Freeze-fracture Electron Microscopy on Nanostructures for Drug & Gene Delivery

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ABSTRACT

The morphology of a variety of nanostructures suitable for drug and/or gene delivery such as liposomes, niosomes, cochleate cylinder, depofoam particles, and liposome/DNA complexes has been studied under biologically relevant conditions by using freeze-fracture electron microscopy. This study shows that freeze-fracture electron microscopy is not only a powerful technique to characterize drug/gene carrier but also the method of choice to study their fate related to the incubation milieu and drug/gene load, and during their interaction with cells on a nanometer resolution scale. Furthermore, parallel studies of the morphology and transfection activity help us to understand the fundamental difference between *in vitro* and *in vivo* transfection activity of cationic liposome/ DNA complexes as non-viral gene vectors.

Keywords: Electron microscopy, freeze-fracture technique, drug carrier, gene delivery, cell interaction.

1 INTRODUCTION

The potency of drug/gene-loaded carriers as well as their uptake by cells is frequently depending upon their morphology adopted in a biological relevant environment [1,2]. While Small Unilamellar Vesicles (SUV) are suitable for systemic applications, Multilamellar Vesicles (MLV) are excellent depots for dermatological use [2,3]. Cochleate cylinder are stable under acidic stomach conditions and therefore they are suitable for oral applications [2,4]. DepoFoam (DF) is a multi-vesicular, drug delivery system most suitable for the encapsulation and sustained release of water-soluble drugs [5]. Cationic Liposome/DNA Complexes (CLDC) are extensively used as non-viral gene vectors [6].

2 NANOSTRUCTURE MORPHOLOGY

Using freeze-fracture electron microscopy (ff-em) we studied the morphology of a variety of liposomal and/or lipid-based drug and gene carriers such as SUV, MLV, CLDC, cochleate cylinders, and DF particles but also non-lipid carriers such as niosomes. Furthermore we investigated the structural transformation of cationic liposomes under the influence of the type and concentration of the helper lipid used for complex formation, the ionic strength of the incubation media, as well as of the DNA/oligonucleotide type encapsulated. Additionally, morphology-transfection activity relationships of CLDC were established to determine the transfection-active structure/s. Finally we recorded the interaction of liposomes, CLDC, and cochleate cylinders with bacteria and/or cultured skin cells.

2.1 Methods

For ff-em the samples were quenched using sandwich technique and liquid nitrogen-cooled propane. Using this technique a cooling rate of 10,000 degree per second is reached avoiding ice crystal formation and artifacts possibly caused by the cryofixation process. The fracturing process was carried out in JEOL JED-9000 freeze-etching equipment and the exposed fracture planes were shadowed with Pt for 30 sec in an angle of 25-35 degree and with carbon for 35 sec (2kV/ 60-70mA, $1x10^{-5}$ Torr). The replicas produced this way were carefully cleaned with concentrated, fuming HNO_3 for 24 hours followed by repeating agitation with fresh chloroform/methanol (1:1 by vol.) examined at a JEOL 100 CX electron microscope [3].

For *in vivo* delivery and gene expression female DC1 mice received CLDC by tail vein injection and the luciferase expression was

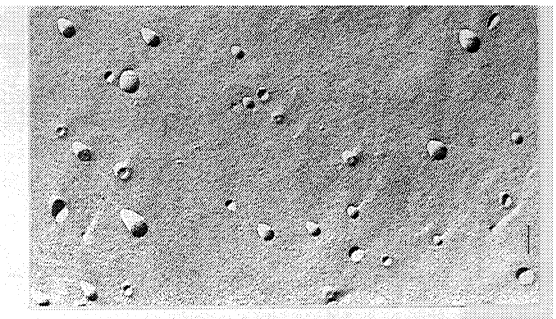

Figure 2: Small unilamellar vesicle (SUV)

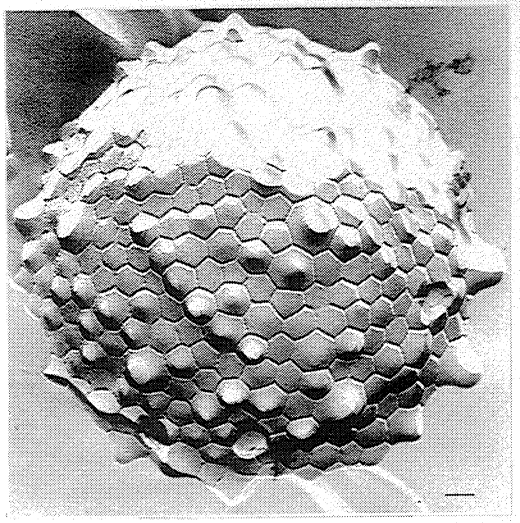

Figure 3: Niosome

Figure 4: Cochleate cylinder

Figure 5: DepoFoam (DF) particle

4 STRUCTURAL POLYMORPHISM OF CLDC AS NON-VIRAL VECTORS

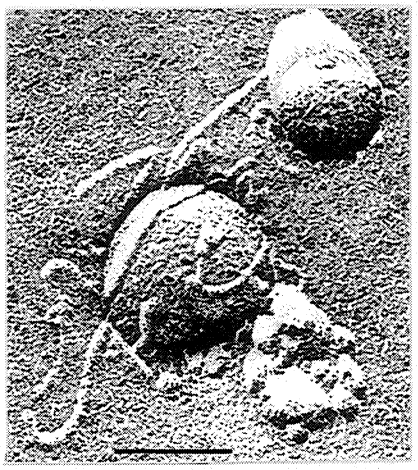

Figure 6: Spaghetti/meatball type structure

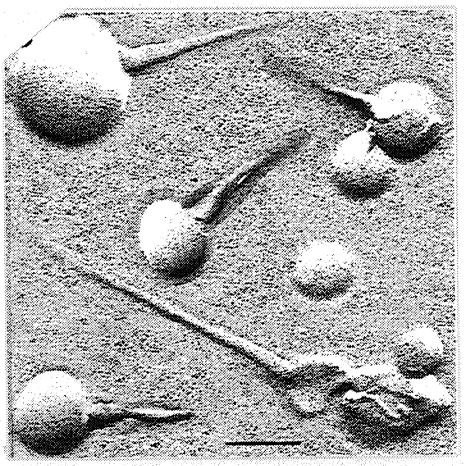

Figure 7: Map-pin structure

measured in homogenized lung extracts. For in vitro delivery and gene expression SK-BR-3 cells were cultured, incubated with CLDC, and assayed for luciferase expression [7,8].

2.2 Results and discussion

While MLV display a multitude of bilayers and diameters of several micrometers, SUV have only one bilayer and can be as small as 15 nm in diameter as seen in Figures 1 and 2 [2,3]. Niosomes are made of non-ionic detergents and look normally very similar to liposomes. Under certain conditions, however, they are able to adopt geodetic sphere structure as shown in Figure 3 [9,2]. Cochleate cylinders are made of negatively charged lipids and form, when calcium ions are added, large cigar-type cylinders, several micrometer in width and several tens of micrometers in length, as displayed in Figure 4 [2-4]. DF particles are prepared by a double emulsification process [8] and display a chambered inner volume and diameters of several micrometers as shown in Figure 5.

Depending upon the type and ratio of the helper lipid, the ionic strength of the incubation medium, and the gene component encapsulated, CLDC are able to adopt structures hugely different in morphology and size. *Spaghetti/meatball type* structures as seen in Figure 6 resemble bilayer-covered DNA tubules (*spaghetti*) and DNA containing liposomal complexes (*meatballs*) and are observed in media with medium to high ionic strength, when DOPE was chosen as helper lipid and super-coiled DNA used as gene component [10,11]. The substitution of DOPE with cholesterol, the addition of a stealth component such as PEG-PE, and the pre-condensation of the DNA by spermidine result in relatively small complexes (100-300 nm) with some proportion of a *map-pin structure*. Map-pins show heads of the size of small liposomes and short, stiff and tapering tails as visible in Figure 7 [7,8]. At high ionic strength as well as high content of DOPE instead of cholesterol, large extended lipid/gene precipitates are observed adopting the *honeycomb structure* or *hexagonal lipid (H_{II}) phase* as displayed in Figure 8 [7,8,11].

Our parallel studies of the morphology and transfection activity of CLDC show clearly that size, morphology, and stability of the complexes profoundly influence their transfection activity under *in vivo* as well as *in vitro* conditions. High *in vivo* transfection activity seems to be related with small, stabilized complexes containing some proportion of the *map-pin structures*, whereas high *in vitro* transfection activity seems to be associated with *hexagonal lipid* precipitates [1,7,8].

Our cell interaction studies of selected drug/gene carriers such as SUV, CLDC displaying the *spaghetti-type* structure, and cochleate cylinder show size/curvature-dependent uptake mechanisms. While small liposomes and spaghettis containing highly curved bilayers are able to pass intact the cytoplasmic membrane of cells, larger liposomes as well as CLDC are taken up by endocytosis [1,12]. Huge cochleate cylinders are not taken up at all by the bacterial strains investigated. However, small bacterial cells are adhered to the cochleate cylinders possibly promoting material/drug exchanged between both structures.

3 FREEZE-FRACTURE ELECTRON MICROGRAPHS OF SELECTED DRUG CARRIERS

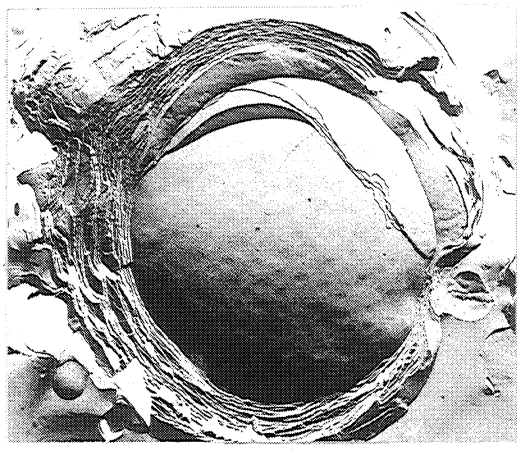

Figure1: Multilamellar vesicle (MLV)
On all freeze-fracture electron micrographs the bar represents 100 nm and the shadow direction is running from bottom to top.

Figure 8: Honeycomb structure or H_{II} phase

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