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Construction of nanoscale multicompartment liposomes for combinatory drug delivery

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Abstract

Liposomes are clinically used delivery systems for chemotherapeutic agents, biological macromolecules and diagnostics. Due to their flexibility in size and composition, different types of liposomes have been developed varying in surface and structural characteristics. Multicompartment liposomes constitute an attractive drug carrier system offering advantages in terms of inner vesicle protection, sustained drug release and possibility for combinatory (cocktail) therapies using a single delivery system. However, all previously described methodologies for multicompartment or multivesicular liposomes resulted in micrometer-sized vesicles limiting most pharmaceutical applications. In this work we report formulation of nanoscale multicompartment liposomes which maybe applicable for systemic administration. A small unilamellar vesicle (SUV) aqueous dispersion (DOPC:DOPG:CHOL) was used to hydrate a dried film of different lipid contents (DMPC:CHOL), followed by extrusion. The system was characterised by techniques such as photon correlation spectroscopy (PCS), zeta potential measurement, transmission electron microscopy (TEM) and laser scanning confocal microscopy (LSCM). We observed a single, multicompartment vesicle population composed of the two different bilayer types of approximately 200 nm in mean diameter rather than a mixture of two independent vesicle populations. In the case of tumour therapy, such multicompartment liposome systems can offer a single carrier for the delivery of two different modalities.
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Keywords: Multicompartment liposomes; Cancer therapy; Combinatory chemotherapy; Multimodal delivery; Theranostic

Liposomes are the most clinically established nanometer-scale systems that are used to deliver cytotoxic drugs, genes, vaccines, as well as imaging agents (Lasic and Papahadjopoulos, 1998). Liposomes consist of single or multiple concentric lipid bilayers (called lamellae) encapsulating an aqueous compartment. Most clinically used liposomes encapsulate a hydrophilic therapeutic agent (such as anthracyclines) in their aqueous compartment surrounded by a lipid bilayer that may contain polymers or targeting ligands (e.g. antibodies) on their surface (Torchilin, 2005). The pharmacokinetics of such systems have been thoroughly studied (Allen et al., 1995). More recently, sophisticated systems such as liposomes responsive to external or environmental stimuli (e.g. pH, temperature) have been described (Andresen et al., 2005). Moreover, the administration of combination chemotherapy treatment regimens using a single delivery system is thought to significantly enhance efficacy (Ramsay et al., 2005). Therefore, the engineering of new types of multicompartment liposomes is needed in order to allow adoption of such novel design features in a single liposome carrier system.

Multivesicular systems were first described in 1982 (Kim et al., 1983) having an average diameter ranged between 6 and 30 μm. These were large clusters of smaller lipid bilayers seemingly 'glued' to each other. Multivesicular structures consisting of lipid bilayers encapsulating intact liposomes of smaller mean diameters have been further developed by various laboratories (Katayama et al., 2002); however, the minimum average diameter described for any multivesicular system is in the range of a few micrometers (Mantripragada, 2002; Yamabe et al., 2003a). This size disparity inhibits the use of multivesicular systems against systemic diseases such as cancer, whereby blood circulation of the delivery systems is required. Micrometer-

Abbreviations: DOPC, dioleoylphosphatidylcholine; DOPG, L-α-phosphatidyl-DL-glycerol-dioleoyl; DMPC, dimyristoylphosphatidylcholine; CHOL, cholesterol

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sized multivesicular systems have been recently reported as oral (Yamabe et al., 2003b) and local (intratumoural) (Xiao et al., 2004) drug delivery systems, providing a sustained drug release profile, inner liposome protection and a higher drug encapsulation efficiency. An alternative multivesicular system, called vesosome, has been developed by Zasadzinski and co-workers using the self-assembly properties between streptavidin-coated cochleate cylinders (Lasic, 1997; Walker et al., 1997; Kisak et al., 2002; Yamabe et al., 2003a,b) or ethanol interdigitated phospholipid bilayer sheets (Kisak et al., 2002) and biotin-coated smaller liposomes. Such micrometer-sized vesosome systems were very recently reported as vaccine delivery systems following topical (skin) immunization (Mishra et al., 2006).

The present work reports a new nanoscale multicompartment liposome (MCL) system. The novel feature of our MCL system is that a vesicle population with a mean diameter of 200 nm and a narrow Gaussian size distribution is structurally composed of two different types of vesicles connected through a tight bilayer interface.

Lipid films of DOPC:DOPG:CHOL (80:10:10 molar ratio) and DMPC:CHOL (90:10) (Lipoid Co., Germany) were hydrated using phosphate buffered saline (PBS, pH 7.4). Small unilamellar vesicles (SUV) of DOPC:DOPG:CHOL were prepared after 5 min of probe sonication (Sonipep 150, Sanyo, Japan). Lipid film hydration and sonication were carried out above the phase transition temperature of the lipids. The SUV suspensions were centrifuged at 13,000 rpm for 10 min (Biofuge, Heraeus Sepatech, Germany) to remove any titanium particles. DMPC:CHOL large unilamellar vesicles (LUV) were prepared by extrusion (Northern Lipid, Canada) at 40 °C 5 times through 800 nm polycarbonate membranes, followed by 15 times through 400 nm membranes (Whatman, UK). The LUV suspension was left to anneal for 30 min at room temperature.

MCL were prepared by hydration of a DMPC:CHOL (90:10) lipid film using the SUV suspension consisting of DOPC:DOPG:CHOL, at 2:1, 5:1, 10:1, and 20:1 lipid film:SUV molar ratios. The MCL size was further reduced by serial extrusions through polycarbonate membranes (as described above for LUV formation). Control vesicle systems were prepared by mixing preformed SUV (DOPC:DOPG:CHOL) and LUV (DMPC:CHOL) at the same LUV:SUV molar ratios as these used for MCL preparation. Liposome mixtures were incubated at room temperature for the same time required for MCL preparation.

The MCL structures were determined using a Philips CM 120 BioTwin transmission electron microscope (TEM) (Eindhoven, the Netherlands). All liposome samples were stained with 1% phosphotungestic acid (PTA). MCL and LUV:SUV mixed control systems were also examined under laser scanning confocal microscopy (LSCM) (Zeiss, Germany). SUV were fluorescently labelled with CF-DOPE (green channel; 0.75 mol%) (Avanti, USA) and LUV with DiI (red channel; 0.02 mol%) (Molecular Probes, USA). Hydrodynamic *z*-average diameter and zeta potentials were measured using the Zetasizer 3000 (Malvern Instrument, UK) laser light scattering instrument.

Fig. 1 shows a characteristic LSCM image of the vesicular system immediately upon hydration of a (DMPC:CHOL) lipid film by an SUV (DOPC:DOPG:CHOL) aqueous suspension (50 nm). Large (micrometer-range) vesicle aggregate structures were formed, consisting of SUV (green signal) around large hydrated vesicles from the DMPC:CHOL lipid film (red signal). These micrometer-sized structures can be considered as precursors of the nanoscale MCL system that formed upon their extrusion.

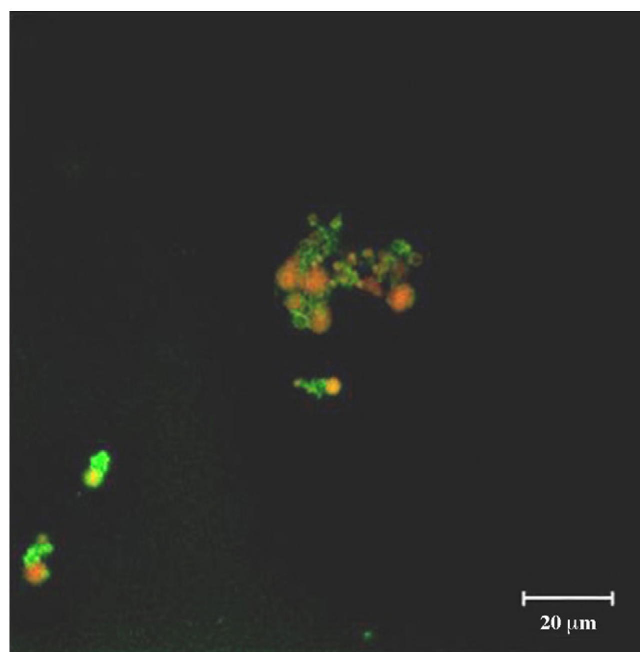

Fig. 1. LSCM image of DiI-labelled DMPC:CHOL lipid film (red) hydrated at 40 °C with CF-DOPE-labelled DOPC:DOPG:CHOL SUV (green) suspended in PBS buffer. Confocal microscopy was performed at excitation wavelengths 488 and 543 nm for the CF-DOPE and DiI dyes, respectively. Emission was collected using a band pass filter between 505 and 530 nm for CF-DOPE and 560 nm long pass filter for DiI dye. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Serial extrusions of the vesicular structures shown in Fig. 1 led to vesicles of considerably smaller mean diameter. Laser light scattering determined that the mean diameter for the MCL system ranged around 200 nm (Fig. 2, squares). The mean vesicle diameter for the LUV:SUV mixed control systems

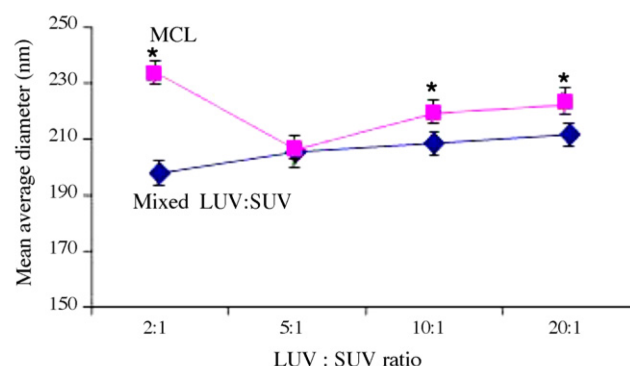

Fig. 2. Mean average diameter of mixing (♦) and MCL (■) systems. Photon correlation spectroscopy measurements of 2:1, 5:1, 10:1 and 20:1 molar ratios of LUV:SUV mixing or lipid film:SUV MCL systems. (*) P value <0.005 obtained by Student's t-test ($n = 3$).

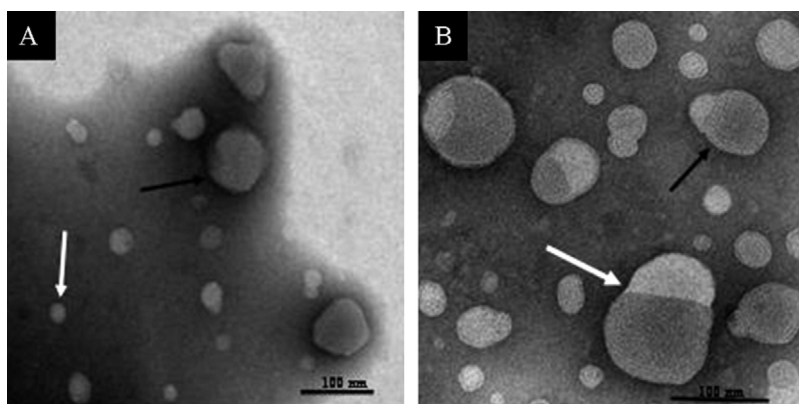

Fig. 3. Nanoscale multicompartment liposome (MCL) consists of small unilamellar vesicles (SUV) (white arrow) separated from multilamellar vesicles (MLV) (black arrow) by interface, prepared by extrusion of micrometer-sized MCL. Transmission electron microscopy image (TEM) of 1% PTA stained LUV:SUV mixing system (A) and MCL system (B).

(Fig. 2, diamonds) resulted in an 'averaging' effect between the two liposome populations indicated by the linear increase observed for higher LUV:SUV molar ratios (more LUV particles added). This was not observed for the MCL system suggesting a more complex interaction between the two lipid bilayer populations. Determination of vesicle surface charge, indicated a high negative surface charge for the SUV system (−41 mV ζ-potential). Interestingly, surface charge values for all MCL and LUV:SUV mixed systems were almost identical to that of the (DMPC:CHOL) LUV control system (−2 mV). This was thought to indicate that the DMPC:CHOL bilayers for both the MCL and the vesicle control systems were overwhelmingly exposed to the external vesicle aqueous phase. The mean average diameter and surface charge for both MCL and LUV:SUV mixed control were stable over 24 h (data not shown).

The structural characteristics of MCL and the LUV:SUV mixed control systems were determined by TEM (Fig. 3A and B). Mixing of LUV with SUV led to distinct vesicle populations with minimum interaction as evidenced in Fig. 3A (see black and white arrows). The MCL structures obtained after 24 h of preparation by TEM indicated a single population of multicompartment liposomes in the order of 200 nm. Very interestingly, multilamellar (concentric bilayer) vesicles seemed connected to unilamellar vesicles (Fig. 3B) through a lipid bilayer interface.

The data presented in this communication indicated that nanoscale multicompartment liposomes (MCL) can be formed by self-assembly and extrusion. The association between SUV and the lipid bilayer vesicles immediately upon hydration indicated that hydration of lipid films with an SUV-containing aqueous phase does not lead to entrapment of the SUV. These results indicate the importance of hydrophobic interactions between the hydrated lipid (DMCP:CHOL) film and the preformed SUV (DOPC:DOPG:CHOL). Similar interactions have been reported for previously described multivesicular systems that contained triolein (Fig. 4A) prepared by the double emulsification technique, where the vesicles were 'glued' together by triolein-rich hydrophobic regions to form a closely packed 'foamy' micrometer-sized bilayer structure (Kim et al., 1983). The MCL system we constructed here can be schematically represented in Fig. 4B, by hypothesising that the interface region connecting the two vesicle types is held together by hydrophobic association, forming junctions that may be rich in cholesterol. The mean diameter of the MCL system developed in this study is in the 200 nm range, two orders of magnitude smaller than the system described previously (Fig. 4A), suitable for pharmaceutical applications that may involve systemic circulation. Further work is needed to optimise the construction of such nanoscale MCL systems and determine their pharmacokinetic and tissue biodistribution profiles. Nanoscale MCL are developed as single-vehicle delivery systems for combinatory chemotherapeutic regiments and multimodal agents.

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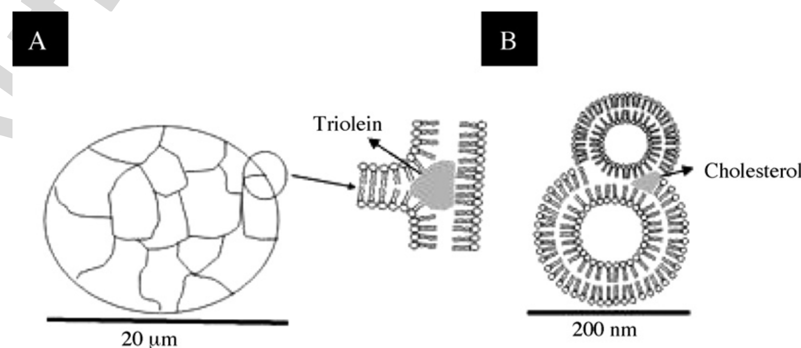

Fig. 4. Schematic diagram of: (A) multivesicular liposomes containing triolein prepared by the double emulsion technique as previously described (Kim et al., 1983; Mantripragada, 2002) and (B) the nanoscale MCL system described in this study formed by lipid film hydration and extrusion.

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