Journal of Structural Biology 185 (2014) 405–417

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Chemically functionalized carbon films for single molecule imaging

Marc C. Llaguno [a], Hui Xu [a], Liang Shi [a], Nian Huang [b,c,1], Hong Zhang [b,c], Qinghua Liu [b], Qiu-Xing Jiang [a,*]

[a] Department of Cell Biology, UT Southwestern Medical Center, 6000 Harry Hines Blvd, Dallas, TX 75390, USA
[b] Department of Biochemistry, UT Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390, USA
[c] Department of Biophysics, UT Southwestern Medical Center, 6000 Harry Hines Blvd, Dallas, TX 75390, USA

ARTICLE INFO

Article history:
Received 17 August 2013
Received in revised form 8 January 2014
Accepted 11 January 2014
Available online 21 January 2014

Keywords:
Chemical functionalization
Carbon films
Biological ligands
Single particle cryoEM
C3PO/RNA complex
ChemiC

ABSTRACT

Many biological complexes are naturally low in abundance and pose a significant challenge to their structural and functional studies. Here we describe a new method that utilizes strong oxidation and chemical linkage to introduce a high density of bioactive ligands onto nanometer-thick carbon films and enable selective enrichment of individual macromolecular complexes at subnanogram levels. The introduced ligands are physically separated. Ni-NTA, Protein G and DNA/RNA oligonucleotides were covalently linked to the carbon surface. They embody negligible mass and their stability makes the functionalized films able to survive long-term storage and tolerate variations in pH, temperature, salts, detergents, and solvents. We demonstrated the application of the new method to the electron microscopic imaging of the substrate-bound C3PO, an RNA-processing enzyme important for the RNA interference pathway. On the ssRNA-linked carbon surface, the formation of C3PO oligomers at subnanomolar concentrations likely mimics their assembly onto ssRNA substrates presented by their native partners. Interestingly, the 3D reconstructions by negative stain EM reveal a side port in the C3PO/ssRNA complex, and the 15 Å cryoEM map showed extra density right above the side port, which probably represents the ssRNA. These results suggest a new way for ssRNAs to interact with the active sites of the complex. Together our data demonstrate that the surface-engineered carbon films are suitable for selectively enriching low-abundance biological complexes at nanomolar level and for developing novel applications on a large number of surface-presented molecules.

© 2014 Elsevier Inc. All rights reserved.

1. Introduction

Cells function by a fine interplay of macromolecular complexes (Alberts et al., 2007). Quantitative studies of the structures and functions of these complexes at molecular, cellular, and system levels derive deeper and broader insights in almost every frontier of biomedical research. However, many of these complexes have low copy numbers inside cells. Different techniques have been developed to enrich them for experimental investigations (Dong et al., 2008). We are interested in engineering a selection surface that allows affinity-based retention of macromolecular complexes in high density. Such a modification will introduce very little mass to the surface of the substrate, and thus very low background noise, but will be capable of selecting target complexes of low natural abundance or with a low expression level in heterologous systems. The enriched molecules would be suitable for many single molecule studies and for assembling multi-component biological processes in a controlled manner.

To develop such a method in a quantitative manner, we need to examine the selective enrichment of macromolecules on a substrate at the nanometer scale, and need to use electron microscopy to visualize them and quantify their surface density. Nanometer-thick carbon or graphene films appear to be the ideal substrates because of their mechanical strength and chemical stability. In the meantime, it is quite clear that the new substrates will be useful for preparing cryoEM specimens.

The current sample preparation method adopted for cryoEM was pioneered by Dübochet et al. (Adrian et al., 1984; Dubochet et al., 1982). It has, in the past three decades, generated great success in cryoEM study of biological complexes that are available at microgram or milligram levels, and helped resolve atomic-resolution structures of a few high-symmetry particles (Yu et al., 2008; Zhang et al., 2008). However, this powerful technology usually leads to the loss of more than 95% of the biological preparations. Alternative ways have been designed to deal with this issue. Double-stranded DNA scaffolds (Selmi et al., 2011), 2D streptavidin crystals (Wang et al., 2008), and lipid monolayers (Kelly et al., 2008, 2010) are three

* Corresponding author. Fax: +1 214 648 5814.
E-mail address: qiu-xing.jiang@utsouthwestern.edu (Q.-X. Jiang).
[1] Present address: Hauptman-Woodward Institute, 700 Ellicott Street, Buffalo, New York 14203, USA.

http://dx.doi.org/10.1016/j.jsb.2014.01.006
1047-8477/© 2014 Elsevier Inc. All rights reserved.

examples. But these three methods all introduce significant amounts of biomass into the final specimens and need specific conditions to maintain the integrity of the introduced materials. In contrast, the carbon-based engineering method to be described next introduces negligible biomass, and can tolerate a broad range of pH variation, harsh solvents (including some organic ones), high/low temperatures, and detergents. We developed procedures to introduce reactive carboxylate groups on the surface of 3–5 nm thick carbon films at a high surface density. We introduced new procedures to keep the chemically modified carbon films stable and clean for biological applications. We optimized reaction conditions to introduce Ni-NTA, DNA/RNA oligos, and Protein G to the surfaces of these carbon films. We quantified the surface density and binding affinity of these introduced groups separately. These surface-presented biological ligands retain their activities and are suitable for selective enrichment of target biological complexes.

To demonstrate the practical application of the new surface engineering method for single molecule studies, we used it to study single-stranded (ss)RNA-induced assembly of C3PO, component 3 Promoter of RNA Interference Silencing Complex (RISC), by single particle electron microscopy. C3PO is a newly characterized ribonuclease and its active complex is believed to contain six translin subunits and two TRAX subunits that form a hetero-octameric barrel with two active sites located inside (Ye et al., 2011). The endonucleolytic activity of C3PO is important for activating duplex small-interfering (si)RNA-initiated RISC, the effector complex of RNA interference (RNAi). In an active RISC, a single-stranded siRNA (called guide strand) directs Argonaute2 (Ago2) endonuclease to cleave complementary mRNAs (Liu and Paroo, 2010; Miyoshi et al., 2005). While it has been shown by several published studies that C3PO is active only in the hetero-octameric form, it remains unclear how a substrate ssRNA is able to reach the interior of a C3PO barrel where its active sites are located (Tian et al., 2011; Ye et al., 2011). Recent crystallographic and EM studies have resolved several structures of C3PO in the absence of ssRNA. The crystal structure of the full-length human C3PO exhibits an asymmetric hetero-octamer made of six translin and two TRAX subunits (6:2 translin/TRAX) (Ye et al., 2011), which was proposed to be the active conformation based on mutagenesis and functional studies in solution. While the crystal structure of a truncated *Drosophila* C3PO mutant was hexameric [4:2 translin/TRAX; see (Tian et al., 2011)], the EM reconstruction of its full-length version appeared octameric (6:2 or 5:3 translin/TRAX). Intriguingly, in both cases, the RNA-binding sites and the catalytic residues for the C3PO RNA-processing activity are located at the interior surface of the octamer. It was proposed that C3PO might cleave short ssRNAs within its fully enclosed barrel. However, a challenging question is how an ssRNA is recruited to the interior of a C3PO complex. Our new carbon-based engineering technology makes it possible to present individual RNA or DNA molecules at spatially separated sites, similar to the presentation of the passenger RNA strands on the surface of individual Ago2/nicked dsRNA complexes. We were able to use these anchored ssRNAs to guide the assembly of C3PO complexes. It is possible that the C3PO complexes assembled on individual RNA oligos will recapitulate the properties of their *in vivo* assemblies on inactive Ago2 complexes. Single particle reconstruction of C3PO by negative-stain EM showed an olive-shaped structure, which resembles the asymmetric octamer (6:2 translin/TRAX) of an RNA-free human C3PO. A clear difference is that on one side, the EM map has a sizable opening, which is large enough for ssRNA molecules to bind or pass through. A cryoEM map at 15 Å resolution showed extra density above the side port, which likely came from the ssRNA bound to the C3PO complex laterally. Our results suggest that the enclosed octameric barrel of an RNA-free C3PO needs significant rearrangements in order to create such a lateral opening and allow an ssRNA to reach the enzymatic active sites from outside. The successful study of C3PO on the functionalized carbon films demonstrates the potential applications of our new technology to the structural and functional studies of many other important biological complexes.

2. Materials and methods

2.1. Grid preparation — ChemiC-coated copper grids

Copper grids were purchased from SPI. They were pre-cleaned with chloroform, 1.0% SDS and 100% ethanol. After air drying, they were stored at room temperature on a filter paper inside a covered petri dish. Immediately prior to use, both sides of the grids were negatively glow-discharged for 1.5 min (EMS 100 Glow Discharge Unit).

Carbon films were thermally evaporated onto freshly cleaved mica sheets from a pair of sharpened graphite carbon rods (Ted Pella, CA) that were heated to melting temperature at a high vacuum of 2.0×10^{-7} Torr inside a Denton Explorer 14 unit. The carbon films on mica sheets were stored at room temperature inside petri dishes for varying amounts of time before being used.

To coat the copper grids, a carbon film on a piece of mica sheet was floated off in a water trough, and slowly settled onto the glow-discharged grids inside the trough. The grids were then slowly dried at 50 °C overnight. Prior to chemical modification, the carbon-coated grids were heated to 200 °C in air for 10 min. We found that this treatment was critical because it allowed the carbon films to adhere very well to the grid surface so that delamination of carbon films was minimized during subsequent steps.

The carbon films on the grids were first oxidized by floating them on top of droplets of 50 μL solution made of 0.40 M $KMnO_4$ and 0.20 M NaOH on a piece of parafilm. After 1.5 h oxidation, the grids were thoroughly washed in a sodium bisulfite solution to remove manganese oxides that were formed during the strong oxidation. We recognized that the sodium bisulfite washing was extremely critical in order to remove electron dense artifacts deposited on the carbon films during the oxidation. We examined the cleanness of the carbon films by EM before moving to the next step. For simplicity, we will call these chemically reactive carbon films the "ChemiC films".

For covalent conjugation reactions, the carboxylate groups on the ChemiC films were first converted into amine-reactive esters by using a zero-length cross-linker, 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide hydrochloride (EDC, Pierce), in the presence of N-hydroxyl-sulfosuccinimide (sulfo-NHS, Pierce). More specifically, the grids were immersed for 10 min in a solution of 5.0 mM EDC, 5.0 mM sulfo-NHS and 0.10 M MES at pH 5.0. In order to quantify the density of amine-reactive groups on the carbon surface, primary amine ($-NH_2$)-containing Quantum Dots (QD 605, amino-modified from Invitrogen) at 1.0 nM in 50 mM borate buffer (pH 8.3) were used as a quality-control assay. We counted the number of QDs in randomly selected areas, and calculated the averaged surface density.

2.2. Ni-NTA-ChemiC films

For selective enrichment of His-tagged proteins to the carbon films, we prepared ChemiC films that were charged with Ni-NTA. 100 μM nitrilotriacetic acid (NTA)-NH_2(AB-NTA from Dojindo Molecular Technologies) was reacted with the NHS-modified ChemiC films for 1 h in a 50 mM borate buffer, pH 8.5. The surface NTA groups were charged for 30 min with 100 μM $NiCl_2$ in water. The resulted Ni-NTA-ChemiC grids were tested for their stability under different pH, temperature, solvents, detergents and salts.

Download English Version:

https://daneshyari.com/en/article/5914038

Download Persian Version:

https://daneshyari.com/article/5914038

Daneshyari.com