Carbon 95 (2015) 767–779

Contents lists available at ScienceDirect

Carbon

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

Review article

Protein functionalized carbon nanomaterials for biomedical applications

Sabrina F. Oliveira [a], Gili Bisker [b], Naveed A. Bakh [b], Stephen L. Gibbs [c], Markita P. Landry [b], Michael S. Strano [b, *]

[a] Department of Microbiology, Federal University of Minas Gerais, Belo Horizonte, 31270-901, Brazil
[b] Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, 02139-4307, USA
[c] Department of Chemical Engineering, University of Florida, Gainesville, 116550, USA

ARTICLE INFO

Article history:
Received 30 June 2015
Received in revised form
20 August 2015
Accepted 22 August 2015
Available online 28 August 2015

Keywords:
Carbon nanomaterials
Bioconjugation
Nano-bio interfaces
Protein nanotechnology

ABSTRACT

Since the discovery of low-dimensional carbon allotropes, there is increasing interest in using carbon nanomaterials for biomedical applications. Carbon nanomaterials have been utilized in the biomedical field for bioimaging, chemical sensing, targeting, delivery, therapeutics, catalysis, and energy harvesting. Each application requires tailored surface functionalization in order to take advantage of a desired property of the nanoparticles. Herein, we review the surface immobilization of bio-molecules, including proteins, peptides, and enzymes, and present the recent advances in synthesis and applications of these conjugates. The carbon scaffold and the biological moiety form a complex interface which presents a challenge for achieving efficient and robust binding while preserving biological activity. Moreover, some applications require the utilization of the protein-nanocarbon system in a complex environment that may hinder its performance or activity. We analyze different strategies to overcome these challenges when using carbon nanomaterials as protein carriers, explore various immobilization techniques along with characterization methods, and present recent demonstrations of employing these systems for biomedical applications. Finally, we consider the challenges and future directions of this field.

Contents

* Corresponding author.
E-mail address: strano@mit.edu (M.S. Strano).

http://dx.doi.org/10.1016/j.carbon.2015.08.076
0008-6223/

1. Introduction

Recent progress in nanotechnology has provided a wide variety of nanoscale materials, including carbon nanomaterial allotropes, which have been applied in numerous applications owing to their basic properties [1]. Up until the 1980s, graphite, diamond, and amorphous carbon, were the only three carbon allotropes known. Since then, fullerenes [2], carbon nanotubes (CNTs) [3] and graphene [4] were discovered and characterized, all of which contributed to the advancement of multiple fields of science. Due to their remarkable structural, chemical, electronic and optical properties, carbon nanotubes have gained enormous interest since their discovery. Carbon nanotubes consist of either a single (single-walled carbon nanotube, SWCNT), double (double-walled carbon nanotube, DWCNT) or multi (multi-walled carbon nanotube, MWCNT) concentric graphene cylindrical layers, resulting in hollow tubes with high aspect ratios. In addition, CNTs can be functionalized with different chemical groups either covalently or non-covalently, making CNTs biocompatible for conjugation with biomolecules and favorable candidates for bioanalytical applications [5,6]. In particular, SWCNTs possess an ideal combination of biocompatibility and an intrinsic near-infrared, non-blinking, non-photobleaching fluorescence [7].

The use of the carbon nanomaterials as scaffolds for biomolecules enhances their utilization potential, especially in biomedical applications. Due the large specific surface area of CNTs, they have been the promising candidates for protein/enzymes immobilization. The development of novel CNT-protein/enzyme conjugates is essential for improving biomedical research and applications, since protein/enzyme immobilization is a critical step in the design of biosensors, which can solve many of the challenges in the healthcare-system [6]. In addition, the unique optical and electrochemical properties of CNTs make them ideal for therapeutic, imaging, sensing and energy applications [8–12].

Graphene, which is a 2-dimensional one-atom thick carbon layer, and its oxidized derivate, graphene-oxide (GO), benefit from large surface area which can be further functionalized with biomolecules for various applications [13]. Both covalent and non-covalent binding have been used to attach proteins, enzymes, peptides, bacteria, cells, and nucleic acids to graphenes and GOs [13,14], for various applications including fluorescence- or electrochemical-based sensors, labeling and imaging, therapy and targeted delivery, and energy storage [15–17].

In this paper, we review the main strategies for the protein-functionalization of carbon nanomaterials and their applications, especially in the biomedical area. We present the recent developments in the synthesis of proteins-, peptides- and enzymes-carbon nanoparticle conjugates, focusing on single-walled carbon nanotubes, as well as the major characterization tools for studying these complexes. In addition, we highlight those applications of SWCNT-protein conjugates involving the use of the optical and electrochemical properties of SWCNTs. Finally, we identify discuss the challenges and give an outlook of this field.

2. Nanoparticle surface immobilization of proteins and enzymes

Owing to their large surface to volume ratio, nanoparticles have a huge loading capacity, while offering a high mobility and functionalization flexibility. Different types of biomolecules, including proteins, peptides, and enzymes, impart new functionalities to the underlying nanoparticles carriers. A loading of the tailored proteins or enzymes on nanoparticles can render them biocompatibility, responsibility to a specific target analyte, or biocatalytic capacity. Here, we focus on carbon nanomaterial scaffolds and discuss various types of surface immobilization for various applications. Most of these carbon nanoparticles are hydrophobic in nature, and proper surface functionalization also promotes their dispersion and colloidal stability in aqueous solution, which is critical to most of the applications discussed here.

One of the primary fields to benefit from nanoparticle-enzyme conjugates is biocatalysis, which is becoming one of the most powerful tools in biotechnology, having a profound social impact on health, food supply, environmental protection and sustainable fuel production [18]. This is due to the fact that the enzymes are capable of accelerating specific reactions to rates not reachable by traditional chemical or physical catalysis. In this context, enzymes can be also applied as selective tools for biomolecule detection, acting as enzymatic biosensors for environmental monitoring, food industry and healthcare application. Particularly, many drawbacks of the healthcare-system can only be solved by new enzymatic-based sensor technology such as point-of-care sensor devices [6,19].

Although natural catalysts are sustainable, selective and efficient, they are often not perfectly adapted for industrial applications. For example, enzymatic biosensors face serious problems of inactivation or loss which affect sensing performance [20,21]. Hence, enzymes immobilization emerges as a key technology that enables practical and commercial viability of such biocatalysts [19,20,22]. Enzyme immobilization is an essential step in the development of efficient industrial biocatalysts which requires increased stability under various thermal, pH, storage, and operating conditions. Moreover the enzyme has to be resistant to heat denaturation, organic solvents or autolysis to a reasonable extent [23,24].

Many biomedical applications also gain tremendously from the development of novel nanoparticle-protein conjugates, which are becoming key players in imaging, targeting, sensing, drug delivery and therapy. In several cases, the main role of surface immobilized biomolecules is to enhance biocompatibility and targeting ability for in-vivo application [25,26]. In sensing application, on the other hand, the surface coating must render the nanoparticle selectively responsive to the target of interest [27], while being the signal transducer of a binding event. Finally, in therapy applications, the protein or enzyme coating is utilized either as the targeting agent accompanying the drug payload, or the therapeutic agent itself [28].

In general, the immobilization strategy assists in the

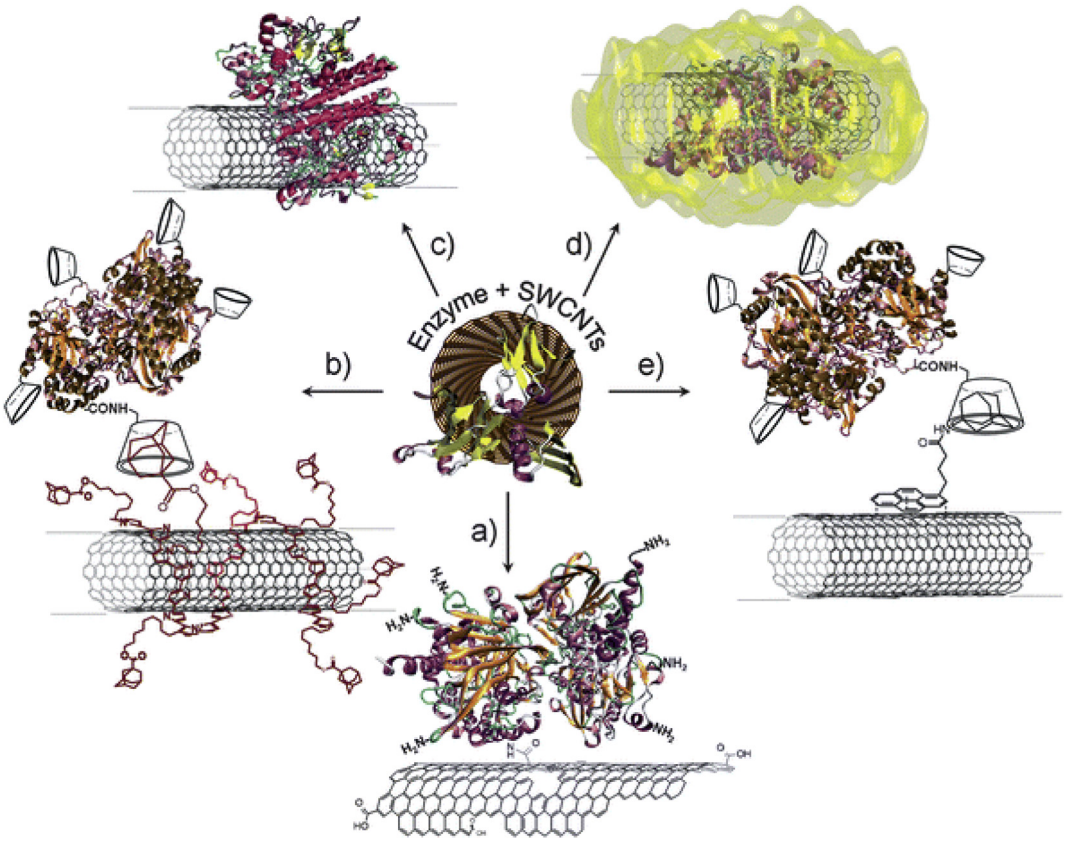

Fig. 1. Immobilization strategies of enzymes on SWCNTs: (a) covalent binding via amide coupling with the carboxylic acid groups of oxidized nanotubes; (b,e) immobilization via interactions onto functionalized nanotubes with different linker molecules; (c) adsorption of enzymes on SWCNTs via hydrophobic or electrostatic interactions; (d) entrapment of enzymes in a polymer matrix formed around SWCNTs. Reprinted from Ref. [29], with permission from The Royal Society of Chemistry. (A colour version of this figure can be viewed online.)

development of new enzymatic and biomedical tools with appropriate performances, high sensitivity, high selectivity, short response time and high reproducibility [19]. Further, the immobilized biomolecules have to maintain their structure, biological activity, and tightly bound to the surface during their application [19,24]. In order to explore the complete potential of the protein-carrier complex, it is essential to optimize the immobilization methods. Even today, the design of effective immobilization methods is one of the main hurdles that hamper industrial-scale synthesis and processes [23], where the best immobilization procedure varies depending on the priority of the desired properties, whether it is maximal sensitivity, increased stability, reproducibility, cost, or protocol complexity [19].

The most frequently used immobilization techniques fall into four categories: a) non-covalent adsorption (physical interactions); b) covalent attachment (tethering), c) cross-linking of a protein and d) entrapment in a polymeric gel or capsule. In this review, we focus in proteins, peptides, and enzymes binding to carbon nanomaterials, including carbon nanotubes, graphene oxide nanoparticles and carbon dots, by physical (such as hydrophobic, Van der Waals, and electrostatic interactions) or covalent interactions (Fig. 1).

2.1. Non-covalent immobilization

Non-covalent enzyme or protein adsorption onto solid supports represents the easiest method of physical immobilization [30]. The adsorption on carbon nanoparticles typically involves solution phase incubation, or direct sonication, followed by a washing step to remove the unbound proteins. The physical adsorption of proteins onto carriers can be facilitated via different types of interactions, based on weak bonds such as Van der Waals forces and electrostatic and/or hydrophobic interactions.

For CNTs, which consist of sp2 hybridized honeycomb carbon lattice, the direct physical adsorption method is predominantly hydrophobic, where the hydrophobic regions of the protein or enzyme can interact with the nanotube wall, with additional contribution from $\pi-\pi$ stacking [31,32]. In addition to hydrophobic and $\pi-\pi$ interactions, hydrogen bonds between proteins and carboxylates of CNTs or side groups of graphene oxide also support surface adhesion [33].

Depending on the pH of the solution and the isoelectric point of the protein, its conformation and charge can vary. The protein can be negatively (for instance carboxylate) or positively charged (for instance protonated amino groups), therefore, an ion exchanger can act as carrier via ionic and strongly polar interactions [20,34]. Techniques of layer-by-layer deposition and electrochemical doping method based on electrostatic adsorption have been employed to develop enzymatic biosensors [19]. Chen and coworkers have developed a highly sensitive SWCNT-based enzyme biosensor for the detection of the mycotoxin sterigmatocystin, where the enzyme aflatoxin-oxidase was immobilized onto SWCNT-modified gold electrode via the electrostatic and hydrophobic interactions. Ionic interaction also has been demonstrated to play an important role in the adsorption of lysozyme on SWCNTs [35]. Lysozyme interacts with the nanotubes via amine adsorption at pH levels higher than its isoelectric point, whereas at lower pH values, the enzyme interacts through protonated amino moieties

with defect sites on the nanotube surface [31].

The physical adsorption of polymers or biomolecules onto CNTs can be achieved with the assistance of surfactants and linking molecules [5,19]. A recent study demonstrated 57% enzymatic activity was remained when the enzyme was conjugated to SWCNT via a linker molecule, versus 27% activity upon direct adsorption [36].

Surface functionalization is extremely important in biosensors development, especially for carbon nanotube based sensors, which are highly hydrophobic and are not soluble in biological environments [5,6,37]. Single walled carbon nanotubes dispersion can be assisted by surfactants like sodium dodecyl sulfate (SDS) or sodium cholate (SC) [38], DNA or RNA [39,40] or other amphiphilic copolymers [27]. Tsai and co-workers, for example, immobilized glucose oxidase (GOx) onto SWCNTs and determined that the enzyme retained 75% of its activity upon adsorption. The resulting GOx–SWCNTs were used in layer-by-layer electrochemical biosensors for glucose, demonstrating enhanced sensor response [41]. Moreover, highly dispersed and debundled SWCNTs coated by the concanavalin-A (con-A) protein were prepared by dialyzing sodium cholate suspended SWCNT in the presence of the protein, resulting in surfactant removal and con-A adsorption [42]. However, the protein sensitivity to chemical perturbations should be considered since this limits the possibilities for chemical functionalization of both carbon nanoparticles and proteins [43].

Despite of its simplicity and other advantages such as improved protein conformation stability, and retention of the intrinsic electronic and optical properties of the carbon nanoparticles, physical binding may be insufficient for the stable immobilization under harsh industrial conditions. Durability and leaching are always a concern for the application of adsorption methods [34,44]. Additionally, there is the risk of protein partial unfolding, since hydrophobic regions normally buried within the protein's tertiary structure may be exposed upon hydrophobic interaction with the carbon nanoparticle. In turn, this partial unfolding can interfere with biological function [43]. When considering protein-carbon nanostructure conjugates, one must consider many variables that contribute to the conjugate's stability and viability. Researchers must consider the structural features and properties of both the nanomaterial and the protein when considering immobilization strategies and final applications of the conjugate [43,45].

2.2. Covalent linking

Most commonly, covalent binding of an enzyme or a protein to a nanoparticle carrier is based on chemical reactions between the side group of amino acid residues located on the protein surface and functionalized group available on the particle surface. Often, carriers are activated before binding to enzymes [34], usually by directly introducing electrophilic functionalities on the support surface [34,46].

Various functional groups can be easily incorporated on the surface of carbon nanotubes by chemical modification, such as carboxylation, acylation, amidation, esterification, PEGylation, or via polymers wrapping, some of which are non-covalent while the rest are covalent functionalization. In addition, such functionalization can also be accomplished by physical adsorption and ionic interactions [5,6]. It is important to note that introducing chemical handles or functional groups onto the SWCNT surface for subsequent modification purposes can potentially disrupt their native structure and introduce defects that affect the unique optical and electronic characteristics, which can pose a limitation for some application [11,47,48].

In general, covalent protein immobilization on carbon nanotubes has been demonstrated by inducing a reaction between the free amine groups on the protein surface and carboxylic acid groups on the sidewall of oxidized CNTs and subsequent activation using carbodiimide chemistry [46,49–51]. Carbodiimides are reagents capable of activating –COOH functions, rendering them reactive towards nucleophilic groups (such as lysine ε-NH_2). However, the drawback of this approach is the loss of the sp2 hybridization at the reaction sites which impairs the electronic properties of the SWCNTs [52]. An amperometric biosensor for H_2O_2 detection was described by immobilizing horse radish peroxidase onto the SWCNTs ends by using carbodiimide to promote amide linkages between carboxyl-terminated nanotubes and lysine residues of the enzyme [49]. Similarly, a glucose biosensor based on covalent immobilization of GOx on SWCNTs was developed [50], demonstrating good operational and storage stability, with 90% of activity retained after 4 months.

Another approach includes a crosslinking molecule that binds to carbon nanotubes through non-covalent functionalization such as hydrophobic and π–π interactions [58,59], and covalently bind the protein through, for example, an amide bond. For example, a bifunctional pyrene molecule can irreversibly adsorb through π–π stacking and be used as a reactive linker for bioconjugation [46,60]. This approach is advantageous for proteins immobilization on carbon nanotubes since the non-covalent functionalization preserves the electronic properties of the SWCNTs, while enabling non-direct binding to the protein. Kim and co-workers developed a SWCNT-based sensor for ATP using non-covalent functionalized with phospholipid (PL)-PEG-COOH and subsequent conjugation to luciferase enzyme [61]. The oxidized production of D-luciferin, oxyluciferin, quenched the near infrared fluorescence of the SWCNT, enabling the detection of ATP with a detection limit of 240 nM.

Although most of the covalent immobilization methods are non-specific, new strategies have overcome this problem [53] using direct enzyme modification, such as bio–orthogonal reactions for site-specific protein labeling [54,55]. “Click chemistry”, for example, has been used extensively used in modern chemistry, especially for immobilizing azido- or alkyne-containing proteins onto alkyne- or azido-coated surfaces, respectively [56]. Adronov and co-workers coupled alkyne-functionalized spacers to SWCNTs via amine groups, where the use of click chemistry for chemical functionalization in this case granted a greater level of control over the orientation and the density of the polymer attached to the nanotube surface while reducing the probability for side reactions [57].

Among all these methods, covalent immobilization generally ensures the highest binding strength between the support nanoparticle and the protein, while minimizing leakage issues, and increasing operational stability towards heat, pH, organic solvents, and storage. On the other hand, covalent immobilization may result in steric modifications of the protein or enzyme, leading to a decrease of protein functionality or enzymatic activity. However, the use of appropriate crosslinking molecules between protein and carbon nanomaterial can often reduce or avoid loss of enzymatic activity [34]. In addition, if an enzyme is irreversibly deactivated upon covalent binding to a nanoparticle, both the enzyme and the support are rendered unusable [34,46].

2.3. Characterization of protein-carbon nanoparticle conjugates

To confirm the effectiveness of conjugating proteins to carbon-based nanoparticles, there is a wide array of techniques that have been used to measure the characteristics of the resulting conjugate. While the applications for these materials are diverse, there are many common features that researchers look to analyze after conjugation. Herein, the techniques that reveal those features are reviewed.

First, knowledge of the protein structure after binding is essential as it is closely related to protein function and activity. By obtaining an image through Transmission Electron Microscopy (TEM), one can view the morphology of the carbon nanoparticle (CNP) as well as measure its size before and after conjugation. Increased size indicates conjugation of protein to the CNP surface. Furthermore, if the length of the unbound protein is known, one can assert whether the protein has unfolded or maintained its structure upon binding by measuring the size increase of the CNP [62–66]. Atomic Force Microscopy (AFM) can be used very similarly to TEM [64,65,67]. Additionally, Circular Dichroism (CD) can be used to determine a protein's secondary structure. More specifically, there are particular CD spectra that are associated with random coil, alpha-helix, and beta-sheet folding. Therefore, it is possible to determine quantitatively the degree to which the protein is folded in any one of those conformations [62–64,66,67]. In fact, Fourier Transform Infrared (FTIR) Spectroscopy can also be used to monitor secondary structure because for certain proteins there are known absorption peaks which correspond to their vibrational modes when folded in a certain way [64].

Beyond measuring protein structure, many spectroscopic techniques can help to monitor protein and CNP conjugation through identification of functional groups on the CNP surface. For example, Ultraviolet Visible Spectroscopy (UV–vis) spectra of the CNP can be obtained before and after protein conjugation [62,63,68]. Characteristic spectroscopic peaks of a desired functional group will appear after conjugation, confirming their successful incorporation onto the CNP surface. FTIR can also be used similarly to UV–vis, for spectroscopic characterization that requires excitation of the sample in the infrared [62,65,66].

Elemental analysis can also be helpful in learning about the surface chemistry of carbon nanoparticle probes that have been covalently functionalized. Atomic composition of the CNP can be found through various methods such as Thermogravimetric Analysis (TGA) [66], X-ray Photoelectron Spectroscopy (XPS) [65,66], and Energy Dispersive X-ray Spectroscopy (EDX) [64].

Particularly for biological applications, and in the case of protein-suspended CNPs, it is useful to quantify these conjugates' dispersion in a given solvent. Three primary techniques are employed that can quantify the dispersion of a sample [64]. In UV–vis spectroscopy, there is a characteristic absorption region for aggregation sites of nanoparticles. Raman Spectroscopy shows the same selectivity with certain wavelengths of scattered light. Finally, visualization with AFM can be used to see how well dispersed the CNPs are in the sample, albeit typically in solid-state.

While finding the structure, surface chemistry, and dispersion of protein-CNP conjugates gives much helpful information, it is necessary to find out if the proteins can continue with their function. The final area of characterization involves kinetic and enzyme activity study. There are many ways to monitor a reaction with these conjugates and to choose one involves knowing the properties of the molecules involved. One way is to measure the varying fluorescence of a certain molecule throughout the experiment. Recent studies even have methods to differentiate dynamic from static fluorescence quenching [62]. Another method of monitoring reaction kinetics used in this area recently is a colorimetric method which reveals the residual activity of a particular conjugated enzyme in comparison to its unbound counterpart [66]. Other methods for monitoring catalytic activity in the area of protein-CNP conjugates can be found elsewhere [63,67,69].

3. Carbon nanotubes as protein carriers

Several long-standing challenges in the delivery of biological materials such as proteins have been addressed via the scaffolding of these biomaterials to nanomaterials. Nanomaterials are of a similar size-scale as many biologicals, and are therefore well-suited to their assisted delivery into organelles, cells, and tissues. Nanomaterials such as graphene, MWCNTs, and SWCNTs have a high surface area-to-mass ratio, which maximizes the scaffolding potential for biological cargoes. Significant progress has been made in the use of graphene [70], MWCNT [71], and SWCNT in cell studies. However, the majority of cases using CNP-protein conjugates for delivery have hinged on the use of SWCNT, which will be the focus of this section. As such, carbon nanomaterials such as SWCNTs have played a central role in developing delivery scaffolds for proteins, and could represent a promising platform for the development of molecular transporters.

3.1. CNTs as protein transporters

CNTs, in particular, have shown a largely ubiquitous ability to transport into a variety of organelles [72], cells [27,73], and tissues [74], likely due to their large aspect ratio and the ease with which they can be functionalized. A leading effort in the field has been put forth by the Dai group, who has developed multiple CNT-based protein delivery platforms. Using chemically oxidized SWCNT, the group has shown electrostatic scaffolding of proteins to SWCNT which are then internalized into cells via endocytosis [75]. These results show endosomal release of the SWCNT-protein conjugate into the cytoplasm, where a variety of protein-directed functions are observed. Subsequent work from the same group has explored the interaction of proteins using SWCNT as a scaffold with human cancer cells CHO and 3T3 [76]. Encouragingly, fluorescently labeled streptavidin alone does not enter cells, SWCNT-scaffolded streptavidin does via adsorption-mediated endocytosis. This study further revealed controlled apoptosis of mammalian cells via SWCNT-mediated delivery of cytochrome c. These results are encouraging for SWCNT usage as a generic protein scaffolding and transportation tool for intracellular delivery.

3.2. Uptake mechanism of SWCNT-protein conjugates

There exist several opinions on the mechanism by which SWCNT-protein conjugates are uptaken into cells. The two primary contending mechanisms are an energy-dependent endocytotic SWCNT-conjugate internalization mechanism, and an energy-independent passive internalization mechanism. The vast amount of literature supporting either mechanism suggests that both are likely contributors to cellular SWCNT internalization [77]. A single mechanistic pathway applicable to all nanoparticle variants, cell lines and culture conditions is extremely unlikely. Rather, the diversity of methods used to functionalize SWCNT, and attach cargoes to SWCNT, determine the internalization mechanism in a manner that remains to be mapped in detail. Previous studies have shown that the functionalized SWCNT conjugate can either fully enable passive and irreversible internalization of nanoparticles into cellular organelles [72], or have no quantifiable permeability into cellular organelles, simply by tuning the functionalized SWCNT conjugate's zeta potential. Similar studies looking into SWCNT cellular internalization have observed “needle-like” penetration of the nanoparticle into cells [77,78]. Conversely, clathrin-mediated endocytosis has been observed for protein-SWCNT conjugates [79]. While many of the studies showing energy-independent SWCNT internalization were performed with DNA- or polymer-coated SWCNT, it is likely that the much larger size and complexity of protein-SWCNT conjugates necessitates an alternate and energy-dependent cellular internalization pathway. Kinetic studies of such pathways have been undertaken, sometimes suggesting an exocytosis contribution to the kinetics of protein-SWCNT

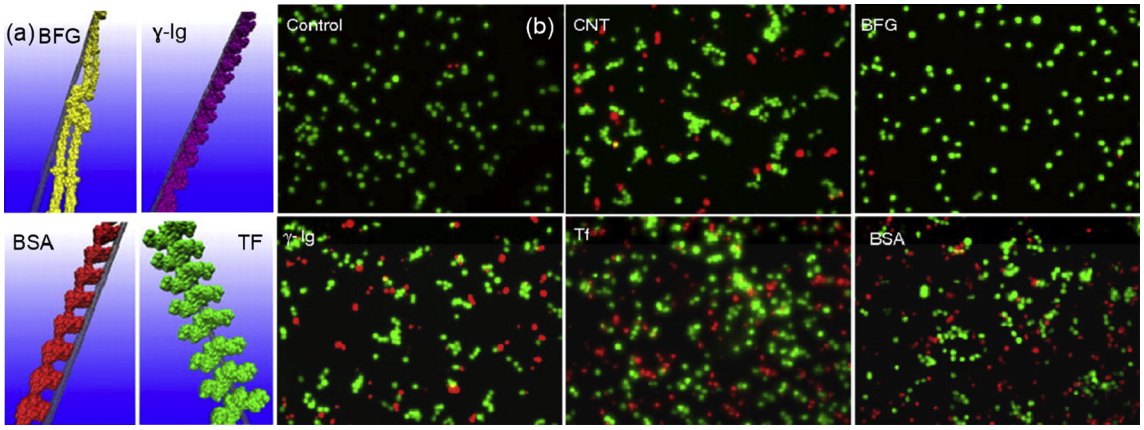

Fig. 2. (a) Illustration of molecular models representing the interaction of proteins BFG, γ-Ig, BSA, and TF, following a 5-h incubation with bare SWCNT. (b) Fluorescence imaging of viable (green) and non-viable (red) THP-1 cells following a 12-h treatment with, from left to right, no SWCNT, only SWCNT, BFG-encapsulated SWCNT, γ-Ig-encapsulated SWCNT, Tf-encapsulated SWCNT, and BSA-encapsulated SWCNT. Reprinted from Ref. [84] with permission. (A colour version of this figure can be viewed online.)

conjugate intracellular transport [80]. Several groups have also undertaken detailed studies of how carbon nanotube size and surface modification can greatly affect the adsorption of proteins. For instance, Marchetti and co-workers found that human surfactant protein D, found in human respiratory secretions, adheres to varying extents on carbon nanotubes depending primarily on the nanotube functionalization with charged groups [81]. Because surfactant protein D is central to the lung's inflammatory response, these findings are suggestive that nanotube functionalization can affect not only the delivery fate of protein-nanotube, but also the toxicity of the conjugate. Groups have also modeled the binding of proteins and peptides on SWCNT, based on the protein's hydrophilic:hydrophobic side chain ratio. For such modeling studies, endocytosis was also a preferred pathway for the update of protein-SWCNT conjugates. These studies investigated the molecular-scale details of how protein-SWCNT interactions modulate SWCNT protein-carrying capacity, and also toxicity [82].

3.3. *Toxicity of delivered SWCNT-protein conjugates*

The use of SWCNT-protein conjugates for the delivery of biological cargoes relies on a thorough understanding of their toxicity. The increased use of SWCNT in therapeutic and biosensing applications has also spawned an interest in understanding the toxicity of these conjugates. Much like the internalization pathway of SWCNT conjugates was found to vary greatly depending on the conjugate's functionalization, the toxicity of said conjugates also varies depending on their functionalization [83]. Studies have shown through extensive analytical characterization, that SWCNT toxicity can be mediated by binding of blood serum proteins to the otherwise hydrophobic surface of SWCNT. Work by Ge and co-workers showed that the different binding capacities of blood proteins to SWCNT led to a competitive protein binding process mediated by each protein's adsorption capacity, as shown in Fig. 2 [84]. Proteins such as bovine fibrinogen, gamma globulin, transferrin, and bovine serum albumin were found to bind to SWCNT with adsorption rates that increase with the order in which these proteins are listed. Further analysis into the structure of these proteins showed that proteins with higher SWCNT adsorption rates also had more hydrophobic surface residues, suggesting that those proteins with more hydrophobic surface residues (Trp, Phe, Tyr) are likelier to bind to SWCNT [84]. In turn, SWCNT functionalized with different surface coverage of each blood protein mediated the cellular translocation pathways and cellular toxicity in human acute monocytic leukemia cells, and human umbilical vein endothelial cells [84]. Another study by Sacchetti and co-workers studied the adsorption of various blood plasma proteins onto SWCNT that had been pre-functionalized with varying quantities of PEG [85], along with Liu and co-workers [86]. Highly and sparsely PEGylated SWCNT were injected into mice, and proteomics analysis were then performed to determine the composition of the protein corona that had formed on the SWCNT surface. The study concluded that the pharmacokinetic profile of PEGylated SWCNT affected the competitive adsorption of blood proteins to the PEGylated SWCNT, which in turn shifted the fate of these complexes in living mice.

Significant progress has been made in the elucidation of protein-SWCNT internalization mechanisms, from cells to model organisms. However, there is lack of, and need for, a systematic and predictable understanding of the parameters that determine protein-SWCNT fates in their interactions with biological systems. Advances in experimental and theoretical tools to study SWCNT-biomolecule conjugate systems at the molecular scale [37,40,87] have emerged, but many remain to be extended for molecular-scale SWCNT-protein studies. The risk of CNPs triggering an immune response should be also considered, since the protein components of the complement system can bind to carbon nanotubes. Certain studies have focused on how the functionalization of carbon nanoparticles, often with protein coronas, can attenuate or abate the immune response of nanoparticles in biological applications [88]. Such studies are particularly promising in suggesting that the fate, toxicity, lifetime, and therefore the utility of CNPs is highly tunable and can be engineered by the user via the nanoparticle corona.

In the case of CNTs, the polydispersity in CNT length has given rise to questions of how CNT length can affect CNT fate and toxicity *in vivo*. Asbestos-like pathogenicity has been observed for MWCNTs in the mesothelial lining of mouse body cavities, particularly for longer CNTss [89]. This length-dependent toxicity has spurred further research into how to circumvent these length-dependent toxic effects. Studies show that proper functionalization of long CNTs can be used to alleviate the asbestos-like effects of long carbon nanotube species [90], which is again promising for a range of applications wishing to utilize the remarkable properties of carbon nanomaterials.

Future studies to broaden our understanding of how to

rationally design and predict protein coronas for SWCNT conjugates are needed to further the field of protein-SWCNT conjugates for sensing, bioengineering, and therapeutic applications.

4. Applications of protein-SWCNT conjugates

Carbon nanomaterials have attracted increasing attention from the scientific community following their discovery. First, the 0-dimensional fullerenes were discovered in 1985, followed by 1-dimensional carbon nanotubes in 1991, and 2-dimensional graphene in 2004 [1]. Based on extensive research of the basic properties of these materials, numerous applications of the different allotropes have started to emerge utilizing their physical, chemical, electronic, and optical features. Further functionalization of these high surface area nanoparticles has elevated their potential prospect in biomedical application. Here we focus of bio-macromolecular functionalization including protein, peptides, and enzymes and present recent advances in the field.

4.1. Utilizing the optical properties of the carbon materials scaffold

Graphene oxide (GO) nanoparticles, or carbon nanodots, provide the benefit of strong ultraviolet absorption, bright fluorescent in a broad wavelength range starting from ultraviolet up to the near infrared part of the spectrum, and high surface area [91,92]. SWCNTs have intrinsic near-infrared, non-blinking, non-photobleaching, fluorescence [7] which corresponds to the tissue transparency window in this wavelength range [6,93–95]. Protein functionalization of carbon nanomaterials renders them biocompatible, enhances stability in biological environment, enables further conjugation to target receptors or sites, and allows for cell internalization. Moreover, their size range from several nanometers to hundreds of nanometers is comparable to the physical size of many biomolecules such as proteins, peptides, enzymes, and hormones [96]. Along with the unique optical, physical, and chemical properties of these carbon nanomaterials, the bio-interface makes them appealing for therapeutic, imaging, sensing and biomedical application [6,70,97–99]. In addition to direct covalent or non-covalent surface functionalization, the Strano group has recently suggested a theoretical framework for designing a helical wrapping of cylindrical nanoparticles, such as CNTs, that would specifically recognize a bio-molecule analyte, leading to its conjugation to a tailored binding pocket on the surface of the nanoparticle [100].

One cardinal advantage of exploiting the optical properties of carbon nanoparticles is the ability to utilize both the spatial and temporal degrees of freedom. Upon labeling with functionalized nanoparticles, their fluorescence can be tracked and imaged in real-time enabling their transient localization in 3 dimensions. For sensing, their optical signal transduction can be mapped to the target analyte location with high temporal resolution, whereas in therapy application, their absorption can be utilized for localized thermal effects in the region of interest. In this section, we review recent demonstrations of these applications using a variety of carbon nanomaterial.

4.1.1. Imaging and tracking

SWCNTs have been used as a fluorescent tag of an intercellular motor protein for tracking dynamic processes within the cytoskeleton [101]. DNA-wrapped SWCNTs were covalently linked to kinesin-1 motor Kif5c expressed in COS-7 cells. The SWCNT label of a motor protein enabled the tracking of intercellular dynamics in real-time using fluorescent microscopy, including kinesin transport along microtubules, and microtubule-network fluctuation [101].

SWCNTs directly suspended by bovine serum albumin (BSA) were utilized for *in vivo* imaging of Drosophila melanogaster larvae [102]. The larvae were fed with the protein-coated SWCNT, which were then fluorescently imaged in the digestive system, clearly showing peristaltic movements [102]. Moreover, transferrin proteins were used to covalently functionalize carbon nanodots through carbodiimide chemistry and their amine terminal groups. The functionalized nanodots were internalized by overexpressing transferrin receptors in cancerous HeLa cells, and imaged under fluorescent microscopy [103].

4.1.2. Optical biosensors

A flux based glucose sensor was demonstrated using SWCNT non-covalently functionalized by glucose oxidase enzyme [104]. The addition of glucose resulted in the modulation of the fluorescent emission of the glucose oxidase-SWCNT, allowing for real-time monitoring of glucose concentrations (Fig. 3a) [104]. Additional glucose sensor was demonstrated by glucose-binding protein covalently bound to carboxylated poly(vinyl alcohol)-wrapped SWCNTs, where the addition of glucose resulted in a conformational change of the protein leading to exciton quenching and a decrease in fluorescent emission intensity (Fig. 3b) [105].

A different approach for protein or enzyme functionalization of SWCNT for sensing applications was recently proposed by the Strano group, where a Hexahistidine-tagged capture protein is tethered by a nickel chelation group conjugated to chitosan wrapped SWCNTs [6,109,110]. This approach enables a label free detection and sensing of target analytes by monitoring the fluorescent signal intensity modulation upon analyte binding. Earlier work demonstrated the capture of his-tagged proteins, expressed using cell-free synthesis, by a nickel chelation group on chitosan wrapped SWCNT sensors array, followed by the detection of a model analyte of anti-histag antibody (Fig. 3c) [106]. A follow up work demonstrated glycoprofiling using a similar platform, where his-tagged recombinant lectins were used as the capture protein, successfully detecting streptavidin-tethered biotinylated monosaccharides [111,112].

Finally, nitroaromatics detection was enabled by bombolitin peptide wrapped SWCNTs. The peptides were non-covalently bound to the nanotubes by direct sonication, and underwent a conformation change upon analyte binding, resulting in a solvatochromatic shift in the fluorescent emission [113].

4.1.3. Therapy

PEGylated GO nanoparticles functionalized with transferrin proteins were used to target gastric cancer cells (AGS) which overregulate transferrin receptors [77]. The transferrin-GOx conjugates were used as optical probes for the targeted malignant cells using their two photon photoluminescence, as well as therapeutic agents for photothermal therapy, causing cell damage under high-power laser irradiation [77]. In addition, bovine serum albumin (BSA) reduced GO nanoparticles (Fig. 3d) were intravenously injected into the tail of mice bearing MCF-7 breast cancer tumor, whose cells underwent thermal induced necrosis following laser irradiation [107].

Targeted drug delivery was also demonstrated *in vivo* using oxidized SWCNT functionalized with epidermal growth factor (EGF) and an anticancer drug, cisplatin (Fig. 3e) [108]. Following the injection of the EGF-SWCNT conjugated into mice with head and neck squamous carcinoma tumors, the nanoparticles targeted the EGF receptors overexpressed on the tumor cells, resulting in slower tumor growth [108].

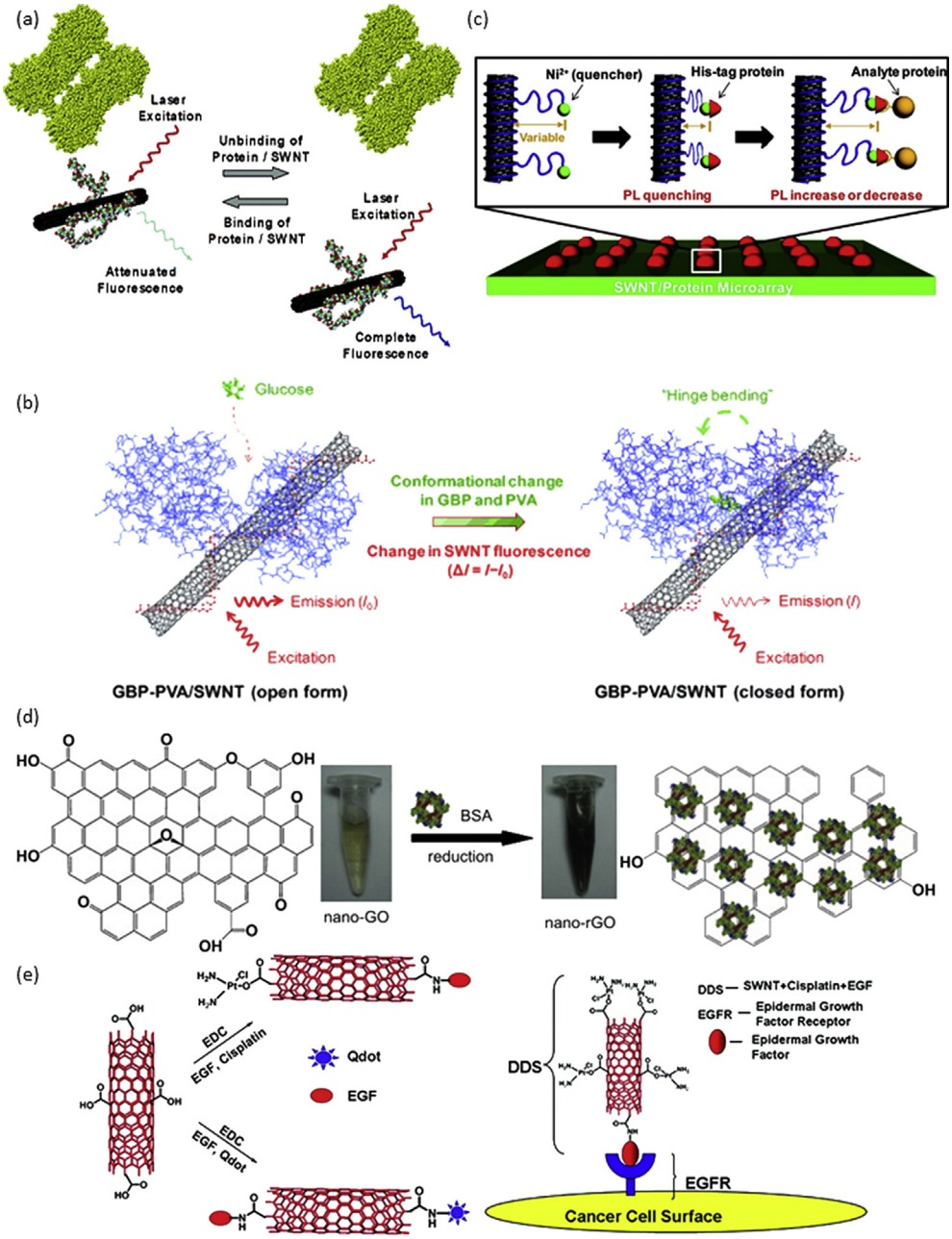

Fig. 3. (a) Illustration of a flux-based glucose sensor. SWCNTs wrapped with dextran, which is a glucose analogue, bind to a glucose specific protein (glucose-oxidase or concanavalin A). Upon the interaction with glucose, the proteins desorbs from the SWCNT resulting in a fluorescent signal increase. Reprinted (adapted) with permission from Ref. [104]. Copyright (2005) American Chemical Society. (b) Glucose oxidase covalently bound to poly(vinyl alcohol)-wrapped SWCNTs undergoes conformational change upon glucose binding, resulting in fluorescence quenching. Reprinted from Ref. [105] with permission. (c) Illustration of the sensor array based on SWCNT functionalized with a nickel chelation group, conjugated to a his-tagged capture protein, where analyte binding results in fluorescent intensity modulation. Reprinted from Ref. [106] with permission. (d) Reduction of graphene oxide with bovina serum albumin (BSA) to produced reduced-GO. Reprinted from Ref. [107], with permission from Elsevier. (e) Oxidized SWCNT functionalized with epidermal growth factor (EGF) for targeting, Cisplatin for therapy, and quantum dots (Qdot) for imaging. The SWCNT- Cisplatin- EGF is a drug delivery system (DDS) capable of targeting EGF receptors (EGFR) overexpressed in cancerous cells. Reprinted from Ref. [108] with permission. (A colour version of this figure can be viewed online.)

4.2. Utilizing the electrochemical properties of carbon materials scaffold

CNTs have several properties that make them attractive to localize at electrode interfaces. For example, they generally have high electrical conductivity and high aspect ratios to allow for fast electron transfer at distances of hundreds of nanometers from the electrode surface [114]. Additionally, they increase the surface area of the electrode which can help with increase the reaction rate and current at the electrode surface. These features make CNTs ideal for certain electrochemical applications.

4.2.1. Electrochemical biosensors

Electrochemical detection of a variety of biomolecules has become a popular analytical technique and CNTs have been used to increase the sensitivity and electron transfer kinetics of these electrochemical measurements for such analytes as dopamine, serotonin, tyrosine, and insulin [115–119]. However, many relevant

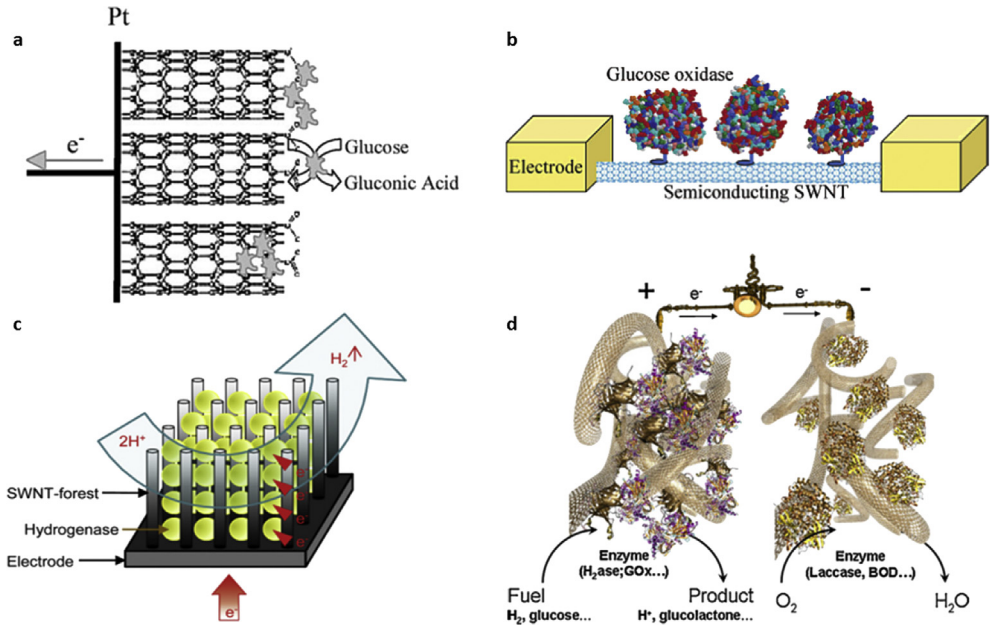

Fig. 4. (a) Direct conjugation of GOx on CNT-electrodes allows for both enzyme immobilization and direct electron transfer from the electrode surface to the enzyme active site acting as a mediator. Figure was taken with permission from Ref. [127]. (b) Conjugation of GOx to the side walls of a SWCNT fixed to electrodes results in a change of SWCNT conductance upon enzymatic reaction, which allows glucose detection at the single nanotube scale. Figure was taken with permission from Ref. [128]. (c) Attaching a SWCNT forest to an electrode surface allowed for hydrogenase to spontaneously attach to the side walls of the nanotubes. The electrode donates electrons to the hydrogenase to catalyze the production of H_2 from protons. Figure was taken with permission from Ref. [129]. (d) Schematic of a biofuel. Fuel is oxidized by at the anode through enzymatic catalysis and O_2 is reduced through an enzymatic reaction at the cathode. Reprinted with permission from Ref. [130]. (A colour version of this figure can be viewed online.)

biomolecules are not electrochemically active at reasonable potential windows, and electrochemical testing would result in the oxidation or reduction of interfering molecules [120]. One solution to this problem is to conjugate enzymes to the CNTs and connect them to the electrode surface, then the electrode can either act on the products of the enzymatic reaction, or directly donate or receive the electron from the redox center of the enzyme [120]. One of the most common biomolecules detected with CNT-enzyme electrochemistry is glucose, due to its clinical importance for diabetes treatment [120,121].

Indirect measurement of glucose is accomplished by measuring the enzymatic products electrochemically. The two main products of interest for indirect detection are hydrogen peroxide and nicotinamide adenine dinucleotide (NADH), both of which can conveniently be measured electrochemically using CNT modified electrodes [121–123]. Hydrogen peroxide is a byproduct of glucose oxidation from glucose oxidase (GOx) and NADH is formed from the reaction of glucose dehydrogenase (GDH). CNT modified electrodes have the advantage of lowering the redox potential of the molecule of interest to a different degree than other interfering molecules, thus increasing the sensitivity and selectivity [124,125]. CNT paste electrodes have utilized MWCNTs dispersed in mineral oil to form an electrode with good electrochemical activity with hydrogen peroxide, and additional incorporation of GOx into the electrode matrix resulted in a selective biosensor [123].

Additionally, direct electron transfer between the redox centers of the enzymes and electrode surfaces have been shown, which utilize the advantageous electrical properties of CNTs to act as molecular wires [126]. CNTs can align normal to the electrode by self-assembly to facilitate electron transfer with redox centers of enzymes, allowing enzymes (and active sites) farther away from the electrode surface to quickly transfer electrons at this interface without the need of a mediator [114]. Direct conjugation of GOx on MWCNTs grown on a platinum substrate have been used to create biosensing arrays where the CNTs both immobilize the enzymes and act as mediators (Fig. 4a) [127]. GOx has also been selectively attached to a SWCNT wall and enzymatic action results in a change in conductance that allows glucose detection at the single nanotube scale (Fig. 4b) [128].

There have also been combinations of GOx and horse radish peroxidase (HRP) coupled to nanotubes and the electrode through a nafion polymer film [131]. In such systems, the HRP directly undergoes electron transfer with the electrode to indirectly measure the glucose concentration through hydrogen peroxide detection [131]. More recently, a more stable and sensitive biosensors have been developed for glucose using a chitosan-bovine serum albumen cryogel incorporated with MWCNTs, GOx, and ferrocene [132].

CNT based biosensors have been developed to indirectly measure a wide range of biomolecules. Galactose has been detected by immobilizing galactose oxidase to a chitosan polymer containing SWCNT with glutaraldehyde [133]. Cholesterol biosensors have been developed which immobilize cholesterol oxidase onto a gold-MWCNT electrode covered with a cross linked matrix of chitosan-room temperature ionic liquid [134]. Ethanol dehydrogenase was also encapsulated in an MWCNT-teflon matrix to create an ethanol biosensor [122]. The strategy of electrochemically measuring hydrogen peroxide or NADH can also be applied to many other analytes using the proper oxidases and dehydrogenases [122].

Biosensors have also been fabricated by interfacing graphene with enzymes at electrode surfaces [135–138]. Direct electron transfer between graphene and GOx has also been shown by cyclic voltammetry measurements that show reversible peaks for the graphene-GOx electrode characteristic of the redox center, while only GOx did not show such peaks [139]. Graphene-enzyme based biosensors, similar to CNT based biosensors, have been developed for a wide variety of molecules [137].

It is also important to highlight the application of the amyloid-carbon nanomaterial hybrids, especially amyloid-graphene nanocomposites, that show desirable electron conductivity that can be also used to design efficient biosensors [140,141]. Furthermore, the

application of amyloid–carbon nanomaterial hybrids may open new strategies in others biomedical areas, such as tissue engineering, drug delivery, nanomedicines, molecular separations and functional nanocomposites [140].

Other interesting carbon nanomaterial hybrids involve the capture of CNTs into virus-like protein cages. Protein coatings, or capsids, of many viruses provide an alternative to encapsulate nanoparticles into closed biocompatible structures with greater shell thicknesses and higher stabilities. Li and co-workers [142] showed that changing the process conditions allows control of the shell thickness and hence permits the complete suppression of cytotoxicity towards to fibro sarcoma cell line. Additionally, the high aspect ratio and rigidity of the CNTs permit the hybrid solutions be filtered into nanocomposite films. This approach may also open new niches for applications in biomedicine, biotechnology and material science areas.

4.2.2. Energy

The same principles that allow direct electron transfer between enzymes and electrodes using CNTs can be used to form products rather than detect analytes. Hydrogenase is an enzyme that catalyzes the reversible oxidation of hydrogen through a [NiFe] or [FeFe] active site [143]. Furthermore, it has been shown through Raman spectroscopy and photoluminescence excitation that surfactant suspended SWCNT can self-assemble with hydrogenase to form a catalytically active system, where the SWCNT facilitates direct electron transfer to and from the iron center of the enzyme [143]. One interesting application of this technique is using the hydrogenase enzyme along with CNT-electrodes to catalyze the production of hydrogen gas, which is of great interest for alternative energy applications [129]. SWCNTs were deposited onto the electrode forming a vertically aligned and dense “forest” of SWCNT. The hydrogenase spontaneously incorporated into the SWCNT forest by interacting with the side walls of the nanotubes creating a stable enzyme-SWCNT-electrode system in aqueous solution that could electrochemically produce hydrogen without a mediator at an electron transfer efficiency of 30% (Fig. 4c) [129].

The ability for CNT based electrodes to facilitate electron transfer to redox enzymes have been utilized to design biofuel cells, where the enzyme system catalyzes the redox reactions that take place at the electrode surfaces, which consist of the oxidation of a fuel at an anode and the reduction of oxygen at the cathode (Fig. 4d) [130]. Due to the abundance of enzymes capable of oxidizing different molecules at the anode, there have been a variety of different fuels for these biofuel cell systems [130]. The most common fuel used is glucose due to the desire to use biofuels in implantable devices, however there are also biofuel cells that utilize ethanol, and other sugars [130,144–146]. Glucose/air biofuel cells have been designed using GOx at the anode and laccase at the cathode to reduce air. Redox polymers containing osmium centers have been used as mediators to aid electron transfer in the absence of CNTs [147]. However, addition of CNT to the electrodes along with enzyme incorporation by simple mechanical compaction significantly increased the performance of the glucose/air biofuel cells [146].

5. Challenges

Immobilized protein systems are of great scientific and commercial interest in a wide range of applications. However, despite recent advances in understanding the mutual interaction between the protein and support surfaces, there are some drawbacks that need to be elucidated.

Protocols for immobilizing the proteins of interest are usually developed based on empirical experience. In most cases, the choice of a suitable carrier is biased by particular polymers that have been shown to be effective with a large number of proteins [148]. Considering enzymes, this may result in non-optimal exploration of their catalytic efficiency. In each case, immobilization protocols seek to find a balance between preserving catalytic activity and technological advantages expected from the immobilization. A considerable amount of scientific data published since the 1970s in enzyme immobilization provides a wide range of technological options for the optimization of immobilized biocatalysts. However, since numerous variables affect the performance of immobilized biocatalysts, some of which are difficult to measure directly, the rational planning of immobilization protocol remains a challenge [24,149]. The protein structure properties must be taken under consideration before selecting an immobilization protocol [149], including the protein orientation based on the surface exposed amino acids available for the conjugation.

An additional important aspect is the mismatch between the heterogeneous enzyme-nanoparticle system and traditional models for measuring enzyme activity, which are designed for homogeneous enzyme-substrate systems. Hence, factors contributing to the heterogeneity of the enzyme-nanoparticle system, such as size, shape, structure, surface area, and chemical functionality, should be considered [150].

Nanoparticle surface immobilized protein conjugates involve a number of components, such as the protein or enzyme, the carrier, the substrates, cofactors, ions, etc., where the modification of one can result in a global change of the whole system. Therefore, a powerful approach would be to consider the nanoparticle-protein complex as a whole [151]. The carbon nanomaterial support must be fully characterized at the molecular level in order to assist efficient protein immobilization. Another important contribution that is unique CNTs is the consideration of CNT chirality. The relative alignment of the carbon atoms along the SWNT lattice is known to influence their electronic properties and interfacial interactions, hence it influences protein conjugates and the eventual application of the SWNT-protein conjugate. Researchers have begun to undertake the task of examining the role of carbon nanotube chirality, curvature, and structure in its non-covalent interactions with the nanotube corona and the protein corona [152]. In addition, nanotoxicity concerns need to be addressed when aiming for applications involving biological cells, tissues, or animal models [153,154]. Finally, commercial viability of protein-carbon nanoparticle systems requires cost-effective synthesis and manufacturing, and competitiveness with regard to other commercially available products [21,154].

6. Conclusions and prospects

Carbon nanomaterials have great advantages for utilization in biomedical research and application. They have been proven to be leading candidates for scaffolding and carrying biological molecules in a wide diversity of successful implementations and there is a growing amount of demonstrations presented in the scientific literature.

The immobilization of proteins on CNT surfaces is critical to the success of any application of nanotube-protein conjugates. Different applications require different immobilization strategies. Non-covalent immobilization is the most simple and desirable of these strategies when effective. However, some applications such as the direct electron transfer to certain enzymes require a covalent linkage between the nanomaterial and the protein of interest. But not all enzymes require a covalent linkage for direct electron transfer. Due to the wide range of applications of nanotube-protein conjugates, a deep understanding of protein immobilization on nanotube scaffolds is needed to achieve a working system.

The carbon nanotube-protein conjugates could represent a promising platform for the development of molecular transporters. In particular, the recent studies have showed that SWCNT successfully can be used as a generic protein scaffolding and transportation tool for intracellular delivery.

The optical properties of carbon nanomaterials, including their photoabsorption and intrinsic or chemically induced fluorescence, have been successfully employed upon proper surface functionalization in imaging, sensing and therapy applications, in which the nanoparticles were utilized as contrast agents for microscopy, signal transducers for analyte detection, light absorbing agent for photo-therapy, and drug carriers for targeted delivery, respectively.

Moreover, the electrochemical properties of carbon nanotubes make them ideal for use in electrochemical systems. Their ability to increase electrode surface area and act as a protein scaffold makes them ideal candidates to localize at an electrode surface. Furthermore, carbon nanotubes can act as mediators, to directly transfer electrons to and from enzyme active sites making CNT-enzyme-electrode systems applicable as biosensors, catalysts, and biofuel cells.

Though significant advancements have been realized in this field, there is still a big challenge to understand more deeply the mutual interaction between the protein and carbon nanomaterials surface. Further studies that seek to develop rationally design for carbon nanotubes-proteins conjugates, considering the toxicity and the cost-effective of this conjugate, are crucial for their application in sensing and biomedical area.

Acknowledgments

We gratefully acknowledge discussions with Strano group members regarding this manuscript. S.F.O. acknowledges funding from CAPES foundation – coordenação de aperfeiçoamento de pessoal de nível superior – Brazil. M.P.L. Acknowledges an NSF postdoctoral research fellowship under award no. 1306229, a NARSAD Young Investigator Award, and a Burroughs Wellcome Fund Career Award at the Scientific Interface (CASI) under award number 1014567. M.S.S acknowledges support by the National Science Foundation under award number 1213622, and the Juvenile Diabetes Research Foundation.

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