[13-15]

Optimization of drug concentration

To study the effect of variable drug concentration on conjugation of nEs, the various concentrations representing 1.0, 1.5, 2.0, 2.5, 3.0 mg/ml of PMA were used. Other experimental conditions such as pore diameter of extruder membrane (0.4 μm), gluteraldehyde concentration (0.03%), incubation period (45 min), and temperature (37±1°C) were kept constant. Packed loaded nEs were washed with PBS (pH 7.4) and deproteinized with acetonitrile (2.0 ml) and subjected to centrifugation at 2500 rpm (Remi, India) for 10 min. The clear supernatant was withdrawn and analyzed by HPLC using C-18 column and the mobile phase consisted of phosphate buffer (0.05 M, pH 5):acetonitrile:concentrated perchloric acid (750:300:2.5, v/v/v) at a flow rate of 1 ml/min.[16] The percent drug loading was determined using the following equation:

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

Surface morphology

The shapes of normal erythrocytes and erythrocyte ghosts were visualized using an optical microscope (Leica, Germany). Scanning electron microscopy (SEM Philips XL30, the Netherlands) was performed to evaluate the surface morphology of DnEs. A small amount of DnEs were stuck on a double-sided tape attached on a metallic sample stand, coated in argon atmosphere with a thin layer of gold, using a POLARON E5100 SEM coating unit, and visualized for surface morphology. The vesicles size was determined by the dynamic light scattering (DLS) method in a multimodal mode using a computerized inspection system (Malvern Zetamaster, ZEM5002, Malvern, UK).

Viscosity and sedimentation volume

A rotatory Brookfield (Synchro Lectric LVT, USA) viscometer was used to determine the viscosity of the formulation. Sedimentation volume of formulation was measured in a graduated measuring cylinder from the height of sediment using the formula, $F = V_u/V_o$, where F = sedimentation volume, V_u = ultimate volume of sediment, and V_o = original volume of formulation.

In vitro release rate

In vitro release studies were conducted using the dialysis membrane MW cut off 1000 Da (Sigma, USA). Free drug from formulation was removed by centrifugation at 18000 rpm for 20 min at 4±1°C. After removal of free drug 1 ml of formulation was introduced into the dialysis membrane and it was placed in a beaker containing 100 ml of 0.1 N HCl and ethanol mixtures. The beaker was kept over a magnetic stirrer and the temperature of the assembly was maintained at 37±1°C throughout the study. Samples were withdrawn at defined time intervals, replenished with same volume of fresh medium, and analyzed for drug content by HPLC.[16] Same procedure was repeated for *in vitro* release rate of free drug.

Stability studies

Jain and Jain (1995)[7] reported that DnEs can be stored without loss of physical integrity when suspended in phosphate buffer saline at 4°C for 4 weeks. Storage stability was accessed at different temperature viz 4±1°C, room temperature, and 37±1°C. Formulation (DnEs) was kept at variable temperature and changes in sedimentation volume and relative turbidity were noted periodically.

Centrifugal stress

The effect of centrifugation on sedimentation volume and drug leakage was studied by centrifuging the formulation in centrifuge tubes at different rpm for 15 min at 4±1°C. Drug leakage in the supernatant solution was estimated by HPLC.[16]

Turbulence shock

It is the measure of simulating destruction of loaded nEs during injection. The formulation was passed through a 23 gauge hypodermic needle at a flow rate of 10 ml/min, which is comparable to the flow rate of blood. The number of passes was varied as a function of turbulence. The drug leakage in supernatant was estimated by HPLC after fixed number of passes.

In vivo evaluation

The formulated products with promising *in vitro* performance were evaluated for their *in vivo* performance on albino rates. All the animal studies were conducted in accordance with the protocol approved by the Institutional Animal Ethical Committee of Dr. H.S. Gour University, Sagar. The albino rats of either sex (average weight 250±25 g) were divided into three groups each comprising six rats. The first group was administered drug solution equivalent to 250 μg of PMA (calculated at drug dose level 1 mg/kg). The second group was administered with DnEs containing equivalent an amount of PMA through caudal vein. The third group served as control. The blood samples were collected at different time intervals from retro-orbital plexus using heparinized syringe and estimated for drug concentration by HPLC.

Tissue distribution studies of PMA-loaded nEs were also performed using albino rats of either sex (250±25 g). The rats were divided into three groups each comprising six rats. The animals of first group were given drug solution (equivalent to 250 μg) of PMA through caudal vein. The animals of second group were administered DnEs (equivalent to 250 μg) through caudal vein. The third group served as control. After 3 and 24 h, rats of each group were sacrificed. The body organs (liver, lung and kidney) were removed, washed to get rid of adhering debris and dried with tissue paper. The organs were homogenized and drug concentration was determined by HPLC.

Statistical analysis

Data are expressed as the mean standard deviation (SD) of the mean and statistical analysis was carried out employing analysis of variance (ANOVA).

RESULTS AND DISCUSSIONS

The present investigation was aimed at developing a new family of natural nanoparticulate nEs for delivery of antimalarial drug, PMA. The formulation was prepared by the extrusion method and drug was conjugated to nEs with the help of gluteraldehyde, which served as a cross-linking agent. Glycine was added to stop the reaction of gluteraldehyde. The formulation was optimized for effective drug loading at variable drug concentration. The maximum percentage drug loading (25.20±1.3) was obtained with 2.5 mg/ml drug solution. Percentage loading was found to be nearly same using a 3.0 mg/ml drug solution indicating saturation of conjugation sites [Table 1].

Surface morphological studies displayed a spherical shape with a smooth surface and no aggregation was observed [Figure 1]. No difference was observed in the morphological properties of drug-encapsulated erythrocytes (DnEs). The size of the DnEs was found to be 125±24 nm. The viscosity of DnEs (29.3±1.4) was almost similar to that of nEs (29.2±1.2). Sedimentation volume of unity in each case revealed very good uniformity of the formulation. The *in vitro* release from the dialysis membrane was found to be 10.02±0.8 and 53.2±1.1% after 8 h for DnEs and free drug, respectively. It was found that the 98.51±2.4% of the free drug was released from the dialysis membrane in 20 h while only 25.57±0.9% drug was released in the case of DnEs in 20 h [Figure 2]. The

Table 1: Effect of drug concentration on drug loading

Formulation	Drug concentration (mg/ml)	Drug loading (%)
DnEs	1.0	14.31±1.2
DnEs	1.5	17.02±0.9
DnEs	2.0	20.72±0.7
DnEs	2.5	25.20±1.3
DnEs	3.0	25.13±1.4

Values represented as mean ± SD (n=3)

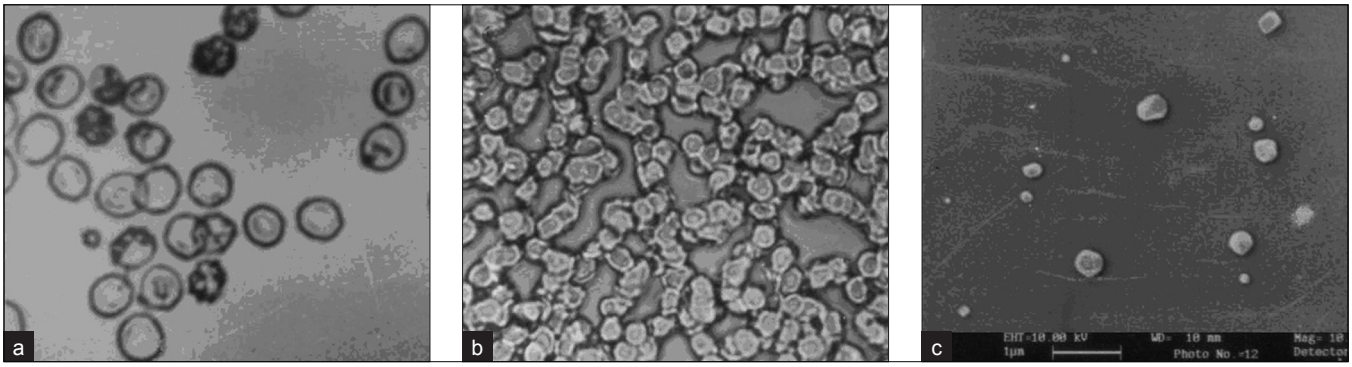

Figure 1: (a) Photomicrograph of normal erythrocytes, (b) photomicrograph of erythrocytes ghosts, and (c) SEM of drug-loaded nEs

decreased drug release rate can be ascribed to cross-linking of drug to nEs by gluteraldehyde.

Stability of formulation was assessed at 4±1°C, at room temperature, and at 37±1°C over time. Formulations exhibited relatively little change in turbidity at 4±1°C but more at room temperature and 37±1°C indicating that the formulation is most stable at 4±1°C. The effect of temperature on sedimentation volume with aging was found to be negligible [Table 2]. Sedimentation volume of formulation after centrifugation at 2500, 5000, and 7500 rpm for 15 min remained unity. This indicated stability of formulation upon centrifugal stress.

The formulation was stable against centrifugal stress as only 2.72% of drug was released after centrifugation at 7500 rpm for 15 min from DnEs [Table 3]. The DnEs formulation was assessed for stability during injection by subjecting to turbulence shock and was also found to withstand turbulence shock as only 0.882% of drug was found to be released after 15 passes through 23 gauge needle [Table 3]. These studies suggested that DnEs are quite stable in nature.

Table 2: Effect of aging on turbidity and sedimentation volume of DnEs

Temperature	Time (hour)	% Relative turbidity	Sedimentation volume (DnEs)
4±1°C	0	100.0	1
	12	101.2	1
	24	101.4	1
	48	102.4	1
	72	103.2	1
Room temperature	0	100.0	1
	12	101.2	1
	24	102.4	1
	48	104.2	1
	72	106.2	1
37±1°C	0	100.0	1
	12	102.3	1
	24	103.2	1
	48	105.4	1
	72	107.2	1

Table 3: Effect of centrifugal force and turbulence shock on stability of DnEs

Effect of centrifugal stress			Effect of turbulence shock	
Centrifugal stress (rpm)	Sedimentation volume	Drug conc. (%)	No. of passes	Drug conc. (%)
2500	1	0.81±0.6	5	0.354±1.2
5000	1	1.62±1.2	10	0.653±1.0
7500	1	2.72±1.0	15	0.882±0.8

Values represented as mean ± SD (n=3)

The formulated product with promising *in vitro* performance was evaluated for *in vivo* performance on albino rats. The blood serum concentration of drug solution and formulation after IV administration has been shown in Figure 3. After 24 h 7.03±1.1 μg/ml of PMA was found in blood using a drug solution, while it was found to be 5.02±0.5 μg/ml for DnEs. It shows that developed formulation has capacity for controlled drug delivery. The tissue distribution data of PMA [Figure 4] suggested that rapid accumulation of drug occurred in target organ such as liver (17.34±1.3 μg/ml) in 3 h in the case of DnEs, which was almost 40% higher than free drug (12.82±0.7 μg/ml). A higher amount of drug i.e. 13.14±0.9 μg/ml was still found in liver in the case of DnEs as compared to free drug concentrations of 9.72±0.5 μg/ml, respectively after 24 h

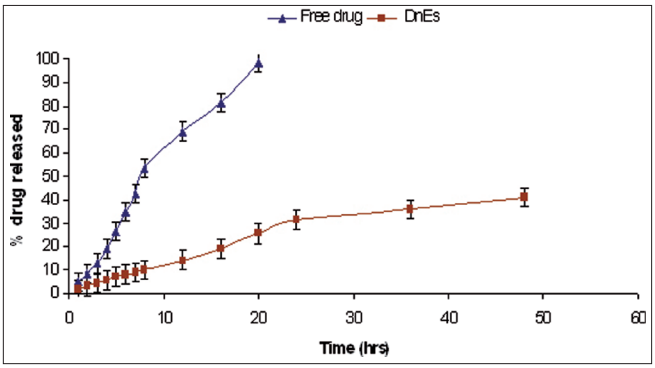

Figure 2: Cumulative percentage PMA released from the free drug solution and DnEs DnEs= PMA-loaded nEs

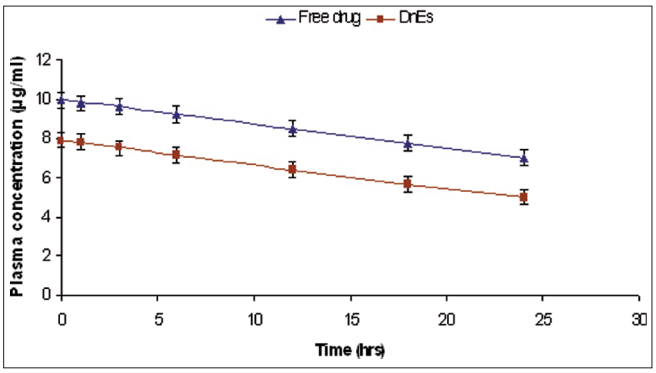

Figure 3: The blood serum concentration of the free drug solution and DnEs DnEs= PMA-loaded nEs

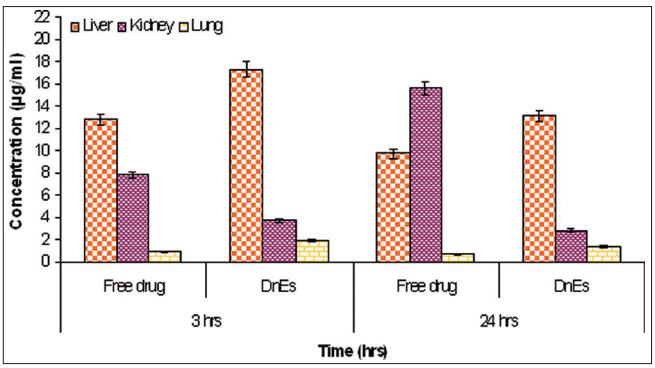

Figure 4: Tissue distribution of PMA at various time intervals (n=3)

[Figure 4]. Talwar and Jain[13] also reported targeted delivery of anti-malarial drug (primaquine) by erythrocytes and found similar higher hepatic localization of drug by erythrocytic carriers. The higher localization of drug in target organ in the case of DnEs might be due to removal of the carrier system by Kupffer cells of liver. Drug distribution studies in kidneys suggest higher accumulation of drug (7.83 ± 1.1 and 15.63 ± 1.0 μg/ml) in kidney at 3 and 24 h, respectively in the case of free drug as compared to 3.72 ± 0.5 and 2.81 ± 0.5 μg/ml in the case of DnEs [Figure 4]. The carrier system has also retarded the excretion of PMA from kidneys.

CONCLUSION

Formulation, characterization, *in vitro* release profile, and *in vivo* targeting ability of the PMA-loaded nEs formulation were evaluated in the present study. The investigations revealed an enhanced controlled release *in vitro* and *in vivo*, higher accumulation of the drug in the case of DnEs as compared to free drug in the target organs. In conclusion, PMA-loaded nEs as a new carrier hold promise for targeting and systemic controlled release.

REFERENCES

1. World Malaria Report. WHO Expert Committee on malaria; Geneva, Switzerland; 2008.
2. Farooq U, Mahajan RC. Drug resistance in malaria. J Vector Borne Dis 2004;41:45-53.
3. Molineaux L. Malaria and mortality: Some epidemiological considerations. Ann Trop Med Parasitol 1997;91:811-25.
4. Murphy SC, Breman JG. Gaps in the childhood malaria burden in Africa: Cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. Am J Trop Med Hyg 2001;64:57-67.
5. Rollo I. Resistance of Plasmodium falciparum to pyrimethamine. Transac Royal Society Trop Med Hyg 1955; 49: 94-95.
6. Fullerton DS. Antimalarials. 8th ed. Wilson and Gisvold's Text book of Organic Medical and Pharmaceutical Chemistry. In: Doergae RF, editor. Lippincott, Philadelphia. 1982. p. 205-6.
7. Jain S, Jain NK. Engineered erythrocytes as a drug delivery system. Indian J Pharm Sci 1997;59:275-81.
8. Gaudreault RC, Gicquaud C, Poyet P. Nanoerythrosome as bioactive agent carrier. US patent 5653999. Published date 5 August 1997.
9. DeLoach JR, Barton C. Circulating carrier erythrocytes: Slow-release vehicle for an antileukemic drug, cytosine arabinoside. Am J Vet Res 1982;43:2210-2.
10. Jain S, Jain SK, Dixit VK. Erythrocytes based delivery of isoniazid: Preparation and *in vitro* characterization. Indian Drugs 1995;32:471-6.
11. Jain S, Jain NK. Resealed erythrocytes as drug carriers. In: Jain NK, editor. Controlled and Novel Drug Delivery. New Delhi: CBS Publishers and Distributors; 2002. p. 256-7.
12. Lejeune A, Moorjani M, Gicquaud C, Lacroix J, Poyet P, Gaudreault R. Nanoerythrosomes, a new derivative of erythrocyte ghost: Preparation and antineoplastic potential as drug carrier for daunorubicin. Anticancer Res 1994;14:915-9.
13. Talwar N, Jain NK. Erythrocytes as carriers of primaquin preparation: Characterization and evaluation. J Control Rel 1992;20:133-42.
14. Talwar N, Jain NK. Erythrocytes as carriers of metronidazole: In-Vitro characterization. Drug Dev Ind Pharm 1992;18:1799-812.
15. Lejeune A, Poyet P, Gaudreault RC, Gicquaud C. Nanoerythrosomes, a new derivative of erythrocyte ghost: III. Is phagocytosis involved in the mechanism of action? Anticancer Res 1997;17:3599-603.
16. Minzi OM, Massele AY, Gustafsson LL, Ericsson O. Simple and cost-effective liquid chromatographic method for determination of pyrimethamine in whole blood samples dried on filter paper. J Chromatogr B Analyt Technol Biomed Life Sci 2005;814:179-83.

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