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RGD-anchored magnetic liposomes for monocytes/neutrophils-mediated brain targeting

Sanyog Jain [a], Vivek Mishra [a], Paramjit Singh [a], P.K. Dubey [a], D.K. Saraf [b], S.P. Vyas [a,*]

[a] *Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences, Dr. Harisingh Gour University, Sagar, M.P. 470 003, India*
[b] *Department of Zoology, Dr. Harisingh Gour University, Sagar, M.P. 470 003, India*

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Abstract

Negatively charged magnetic liposomes were prepared using soya lecithin (Soya PC), cholesterol and phosphatidyl serine (PS) for their preferential presentation to circulating blood phagocytes (monocytes and neutrophils). PS ratio was optimized in terms of drug and magnetite loading, in vitro magnetic responsiveness and ex vivo monocytes/neutrophils uptake. RGD peptide was covalently coupled to the negatively charged liposomes composed of PC, cholesterol, PS and phopsphatidyl ethanolamine (PE) via carbodiimide-mediated coupling. In vivo cellular sorting study under magnetic guard indicated an increase in relative count of neutrophils and monocytes. Results suggest that selective uptake of RGD-anchored magnetic liposomes by these cells imparts them magnetic property. High levels of a model drug diclofenac sodium was quantified in target organ brain. In case of negatively charged uncoated magnetic liposomes brain levels of the drug was 5.95-fold compared to free drug and 7.58-fold in comparison to non-magnetic formulation, while for RGD-coated magnetic liposomes this ratio was 9.1-fold compared to free drug solution, 6.62-fold compared to non-magnetic RGD-coated liposomes and 1.5-fold when compared to uncoated magnetic liposomes. Liver uptake was significantly bypassed (37.2% and 48.3% for uncoated and RGD-coated magnetic liposomes, respectively). This study suggested the potential of negatively charged and RGD-coated magnetic liposomes for monocytes/neutrophils-mediated active delivery of drugs to relatively inaccessible inflammatory sites, i.e. brain. The study opens a new perspective of active delivery of drugs for a possible treatment of cerebrovascular diseases.

Keywords: Magnetic liposomes; Brain targeting; RGD; Blood–brain barrier; Monocytes; Neutrophils

1. Introduction

Blood–brain barrier (BBB) constitutes the major physiological barrier that effectively screens the blood components present in the blood stream before they are allowed to penetrate and reach the brain compartment. Unlike the endothelia of many peripheral tissues, cerebrovascular system has tight intercelluar junctions (Reese and Karnovsky, 1967; Brightman and Reese, 1969). Moreover, because of its highly lipophilic nature, many hydrophilic compounds fail to enter the brain following systemic administration. Hence, management of brain related diseases with presently available therapeutic

* Corresponding author. Tel.: +91-7582-226525; fax: +91-7582-226525.
E-mail address: vyas_sp@rediffmail.com (S.P. Vyas).

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systems is often a formidable therapeutic attempt. The brain being the major organ of interest for selective delivery of various therapeutically active compounds for effective management of cerebrovascular diseases.

The use of liposomes for drug delivery across the brain capillaries has been documented and examined. Small unilamellar vesicles (SUVs) as well as cationic liposomes coupled with brain drug transport vectors may be transported through the BBB by receptor-mediated or absorptive-mediated transcytosis (Umezawa and Eto, 1988; Wang et al., 1995; Huwyler et al., 1996; Shi and Pardridge, 2000; Shi et al., 2001; Mora et al., 2002; Thole et al., 2002; Zhang et al., 2002). These colloidal carriers are however subjected to conductive opsonization and subsequent opsonic phagocytosis by circulating phagocytes (monocytes and neutrophils) and by macrophages of liver and spleen (Kirsh et al., 1987; Scherphof, 1991). One of the strategies to overwhelm this problem is to magnetize the drug loaded carrier so that it can be retained at or guided to the target site with the help of an external magnetic field of appropriate strength. Retention of magnetic carrier at target site apparently delays reticuloendothelial clearance and neodisposition of the carrier and contained drug. Magnetic liposomes have been investigated for targeted drug carrying potentials (Kiwada et al., 1986; Shinkai et al., 1995, 1996; Viroonchatapan et al., 1995; Yanase et al., 1996; Kubo et al., 2000). However, targeting of the drugs to brain using these magnetic liposomes remains an unmet goal because of the stern limitations imposed by impervious capillaries that supply to brain. These capillaries are the major constituents of BBB. Even the liposomes with a diameter as small as 100 nm fail to penetrate via free diffusion through BBB (Sakamoto and Ido, 1993).

It is becoming increasingly apparent that many neurological diseases, such as Alzheimer's disease, Parkinson's disease, Prion disease, meningitis, encephalitis and AIDS related dementia, have in common an inflammatory component (Perry et al., 1995). The process of inflammation is characterized by extensive leukocytes (neutrophils and monocytes) recruitment. These cells have unique property of migrating toward inflammation site via the processes known as diapedesis and chemotaxis (Kuby, 1994; Levinson and Jawetz, 1994). Leukocytes are reported to cause BBB breakdown following brain inflammation (Anthony et al., 1997, 1998; Bolton et al., 1998; Blamire et al., 2000). Studies revealed that leukocytes can cross an intact BBB in healthy condition (Perry et al., 1997).

Phagocytic and exclusive extravasation property of leukocytes make it possible to exploit these cells as carrier system for targeted delivery. Magnetic neutrophils have been prepared in vitro and targeted to lungs under magnetic guidance following intravenous injection (Ranney and Huffaker, 1987). It was therefore envisaged that drug loaded magnetic liposomes can be devised for selective and preferential presentation to blood monocytes/neutrophils that result in both drug and magnetite incorporation into these cells, which subsequently become magnetized cells responding to magnetic field. These magnetic monocytes/neutrophils can then be guided in vivo to the target site, i.e. brain by applying an external magnetic field of appropriate strength. Thus, co-ordinated carrier-biocell strategy, controllable at an external level could be designed with distinctive targeting and therapeutic potentials. Studies revealed that negatively charged liposomes are generally taken up by blood monocytes/neutrophils preferentially and rapidly compared to neutral or positively charged lipid vesicles (Juliano and Stamp, 1975; Poste et al., 1982). Furthermore, these cells (monocytes/neutrophils) express integrin receptors on their surface that selectively bind to small peptide domain Arg-Gly-Asp (RGD) (Aznavoorian et al., 1990; Odekon et al., 1991; Hauzenberger et al., 1993; Saiki et al., 1996). Exquisitely designed studies have exhibited that interaction between RGD domain on integrin molecule and integrin receptor on leukocytes stimulates phagocytosis by polymorphonuclear cells (e.g. neutrophils) (Senior et al., 1992). Furthermore, localized delivery of bioactives to inflammatory sites (rich in integrin molecules or RGD domain) has been achieved using these cells as delivery vehicle (Kao et al., 2002).

In the present study, an anti-inflammatory drug Diclofenac sodium, a model drug was selected for developing drug loaded negatively charged and RGD-coated magnetic liposomes for their preferential presentation to blood phagocytes (monocytes/neutrophils) and evaluating their subsequent

targetability to the brain under inflammatory condition.

2. Materials and methods

2.1. Materials

L-α-Soya phosphatidyl choline (Soya PC), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), cholesterol (Chol), RGD peptide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), Sephadex G-50, human recombinant interleukin (IL-1β), concanavallin A and Triton X-100 were purchased from Sigma Chemical Co. (USA). Dextran-Magnetite (DM) was procured from Meitosangyo (Nagoya, Japan). Diclofenac sodium was a kind gift from M/s Promise Pharmaceuticals, India. All other chemicals used were of analytical grade unless mentioned.

The specification of DM were as follows: a hematite impurity was under a detection limit according to the results of X-ray diffraction; an average molecular weight of dextran in DM was 4000; a dextran/magnetite ratio was 0.43 and its average core size was 8 nm ranging between 5 and 10 nm.

2.2. Preparation of magnetic liposomes

Magnetic liposomes were prepared by reverse phase evaporation method using different molar ratios of soya PC:Chol:PS (Szoka and Papahadjopoulos, 1978; New, 1990). Lipids were dissolved in diethyl ether, followed by emulsification with aqueous solution of drug (diclofenac sodium) (10 mg) and DM dispersion (100 μM as magnetite) by probe sonication for 5 min at 40 kc/s (Soniweld, India). The organic solvent was evaporated in a rotary flash evaporator at 37 °C under reduced pressure (260–400 mm Hg). The lipid gel so formed was collapsed and transformed into a fluid on continual vigorous mechanical agitation on a vortex mixer. Magnetic liposomes were separated from non-magnetite loaded ones following the procedure described by Gallo et al. (1989). The liposomes were transferred into a tarred centrifuge tube in the presence of a 300 G bar magnet placed adjacent to the decanting vessel. The free magnetite was effectively retained in the vessel due to its high magnetic responsiveness relative to the liposomes encapsulated magnetite.

2.3. Optimization of process variables

2.3.1. Optimization of negatively charged lipid (PS) ratio

Magnetic liposomes loaded with drug were prepared using different molar ratios of lipids (PC:Chol:PS; total amount of lipid used was 50 μmol). Prepared liposomes were characterized for percent drug and magnetite entrapment, in vitro magnetic responsiveness and ex vivo monocytes/neutrophils uptake to optimize PS concentration.

2.3.1.1. Determination of drug and magnetite content. The liposomal suspension was passed through Sephadex G-50 mini-column by centrifugation at 3000 rpm for 3 min to remove unentrapped drug. The liposomes were lysed using minimum amount of Triton X-100 (0.5% w/v) and drug content was determined spectrophotometrically at 276 nm using Shimdazu 1601 double beam spectrophotometer. Magnetite was estimated following colorimetric method developed in our laboratory (Jain et al., 2002).

2.3.1.2. In vitro magnetic responsiveness. Drug loaded magnetic liposomes were evaluated for magnetic responsiveness following the procedure described by Kiwada et al. (1986). Briefly, 1.0 ml of liposomal suspension was injected as a bolus through the top of silicone capillary tubing (internal diameter 1 mm) during the infusion of PBS with an infusion pump (Model M361, Orion Research, MA). The electric magnet (Model MCD-1B, JASCO, Tokyo, Japan) was located at middle of the capillary so that fixed liposomes would not be washed away by the water stream. Magnetic field of 8.0 kG strength at a flow rate of 0.5 ml/min was applied. The effluent from the bottom of the capillary was collected in 1 ml fractions and the drug (diclofenac sodium) content in each fraction was determined spectrophotometrically. After collection of 10 fractions, the medium retained in the capillary was washed and drug content was measured. In vitro magnetic responsiveness was calculated as the percentage of drug retained in the capillary per dose after the infusion of 10 ml of medium.

2.3.1.3. Ex vivo cellular uptake. Drug loaded magnetic liposomes (1 ml) possessing different magnitude of negative charge were mixed with fresh

heparinized whole human blood (2 ml) and incubated at 37 ± 1 °C. After 2 h samples were added to agarose and fucose (2%) dispersion media in a microsyringe centrifugation column and centrifuged at 10,000 rpm for 10 min. The sedimented cells were separated and observed under microscope. To the column 2% solution of concanavallin A was added and contents of the microsyringe gradient column were again centrifuged for 10 min at 4000 rpm. The separated sedimented cells were identified microscopically as monocytes/neutrophils (after Leishman's staining) (Rebasamen et al., 1987). To 150 μl of cell suspension equal volume of 10% trichloro acetic acid in water was added and mixed by vortexing for 30 s. The mixture was then centrifuged at 5000 rpm for 5 min and supernatant was filtered through 0.45 μm membrane filter and drug content was determined following HPLC method reported by Sayed et al. (1988). Pharmacia LKB's System (Bromma, Sweden) equipped with a C_8 reverse-phase column (RP-C_8) with UV detection at 280 nm was used. Flufenamic acid (Sigma, USA) was used as internal standard, while water being a mobile phase.

2.3.2. Optimization of magnetic field strength

Since magnetic retention of liposomes is influenced by the flow rate of the suspension and the strength of the magnetic field (Kiwada et al., 1986), the minimum magnetic field strength required for effective in vivo targeting of magnetic liposomes under the guidance of external magnetic field was optimized. This was studied by testing in vitro magnetic responsiveness of the optimized formulation at different flow rates (0.5–8.0 ml/min) under the effect of magnetic field of various strengths (2–12 kG) as described earlier.

2.4. Anchoring of RGD peptide

Coating of magnetic liposomes with RGD peptide was performed via covalent coupling of carboxylate group on RGD and amine group on PE lipid fraction of liposomes using carbodiimide (EDC) as coupling agent (Jansons and Mallett, 1980; Hermanson, 1996). PE based magnetic liposomes containing diclofenac sodium were prepared using the molar ratio of PC:PE:Chol:PS (4:1:3:2) following the procedure described previously. One milliliter of liposomal suspension was added to 0.2 ml of RGD solution (10 mM in PBS, pH 7.4) in order to achieve a molar ratio of PE to peptide approximately 1:2. EDC in optimized concentration was then added to the mixture, solubilized in to the dispersion using vortex mixer and incubated for 2 h at room temperature. The excess peptide was removed by gel filtration using Sephadex G-50 minicolumn (Hermanson, 1996). The amount of EDC added was optimized in terms of vesicle aggregation. The prepared liposomes were characterized for percent drug and magnetite loading, in vitro magnetic responsiveness and ex vivo monocytes/neutrophils uptake as described previously.

2.5. Vesicle morphology and size analysis

The liposomal formulations were characterized for their shape and morphology by transmission electron microscopy (JEM-200 CX, JEOL, Tokyo, Japan). Samples were not stained because magnetite can be visualized in the electron microscope by virtue of its high electron density. Vesicle size and size distribution were determined by using particle size analyzer (CILAS, 1064, France).

2.6. In vivo studies

In vivo studies were conducted on albino rats (Wister origin) of either sex having average weight 150–200 g.

2.6.1. Monocytes/neutrophils sorting under magnetic guard

Animals were divided in three groups of six rats each. To all groups 0.2 ml of liposomal formulations (RGD-coated magnetic, uncoated magnetic and plain) were injected intravenously through caudal vein. To the first and second groups the magnetic field of 8.0 kG was applied at the selected target portion of tail. To the third group no magnetic field was applied and this group was used as control group. After 2 h injection blood samples were collected from tail segment of caudal vein, where the magnetic field was applied. Differential leukocyte count (DLC) of the collected blood samples was carried out and results of the two groups were compared for any change in count.

2.6.2. In vivo organ distribution studies

Animals were divided in five groups of three rats each. Animals received intra-striatal microinjection (100 units) of human recombinant IL-1β to produce brain inflammation as described by Anthony et al. (1997). Different formulations (plain drug solution, non-magnetic uncoated and RGD-coated liposomes and uncoated and RGD-coated magnetic liposomes) were administered intravenously through caudal vein in equivalent dose (1 mg/kg body weight). The magnetic field of 8.0 kG strength was turned on and was applied near the brain of animals receiving magnetic liposomal formulations. Animals were sacrificed after 4 h of administration. Blood was collected by cardiac puncture method. Different organs (liver, spleen, lung, kidney and brain) were excised, isolated, washed with Ringer's solution and dried using tissue paper. Drug present in serum and different organs was measured.

2.6.2.1. Drug estimation in serum. The collected blood was centrifuged at 5000 rpm for 3 min. Serum was harvested from supernatant. To 150 μl of serum, an equal volume of 10% trichloro acetic acid in water was added and mixed by vortexing for 30 s. The mixture was then centrifuged at 5000 rpm for 5 min, supernatant was filtered through 0.45 μm membrane filter and estimated by HPLC method as described previously.

2.6.2.2. Estimation of drug in different organs. Various organs (liver, spleen, lung, kidney and brain) after drying were weighed and cut into small pieces. One gram of each organ or whole organ (in case organ weight less than 1.0 g) were homogenized with 2 ml PBS (pH 7.4). Tissue homogenate was processed and analyzed for drug content in similar manner as described for serum.

2.6.3. Fluorescence microscopy

Fluorescence marker (6-carboxy fluorescein) was encapsulated in different formulations in place of drug. Formulations were administered intravenously through caudal vein after intra-striatal microinjection (1 μl) of human recombinant IL-1β. The magnetic field of 8.0 kG strength was turned on and was applied near the brain of animals receiving magnetic liposomal formulations. Animals were sacrificed after 4 h of administration. Brain was excised, isolated and microtomy was performed. Transverse sections were mounted on glass slide and observed under a fluorescence microscope (Leitz-Biomed, Germany).

3. Results and discussion

This study attempted to establish the optimized conditions for preparation of magnetic liposomes in order to develop an effective biophysically modulated carrier-cellular co-ordinated targeting strategy. Magnetic liposomes containing diclofenac sodium and dextran magnetite (DM) could be prepared by reverse-phase evaporation method. The prepared system was firstly optimized for the amount of negatively charged lipid (PS). Magnetic liposomes were prepared using different molar ratio of lipids (PC:Chol:PS) and evaluated for percent drug and magnetite entrapment, in vitro magnetic responsiveness and ex vivo monocytes/neutrophils uptake. The cellular distribution of drug loaded magnetic liposomes was determined by employing modified gradient centrifugation technique, which allowed separation of these cells from the rest. The technique basically involved affinity retention of these cells by fucose, a sugar that efficiently binds with lectin receptors present on these cells. Monocytes and neutrophils express lectin-receptors in excess compared to other cells. However, when free lectin (concanavallin A) was added, this could effectively replace the cells by binding itself to fucose in the column due to its higher affinity towards sugars and thus allows cells to sediment under centrifugation (Rebasamen et al., 1987).

It was observed that at lipid molar ratio 5:3:2 (PC:Chol:PS) maximum drug (59.8 ± 4.3%) and magnetite loading (46.7 ± 3.4%) were attained (Table 1). Magnetite encapsulation ratio per mmol lipid was also calculated and found to be 224.1 ± 14.5 mg magnetite per mmol lipid. Percent in vitro magnetic responsiveness measured at 0.5 ml/min flow rate and 8.0 kG magnetic field strength was also maximum and found to be 86.4 ± 6.5%. At this ratio monocytes/neutrophils uptake of the system was fairly good as 57.8 ± 4.1% drug could be recovered from the cells after 2 h incubation. The monocytes/neutrophils uptake of the system increased with increasing PS ratio, which is consistent with the findings of Juliano and Stamp, 1975 and Poste et al., 1982. However, drug and

Table 1
Optimization of PS ratio

Molar ratio (PC:Chol:PS)	Drug entrapment (%)	Magnetite entrapment (%)	In vitro magnetic responsiveness (%)	Ex vivo monocytes/neutrophils uptake of drug after 2 h (%)
7:2:1	51.6 ± 4.9	35.4 ± 2.4	71.8 ± 5.2	36.5 ± 2.6
6:3:1	53.5 ± 4.3	39.3 ± 2.8	76.9 ± 5.4	41.4 ± 3.7
5.5:3:1.5	57.4 ± 4.5	43.6 ± 3.7	82.6 ± 6.7	48.6 ± 3.9
5:3:2	59.8 ± 4.3	46.7 ± 3.4	86.4 ± 6.5	57.8 ± 4.1
4.5:3:2.5	54.1 ± 4.1	37.2 ± 2.9	74.7 ± 5.6	60.1 ± 4.8

Values are expressed as mean ± S.D. ($n = 6$).

magnetite loading and in vitro magnetic responsiveness were found to decrease with increase in PS amount. On the basis of these observations PC:Chol:PS ratio, 5:3:2 (on molar basis) was finalized for further studies, which in fact was a compromise between phagocytic uptake and other optimized parameters.

Fig. 1 illustrates the percentage retention of magnetic liposomes under varied conditions of magnetic field strength and fluid flow rate (Fig. 1). It is evident that the retention of magnoresponsive vesicles increases with an increase in magnetic field strength and decreasing fluid flow rate. At 8.0 ml/min flow rate, however, magnetic liposomes were not retained (% retention was only 1.5 ± 0.39% even at 12 kG field strength). Retention at 4.0 ml/min fluid flow rate was also low as only 10.8 ± 1.2% vesicles were retained at 12 kG field strength. Magnetic liposomes were responded to magnetic field with the highest percentage retention with magnetic field strength ≥8.0 kG and fluid flow rate 0.5–1.0 ml/min. At 8.0 kG field strength and 0.5 ml/min flow rate 86.4±6.5% retention was observed and it was 94.7 ± 7.4% at 12 kG field strength and 0.5 ml/min flow rate. This flow rate corresponds to mammalian blood flow in capillaries (Eckert et al., 1988). These findings suggest that magnetic liposomes could be effectively targeted in vivo with the help of external magnetic field of 8.0 kG.

The optimized formulation of negatively charged magnetic liposomes was surface appended with RGD peptide using carbodiimide activated carboxylate group of RGD with underivatized PE of preformed vesicles. The water soluble EDC activates carboxylate groups to form active ester intermediates that can react with amine group of PE to form an amide linkage (Martin et al., 1990). EDC coupling of proteins to liposomes is generally reported to cause protein to protein cross linking and vesicle aggregation by protein coupling to more than one vesicle, since proteins contain an abundance of both amines and

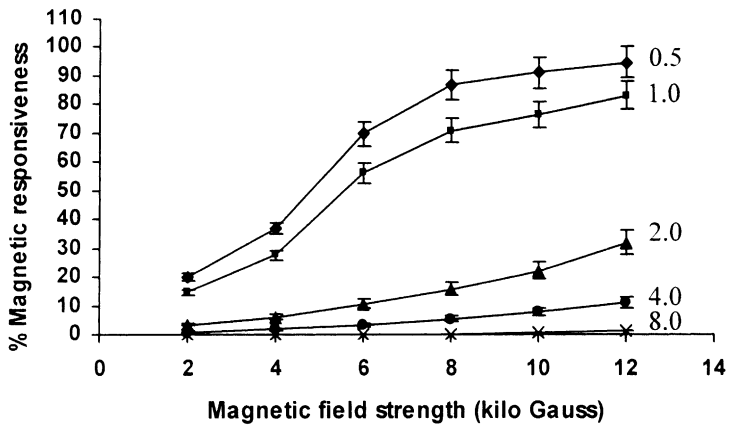

Fig. 1. Correlation between the percent retention of magnetic liposomes and applied field strength at different flow rates. All values are expressed as mean ± S.D. ($n = 6$). The number corresponding to each line denotes fluid flow rates in ml/min.

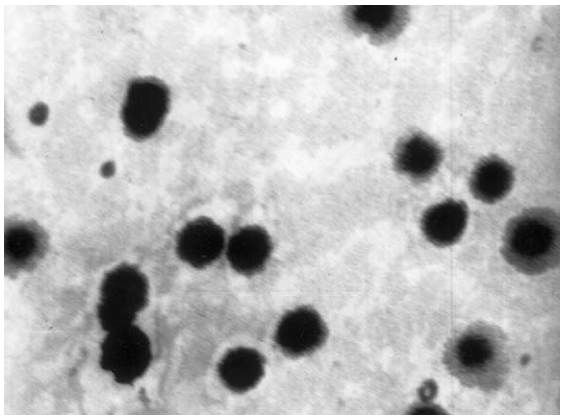

Fig. 2. Transmission electron micrograph of magnetic liposomes (18,500×).

carboxylates. Blocking the amine groups of proteins with citraconic acid prior to reaction is suggested to avoid this polymerization problem (Jansons and Mallett, 1980; Martin et al., 1990). However, in the present study, a small tripeptide RGD was used that contains only two carboxyl and two amine groups in its molecule. Hence, there was little risk of extensive cross linking and vesicle aggregation leading to vesicle instability. Otherwise, if some polymerization takes place it will form poly RGD that will be of course a better ligand for monocytes/neutrophils, since integrin molecules contain repeat units of this small peptide sequence (Aznavoorian et al., 1990; Odekon et al., 1991; Hauzenberger et al., 1993; Saiki et al., 1996). Therefore, blocking of amine group of RGD was not appeared essential in this case. However, EDC concentration was optimized in order to ensure that coupling of peptide do not result in vesicle–vesicle aggregation or extensive cross linking. It was observed that at an amount of 3 mg per ml of peptide/lipid mix, EDC did not cause any instability. Beyond this amount, however, the vesicle aggregation was noticed.

In the transmission electron photomicrograph (Fig. 2), almost completely filled zone of magnetite was observed within the vesicles. This is in accordance with the findings of Viroonchatapan et al. (1995) who incorporated different quantities of DM within liposomes. Presence of coating could not be visualized very clearly in TEM probably because RGD is a very small molecule. However, RGD-coupled formulations after removal of free uncoupled peptide followed by subsequent palletization (centrifugation at 60,000 rpm for 4 h) gave a violet color on treatment with ninhydrin solution. This clearly indicated the presence of the peptide on the surface of liposomes.

Results of other characterization parameters including percent drug and magnetite encapsulation, vesicle size, vesicle count, in vitro magnetic responsiveness and ex vivo monocytes/neutrophils uptake for both negatively charged uncoated and RGD-coated magnetic liposomes are recorded in Table 2. In both types of formulations percent drug and magnetite entrapment, magnetite encapsulation ratio and vesicle count were found to be satisfactory. Percent drug and magnetite entrapment were found to be 59.8 ± 4.3% and 46.7 ± 3.4% in case of uncoated magnetic liposomes, while in the case of RGD-coated formulations these values were found to be 57.6 ± 4.8% and 43.2 ± 3.6%, respectively. Magnetite encapsulation ratio in both uncoated and RGD-coated formulations was 224.1 ± 14.5 mg magnetite/mmol lipid and 213.9 ± 15.1 mg magnetite/mmol lipid, respectively. Both formulations exhibited good magnetic responsiveness to the magnetic field of 8.0 kG

Table 2
In vitro characterization of various formulations

Characterization parameters	Uncoated magnetic liposomes	RGD-coated magnetic liposomes
% Drug entrapment	59.8 ± 4.3	57.6 ± 4.8
% Magnetite entrapment	46.7 ± 3.4	43.2 ± 3.6
Magnetite encapsulation ratio (mg/mmol lipid)	224.1 ± 14.5	213.9 ± 15.1
Vesicle size (μm)	1.19 ± 0.07	1.21 ± 0.09
Vesicle count (×1000 per mm^3)	78.2 ± 5.4	76.7 ± 5.6
In vitro magnetic responsiveness (%)	86.4 ± 6.5	81.3 ± 6.1
Ex vivo monocytes/neutrophils uptake[a]	57.8 ± 4.1	73.5 ± 5.8

Values are expressed as mean ± S.D. ($n = 6$).
[a] Expressed as percent fraction of drug recovered from monocytes/neutrophils as compared to total drug loaded in the liposomes.

Table 3
Results of in vivo cellular sorting

Type of leukocytes	Normal cell count of plain blood (%)	Relative cell count (%) after 2 h		
		Plain liposomes	Uncoated magnetic liposomes	RGD-coated magnetic liposomes
Neutrophils	49 ± 3.8	49 ± 3.5	60 ± 5.3	69 ± 5.8
Lymphocytes	45 ± 3.1	45 ± 3.6	34 ± 2.8	22 ± 1.4
Monocytes	2 ± 0.21	2 ± 0.15	5 ± 0.33	8 ± 0.63
Eosinophils	4 ± 0.32	4 ± 0.35	1 ± 0.08	1 ± 0.06
Basophils	ND	ND	ND	ND

Values are expressed as mean ± S.D. ($n = 6$). ND: not detected.

strength at fluid flow rate 0.5 ml/min (86.4 ± 6.5% and 81.3 ± 6.1% in case of uncoated and RGD-coated formulations). In general, the recorded values of all parameters in the case of RGD-coated formulation are slightly less than those of uncoated ones. This may be attributed to leakage of some amount of drug as well as magnetite during the coating process. However, these differences were minute and insignificant. Mean vesicle size of both formulations was found to be 1.19 ± 0.07 μm and 1.21 ± 0.09 μm. The change in vesicle size due to coating is negligible and insignificant. Amount of drug recovered from monocytes/neutrophils was 57.8 ± 4.1% and 73.5 ± 5.8% respectively, for negatively charged uncoated and RGD-coated liposomes. Results clearly showed better localization of RGD-coated vesicles within these cells.

In vivo cell sorting studies exhibited a significant increase in relative neutrophils and monocytes count in blood samples collected from animals administered with uncoated and RGD-coated magnetic liposomal formulations (Table 3). Initial neutrophils count was 49 ± 3.8% and increased up to 60 ± 5.3% and 69 ± 5.8% following administration of uncoated and RGD-coated magnetic liposomes, respectively at the region of tail segment placed under the magnetic field. Similarly, initial monocytes count was 2 ± 0.21% in normal condition and increased up to 5 ± 0.33% and 8 ± 0.63% after 2 h following administration of uncoated and RGD-coated magnetic liposomes, respectively. At the same time the relative percent count of other cells (lymphocytes, eosinophils and basophils) were reduced. No change in DLC was recorded after 2 h administration of plain formulation. These results strongly suggest that only monocytes and neutrophils became magnetic due to selective uptake of magnetic liposomes and they responded to magnetic field. Therefore, under the influence of external magnetic field these cells remained localized at the site of magnet field application, while others could circulate through out the blood. This localization was well reflected in monocytes/neutrophils counts of circulating blood. Further, the increase in monocytes and neutrophils count was greater in the case of RGD-coated magnetic liposomes. RGD serves as ligand for the integrin receptors on neutrophils and monocytes and thus facilitates receptor-mediated endocytosis, which results in higher uptake of the liposomes (Senior et al., 1992). The results of in vivo organ distribution studies are best correlated with these ex vivo observations.

Intra-striatal injection of IL-1β produced a considerable increase in BBB permeability and recruitment of leukocytes into brain within 4 h. This effect is also accompanied by a marked meningitis (Anthony et al., 1997; Blamire et al., 2000). Leukocyte recruitment can trigger signal transduction cascades leading to junctional disorganization and loss of tight junction proteins occludin and zonula occludens-1 from cerebral vascular endothelium (Bolton et al., 1998). Results of in vivo organ distribution studies showed that free drug remained mainly in circulation and very little quantity (only 2.37 ± 0.20%) reached the brain (Table 4). Non-magnetic formulations (both negatively charged uncoated and RGD-coated) were mainly taken up by organs of RES, therefore little concentration was found in blood (7.63 ± 0.59% and 6.78 ± 0.57%, respectively) and maximum drug was accumulated in liver (47.32 ± 3.12% and 51.43 ± 4.21%, respectively) and spleen (8.57 ± 0.65% and 10.53 ± 0.92%, respectively). Very less quantity (1.86 ± 0.12% and

Table 4
Percent relative in vivo organ distribution of drug from various formulations

Organ	Free drug	Non-magnetic liposomes		Magnetic liposomes	
		Uncoated negatively charged	RGD-coated	Uncoated negatively charged	RGD-coated
Serum	56.80 ± 4.53	7.63 ± 0.59	6.78 ± 0.57	10.86 ± 0.86	13.73 ± 1.32
Liver	19.32 ± 1.11	47.32 ± 3.12	51.43 ± 4.21	29.68 ± 2.14	26.58 ± 0.21
Lung	3.61 ± 0.34	4.76 ± 0.46	4.17 ± 0.35	4.23 ± 0.34	3.84 ± 0.29
Spleen	2.14 ± 0.26	8.57 ± 0.65	10.53 ± 0.92	6.42 ± 0.59	5.27 ± 0.47
Kidney	4.56 ± 0.38	2.83 ± 0.21	2.86 ± 0.22	2.65 ± 0.23	2.35 ± 0.18
Brain	2.37 ± 0.20	1.86 ± 0.12	3.25 ± 0.27	14.11 ± 1.08	21.53 ± 1.94

Values are expressed as mean ± S.D. ($n = 3$).

3.25 ± 0.27%, respectively of the administered dose) reached the target site brain via their uptake by neutrophils and monocytes. This distribution pattern was reversed for these formulations under the influence of external magnetic field. Liver uptake was avoided to a significant extent and relative reduction in accumulation level was 37.2% and 48.3% for uncoated and RGD-coated magnetic liposomes, respectively when compared to their non-magnetic versions. Percent dose reached to liver was 29.68 ± 2.14% for uncoated magnetic liposomes and 26.58 ± 0.21% for RGD-coated magnetic liposomes. These observations suggest that application of external magnetic field tend to accumulate carrier systems at the target site and delay their RES clearance. Much higher quantity of the drug was found at target site (brain) after administration of magnetic formulations and application of magnetic field. Brain drug level for negatively charged uncoated magnetic liposomes was 14.11 ± 1.08% after 4 h. When compared with free drug and non-magnetic counterpart of the same formulation this level was approximately 5.95 and 7.58 times higher (Fig. 3). In the case of RGD-coated magnetic liposomes accumulation in the brain was found to be highest (21.53 ± 1.94%) and this ratio was approximately 9.1 times of the free drug solution, 6.62 times of the non-magnetic version of RGD-coated liposomes and 1.5 times of the uncoated negatively charged magnetic liposomes. RGD-coated liposomes were more preferentially taken up by neutrophils and monocytes via receptor-mediated endocytosis as compared to simple negatively charged liposomes hence reached the brain in relatively more concentration along with monocytes/neutrophils following brain inflammation under the guidance of external magnetic field. Further, fluorescence photomicrographs explicitly suggest that all the formulations were distinctively localized within the brain tissues (Fig. 4a–c). Moreover, the fluorescence

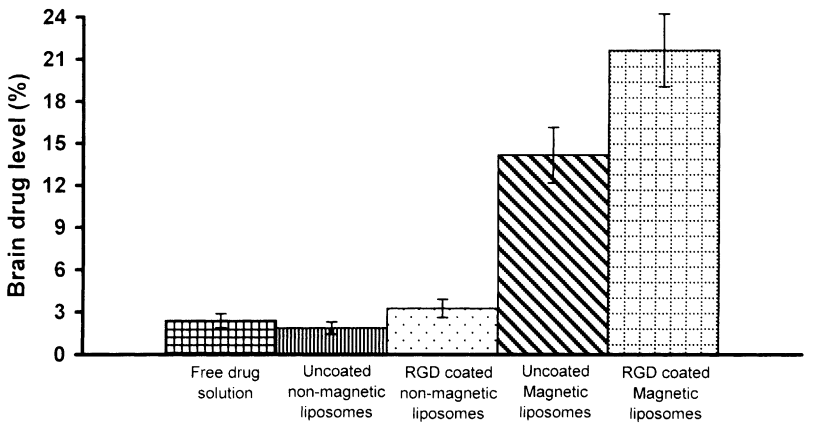

Fig. 3. Relative brain uptake of various formulations. All values are expressed as mean ± S.D. ($n = 3$).

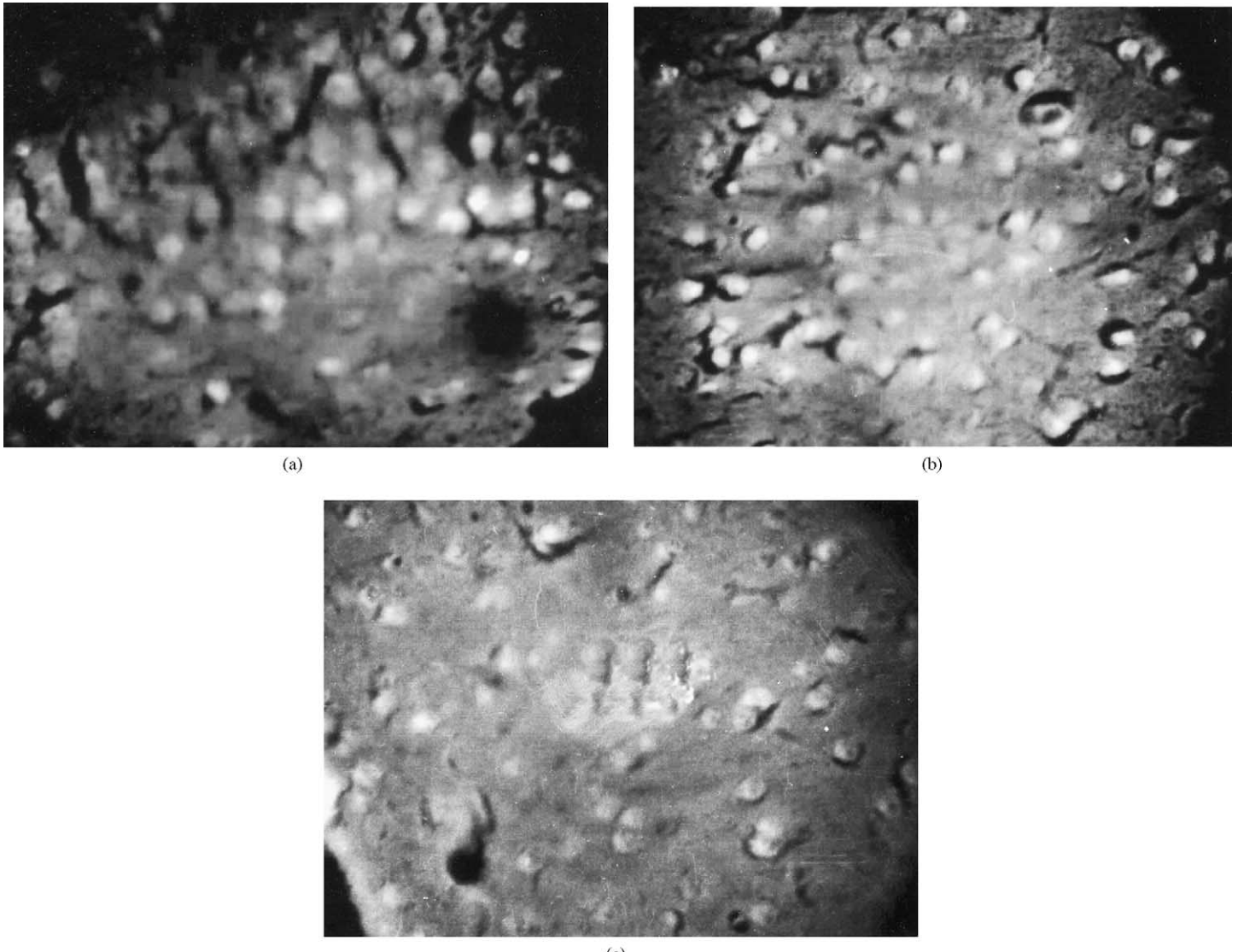

Fig. 4. Fluorescence image of T.S. of brain of albino rat after 4 h of administration of 6-carboxyfluorecein loaded: (a) RGD-coated magnetic liposomes; (b) negatively charged uncoated magnetic liposomes and (c) RGD-coated non-magnetic liposomes (600×).

intensity was much higher in case of magnetic liposomes (both uncoated and RGD-coated) as compared to RGD-coated non-magnetic liposomes, hence, it can be concluded that magnetic liposomes under magnetic guidance were better localized within the brain as compared to non-magnetic liposomes.

4. Conclusion

The negatively charged magnetic liposomes and RGD-coated magnetic liposomes developed in the present study were shown to be selectively taken up by the circulating blood monocytes/neutrophils thus avoiding the uptake by fixed macrophages of the liver. Coating of the liposomes with RGD peptide further increased their uptake via receptor-mediated endocytosis. This uptake in turn imparts magnetic property to these cells and because of their exclusive migration tendency towards inflammatory sites and under the guidance of external magnetic field the drug can be actively targeted to any poorly accessible inflammatory site, i.e. brain. Such systems can be useful in treatment of neurological diseases, such as Alzheimer's disease, Parkinson's disease, Prion disease, meningitis, encephalitis and brain tumors, etc. with an inflammatory component. Further studies are suggested to establish the possible application of such systems in targeting of anticancer drugs to poorly accessible tumor sites. Moreover, studies are also required for effective quantitation and optimization of charge and ligand (i.e. RGD) on to the liposomal surface. The strategy is seemingly useful in two ways, firstly, it negotiates targeting of drug and secondly, it leads to the recruitment of leukocytes at the site of infection to combat infection by playing a major role in natural body defense mechanism.

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