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Covalent Immobilization of Proteins on Carbon Nanotubes Using the Cross-Linker 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide—a Critical Assessment

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Functionalization of carbon nanotubes (CNTs) with proteins is often a key step in their biological applications, particularly in biosensing. One popular method has used the cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to covalently conjugate proteins onto carboxylated CNTs. In this article, we critically assess the evidence presented in these conjugation studies in the literature. As CNTs have a natural affinity for diverse proteins through hydrophobic and electrostatic interactions, it is therefore important to differentiate protein covalent attachment from adsorption in the immobilization mechanism. Unfortunately, many studies of conjugating proteins onto CNTs using EDC lacked essential controls to eliminate the possibility of protein adsorption. In studies where the attachment was claimed to be covalent, discrepancies existed and the observed immobilization appeared to be due to adsorption. So far, bond analysis has been lacking to ascertain the nature of the attachment using EDC. We recommend that this approach of covalent immobilization of proteins on CNTs be re-evaluated and treated with caution.

1. INTRODUCTION

The discovery and subsequent large-scale production of carbon nanotubes (CNTs) have opened a new door in nanotechnology (*1*). Due to their nanosize, exceptional physical strength, chemical stability, and conductive properties, CNTs are being explored for a wide range of applications (*2*). Among these, CNT-based biosensing has drawn particular interest with some extensive reviews published recently (*3*−*7*).

A key step in CNT-based biosensing is the immobilization of a biomolecule on the surface of the CNTs in devices such as electrodes and field effect transistors. The biomolecules are often proteins such as an antibody (*8*), antigen (*9*), or enzyme (*10*). They typically bind to, catalyze a reaction of, or are inhibited by the target molecule. The nanostructure of CNTs, together with their conducting properties, enables tiny signals to be detected and transmitted, leading to the detection of target molecules at extremely low levels. Noncovalent (e.g., adsorption) and covalent conjugation have been reported for the immobilization of various proteins. Noncovalent attachment preserves the unique properties of the CNTs, but the immobilized protein is in equilibrium in a solution and can therefore be gradually lost during the use of the CNT biosensor. On the other hand, covalent conjugation provides durable attachment, but the harsh oxidation step (e.g., with strong acids) disrupts the π-networks on the CNT surfaces and may diminish their mechanical and electronic properties (*11*). Covalent

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Table 1. Some Proteins That Have Been Adsorbed or Claimed to Be Covalently Attached onto CNTs[a]

protein	adsorption (ref)	covalent attachment (ref)
AChE	(*56*)	(*51*)
Antibody		(*17, 18, 20, 21*)
Avidin	(*9*)	(*57*)
BSA	(*9, 32, 33*)	(*12, 13, 53*)
Chymotrypsin	(*30*)	
Cytochrome C	(*23, 24, 26, 58*)	(*26*)
Ferritin	(*26, 27*)	(*13, 26, 27, 52*)
Fibrinogen	(*59*)	
Glucose oxidase	(*26, 34, 60, 61*)	(*15, 26*)
α-glucosidase	(*9*)	
β-glucosidase	(*62*)	
Hemoglobin	(*28, 63*)	
HRP		(*16, 17, 19*)
HupR	(*25*)	
Hydrogenase	(*64*)	(*22*)
β-lactamase	(*23*)	
Lipase	(*32*)	(*50*)
Lysozyme	(*33*)	
Metallothionein	(*23, 24*)	
Myoglobin	(*64*)	(*16*)
OPH	(*65*)	
SBP	(*30*)	(*50*)
Streptavidin	(*9, 25, 29, 66−68*)	(*14*)

[a] AChE, acetylcholinesterase; OPH, organophosphorus hydrolase.

attachment of proteins on carboxylated CNTs using the popular cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was first reported by Huang et al. (*12*) and Jiang et al. (*13*). Since then, it has been widely used in the covalent immobilization of proteins on carboxylated CNTs in biosensing studies (*14*−*22*).

The validity of covalent conjugation of proteins onto carboxylated CNTs using EDC appears to have been accepted without due scrutiny. In this article, we critically examine the evidence presented in the conjugation studies in the literature. We find that conclusive evidence for covalent immobilization is lacking, and that the protein immobilization in most of these studies may be largely due to adsorption.

2. ADSORPTION OF PROTEINS ONTO CNTS

Before we proceed to consider EDC-mediated covalent attachment of proteins on CNTs, it is necessary to briefly look at protein adsorption first.

Tsang and colleagues first reported that small proteins such as metallothionein, cytochrome C, and *β*-lactamase were able to adsorb on the internal as well as outer walls of CNTs after the ends were opened by treatment with concentrated nitric acid (*23, 24*). Since then, both multiwalled (MWCNT) and single-walled CNTs (SWCNT), whether dispersed or oxidized, have been demonstrated to have the ability to adsorb diverse proteins (Table 1). The adsorbed proteins form a coat on the CNT surface when visualized by atomic force microscopy (AFM) or electron microscopy (*25*−*28*). Streptavidin can almost completely cover the surface of MWCNT where it forms a highly ordered helical structure (*25, 29*). Some proteins, such as *β*-glucosidase, chymotrypsin, and soybean peroxidase (SBP), are adsorbed at very large quantities, i.e., at 630, 670, and 575 *μ*g protein per mg of CNTs, respectively (*30, 31*). Bovine serum albumin (BSA) is also adsorbed well and has been reported as an effective agent in dispersing CNTs (*9, 32, 33*).

The adsorption is spontaneous when the protein comes in contact with CNTs in a solution, and follows a pseudosaturation behavior (*30, 31, 34*). The interaction between the CNTs and the protein can be very strong. Using techniques such as FT-IR and AFM, the conformation of the adsorbed protein is observed to be distorted or partially unfolded due to the strong interaction with the CNTs (*23, 30*). As a result of such structural perturbations and other mechanisms, the activities of adsorbed enzymes are reduced by 70−99% for *β*-lactamase, *β*-glucosidase, chymotrypsin, and SBP as compared with the free enzyme in solution (*23, 30, 31*).

The interacting force between CNTs and a protein is predominantly hydrophobic interaction (*25, 29, 33*), although electrostatic interaction and hydrogen bonding can also play a role in some situations (*31, 35*). The relationship between the structure of a protein and its binding to CNTs has been further studied using peptides. Ring structures in aromatic amino acids (e.g., phenylalanine and tryptophan) are important for the interaction between the peptide and CNTs through *π*-stacking (*35*−*37*). However, due to protein folding, the binding ability of a protein to CNTs cannot be predicted by its number of hydrophobic or aromatic residues in the primary structure (*33*).

3. COVALENT IMMOBILIZATION OF PROTEINS ON CNTS USING EDC

3.1. Chemistry of EDC Conjugation. In order to better understand the studies in which EDC is used to covalently attach proteins onto CNTs, it is helpful to look at the conjugation mechanism. EDC is a zero-length cross-linker widely used in protein conjugations (*38*). The conjugation reactions occur in two sequential steps (Figure 1). EDC first reacts with a carboxyl group, forming an amine-reactive *O*-acylisourea intermediate which subsequently reacts with an amine group to produce a stable amide bond. However, the *O*-acylisourea intermediate is very unstable and susceptible to hydrolysis. Such instability results in low coupling efficiency. The addition of NHS (*N*-hydroxysuccinimide or its more water soluble analogue Sulfo-NHS) stabilizes the intermediate by converting it to a semistable amine-reactive NHS ester, thus increasing the coupling efficiency by 10−20 fold (*39, 40*).

The coupling of a protein onto carboxylated CNTs can be achieved in a single-step or a two-step procedure. In the former case, the protein, CNTs, and EDC are mixed in a single tube. The use of NHS is conceptually not essential but will greatly improve the coupling efficiency. In this single-step procedure, interprotein conjugation also takes place and can lead to protein supramolecules or even aggregates. To overcome this problem, a two-step conjugation procedure can be used. The carboxylated CNTs are first reacted with EDC in the presence of NHS (or sulfo-NHS) to yield a semistable amine-reactive NHS-ester. After excess EDC is washed off or deactivated by the addition of *β*-mercaptoethanol (*41*), the activated CNTs are then reacted with a protein. In this case, interprotein conjugation is eliminated because only the CNTs are exposed to the cross-linker. The only covalent bond formed is between the solid CNT support and the protein.

The buffer used in EDC coupling is also an important consideration. The coupling is typically performed at slightly acidic pH in MES buffer (2-morpholinoethanesulfonic acid). The use of phosphate buffer should be avoided, as phosphate reacts with EDC and reduces its stability from a $t_{1/2}$ of 20 h in MES buffer to 0.4 h at pH 6.0 (*42*). If phosphate buffer has to be used, a large excess of EDC can be used to compensate for the instability.

3.2. Conjugation of Proteins onto Carboxylated CNTs Using EDC. Due to the chemical inertness of CNTs, any covalent immobilization of molecules will require chemical modifications to create reactive groups on the surface first. Popular methods include sonicating CNTs in a mixture of sulfuric acid and nitric acid (typically 3:1 v/v) or refluxing in concentrated nitric acid. Such oxidative treatments create carboxylic groups, among others, at the ends and defect sites

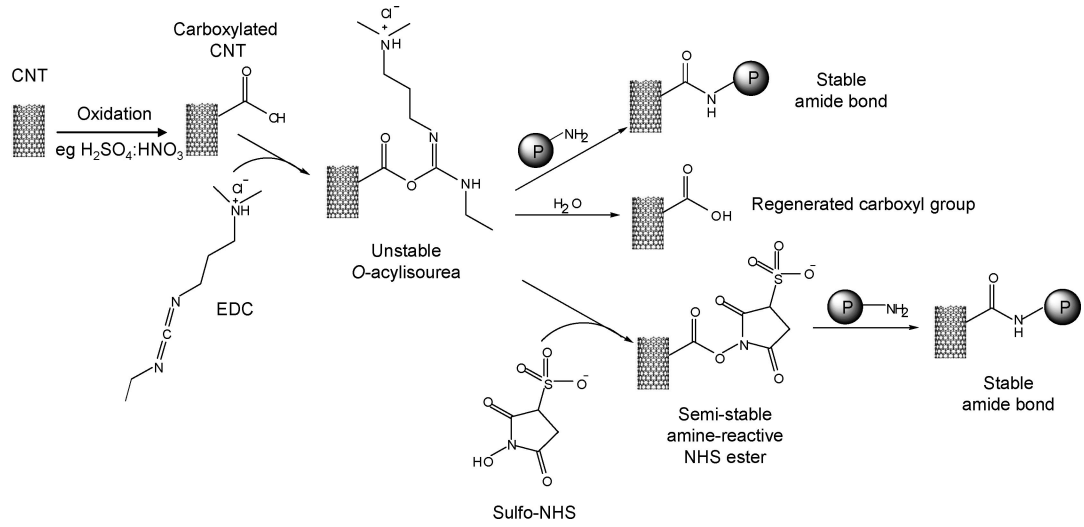

Figure 1. Theoretical conjugation of proteins to carboxylated CNTs using EDC in the presence or absence of sulfo-NHS.

on the sidewalls of the CNTs (*43, 44*). The presence of carboxylic groups has been verified by titration experiments (*45*−*47*) and IR spectroscopy (*48, 49*).

The carboxylic groups on the CNTs provide, in theory, ideal anchoring points for the covalent attachment of proteins using the cross-linker EDC. Indeed, this has been the method of choice by many researchers in recent years, although other cross-linkers have been occasionally used. Table 1 lists proteins that have been claimed to be covalently coupled onto CNTs using EDC. However, many of these proteins can also be adsorbed onto CNTs. This raises a question whether the observed protein immobilization in the coupling studies is actually due to covalent attachment or due to adsorption.

Huang et al. (*12*) were the first to specifically report the covalent coupling of a protein onto CNTs using EDC. In their work, acid-treated SWCNTs were sonicated for two hours in phosphate buffer containing 50 mM EDC but in the absence of NHS. BSA was then added to the solution and the coupling was allowed to proceed for 24 h at room temperature. BSA immobilization was observed by AFM to be at various points on the CNTs, which seemed to be consistent with the notion that carboxylic groups are at defect sites on the sidewall. However, the evidence presented to support the claim that BSA was covalently attached to the CNTs was inconclusive, for the following reasons. First, the comparison of CNT-conjugated BSA with free BSA on a SDS-polyacrylamide gel actually showed that the two behaved similarly (Figure 5 in (*12*)). This indicated that the CNT−BSA complex was dissociated under the denaturing electrophoretic conditions, revealing their non-covalent association nature. Second, the study lacked essential controls. As various studies have demonstrated that CNTs have great affinity for BSA, it is imperative that negative controls, i.e., reactions in the absence of the cross-linker, be incorporated to determine whether the immobilization is due to protein adsorption. Third, very unfavorable coupling conditions were used in this study. The omission of NHS in the reaction, the use of phosphate buffer, and the two hour time delay before BSA was added to the reaction would have collectively resulted in very low conjugation efficiency in a coupling reaction.

Jiang et al. (*13*) also specifically studied covalent coupling of proteins onto CNTs using EDC but using a two-step procedure. Acid-treated MWCNTs were first activated with EDC/NHS and then reacted with BSA or ferritin. Under TEM, the immobilized ferritin was observed to distribute relatively evenly on the sidewalls of the CNTs (Figure 2 in (*13*)). The authors claimed, but without presenting the data, that ferritin did not bind to the CNTs when EDC or NHS was omitted in the reactions. For BSA conjugation, the only evidence presented was two AFM images showing BSA on the CNTs. These results led to the conclusion that the attachment of ferritin and BSA onto the CNTs was mediated by EDC/NHS. This conclusion is open for further discussion. First, the uniform distribution of ferritin on the side wall may be inconsistent with carboxylic groups which are only located at the tips and defect sites on the sidewall of the oxidized CNTs. Second, it is unclear whether or not ferritin adsorbs onto CNTs. Azamian et al. and Lin et al. have shown that ferritin adsorbs efficiently to oxidized SWCNT, and that adding EDC to the binding solution does not alter the immobilization (*26, 27*). Third, proper controls, which were not reported in the study, were needed to eliminate that the observed BSA immobilization was not due to physical adsorption.

A few other studies have used EDC to conjugate other proteins onto CNTs. Lee et al. observed that horseradish peroxidase (HRP) could be adsorbed on MWCNT and the use of EDC increased its immobilization by up to 20% (*19*). It was unknown, however, whether this small increase was significant because no statistical analysis could be performed. It is interesting to note that this increase occurred only at 10−40 mM but not at 60−100 mM of EDC. As unfavorable coupling conditions were employed in this study, (i.e., the use of phosphate buffer in the absence of NHS), one would expect that covalent conjugation should have increased with increasing EDC concentration. Asuri et al. reported that 170 μg of SBP could be coupled to 1 mg CNTs, and a control reaction in the absence of EDC/NHS showed that nonspecific binding accounted for less than 5% of total immobilized activity (*50*). Similarly, Wohlstadter et al. reported, without presenting the data, that EDC/NHS coupling tripled the amount of streptavidin immobilized on the CNTs (*14*). However, as both SBP and streptavidin can adsorb to CNTs spontaneously in large quantities resulting in complete coverage of the CNT surface, it is difficult to imagine how these proteins could still be conjugated by the much slower covalent process. More studies are needed to reconcile these studies. Some of these studies have used a two-step conjugating procedure where the only amide bonds formed would have been between the protein and the CNT surface. Functional group analysis by techniques such as FT-IR or Raman could potentially assist in determining the nature of the attachment.

EDC has been used to immobilize a number of enzymes and antibodies on CNTs using diverse conditions in biosensing studies (*15*−*18, 20, 21, 51*−*53*). These studies generally did not include any necessary control reactions to differentiate adsorption from covalent conjugation and did not characterize

the immobilization. Protein immobilization on the CNTs in these studies was simply assumed to be due to the action of EDC.

Therefore, no conclusive evidence so far exists to show that proteins have been covalently conjugated onto carboxylated CNTs by the use of carbodiimide chemistry. Given the affinity of CNTs for many proteins including those used in the conjugating studies, proteins immobilization observed using EDC may be predominantly due to adsorption. If this is true, it is necessary to rethink the value of this "covalent" approach. If it does not significantly contribute to the amount of protein immobilization, the drawback would be, apart from labor and chemical cost, the likely loss of protein activity resulting from the many steps of handling in the conjugation process and the formation of cross-linked protein aggregates (if a one-step conjugation protocol is used). For many applications, it may make little difference whether the protein is adsorbed or covalently bound as long as adequate activity is maintained.

In contrast to proteins, small molecules such as short peptide nucleic acid (*54*) and amine-containing quantum dots (*55*) have been conjugated to acid-oxidized CNTs by EDC/NHS chemistry. Through the use of proper negative controls, FT-IR analysis, and localization by SEM imaging, the immobilization of these substances appears to be covalent. The difficulty with covalent protein coupling may be due to a number of factors. First, for many proteins, there is a competition between adsorption and covalent conjugation for surfaces of the CNTs. Adsorption, due to its strong affinity and spontaneity, is probably the dominating process over the much slower covalent reactions. Once occurred, the adsorbed protein may shield adjacent carboxylic groups from reacting with other protein molecules. Second, the carboxylic groups on oxidized CNTs are directly associated with the CNTs (i.e., without a spacer arm); this may lead to steric hindrance in the reactions between a soluble protein and the solid surface of CNTs, resulting in low conjugation efficiency.

4. CONCLUSIONS

Covalent conjugation of proteins on carboxylated CNTs using the coupling agent EDC has been widely used in recent years. However, many proteins used in such conjugation studies have also been shown to adsorb onto CNTs. So far, conclusive evidence is lacking to show that EDC could significantly improve protein immobilization on CNTs above the level of adsorption. It is necessary to re-evaluate this approach of protein conjugation onto CNTs. The unnecessary use of EDC chemistry will not only increase the cost of the work, but also may lead to some loss of protein activity resulting from the handling in the process and the possible formation of cross-linked protein aggregates. We recommend that this approach be treated with caution.

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