

## Technical Note

## A method to achieve homogeneous dispersion of large transmembrane complexes within the holes of carbon films for electron cryomicroscopy

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## ABSTRACT

Difficulties associated with using X-ray crystallography for structural studies of large macromolecular complexes have made single particle cryo-electron microscopy (cryoEM) a key technique in structural biology. The efficient application of the single particle cryoEM approach requires the sample to be vitrified within the holes of carbon films, with particles well dispersed throughout the ice and adopting multiple orientations. To achieve this, the carbon support film is first hydrophilised by glow discharge, which allows the sample to spread over the film. Unfortunately, for transmembrane complexes especially, this procedure can result in severe sample adsorption to the carbon support film, reducing the number of particles dispersed in the ice. This problem is rate-limiting in the single particle cryoEM approach and has hindered its widespread application to hydrophobic complexes. We describe a novel grid preparation technique that allows for good particle dispersion in the ice and minimal hydrophobic particle adhesion to the support film. This is achieved by hydrophilisation of the carbon support film by the use of selected detergents that interact with the support so as to achieve a hydrophilic and neutral or selectively charged surface.

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### 0. Introduction

The popularity of cryoEM for structural analysis of macromolecular assemblies is due to the speed and ease of the data collection procedure. Difficulties associated with crystallisation have limited application of X-ray crystallographic techniques to a handful of large complexes. For membrane-derived macromolecular complexes, which show an even lower propensity to crystallise, cryoEM is often the only option (Chandran et al., 2009; Lau and Rubinstein, 2012; Yusupov et al., 2001).

Single particle cryoEM involves the suspension of macromolecules in vitrified ice, acquisition of data at low temperature and low electron dose and computerised processing of the images. This technique retains the complexes in a hydrated state, giving a good representation of their native structure. Due to technical advances, the achievable resolution is now sub-nanometre (Jiang and Ludtke, 2005).

For cryoEM, the highest quality images, in terms of contrast, signal to noise ratio (S/N) and resolution, are attained when the sample is applied to a holey carbon support film and particles are dispersed in the ice within the holes of the carbon. However, continuous carbon support films are often used because particles do not distribute efficiently into the holes. This improves particle spread but limits the observable particle orientations. The carbon also makes the image contrast of the particles lower, impairing high-accuracy alignment required for extraction of high-resolution image information.

### 1. Consequences of grid preparation by glow discharge

Carbon support films are generally hydrophilic immediately after production. However, adsorption of organic molecules and/or oil vapour from the environment gradually diminishes surface charges, eventually rendering support films hydrophobic (Dubochet et al., 1985; Sogo et al., 1975). To achieve spreading of the sample over the grid, the grid surface is first hydrophilised by ion bombardment. In a reduced air environment, glow discharging creates a hydrophilic surface by removal of adsorbed impurities and deposition of electrons onto the carbon surface, thereby creating a negatively charged surface (Dubochet et al., 1985; Hayat,

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2000). Charging the grid surface also increases charge interactions between the carbon and protein, further aiding sample spreading.

The key difference between holey and continuous carbon supports is the role of the carbon. With continuous supports, the carbon acts as a support for the sample itself. Therefore, anything that increases the interaction between the support and sample is advantageous. However, for holey carbon supports, the carbon and holes provide an aqueous environment for the sample. The goal is to have particles dispersed within thin sample solution films formed in the holes before freezing. Here, anything that increases interactions between the support and sample results in sample adsorption to the carbon support, inhibiting particle dispersion within the holes.

## 2. Transmembrane macromolecular complexes and cryoEM

In an aqueous environment, macromolecules exist in an ionised state, with the degree of ionisation dependent on buffer constituents, solution pH, temperature etc. For this reason, macromolecular complexes often adsorb strongly onto hydrophilic surfaces (Dubochet et al., 1985). Transmembrane protein complexes are removed from lipid bilayers by solubilisation with a detergent, resulting in large protein–detergent complexes. Along with vast hydrophobic regions, all transmembrane complexes will have hydrophilic regions exposed to the cytosol, periplasm and/or extracellular space. The complexity of surface charges of the solubilised complexes further exasperates adsorption onto hydrophilic surfaces. Sample adsorption to glow discharged holey carbon films prevents particle dispersion within the holes and has become a common obstacle in cryoEM studies of transmembrane macromolecular complexes.

## 3. Principle of the new method

The propensity of transmembrane complexes to adsorb to glow discharged carbon surface may indicate a problem not with glow discharge *per se*, but with surface ionisation in general. Any method

that serves to increase surface hydrophilicity by inducing ionisation is likely to have the same adsorption effect on transmembrane complexes. With samples where the adsorption to the support film is almost complete, it is often better to apply the sample to a non-glow discharged, hydrophobic grid as this may yield at least some dispersed particles. However, a more logical workaround would be to create a hydrophilic surface with an overall neutral or selected charge. To achieve such a surface, we thought to pretreat the carbon support with detergents.

The amphipathic nature of detergent molecules makes them ideal for altering the properties of a surface. If applied to a hydrophobic surface, a detergent molecule should orient in the most energetically favourable manner, i.e. with the non-polar tail group facing the surface and the polar head group facing away from the surface. The wide variety of commercially available detergents with differing head group polarities (non-ionic, anionic, cationic and zwitterionic) allows one to obtain a surface charge that best suits the requirements of the specimen.

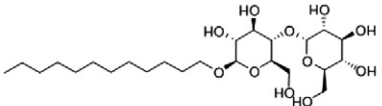
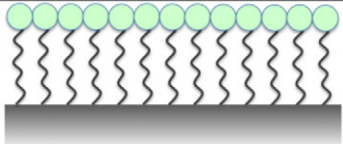
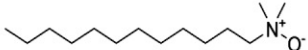
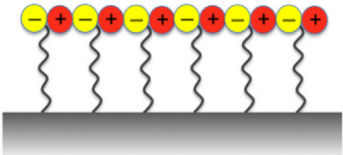
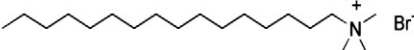
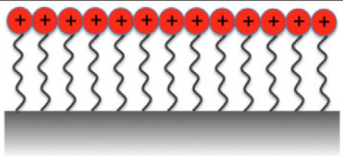
## 4. Grid types used

We purchased carbon-coated grids, with a defined hole size (R 0.6/1, Molybdenum, 200 mesh; Quantifoil Micro Tools GmbH, Germany). Newly purchased grids are assumed to be hydrophobic, and any ionisation of the carbon support should be avoided to assure this. For reproducibility, impurities from the manufacturing process were removed by incubating grids overnight on filter paper soaked in chloroform, with the carbon side facing away from the filter paper. This was followed by an overnight wash in toluene, using the same procedure. This wash was conducted in advance and the grids stored under reduced-humidity conditions.

## 5. Detergent selection and concentration

Detergent molecules in solution form micelles once the detergent concentration surpasses the critical micelle-forming concentration (CMC). At any detergent concentration above the CMC,

**Table 1**  
Summary of the properties of the detergents used in this study and of their proposed adsorption to a hydrophobic surface.

Molecular structure	Head group	Critical micelle-forming concentration (mM)	Predicted adsorption to a hydrophobic surface
<p><i>n</i>-dodecyl-<math>\beta</math>-D-maltopyranoside (DDM)</p> 	Non-ionic	0.2	
<p><i>N,N</i>-dimethyldodecylamine-<i>N</i>-oxide (LDAO)</p> 	Zwitterionic	2.2	
<p>Cetyltrimethylammonium bromide (CTAB)</p> 	Cationic	0.9	

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