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BRIEF COMMUNICATION

Capture of unstable protein complex on the streptavidin-coated single-walled carbon nanotubes

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Abstract Purification of unstable protein complexes is a bottleneck for investigation of their 3D structure and in protein–protein interaction studies. In this paper, we demonstrate that streptavidin-coated single-walled carbon nanotubes (Strep•SWNT) can be used to capture the biotinylated DNA–*Eco*RI complexes on a 2D surface and in solution using atomic force microscopy and electrophoresis analysis, respectively. The restriction enzyme *Eco*RI forms unstable complexes with DNA in the absence of Mg^{2+}. Capturing the *Eco*RI–DNA complexes on the Strep•SWNT succeeded in the absence of Mg^{2+}, demonstrating that the Strep•SWNT can be used for purifying unstable protein complexes.

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Introduction

Biomacromolecular interactions determine all of the biologic processes by forming protein complexes, and structural characterization of these complexes gives vital information on the mechanisms of the interactions (Phizicky and Fields 1995). However, most protein interactions are dynamic and the protein complexes are not stable over time (Berggard et al. 2007). Conventional protein purification methods are either time consuming (such as tandem affinity purification (Burckstummer et al. 2006) and multiple chromatographic separation steps (Austin and Biggin 1996)) or require elution processes, which may lead to protein denaturation (such as the streptavidin beads or the Ni–NTA–6His beads); therefore, they are only suitable for stable complexes (Chen 1990). In our previous papers, a novel protein purification method was described using the streptavidin-coated single-walled carbon nanotube (Strep•SWNT) and graphene oxide (Strep•GO) (Liu et al. 2010a, b). This method is fast as it does not require an elution process and the captured protein is allowed to be immediately characterized by 3D single-particle analysis using cryo electron microscopy (cryo-EM). In this method, the biotinylated species can be captured on

the Strep•SWNT or the Strep•GO via the biotin–streptavidin interaction and can be separated from other soluble compartments because of the large size of SWNT and graphene. More importantly, because of the electron transparency of the affinity substrate of SWNT and GO, the captured protein or protein complex is ready for the cryo-EM observation without the need for any elution steps. Therefore, the purification, sample preparation, and the following structural characterization are rolled in one step using SWNT or GO. Using the Strep•SWNT, we captured a stable protein complex (nucleoid protein HU–DNA complex) (Dame and Goosen 2002; Rouviereyaniv and Gros 1975) on a mica surface and in solution (Liu et al. 2012). In order to show that the Strep•SWNT can also be used for the capture of unstable protein complex for the further applications in 3D structural reconstruction using single-particle cryo-EM analysis (Zhou 2008; Frank 2002; van Heel et al. 2000; Tao and Zhang 2000), in the current study, we tested the capture of an unstable protein complex (DNA–*Eco*RI complex) (Sorel et al. 2006) on the Strep•SWNT and characterized these complexes with atomic force microscopy (AFM) and electrophoresis gel analysis. For the first time, we show that the unstable protein complexes can indeed be captured on these Strep•SWNT, and this paves the way for the cryo-EM analysis for 3D structural characterization of unstable protein complexes.

Results and discussion

The Strep•SWNT was prepared according to our previous method (Liu et al. 2010a). Briefly, the SWNTs were oxidized using dilute HNO_3 biotinylated via a carbodiimide cross-linking reaction to form SWNT–biotin. By adding this product to a vast surplus of the streptavidin to saturate the biotin without inducing cross-conjugation between the SWNTs, the well-dispersed Strep•SWNT was prepared (Liu et al. 2010a). The successful biotinylation and the streptavidin complexation can be confirmed from the height change in AFM (Fig. 1a–c) and the FTIR analysis (Fig. 1d). In AFM, the SWNTs show a diameter of 1–3 nm (Fig. 1a); after biotinylation, the diameter increases to $\sim$2 to 5 nm, which should be due to the addition of biotin–PEG_8-segment on the SWNT's side wall. Then, after complex formation with the streptavidin, the diameter increases to 5–9 nm. Please note that the height of proteins is always underestimated in AFM, possibly caused by their flexibility (Wagner et al. 2009). It can be seen from the AFM image that the streptavidin fully covers the SWNT surface. Using a Bradford assay, we determined the streptavidin content to be 5.3 mg mg^{-1} of SWNT, indicating full coverage of the SWNTs with the streptavidin.

The restriction enzyme *Eco*RI binds to DNA by recognizing the nucleotide sequence GAATTC and cuts the DNA in the presence of Mg^{2+} (Thomas and Davis 1975). It has been shown that *Eco*RI forms unstable complexes with DNA in the absence of Mg^{2+}, with a dissociation constant K_d of 10^7 M and 2.6×10^6 M at low and high ionic strength, respectively. The *Eco*RI–DNA complex easily dissociates in a high ionic strength buffer solution or when adsorbing to mica (with the abundance of negative charges on its surface) and hence was used as a model protein complex to study weak protein–protein interactions (Sorel et al. 2006). Therefore, in this paper, the *Eco*RI–DNA complex was used to check if an unstable protein complex can be captured by the Strep•SWNT. A 1,000-base pair (bp) DNA having GAATTC sequence at one end and a biotin moiety at the other end was prepared by a polymerase chain reaction (PCR) method, allowing its capture on the Strep•SWNT and the subsequent complex formation with *Eco*RI, as shown in Fig. 2a. The DNA was shown to form a complex with *Eco*RI without Mg^{2+} on the mica surface, as shown by AFM (Fig. 3a, b). The white arrows point out the *Eco*RI molecules complexed with DNA and the black arrows point out the free *Eco*RI molecules.

Then, we did a titration of *Eco*RI on DNA on agarose gel to further check the formation of the *Eco*RI–DNA complexes at low ionic strength. As expected, we failed to observe a band shift of the DNA band with increasing *Eco*RI amount, as the *Eco*RI–DNA complex is not very stable (Fig. 4a). This may also be because *Eco*RI has a very small molecular weight (31 kDa) compared to the 1,000-bp DNA (660 kDa). Therefore, we used a shorter DNA (340 bp), which has three *Eco*RI-recognition sites and a biotin moiety at one end. If all of the three recognition sites are occupied, there should be three *Eco*RI (91 kDa) molecules capturing one 340-bp DNA molecule (224 kDa). The band shift assay of

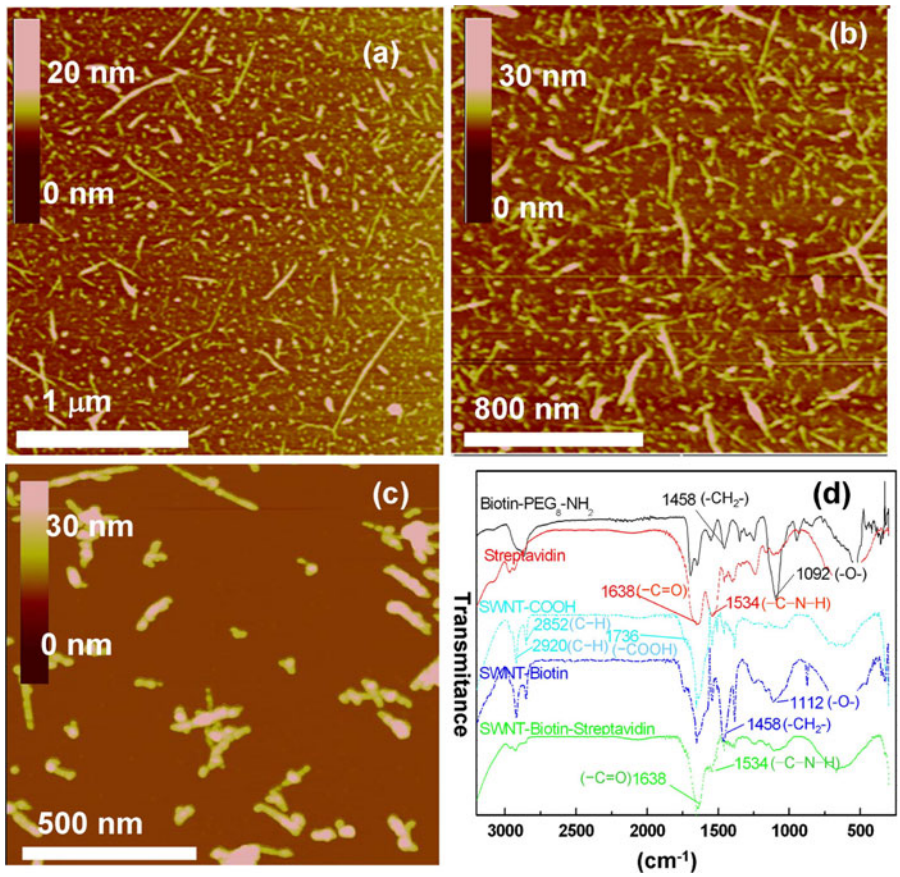

Fig. 1 AFM images of **a** SWNT (average height 1–3 nm), **b** SWNT–biotin (average height 2–5 nm), and **c** the Strep•SWNTs (average height 5–9 nm). The increase in average height confirms the decoration of the SWNTs with biotin and the streptavidin. **d** FTIR analysis confirms that the streptavidin binds to the biotinylated SWNTs. The spectra of the Strep•SWNTs, SWNT–biotin, biotin–PEG_8–NH_2, the streptavidin, SWNT–COOH are shown. The presence of the biotin–PEG_8–NH_2 was confirmed with peaks at 1,458 cm^{-1} (corresponding to CH_2 bending) and at 1,092 cm^{-1} (corresponding to the vibration of the ether group in the PEG segment), which also exist in the FTIR spectrum of SWNT–biotin (1,458 and 1,112 cm^{-1}). The streptavidin shows strong peaks at 1,638 cm^{-1} (corresponding to amide I: –C=O stretch of protein) and 1,534 cm^{-1} (corresponding to amide II: –C–N stretch and –C–N–H deformation), which were also present in the FTIR spectrum of the Strep•SWNTs (ν = 1,638 and 1,534 cm^{-1}) (Park et al. 2008). *Note*: peaks from 2,000 to 2,500 cm^{-1} come from the moisture and are artificially removed from the image

*Eco*RI on 340-bp DNA is shown in Fig. 4b, using 280-bp DNA without any *Eco*RI-recognition site as a control. It can be seen that after lane 3 that the band of the 340-bp DNA gets less bright and new bands appeared, and after lane 5, the band of the 340-bp DNA almost disappeared, as opposed to the 280-bp DNA, indicating successful formation of the specific DNA–*Eco*RI complex in these conditions.

The capture of the *Eco*RI–DNA complex on the Strep•SWNT was achieved via a two-step process: First, the biotinylated DNA (bio-DNA) was captured on the Strep•SWNT and then incubated with *Eco*RI to form the complex with DNA. We have previously shown that a high density of bio-DNA can be captured on the Strep•SWNT and subsequently visualized using AFM (Liu et al. 2010a). Alternatively, the Strep•SWNT coated on the mica was incubated with DNA solution and a high density of DNA was captured on the Strep•SWNT. In that case, individual DNA duplexes can hardly be recognized because the DNA forms a very compact structure through the Maranon effects (Liu et al. 2010a). Here, we used yet another approach, as shown in Fig. 2b. A mica surface covered with the Strep•SWNT was incubated in a dilute DNA (1,000 bps) solution and shaken for 2 min. During this process, the DNA duplexes bind to the Strep•SWNT, as shown in the AFM images in Fig. 3c, d. In this process,

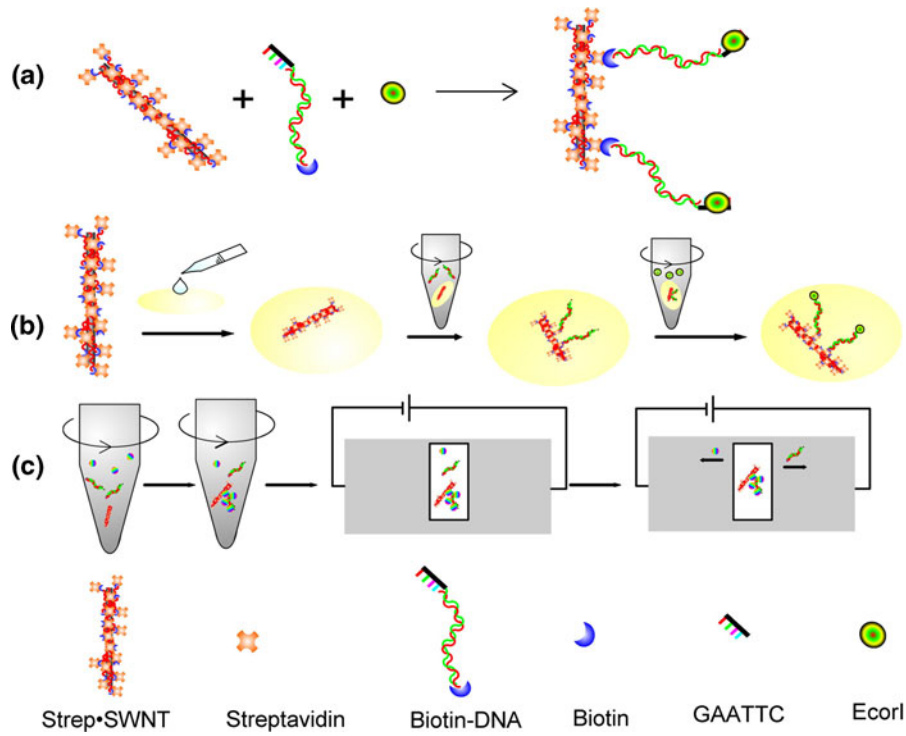

Fig. 2 **a** Schematic representation of the capturing principle of the *Eco*RI–DNA complex on the Strep•SWNTs; **b** schematic representation of absorbing the Strep•SWNTs on mica, capturing biotinylated DNA (bio-DNA), and complexation with *Eco*RI; **c** schematic representation of the purification process of the *Eco*RI–DNA complex captured on the Strep•SWNTs via electrophoresis

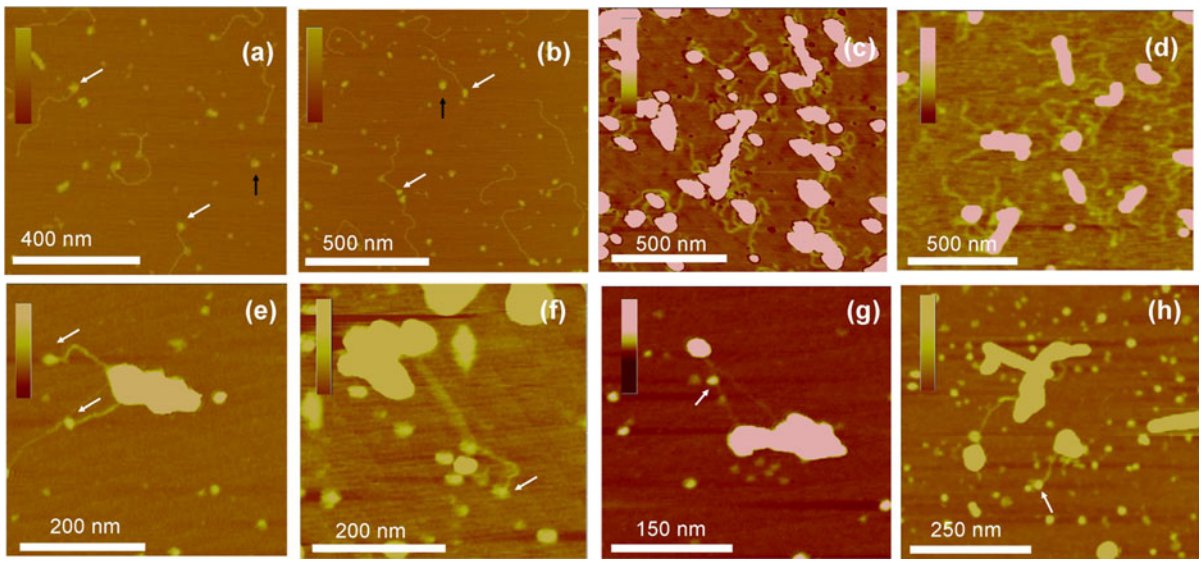

Fig. 3 **a**, **b** AFM images of the *Eco*RI–DNA complexes (pointed by the *white arrows*) and free *Eco*RI molecules (pointed by *black arrows*); **c**, **d** DNA captured on the Strep•SWNTs; **e**–**h** the *Eco*RI–DNA complexes captured on the Strep•SWNTs. *Z scale* 2 nm

a suitable density of the Strep•SWNT on mica is also crucial to obtain clearly recognizable DNA capture (Fig. 3d, as compared to 3c).

Then, the Strep•SWNT•DNA captured on mica was incubated with *Eco*RI to form the DNA–*Eco*RI complex, followed by washing and N_2 drying. The AFM images are shown in Fig. 3e–h. It can be seen that the *Eco*RI–DNA complexes were successfully captured on the Strep•SWNT. As pointed by the white arrows, the *Eco*RI molecules attached to the end of the

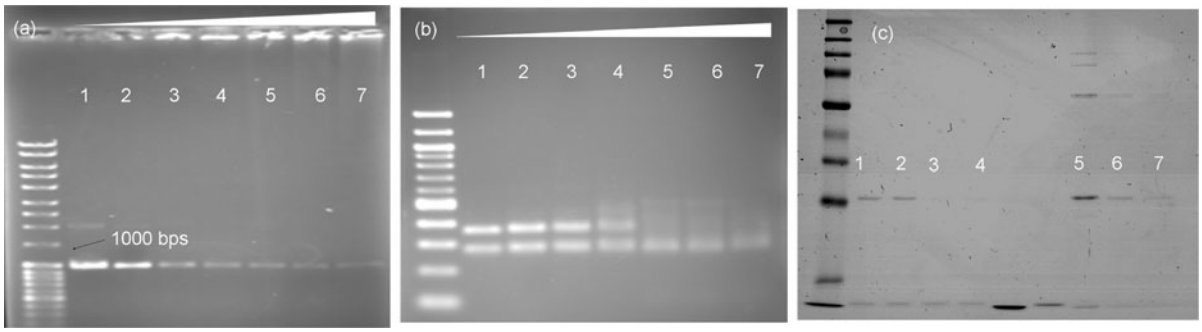

Fig. 4 **a** Band shift assay of bio-DNA captured by the Strep•SWNTs (*lanes 1–7*: bio-DNA 1,000 bp, 50 ng; the Strep•SWNTs (μL), 0, 0.0375, 0.075, 0.1125, 0.15, 0.3, 0.6.); **b** band shift assay of bio-DNA complexed with *Eco*RI (*lanes 1–7*: bio-DNA 340 and 280 bp, 50 ng; *Eco*RI (Units), 40, 80, 200, 400, 600, 800, total volume: 20 μL); **c** SDS PAGE of the bio-DNA–*Eco*RI complexes captured on the Strep•SWNT after electrophoretic "filtration" (*lanes 1* and *2*), control experiments using a mixture of *Eco*RI and the Strep•SWNT after the electrophoretic "filtration" (*lanes 3* and *4*), the Strep•SWNT (*lane 5*), *Eco*RI 200 U (*lane 6*), *Eco*RI 40 U (*lane 7*), indicating successful capture of the DNA–*Eco*RI complexes by the Strep•SWNT

DNA duplexes, which is captured on the Strep•SWNT at the other end. Although there are free *Eco*RI molecules adsorbed non-specifically on the mica (as is the case of the *Eco*RI–DNA complex without using the Strep•SWNTs, Fig. 3a, b), which are not removed by the washing step, the successful capture of the DNA–*Eco*RI complexes on the Strep•SWNT shows a strong basis for further purification of unstable protein complexes using the Strep•SWNT.

In order to provide further evidence of the successful capture of the DNA–*Eco*RI complex on the Strep•SWNT, we purified the DNA–*Eco*RI complex captured on the Strep•SWNT using a filtration process and checked using the SDS PAGE electrophoresis. The filtration process is schematically shown in Fig. 2c. First, the Strep•SWNT, DNA (340 bp), and *Eco*RI were mixed together and loaded on agarose gel for electrophoresis. Upon switching on the current, both the unbound DNA and the *Eco*RI move into the gel, while the DNA–*Eco*RI complex captured on the Strep•SWNT stays in the well as it is too large to migrate into the agarose. Such a "filtration" process only takes about 1 min. The solution that stays in the well was taken out and analyzed by the SDS PAGE assay.

There are strong *Eco*RI bands (lanes 1 and 2) in the presence of DNA, indicating that *Eco*RI is specifically captured by the bio-DNA•Strep•SWNT. In the absence of DNA (lanes 3 and 4), only a very faint band at the position of *Eco*RI is visible, which may be caused by the non-specific adsorption of *Eco*RI onto the Strep•SWNT. Also, note that the impurity proteins present in the original *Eco*RI solution (lanes 5, 6, and 7) are no longer present in the electrophoresis-purified system (lanes 1 and 2). This indicates that this process not only captures the *Eco*RI–DNA complex but also removes the impurities.

In conclusion, the *Eco*RI–DNA complex can be successfully captured on the Strep•SWNT, and this was validated by capturing the complexes on mica and analyzing them in solution using an electrophoretic filtration process. This shows a solid basis for developing efficient purification procedures for other unstable protein complexes, which are compatible with structural characterization with techniques such as cryo-EM, AFM, or mass spectroscopy.

Experimental

The Strep•SWNTs were prepared according to the literature (Liu et al. 2010a). *Eco*RI was purchased from Sigma-Aldrich. Biotinylated DNA was obtained by PCR using the biotinylated primers. The resulting DNA fragments were purified over Micro Bio-Spin 6 chromatography columns (Bio-Rad, Hercules, CA) and further purified using agarose gel.

The Strep•SWNT-covered mica surface was prepared by incubating the Strep•SWNT (10 ng μL^{-1} of SWNT) on freshly cleaved mica for 1.5 min, followed by H_2O rinsing. Capture of DNA on the Strep•SWNT on the mica surface was achieved by putting the

Strep•SWNT-covered mica (diameter: 3 mm) in a DNA solution (2 ng μL, 100 μL, 10 mM Tris, 20 mM NaCl, 1 mM EDTA, pH = 7.5, the same in the following experiments) and shaking for 2 min, followed by H_2O rinsing and N_2 drying.

Capture of bio-DNA by the Strep•SWNT in solution for the band shift assay was achieved by shaking a mixture of the Strep•SWNT and the DNA for 1 h, loading on agarose gel (1 % for 1,000 bp, 2 % for 340-bp DNA) for electrophoresis at 80 V, and staining with ethidium bromide. Band shift titration of the DNA with *Eco*RI was performed by incubating the DNA with *Eco*RI at 37 °C for 20 min and then separating complexes on agarose gel (1 %).

Capture of the DNA–*Eco*RI complex was done in two different ways. (i) The Strep•SWNT-covered mica capturing DNA was put into the *Eco*RI solution (400 U) and incubated at 37 °C for 20 min. (ii) The DNA (5 ng μL^{-1}) and the *Eco*RI (400 U) were incubated at 37 °C for 20 min, and then the Strep•SWNT (12 ng μL^{-1} for the SWNT) was added and the solution was shaken for 5 min. This solution was mixed with the DNA-loading buffer and loaded on an agarose gel (1 %) before electrophoresis at 80 V for 3 min. Then, the solution in the well was taken out for the SDS PAGE analysis. For the SDS PAGE analysis, the sample solution (20 μL) was mixed with the 4× protein-loading buffer (5 μL) and incubated at 95 °C for 5 min and then loaded on a polyacrylamide gel (15 %) for the electrophoresis analysis at 200 V for 1 h, followed by the silver staining.

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