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Traumatic brain injury opens blood–brain barrier to stealth liposomes via an enhanced permeability and retention (EPR)-like effect

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Abstract

The opening of the tight junctions in the blood–brain barrier (BBB) following traumatic brain injury (TBI) is hypothesized to be sufficient to enable accumulation of large drug carriers, such as stealth liposomes, in a similar manner to the extravasation seen in tumor tissue via the enhanced permeability and retention (EPR) effect. The controlled cortical impact model of TBI was used to evaluate liposome accumulation in mice. Dual-radiolabeled PEGylated liposomes were administered either immediately after induction of TBI or at increasing times post-TBI to mimic the likely clinical scenario. The accumulation of radiolabel in the brain tissue ipsilateral and contralateral to the site of trauma, as well as in other organs, was evaluated. Selective influx of liposomes occurred at 0–8 h after injury, while the barrier closed between 8 and 24 hr after injury, consistent with reports on albumin infiltration. Significantly enhanced accumulation of liposomes occurred in mice subjected to TBI compared to anaesthetized controls, and accumulation was greater in the injured versus the contralateral side of the brain. Thus, stealth liposomes show potential to enhance drug delivery to the site of brain injury with a wide range of encapsulated therapeutic candidates.

Keywords

Albumin, blood–brain barrier, extravasation, stealth liposomes, traumatic brain injury

History

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Introduction

Traumatic brain injury (TBI) is a complex heterogeneous condition comprising the physical injury to tissues, and the temporal and spatial biochemical response to the injury [1]. Changes in the biochemical nature of the blood–brain barrier (BBB) have been investigated [2,3] and involve a wide range of pathways that typically result in loosening or physical disruption of the tight junctions in the vascular bed, allowing infiltration of protein and fluid from the blood. It has been shown that an influx of albumin from the vasculature to the brain occurs during a window of approximately 3 h after cerebral ischemia [4] and following TBI [5]. Aside from the initial physical injury, the resulting edema is believed to be the major damaging pathology [6] and thus strategies to reduce or eliminate edema are considered the best route to reduce long-term damage to the brain neurons after the injury [7].

The opening of the BBB after TBI is a serious clinical concern for which there are limited options, although recent studies have implicated changes in levels of key proteases, including the matrix metalloproteinase (MMP) levels and the plasminogen activating system [5,8,9]. Indeed, antagonists of these proteases or blockade of downstream signaling events initiated by these enzymes have been shown to have therapeutic benefit in rodent models of TBI when administered either orally or following direct intracortical injection [5]. However, a more appropriate approach would be one that preferentially targets these drugs directly to the site of brain injury to reduce BBB permeability and with less off-target consequences.

The extravasation of albumin into the brain tissue after injury is somewhat reminiscent of the “enhanced permeability and retention” (EPR) effect in tumors, whereby macromolecules and colloidal particles can extravasate into the interstitial tumor tissue [10], and this process has been well-recognized in the drug delivery field as a possible means of providing selective delivery of chemotherapeutic drugs to
tumor tissues [11]. In particular ‘‘stealth liposomes’’, which have enhanced plasma circulation times compared to naked liposomes by virtue of a polyethylene glycol (PEG)-coating on their surface, are already used in the clinic in a number of marketed products to carry drug to tumor tissues by selectively extravasating in regions of leaky vasculature.

The analogy between the extravasation of macromolecules via the EPR effect and the extravasation of albumin after TBI indicates a potential means to enhance selective uptake of drug into the brain upon injury using a colloidal or macromolecular carrier. Hence, it is hypothesized that stealth liposomes will selectively accumulate in the brain after TBI and ultimately provide a means to deliver a payload of therapeutic drugs for TBI directly to the site of action via an EPR-like effect.

Hence, in this study, we have taken the first step towards evaluating the potential for liposomes to act as a carrier for enhanced uptake into the damaged brain by evaluating the biodistribution of PEG-liposomes administered to mice following TBI. Specifically, radiolabeled liposomes, dual labeled with cholesterol and phospholipid, were administered intravenously at various time points with or without TBI, and distribution to the ipsilateral and contralateral sides of the injured and control brain, as well as other major organs, was determined.

**Material and methods**

**Materials**

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt) (PEG2000-DSPE) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was from Sigma Aldrich (Sydney, NSW, Australia). Sterile saline was from Baxter Healthcare (Old Toongabbie, NSW, Australia). Isopropanol and hydrogen peroxide solution (30%) were from Sigma Aldrich (Sydney, NSW, Australia).

**Preparation of PEGylated liposomes and albumin solution**

Liposomes (10 µmol phospholipid/ml) were prepared in saline similar to those previously reported [12] with the following composition: PEG2000-DSPE:DOPE:cholesterol at a mole ratio of 0.15:1.5:1.0. Lipid mixtures (DSPE-PEG2000, DOPC and cholesterol) were prepared by weight into a 5-ml Covaris® glass vials (Covaris Inc, Woburn, MA) and approximately 0.5 ml chloroform added to enable homogeneous mixing. The 14C-cholesterol and 3H-DPPC (~2 µCi) were added where required to the mixture, and the organic solvent evaporated under a stream of nitrogen overnight to provide a thin lipid film. Saline (2 ml) was added to the vial and the vial capped with a Teflon-lined cap before vortex mixing to provide a coarse dispersion. The dispersion was then homogenized by ultrasonication (Covaris S220X, Covaris Pty. Ltd., Woburn, MA) for 20 cycles (10 s on/off each cycle) peak power = 400, duty factor = 25.5, cycles/burst = 600 at 4°C.

For the albumin containing systems 3H-albumin (1 µCi) was added to liposomes prepared as described above without addition of 3H-DPPC. Hence, in this case, the dispersion contained 3H-albumin and single labeled (i.e. 14C-labeled) liposomes.

**Particle size distribution of dispersions**

The particle size distributions of the liposomes were measured by dynamic light scattering using a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK) at 25°C assuming a viscosity of pure water. The dispersion samples were diluted 100-fold (v/v) with Milli-Q water prior to particle size measurement for optimal measurement sensitivity. The measurement was carried out using automated settings in low-volume polystyrene cuvettes.

**Determination of radioactivity in materials for administration**

To determine the activity of the liposome and albumin preparations, 10 µl of the dispersion was mixed with 2 ml of scintillation cocktail (Ultima Gold, Perkin Elmer, Waltham, MA) in a 6-ml polypropylene scintillation vial and vortex mixed. The sample was then analyzed by scintillation counting on a Packard Tri-Carb 2000CA liquid scintillation analyzer (Meriden, CT) counter. All samples were run twice: once for 3H and once for 14C to avoid problems with overlap in counting energies.

**Animal procedures**

All animal procedures were undertaken in accordance with the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. Experiments were performed with adult male mice in the C57/Bl6 background aged 8–12 weeks and approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee. Mice were maintained under specific pathogen-free facilities at a maximum of six per cage, on a 12-h light/dark cycle and had ad libitum access to food and water. Animals were randomly assigned to either trauma or non-trauma groups. Following trauma surgery, all animals were housed individually in cages fitted with dividers and split-feeders. Animals were monitored regularly, and no adverse events related to the trauma procedure were observed.

The appropriate treatment (liposomes or liposomes + albumin) was administered as a 100-µl intravenous bolus injection via the tail vein.

**Controlled cortical impact (CCI) model of TBI**

TBI was induced in anaesthetized mice using the controlled cortical impact model as described [9] using an impact depth of 2 mm and a dwell time of 150 ms. The exposed site was then covered with bone wax, the scalp was sutured, and the animals were allowed to recover on a 37°C heat pad. This model has been shown to be highly reproducible and with low (<1%) mortality. Control animals were anaesthetized with
avertin but were not subjected to the scalp incision or to the CCI injury.

Harvest and treatment of tissue samples to determine radioactivity

At defined time points post-administration, mice were anaesthetized with urethane (3.3 g/kg), and transcardially perfused with 30 ml of PBS pH 7.3. The injured (ipsilateral) and uninjured (contralateral) hemispheres were separated by dissection. The two brain samples, as well as the heart, lung, spleen, kidneys, liver, gastrocnemius muscle and plasma, were transferred to 20 ml polypropylene scintillation vials. Tissues were then treated as previously described [13]. Briefly, soluene (2 ml, Perkin Elmer) was added and the tissue digested by heating overnight at 60°C. Isopropanol (2 ml) and hydrogen peroxide (0.2 ml) were added to the vial to clarify the sample prior to scintillation counting to determine total reactivity. Scintillation cocktail (10 ml) was added to the vials, and the vials were stored undisturbed at 4°C for at least 24 h prior to scintillation counting as described above to suppress background chemiluminescence.

Statistical treatment

Where indicated, significance was assessed using either a one-way ANOVA with a Tukey post-hoc test or a Student’s t test using GraphPad Prism software. Minimum cohorts of five mice were used in each experiment. p < 0.05 was considered significant.

Results

The PEG-liposomes administered to the animals had a mean particle size of 82 nm and polydispersity index of 0.18. This size distribution is typical of this type of liposomes [12]. The tissue distribution across the major organs after administration of the liposomes (see Supplementary Figure S1) was typical of that for PEGylated liposomes [14], with the major organs in which the liposomes accumulated being the liver, spleen and lung. The approximate plasma half-life, based on the limited data points available, was 24 h (23.1 ± 3.2 h for TBI cohort and 26.3 ± 3.3 h for control cohort, Figure S2), also consistent with previous reports at 23.6 h in rats [14], confirming that the liposomes were behaving as expected as stealth long-circulating particles. There was no difference in the plasma circulation of liposomes between TBI or control animals.

Administration of liposomes and albumin immediately following injury

Distribution in the brain

The accumulation of radioactivity in brain tissue after perfusion is expressed as a fraction of the administered dose per weight of tissue in Figures 1 and 2. When PEG-liposomes were administered in control animals, accumulation of liposomes was identical on the ipsilateral and contralateral sides of the brain when assessed at 8 h (p = 0.7790, Figure 1). In contrast, liposomes administered into animals 10 min following TBI displayed significantly enhanced levels of radioactivity in the ipsilateral side of the brain relative to contralateral side at the same time point (p < 0.0001). Thus, in animals sacrificed at 8 h after injury and administration, there was significantly enhanced accumulation of liposomes in the ipsilateral side of the injured brain. Slightly enhanced accumulation in the tissue contralateral to the injury, compared to control tissues, likely indicates that the impacting injury induces some level of response in the contralateral side of the brain, albeit less than the ipsilateral side.

Importantly, the patterns of distribution were the same between the 3H-phospholipid and 14C-cholesterol determinations at 8 h following TBI (and indeed at all time points, see Supplementary Figure S3 for data at each individual time point) indicating that the liposomes remained intact during circulation and accumulation, i.e. the cholesterol and phospholipid remained co-located, and one or other component did not dissociate from the liposome carrier. Consequently, from here on in this manuscript, the accumulation behavior will be referred to as accumulation of liposomes, rather than radioactivity.

Figure 2 illustrates the time course for accumulation of liposomes in the brain tissue, expressed as a ratio of ipsilateral to contralateral accumulation in the injured brain. For this form of data presentation, it is expected that the ratio = 1 in control animals, i.e. in the absence of selective delivery to the site of injury, which indeed is the case in the data. The time course for injured animals on the other hand provides evidence for a maximum accumulation already occurring at approximately 8 h, with similar levels of accumulation of liposomes evident at the 24 and 48 h timepoints. By 96 h after injury, the overall levels of radioactivity had
significantly declined from the brain presumably through natural clearance mechanisms for the phospholipid and cholesterol (Figure SI-3), but the contra relative levels were similar to the other time points (Figure 2).

Administration of albumin

Radiolabeled $^{3}$H-albumin was also administered with $^{14}$C-labeled liposomes, in part to exclude a significant active transport contribution to the process, whereby albumin may have been selectively transported out of the vasculature after TBI, and also to confirm analytically the previous results demonstrating endogenous albumin extravasation measured by immunoassay [5,9]. Albumin also serves as a model macromolecular construct which is significantly smaller than the liposomes (~7–10 nm diameter [15,16]), but may represent the physical size of dendrimers or other polymer-based drug carriers also known to have long circulating properties when functionalized with PEG groups [17].

Albumin preferentially accumulated in the ipsilateral side of the brain 24 h after trauma, with very similar behavior to the liposomes (Figure 3). Albumin extravasated to a slightly greater extent than the liposomes, indicated by the slightly higher values in Figure 3, panel A versus panel B.

Although Figure 3 indicates that there was a slightly greater accumulation of albumin compared to liposomes across all samples, there was a correlation between the accumulation of liposomes and albumin in the brain ($R^2 = 0.72$, Figure S4), indicating that there was no significant size selectivity up to the size of the liposome and that the same physical extravasation process most likely governed the accumulation, precluding a significant contribution from transport processes.

Delayed administration of liposomes and albumin following injury

To mimic the likely clinical scenario, whereby a patient presents some time after the traumatic injury has occurred, and treatment is provided some hours into the trauma, liposomes were administered at 3, 6 and 24 h after the injury. Animals were sacrificed at 24 h after administration, and radioactivity in tissues was determined. This protocol is also of interest in understanding the behavior of the BBB at different times after injury; a closing of the barrier is anticipated to prevent further access of particles and macromolecules into the brain, so administration after this time would result in reduced brain uptake.

The data in Figure 4 clearly indicate reduced access of the liposomes to the damaged brain tissue between 6 and 24 h after the injury. Administration at 3 and 6 h provided similar distribution behavior to that observed when the liposomes were administered immediately after the injury, with significantly higher accumulation of the radioactivity in the ipsilateral side of the brain relative to the contralateral side and the control animals. In contrast, when liposomes were administered at 24 h after the injury and the tissue sampled at 48 h after the initial injury, there was reduced accumulation in the injured brain, relative to the control brain, and substantially reduced accumulation relative to the 8-h time point illustrated in Figure 1. This indicates not only that the BBB appeared to have closed in the injured animals at some time point between 6 and 24 h, but further that there may be an over compensation in BBB closure as a protective measure after the immediate opening of the barrier. As expected, the accumulation in the control animals was independent of ipsilateral or contralateral sampling in uninjured animals, and...
independent of the time when the dose was administered, as there is no time-dependent change in the behavior at the BBB.

Hence, in summary, the results show that liposomes preferentially accumulate in the injured region of the brain compared to non-injured and control, although some accumulation in non-injured side of the injured cohort was also apparent relative to control. Administration immediately after injury led to an increase in liposome accumulation which did not increase beyond 8 h. Administration at 24 h after the injury, when the barrier appeared to have closed, resulted in dramatically lower accumulation than when administered immediately after the injury or at 3 or 6 h after the injury.

Discussion

In this study, we have evaluated the potential utility of PEG liposomes as a delivery modality in a mouse model of TBI. We took the view that the time frame of albumin extravasation following TBI provided reasonable grounds to test the hypothesis that liposomes would preferentially enter the damaged brain following TBI via an EPR effect.

To the best of our knowledge, intravenous delivery of liposomes in TBI has not been previously evaluated. However, liposomes containing the drug fasudil hydrochloride have been reported to provide sustained levels of the rho-kinase inhibitor in the cerebrospinal fluid for over 24 h, resulting in improved protection against cerebral ischemia compared to an equivalent dose of free drug [18]. However, in that study, the liposomes were administered intrathecally, hence passage across the BBB was not evaluated, and this mode of treatment is invasive and may not concentrate in the traumatized area of the brain compared with other routes of administration. Hence, we believe this study to be the first to evaluate the potential of any drug delivery system to extravasate into brain tissue after TBI via intravenous administration. Liposomes have also been reported to be absorbed into the systemic circulation after subcutaneous administration at levels approximating 30% of the dose [19], indicating potential for subcutaneous treatment with liposome-encapsulated drugs for treatment of TBI.

The model used in this study (CCI) delivers a controlled impact trauma locally to one side of the brain [9]. One hypothesis in embarking on this study was that accumulation would be preferentially localized to the side of the brain that was directly injured. Interestingly, it is apparent that liposomes also have a propensity to accumulate in the uninjured contralateral side of the brain, albeit at lower levels than the injured side. This indicates that the response observed in these studies is primarily biochemical rather than physical in nature, and that local delivery of agents to the site of trauma may limit effectiveness in treating the entire cohort of affected tissues in TBI.

Although clearly favoring accumulation in the brain of animals with TBI compared uninjured animals, the level of liposome accumulation was relatively low (1–1.5% of the administered dose). This is lower than the accumulation seen in tumor tissues for PEGylated polymers, where around 10% of the dose is often found to have accumulated in tumor tissue [20–22], but is consistent with doxorubicin accumulation in tumors delivered using PEGylated liposomes [14]. In the case of tumor tissues, the endothelial gaps through which the liposomes can escape the vasculature remain open while they circulate for several days, while in the case of TBI, the data in the present study indicate a closing of the barrier after 6 h, which is also expected to limit their potential for higher accumulation. This time window could be different in the human brain following severe TBI. The biodistribution profiles for the liposomes showed preferential accumulation in organs of the reticuloendothelial system such as the liver and spleen, and also the lung (Figure SI-1), consistent with studies in tumor models [14]. It should, however, be kept in mind that the carrying capacity of liposomes for water soluble drugs or poorly-soluble ionizable drugs such as fasudil is high. For example, Ishida et al. [23] loaded liposomes at a drug to lipid ratio of 0.36. There is also potential to load drug as a suspension at even higher concentrations, as is the case with doxorubicin in Doxil®, the marketed anticancer liposomal product. Thus, for a 1-ml IV injection at 10% w/ w lipid, 3% w/w of the formulation (approximately 3 mg) would be drug and 30 μg of drug would therefore be expected to accumulate specifically at the site of injury. It should be kept in mind that the liposomes of the current study were relatively dilute, and that the dose per volume could be increased at least 5-fold in preparation of a therapeutic treatment containing drug.

Figure 4. Accumulation of liposomes in ipsilateral and contralateral sides of the brain at 24 h after administration, when administration of liposomes is delayed after the injury (data are mean ± SEM, n = 5). Panel A: 3 h, panel B: 6 h, panel C: 24 h (injection post-TBI). At 3 and 6 h, the accumulation of liposomes in the ipsi hemisphere for TBI animals was statistically significant compared to control animals.
The uptake of albumin was similar to or slightly greater than that of the liposomes. Drug–polymer conjugates, including drug–dendrimer conjugates, are receiving much attention as potential delivery agents of chemotherapeutic drugs to tumors via utilization of the EPR effect. However, they also have a limited carrying capacity, conjugation of the drug can greatly reduce their circulation times and requires covalent bonds that often leave a residual functional group on the parent drug, complicating their toxicological and regulatory assessment. The residual polymer is also a complicating factor in the approval process. Consequently, as yet no drug–polymer conjugates form the basis of market approved intravenous dose forms.

The intention of the perfusion step prior to further processing of the tissues was to remove any vascularity localized radiolabeled material from the brain at the end of the experimental period. There are some reasons why this may not be complete, particularly for albumin, leading to the non-zero background in, e.g. Figure 3. It is known that some proteinaceous material can bind to brain capillary endothelium [24]. Radiolabeled albumin may also be processed in the body during the experimental period through normal albumin turnover resulting in liberation of radiolabeled amino acids and reincorporation into proteins that are subsequently absorbed into the brain parenchyma [13]. Finally, access of albumin to the CSF has been reported, which may result in some appearance of the radiolabel in the brain [25]. Although any or all of these mechanisms may be responsible for the background albumin levels in control animals, the comparison with CCI-treated animals is compelling and future studies with small molecule drugs may help to discriminate between these effects.

Some uptake of PEG liposomes was also evident in non-TBI mice. Thorne and Nicholson have reported measurements of diffusivity of PEGylated 35 nm quantum dots in the brain to probe particle penetration of the BBB and to estimate the size of the extracellular gaps in healthy brain [26]. Using a theoretical approach based on diffusivity, they establish that the extracellular gaps are likely to be of the order of 38–64 nm. It should be recognized that although the mean diameter of the liposomes in this study was 82 nm, it is always a distribution about the mean and that a significant proportion of particles may have diameter close to 64 nm. It must also be realized that dynamic light scattering measurements provide a hydrodynamic diameter, and that together with the deformability of the liposomes as a soft particle, that strict comparison with the diameter of hard metallic quantum dots is fraught with a large degree of uncertainty. Hence, the study of Thorne and Nicholson does not preclude the possibility of liposome uptake in the healthy brain.

The clinical scenario in which the liposomes of the present study would be applied would most likely entail administration at the site of the trauma, during transport to hospital or in the emergency room at the hospital. Based on the behavior of the liposomes, application before the 8-h timepoint would be most beneficial, a time frame of clinical relevance. However, it remains to be demonstrated whether drug accumulates in the same manner as expected from the “empty” liposome carriers and can be released at the site of injury to provide a consequent therapeutic effect. Future studies in this regard will build on this promising behavior of the drug carrier towards a new treatment approach to TBI.

Conclusion
PEGylated liposomes accumulated selectively at the site of TBI in a similar manner to albumin, suggesting implication of opening of the BBB after injury and accumulation via an EPR-like mechanism. Liposomes were hindered from access to injured brain after approximately 8 h after injury, apparently due to closing of the BBB, while there was no difference in injured control brains. The results indicate a potential role of PEGylated liposomes as a carrier for selective delivery of therapeutics to the injured brain.

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Declaration of interest
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Supplementary material available online
Supplementary Figure S1–S4