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Biotinylated methotrexate loaded erythrocytes for enhanced liver uptake. 'A study on the rat'

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Abstract

To serve as liver specific delivery system, methotrexate (MTX) loaded erythrocytes were modified by attachment with *N*-hydroxysuccinimide ester of biotin (NHS-biotin) and in vitro macrophage uptake and in vivo studies were carried out in the rat. Surface bound biotin was quantitated indirectly using an avidin support and measuring the change in absorbance at 500 nm by the interaction with 2-(4′ hydroxyazobenzene) benzoic acid (HABA). A concentration course study of biotin binding reaction showed biotin molecules bound to the erythrocytes as a function of the NHS-biotin concentration. Glycerol lysis time study revealed enhanced stability of biotinylated cells (BT) compared with nonbiotinylated drug loaded cells (NB). These surface modified erythrocytes were characterized for in vitro macrophage uptake. The macrophage uptake and phagocytic index of these modified erythrocytes were enhanced by more than two-fold compared with NB cells ($P < 0.05$). In vivo organ localization was assessed by recording amount of drug present in different organs. The modified carrier induces substantial liver targeting as drug therapeutic index in liver was found to be 3.1. These findings support the use of carrier erythrocytes, exploiting the targeting properties imparted by biotinylation. Consequently, it can spare surgical intervention to place hepatic arterial catheters for locoregional treatment of liver neoplasms.

Keywords: Avidin; Biotin; Chemical modification; Methotrexate; Red cell carriers

1. Introduction

From the last few decades erythrocytes are potentially recognized as a drug delivery system (Pinilla et al., 1994) and their physiological characteristics, viability and being a part of viable endogenous tissue, support their utilization (Ihler et al., 1975; Magnani et al., 1989). Several reports revealed targeting of drugs to phagocytic cells, like normal erythrocytes coated with antibodies result in the reduction of circulation half-life (Berman and Gallalee, 1985). In fact, the efficacy of this system can be dependent on the use of several chemical treatments, which can react with cell membrane proteins. Gluteraldehyde has been the most extensively used crosslinker in erythro-

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cytes for drug localization in the reticuloendothelial system (RES) (Talwar and Jain, 1992). Targeting of mouse erythrocytes to RES has also been reported using bis (sulfosuccinimidyl) suberate (BS^3) and 3-3′ dithiobis (sulfosuccinimidyl) propionate (DTSSP) (Jordan et al., 1997).

Systemic chemotherapy has only marginal role in the treatment of unresectable hepatocellular carcinoma or secondary liver metastases from gastrointestinal malignancies, producing at least a 20% response rate. Red blood cells (RBCs) with incorporated xenobiotics have unique potential utility because they are part of a viable endogenous tissue, lack nuclear material and associated oxidative enzymes, and are poorly permeable to many complex molecules including enzymes, other polypeptides and most phosphorylated compounds and can be targeted using suitable chemical modification.

In a previous report we have successfully achieved enhanced macrophage uptake using desialation technique and phenylhydrazine treatment (Mishra and Jain, 2000). Additionally we have studied biotinylation of erythrocytes, which is another alternative method for carrier modification, having potential for targeting to the liver with different mechanism. Our aim has been to study, how biotinylation of erythrocytes alter biodistribution pattern of drug loaded erythrocytes. In the present study we present evidence that on altering the configuration of carrier erythrocytes, the survival of erythrocytes dramatically changes. Biotinylation of RBC is a simple procedure in which succinimide ester of biotin is covalently attached to erythrocytes using succinylated bovine serum albumin (BSA) as spacer. RBC modified with succinimide ester of biotin was used to trace normal senescent and oxidized RBC in the circulation (Suzuki and Dale, 1987). RES is comprised of a set of mononuclear phagocytic cells. These cells originate from precursors in bone marrow, enter the blood stream as monocytes and then pass into various tissues where they differentiate into macrophages (Meuret, 1981; Silverstein et al., 1977). Biotinylation of erythrocytes and thus involvement of complement mechanism is a new approach for methotrexate delivery. In vitro and in vivo behavior of these modified cells have been studied and compared with recognition by macrophages. Thus we described conditions for using these modifications for targeting to organs and macrophages. This sort of surface modification renders them more biocompatible and this attachment is reported to be stable on erythrocytes (Suzuki and Dale, 1987). These chemically modified cells are expected to be useful in effective treatment of RES malignancies, and the use of other bifunctional reagents like gluteraldehyde which is difficult to remove and is reported to be carcinogenic.

2. Materials and methods

2.1. Materials

Methotrexate (MTX) was received as a gift sample from M/s Biochem Industries, Mumbai India; *N*-hydroxysuccinimide ester of biotin (NHS-biotin); avidin; 2-(4′ hydroxyazobenzene)-benzoic acid (HABA) (Sigma, USA); sodium phosphate dibasic; potassium dihydrogen orthophosphate, sodium chloride; thiobarbituric acid; glycerin GR (Loba Chemie, Mumbai, India); acetonitrile; methanol (Ranbaxy Lab, Delhi, India); dimethyl formamide (DMF); bovine serum albumin (BSA); Brewer's Thioglycolate medium and RPMI 1640 (Himedia Lab, Mumbai, India); and Heparin sodium (Biological Evans, Mumbai).

2.2. Separation of erythrocytes, serum and drug encapsulation

Blood was collected from descending aorta of rats weighing 250–300 g and loaded with MTX using preswell dilution method as reported (Mishra et al., 1996), the amount of drug entrapped was determined spectrophotometrically at 259 nm.

To obtain serum, nonheparinized blood was collected from descending aorta. After 2-h incubation at 4 °C, serum was separated by centrifugation.

2.3. Collection of macrophages

Autoclave sterilized Brewer's thioglycollate medium was stored in dark for 1 week before use. Rats were injected with 2 ml of thioglycollate medium intraperitoneally. Three days later, midline incision was made and the peritoneal fluid was collected by lavaging with 5×10 ml heparinised phosphate buffered saline (PBS pH 7.4) taking aseptic precautions. The peritoneal fluid was withdrawn by suction into a collecting vessel on ice, centrifuged at 1500 rpm for 15 min at 4 °C and supernatant was discarded. The cells were resuspended in RPMI 1640 medium supplemented with heat inactivated 20% fetal calf serum (FCS) and subsequently washed thrice with the same medium. Total cell counts were performed in hemocytometer chamber and differential cell counts obtained by staining smears with leishmans, haematoxylin and eosin. Cell viability was assessed by the ability of cells to exclude trypan blue. Cells were counted in a haemacytometer chamber using trypan blue (0.17% in PBS, pH 7.4) as the cell diluting fluid. Out of the total cells more than 95% ($95.4 \pm 3.9\%$, $n = 3$) of the cells were macrophage (Foong and Green, 1988).

2.4. Biotinylation of erythrocytes

Biotinylation of erythrocytes was carried out by the reported method (Suzuki and Dale, 1987) with slight modification. Succinylated BSA was prepared as described by Chu et al. (1969) with slight modification. Briefly, 1 g of BSA was dissolved in 100 ml of 0.5 M $NaHCO_3$, pH 8.5. Succinic anhydride (800 mg) was added to the BSA solution in four equally divided aliquots up to 1 h at room temperature and the pH maintained at 8.0 with sodium hydroxide. The product obtained was dialyzed against 1 l of PBS (pH 7.4) at 4 °C. Succinylated BSA was stored at a protein concentration of 12 μmol 100 ml^{-1}. MTX loaded and control cells were washed in PBS (pH 7.4) three times, buffy coat was removed and suspended to a 20% hematocrit in PBS (pH 7.4) containing 15 mmol l^{-1} glucose. Two milliliter of succinylated BSA (equivalent to NHS-biotin concentration), was then added to the cell suspension and shaken for 5 min at room temperature. The succinylated BSA serves as a non-nuclophilic carrier for the hydrophobic NHS-biotin. In the presence of succinylated BSA, the NHS-biotin is presumably prevented from intercalating into the erythrocyte membrane and causing a reversible shape change. Finally different concentrations of NHS-biotin (100, 200, 300, 400 μg stock solution; 1 mg ml^{-1} in DMF) were added to the mixture, followed by incubation at 37 °C for 1 h.

3. In vitro characterization

3.1. Extent of modification

To determine the substitution level of biotin molecules per erythrocyte, a slightly modified method reported by Green (1970) was adopted. Briefly, 100 μl of supernatant was washed thrice with PBS (pH 7.4) and suitably diluted. To this, 2 ml of avidin–HABA complex (0.2 mg ml^{-1} of avidin, 0.25 mM HABA in 0.1 M phosphate buffer, pH 7.0) was added and the biotin bound to erythrocytes was quantitated indirectly by measuring absorbance of free avidin–HABA complex in the supernatant at 500 nm. The number of biotin molecules bound per erythrocyte was calculated by taking concentration and molecular weight of avidin and total number of cells used.

3.2. Glycerol lysis time

Glycerol lysis time (GLT_{50}) was determined by measuring the time required for hemolysis of 50% of the erythrocytes in standardized glycerol mixture (Gottfield and Robertson, 1974; Mishra and Jain, 2000). Briefly, 100 μl of blood was diluted to 20 ml with isotonic PBS (pH 7.4). One milliliter of this diluted suspension was pipetted into standard spectroscopy cuvette (10 mm light path). To sample operating in kinetic mode (at 625 nm) of UV-1601 spectrophotometer, 2 ml of glycerol reagent (0.3 M in deionized water) was added quickly using syringe pipette. Using completely hemolyzed blood sample as a blank, the median glycerol lysis time corresponds to the time required for the absorbance to come down to the half of the initial value.

3.3. Viscosity

The viscosity of the cell suspensions (NB and BT cells) at the hematocrit value of 75% was determined in Brookfield viscometer (Model RVT 73042) at the shear rate of 2.3 per second.

4. Targeting of modified erythrocytes to macrophages

In vitro macrophage uptake of normal drug (NB cells) loaded and treated drug loaded (BT cells) erythrocytes were studied both in serum and in PBS–BSA (PBS containing 2 mg ml^{-1} BSA) on rat peritoneal derived macrophages, by layering modified erythrocytes on macrophages in the ratio of 100:1 and phagocytosis was conducted for 24 h. Noningested RBCs were removed by extensive washing (three times) with RPMI 1640 medium and then washed with 0.18 M ammonium chloride. The phagocytosis index of these carrier systems was determined both in terms of percent drug assay and visual examination under optical microscope. The amount of drug associated with macrophages was estimated using reported polarographic method (Dias and Dhadke, 1994) after extraction of drug with ethyl acetate and propan-2-ol in the ration of 10:1. The phagocytosis index was then calculated by reported method (Mc Evoy et al., 1986). In order to investigate the uptake of NB cells by macrophages, these cells were incubated with macrophage culture and observed in random microscopic fields in parallel cultures in the ratio of 100:1.

5. In vivo studies

For biodistribution studies 18 rats were divided into three groups. The first group received free MTX solution in PBS (1.0 mg kg^{-1}) whereas the second group received drug loaded nonmodified erythrocytes (NB cells) while the third group was administered biotinylated erythrocytes (BT cells) containing equivalent amount of drug through caudal vein. At 1 and 12 h post injection, 200 μl of blood was collected from retro-orbital plexus and animals were humanely sacrificed (three animals from each group) and internal organs were collected. The organs were rinsed thoroughly in saline to remove adhering debris, and dried with tissue paper. The organs were sliced and minced and then finally homogenized in Micro Tissue Homogenizer (MAC, Virtis Type). The drug was extracted using ethyl acetate:propan-2-ol (10:1) mixture. The samples were centrifuged and supernatants were analyzed for MTX content polarographically (Dias and Dhadke, 1994). For MTX determination a polarograph composed of polyflex galvanometer (sensitivity 8.1×10^{-9} amp./div) was used. Dropping mercury electrode (DME) served as reference electrode. Capillary characteristics of DME were 2.3 $mg^{2/3}$ $s^{-1/2}$ at 50 cm effective wave height of mercury column. Diffusion current was found to vary linearly with concentration. MTX gave a distinct polarographic wave in Clarks and Cubb's buffer at pH 10 ± 0.2 with half wave potential of $E_{1/2} = 0.85$ V versus standard calomel electrode (SCE). The sensitivity of the proposed method of analysis is 100 ng ml^{-1}. The between-run reproducibility and accuracy of the method were examined on three different occasions in various tissues. Over the range of 100–600 ng ml^{-1}, the coefficient of variation was being in the range of 0.4–3.0%. Compared with predicted concentration, the observed concentrations of MTX also reflected the excellent accuracy of method with relative errors (R.E.) falling in the range of 0.2–2.5%. Drug targeting index (DTI) was calculated by the following formula.

$$\mathrm{DTI} = \frac{\%\text{ drug concentration in liver at time '}t\text{' after administration of surface modified erythrocytes}}{\%\text{ drug concentration in liver at time '}t\text{' after administration of drug as solution}}$$

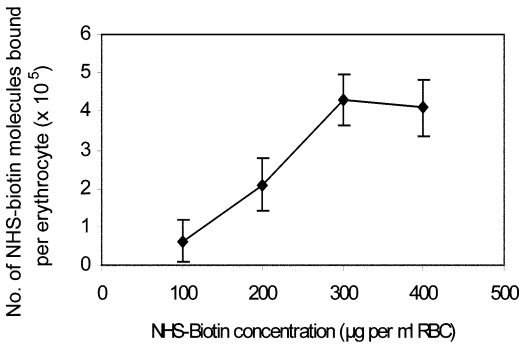

Fig. 1. Effect of different concentration of NHS-biotin on erythrocyte binding.

6. Results

There are several reports, where enhanced RES uptake has been demonstrated using erythrocytes as a carrier (Talwar and Jain, 1992; Jordan et al., 1997; Mishra and Jain, 2000). However, we have studied another possible way (biotinylation) to deliver MTX to the liver with a potential for therapeutic application. The results of in vitro studies suggest that recognition of erythrocytes by macrophages can be regulated by chemical modification of erythrocytes using NHS-biotin. The biotinylation of erythrocytes was carried out by incubating different concentrations of NHS-biotin. On treatment with 100, 200, 300, 400 μg of NHS biotin, approximately $(0.63, 2.1, 4.3, 4.1) \times 10^5$ biotin molecules were bound per erythrocyte, respectively (Fig. 1). Maximum binding occurs at the concentration of 300 μg of NHS-biotin. Therefore, 300 μg of NHS-biotin concentration was used for biotinylation throughout the experiment.

Methotrexate was encapsulated in erythrocytes by preswell dilution procedure using optimized parameters as reported earlier (Ihler et al., 1975; Mishra and Jain, 2000). The optimized parameters for maximum percent encapsulation ($47.13 \pm 0.76\%$) in terms of volume of drug solution, concentration of drug solution and temperature were 300 μl, 5 mg ml^{-1} and 37 °C, respectively. The shape of the erythrocytes were normal biconcave after biotinylation and no lysis was detected when observed under phase contrast microscope, which is in agreement with our earlier report (Mishra et al., 1996). On using 6 and 7 mg ml^{-1} drug concentration, the percent encapsulation was reduced to 26.41 ± 0.82 and $18.34 \pm 0.46\%$, respectively. The median GLT_{50} of BT cells was also much higher than the normal cells (Table 1), which indicates that biotinylation stabilizes the erythrocyte membrane. The viscosity of BT cells was slightly higher than NB cells, which may be attributed to hardening of the cell surface due to biotinylation.

In vivo behavior showed that surface modification rendered erythrocytes, on injection into rats, are predominantly targeted to liver. Spleen also showed significant uptake of carriers. As it can be observed (Fig. 2), there was increase in percent of unmetabolized drug in liver and spleen as a function of time. BT cells showed almost three-fold (37.2%) increased drug level in liver compared with free drug (11.7%) and almost 1.8 fold compared with NB cells (20.4%) after 1 h. However, at 12th hour, 23.5% of unmetabolized drug was recovered in liver after administration of BT cells while only 1.7% of drug was recovered after free drug administration. DTI in liver was found to be 3.1 on administration of BT cells after 1 h.

Table 1
Characterization of surface modified erythrocytes

Type of cells	Extent of biotinylation[a] (number of NHS-biotin molecule per RBC)	GLT_{50} (s)[b] ($n = 3$)	Viscosity (centipoise) ($n = 3$)
Normal	–	36 ± 3.2	51 ± 1.2
NB	–	46 ± 2.4	52 ± 1.7
BT	4.3×10^3	105 ± 4.1	63 ± 3.1

[a] Concentration of NHS-biotin used was 300 μg ml^{-1} RBC. NB, nonbiotinylated drug loaded cells. BT, biotinylated drug loaded drugs.

[b] Glycerol lysis time (GLT_{50}) is the time required for hemolysis of 50% of the erythrocytes in standardized glycerol mixture.

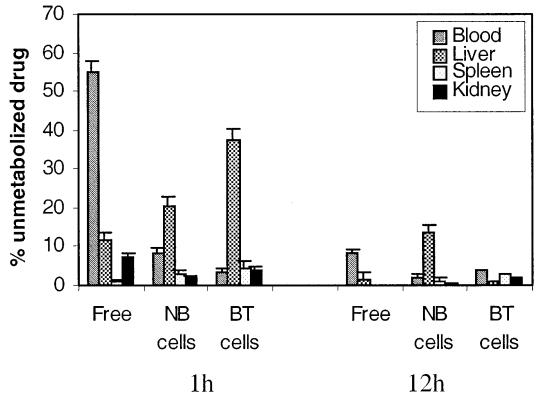

Fig. 2. In vivo organ localization of methotrexate after i.v. administration of free drug, NB and BT cells (1.0 mg kg^{-1}) in albino rats. Three animals were taken from each group for analysis ($n = 3$). NB, nonbiotinylated drug loaded cells; BT, biotinylated drug loaded cells.

In vitro macrophage uptake was studied on rat peritoneal derived macrophages by layering modified erythrocytes on macrophages in the ratio of 100:1 and phagocytosis was conducted for 24 h. The number of erythrocytes ingested by macrophages in random microscopic fields in parallel cultures were compared. Cultures were fixed and stained for hemoglobin and it was found that NB cells containing macrophages culture contained one or two internalized erythrocytes per cell (both, in PBS–BSA, and serum), while macrophages in culture that had been presented with BT cells (with serum) contained on an average two to three phagocytosed erythrocytes. Table 2 shows that when biotinylated RBCs were added with PBS–BSA, the phagocytosis index was nearly same as that of NB cells (with or without serum), while addition of biotinylated RBC along with rat serum enhanced the index ($P < 0.05$) almost by 1.8 times compared with NB cells.

To confirm these values from visual examination of macrophages, phagocytosis by entire population was measured quantitatively by using NB (with serum) and BT cells (with serum), which were incubated with macrophages for 24 h and noningested erythrocytes were lysed as explained in the experimental section. The percent of drug added that remained associated with macrophages after lysis was used as a measure of phagocytosis. The percent of drug associated with macrophages in case of BT cells (with serum) was found to be more than three times as compared with nonbiotinylated drug loaded (NB) cells. In contrast to this, the percent of drug associated with macrophages in case of BT cells (with PBS–BSA)

Table 2
Phagocytosis of normal and surface modified drug loaded erythrocytes by macrophages

Cell type	Visual assay	% Drug assay		
	Number of cells ingested/macrophage[a]	Phagocytosis index[b]	% Drug ingested by macrophage[c]	Phagocytosis index[b]
N cells	0.5	1.0 ± 0.25	0.43	1.0 ± 0.32
NB cells (with PBS–BSA)	1.1	2.2 ± 0.61	0.79	1.80 ± 0.42
NB cells (with serum)	1.1	2.2 ± 0.62	0.78	1.81 ± 0.57
BT cells (with PBS–BSA)	1.2	2.4 ± 0.78	0.81	1.88 ± 0.62
BT cells (with serum)	2.1	4.2 ± 0.41	1.83	4.30 ± 0.31

N, normal cells; NB, nonbiotinylated drug loaded cells; BT, biotinylated drug loaded cells. $P < 0.05$ for NB cells (with serum) compared with N cells and $P < 0.01$ for BT cells (with serum) compared with NB cells (with serum).

[a] Average number of cells ingested per macrophage from one representative experiment in which 200 macrophages in each of triplicate culture were examined. Only one experiment is given since these absolute numbers varied (from 0.2 to 1.0) for normal cells depending upon the population of macrophages used.

[b] Mean ± S.D. of three separate experiments.

[c] Average % of drug associated in triplicate wells from one representative experiment. These absolute numbers varied upon the population of macrophages used.

was nearly same as that of BT cells (with PBS–BSA), which is fully complied with visual examination of macrophage uptake.

7. Discussion

Among the various carrier systems proposed for transportation and delivery of pharmacologically active substances for improving their therapeutic effectivity, erythrocytes have attracted much attention in recent years, as these are biodegradable and biocompatible carriers for a variety of bioactive agents. Further, erythrocytes also have potential application for the delivery of drugs to macrophages.

In the present investigation we have developed original protocols on the alternate application of biotinylated erythrocytes, which may provide practical application in the future. We are exploiting erythrocytes as a carrier system for targeting to RES apart from treatment with gluteraldehyde, which has been extensively reported as a protein crosslinker which renders the carrier system to be taken up quickly by the liver or spleen phagocytes. But unreacted gluteraldehyde is very difficult to remove and is likely to be carcinogenic. In the present report we have gathered evidence on the enhanced RES uptake and superior therapeutic efficacy through biotinylated erythrocytes bearing MTX.

The coating of drug loaded erythrocytes with BSA prior to biotinylation resulted in reversible shape change of cells (Chu et al., 1969), which may be due to the fact that BSA serves as a non-nucleophilic carrier for NHS-biotin that presumably prevents intercalation with erythrocyte membrane. The biotin molecules attached to erythrocytes were recovered using an avidin support. Avidin–biotin interaction was exploited for the quantitation of surface bound biotin. The avidin has high affinity for the biotin molecule, which forms highly stable noncovalent bond with biotin (Suzuki and Dale, 1987). As shown in Fig. 1, there was continuous increase in biotin molecules bound per erythrocyte, on increasing NHS-biotin concentration up to 300 μg ml^{-1} of RBC but on further increasing the NHS-biotin concentration (400 μg), the number of attached biotin molecules sharply decreased which may be ascribed to the surface saturation of erythrocytes. The decline in percent encapsulation of MTX on using higher concentration (6 and 7 mg ml^{-1}) can be ascribed to the hemolytic effect of aqueous solution as well as drug itself. Thus the drug concentration of 5 mg ml^{-1} was considered to be the optimal.

In vivo behavior showed that on injection into rats, surface modified erythrocytes were predominantly targeted to liver. Spleen also showed significant uptake of carriers. To clarify the mechanism of clearance of biotinylated RBC in vivo, we have performed in vitro macrophage (rat peritoneal derived macrophages) interaction with formulations (with and without serum). The phagocytic index of BT cells (with serum) was enhanced almost by 1.8 times compared with NB cells (with and without serum) and BT cells (without serum). Furthermore, inactivation of serum complement by heating abolishes uptake (data not shown). These results strongly suggest the possibility of the involvement of complement in enhanced uptake of BT cells by the RES. This interesting finding suggests another possible way to deliver drug to the liver with a potential therapeutic use.

The major cell types, which are responsible for the elimination of modified cells, are hepatic and splenic macrophages. It is reported that lower levels of biotinylation result in normal in vivo survival as compared with normal erythrocytes (Suzuki and Dale, 1987) while extensive biotinylation renders them susceptible to deposition of C3b on the RBC membrane (Zaltzman et al., 1995). The targeting index of 3.1 may be due to the deposition of C3b, which possibly opsonizes biotinylated RBCs in vivo. It is well known that C3b-opsonized particles (including RBCs) are cleared by the liver and spleen in a process that presumably depends upon C3b receptors on liver macrophages (Muzykontov et al., 1991a,b,c) and thus leads to C3b mediated uptake of MTX loaded RBCs by tissue macrophages in the RES system (Muzykontov et al., 1996). There are multiple signals for recognition by the RES. In case of liposomes, if the phospholipids of the bilayer are not tightly packed or if modest levels of

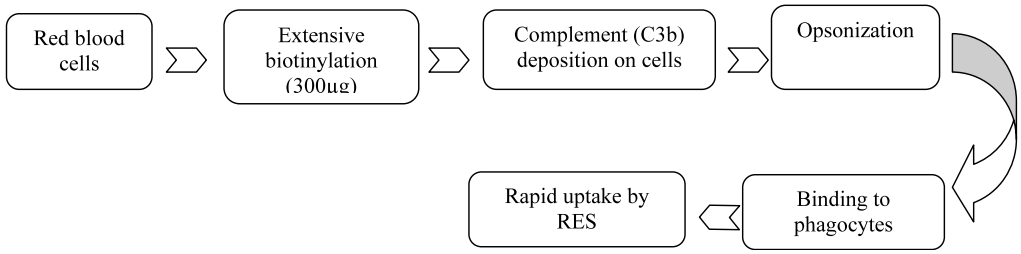

Fig. 3. Proposed mechanism of sequential macrophage uptake post biotinylation.

phoshotidylsphingomyelin (PS) are exposed, they are rapidly removed from circulation. Apart from this different levels of RES targeting could also be achieved in case of colloidal based drug delivery systems based on physical targeting (particle size). Thus it can be concluded that this preferential targeting of carriers to the liver is possibly due to binding of biotinylated erythrocytes to macrophages via C3b-mediation in liver. The proposed sequential complement dependent enhanced macrophage uptake is illustrated in Fig. 3.

If liver targeting is also achieved by systemic administration, the optimal use of targeted carriers would spare surgical intervention to place hepatic arterial catheters for locoregional treatment of liver neoplasms. Although major of the carrier erythrocytes will be taken up by RES after biotinylation. But it does not necessarily prevent the drug from reaching the tumor cells either due to intracellular uptake and finally release of active drug inside the cell or breaking up of the carrier system in the vicinity of the tumor cells, which would cause the drug to be taken up by the tumor cells. The major of the carrier erythrocytes are taken up by the Kupffer cells, which acts as a reservoirs from where the drug will be released slowly due to massive diffusion and transportation in the vicinity of the tumor cells. Apart from this, it can hold an advantage compared with gluteraldehyde treated cells for enhanced liver uptake, as free gluteraldehyde is difficult to remove and may be carcinogenic.

In conclusion, an alternative technique has been developed in order to ascertain the possibility of enhanced uptake of drug by macrophages by modified cells. This approach can also be used to target the carriers to macrophages and may prove superior in case of RES tumors.

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