Fabrication of biotin functionalised SiO$_2$ EM grid for studying biotin tagged biomolecules

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A biotin functionalised SiO$_2$ electron microscope grid has been developed for studying various biotin/avidin tagged biomolecules onto the grid surface under electron microscope. As an application, we have shown successful immobilisation of streptavidin gold nanoparticles and biotinylated microtubules through biotin-streptavidin-biotin interaction. This grid will be useful for studying the structures of various biotin-tagged proteins and their complexes under electron microscope.

**Keywords:** Electron microscopy, Surface functionalisation, Silica EM grid, Gold nanoparticles, Nanoparticles, Microtubules, Biotin

Surface chemistry plays a crucial role in surface modification of various materials through covalent chemical functionalization for development of biomedical and biotechnological devices. Biomolecules are highly sensitive and maintaining their functionality in unnatural environment is a challenging task. Therefore, development of biocompatible surface for studying various biomolecular interactions is of high importance.

Long chain polyethylene glycol (PEG) plays a key role in surface modification, because of its hydrophilic nature. Some functional groups like hydroxyl, carboxyl, epoxide, etc. are required for surface functionalisation through covalent chemical reaction, which help in initial covalent bond formation with long chain polymer/PEG. In addition to the biocompatibility of the surface, covalent chemical attachment of various molecules like multivalent head groups, containing tris-nitrilotriacetic acid (Tris-NTA), a chelator for Ni$^{2+}$, sugar, peptide and biotin, has huge importance in various in vitro biochemical studies. These molecules are able to capture various biomolecules on the surfaces and help in studying protein-protein, antibody-antigen and DNA-protein interactions under fluorescence microscopy.

Interactions between fluorophore tagged biomolecules are commonly studied by fluorescence microscopy, but it has limitation of visualising molecules at very high resolution. There are other techniques like crystallography, atomic force microscopy (AFM) and electron microscopy (EM) for visualizing molecules at very high resolution up to the atomic level. Obtaining crystals of large molecules is challenging with low probability and requires longer time. AFM is also a good microscopic technique based on force measurements, it has the limitation of slow scan speed. In this context, EM has many advantages, especially, cryo-EM imaging followed by 3D reconstruction gives atomic resolution structures of molecules and macromolecular complexes. Sample preparation for EM is generally performed in solution, followed by loading onto the grid and imaging.

However, in the case of studying protein-protein or protein-DNA interactions, for understanding the interaction between two different proteins on surface one needs to follow the method of immobilisation of one protein on the surface and visualisation its interaction with another protein, which is present in the solution. Immobilisation of proteins on the surface of EM grid requires functionalisation with a functional group of choice, depending on the tag attached on the protein of interest. Silanisation on SiO$_2$ and amine formation from silicon nitride surface of EM grid for DNA and nanoparticle assembly has been attempted before. Recently, we have developed a method of tris-NTA functionalisation on EM grid for immobilisation of oligohistidin-tagged functional
proteins like microtubule polymerase, XMAP215, and shown the microtubule nucleation from XMAP215 immobilised grid. This method of EM grid functionalisation is a very good platform for immobilisation of oligohistidine-tagged proteins. Now the question remains, how one can study the protein-protein interaction on surface, if the protein is tagged with biotin instead of decahistidine. To solve this problem, herein we have developed a method of functionalisation of SiO₂ EM grid with biotin.

Materials and Methods

Silicon dioxide (SiO₂) 40 nm TEM SQ 100 μ nine window grids were purchased from SPI Supplies Division of Structure Probe Inc. Sulphuric acid (98% GR) and MgCl₂ were purchased from Merck. 3-Glycidoxypropyltrimethoxysilane (GOPTS) was purchased from Fluka. Acetone (analytical reagent grade) was purchased from Rankem. Diamino-polyethylene glycol with MW 3000 (H₂N-PEG₃₀₀₀-NH₂) was purchased from Rapp Polymer. Dry N,N'-dimethylformamide (DMF), taxol, GTP, PIPES and EGTA were purchased from Sigma Aldrich. HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) was purchased from Himedia. EZ-Link NHS-Biotin was purchased from Thermo Scientific. Neutravidin was purchased from Invitrogen. Rhodamine avidin dye was purchased from Vector Laboratories. Confocal dishes (cover glass bottom dish 35×10 mm) were purchased from SPL Life Sciences. Streptavidin gold nanoparticle and uranyl acetate dihydrate were purchased from Nanocs and Spectrochem respectively. All the commercially purchased chemicals were used without further purification. Fluorescence microscopic imaging was done by 10× objective using Nikon imaging solution (NIS) software of Nikon Eclipse Ti microscope.

To study the protein biochemistry, tubulin from goat brain was purified and stored in liquid nitrogen. Biotin labelled tubulin was prepared following previously described method, and stored in liquid nitrogen at two concentrations, i.e., 200 and 150 μM. Tubulin concentrations (UV absorbance) refer to tubulin dimers.

For electron microscopy, the imaging was done using a Tecnai G2 Spirit Biotwin Czech Republic 120 kV electron microscope.

Surface chemistry

Biotin functionalisation on the PEGylated SiO₂ thin (40 nm) film coated TEM window grids is shown schematically in Scheme 1. Functionalisation of SiO₂ EM grids up to PEGylation was achieved as described before. PEGylated SiO₂ thin film coated TEM window grids were reacted with dry DMF solution of EZ-Link Biotin-NHS (25 mg/mL) at 75 °C for 4 hours and were washed with plenty of DMF and MilliQ water followed by drying under the steam of nitrogen gas. The grids were stored at 4 °C.

Transmission electron microscopic studies

For transmission electron microscopic imaging after immobilisation of streptavidin gold nanoparticles onto the biotin functionalised SiO₂ EM grid, 1 μL of streptavidin gold nanoparticle solution was diluted in 9 μL BRB80 buffer and 5 μL of the solution was loaded onto the biotin functionalised SiO₂ grid and incubated for 2 min. The grid was then extensively washed with BRB80 buffer followed by immediate staining with 2% uranyl acetate for 30s and air-dried and imaged under 120 kV electron microscope. Similar procedure was followed using non-biotinylated SiO₂ grid for control experiment.

For preparation of biotin labeled taxol stabilized microtubules, two mixtures A and B were prepared. Mixture A contained 0.5 μL biotin tubulin (15 mg/mL), 2 μL tubulin (27 mg/mL), 2.3 μL BRB80 and 0.2 μL...
GTP-Mg (25 mM). Mixture B contained 180 µL of BRB 80 and 0.36 µL taxol (20 µM). Mixtures A and B were separately warmed at 37 °C for 20 min and then 45 µL mixture B was added into mixture A and mixed carefully. The mixture was centrifuged for 8 min at 12000 rpm at 37 °C. Then the supernatant was carefully discarded and the red colored pellet was dissolved into 10 µL mixture B covered with aluminum foil and stored at room temperature.

For TEM imaging after loading taxol stabilised biotinylated microtubules onto the biotin functionalised SiO$_2$ EM grid, 10 µL neutravidin solution (100 times diluted in BRB80 buffer) was loaded onto biotin functionalised SiO$_2$ TEM grid, kept for 5 min and washed with ice cold BRB80 buffer. Then 10 µL of taxol stabilised biotinylated and Alexa 568 labelled microtubules (previously prepared and diluted in BRB80 buffer at room temperature) were loaded onto the grid and incubated for 10 min. The grid was then washed extensively with BRB80 buffer (room temperature) and Milli Q water, followed by immediate negative staining with 2% uranyl acetate for 30 s. The grid was dried in dessicator and imaged under 120 kV electron microscope. Similar procedure was followed using non-biotinylated SiO$_2$ grid for control experiment.

**Fluorescence studies**

For fluorescence microscopic imaging after immobilisation of rhodamine avidin d dye onto the biotin functionalised SiO$_2$ TEM grid, 5 µL solution of rhodamineavidin d dye (1 µL stock solution diluted in 1000 µL HEPES buffer) was loaded onto the biotin functionalised SiO$_2$ TEM grid and incubated for 5 min. Unbound dye was washed out with HEPES buffer and placed in a confocal dish with functionalised side down, filled with HEPES buffer. The grid was visualised under fluorescence microscope (Nikon Plan Fluo 10X DIC objective). Similar procedure was followed using non-biotinylated SiO$_2$ grid for control experiment.

**Results and Discussion**

Biotin functionalization through covalent chemical reaction with SiO$_2$ EM grid was confirmed by immobilizing rhodamine avidin d red dye onto the grid surface, followed by fluorescence microscopic imaging. We observed red coloured fluorescence from the grid at 561 nm laser light, which indicated that rhodamine avidin d dye was successfully immobilised onto the grid through classical biotin-avidin interaction. It is well known that biotin binds with streptavidin and this interaction has high importance in various protein-protein interaction studies. Intense red coloured fluorescence signal was observed from the thin SiO$_2$ film, which was not attached with the support of the grid (Fig.1(a, b)) on the other hand very low intense red fluorescence was observed from the SiO$_2$ film, adhered onto the support (data not shown) due to the fact that light can only pass through the thin film of functionalized SiO$_2$ and cannot pass through the support. Experiments were also carried out to show that immobilisation of rhodamine avidin d dye is not a consequence of non-specific sticking of the dye with SiO$_2$ EM grid. For this purpose, we incubated rhodamine avidin d dye with non-biotinylated SiO$_2$ EM grid following previously described method and found no red fluorescence onto the grid (Fig. 1(c, d)). Therefore, fluorescence microscopic images clearly indicate successful functionalization of biotin onto the SiO$_2$ EM grid. The above results from fluorescence microscopy clearly indicate that biotin functionalized...
SiO₂ EM grid can serve a platform for studying protein-protein interaction on EM grid by fluorescence microscope.

Our ultimate goal was to develop functionalized grids for studying protein-protein, DNA-protein and other biomolecular interactions on the surface, which can be studied by electron microscope (EM). Therefore, after successful characterization of biotin functionalization of SiO₂ grid through fluorescence microscopy, we have performed electron microscopy. For that purpose, as first proof of the principle experiment, we immobilised streptavidin coated gold nanoparticle onto the biotin functionalised EM grid and observed that the nanoparticles were immobilised onto the grid (Fig 2(b, d)). Similarly, we also checked whether streptavidin coated gold nanoparticle was immobilised onto the non-biotinylated SiO₂ EM grid or not, following the previous method and found after scanning the whole grid that nanoparticles nonspecifically bind onto the grid rarely (Fig 2 (a, c)). This clearly indicates that biotin functionalised SiO₂ EM grid can be used for immobilisation of nanoparticles for various biotechnological applications. To check whether biotin functionalised grid can be used for immobilisation of some other proteins or not, preformed biotinylated microtubules were used. Microtubules are 25 nm wide cytoskeletal filaments, which are formed by polymerisation of αβ tubulin in presence of guanosine 5'-triphosphate (GTP) and are highly dynamic. They play a crucial role in cell division as well as in other cellular functions. Herein, we polymerized biotinylated microtubules in Brinkley Reassembly buffer 80 (BRB 80) containing GTP, unlabelled tubulin and biotin labelled tubulin at 37°C for 20 min. The microtubules were then stabilised by taxol solution in BRB80. Prepolymerised microtubules were incubated with neutravidin immobilized SiO₂ EM grid followed by washing with BRB80. Then the grids were negatively stained with uranyl acetate and imaged under EM, which clearly indicate immobilization of microtubules onto the grid (Fig 3b). Control experiments were carried out to confirm immobilisation of biotin-tagged microtubules only through biotin-avidin interaction and not by non-specific binding with EM grid. For this, we incubated non-biotinylated SiO₂ EM grid with biotin tagged microtubules by the same method as mentioned before and imaged under electron microscope. However, we did not find microtubules on the grid after scanning the whole grid (Fig. 3a), which indicates the binding of microtubules through biotin functionalisation and not through non-specific attachment (Fig. 3c).

Fig. 2 – Electron microscopic characterisation of biotin functionalisation on SiO₂ EM grid. [(a) Snapshot of non-functionalised grid; (b) Snapshot of biotin functionalised grid; (c) Snapshot of non-functionalised grid after incubation with streptavidin conjugated gold nanoparticles; (d) Snapshot of biotin-functionalised grid after incubation with streptavidin conjugated gold nanoparticle].

Fig. 3 – Microtubule immobilisation on biotinylated SiO₂ EM grid surface at room temperature. [(a) Snapshot of non-biotinylated grid after incubation with biotin tagged microtubules reveals absence of microtubules on grid; (b) Microtubules immobilised on biotin functionalized grid through biotin-neutravidin-biotin interaction mode; (c) Cartoon diagram shows immobilisation of biotin tagged microtubule onto biotin functionalised SiO₂ grid through neutravidin].
Conclusions

We have developed biotinylated SiO$_2$ EM grid by a simple chemical reaction and fully characterised the biotin functionalisation by fluorescence and electron microscopic imaging, which clearly demonstrates immobilisation of biotin/avidin-tagged proteins or biomolecules onto this grid. Successful immobilisation of biotin tagged microtubules onto the grid indicates that biotin functionalised SiO$_2$ EM grids are highly biocompatible. Finally, this simple method of biotinylation of SiO$_2$ grid will be useful for studying biomolecular interactions and visualization of complex biomolecular structures on EM grid by first immobilization of biotin-tagged protein and then loading another protein which is known to interact with immobilized protein.

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