Characterization of liposomes using negative staining and freeze fracture transmission electron microscopy

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Lipid nanoparticles such as liposomes have attracted increasing interest in different fields of drug or gene delivery, molecular imaging or cosmetics [1-2]. Liposomes are small, spherical vesicles made of phospholipid bilayers. They are biodegradable, biocompatible and usually not toxic. Due to their hydrophilic and hydrophobic parts, liposomes can be used to carry a variety of compounds, either within the hydrophilic core or within the hydrophobic regions in-between the bilayer. An important tool to characterize such particles is electron microscopy as it allows various settings and methods for the tasks required. It was therefore the aim of the present work to use electron microscopy to answer different questions about structure and to visualize different domains of lipid vesicles.

For all our studies we used either negative staining or freeze fracture electron microscopy. For negative staining 10 µl of liposomal suspension were placed on a carbon-over-Pioloform-coated copper grid. After one minute the excess of the sample was blotted with filter paper and immediately replaced by 10 µl of staining agent, which was allowed to settle for 2 minutes, and then blotted again. Ammoniummolybdate (5%), phosphotungstic acid (1%) and uranyl acetate (2%) were tried as staining agents. For freeze fracture electron microscopy, liposomes were mixed with 30% glycerol (v/v), frozen in liquid propane and stored in liquid nitrogen until further use. Samples were fractured in a Balzers BAF400D freeze-etching apparatus (Balzers, Liechtenstein) under vacuum with a pressure between 1.3 * 10⁻⁴ to 1.3 * 10⁻⁵ Pa. Replicas were produced by vacuum-deposition of the surface with platinum and carbon, controlled with a quartz crystal thin-film monitor. To clean the replicas they were put into sodium hypochlorite solution for about three hours and stored overnight in 50% NaOH. Before mounting them on an uncoated copper grid, replicas were washed with distilled water at least three times.

Firstly, negative staining electron microscopy was used to reveal whether ultra small superparamagnetic iron oxide (USPIO) nanoparticles are efficiently entrapped within liposomes. Ammonium molybdate, phosphotungstic acid and uranly acetate were used as staining agents. The technique worked very well for visualizing the iron oxide nanoparticles (see Figure 1) but had some problems in maintaining the structure of lipid vesicles due to different salt concentrations within the liposome suspension and the staining solution and required drying steps. It is therefore difficult to get reliable information about the shape of lipid vesicles by using this technique.

Another question concerned the visualization of a coating around the liposome which is commonly used to protect the vesicles, or to target them to a certain tissue, or to extend the residence time in the blood. [3-4] As a first attempt, negative staining was tried but this technique was not successful in revealing the coating layer around the liposome. Therefore freeze fracture electron microscopy was selected as a powerful tool to visualize even a very thin coating layer. However, using this technique one should keep in mind that the size distribution of the vesicles on the image does not reflect the vesicle size distribution in solution. The polymer, which was used as coating material in this study, could be detected surrounding the liposomes as well as crosslinking several vesicles, so that the vesicles formed clusters (see Figure 2).

In a third project we synthesized liposome-based chemical exchange saturation transfer agents for magnetic resonance imaging by encapsulating a hydrophilic paramagnetic lanthanide (III)-based
shift reagent, Tm-DOTA, in the inner core of spherical liposomes, and incorporating an amphiphilic lanthanide-complex in the phospholipid bilayer. To increase the paramagnetic shift of intraliposomal water protons and with it also the contrast efficiency, the vesicles were shrunken by osmotic stress. Liposomes were prepared at low ionic strength conditions and dialyzed against isotonic buffer to induce a change in shape from spherical to elliptical. This conversion was visualized by freeze fracture technique.

To conclude from these studies, negative staining seems to be only of limited suitability for studying liposome characteristics. But if the main intention is to detect substances that are entrapped within the vesicles, it may still be the method of choice since it is quick, easy and does not require much equipment. For more complex problems, especially concerning shape or surface modifications, freeze fracture electron microscopy was found to be an ideal method and enabled us to get accurate information about the liposome morphology.

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References

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Figure 1: TEM-micrographs of (a) dextran-coated USPIOs in aqueous buffer (b) vesicles loaded with USPIOs (after extrusion and purification) and (c) extruded control liposomes without magnetite.

Figure 2: Transmission electron micrographs using freeze fracturing of (A) uncoated POPC/DOPE-MCC liposomes and (B) POPC/DOPE-MCC liposomes coated with polymer. Arrows indicate the polymer coat. Magnification: 30.000x. Scale bar indicates 200nm.