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European Journal of Pharmaceutics and Biopharmaceutics 59 (2005) 359–366
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Targeting of sterically stabilised pH-sensitive liposomes to human T-leukaemia cells

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Received 25 May 2004; accepted in revised form 24 August 2004
Available online 23 November 2004

Abstract

The main aim of this work was to develop novel targeted sterically stabilised pH-sensitive liposomes tailored to promote efficient intracellular delivery of therapeutic molecules into human T-leukaemia cells. Our results indicate that the targeting moiety (thiolated transferrin) was successfully coupled to the distal reactive maleimide terminus of poly(ethylene glycol)–phospholipid conjugates incorporated in the liposomal bilayer. Results from atomic force microscopy studies, performed to characterise vesicle surface topology, indicated that, to a certain extent, thiolated transferrin has the ability to associate in a non-specific manner with the lipid membrane of pegylated liposomes. This is an issue not commonly reported in the literature but which is crucial to demonstrate the targeting proof of principle. Nevertheless, fluorimetric studies together with confocal microscopy clearly demonstrate that liposomes bearing covalently coupled transferrin associate more extensively to human T-leukaemia cells in vitro than non-targeted liposomes. Cell mechanistic studies indicate that targeted liposomes bind specifically to transferrin receptors and are internalised via receptor-dependent endocytotic pathway. In addition, the biophysical features exhibited by the developed liposomes, namely their ability to promote pH-triggered cytoplasmic delivery of loaded material, make them promising delivery systems for in vivo targeting of therapeutic molecules to tumours.

Keywords: Nanotechnology; pH-sensitive liposomes; Targeting; Transferrin; Human T-leukaemia cells; Cancer therapy

1. Introduction

The success of novel strategies for cancer therapy strongly relies on the development of formulations capable of improving the therapeutic index of biologically active molecules, namely by increasing their concentration at desired target sites while avoiding normal tissues. Among other nanovesicular vectors currently under investigation, liposomes (phospholipid bilayer vesicles) have attracted considerable interest as site-specific delivery systems, because of their biocompatibility and chemical and structural versatility which allow manipulation of their fate not only in vivo but also at an intracellular level [1,2]. Targeting to cancer cell surface antigens constitutes a promising, although challenging approach that can be applied to liposomes in order to promote specific drug delivery into tumours [3]. In this context, covalent coupling of antibodies or other ligands to the distal reactive end of poly(ethylene glycol)–phospholipid conjugates incorporated in the liposomal bilayer has resulted in formulations exhibiting both prolonged circulation half-lives in blood and cell targeting specificity [4,5]. However, the clinical success of such approaches depends on the selection of appropriate ligands, lacking immunogenic potential and with the ability to mediate cargo internalisation by the target cells [3]. Moreover, upon nanovesicle–cell interaction, it is still desirable to ensure an efficient intracellular delivery of

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doi:10.1016/j.ejpb.2004.08.012

the therapeutic cargo. In this regard, pH-sensitive liposomes, that are stable at physiological pH but undergo destabilisation and acquire fusogenic properties under acidic conditions, thus leading to the release of their aqueous contents, constitute a promising strategy [2,6]. The concept of pH-sensitive liposomes emerged from the fact that certain enveloped viruses developed strategies to take advantage of the acidification of the endosomal lumen to infect cells, and from the observation that some pathological tissues (tumours, sites of inflammation and infected areas) exhibit an acidic environment as compared to normal tissues [6–8]. Different classes of pH-sensitive liposomes have been described in the literature according to the mechanism triggering pH-sensitivity [2,7,9]. In our laboratory, very promising results have been obtained with sterically stabilised liposomes composed of phosphatidylethanolamine (PE) (or its derivatives) with compounds containing an acidic group (e.g. carboxylic group) that act as a stabiliser at neutral pH [6,10]. This is a simple, non-expensive and easily scaled-up formulation that avoids the use of pH-sensitive peptides and polymers, which may be responsible for immune responses and toxicity [6,9].

In this work, transferrin, a glycoprotein which transports ferric ion into cells, was tested as a ligand to target sterically stabilised pH-sensitive liposomes to human T-leukaemia cells. Two main reasons dictated the choice of this ligand: (i) the over-expression of its receptor on these cells, and (ii) the ability of transferrin to promote receptor-mediated endocytosis, which would enhance intracellular delivery of the carried material [11]. In addition, transferrin is non-immunogenic and can be conjugated without losing its biological activity, which make this protein an interesting and promising tumour-targeting ligand [12]. Surface topology analysis of the developed targeted liposomes indicated that currently used coupling procedures may lead to non-specific adsorption of ligands to liposomes surface. This is an issue not commonly reported in the literature but which is crucial to demonstrate the targeting proof of principle. However, cell association studies, performed in the presence or absence of drugs that interfere with the endocytotic pathway, demonstrated that targeted liposomes bind specifically to cell surface Trf receptors and are internalised via a receptor-dependent endocytic pathway.

2. Materials and methods

2.1. Materials

Dioleoylphosphatidylethanolamine (DOPE), methoxy-poly(ethylene glycol)-distearoylphosphatidylethanolamine (M_w 2000) ($mPEG_{2000}$-DSPE), maleimide-derivatised poly(ethylene glycol)-distearoylphosphatidylethanolamine ((Mal)PEG_{2000}-DSPE), and rodamine labelled-phosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesteroyl hemisuccinate (CHEMS), human holo-Transferrin (Trf), 2-iminothiolane, bicinchoninic acid (BCA) protein kit, Sepharose CL-4B, Sephadex G-25, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) were purchased from Sigma Chemical Co. (St Louis, MO).

2.2. Preparation of liposomes

Liposomes composed of DOPE:CHEMS:PEG_{2000}-DSPE:(Mal)PEG_{2000}-DSPE (6:4:0.06:0.24, molar ratio) or DOPE:CHEMS:PEG_{2000}-DSPE (6:4:0.3, molar ratio) at a DOPE concentration of 10 mM, were prepared by the reverse phase method previously described by Szoka and Papahadjopoulos [13]. When necessary, 5 mol% of Rh-PE relative to DOPE concentration was also included in the lipid bilayer. Briefly, a mixture of the appropriate amounts of lipids from stock solutions in chloroform was dried in a rotary evaporator under reduced pressure in order to obtain a thin lipid film. The film was dissolved in 800 μl of pre-washed diethyl ether, and 300 μl of HEPES buffer (75 mM HEPES, 2 mM EDTA, 290 mOsm NaCl, pH 7.2) were then added. This mixture was sonicated briefly to form a stable emulsion. The ether was removed in a rotary evaporator under controlled vacuum at 37 °C, to obtain a semi-solid gel. An additional aliquot of 700 μl of HEPES buffer was added to the gel, which was then broken by vigorous mechanical shaking with a vortex mixer. Evaporation was continued to remove any residual ether. Vesicles were extruded (21×) through two polycarbonate membranes of 80 nm pore diameter using a LipoFast mini extruder (Lipofast, Avestin, Toronto, Canada) to obtain a uniform size distribution. Final lipid concentrations were determined based on lipid phosphorous assay by Fiske and Subarrow [14].

2.3. Preparation of thiolated transferrin and coupling procedure

Transferrin and 2-iminothiolane were both dissolved in HEPES buffer (25 mM HEPES, 140 mM NaCl, pH 8) and mixed at a 1:10 molar ratio. This mixture was left to react for 1 h, at room temperature, under dark conditions and under an inert N_2 atmosphere. Upon purification by gel chromatography (Sephadex G-25 spin column), 50 μl of thiolated-transferrin was immediately added to 400 μl of freshly prepared liposomes (final phospholipid concentration of 10 mM) at different Trf-SH:phospholipid molar ratios as indicated in the legend to the Fig. 1. The resulting suspension was left to react overnight at room temperature under magnetic stirring, dark conditions and under an inert N_2 atmosphere. Free maleimide groups were quenched with a 5-fold excess of 2-mercaptoethanol (50 μl of a 4 mM solution) relative to (Mal)PEG-DSPE concentration in the final reaction mixture, for 30 min at room temperature. Free transferrin was separated from liposomes by size exclusion chromatography using a Sepharose CL-4B column equilibrated in 25 mM HEPES 140 mM NaCl, pH 7.4.

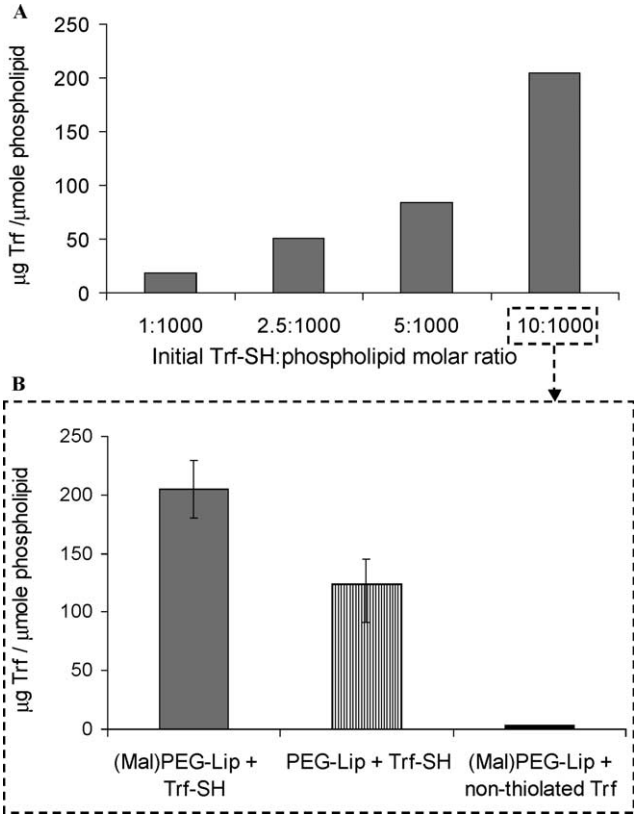

Fig. 1. Effect of the initial Trf-SH:phospholipid molar ratio on the amount of Trf coupled to liposomes. Liposomes were prepared according to the reverse phase evaporation procedure previously described by Szoka and Papahadjopoulos [13]. Liposomes composed of a mixture of DOPE:CHEMS:PEG_{2000}-DSPE:(Mal)PEG_{2000}-DSPE (6:4:0.24:0.06) or DOPE:CHEMS:PEG_{2000}-DSPE (6:4:0.3) at a DOPE concentration of 10 mM, were incubated with (A) various amounts of thiolated Trf at Trf-SH:phospholipid molar ratios ranging from 1:1000 to 10:1000 or (B) with thiolated or non-thiolated Trf at a Trf:phospholipid molar ratio of 10:1000. In both cases (A and B), free transferrin was separated from liposomes by size exclusion chromatography using a Sepharose CL-4B column equilibrated in HEPES buffered saline solution (25 mM Hepes, 140 mM NaCl, pH 7.4). The amount of protein coupled to liposomes was determined by the bicinchoninic acid method (BCA).

The amount of protein bound to liposomes was determined by the bicinchoninic acid method (BCA).

2.4. Surface topography analysis

Surface topography analysis was performed by atomic force microscopy (AFM). Liposomes were deposited on freshly cleaved mica and incubated for 30 min in HEPES buffer at pH 7.4 (0.5 mM final concentration of phospholipid). The surface was then rinsed gently with HEPES buffer at room temperature. Samples were kept in HEPES buffer and introduced as such into the AFM fluid cell. All imaging was performed using a Digital Instruments Nanoscope IIIa AFM system (Santa Barbara, CA) and a tapping mode fluid cell. Commercially available cantilever with Si_3N_4 tips were used (Olympus Ltd, Tokyo, Japan). Heights of the images were determined by cross-sectional analysis.

2.5. Mean diameter and surface charge

Mean diameter and polydispersity index of the previously described formulations, were evaluated by photon correlation spectroscopy (Coulter N4 Plus, Coulter Electronics, Hialeah, FL), upon the appropriate dilution of the different liposome formulations in previously filtered HEPES buffer at pH 7.4. Surface charge was determined by Doppler Electrophoretic Light Scattering Analysis (DELSA) using a Coulter DELSA 440 (Coulter Electronics, Hialeah, FL). Samples were diluted in HEPES buffer and placed in the measurement cell, whose position was adjusted to cover a previously determined stationary layer and an electric field of 3.0 mA was applied. Data were recorded and the ζ potential calculated for each scattering angle (8.6, 17.1, 25.6 and 34.2°).

2.6. Cells

Two different human acute limphoblastic leukaemia cell lines were used in this study: CEM and MOLT-3 (DSMZ, Braunschweig, Germany). Cells were cultured in suspension in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biochron, Berlin, Germany) and antibiotics (100 mg/ml streptomycin and 100 unit/ml of penicillin) (Sigma, St Louis, MO) at 37 °C in balanced air humidified incubator (90% humidity) under 5% CO_2. Cells were maintained in an exponential growth phase by periodic dilutions in fresh medium.

2.7. Cell association studies

One million CEM or MOLT-3 cells were incubated for 30 min at 37 °C in RPMI medium in the absence (control) or presence of an excess of free Trf (8 mg/well) or with cytochalasin B (25 µg/ml). Cells were further incubated for 2 h at 37 °C with different liposome formulations (final phospholipid concentration of 300 µM). Cells were then analysed for their fluorescence content following the addition of $C_{12}E_8$ at excitation and emission wavelengths of 570 and 590 nm, respectively. The sample chamber was adjusted to the front face configuration (to prevent light scattering contributions to the fluorescence) and equipped with a magnetic stirrer. The temperature was maintained at 20 °C with a termostated circulating water bath. Fluorescence measurements were performed in a SPEX Fluorolog 2 fluorimeter (SPEX Industries, Edison, NJ).

2.8. Confocal microscopy analysis

Cells (1×10^6/well) were seeded in 48 multi-wells plates and incubated with Trf-SH-(Mal)PEG-Liposomes (targeted-liposomes) or with (Mal)PEG-Liposomes (liposomes without Trf) for 2 h at 37 °C (final phospholipid concentration of 300 µM). In some experiments cells were pretreated for 30 min at 37 °C with inhibitors of endocytosis

(antimycin A (1 μg/ml), sodium azide (0.1%) and sodium fluoride (10 mM)). After incubation, cells were washed twice with cold PBS, fixed with 4% solution of paraformaldehyde in PBS for 15 min and washed twice again with cold PBS. Cells were then observed under a confocal microscope (Bio-Rad, MRC 600, Hercules, CA).

3. Results and discussion

Different methodologies have been described to covalently attach ligands to the reactive distal end of PEG molecules [15,16]. In this work covalent coupling of thiolated transferrin to the maleimide terminus of distearoylphosphatidylethanolamine–poly(ethylene) glycol (DSPE-PEG) molecules ((Mal)PEG-DSPE) was explored [12]. This methodology was chosen because the reaction between thiol and maleimide groups was reported as one of the most efficient reactions in bioconjugate chemistry resulting in a stable thio-ether linkage; it occurs at room temperature and at pH close to neutrality [17,18]. The latter aspect is a crucial issue in this study since the liposomes exhibit pH-sensitivity.

Prior to coupling with maleimide-PEG-grafted liposomes, transferrin was thiolated through incubation with Traut's reagent (2-iminothiolane) at a 10:1 2-iminothiolane:protein molar ratio for 1 h at room temperature. Different Trf-SH to phospholipid molar ratios were then tested in order to optimise the coupling procedure (Fig. 1).

Fig. 1A shows the effect of Trf-SH:phospholipid molar ratio on the transferrin density (μg Trf:μmol phospholipid) at liposomal surface. As can be observed in Fig. 1A, higher Trf-SH:phospholipid molar ratios resulted in higher liposomal surface densities of Trf. When a 10:1000 Trf-SH:phospholipid molar ratio was used, a density of approximately 200 μg of Trf/μmol phospholipid was achieved. Considering that one liposome particle with a mean diameter of 150 nm bears approximately 200,000 lipid molecules, each liposome particle will contain 313 molecules of Trf [19]. A further increase in the Trf-SH:phospholipid molar ratio (20:1000) did not improve the amount of transferrin coupled to the liposomes (data not shown). Therefore, a 10:1000 Trf-SH:phospholipid molar ratio was used in all subsequent studies. To clarify whether the thiolated transferrin was selectively coupled to the maleimide group at the reactive PEG terminus, additional control experiments were performed. As can be observed in Fig. 1B, incubation of non-thiolated transferrin (Trf) with maleimide-PEG-grafted liposomes did not result in a significant association of the protein to liposomes. However, when Trf-SH was incubated with liposomes lacking a maleimide reactive group a significant amount of protein was found to be associated to the liposomes. These results suggest that Trf-SH has the ability to associate in a non-specific manner with PEG-grafted pH-sensitive liposomes.

To observe the liposome surface topology and to investigate whether thiolated transferrin was coupled to the distal maleimide end of PEG chains or non-specifically bound to the liposomal lipid bilayer, AFM studies were performed. AFM is a high-resolution technique that can image biological or model membranes on solid supports under aqueous environments [20,21]. It relies on successfully immobilising biological entities to a surface on which they can be distinguished (the surface must have a roughness that is small compared to the size of the entity) [22]. For this reason, mica was chosen as a solid support for our samples. Since we were dealing with soft samples, imaging was performed in the tapping mode, which is an intermittent-contact mode of operation used to reduce lateral and friction forces. Although this imaging mode has a slightly lower resolution, it has the advantage of reducing imaging artefacts or possible modifications of the surface by the tip [23].

Representative AFM images obtained for the different liposome formulations are shown in Fig. 2.

When (Mal)PEG-Liposomes were deposited on the mica support, the AFM imaging showed that a large area of the mica surface, was covered by a flat and smooth lipid layer, easily distinguished from the rougher mica surface (Fig. 2A). The height of this lipid layer was 5.6 nm as determined by cross-sectional analysis, which is in agreement with previously reported values for lipid bilayers formed upon adsorption of liposomes to solid supports [21,24].

In the case of Trf-SH-(Mal)PEG-Liposomes or samples obtained from the mixture of Trf-SH with PEG-Liposomes (Fig. 2B and C), besides this smooth lipid bilayer additional irregular structures were observed. Such structures could result from transferrin associated to liposomes since they were not observed in control samples (liposomes lacking Trf, Fig. 2A). Furthermore, these structures cannot be attributed to intact liposomes, lipid fragments or aggregates because these are known to be efficiently removed from the samples after the washing procedure [25]. A different distribution pattern of these structures was observed depending on the formulation. In the case of Trf-SH-(Mal)PEG-Liposomes they are mainly located on the top of the lipid bilayer while in the case of the formulation where Trf-SH is mixed with PEG-Liposomes such structures are located in the form of aggregates at the mica surface (height of approximately 2.3 nm, for both formulations). Previous studies on the topology of transferrin molecules on mica surfaces revealed that transferrin exhibits affinity to mica supports and forms structures of approximately 2 nm height [26]. These results corroborate our hypothesis that these structures result from transferrin associated to liposomes. The different distribution pattern observed for the two formulations may be explained by the different location of the transferrin associated to the liposomes. In the case of Trf-SH-(Mal)PEG-Liposomes, Trf is mainly covalently linked to the reactive maleimide group and, therefore, structures were observed predominantly on

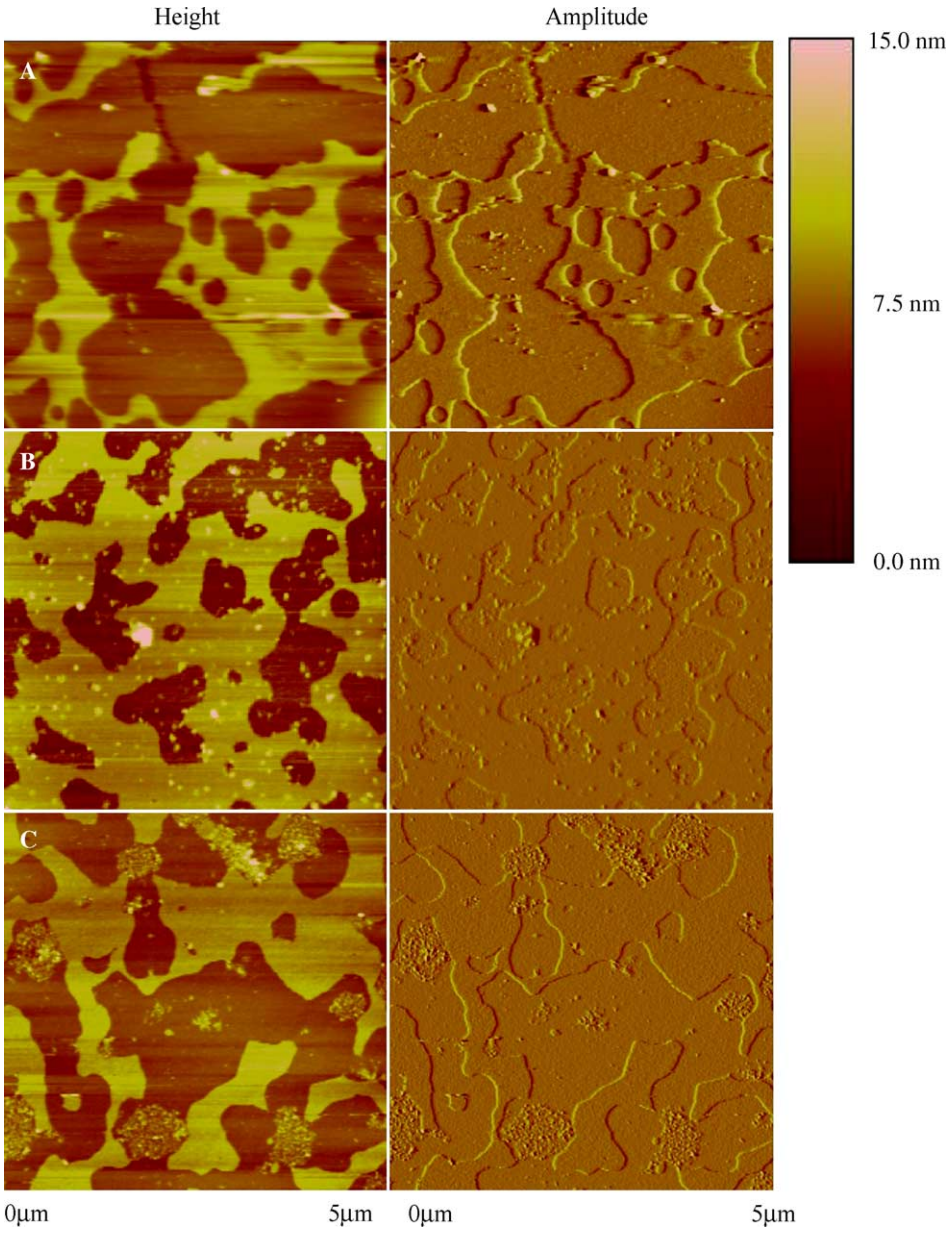

Fig. 2. Surface topography analysis of liposomes by atomic force microscopy. Different formulations of liposomes, (A) (Mal)PEG-Liposomes without transferrin, (B) Trf-SH-(Mal)PEG-Liposomes or (C) Trf-SH mixed with PEG-Liposomes, were deposited on freshly cleaved mica and incubated for 30 min in Hepes buffer (0.5 mM phospholipid concentration). After the washing procedure, samples were kept in HEPES buffer and introduced as such into the AFM fluid cell. All imaging was performed using a Digital Instruments Nanoscope IIIa AFM system (Santa Barbara, CA) and a tapping mode fluid cell.

the top of the lipid bilayer (Fig. 2B), while in the case of the mixture of Trf-SH with PEG-Liposomes, transferrin was only adsorbed on the liposome surface (Fig. 2C). Due to the high affinity of transferrin to the mica surface, it is possible that for the latter case, upon adsorption and attachment of liposomes, transferrin had moved from liposome surface to the mica support.

It is well established that clearance of liposomes from the circulation and their biodistribution depend on the physicochemical properties of the liposomes such as size and surface charge, among others. Small-sized liposomes (100–200 nm) show prolonged circulation times in blood because they are not so easily recognised, as occurs with large vesicles, by the cells in the RES which are responsible for their removal from circulation [27,28]. In addition, smaller liposomes show higher capacity to extravasate into diseased tissues, such as solid tumours, as compared to larger vesicles [29]. Regarding the effect of liposomal net charge, recent studies indicate that positively or negatively charged liposomal surfaces show increased levels of binding to serum proteins and, consequently, are rapidly removed from circulation [27].

Table 1 illustrates the results obtained for particle size distribution and surface charge of the different liposome formulations used in this work.

Table 1
Physicochemical characterisation of different liposome formulations

Formulation	Mean diameter (nm)	Polydispersity index	ζ potential (mV)
PEG-Lip	140	0.13	n.d.
Trf-SH+PEG-Lip	146±1	0.09	n.d
(Mal)PEG-Lip	138±1	0.16	−3.46
Trf-SH-(Mal)PEG-Lip	150±3	0.13	−4.26
Non-thiolated Trf+(Mal)PEG-Lip	136±3	0.16	n.d.

Mean diameter and polydispersity index were measured by photon correlation spectroscopy using a Coulter N4 Plus instrument (Coulter Electronics, Hialeah, FL) and ζ potential was determined by Doppler Electrophoretic Light Scattering Analysis using a Coulter DELSA 440 (Coulter Electronics, Hialeah, FL). n.d., non-determined.

As can be observed, all formulations presented a mean diameter ≤150 nm and exhibited a homogeneous size distribution (polydispersity index <0.16). The presence of transferrin did not significantly affect the final particle size. With respect to surface charge both (Mal)PEG-Liposomes and Trf-SH-(Mal)PEG-Liposomes formulations exhibited a surface charge close to neutrality with values of −3.46 and −4.26 mV, respectively. Overall, these results show that the methodology used for the liposome preparation and the lipid composition selected in this work allowed the formation of liposomes exhibiting biophysical properties suitable for intravenous administration.

In vitro studies previously performed suggested that the transferrin receptor, could be investigated as a possible mediator of liposome uptake by human T-leukaemia cells [30]. Therefore, in order to evaluate whether the covalent attachment of transferrin to (Mal)PEG-Liposomes would selectively target liposomes to cells in culture, cell association studies were carried out using two different human T-leukaemia cell lines (CEM and MOLT-3 cells).

As can be observed in Fig. 3, fluorimetric studies indicate that an increase both in the incubation time and phospholipid concentration resulted in a higher degree of liposome–cell association.

Trf-SH-(Mal)PEG-Liposomes (targeted formulation) showed a 2-fold increase in in vitro cell association as compared to either Trf-SH mixed with PEG-Liposomes or to (Mal)PEG-Liposomes (non-targeted formulations) (Fig. 4).

Competitive inhibition studies indicated that pre-treatment of CEM cells with an excess of free transferrin (at non-toxic concentrations) resulted in a decrease in the extent of cell association of the targeted liposomes but had no effect on the association of the non-targeted formulations. On the other hand, pre-treatment of cells with cytochalasin B, a drug that is known to block phagocytosis but not receptor-mediated endocytosis [4,31] had no significant effect on the extent of cell association of Trf-SH-(MAL)PEG-Liposomes (Fig. 4). Similar results were obtained with the MOLT-3 cell line (data not shown).

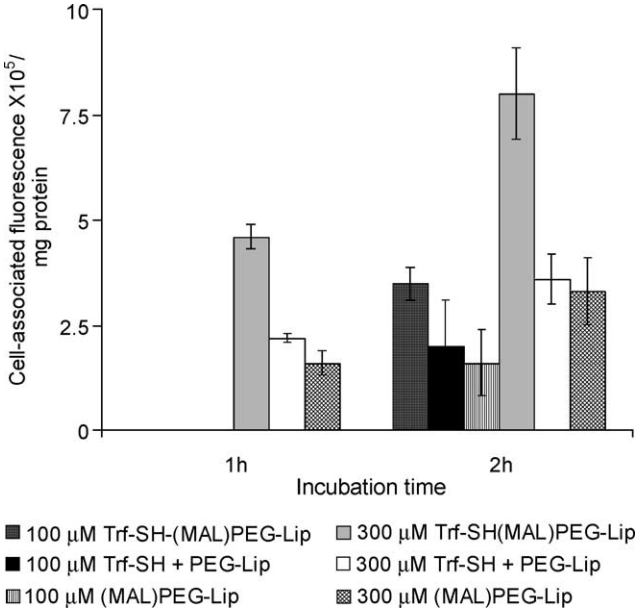

Fig. 3. Effect of incubation time and lipid concentration on the extent of cell association of different liposome formulations. Rhodamine-PE labelled (5 mol% Rh-PE) liposomes (final phospholipid concentration of 100 or 300 μM) were incubated with CEM cells (1×10^6 cells/well) in RPMI culture medium for 1 or 2 h at 37 °C. Then, cells were washed twice, re-suspended in 2 ml of cold PBS and finally analysed for their fluorescence intensity following the addition of 20 μl of $C_{12}E_8$ (20 mM) in a SPEX Fluorolog 2 fluorometer (SPEX Industries, Edison, NJ).

The extent of cell association of the different liposome formulations with CEM cells was further evaluated by confocal microscopy (Fig. 5).

As can be observed in Fig. 5, the results were consistent with those obtained from the fluorimetric studies. Following incubation of CEM cells with Trf-SH-(Mal)PEG-Liposomes

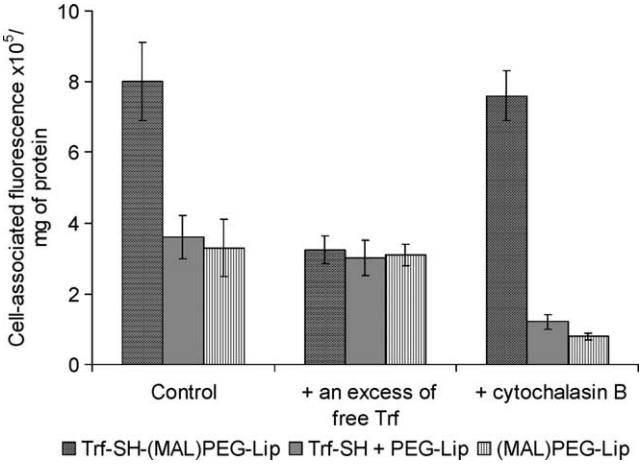

Fig. 4. Cell association studies. CEM cells (1×10^6/well) were incubated for 30 min at 37 °C in the absence (control) or the presence of an excess of free Trf (8 mg/well) or with cytochalasin B (25 μg/ml). Cells were further incubated for 2 h at 37 °C with different liposome formulations (final phospholipid concentration of 300 μM). Then, cells were washed twice, re-suspended in 2 ml of cold PBS and finally analysed for their fluorescence intensity following the addition of 20 μl of $C_{12}E_8$ (20 mM) in a SPEX Fluorolog 2 fluorometer (SPEX Industries, Edison, NJ).

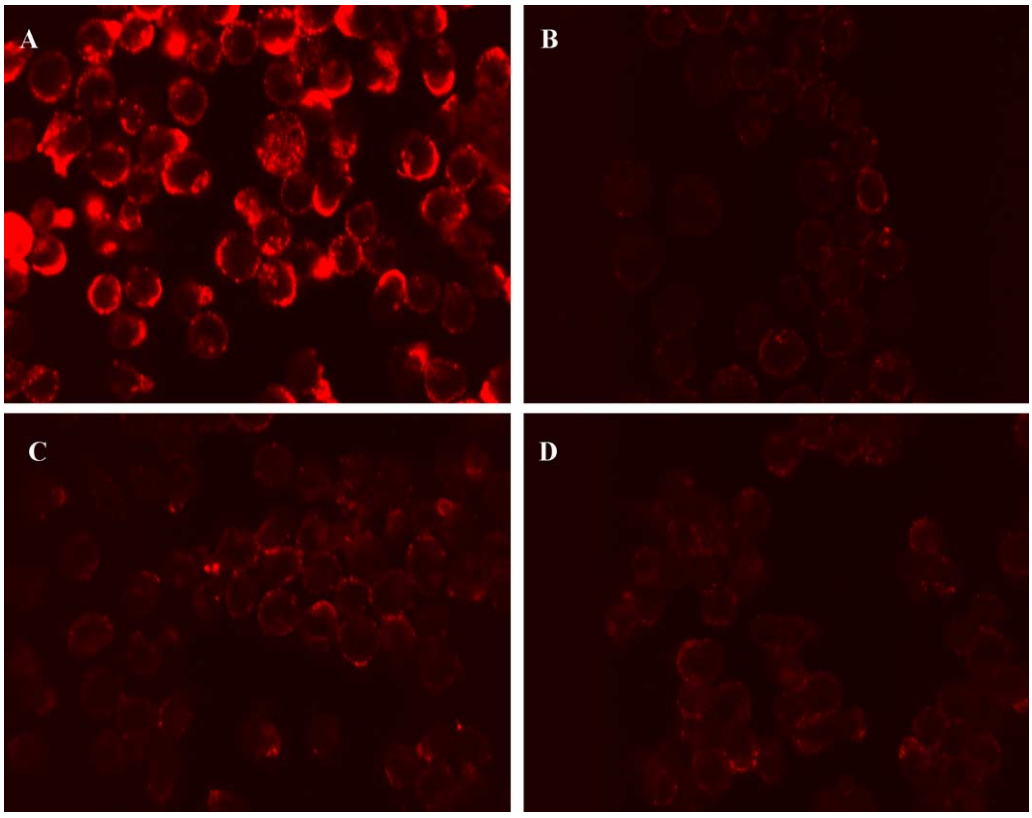

Fig. 5. Confocal microscopy images of CEM cells incubated with different formulations of Rh-PE labelled liposomes (final phospholipid concentration of 300 μM). Cells (1×10^6) were incubated with Trf-SH-(Mal)PEG-Liposomes (targeted-liposomes) (A) in the absence or (B) in the presence of inhibitors of endocytosis composed of a mixture of antymicin A (1 mg/ml), NaF (10 mM) and NaN_3 (0.1%) for 1 h at 37 °C. As additional controls, cells were incubated with (C) (Mal)PEG-Liposomes (liposomes without Trf) or with (D) Trf-SH+PEG-Liposomes (liposomes without (Mal)PEG-DSPE but mixed with Trf-SH). Following incubation, cells were washed twice with cold PBS, fixed with 4% paraformaldehyde and then washed twice again with cold PBS. Cells were observed using a Confocal Microcope (Bio-Rad MRC 600, Hercules, CA).

an intense fluorescence was observed both in the cytoplasm and the cell membrane (Fig. 5A), while for non-targeted formulations the fluorescence intensity was much lower and mainly located at the cell surface (Fig. 5C and D). Furthermore, pre-treatment of the cells with inhibitors of endocytosis followed by incubation with Trf-SH-(Mal)PEG-Liposomes resulted in a drastic decrease of fluorescence intensity, and only traces of puntacted fluorescence were observed at the cell periphery (Fig. 5B). It should be noted that cell viability was not affected by the presence of inhibitors of endocytosis, as assessed by the tetrazolium dye assay of Mosmann (MTT assay) (Fig. 6) [32]. Therefore, the inhibitory effect on cell association observed under this condition cannot be attributed to any cytotoxic effect of the drugs.

Fluorimetric studies and confocal microscopy analysis demonstrated that association of targeted liposomes to leukaemia cells occurs at a significantly larger extent than non-targeted formulations. Notably, (Mal)PEG-Liposomes (liposomes without transferrin) and the mixture of Trf-SH with PEG-Liposomes, exhibited a similar low extent of cell association suggesting that in the later formulation, transferrin is not available to bind to its receptor. These data support our previous hypothesis that in the case of

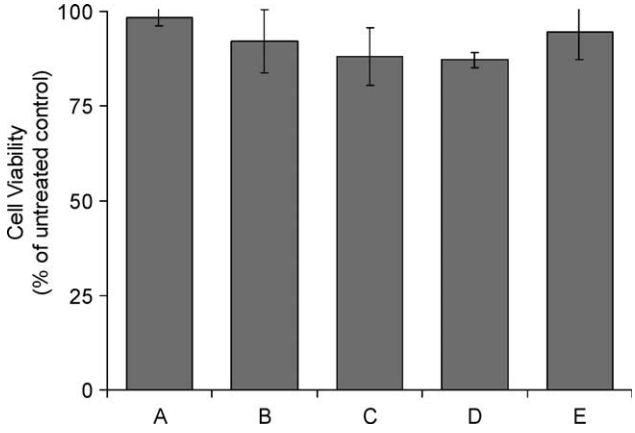

Fig. 6. Viability of CEM cells under different experimental conditions. Cells (1×10^6) were incubated with different formulations of Rh-PE labelled liposomes ((A) Trf-SH-(Mal)PEG-Lip; (B) Trf-SH+PEG-Lip; (C) (Mal)PEG-Lip, final phospholipid concentration of 300 μM), with (D) free Trf (8 mg/well) or with (E) a mixture of inhibitors of endocytosis composed of antymicin A (1 mg/ml), NaF (10 mM) and NaN_3 (0.1%), for 2 h at 37 °C. Cell viability was determined by the MTT assay and expressed as a percentage of untreated control cells.

liposomes lacking the maleimide group, thiolated transferrin may be non-specifically adsorbed to the liposome surface. Most likely, protein molecules become intercalated between PEG chains, thus preventing the interaction between transferrin and its receptor. In contrast, for Trf-SH-(Mal)PEG-Liposomes, transferrin is indeed covalently coupled to the terminal end of PEG-grafted liposomes thus becoming easily accessible for receptor binding.

In conclusion, this work demonstrated that the use of a targeting ligand that binds to receptors which undergo internalisation combined with the ability of the developed formulation to promote pH-triggered cytoplasmic delivery of loaded material leads to a promising strategy for in vivo targeting of therapeutic molecules to leukaemia cells.

Acknowledgements

This work was supported by a grant from Science and Technology Foundation (FCT) Portugal (SFRH/BD/977/2000), SAF01-0363 from CICYT and by the grant FCT/BIO/36202/00.

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