The Transferrin Receptor at the Blood-Brain Barrier - exploring the possibilities for brain drug delivery

Visser, Corine

Chapter 6

Targeting liposomes with protein drugs to the blood-brain barrier *in vitro*

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Abstract

In this study, we aim to target pegylated liposomes loaded with HRP and tagged with Tf to the BBB *in vitro*. Liposomes were prepared with the post-insertion technique. Tf was measured indirectly by measuring iron via atomic absorption spectroscopy. All liposomes were around 100 nm in diameter, contained 5 - 13 μg HRP per μmol phospholipid and 63 – 74 Tf molecules per liposome (lipo Tf) or no Tf (lipo C).

Brain capillary endothelial cells (BCEC) were incubated with liposomes at 4 °C (to determine binding) or at 37 °C (to determine association, i.e. binding + endocytosis) and the HRP activity, rather than the HRP amount was determined in cell lysates. Association of lipo Tf was 2 – 3 fold higher than association of lipo C. Surprisingly, the binding of lipo Tf at 4 °C was 4 fold higher than the association of at 37 °C. This is explained by intracellular degradation of endocytosed HRP.

In conclusion, successful targeting of liposomes loaded with protein or peptide drugs to BCEC may be hampered by intracellular degradation of the drug. However, it may also be an advantage to target the liposomal content to the lysosomes (e.g. in the treatment of lysosomal storage disease). Our experiments suggest that liposomes release some of their content within the BBB, making targeting of liposomes to the TfR on BCEC an attractive approach for brain drug delivery.

Introduction

The central nervous system (CNS) is protected by the blood-brain barrier (BBB). This barrier is located at the interface between blood and brain and its primary function is to maintain homeostasis in the brain. Unique features, such as tight junctions, low vesicular transport and high metabolic activity accomplish this barrier function (1). Drug delivery to the brain is limited due to the BBB. Only small molecules (molecular weight lower than 600 Da) can pass the BBB paracellularly or transcellularly, depending on their lipophilicity (2). However, high molecular weight drugs, such as (recombinant) proteins, peptides or DNA, do not cross the BBB (3).

Over the years, many drug targeting and delivery strategies have been explored. Drug delivery strategies to the brain involve chimeric proteins, in which the protein drug is covalently linked to a transport vector (4). For the delivery of antisense drugs, encapsulation of the antisense in targeted liposomal drug carriers has been applied (4, 5). In our previous work we have shown that drug targeting to the transferrin receptor (TfR) with conjugates of transferrin (Tf) and horseradish peroxidase (HRP) shows accumulation of Tf-HRP in brain capillary endothelial cells *in vitro* (6).

In this study, we extend our investigations to deliver proteins to the BBB. Therefore, we have incorporated HRP in Tf-tagged pegylated liposomes. By using liposomes the amount of drug molecules per targeting vector (i.e. Tf) can be increased. Furthermore, it is not necessary to chemically modify the drug molecule, and the drug molecule is protected from degradation in serum. Also, for targeting drugs to the TfR at the BBB, liposomes have an additional advantage, since it is possible to circumvent the efflux transporter P-glycoprotein (7). HRP was chosen as a model compound, since it does not cross the BBB (8, 9) and is readily quantified. Liposomes were prepared according to the post-insertion technique (10, 11). Tf was tagged to the distal end of the polyethylene glycol chain (PEG) via a maleimide-thiol coupling. Non-tagged (lipo C) and Tf-tagged (lipo Tf) liposomes were prepared, containing 5 - 13 μg HRP per μmol phospholipid. Lipo Tf contained 63 – 74 Tf molecules per liposome.

Primary brain capillary endothelial cells (BCEC) were incubated with liposomes at 4 °C (to determine binding) or at 37 °C (to determine association, i.e. a combination of binding and endocytosis). Incubation was performed based on adding equal HRP concentrations in liposomal form, to be able to compare non-tagged liposomes with Tf-

tagged liposomes. From these studies with Tf-tagged liposomes and from our previous research on Tf-HRP conjugates (6), we are able to distinguish between route and rate of uptake of Tf-tagged liposomes and Tf-protein conjugates, as well as intracellular trafficking and fate of HRP.

Experimental

Cell culture

Primary brain capillary endothelial cells (BCEC) were cultured from isolated bovine brain capillaries as described before (12). Briefly, brain capillaries were seeded in type IV collagen and fibronectin-coated plastic culture flasks and cultured in a 1:1 mixture of DMEM (containing 2 mM L-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin, non essential amino acids and 10% fetal calf serum) and astrocyte-conditioned medium (ACM), supplemented with 125 μg/ml heparin at 37 °C, 10% CO_2 for 4 - 5 days. ACM was obtained as described before (12). ACM is added to the cell culture medium to induce BBB properties of the BCEC, while the TfR is not influenced by addition of ACM (13). At 70 % confluence the BCEC were passaged with trypsin-EDTA and seeded into a type IV collagen coated 96 wells plate at a density of 15,000 cells/well. Subsequently, BCEC were cultured for 4 – 5 days at 37 °C, 10% CO_2.

Preparation of liposomes containing HRP, using the post-insertion technique

Tf was modified with a thiol group using SATA (1:8 molar ratio) as described before (14). Micelles of PEG_{2000}-DSPE and PEG_{2000}-maleimide-DSPE (in a molar ratio of 1:1) were prepared by hydration of the lipid film in 0.25 ml HEPES buffered saline (HBS), pH 6.5. Directly after hydration micelles were incubated with SATA modified Tf (1 or 3 mg Tf per 9 μmol phospholipid) in the presence of TCEP (0.01 mM) for 2 h at room temperature under constant shaking. To block all excess maleimide groups, micelles were incubated with L-cystein for 30 min, subsequently 1 mM NEM was added to block free thiol groups. Free maleimide groups need to be blocked to prevent non-specific reactions with other components during the preparation or the use of liposomes.

Liposomes were prepared of EPC-35 and cholesterol in a 2:1 molar ratio, by resuspending a lipid film in HBS with a final HRP concentration of 300 μg/μmol phospholipid (PL). Liposomes (approximately 10 mM PL) were extruded stepwise, through 200, 100, 80 and 50 nm polycarbonate filters (4 times per filter size) using a hand-extruder from Avanti Polar Lipids (Alabaster, AL, USA). External HRP was removed by ultracentrifugation (60,000 g, 45 min, 10 °C).

Liposomes and PEG micelles were mixed for 2 h at 40 °C to obtain liposomes with 5% total PEG. This method was modified from earlier published protocols (10, 11). After incubation, the mixture was separated on a Sepharose CL4B column (Amersham Pharmacia Biotech, Uppsala, Sweden). The fractions containing the liposomes were concentrated by ultracentrifugation (60,000 g, 30 min, 10 °C). After ultracentrifugation liposomes were resuspended in HBS pH 7.0 and stored at 4 °C. The liposomes were used for experiments within 3 weeks, during which the HRP activity did not change.

Association of liposomes by BCEC

BCEC were checked under the microscope for confluency and morphology (12). BCEC were incubated with liposomes, based on the HRP concentration. BCEC were incubated in 50 µl DMEM with 10 % serum (DMEM+S) with 1 – 12 µg/ml liposomes for 2 h (concentration range), or with 3 µg/ml liposomes for 1 – 8 hour (time range). Incubation was performed at 37 °C, to determine association, which is a combination of binding and uptake, or at 4 °C, to determine binding only.

After incubation, BCEC were washed 6 times with 200 µl ice-cold PBS and solubilised for at least 2 h at room temperature with 40 µl Na-deoxycholic acid (0.1 % in MilliQ) for determination of HRP activity (adapted from (15)) or cellular protein. All data are represented as mean ± SD (performed in quadruplicate). Statistical analysis was performed by an unpaired t-test, using GraphPad InStat version 3.00 (GraphPad Software, San Diego, California, USA).

Analytical Methods

The PL content was determined according to Rouser (16). Size and polydispersity index (p.i.) were determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd. Malvern, UK). The p.i. is a measure of the particle size distribution and can range form 0 (monodisperse) to 1 (polydisperse).

HRP activity was determined using a colorimetric assay. Briefly, cell lysates or liposomes were incubated with TMB liquid substrate for 20 min, after which 0.5 M H_2SO_4 was added to stop the reaction. HRP (0 – 2 ng/ml) was used for a standard curve and absorption was read at 450 nm. HRP activity in 0.1 % Na-deoxycholic acid was not significantly different from samples in MilliQ. Cellular protein contents were determined using Biorad DC reagents and BSA for a standard curve (0 – 400 µg/ml). Absorption was read at 690 nm.

The Tf concentration was determined indirectly, by measuring the iron content with atomic absorption spectroscopy (Perkin Elmer 3100 atomic absorption spectrometer, Boston, MA, USA). Iron was measured at 248.3 nm against a standard curve (0 – 400 µg/ml Fe), samples were prepared in water. The calculation of the Tf concentration is based on the assumption that each Tf contains 2 iron atoms.

The number of Tf molecules per liposome is calculated with

$$(E \cdot 4\pi r^2 \cdot number\ of\ monolayers) / (MW \cdot (PL \cdot SH_{EPC} + CHOL \cdot SH_{CHOL})),$$

in which E is the g protein per mol total lipid, r is the radius of one liposome in nm, the number of monolayers for a 100 nm liposome is 2, MW is the molecular weight of Tf (77,000), PL is the phospholipid in mol, CHOL is the cholesterol in mol and SH is the surface area of EPC-35 and cholesterol headgroups. The surface area of EPC-35 and cholesterol is 0.70 nm^2 and 0.28 nm^2, respectively (17). For this calculation we assume that EPC-35 and cholesterol are spread evenly over the liposomal bilayer and are incorporated in a 2 : 1 molar ratio (17). For HRP the same formula is used, but with a MW of 40,000.

Materials

Culture flasks were obtained from Greiner (Alphen a/d Rijn, the Netherlands) and 96 wells plates from Corning Costar (Cambridge, MA, USA). PBS, DMEM, supplements and fetal calf serum were purchased from BioWhittaker Europe (Verviers, Belgium). Type IV collagen, heparin, trypsin-EDTA, endothelial cell trypsin, HRP, sodium deoxycholic acid, TMB liquid substrate, SATA, TCEP, HEPES, L-cystein and cholesterol were obtained from Sigma (Zwijndrecht, the Netherlands) and fibronectin from Boehringer Mannheim (Almere, the Netherlands). Bovine holo-Tf, BSA and hydroxylamine-HCl were obtained from ICN Pharmaceuticals (Zoetermeer, the Netherlands) and sodium chloride from Merck (Amsterdam, the Netherlands). EPC-35 was purchased from Lipoid GmbH (Ludwigshafen, Germany), PEG_{2000}-DSPE from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and PEG_{2000}-maleimide-DSPE from Shear Water Corporation (Huntsville, AL, USA). Vivaspin columns are obtained from Vivascience AG (Hannover, Germany) and polycarbonate filters for extrusion from Nuclepore (Pleasanton, USA). The Biorad protein assay was obtained from Bio-Rad Laboratories (Veenendaal, the Netherlands).

Results and Discussion

Characterisation of liposomes

Liposomes were prepared according to the post-insertion technique. Liposome batch 1 was prepared with PEG-micelles incubated with 1 mg Tf, while PEG-micelles for batch 2 and 3 were incubated with 3 mg Tf. Table I summarises the PL, HRP and Tf concentrations. All liposomes were around 100 nm and have polydispersion index (p.i.) values lower than 0.2, indicating that the liposomes have a rather narrow size

distribution. Liposomes were used within 3 weeks for experiments, during that time the increase in size and p.d. was less than 10 %. PL and HRP concentrations differed between batches 1, 2 and 3. Batch 1 had a different HRP concentration for lipo C (11.8 μg/μmol PL) and lipo Tf (6.4 μg/μmol PL). The encapsulation efficiency calculated from the final HRP concentration (table I) and the added amount of 300 μg HRP per μmol PL was 1.5 – 4.6 %. Corvo *et al* (18) have shown that for liposomes containing superoxide dismutase the encapsulation efficiency decreased upon extrusion. However, extrusion is necessary to obtain liposomes of approximately 100 nm. When calculating the average number of HRP molecules per liposome, based on the HRP activity assay rather than the amount of HRP, only 10 - 26 HRP molecules per liposome were incorporated. This low incorporation might be due to the extrusion as well as due to loss of HRP activity: we did not determine the amount of HRP by a protein determination due to interference by Tf, but have used the activity of the incorporated HRP for these calculations.

The average number of Tf molecules exposed from the outer surface was 63 – 74 per liposome. Based on previous experiments we expected an approximate 2-fold increase in Tf-tagging when a 3-fold higher amount of Tf was added to liposomes (14). But, the difference in the number of Tf molecules per liposome between batch 1 (1 mg Tf) or batch 2 and 3 (3 mg Tf) was relatively small. Therefore, we have not been able to determine the influence of the number of Tf molecules at the liposome surface on the association of liposomes by BCEC. Although we have not been able to determine the incorporation efficiency of the PEG micelles into the liposomes, this small difference in Tf-tagging between liposome batches may be the result of the post-insertion method that is used for the preparation of HRP containing liposomes.

Tf containing two iron atoms has the highest affinity for the TfR. Recently, we have described the preparation of Tf-tagged liposomes without loosing Fe from Tf, for which we determined the Tf concentration by a protein assay (14). However, the liposomes used for these experiments contain HRP, which interferes with the protein determination of Tf. Therefore, the determination of the iron concentration has been used to indirectly measure the Tf concentration. This method was verified using empty Tf-tagged liposomes in which Tf was determined using a protein assay as well as using AAS to determine Fe (data not shown).

Table I: Characterisation of liposomes, based on their phospholipid (PL) content, size, polydispersion index (p.i., a p.i. < 0.2 indicates a rather narrow particle size distribution), HRP concentration and the number of Tf molecules per liposome. Each batch contained non-tagged liposomes (lipo C) and Tf-tagged liposomes (lipo Tf).

		PL (mM)	Size (nm)	p.i.	HRP/PL (µg/µmol)	Tf molecules per liposome
batch 1	*lipo C*	9.8	110	0.15	11.8	
	lipo Tf	10.8	112	0.09	6.4	63
batch 2	*lipo C*	9.5	81	0.20	4.5	
	lipo Tf	8.9	98	0.16	5.7	70
batch 3	*lipo C*	5.0	101	0.09	13.7	
	lipo Tf	4.8	113	0.16	11.1	74

Comparing Tf-HRP conjugates (1:1 ratio) and Tf-tagged liposomes containing HRP, there is no increase in the amount of drug (HRP) per targeting vector (Tf), when using liposomes for protein drugs. However, Vingerhoeds *et al* (19) and Mastrobattista *et al* (20) have shown that liposomes with diphtheria toxin chain A (MW 21,000) are effective at the target site. Therefore, incorporation of a few potent protein drug molecules may be efficient if the drug is potent enough at low concentrations.

Binding and association of liposomes by BCEC

BCEC were incubated with liposomes. The chosen concentrations were based on equal HRP concentrations for comparison of non-tagged and Tf-tagged liposomes. We ascertained that the amount of liposomes (based on their PL concentration) that was added to the cells did not affect the cells at the concentrations used (data not shown).

After 2 hours of incubation of BCEC with liposomes at 37 °C, the difference in association of lipo C and lipo Tf was 2 – 4 fold (figure 1 A). This was found consistently in all experiments and was independent of the batch of liposomes used. Extensive concentration and time studies at 37 °C with all batches of liposomes were performed. For this, BCEC were either incubated with 1 – 12 μg/ml HRP, for 2 hours or with 3 μg/ml HRP for 1 – 8 hours. In two experiments the association of lipo Tf seemed saturated after 4 hours of incubation, but this was not reproducible. In general, the association of lipo Tf by BCEC increased both depending on time and concentration (data not shown), while association of lipo C was less dependent on time and concentration. This indicates that Tf-tagged liposomes are associated by BCEC via a different mechanism than non-tagged liposomes. We found that the difference in association between lipo C and lipo Tf was highest after 2 hours of incubation with liposomes containing 3 μg/ml HRP (figure 1A).

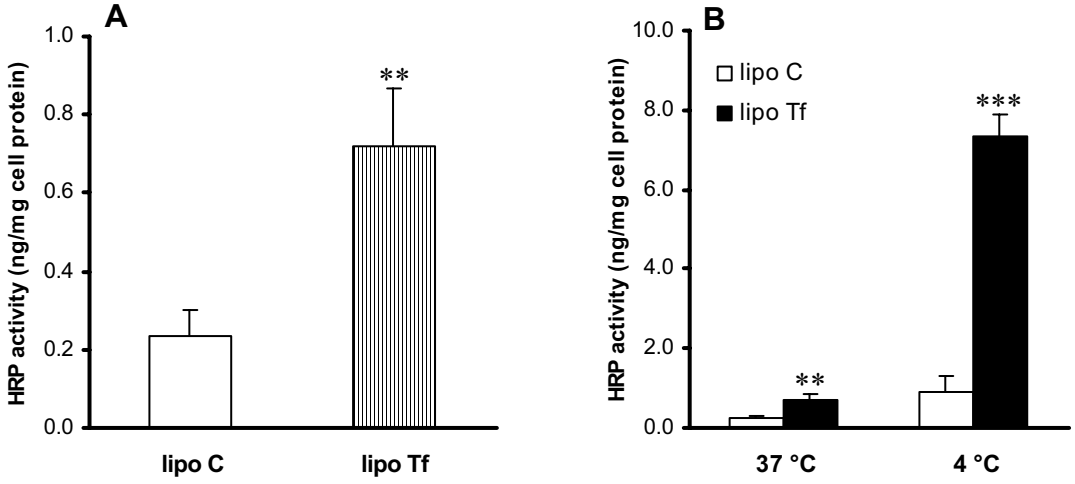

Figure 1: BCEC were incubated with liposomes (3 μg/ml HRP) for 2 hours at 37 ºC (to determine association) or at 4 ºC (to determine binding). Figure **1A** shows the association of liposomes, in figure **1B** the association is compared to the binding of liposomes. Tf-tagged liposomes show higher binding than association, please notice the difference in Y-axis. These graphs are representative for all data generated during these experiments. ** $P < 0.01$, *** $P < 0.001$, unpaired t-test.

Binding studies at 4 °C (figure 1B) with liposomes (3 μg/ml HRP) show that lipo Tf displayed a 4 – 8 -fold higher binding to BCEC than lipo C. All experiments were performed in the presence of serum and thus in the presence of endogenous Tf. Tf is, in fact, a high abundance protein in serum (21). The concentration Tf in serum is between 1.7 and 2.7 g/L (22). In our cell culture and in the binding and association studies we used 10 % serum in the incubation medium. Previously, we have reported that DMEM with 10 % serum contained 5.2 μM Tf, which is approximately 400 μg/ml (13). The liposome solution contains 290 – 500 μg/ml Tf, which is approximately 20 times further diluted in the experiments, indicating that liposomal Tf is lower than serum Tf. Therefore, the TfR might already be saturated with Tf. We have performed experiments with liposomes in the presence of serum to mimic *in vivo* conditions and to minimise the association of non-tagged liposomes. BCEC under physiological conditions show a very low, non-specific vesicular uptake of solutes from the blood (1). However, we found that when the experiments were performed in DMEM without serum or in PBS, non-tagged liposomes display a high association (data not shown). When incubating BCEC with liposomes in the presence of serum, the association of non-tagged liposomes was low.

The binding at 4 °C of lipo Tf (data not shown) was not time dependent, while the association (37 °C) was. The experiments with Tf-HRP showed saturation of both binding and association by BCEC after 1 hour incubation (6). This is consistent with the binding of Tf-tagged liposomes, which did not change between 1 and 8 hours. The association at 37 °C of Tf-tagged liposomes did increase with time, indicating that Tf-tagged liposomes may also be endocytosed via a different process than receptor mediated endocytosis. Although we have not been able to determine which mechanisms were involved, we consistently found a higher HRP activity after incubation with lipo Tf than after incubation with lipo C.

Intracellular trafficking of Tf-tagged liposomes

The binding of Tf-tagged liposomes is 10–fold higher than the association (figure 1B). This may be explained by intracellular degradation of HRP. This phenomenon becomes apparent, since we determine the activity of HRP rather than the amount of

endocytosed HRP. From literature it is known that liposomes are intracellularly routed towards the lysosomes (23). In these lysosomes HRP (and other proteins) are subject to degradation (8, 9). Normally, the TfR-Tf complex is not directed towards lysosomes, but recycles from endosomes back to the cell surface (24, 25). However, when Tf is attached to a 100 nm liposome it has not been described what the effect is on the intracellular trafficking of the TfR-Tf-liposome complex.

To determine the mechanism of endocytosis of Tf-tagged liposomes, we have applied phenylarsine oxide (inhibitor of clathrin-coated vesicles associated with the TfR) and indomethacin (inhibitor of caveolae formation) (6). However, these inhibitors did not have a conclusive effect. We expected that by inhibition of endocytosis we would find a higher HRP activity, since HRP would not be intracellularly degraded. However, this was the case in one experiment, but not in another experiment with a different batch liposomes. Also, the use of chloroquine or NH_4Cl to prevent acidification of endosomes and therefore fusion with lysosomes did not have an effect on HRP activity after association of Tf-tagged liposomes by BCEC. Therefore, it was not possible to determine the mechanism of endocytosis.

Although we have incubated BCEC in the presence of endogenous Tf, we have tried to use an even higher excess of free Tf to further block TfR-mediated endocytosis. BCEC were pre-incubated for 30 min with 0.5 – 5 mg/ml Tf in DMEM+S (0.4 mg/ml Tf) before incubation with liposomes (3 μg/ml HRP). No significant inhibition of association of Tf-tagged liposomes was detected (data not shown). Since higher concentrations of Tf showed a decrease in cellular protein, we have not increased the concentration of free Tf beyond 5 mg/ml. Therefore, we have not been able to show that Tf-tagged liposomes are exclusively endocytosed via a TfR mediated mechanism.

Comparison of Tf-tagged liposomes and Tf-HRP conjugates

The binding (4 °C) of Tf-tagged liposomes was higher than the association (37 °C, figure 1B). In contrast, the association of the Tf-HRP conjugates, which we have described recently was 5.2 ng HRP per mg cell protein (6) and was approximately 4-fold higher than the binding. For this BCEC were incubated for 2 hours with Tf-HRP conjugates, based on 3 μg/ml HRP. The association at 37 °C of Tf-HRP was

approximately 7.5–fold higher than the association of Tf-tagged liposomes. The experiments with Tf-HRP were performed in PBS without serum, in order to eliminate potential interference with endogenous Tf. However, experiments with liposomes were performed in DMEM+S containing endogenous Tf. We found that the addition of serum was essential to minimise non-specific association of non-tagged liposomes. Still, the difference between PBS and DMEM+S is unlikely to cause the large difference between association of the Tf-HRP conjugates and the Tf-tagged liposomes. Furthermore, the binding of Tf-tagged liposomes is higher than the association, which indicates that the difference in association between Tf-HRP and Tf-tagged liposomes is most likely not caused by differences in TfR binding and endocytosis, but to a difference in intracellular trafficking. Furthermore, we assume that the rate of internalization is slower for the larger Tf-tagged liposomes than for Tf-HRP conjugates.

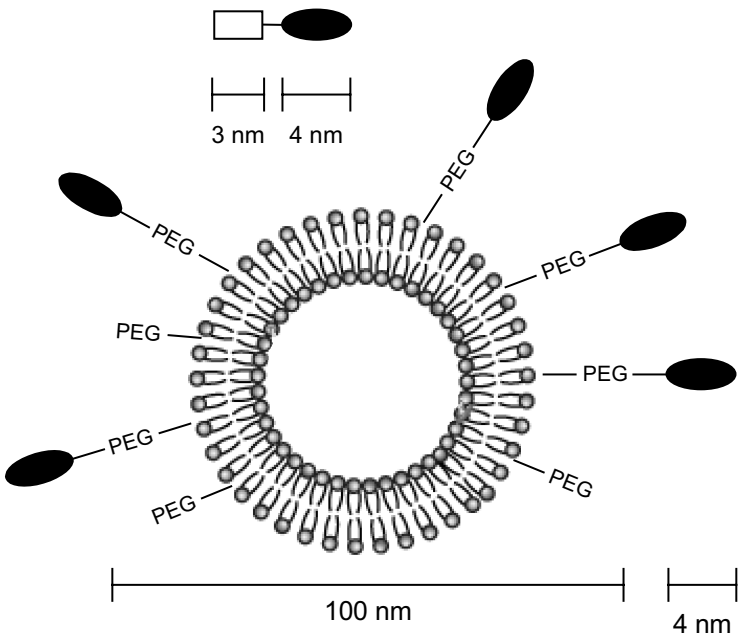

Figure 2: Schematic comparison between HRP encapsulated in a Tf-tagged liposome and a Tf-HRP conjugate. Tf (black oval) is approximately 4 nm (Enns and Sussman, 1981). HRP (square) has a similar size (approximately 3 nm (Ojteg *et al.*, 1987)), while a liposome is 100 nm in diameter. This picture is not to scale, the liposome should be 4 times larger for real comparison with Tf. Liposomes prepared for this research contained 63 – 74 Tf molecules per liposome.

The difference in association of Tf-tagged liposomes and Tf-HRP conjugates may be explained by the difference in size. HRP has a Stokes-Einstein radius of approximately 3 nm (26), and is thus much smaller than a 100 nm-liposome. In addition, the Stokes-Einstein radius of Tf is approximately 4 nm (27). Figure 2 gives a schematic representation for Tf-HRP conjugates and Tf-tagged liposomes. We hypothesise that the intracellular trafficking of Tf-HRP conjugates is similar to endogenous Tf, while the intracellular trafficking of the larger Tf-tagged liposomes is mainly determined by the liposomes. Tf and Tf-HRP are both endocytosed via clathrin-coated vesicles that are associated with receptor-mediated endocytosis (6, 13). For the endocytosis of HRP loaded Tf-tagged liposomes multiple processes, such as non-specific vesicular endocytosis (pinocytosis) may play a role. However, the high binding of Tf-tagged liposomes compared to non-tagged liposomes suggests that TfR mediated processes play a role.

Conclusions and Perspectives

In this paper we described the preparation, as well as the binding and association of Tf-tagged liposomes. Furthermore, we compared these results with the association of Tf-HRP conjugates. This enabled us to determine the differences in internalisation and intracellular trafficking of the protein drug. By using liposomes, we do not need to chemically modify the drug molecule as for the conjugates and after injection the drug is protected against degradation in serum. In addition, by using liposomes it is possible to circumvent the efflux-transporter P-glycoprotein (7) which is an advantage for drug targeting to the brain.

We found that the binding at 4 °C of Tf-tagged liposomes is higher than the association at 37 °C. We assume that this difference is caused by intracellular degradation of HRP. Therefore, it is important to realise that we have determined the HRP activity, rather than the amount of HRP. By doing so, we obtain a more realistic value for association of an active protein or enzymatic drug. Alternatively, Mumtaz and Bachhawat (28) have shown that stabilisation of HRP with dextran improves the intracellular stability of HRP. They have incorporated dextran-HRP into liposomes to target the lysosomes for the treatment of metabolic disorders, such as lysosomal storage

disease. Other information about differences in intracellular trafficking routes were published by Beyer *et al* (29). They have found that liposomal doxorubicin showed less endocytosis than free doxorubicin, but that the intracellular distribution was similar to free doxorubicin. In contrast, Tf- and BSA-doxorubicin conjugates were differently distributed. These findings are similar to our results and could imply that depending on the intracellular target, a Tf-drug conjugate or a Tf-tagged liposome should be used.

The association of Tf-tagged liposomes is significantly higher than non-tagged liposomes. Schreier *et al* (30) have shown that the lipid composition of the liposome can influence the intracellular trafficking. Furthermore, the size of liposomes can determine the route of uptake (31, 32). Although we were not able to determine the exact mechanism of endocytosis and intracellular fate of Tf- and non-tagged liposomes, it is still possible to increase the intracellular delivery of drugs by using Tf-tagged liposomes. Over the years, new techniques have been developed which allow release of the liposomal content in the cytosol, rather than in the lysosomes. For example, by using pH-sensitive liposomes the intra-endosomal and intra-lysosomal degradation of the encapsulated drug can be avoided. Soon after internalisation these liposomes fall apart when the endosomal pH decreases, thereby releasing their content. Subsequent endosomal escape might be induced by adding pH dependent fusogenic peptides, such as GALA (33, 34).

In conclusion, targeting liposomes encapsulating protein or peptide drugs to the TfR on BCEC is possibly limited due to intracellular degradation of the protein drug. However, as not all drugs are susceptible to degradation, it may be an advantage to target liposomal contents to the lysosomes for those drugs that can withstand the harsh conditions in the lysosomes (e.g. to treat lysosomal storage disease). Furthermore, our experiments suggest that liposomes release some of their content within the BBB, making targeting of liposomes to the TfR on BCEC an attractive approach for brain drug delivery.

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