BBB project

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Purpose:

1) To study immuno-surveillance of the brain in brain-associated inflammatory disorders, including multiple sclerosis and Alzheimer's disease, which are characterized with increased passage of immune cells across the BBB.
2) To study the transport of drugs encapsulated in nanoparticles across the BBB by mononuclear leukocytes.

Abstract of recent research

Many drugs are not able to enter the brain due to the presence of the blood-brain barrier (BBB) and therefore cannot be used in the treatment of diseases of the brain. Since it is now known that the brain is under immunological surveillance, we hypothesized that phagocytic cells of the innate immune system, mainly neutrophils and monocytes, can be exploited as transporters of drugs to the brain. To target circulating mononuclear phagocytic cells, negatively-charged nano-sized liposomes were formulated encapsulating serotonin, a BBB impermeable neurological drug. Brain uptake, biodistribution, and the mechanism of brain transport were examined *in vitro* and in rats and rabbits by utilizing double-radiolabeled ^{3}H (in the membrane) and ^{14}C-serotonin (in the core), and liposomes with fluorescent markers (membrane and core). The brain uptake of liposomal serotonin was significantly higher (0.138%±0.034 and 0.097%±0.011, vs. 0.068%±0.02 and 0.057%±0.01, 4hr and 24hr after IV administration in rats, serotonin liposomes and in solution, respectively). The same brain uptake of both empty and serotonin liposomes, the co-localization in the brain of both markers, and the unchanged ratio of ^{3}H:^{14}C suggest that intact liposomes entered the brain. Since treatment of animals by liposomal alendronate resulted with inhibition of monocytes but not of neutrophils, and with no brain delivery, it is suggested that monocytes are the main transporters of liposomes to the brain.

Key words: Blood-Brain Barrier, drug delivery, liposomes, nanoparticles, leukocytes monocytes, neutrophils, brain transport.

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Delivery of serotonin to the brain by monocytes following phagocytosis of liposomes

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ABSTRACT

Many drugs are not able to enter the brain due to the presence of the blood-brain barrier (BBB) and therefore cannot be used in the treatment of diseases of the brain. Since it is now known that the brain is under immunological surveillance, we hypothesized that phagocytic cells of the innate immune system, mainly neutrophils and monocytes, can be exploited as transporters of drugs to the brain. To target circulating mononuclear phagocytic cells, negatively-charged nano-sized liposomes were formulated encapsulating serotonin, a BBB impermeable neurological drug. Brain uptake, biodistribution, and the mechanism of brain transport were examined *in vitro* and in rats and rabbits by utilizing double-radiolabeled ^{3}H (in the membrane) and ^{14}C-serotonin (in the core), and liposomes with fluorescent markers (membrane and core). The brain uptake of liposomal serotonin was significantly higher (0.138%±0.034 and 0.097%±0.011, vs. 0.068%±0.02 and 0.057%±0.01, 4 h and 24 h after IV administration in rats, serotonin liposomes and in solution, respectively). The same brain uptake of both empty and serotonin liposomes, the co-localization in the brain of both markers, and the unchanged ratio of ^{3}H:^{14}C suggest that intact liposomes entered the brain. Since treatment of animals by liposomal alendronate resulted with inhibition of monocytes but not of neutrophils, and with no brain delivery, it is suggested that monocytes are the main transporters of liposomes to the brain.

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1. Introduction

The limiting factor in the development of new drugs for brain diseases is the blood-brain barrier (BBB) [1]. The brain microvessel endothelial cells form a continuous layer of cells and extracellular matrix, tight extracellular junctions, and reduced levels of pinocytic activity. In addition, solutes crossing the cell membrane are subsequently exposed to degrading enzymes present in large numbers inside the endothelial cells and to active efflux pumps. Even small molecules do not cross the BBB in pharmacologically significant amounts, unless the molecule is both lipid soluble and has a molecular weight (MW) <400 Da [2]. Most drugs lack these dual molecular characteristics, and do not cross the BBB, including all products from biotechnology or gene therapy [3].

The delivery of drugs to the brain has traditionally been approached with medicinal chemistry or barrier disruption and neurosurgical based invasive brain drug delivery [1,4,5]. Although some of the more recent methods are promising, no method has yet proved to be efficient, and the invasive procedures are by nature severely limited. Additional approaches that have been studied for BBB delivery are special lipid based delivery systems [6], targeted liposomes [7–10], and immunoliposomes [11]. However, some of these methods were not successfully reproduced, and were found more effective in inflammatory or pathological conditions when the permeability of the BBB is increased [9,12,13]. Polymeric nanoparticles (NP) have shown some promise [14–18]. However, the transport mechanism remains controversial; it is unclear whether they penetrate by low-density lipoprotein receptor-mediated pathway [19], receptor-mediated endocytosis of apolipoprotein-coated particles [6], caveolae-mediated pathway [20], or mediated by disruption of the BBB due to a toxic effect [21,22].

The brain has often been considered an immunologically privileged organ, and the presence of the BBB was thought to prevent the entry of immune cells from the peripheral circulation into the brain. However, it is now accepted that the brain is under immunological surveillance [23,24]. The BBB allows the selective entry of leukocytes into the central nervous system including monocytes, neutrophils and lymphocytes [25–29].

We hypothesized that phagocytic cells of the innate immune system, mainly neutrophils and monocytes can be exploited as transporters of drugs to the brain. Loading these cells with the drug can be achieved by administering the drug of choice in a particulated dosage form such as liposomes or polymeric NP, which are

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phagocytized in circulation by these cells [30–37]. The so-called, 'conventional' liposomes, are not hydrophilic ('pegylated'), do not have a neutrally-charged membrane, and are not of ultra-small size [30,33,38–40]. Thus, they are most suitable for effective phagocytosis in the circulation by monocytes and neutrophils. However, the formulation should not inhibit the transporting cells but rather activate the mononuclear phagocytic system (MPS). In addition, the relatively long circulation time of the liposomes in circulating phagocytic cells rather than rapid disposition in macrophage-rich organs (e.g., liver and spleen) is advantageous for brain transport. Last but not least, it is expected that if the drug is not metabolized in the lysosome, it will be excreted from the endocytosing cell in the brain.

In this work, serotonin, a brain impermeable physiological neurotransmitter [41], was encapsulated in negatively-charged liposomes. The biodistribution and brain uptake of empty liposomes and liposomal serotonin was studied in rabbits and rats. In addition, the mechanism of transport was elucidated by studying uptake in monocytes and neutrophils *in vitro* and *in vivo*.

2. Materials and methods

2.1. Liposomes preparation

Liposomes were formulated with distearoylphosphatidylcholine (DSPC), distearoyl phosphatidyl glycerol (DSPG) (Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Sigma, St. Louis, MO). The liposomal formulations were prepared by the modified thin film hydration method [42]. Phospholipids and cholesterol (DSPC:DSPG: CHOL, 3:1:2 molar ratio) were dissolved in *tert*-butanol and lyophilized to produce a film. A homogeneous aqueous solution of serotonin (50 mM) (as the binoxalate salt; Sigma, St. Louis, MO) was added to the film, and the liposomes thus obtained were homogenized to 200 nm by means of an extruder (Lipex Biomembranes, Vancouver, Canada). To remove un-encapsulated drug, the liposomes were passed through a Sephadex G-50 column and eluted with MES/HEPES buffer pH 7.2 (50 mM MES, 50 mM HEPES, 75 mM NaCl). The formulation volume was adjusted to 8.0 ml.

Double-radiolabeled serotonin liposomes were prepared similarly with ^{3}H-Cholesterylhexadecylether (1.5 μCi) (PerkinElmer Boston, USA) and an aqueous solution of ^{14}C-serotonin (50 mM, 70 μCi; PerkinElmer Boston, USA). Fluorescent liposomes were prepared similarly with rhodamine and 1-hydroxypyrene-3,6,8 trisulfonic acid (Avanti Polar Lipids, Alabaster, AL). Alendronate liposomes for studying brain uptake under monocytes depletion, were prepared similarly with sodium alendronate (200 mM) as reported previously [37,43].

2.2. In vivo brain uptake studies

New Zealand White rabbits and male Sprague Dawley rats (Harlan Laboratories, Jerusalem, Israel), weighing 2.5 to 3.5 kg and 140 to 210 g, respectively, were used in accordance with the guidelines for animal care of the Hebrew University of Jerusalem and National Institutes of Health (USA). Animals were fed standard laboratory chow and tap water ad libitum. Experiments were performed under anesthesia by an IP injection of ketamine (80 mg/kg, Fort Dodge Animal Health, USA) and xylazine (5 mg/kg, V.M.D. NV, Belgium) solutions.

2.2.1. Quantitative brain penetration

Male rats (Sprague Dawley™, Harlan Laboratories, Jerusalem, Israel) weighing 220–240 g were used for brain penetration studies. Liposomes containing ^{14}C-labeled serotonin (0.5 μCi, n=10), empty ^{3}H-labeled liposomes (1.5 μCi, n=8) and ^{14}C-serotonin solution (0.5 μCi, n=10) were IV injected into the tail vein of rats (after gentle swabbing with warm water). One half of the rats in each group were euthanized 4 h or 24 h post-injection, followed by perfusion with saline (23G needle and a 50 ml syringe) directly to the left ventricle and subsequent severing of the jugular vein for 10 min or until the perfusate was clear. The brains were excised, and dissolved in 1 ml Solvable per 0.1 g of tissue (Perkin Elmer, The Netherlands), and incubated overnight at 60 °C until complete solubilization. The obtained solution was decolorized with 0.2 ml of 35% hydrogen peroxide per 1 ml of solvable until the solution turned to pale yellow. The excess of hydrogen peroxide was inactivated by heating the samples at 60 °C overnight. After mixing with 10 ml of Ultima Gold scintillation cocktail (Perkin Elmer, The Netherlands), the radioactivity (DPM) was counted by means of a liquid scintillation analyzer (Packard, Tri-carb 2900TR, USA) against a calibration curve of R^2=0.997.

2.2.2. Qualitative brain penetration

Qualitative assessment of liposome disposition in brain tissue was carried out in rabbits (n=8) following IV injections of fluorescent liposomes. Animals were randomly assigned to treatment groups of single labeled fluorescent liposomes (Rhodamine-DSPE, membrane marker), double-labeled fluorescent liposomes of Rhodamine-DSPE and 1-hydroxypyrene-3,6,8 trisulfonic acid (hydrophilic core marker), Rhodamine-DSPE liposomal alendronate (3 mg/kg), and saline (control group). The animals were sacrificed 4 h post-injection after which the harvested brain tissue was rinsed with saline and n-octyl β-D-gluco-pyranoside (OCT)(Sakura, U.S.A), flash frozen in liquid nitrogen, and stored at −70 °C until analysis. Tissues were sectioned (frontal lobe, 2 sections, and temporal lobe, parietal lobe and occipital lobe) and mounted on slides. Liposome uptake was observed and recorded by means of confocal microscopy (Zeiss LSM 410, Germany).

2.2.3. Body distribution

Sprague Dawley™ rats (140–210 g), housed in metabolic cages, were IV injected (tail vein) with double-radiolabeled liposomes (^{3}H-Cholesteryl-hexadecy-lether and ^{14}C-serotonin, 0.4 μCi and 1 μCi, respectively, injected to each rat, n=9), or with ^{14}C-serotonin solution (1 μCi, n=6), and were sacrificed 4 h later. Blood samples were collected before injection and at sacrificing. Urine was collected, and the following organs were removed completely at sacrifice: brain, spinal cord, kidneys, heart, lungs, liver, spleen, tibia bone, and pancreas. Muscle specimens were excised near the femoral. Each specimen was weighed and frozen at −70 °C. Organs or tissues were burned and analyzed by means of a Sample Oxidizer (Packard, model 307, Switzerland). Eluting solutions and scintillation cocktails were, Monophase for ^{3}H, and Carbosorbe+Permafluore for ^{14}C (PerkinElmer, MA, USA). The scintillation bottles were left overnight and counted by means of a liquid scintillation analyzer (Packard, Tri-carb 2900TR, USA), against calibration curves of R^2>0.962.

2.3. Endocytosis of liposomes by monocytes and neutrophils

2.3.1. Animal studies

Qualitative assessment of liposome uptake by monocytes and neutrophils was carried out *in vivo* by injecting fluorescent-labeled liposomes to rabbits (n=4). At 4 h post-injection, cells were isolated from blood specimens using Ficoll and 70% dextrose gradients (Sigma-Aldrich, Israel), and were centrifuged (4 °C, 1500 RPM, 5 min). The cells were washed 3 times with PBS, fixed with formaldehyde (4%, 4 min), and further washed 3 times with PBS. Liposome uptake was observed and recorded by means of confocal microscopy.

To quantify the internalization of liposomes by monocytes and granulocytes, liposomes labeled with dextran-FITC (Sigma-Aldrich, Israel) were injected IV to rabbits (n=3). Blood samples were taken after 4 h, 24 h, 48 h and 7 days, and analyzed by fluorescence activated cell sorting (FACS), as previously described [35]. Whole blood (100 μl) specimens were labeled with mouse anti-human R-phycoerythrin-conjugated anti-CD14 (Dako, Denmark). Red blood cells were lysed

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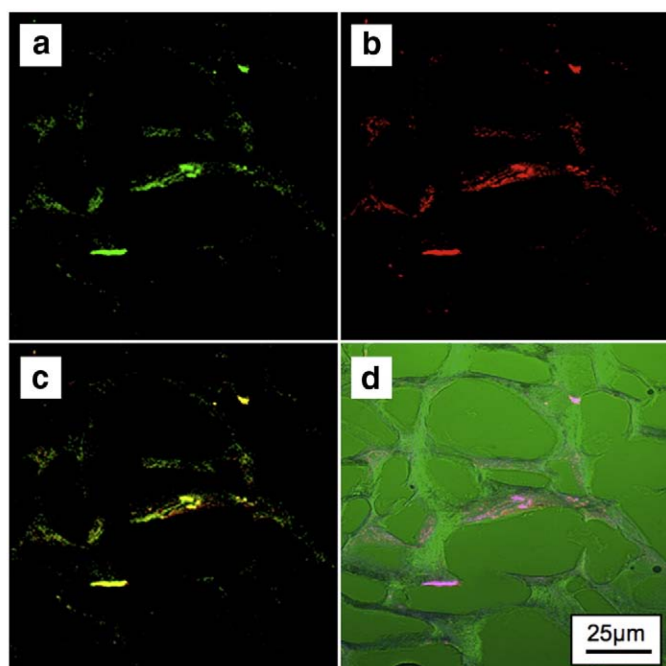

Fig. 1. Representative confocal microscopy images depicting rabbits' brain sections (Frontal lobe, Broca's area) 4 h after IV injection of double-labeled fluorescent liposomes. a) Fluorescent core marker (1-hydroxypyrene-3,6,8 trisulfonic acid, green; b) lipids fluorescent marker (Rhodamin-DSPE, red; c) merged images demonstrating overlap of liposomes core and lipids (yellow) and their co-localization (n=2); d) Background, differential interference contrast (Nomarski's) image of the cells shown (×40). Similar images were obtained in other regions.

(FACS lysing solution, B&D, USA), and the cells suspension was washed twice with PBS containing 1% fetal calf serum. The accumulation of fluorescent liposomes within monocytes and granulocytes, and their percentage of total white blood cells were determined on the basis of relative size, side scattering and fluorescence by FACS (Becton-Dickinson, USA). Similarly, the inhibitory effect of alendronate liposomes on monocytes and granulocytes was evaluated following IV injection (3 mg/kg, n=3).

2.3.2. Human monocytes and neutrophils

Human monocytes and neutrophils were isolated from the blood of human donors (n=3) using Ficoll and 70% dextrose gradients (Sigma-Aldrich, Israel), and centrifuged (4 °C, 1500 RPM, 5 min). Cells were grown in RPMI (Beit Haemek, Israel) enriched with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, 100 units/ml penicillin, 0.1 mg/ml streptomycin, adjusted to contain 4.5 g/l glucose and 1.0 sodium pyruvate, and incubated at 8% CO_2, 37 °C. Qualitative cellular uptake was observed by confocal microscopy.

Table 1
The tissue biodistribution of serotonin solution and liposomal serotonin 4 h following IV injection to rats (^{14}C serotonin counts)

	Free serotonin	Serotonin liposomes
	%, (DPM/g tissue)/dose±SD	%, (DPM/g tissue)/dose±SD
	n=6	n=9
Spinal cord	0.07±0.02	0.10±0.03*
Lungs	0.43±0.05	0.55±0.18
Kidneys	0.35±0.06	0.32±0.08
Pancreas	0.09±0.03	0.25±0.11*
Liver	0.27±0.07	0.98±0.15*
Spleen	2.39±0.45	17.16±1.97*
Urine	20.89±5.96	5.61±1.39*
Plasma	0.08±0.045	1.25±0.21*

*p<0.05.

2.4. Statistical analysis

Comparisons among treatment groups were made by the unpaired t-student's test or by 2-way analysis of variance (ANOVA) followed by Tukey test. Differences were determined statistically significant with p<0.05. Data are expressed as mean+SD.

3. Results

3.1. Serotonin liposomes

The serotonin liposomes obtained were composed of distearoyl-phosphatidylcholine (DSPC), distearoyl phosphatidyl glycerol (DSPG) and cholesterol [DSPC: DSPG: CHOL, 3:1:2 molar ratio), size of 169.32±36.32 nm, zeta potential of −29±1.9 mV, and a 10% encapsulation yield of the added serotonin concentration (50 mM). The formulation was found stable for over one month (no physicochemical changes, including no aggregation, and no leakage of the drug).

3.2. Brain transport

Brain penetration was evaluated qualitatively by confocal microscopy following injection of double-labeled liposomes with the hydrophobic (Rhodamine-DSPE) and hydrophilic (1-Hydroxypyren-3,6,8-Trisulfonic acid) fluorescent markers, embedded in the liposome membrane and in the aqueous core, respectively. It was found that liposomes with both markers were transported to the brain. Co-localization images demonstrated that intact liposomes penetrated the brain (Fig. 1).

Next we examined quantitatively the brain transport of empty and serotonin liposomes. A significantly higher uptake of serotonin was detected in the brain of rats following its administration in liposomes in comparison to solution (Fig. 2). Serotonin concentration in the brain 4 h after administration was 0.138%±0.034 and 0.068%±0.02, serotonin liposomes and serotonin in solution, respectively. Similarly,

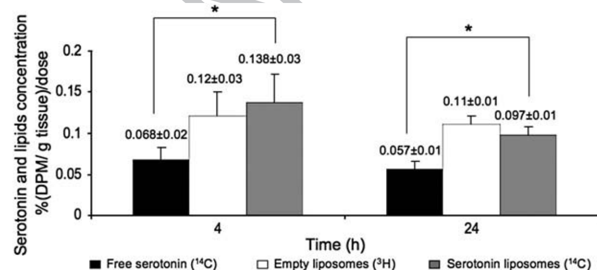

Fig. 2. Serotonin concentration in the brain of rats after IV administration of ^{3}H-empty liposomes (n=4, at each time point), ^{14}C-serotonin in liposomes (n=5, at each time point), and in solution (n=5, at each time point). (*p<0.05.)

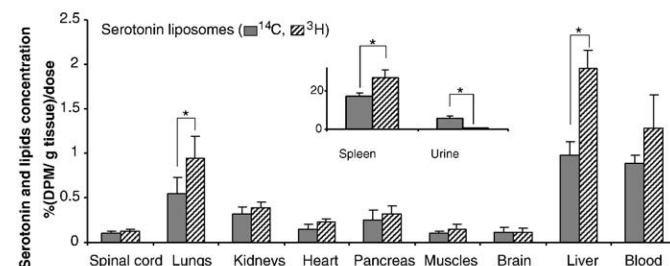

Fig. 3. Tissue biodistribution of double-radiolabeled serotonin liposomes (^{3}H in the lipid membrane, and ^{14}C-serotonin) analyzed for both radiolabels, 4 h following IV injection to rats (n=9, *p<0.05).

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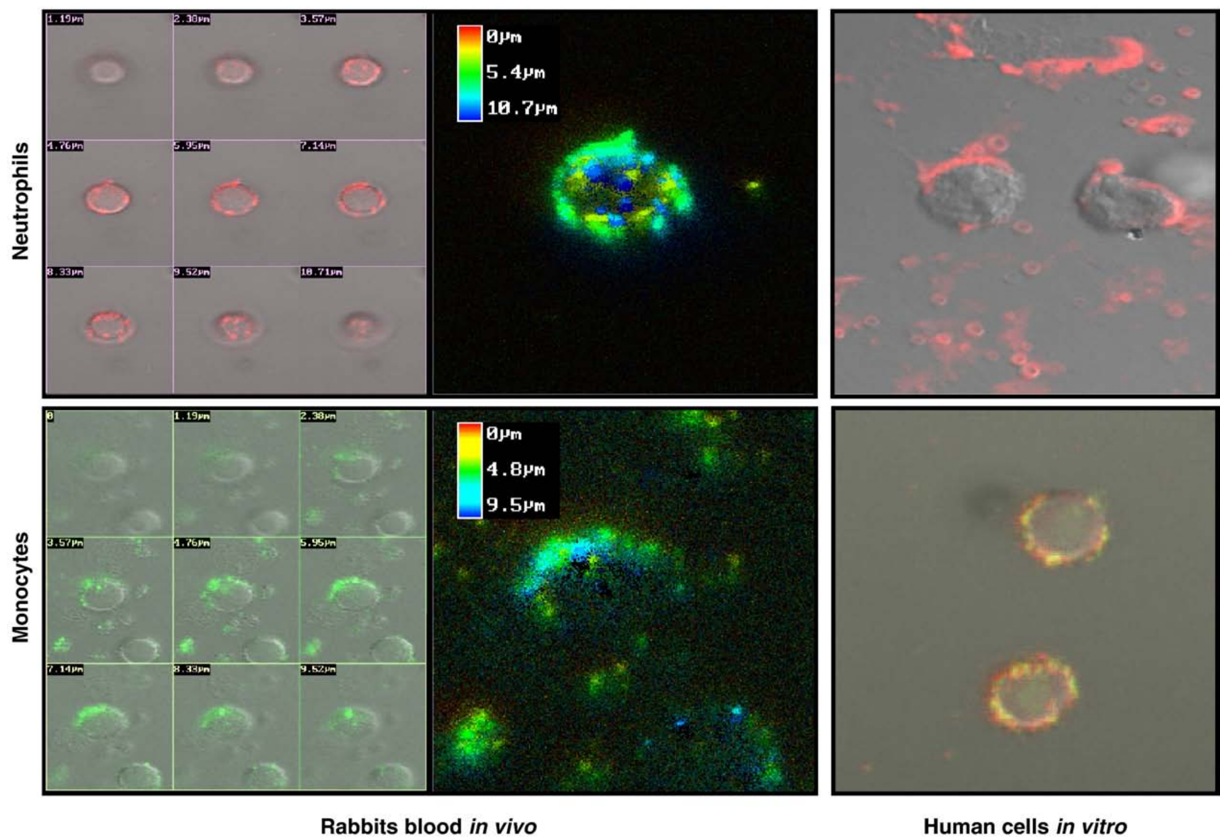

Fig. 4. Qualitative assessment of liposome uptake by monocytes and neutrophils in rabbit blood 4 h after IV injection (left panel), and in human monocytes and neutrophils after 4 h of incubation (right panel). The confocal microscopy images depict internalization of fluorescent-labeled liposomes with Rhodamin-DSPE (liposome membrane, red) and 1-hydroxypyrene-3,6,8 trisulfonic acid (aqueous core, green). Confocal cross-sections verified cell internalization (middle panel).

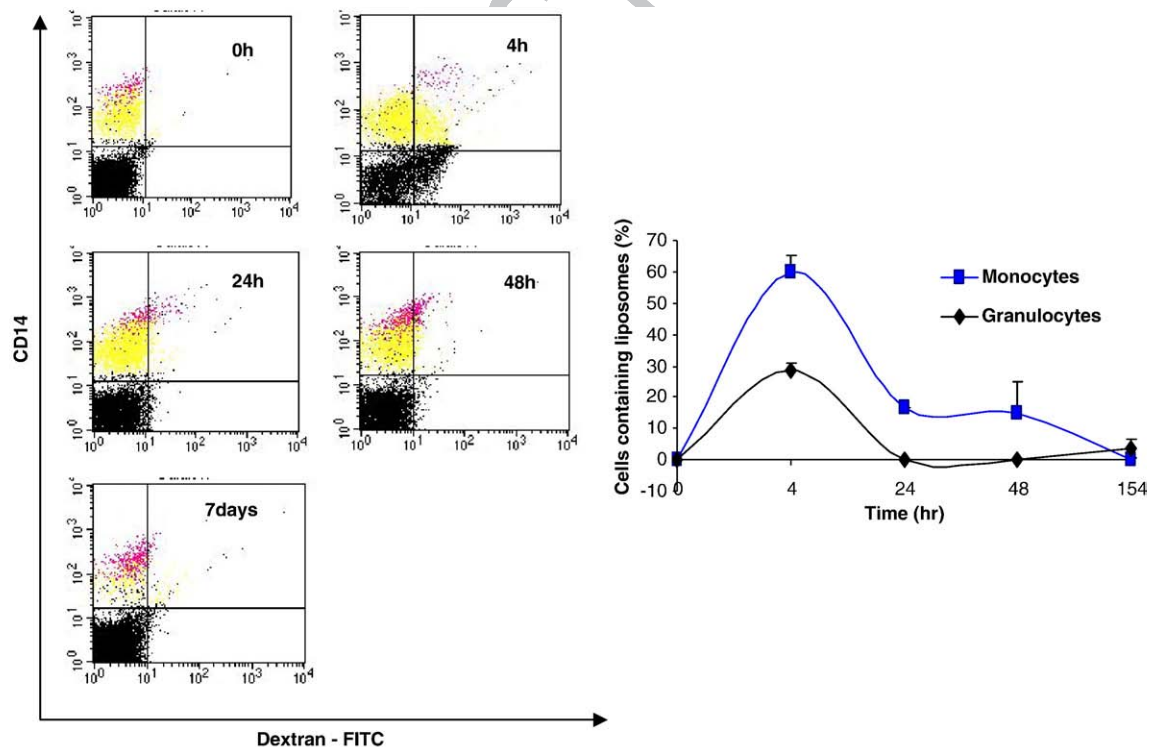

Fig. 5. Quantification of liposomes internalization by circulating monocytes and granulocytes (neutrophils) in rabbit blood by means of fluorescence activated cell sorting (FACS). Liposomes were labeled with dextran-FITC in the liposome core ($n=3$). Positive CD14 cells (monocytes, red, granulocytes, yellow) endocytosing dextran-FITC liposomes are shown in the upper right quadrant.

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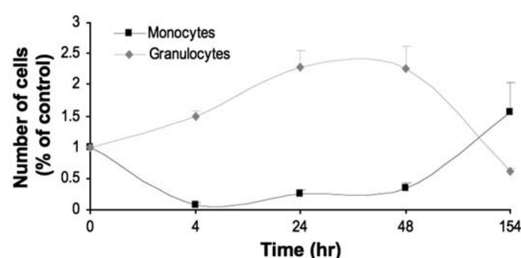

Fig. 6. Monocytes and neutrophils levels in rabbit blood over time after the administration of liposomes containing alendronate (IV, 3 mg/kg, $n=3$). Positive CD14 cells (monocytes and granulocytes) were analyzed by fluorescence activated cell sorting (FACS). Note the marked depletion of monocytes in contrast to the increased number of granulocytes returning to basal levels after 7 days.

after 24 h, 0.097%±0.011 and 0.057%±0.01, serotonin liposomes and serotonin in solution groups, respectively, were found in the animals' brains. The encapsulation of serotonin in liposomes did not affect liposome transport to the brain and was dependent on liposome penetration to the brain. Brain uptake of empty liposomes was, 0.12%±0.03 and 0.11%±0.01, after 4 h and 24 h, respectively, similar to the uptake of loaded liposomes after 4 h and 24 h, 0.138%±0.034 and 0.097%±0.011, respectively (Fig. 2).

3.3. Body distribution

After evaluating brain transport we assessed the body distribution of liposomes in order to validate the altered biodistribution of serotonin. As expected, a profound accumulation of liposomes in the spleen and liver was detected (Table 1). A significant difference in the biodistribution of serotonin in liposomes vs. in solution was found in several organs: spleen (17.16%±1.97 vs. 2.39%±0.45), liver, (0.98%±0.15 vs. 0.27%±0.07), spinal cord (0.10%±0.03 vs. 0.07%±0.02), pancreas (0.25%±0.11 vs. 0.09%±0.03), plasma (1.25%±0.21 vs. 0.08%±0.045), and urine (5.61%±1.39 vs. 20.89%±5.96), respectively (Table 1).

To further validate that intact serotonin liposomes are transported to the brain, the uptake and biodistribution of both serotonin and lipids was examined following the administration of double-radiolabeled liposomes, ^{14}C-serotonin and ^{3}H lipids. Harvested organs were measured for ^{3}H to ^{14}C ratio normalized to the injected dose (the ratio between ^{3}H radiotracer and ^{14}C in the injected formulation was 1:1). Insignificant differences were found between the ^{3}H and ^{14}C counts in the spinal cord, kidneys, muscles, blood, pancreas and brain (Fig. 3). These findings indicate that intact liposomes accumulated in these organs. In contrast, higher counts of ^{3}H than ^{14}C were detected in the lungs, spleen and liver indicating elimination of serotonin from these metabolic organs following liposomal degradation and discharge of the drug (Fig. 3).

3.4. Uptake of liposomes by monocytes and neutrophils

To elucidate the role of circulating monocytes and/or neutrophils in transporting liposomes, confocal microscopy and flow cytometry were utilized. Endocytosis of liposomes by both circulating monocytes and neutrophils was detected by confocal microscopy 4 h post-IV injection to rabbits (Fig. 4, left). Following incubation of isolated human monocytes and neutrophils with fluorescent-labeled liposomes, liposomes were endocytosed by both monocytes and neutrophils (Fig. 4, right).

Next, we examined the extent of liposome endocytosis by circulating monocytes and neutrophils (granulocytes). FACS analysis of rabbits' ($n=3$) blood 4 h post-administration of liposomes labeled with dextran-FITC (equivalent to a dose of 3 mg/kg alendronate liposomes), demonstrated that liposomes were endocytosed by 60.1%±17 and 28.5%±2.1, monocytes and granulocytes, respectively. Furthermore, 24 h and 48 h post-injection, 16.6%±0.3 and 14.7%±12 of monocytes, but no granulocytes, endocytosed liposomes, respectively (Fig. 5).

3.5. Differentiating between monocytes and neutrophils as transporters to the brain

To differentiate between blood monocytes and neutrophils as the possible transporters of liposomes to the brain, we examined the cellular uptake and biodistribution following depletion of monocytes by liposomal alendronate treatment (3 mg/kg, $n=3$), which results in depletion of circulating monocytes [36,37]. Blood monocytes were depleted following IV injection of alendronate liposomes by, 92.8%±3, 76.3%±9 and 65.7%±9, after 4 h, 24 h and 48 h, respectively, returning to basal levels after 7 days. In contrast, liposomal alendronate treatment resulted in increased granulocyte numbers by 50%±8.6, 128.6%±26 and 126.2%±35, 4, 24 and 48 h post-injection, respectively (Fig. 6). Since treatment of isolated human neutrophils with liposomal alendronate in cell culture had no effect on their viability (data not shown) it was concluded that liposomal alendronate has no inhibitory effect on circulating neutrophils.

Additional indirect evidence demonstrating that circulating monocytes are the carriers of the liposomes to the brain was obtained from an experiment comparing the injection to rabbits of either

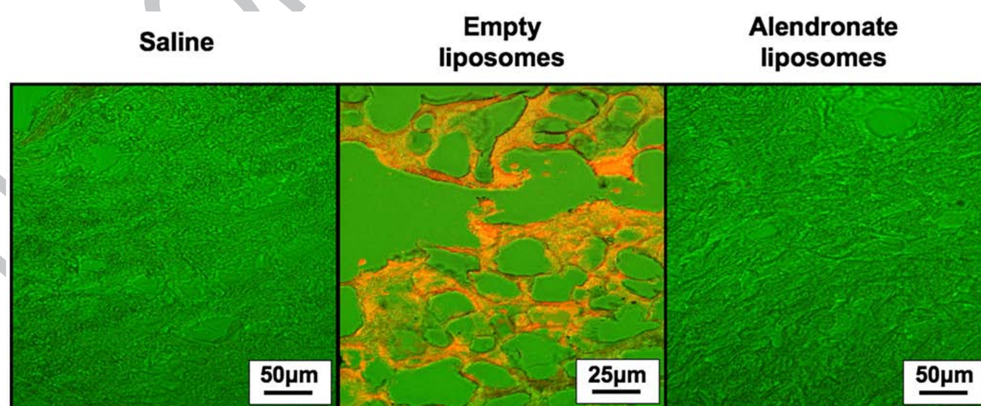

Fig. 7. Representative images of rabbit brain sections (Frontal lobe, Broca's area) after administration of empty fluorescent liposomes (1.5 ml/kg which is equivalent to liposomal alendronate dose of 3 mg/kg, Rhodamin-DSPE and 1-hydroxypyrene-3,6,8 trisulfonic acid) or fluorescent liposomes containing alendronate (3 mg/kg), in comparison to saline ($n=1$). Note that liposomes were deposited in the brain only after injection of empty liposomes and are markedly reduced after treatment with liposomal alendronate ($n=2$ in each group). Image magnifications, ×40 and ×10. Similar images were obtained in other regions.

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fluorescently labeled (1-Hydroxypyren-3,6,8-Trisulfonic acid) alendronate liposomes or empty liposomes ($n=2$ in each group). Treatment with liposomal alendronate resulted in no fluorescence in the brain tissue 4 h post-injection (Fig. 7).

4. Discussion

The major findings of the present study are that negatively-charged liposomes enter the brain of animals under normal physiologic conditions in an amount two folds higher than free drug, and that activated monocytes are the transporters.

We hypothesized that phagocytic cells of the innate immune system, mainly neutrophils and monocytes could be exploited as transporters of drugs to the brain. It was expected that the encapsulation of a drug of choice in liposomes, that are not hydrophilic, do not have a neutral membrane, and are not an ultra-small size [30,39,40], will result in efficient uptake by phagocyte cells (monocytes and neutrophils). These liposomes, similar to other circulating particles, are rapidly cleared by the cells of the MPS, blood monocytes, neutrophils and macrophages of the liver, spleen, and bone marrow [38]. The phagocyte cells will transport the drug across the BBB with the drug then being released from the cells in the brain. It is expected that if the drug is not metabolized in the lysosome it will be excreted from the endocytosing cell in the brain. The formulated liposomes in our work were not specifically targeted to macrophages as with mannosylated liposomes [7]. Large vesicles (>1 µm) are known to cause adverse effects after injection accumulating in the lungs and causing thrombosis [44], whereas vesicles smaller than 100 nm are prone to non-phagocytic cells escaping the MPS [39]. Since our target cells are circulating monocytes, a liposome size of 100 to 250 nm is preferable because vesicles larger than 100 nm are eliminated from the blood stream exclusively by monocyte/macrophage uptake [30,45]. In addition, the examined liposomal formulation can be filter sterilized.

Brain penetration was evaluated qualitatively by confocal microscopy 4 h following IV injections of fluorescently double-labeled liposomes. It was demonstrated that liposomes crossed the BBB in intact animals (Fig. 1).

Serotonin (5-Hydroxytryptamine) is a chemical neurotransmitter of the central and peripheral nervous system, but it does not cross the BBB [41]. As expected, the biodistribution of serotonin was significantly altered when administered in liposomes (Table 1). A significantly higher (two-fold) brain uptake was observed following the administration of serotonin liposomes in comparison to the free drug in solution (Fig. 2). These results appear to be in contrast with the report that only mannosylated liposomes cross the BBB [46]. However, they studied brain uptake in animals depleted of monocytes. In addition, the BBB was most probably impeded in the inflammatory rat encephalomyelitis model utilized, enabling the transport of various molecules [9,12,13], and the transport was limited to the perivascular lesions of the CNS [47].

Co-localization images showed that intact liposomes were transported into the brain (Fig. 1). To validate that intact serotonin liposomes penetrated the BBB, brain uptake and body distribution of serotonin and lipids were examined by double-radiolabeled serotonin liposomes (^{14}C serotonin in the aqueous core, and ^{3}H-lipids in the membrane). The ratio between ^{14}C and ^{3}H found in the brain following liposomal serotonin administration was similar to that of the pre-injected formulation, indicating that liposomes penetrated into the brain as intact vesicles. In addition to the brain, the original isotopes ratio was also preserved in the spinal cord, kidneys, muscles, pancreas and blood (Fig. 3). In contrast, significantly higher ^{3}H-lipid counts than ^{14}C-serotonin counts were found in the spleen, liver and lungs. Since liposomes are known to accumulate extensively in macrophages of these organs [48,49], it is suggested that following lysosomal degradation serotonin was released to the blood followed by urine excretion.

To identify the transporting cells of liposomes to the brain fluorescently labeled liposomes were injected to rabbits and incubated with human monocytes and neutrophils. Liposomes were endocytosed by both monocytes and neutrophils, but a higher uptake was noted in the former cells (Figs. 4 and 5). Since liposomal alendronate treatment resulted in a reduced number of monocytes and an increased number of granulocytes (neutrophils), which was accompanied with no brain uptake (Fig. 7), it is suggested that monocytes are the transporters of liposomes to the brain.

Despite the two-fold higher uptake of serotonin following its administration in liposomes vs. in solution, the clinical relevance is limited. An order of magnitude or more of brain uptake seems required to serve as a viable solution for brain drug delivery. Since several studies demonstrate increased passage of immune cells across the BBB in various pathological conditions [50,51], including multiple sclerosis [52,53] and Alzheimer's disease [54], the suggested delivery systems might be found more effective in brain-associated inflammatory disorders. It should also be noted that experimental treatments with 'conventional' liposomes might be associated with CNS side effects, depending on the drug type and potency, since the liposomes are transported to the brain.

5. Conclusions

IV administration of negatively-charged serotonin liposomes exhibited two times higher uptake than the free drug. Intact liposomes were transported to the brain of rats and rabbits. The encapsulation of serotonin in liposomes did not affect liposome transport to the brain. The biodistribution of serotonin was altered following its administration in liposomes, and circulating monocytes were identified as the main carriers of liposomes to the brain.

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