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Energy-filtered transmission electron microscopy of biological samples on highly transparent carbon nanomembranes

Daniel Rhinow [a,*], Matthias Büenfeld [b], Nils-Eike Weber [b], André Beyer [b], Armin Gölzhäuser [b], Werner Kühlbrandt [a], Norbert Hampp [c], Andrey Turchanin [b]

[a] Max-Planck-Institute of Biophysics, Department of Structural Biology, Max-von-Laue-Straße 3, D-60439 Frankfurt, Germany
[b] University of Bielefeld, Department of Physics, Universitätsstraße 25, D-33615 Bielefeld, Germany
[c] University of Marburg, Department of Chemistry, Hans-Meerwein-Straße, D-35032 Marburg, Germany

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ABSTRACT

Ultrathin carbon nanomembranes (CNM) comprising crosslinked biphenyl precursors have been tested as support films for energy-filtered transmission electron microscopy (EFTEM) of biological specimens. Due to their high transparency CNM are ideal substrates for electron energy loss spectroscopy (EELS) and electron spectroscopic imaging (ESI) of stained and unstained biological samples. Virtually background-free elemental maps of tobacco mosaic virus (TMV) and ferritin have been obtained from samples supported by ~1 nm thin CNM. Furthermore, we have tested conductive carbon nanomembranes (cCNM) comprising nanocrystalline graphene, obtained by thermal treatment of CNM, as supports for cryoEM of ice-embedded biological samples. We imaged ice-embedded TMV on cCNM and compared the results with images of ice-embedded TMV on conventional carbon film (CC), thus analyzing the gain in contrast for TMV on cCNM in a quantitative manner. In addition we have developed a method for the preparation of vitrified specimens, suspended over the holes of a conventional holey carbon film, while backed by ultrathin cCNM.

1. Introduction

Electron cryo-microscopy (cryoEM) of vitrified biological specimens is a powerful method for the analysis of macromolecular structures, ranging from single particles up to tomographic volumes of whole cells [1–4]. Biomolecules are phase objects and image contrast is degraded by radiation damage, inelastic scattering, electrostatic charging, and specimen movement [5]. Among the technical efforts to increase the notoriously low signal-to-noise ratio of EM images of biological macromolecules the development of support films other than the routinely used amorphous carbon has attracted interest of several groups. Various new materials have been tested recently as support films for electron microscopy, among them conductive amorphous TiSi alloys [6] as well as ultrathin carbonaceous substrates like graphene [7], graphene oxide [8,9], and carbon nanomembranes [10–12].

The implementation of energy filters to remove inelastically scattered electrons has considerably improved structural analysis of ice-embedded biological specimens [13,14]. Furthermore, an energy filter converts a conventional electron microscope into a powerful analytical tool for electron energy loss spectroscopy (EELS) and electron spectroscopic imaging (ESI) [15]. Widely used in materials science, there is growing interest in soft matter and biological applications of EELS and ESI [16–20].

In this work we combine the advantages of ~1 nm thin carbon nanomembrane (CNM) support films with the potential of energy-filtered transmission electron microscopy (EFTEM) and demonstrate that CNM are well-suited for background-free mapping of chemical elements within stained and unstained biological specimens. Furthermore, using two different ways of specimen preparation, we demonstrate that conductive carbon nanomembranes (cCNM) are promising support films for cryoEM of ice-embedded specimens.

2. Materials and methods

2.1. Fabrication of CNM and cCNM support films

Ultrathin carbon nanomembranes (CNMs) were fabricated by transferring crosslinked self-assembled monolayers (SAMs) of 1,1′-biphenyl-4-thiol (BPT, Platte Valley Scientific) onto TEM grids. The BPT SAMs were prepared on gold/mica substrates with a thickness of the gold layer of 300 nm (G. Albert PVD, Silz, Germany). The substrates were cleaned in a UV cleaner (UVOH 150 LAB from FHR, Germany) for 5 min, rinsed with ethanol and

* Corresponding author. Tel.: +49 69 6303 3050.
E-mail address: daniel.rhinow@biophys.mpg.de (D. Rhinow).

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dried in a stream of nitrogen. Afterwards they were immersed in a 1 mM solution of BPT in N,N-dimethylformamide (DMF p.a., Sigma-Aldrich, dried with 0.4 nm molecular sieve) at room temperature (RT) for 3 days. Subsequently the samples were rinsed with DMF, ethanol (p.a., VWR) and finally dried in a stream of nitrogen. This preparation results in densely packed BPT SAMs on Au with a thickness of $\sim$1 nm [21]. The BPT SAMs were crosslinked by electron exposure [22] with a flood-gun (Specs FG20) in high vacuum ($<5\times10\exp(-7)$ mbar). Electron energy of 100 eV and a dose of $\sim$50 mC/cm^2 (80 e$^-$/Å^2) were applied to achieve complete crosslinking [23] and to transform a SAM into a molecular nanomembrane consisting of disordered aromatic carbon rings. The dose was measured with a Faraday cup in close proximity to the sample.

To fabricate cCNMs the cross-linked BPT SAMs were annealed at high temperatures under UHV conditions. To this end, cross-linked BPT SAMs (nanomembranes) with the underlying gold layer were first cleaved from the mica by immersion in hydrofluoric acid (48%) for 15 min and transferred onto a clean quartz substrate using a spin-coated (500 nm) and baked layer of polymethylmethacrylate (PMMA) for stabilization [11]. The PMMA layer was then dissolved in acetone to yield a clean nanomembrane surface. The samples were annealed at $\sim$1200 K in Mo sample holders with a resistive BN-heater using a heating/cooling rate of $\sim$150 K/h and an annealing time of $\sim$0.5 h. Annealing of the nanomembranes on the Au/quartz substrates results in substantially lower defect density in cCNMs in comparison to the annealing on the original Au/mica substrates [11]. Annealing temperature was controlled with a Ni/Ni–Cr thermocouple and two-color pyrometer (SensorTherm). This procedure transforms insulating CNMs into cCNMs with a sheet resistivity of $\sim$100 kΩ/sq at RT [11]. Accounting for the thickness of cCNM, which was determined to 0.7 nm in former work [11], this corresponds to a bulk resistivity of $\sim7\times10\exp(-5)$ Ωm.

Transfer of both CNMs and cCNMs from the gold surfaces onto TEM grids was done by removing a PMMA/nanomembrane/Au stack from the underlying mica or quartz surface as described above, etching gold in an I_2/KI-etch bath (15 min) and transferring the nanomembrane/PMMA to TEM grids with the holey carbon or lacey carbon films (Plano). Afterwards the PMMA was dissolved in acetone using a critical point dryer to yield clean nanomembranes. Such a transfer procedure is very effective and results in large-scale and robust nanomembranes with the size of suspended areas more than 200 μm [24,25].

2.2. Conventional TEM of negatively stained TMV on CNM

Tobacco mosaic virus (TMV) was a kind gift of Ruben Diaz-Avalos (New York Structural Biology Center). TMV was prepared

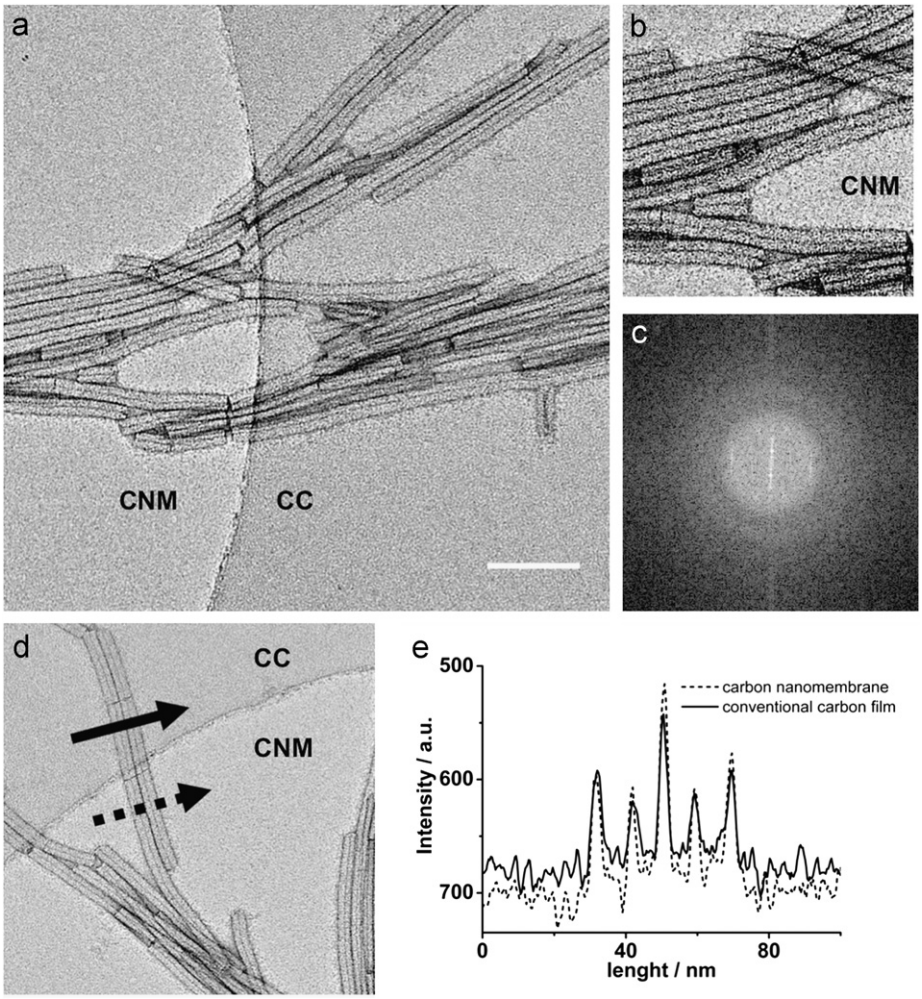

Fig. 1. TEM images (non-filtered) of negatively stained TMV at room temperature on CNM support films. (a) The contrast of stained TMV on CNM is significantly higher compared to TMV on conventional carbon (CC). The scale bar is 100 nm. (b) Image and (c) corresponding Fourier transform of TMV on CNM. (d) and (e) Image intensity measured along line sections perpendicular to TMV on CNM (dashed arrow) and CC (solid arrow) supports.