Observation by transmission electron microscopy of organic nano-tubular architectures

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Through interdisciplinary collaborations such as those between nano-material scientists or molecular biologists, transmission electron microscopy (TEM) has played a very important role in providing adequate morphological information at each level of organization within the specimen. To meet the demands required from such collaborations, the development of quick and effective techniques for acquiring clear electron microscopic images are requisite. The traditional method of negative staining is a very rapid and simple method for providing high contrast and radiation protection of the biological specimen. It has been widely applied since the late 1950s to a broad range of investigations on biological molecules. This review will critically evaluate the potential of the simple staining method in TEM, focusing on how negative staining methods meet the requirements arising in nano-structure science. We will take as illustrative examples our earlier work on galactosylceramides (GalCer) lipid nanotubes, and GroEL chaperonin nanotubes (as negative staining cases); and graphitic nanotubes (as a non-negative-staining case).

Keywords galactosylceramide; GroEL; chaperonin; Hexa-peri-hexabenzocoronene; HBC; nanotube

1. Introduction

Helicity is one of the most essential structural elements in nature as well as in cellular biology. However, artificially synthesizing a proper helical architecture having a desired scale such as a certain diameter and length is still challenging. Once a technique is established for promoting the formation of helical arrays with a desired protein, electron micrographs of the arrays will be useful for helical image analysis and three-dimensional reconstruction of the protein [1–3].

Some variety of lipids such as galactosylceramides (GalCer) is known to spontaneously form tubular vesicles having a unique diameter under certain conditions. This can then be a suitable cylindrical template for proteins that can align in a helical array on the surface [4]. There are many well-known tubular nano-structures in the living cell. Apart from those, one example is the Escherichia coli GroEL chaperonin, a biological macromolecule having a unique function, which can be altered artificially to link one dimensionally to form nano-tubules [5]. Each stage of tubular construction has been evaluated, and the simple and rapid technique of negative staining combination with traditional TEM is still facile, practical, and very useful [6–9]. On one hand, organic nano-tubules having intrinsic conductivity such as graphitic nanotubes which consist of Hexa-peri-hexabenzocoronene (HBC) derivatives need not always be stained negatively with heavy metal salts in electron microscopy [10]. At any rate, such organic tubular materials have attracted keen interests due to the potential application in medical, pharmaceutical, food science, and nano-material fields. With the promising negative staining technique, TEM can provide a host of valuable structural information, which are discussed in detail in the following applications.

2. Electron microscopy of lipid nanotubes

2.1 Overview of the ceramide

Ceramide is a sphingolipid which accounts for approximately 50% of the lipids between the cells of the stratum corneum of the skin. Because the lipid ceramide is a kind of oil it has the familiar hydrophilic and lipophilic group as its chemical structure (Fig. 1). Under some conditions it forms tubular liposomes as well as spherical ones. Thus, they are, by their characteristics, have attracted much attention as a promising material.
2.2 Materials and methods

Mixed galactosylceramides (GalCer) was purchased from Sigma and the nickel lipid (1,2 dioleoyl-sn-glycero-3-[N(5-amino-1-carboxypentyl) iminodiacetic acid] succinyl] (Nickel salt): DOGS-NTA-Ni) was custom synthesized by Avanti Polar Lipids (Fig. 1).

Aliquots (1 – 10 μl) of synthetic GalCer in chloroform/methanol (1:1) at 10 mg/ml were dried down under argon and rehydrated in the buffer (50 mM Tris-Cl, pH 7.6) at 1 mg/ml. Tubes containing Nickel-lipid were prepared in a similar manner mixing 10 – 60 % DOGS-NTA-Ni in chloroform with Ga lCer in chloroform/methanol (1:1). This solution was sonicated for 1 min in a water bath at room temperature, then dried down, and resuspended in the buffer (50 mM Tris-Cl, pH 7.6) at 1 mg/ml. Conditions for helical crystallization of a His-tagged protein were investigated by incubating the His-tagged protein with GalCer tubes doped with 10 – 60 % DOGS-NTA-Ni (0.5 mg/ml) in 50 mM Tris-Cl (pH 7.6) and 200 mM NaCl. Influence of pH (range of 5.8 – 8.5) on the formation of the tubes and on the interaction between the tubes and the His-tagged protein were also examined in a similar manner. The protein concentration during the incubation with the lipid tube was also checked in a range of 25 – 500 μg/ml to see how it influenced the binding process of the protein onto the tube. After incubation, aliquots of the solution were applied onto EM specimen copper grids covered with a thin carbon support film which had been made hydrophilic by glow-discharge (JEOL HDT-400). Grids either were negatively stained with 0.5 – 1.0 % uranyl acetate. Electron micrographs were recorded on a Zeiss 10A (Carl Zeiss, Inc., Germany) operating at 80 kV at nominal magnification of 10,000×, or 20,000×, and on a FEI Tecnai F20 (FEI Company, the Netherlands) operating at 120 kV at magnification of 9,600×, or 25,000×. Some tube images which appeared to have a potential capability for diffracting a He-Ne laser beam (λ = 632.8 nm) were examined closely by optical diffraction method.

2.3 Results and discussion

Lipid tubes prepared with GalCer alone tended to clump together and form precipitates. Additions of DOGS-NTA-Ni to the GalCer tube were less aggregated. Formation of the GalCer tubes doped with DOGS-NTA-Ni was achieved within several trials. The method employed showed high reproducibility and the tubes once formed were able to stay stable for about one week at room temperature. There were found two tubes having different diameters (25 nm and 40 nm) when the tubes were prepared from GalCer and DOGS-NTA-Ni at 1:1 (w/w). The tube beyond one and a half weeks appeared to melt changing the forms into narrow sheet-like structures. Nickel-lipids provide a common moiety that allows specific binding of His-tagged proteins. The interaction has been used commonly to facilitate protein purification from amplified expression systems, and to adsorb protein directly to planar lipid promoting the formation of two-dimensional crystals at an air/water interface. The tubes modified with Ni-residues were mixed with His-tagged NtrC [11, 12] (its domain structure is shown in Fig. 2) or His-tagged Fab fragment one day after the formation of the tubes and observed by TEM at several different time course of incubation at room temperature. The images of tubes which were densely bound with His-tagged proteins could be seen in the early stages of incubation at each different pH conditions. Though we have not yet fully succeeded in helical array formation as to His-tagged NtrC and its derivatives on the surface of the tubular vesicles, many useful data have been gathered. Typical results as electron micrographs are shown in Fig. 3. In the case with His-tagged Fab fragment, helical array formation has been observed (Fig. 3(e)). Lowering the concentration of His-tagged proteins to around 25 – 50 μg/ml appeared to facilitate the attachment of proteins in a...
mono-layer on the surface of the tubes. Further study is necessary to work out conditions under which the His-tagged proteins form ordered arrays on the surface of tubular vesicles.

In the structural study of protein molecules on the helical assemblance, negative staining appears to dry as an amorphous electron-dense supportive layer and there is a generally quoted resolution limit of around 20 Å [13]. Lipid membranes themselves are generally more resistant to irradiation due to their different chemical organization. A carbohydrate such as glucose or trehalose would be included for obtaining some retention of water in the specimen. Cryogenic electron microscopy of thin unstained vitrified specimens has made possible the observation by electron microscopy of numerous biological particles and macromolecules in their native fully aqueous environment. This means preserving specimens in their native quaternary structure at near atomic resolution level (~ 3 Å) [14]. Thus, cryo-TEM is a promising approach for protein structure determination and analysis at this resolution. However, it requires ready access to rapid freezing and cryo-transfer facilities. The contrast of unstained biological macromolecules in an aqueous vitrified environment is very low and hardly recognizable by eye on the microscope. Furthermore, the hydrated biological specimens in an aqueous vitrified condition are electron beam sensitive. At this point, negative staining still has some advantage in the fact that the stain envelope around the embedded biological particle protects the particle, and leads to sharp high contrast images. Although the procedure is similar to cryo-embedding method, cryo-negative staining technique had been introduced in cryo-TEM [15, 16]. To obtain superior images at higher resolution level by TEM will always remain difficult whichever approach was taken.

![Diagram of NtrC](image)

**Fig. 2** Domain structure of NtrC.

![Electron micrographs](image)

**Fig. 3** Nickel functionalized lipid nano-tubules, and His-tagged proteins on nickel functionalized lipid tubules. Electron micrographs of (a) the lipid nano-tubules preserved in the buffer containing 90% GalCer and 10% DOGS-NTA-Ni; (b), (c) His-tagged protein (His)$_8$NtrC adsorbed on nickel functionalized tubule. (b) and (c) are prepared under different incubation time. Visibility of inner wall of the tubule is due to the existence of permeated buffer in the cavity. (d) His-tagged protein (His)$_8$-N-NtrC (N-terminal domain of NtrC) adsorbed on nickel functionalized tubule. Unbound excess proteins are seen aggregated into chain-like architectures alongside the lipid tubule. (e) His-tagged Fab fragment adsorbed on nickel functionalized tubule in a helical array. Scale bar, 100 nm.
3. Electron microscopy of GroEL\textsubscript{SP/MC} chaperonin nanotubes

3.1 Overview of the chaperonin

Chaperonin is a cytosolic protein that mediates protein folding in an ATP-dependent manner [17, 18]. A higher molecular weight complex of GroEL (∼840 kDa, i.e. 14-mers) has been described as the prominent species in \textit{E. coli} and the asymmetric GroEL-GroES complex has been depicted \textit{in vitro} as soluble fractions from the living cells [18, 19]. X-ray crystallography on GroEL confirms the hollow cylindrical shape constructed with two heptamer rings consisting of 57 kDa subunits stacked with dyad symmetry [20]. The subunit can be subdivided into three domains; that is, equatorial-, intermediate-, and apical-domain, respectively. The equatorial domain is the largest and is responsible for the construction of each heptamer ring and for intersubunit contact between the two stacked rings in GroEL. The end region of the central cavity is constructed with the apical domains of the subunits, which as a domain involved in interactions with a substrate polypeptide and GroES. The apical domain is linked to the equatorial nucleotide-binding domain by the intermediate domain. The formation of a metastable GroEL-GroES complex with a folding intermediate appears to be taking place at the common site for substrates that interact with GroEL [21]. This structure is often referred to as ‘bullet-shaped’ after Thermus thermophilus holo-chaperonin (belonging to group I chaperonins) which was the first protein to have its structure in a functional form observed by TEM [22, 23].

The filamentous complex (one-dimensional assembly) of \textit{E. coli} GroEL and GroES was found to form when GroEL and GroES at relatively low molecular rate (1:1 to 1:3) were incubated at room temperature in the presence of ATP and magnesium [24]. A similar filamentous chain formation of group II chaperonins was found in \textit{Sulfolobus shibatae} [25] and in \textit{Sulfolobus tokodaii} [26]. These filamentous cytoskeleton-like assemblies consisting of chaperonin molecules attract keen interests in nano-biotechnology and biomedical research fields [5, 27].

3.2 Materials and methods

Chaperonin GroEL used in the study was modified site-specifically at the entrance of its central cavity with a number of photochromic (spiropyran/merocyanine) units, which allowed the switching of open/close gating motion of GroEL by ATP and light [27]. The mutant GroEL\textsubscript{SP/MC} was prepared introducing 14 Cys residues in each entrance of the cavity of pre-constructed mutant GroEL (all Cys were replaced with Ala, Lys-311 and Leu-314 were replaced with Cys). Construction of GroEL\textsubscript{SP/MC} was performed according to the previous report [5]. Briefly, after the incubation allowing the reaction of Cys on the mutant GroEL with spirobensazopyran-appended maleimide for 12 h at 4 °C, the color of the mixture turned light-purple indicating that the spontaneous reaction of partial isomerization of SP to MC had occurred. Using the gel filtration chromatography to remove the unreacted substances, the purified protein was used as GroEL\textsubscript{SP/MC}. Suitable conditions for GroEL\textsubscript{SP/MC} to form self-linked one-dimensional assemblies, precisely GroEL\textsubscript{SP/MC} chaperonin nanotubes, were examined assessing the buffer conditions, incubation periods, and incubation temperature. Influence of coexistence of monovalent, or divalent cations, and with/without nucleotides, was monitored by TEM.

In electron microscopy, aliquots of the solution were applied onto EM specimen copper grids covered with a thin carbon support film which had been made hydrophilic by glow-discharge (JEOL HDT-400). Grids either were negatively stained with 1.0 % uranyl acetate. Electron micrographs were recorded by making use of the slow scan CCD camera (Gatan Retractable Multiscan Camera) under low electron dose condition on a FEI Tecnai F20 (FEI Company, the Netherlands) operating at 120 kV at magnification of 50,000×.

3.3 Results and discussion

Figure 4 shows a schematic drawing of the occurrence of GroEL\textsubscript{SP/MC} chaperonin nanotubes. Electron micrographs of GroEL\textsubscript{SP/MC} chaperonin nanotubes are shown in Fig. 5. The typical stripes of GroEL in side-on view are clearly seen perpendicular to the cylindrical long axis under each condition. As shown in Fig. 6, the factor most influenced the formation of the nanotubes was found whether divalent cations co-exist or not. It has been known that the binding of ATP alters the conformation of each GroEL subunit (apical domain) causing it to elongate. This may not critically contribute towards the axial stacking of GroEL\textsubscript{SP/MC} molecules facing at each end-on domain. The interesting and fascinating features of molecularly engineered chaperonin GroEL\textsubscript{SP/MC} into a tubular biocontainer is discussed in detail by Biswas et al. [5]. As they reported, even GroEL\textsubscript{SP/MC} possesses the binding capability of denatured proteins into its central cavity. This means that the tube exactly functions as connected cages to transport the object of purpose within.

As mentioned above, it has been reported that wild-type GroEL forms filamentous assembly with GroES [24]. It appears that this process is of low reproducibility or that it happens under the special conditions. We could hardly reproduce the one-dimensional assembly using \textit{E. coli} wild-type GroEL and GroES. To introduce an artificial joint is critical for engineering, once the conditions were established (though it is actually difficult). Its reliability and reproducibility can be enhanced and thus widen the range of applications.

The clue to design which allows for the spontaneous one dimensional assembly of artificially modified chaperonin GroEL\textsubscript{SP/MC} into micrometer-long hollow cylinders with a remarkable mechanical stability was examined and confirmed. TEM has proven to be very useful for morphological observation of the nano-architectures formed from soft
biomaterials like protein macromolecules. Without the morphology revealed by the technique, we can hardly gain insight into how the ordering is established in the microscopic scale.

Fig. 4 A schematic drawing of 1D assembly of GroEL chaperonin inducing a nanotube.

Fig. 5 Electron micrographs of negatively stained one-dimensionally assembled GroELSP/MC after mixing with (a) 5 mM ATP-Mg, (b) 5 mM ADP-Mg, (c) 5 mM GTP-Mg, (d) 5 mM CTP-Mg, and (e) 5 mM ITP-Mg. Nano-tubular structures with typical stripes of GroEL in perpendicular to the cylindrical long axis are clearly seen under each condition. Scale bar, 50 nm.
Electron micrographs of negatively stained one-dimensionally assembled GroEL SP/MC after mixing with divalent cation, 5 mM Mg\(^{2+}\) (a) after 5 min-, (b) 15 min-, (c) 3 h-incubation at 37 °C. Divalent metal ions such as Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), and Zn\(^{2+}\) triggered 1D assembly of GroEL SP/MC. Monovalent cations such as Na\(^{+}\), K\(^{+}\), and Cs\(^{+}\) hardly induced the assembly. (d) Addition of EDTA (25 mM) resulted in dissociation of the nano-tubules into shorter components. (e) Without Mg\(^{2+}\), no more one-dimensionally assembly happened. Scale bar, 50 nm.

4. Electron microscopy of graphitic nanotubes

4.1 Overview of the graphitic nanotubes

The discovery of carbon nanotubes (CNTs) has vitalized research activities in nanotechnology [28], and a central component of nano-structured materials science [29–31]. The single-walled carbon nanotubes (SWCNTs) have a cylindrical nano-structure with a high aspect ratio and possess a wall consisting of a rolled-up graphene sheet with a large π-electronic surface. Nano-structures consisting of π-conjugated molecules have attracted keen interests because of their potential for use as innovative materials and in electronics, and so forth. Aromatic core based polycyclic hydrocarbons are often utilized as building blocks for these nano-composites [32]. This section will discuss the case of Hexa-peri-hexabenzocorone (HBC) which is an example of a highly extended π-conjugated system.

4.2 Materials and methods

Synthesis of HBC amphiphiles were performed according to the previously reported methods [10, 33]. The suspension containing the HBC derivative of interest was incubated under the optimized condition allowing the tubular formation. Aliquots of sample solution were applied to a specimen grid covered with a thin carbon film. The excess solvent liquid was removed blotted with a torn filter paper and dried at room temperature. Electron micrographs were recorded by making use of the slow scan CCD camera (Gatan Retractable Multiscan Camera) under low electron dose condition at magnifications of 9,600×, 25,000×, 50,000×, and 100,000× in a FEI Tecnai F20 electron microscope (FEI Company, the Netherlands) operated at anode voltage of 120 kV. The images were analyzed on computers using Digital Micrograph (Gatan Inc.).
4.3 Results and discussion

Through a serendipitous observation by TEM of the precipitates in the solution containing HBC derivatives, nanotubular assemblies having a single wall and axial hollow cavity appeared with increasing magnification. In the initial survey stage, we had applied the negative staining method with a modification using ruthenium tetroxide to the bundles of HBC derivatives’ nano-tubules. However, this nano-structure was naturally conductive, and we eventually noticed that it was resistant to electron beam irradiation. It was convenient to observe the target nanotube alone as is, although careful attention was paid in observing and shooting the images as it appeared on the fluorescent screen under the low electron dose mode (Fig. 7).

Carbon is perhaps the only element which has an infinite number of allotropes. Some of them can appear very similar. For example, carbon nano-fibers and carbon nanotubes (both multi-walled and single-walled) look similar when observed with scanning electron microscopy. Former is a solid filled one dimensional nano-structure while the later is a hollow concentric one dimensional nano-tubule. It is almost impossible to distinguish between carbon nano-fibers and carbon nanotubes unless and until one observes such materials by TEM. TEM gives direct insight into the nano-structure of carbon materials. Absence of TEM observations can lead to wrong conclusions in the case of carbon nano-materials. It is the most important and most reliable technique for correctly identifying the nature and the form of carbon nano-materials in academic research and in industry.

However, one must be always keep in one’s mind that overconfidence on TEM images can be perilous. Because TEM image is a two-dimensional projection image of three-dimensional substances, it is hard to tell from a sole projection image such as a helical structure which handedness is true for the original substances. Additional information is necessary and can be achieved by shadow casting, i.e., the TEM image of the same target whose surface has been vacuum-deposited with precious metals. SEM, or AFM images can be used complementarily.

5. Conclusion

The importance of TEM in the evaluation at each organization level of nano-tubular assemblies consisting of the organic lipids, and/or protein macromolecules, and of aromatic core based carbon nano-materials were reviewed. The classic but very useful negative staining has still been attracting wide range demands among electron microscopists dealing with organic nano-substances. If higher accessibility to TEM and cryogenic facilities were available, and an electron microscopist had sufficient time allotted for electron microscopic experiments, it would be better to examine the same target materials by making specimens with the different methods.

Cryo-TEM approaches were developed and put to practical use in early 1980s, which made possible the preservation of biological particles and macromolecules in their native quaternary structure in a fully aqueous environment. The determination of protein structure and function at or near atomic resolution range is a challenge that can be met by cryo-TEM. It must be a promising approach to determine a fine delicate protein structure. In late 1990s, a compromise approach between the conventional negative staining and cryogenic aqueous vitrified fixation, so called cryo-negative staining was presented, using ammonium molybdate as an agent in heavy metal staining [15, 16]. Nonetheless, negative staining has been utilized as a useful and successful fixation method and numerous electron microscopists routinely use these simple, rapid, highly and reproducible methods.
Although negative staining allows for electron microscopic images with sharp high contrast, it is important to realize what is measured in the negatively stained images. In the aqueous vitrified surrounding, the protein molecule itself is visualized, thus the contrast is very low at close to focus. In negative staining, it is not the biological molecule by itself that is visualized but the excluded in the embedded layer of heavy metal salt. We have recently tried to capture high contrast images using the modified negative staining technique with meglumine gadoterate, gadolinium acetate, samarium acetate, and so on, substituting uranyl acetate. TEM with negative staining continues to open new opportunities in nano-composites consisting of protein molecules as a way to study protein functions [34, 35].

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