# Effect of electron microscopy sample preparation protocol on the preservation of liposomes in cell culture

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Background:
Liposomes are powerful vehicles for the delivery of a broad range of drugs and other compounds to specific locations within the human body. This has made them a versatile, and often used, nanoformulation within the pharmaceutical industry. Accurately localizing these liposomes within cells and tissue is vital to understanding the uptake mechanisms and ultimately the efficacy of the liposomes. Electron microscopy is the ideal tool to localize liposomes within cells and tissue, however there are numerous challenges that must be addressed. 1st, Liposomes exhibit a pronounced sensitivity to their surroundings, and the harsh conditions often employed to prepare cells for electron microscopy may result in the dissolution of the liposome of interest. 2nd, Liposomes are composed of carbonaceous material much like the surrounding cellular matrix, making it difficult to distinguish. In this work we aimed to characterize the effect that sample preparation has on the preservation of liposomes within a cell culture sample.

Methods:
100 million CT26 colon carcinoma cells were incubated unloaded stealth liposomes for 3 hours to allow for uptake. The cells were then divided into 4 groups for further processing. Group 1 was processed using a standard chemical fixation, staining and dehydration protocol. The cells were placed in 4% paraformaldehyde, 2% glutaraldehyde in 0.1 M Na Cacodylate Buffer for 1 hour at room temperature. The cells were then pelleted, suspended in 10% gelatin, and stained with 1% osmium tetroxide in water at 4 C for 1 hour. The cells were then stained with 1% uranyl acetate in water overnight before being progressively dehydrated in ethanol and propylene oxide. After dehydration, the cells were embedded in EMBED 812 epoxy resin.

Groups 2-4 were all high pressure frozen using a Leica EM-ICE and underwent 3 different freeze substitution protocols using the Leica AFS2. Group 2 underwent a standard freeze substitution protocol in a 1% osmium tetroxide in acetone solution over the course of 3 days. This was followed by room temperature staining in 1% uranyl acetate for 1 hour before embedding in EMBED 812 Epoxy resin. Group 3 underwent a quick freeze substitution protocol over the course of 4 hours in a 1% Osmium tetroxide, 1% uranyl acetate in acetone solution before being embedded in EMBED 812 Epoxy resin. Group 4 underwent freeze substitution with a 1% uranyl acetate solution and low temperature embedding in Lowicryl HM20 at -50 C.

Ultrathin sections from each sample were prepared and imaged using a Tecnai T12 at DTU Nanolab.

Results:
All 4 samples were successfully prepared into 100 nm sections for imaging. Liposomal structures were observed for all 4 samples with the samples undergoing freeze substitution exhibiting qualitatively the best-preserved liposomes. Imaging is ongoing to obtain enough data to try and definitively state which freeze substitution method will result in the best preservation.

Conclusion:
Freeze substitution appears to provide better preservation of liposomes in cell culture. Additional characterization is ongoing and necessary to determine which freeze substitution method provides the best results for preserving liposomes. Even with improved preservation of the liposomes, it is still very often difficult to ensure that the object being imaged is a liposome and not some other vesicular body within the cell.

**Keywords:**

sample preparation, TEM, Liposomes