

Protein immobilization on carbon nanotubes *via* a two-step process of diimide-activated amidation

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Ferritin and bovine serum albumin (BSA) proteins are chemically bonded to nitrogen-doped multi-walled carbon nanotubes (CN_x MWNTs) through a two-step process of diimide-activated amidation. First, carboxylated CN_x MWNTs were activated by *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), forming a stable active ester in the presence of *N*-hydroxysuccinimide (NHS). Second, the active ester was reacted with the amine groups on the proteins of ferritin or BSA, forming an amide bond between the CN_x MWNTs and proteins. This two-step process avoids the intermolecular conjugation of proteins, and guarantees the uniform attachment of proteins on carbon nanotubes. TEM and AFM measurements clearly confirmed the successful attachment. This approach provides a universal and efficient method to attach biomolecules to carbon nanotubes at ambient conditions.

Carbon nanotubes exhibit interesting electrical, structural and mechanical properties that make them highly promising nanoscale building blocks for the construction of novel functional materials. Many potential applications have been proposed, such as conductive and high-strength composites, field emission displays, fuel cells, sensors, and hydrogen storage media.^{1,2} In addition, biosensors for detecting abnormalities³⁻⁵ and bio-fuel cells⁶ for embedded devices are among the most exciting applications. In order to create the synergy between the biomolecules and nanotubes required to realize these applications, biomolecules, such as proteins and DNAs, must be connected to the carbon nanotubes. This connection can be non-covalent interaction or covalent bonding. There have been several reports on the immobilization of biomolecules on carbon nanotubes,⁷⁻¹³ and most of them use non-covalent interaction. The best stability, accessibility and selectivity, however, will be achieved through covalent bonding because of its capability to control the location of the biomolecule, improve stability, accessibility and selectivity and reduce leaching. In the present study, we report the covalent bonding of proteins to nitrogen-doped multiwalled carbon nanotubes (CN_x MWNTs) *via* a two-step process of diimide-activated amidation between the carboxylic acid groups on CN_x MWNTs and the amine groups on proteins.

To covalently bond molecules to the nanotubes first requires the formation of functional groups on the carbon nanotubes. The carboxylic acid group is often the best choice because it can undergo a variety of reactions and is easily formed on carbon

nanotubes *via* oxidizing treatments, *e.g.*, sonication in sulfuric and nitric acid, refluxing in nitric acid, ozonolysis, and air oxidation. The control of reactants and/or reaction conditions may control the locations and density of the carboxylic groups on the nanotubes, which can be used to control the locations and density of the attached biomolecules. One of the universal methods for connecting biomolecules to other materials is diimide-activated amidation, by direct coupling of carboxylic acid to proteins using *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) or *N,N'*-dicyclohexylcarbodiimide (DCC) as a coupling agent. However, this process leads to undesirable side reactions of intermolecular conjugation of proteins, because most proteins are rich in both amine groups and carboxylic acid groups on their surface. This intermolecular connection can be avoided by using a two-step process: carboxylic acid groups are first converted to active esters *via* diimide-activation, and then the active esters are reacted with the amine groups on proteins without the presence of diimide. Thus, the process can guarantee homogenous attachment of proteins onto carbon nanotubes.

Fig. 1 shows the overview of our covalent attachment process. CN_x MWNTs were acid-oxidized to form carboxylic acid groups on the surface of the carbon nanotubes. Then, the carboxylic acid groups were activated by EDAC, forming a

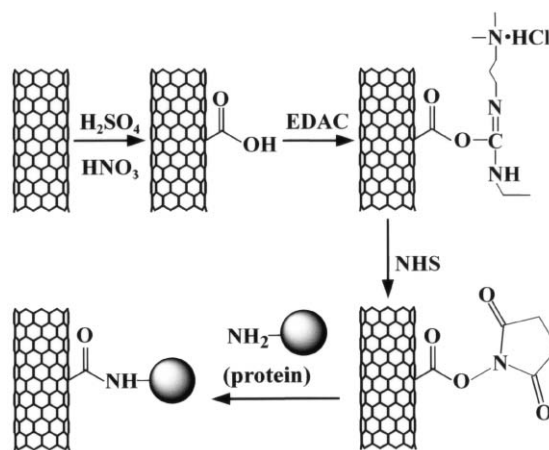


Fig. 1 Schematic view of the attachment of proteins to carbon nanotubes *via* a two-step process of diimide-activated amidation.

highly reactive *O*-acylisourea active intermediate. The intermediate is unstable in aqueous solution, and does not have a sufficient lifetime for the two-step conjugation procedure. However, in the presence of *N*-hydroxysuccinimide (NHS), a more stable active ester (succinimidyl intermediate) can be formed. The active ester undergoes nucleophilic substitution reaction with the amine groups on proteins, resulting in the formation of an amide bond between the CN_x MWNTs and proteins.

CN_x MWNTs were synthesized by pyrolyzing ferrocene/melamine mixtures at 1050 °C in an Ar atmosphere.¹⁴ The as-produced material consists of carpet-like structures containing highly oriented nanotubes of uniform diameter (*ca.* 10–50 nm OD) and length (*ca.* 50–60 μm). The as-produced CN_x MWNTs were suspended in a concentrated sulfuric acid–nitric acid mixture (3:1 v/v) and sonicated in a sonication bath for 2 h. A nanotube mat was obtained after filtration using a 0.45 μm hydrophilized PTFE membrane and washed with deionized water until no acid was detected, followed by drying under vacuum. After the acid-treatment, the CN_x MWNTs dispersed easily in water after sonication for a short period of time. TEM measurements indicated that the acid-treated nanotubes were well separated and shortened (*ca.* 5–10 μm). FT-IR studies revealed that this acid-treatment generated four types of functional groups on the CN_x MWNTs: hydroxyl groups (3424 cm⁻¹), carboxyl groups (1719 cm⁻¹), carbonyl groups (1626 cm⁻¹) and sulfate groups (1384 cm⁻¹).¹⁵

The two-step attachment process is described in detail here. In the first step, 2.0 mg of carboxylated CN_x MWNTs were suspended in 5.0 ml of deionized water by sonicating the mixture for a short period of time. Then, 1.0 ml of a 500 mM MES buffer solution (pH = 6.1) and 2.3 ml of a 50 mg ml⁻¹ NHS aqueous solution were added to the above suspension and mixed. Under fast stirring, 1.2 ml fresh EDAC aqueous solution (10 mg ml⁻¹) was added quickly, and the mixture was continually stirred at room temperature for 30 min. The suspension was then filtered through a 0.45 μm hydrophilized PTFE membrane and rinsed thoroughly with a 50 mM MES buffer solution (pH = 6.1) to remove excess EDAC, NHS and byproduct urea.

In the second step, the esterified carbon nanotubes were re-dispersed in 9.0 ml of a 50 mM MES buffer solution (pH = 6.1) and 1.0 ml of a 10 mg ml⁻¹ protein in MES buffer solution (pH = 6.1) was added. After shaking the mixture on a platform shaker at 150 rpm at room temperature for 1 h, the nanotube suspension was centrifuged and washed with 50 mM MES buffer solution (pH = 6.1) three times to remove unbound protein. The washed protein–nanotube conjugates were dispersed in 50 mM MES buffer solution (pH = 6.1) or deionized water for TEM or AFM measurements. Two kinds of proteins, ferritin from horse spleen and bovine serum albumin (BSA), were attached to CN_x MWNTs in this study.

TEM and AFM studies confirmed the successful immobilization of both ferritin and BSA onto CN_x MWNTs by the aforementioned two-step process. Fig. 2 shows a typical TEM micrograph of ferritin on CN_x MWNTs. Ferritin molecules densely decorate the sidewalls of the carbon nanotubes, and the iron core of each ferritin (~7 nm) can be clearly observed. The decoration is quite uniform and exists as a submonolayer. This indicates that the intermolecular aggregation of the proteins is negligible. The homogeneous positioning of ferritin on the surfaces of the carbon nanotubes is very similar to the attachment of Au particles onto carbon nanotubes that we previously reported.¹⁵ The location of the ferritins is representative of where the carboxylic acid groups were. Because the locations and density of carboxylic acid groups on the carbon nanotubes can be controlled by the oxidation and reaction conditions, we can control the locations and density of the

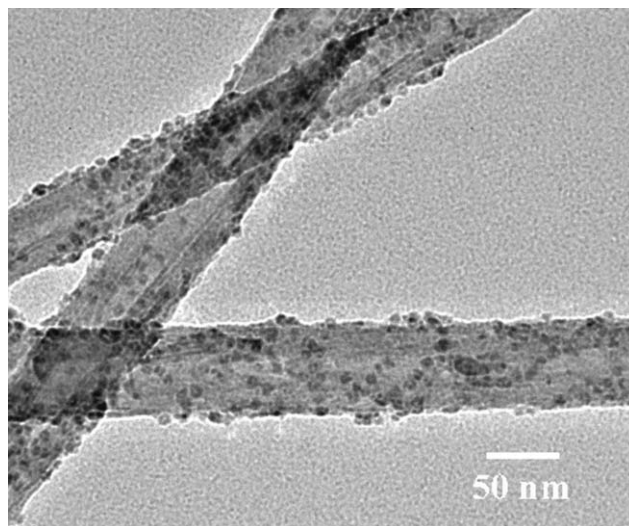


Fig. 2 TEM image of ferritin-CN_x MWNT conjugates.

attached biomolecules, which is extremely useful in some applications for high sensitivities.¹⁶

To confirm the protein immobilization mechanism outlined in Fig. 1, we carried out two control experiments without adding EDAC or NHS. The control experiment without adding EDAC showed that the carbon nanotubes were rather clean with little attachment of ferritin, and the control experiment without adding NHS showed that ferritin aggregates existed and that the carbon nanotubes were almost free of absorbed ferritin on their sidewalls.

Fig. 3 shows representative AFM images of the attached BSA on carbon nanotubes. In Fig. 3(a), a submonolayer of BSA proteins densely decorates the carbon nanotubes and no aggregation is obviously observed. The BSA molecules retain

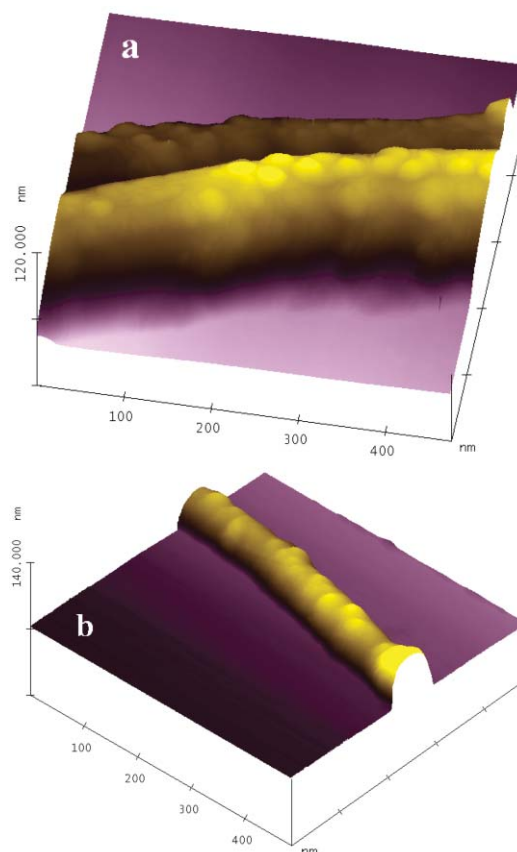


Fig. 3 AFM images of BSA-CN_x MWNT conjugates.

their original globular conformation, and their size estimated from the image is 30 nm. The size discrepancy with the hydrodynamic prolate ellipsoidal shape of BSA, 14×4 nm,¹⁷ is attributed to an AFM tip-broadening effect. In Fig. 3(b), the density of BSA molecules on the sidewall of the carbon nanotube is lower than that in Fig. 3(a). The reason for this is that carbon nanotubes with small diameter may contain fewer structural defects, so they will form fewer carboxylic acid groups through acid-treatment, since carboxylic acid groups are preferably derived from the structural defect sites.^{18,19} Also, carbon nanotubes with small diameter have higher surface curvature, which may not match well with the conformation of BSA molecules.

In summary, we have demonstrated a useful, simple and universal method to attach biomolecules onto carbon nanotubes with covalent bonding. The two-step process was carried out at room temperature in buffer solutions and was accomplished in a short time, which will maximize the survival rate of biomolecules. This method can be easily applied to attach any other useful entities with amine groups, such as nucleic acids, polymers, dendrimers and even inorganic nanoparticles on carbon nanotubes. It is expected that this approach will provide novel nanoscale building blocks for the construction of biosensors, biocatalysts, bio-fuel cells, and other relevant structures.

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