CHAPTER 9

RELEASE ADVANTAGES OF A LIPOSOMAL DENDRIMER-DOXORUBICIN COMPLEX, OVER CONVENTIONAL LIPOSOMAL FORMULATION OF DOXORUBICIN*

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Abstract: Data on the release advantages of a liposomal formulation incorporating a doxorubicin–PAMAM G4 complex in comparison to a liposomal doxorubicin are presented. The liposomes incorporating either doxorubicin-PAMAM complex, or doxorubicin as free drug, were composed of Egg-phosphatidylcholine (EPC): Stearylamine (SA) at a 10:0.1 molar ratio and their size distribution and ζ-potential were characterized. Liposomes incorporating the doxorubicin-PAMAM complex exhibited release properties which were advantageous compared to the conventional type of liposomal doxorubicin in terms of doxorubicin toxicity and its availability to the tumor site. This liposomal formulation may show improved therapeutic properties in vivo

Keywords: Liposome; dendrimer; PAMAM G4; doxorubicin; drug release

1. INTRODUCTION

Liposomes are non-toxic and biocompatible drug delivery systems that have been proven to be very useful in the fight against cancer. Liposomes can increase the therapeutic effectiveness of the encapsulated drugs and decrease their toxicity (Straubinger *et al*. 2004). One of the best-known liposomal drug delivery systems

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is the liposomal doxorubicin. The high cardiotoxicity of free doxorubicin limits its clinical use, despite its high anticancer activity against a variety of tumours. Liposomal doxorubicin is active against many types of cancer and reduces the toxicity of doxorubicin and it is now in clinical use in USA and Europe (Gabizon 2002). Several clinical trials are currently in progress in order to evaluate the use of doxorubicin liposomes either alone or in combination with other anticancer drugs (Toma *et al*. 2002; Syrigos *et al*. 2002).

Despite several advantages, the therapeutic use of liposomes has limitations, which are related to the release of the encapsulated drug that can be only partially delayed by the modification of the membrane composition. Many attempts are made towards a more effective control of the release of the encapsulated drug, using polymers. One novel approach is the entrapment of liposomes in polymeric microspheres and the progressive release of the intact liposomes from the biodegradable matrix (Stenekes *et al*. 2002). Other approaches are based on the encapsulation of liposomes in microcapsules in order to modulate the release of the encapsulated drug (Dhoot and Wheatley 2003) or to produce liposome-like microspheres (Pan *et al*. 2004).

Dendrimers are highly branched macromolecules that, contrary to traditional "linear" polymers, possess fractal architecture, nanoscaled size and unique physicochemical properties. They are small in size, and exhibit a low polydispersity that can contribute to a reproducible pharmacokinetic behavior. However, the main characteristics of dendrimers are their multiple reactive groups, a well-defined structure, and their ability to encapsulate drugs in their void spaces (Cloninger 2002; Aulenta *et al*. 2003). An ideal dendrimer as drug delivery system must be non-toxic, non-immunogenic and biodegradable (Aulenta *et al*. 2003). The first dendrimer family which has been synthesized, characterized and commercialized is the Poly (amidoamine) (PAMAM) dendrimer. These dendrimers are considered safe regarding toxicity and are non-immunogenic and they have been used in the delivery of drugs, antisense nucleotides and gene therapy, both *in vitro* and *in vivo* (Eichman *et al*. 2001). Dendrimers and dendrons have already been proposed for drug complexation and transport; especially lipidic dendrons that can produce higher order lamellar structures called "dendrisomes" (Khuloud *et al*. 2003) or can aggregate to form nanosystems (Singh and Florence 2005).

In this paper a liposomal formulation composed of egg phosphatidylcholine and stearylamine (EPC:SA 10:0.1 molar ratio) and a doxorubicin-PAMAM complex attached to liposomes is compared to a conventional liposomal formulation with the same composition encapsulating doxorubicin by the pH gradient method (Papagiannaros *et al*. 2005; Papagiannaros *et al*. 2006). The main advantage of the liposomal formulation is the controlled and sustained release of the encapsulated drug; the release of which is controlled by the complexation in the dendrimer's internal cavity. The liposomal membrane employed in the formulation is useful for the biocompatibility of the liposomal system and it offers advantages of the liposomal drug delivery. This liposomal system is compared to that of the conventional liposomes of the same lipid composition with respect to the % release of the

encapsulated drug at 37°C, in 50 RPMI culture medium for 48 h period, in order to assess its possible advantages and evaluate its potential applications in cancer therapy.

2. MATERIALS AND METHODS

2.1. Materials

Egg Yolk Phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (AL, USA). Doxorubicin Hydrochloride was purchased from Pharmacia (NJ, USA). Ammonium sulphate, TES (N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid), PAMAM, Poly (amidoamine) 4th generation, Tris (tris (hydroxymethyl) aminomethane), stearylamine (SA), Sephadex G75, chloroform, absolute ethanol and methanol were of spectroscopic grade and were purchased from Sigma (St. Louis, USA).

2.2. Conventional Liposome Preparation, Characterization and Doxorubicin Encapsulation

Liposomes composed of EPC:SA at 10:0.1 molar ratio, were prepared using the reverse phase evaporation method (Szoka *et al*. 1978) while their size and ζ-potential measurements were performed at 25°C and at an angle of 90° in a photon correlation spectrometer (Zetasizer 3000, Malvern U.K.) and analysed by the CONTIN method (MALVERN software). The liposomes were prepared as follows: EPC, and SA were first dissolved in chloroform / methanol and then transferred into a 100 ml round bottom flask. Then a 150 mM ammonium sulphate (pH=5.3) was added to the flask. The mixture was subsequently sonicated for 15 min in a bath sonicator and the organic solvents were removed using a flash evaporator (Bucchi R-480) at 60°C. The liposomal suspension was finally allowed to anneal at 50°C for 1 hour.

Large Unilamellar Vesicles (LUVs) were prepared by sonicating the liposomal suspension in an ice bath, for two cycles of 5 min each (0.7 cycle and 100% amplitude) interrupted by a 5 min resting period, using a probe sonicator (UP 200S, dr. hielsher GmbH, Berlin, Germany). The 150 mM ammonium sulphate buffer (pH=5.3) of the liposomal suspension was exchanged with a 100 mM TES, 100 mM NaCl buffer (pH=7.5) using a Sephadex G75 column. Doxorubicin was subsequently encapsulated into the liposomes using the pH gradient method (Mayer and Bally 1986). Briefly, 854 μl or 0.015 mmole of doxorubicin was added and the preparation was incubated at 60°C for 30 min. Unentrapped doxorubicin was removed by passing the liposomal suspension through a Sephadex G75 column using 100 mM TES, 100 mM NaCl buffer (pH=7.5).

2.3. Determination of Lipids and Doxorubicin

EPC and SA were determined by high performance thin-layer chromatography coupled with a flame ionization detector (HPTLC-FID, Iatroscan MK-5, Iatron Lab.

Inc. Tokyo, Japan) (Goniotaki *et al*. 2004). Hydrogen flow rate was 160 ml/min, airflow rate 1900 ml/min, scan speed 30 s/scan. As stationary phase Chromarods – SII (Iatron Lab. Inc.) in set of 10 rods was used. Doxorubicin concentration of the liposomal samples was measured on a Perkin Elmer UV-vis spectrometer at λ=481 nm by adding absolute ethanol to the samples. Prior to determination, the samples were purified using column chromatography (Sephadex G75).

2.4. Release of Doxorubicin from Conventional liposomes *in vitro*

Equal volumes of liposomal suspension encapsulating doxorubicin in TES (100 mM) and NaCl (100 mM) buffer (pH: 7.5) and in RPMI 1640 culture medium, were mixed and the liposomes were incubated at 37°C. Aliquots of 300 μl were then withdrawn at various time intervals and passed through Sephadex G-75 column, in order to remove the released doxorubicin. Doxorubicin retained in the liposomes was measured by UV-vis spectrometry at λ=481 nm.

2.5. Incorporation of Doxorubicin in PAMAM Dendrimer and Assessment of Doxorubicin Release

An aqueous solution of doxorubicin (122 μl) was mixed with a PAMAM G4 solution (3:1 and 6:1 molar ratio of doxorubicin-PAMAM) in methanol (2 ml) and the solutions were stirred for 12 hours. The solutions were evaporated to dryness at 30°C in vacuum and the PAMAM dendrimer incorporating doxorubicin was extracted overnight using chloroform. Chloroform was evaporated to dryness, the dry residue was dissolved in TES (10 mM, pH: 7.5) and the absorbance of doxorubicin was measured at λ=481 nm using UV-vis spectrometry. In the later case acidification of the solution and buffering to pH=4.5 was performed before measuring the absorbance. The release of doxorubicin was studied in TES at 37°C using dialysis bags (molecular weight cut off 13,000).

2.6. Incorporation of Doxorubicin-PAMAM Complex in Liposomes

Liposomes were prepared by using the thin film hydration method (Gabizon 2002). The doxorubicin-PAMAM complex (3:1 molar ratio; 2.1 μmoles of doxorubicin) was attached to liposomes, composed of EPC:SA 10:0.1 (molar ratio). Briefly, the lipid film was prepared by dissolving EPC (73.6 μmole), SA (0.736 μmole) and doxorubicin-PAMAM complex (3:1 molar ratio; 2.1 μmoles of doxorubicin) in chloroform. The solvent was slowly evaporated in a flash evaporator to form a lipid film, which was dried under vacuum for at least 12 h. Multilamellar vesicles (MLVs) were prepared by hydrating the lipid film with TES buffer (10 mM, pH=7.5) and stirring for 1 h. Small unilamellar vesicles (SUVs) were prepared from the resultant liposomal suspension, which was subjected to sonication for two 5 min periods interrupted by a 5 min resting period, in an ice bath using a probe sonicator (amplitude 100, cycle 0,7 – UP 200S, dr. hielsher GmbH, Berlin, Germany). The resultant

vesicles were allowed for 30 min to anneal any structural defects. Non-incorporated doxorubicin-PAMAM complex was removed by passing the liposomal suspensions through a Sephadex G75 column. The size and ζ-potential of liposomes incorporating the doxorubicin-PAMAM complex (3:1molar ratio; 2.1 μmoles of doxorubicin) were measured using photon correlation spectroscopy (Malvern Zetasizer 3000HS). Doxorubicin concentration was measured on a Perkin Elmer UV-vis spectrometer at λ=481nm after the addition of absolute ethanol to the samples.

2.7. Release of Doxorubicin from the Liposomes Incorporating Doxorubicin-PAMAM Complex

The release of doxorubicin from the MLCRS incorporating the doxorubicin-PAMAM complex (3:1 molar ratio; 2.1 μmoles of doxorubicin) was studied in 50% RPMI culture medium and in TES (100 mM), NaCl (100 mM) buffer (pH 7.5), at 37°C, by placing the liposomal formulations in dialysis bag (molecular weight cut off 13,000). The doxorubicin released at various times, up to 48 h was measured using UV-vis at λ=481 nm.

2.8. Statistical Analysis

Statistical analysis of the effect of liposome type on the size and ζ-potential was performed using one-way ANOVA followed by a post hoc Tukey's HSD test (SPSS for Windows release 11). All the results were from four (n=3) independent experiments.

3. RESULTS

3.1. Encapsulation, Physical Properties and Release of Doxorubicin from Conventional Liposomes

Doxorubicin was encapsulated in liposomes composed of EPC:SA (10:0.1 molar ratio) at a doxorubicin to lipid molar ratio of 0.77±0.01 (initial 0.1). The encapsulation efficiency of doxorubicin into liposomes was 99.1%±1.1. Size measurements for liposomes incorporating doxorubicin, indicated an average size of 91.2±0.74 nm and a ζ-potential of –26±3.3 mV (Table 1).

The release of doxorubicin from the conventional liposome EPC:SA 10:0.1 molar ratio in 50% RPMI cell culture medium at 37°C and in TES buffer after 24 hours is quite fast. The liposomes retained 24.5% of the drug in 50% RPMI cell culture medium and 35.5% in buffer at 37°C after 24 hours (Figures 1 and 2).

3.2. Incorporation and Release of Doxorubicin from the Doxorubicin-PAMAM Complex

The doxorubicin-PAMAM complex was formed using two different pH (*i.e.* 10 mM TES buffer at pH: 7.5 or 10 mM acetate buffer at pH: 4.5) and two different molar

Table 1. Physicochemical characteristics of EPC:SA (10:0.1 molar ratio) liposomes encapsulating doxorubicin and of liposomes (EPC:SA 10:0.1 molar ratio), incorporating doxorubicin-PAMAM complex (3:1 molar ratio)

Liposome formulation	Size (nm)	ζ-potential (mV)
Conventional liposomes: EPC:SA 10:0.1 (molar ratio) encapsulating doxorubicin	91.2±0.74	−26.0±3.3
Liposomes incorporating doxorubicin-PAMAM complex: EPC:SA 10:0.1 (molar ratio) encapsulating doxorubicin as doxorubicin-PAMAM complex (3:1 molar ratio)	116.3±7.8	−8.7±1.7

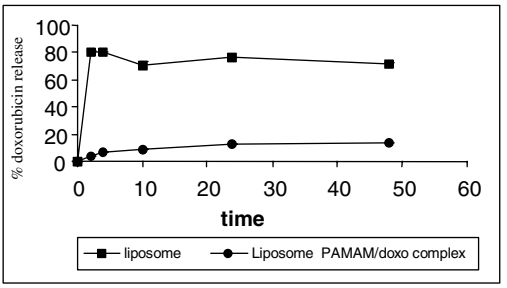

Figure 1. Doxorubicin release from liposomes incorporating doxorubicin-PAMAM complex (•) and from conventional liposomes (■) both composed of EPC:SA 10:0.1 (molar ratio) in 50% RPMI 1640 culture medium at 37°C. Each point represents the mean of three independent experiments (SD never exceeded 5% of the mean value)

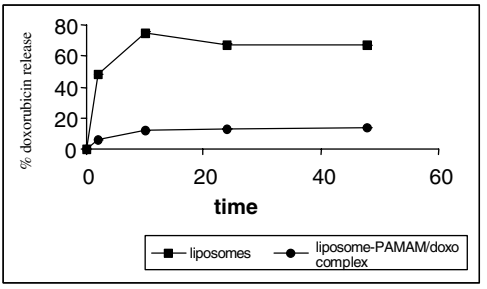

Figure 2. Doxorubicin release from liposomes incorporating doxorubicin-PAMAM complex (•) and from conventional liposomes (■) both composed of EPC:SA 10:0.1 (molar ratio) in TES buffer at 37°C. Each point represents the mean of three independent experiments (SD never exceeded 5% of the mean value)

ratios of doxorubicin to PAMAM (*i.e*. 3:1 and 6:1). The results indicated that a doxorubicin to PAMAM molar ratio of 3:1 was sufficient in order to achieve an almost 97% incorporation of doxorubicin into the dendrimer. Doxorubicin incorporation into PAMAM was higher when the complex was formulated in TES buffer (pH: 7.5) as compared to that of acetate buffer (pH: 4.5). The release of doxorubicin appeared to be quite slow. The lower doxorubicin release (7.4% during 48 h) was observed at a molar ratio of 3:1 of doxorubicin to PAMAM, and the higher (16.5% during 48 h) at molar ratio of 6:1 of doxorubicin to PAMAM in TES buffer (pH: 7.5) at 37°C (Papagiannaros *et al*. 2005).

3.3. Incorporation and Release of Doxorubicin-PAMAM Complex from Liposomes

The incorporation efficiency of doxorubicin-PAMAM complex, (3:1 molar ratio) into liposomes (EPC:SA 10:0.1 molar ratio) was almost 95% while doxorubicin (doxorubicin-PAMAM complex 3:1 molar ratio) to lipid molar ratio was 0.020 (initial 0.028) in TES buffer (pH: 7.5).

The release of doxorubicin (doxorubicin-PAMAM complex 3:1 molar ratio) from the liposomes was quite slow; 13.6% at 37°C (48 h) in TES buffer at pH: 7.5 and 14.0% at 37°C (48 h) in 50% RPMI cell culture medium (Figures 1 and 2).

3.4. Physical Properties of Liposomes Incorporating the Doxorubicin-PAMAM Complex

Size measurements of the doxorubicin-PAMAM complex (3:1 molar ratio) attached to liposomes indicated an average size of 116.3±7.8 nm and a ζ-potential of –8.7±1.7 mV (Table 1)**.** The stability of liposomes was studied for a period up to 26 weeks. The liposomal suspension was kept at 4°C in the dark. No sediment was observed while their average hydrodynamic diameter increased rapidly (>1μm) (Papagiannaros *et al*. 2005).

4. DISCUSSION

A liposome delivery system is proposed for incorporating anticancer drugs, combining the liposomal and dendrimeric technologies. Its ability to modulate the release of the encapsulated drug in a way that is independent of the liposomal membrane but strongly related to the complexation of the drug with the dendrimer, offers advantages over conventional liposomal formulation in terms of the pharmacological activity. The controlled release of the encapsulated cytotoxic drugs is of paramount importance in cancer chemotherapy (Andresen *et al*. 2005). An example is presented in this report, based on the release properties of liposomes encapsulating doxorubicin-PAMAM G4 complex in comparison with the conventional type of liposome encapsulated doxorubicin. This liposomal formulation has shown superior *in vitro* anticancer activity, due to its slow releasing properties

(Papagiannaros *et al*. 2005). It has already been established that the cytotoxic effect of the drug is mediated by the leakage of doxorubicin from the liposomes (Gabizon 2002). However a delayed release of doxorubicin is necessary in order to reduce the toxicity and increase the therapeutic usefulness of the drug (Charrois *et al*. 2004).

The release rate of doxorubicin is an important factor since a slow release is necessary in order to decrease the side effects of doxorubicin and improve its therapeutic index (Gabizon 2002; Horovic *et al*. 1992). A slow release rate can also contribute to the accumulation of the drug in the tumor (Charrois and Allen 2004). The control of the leakage of the encapsulated drug is mainly achieved through modifications in the liposome membrane, mainly by changing the fluidity of the membrane, by addition of cholesterol (Ohvo-Rekila *et al*. 2002) or "rigid state" lipids (Oussoren *et al*. 1998); increasing the rigidity of the liposome membrane also affects the uptake of the encapsulated drug by the tumor cells, therefore reducing the toxicity can also reduce the availability of the drug to the tumor site (Sadzuka *et al*. 2002). On the contrary, doxorubicin incorporated into cholesterol–free liposomes, as a doxorubicin-PAMAM complex, exhibited a slow release rate, at 37°C, after a 48 h incubation period (in 48 hours less than 20% was released). Consequently, it can be expected that this formulation possess reduced doxorubicin side effects. Various drugs encapsulated in dendrimers (Kolhe *et al*. 2005) or incorporated in liposomes together with PAMAM dendrimers (Klopade *et al*. 2002) have shown slow release profiles. The contribution of the doxorubicin-PAMAM complex may not be limited to the delayed release of the encapsulated doxorubicin, since an ibuprofen- PAMAM G4 complex was found to enter lung epithelial cancer cells in 1h (compared to 3h for free ibuprofen) (Kolhe *et al*. 2005), thus the dendrimer could facilitate the cellular entry of the complexed drugs. Furthermore, PAMAM G4 dendrimer conjugated with ibuprofen entered lung carcinoma cells in less than 15 min compared to 1h for free ibuprofen (Kolhe *et al*. 2005) and PAMAM G5 encapsulating methotrexate exhibited four times more activity *in vitro* than the free drug against the KB epidermoidal cancer cell line (Quintan *et al*. 2002).

The encapsulation efficiency of doxorubicin in PAMAM G4 was almost 100%. The presence of dendrimers resulted in a higher encapsulation efficiency and a decreased release rate of the encapsulated drug, although this was achieved by creating a higher and more stable proton gradient across the liposomal membrane (Klopade *et al*. 2002).

Although the average hydrodynamic diameter of the liposomal formulation incorporated doxorubicin-PAMAM complex was almost 116nm immediately after their production, this size increased to the microns (μ) very rapidly with time. This fact was not observed with the conventional liposomal formulation, that does not incorporate the doxorubicin-PAMAM complex, and therefore it might be attributed to the presence of the dendrimer. It has already been observed that dendrimers could facilitate the formation of liposome aggregates (Sideratou *et al*. 2002). The charge of liposomes incorporating doxorubicin – PAMAM complex, did not seem to be involved in the formation of the aggregates suggesting that hydrophobic forces between dendrimers, which are attached to liposomal particles, may be responsible.

Earlier studies using 'dendrons' (partial dendrimers) (Purohit *et al*. 2001) have also reached the same conclusion.

5. CONCLUSIONS

A liposomal drug delivery system incorporating a complex of doxorubicin-PAMAM G4 dendrimers was prepared and compared to conventional liposomal formulation encapsulating doxorubicin with the same lipid composition regarding release properties of the antineoplastic agent. The results suggest that this new controlled release system may be useful in anticancer therapy.

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