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Under the lens: carbon nanotube and protein interaction at the nanoscale

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The combination of the very different chemical natures of carbon nanotubes (CNTs) and proteins gives rise to systems with unprecedented performance, thanks to a rich pool of very diverse chemical, electronic, catalytic and biological properties. Here we review recent advances in the field, including innovative and imaginative aspects from a nanoscale point of view. The tubular nature of CNTs allows for internal protein encapsulation, and also for their external coating by protein cages, affording bottom-up ordering of molecules in hierarchical structures. To achieve such complex systems it is imperative to master the intermolecular forces between CNTs and proteins, including geometry effects (*e.g.* CNT diameter and curvature) and how they translate into changes in the local environment (*e.g.* water entropy). The type of interaction between proteins and CNTs has important consequences for the preservation of their structure and, in turn, function. This key aspect cannot be neglected during the design of their conjugation, be it covalent, non-covalent, or based on a combination of both methods. The review concludes with a brief discussion of the very many applications intended for CNT–protein systems that go across various fields of science, from industrial biocatalysis to nanomedicine, from innovative materials to biotechnological tools in molecular biology research.

1. Introduction

Carbon nanotubes (CNTs) and proteins have very different chemical nature, but they have something in common: a nanostructure that allows for sophisticated electronic, chemical and even biological functions. On the one hand, CNTs have become popular in nanotechnology[1] and, more recently, nanomedicine,[2] because of their unique properties in terms of flexibility, lightness, resilience, and electronic behaviour conferred by their regular chemical structure made of solely carbon atoms. The different ways in which sp^2 carbons are arranged into space give rise to a surprising number of nanotube types, each of well-defined physicochemical character. Distinctive features include (1) helicity (*i.e.*, armchair, zig-zag, or chiral CNTs), (2) electronic behaviour (metallic or semiconducting), and (3) geometry (*i.e.*, single-walled or multi-walled, capped or open-ended). In addition, they can be conveniently functionalised to enhance their biocompatibility[3] and even biodegradation,[4] and indeed chemical derivatisation is an indispensable step to improve their dispersibility for most applications.[5] All these features make them stand out among nanomaterials that find application in the modern fields of sensing[6] and theranostics,[7] as well as electronics,[8] catalysis,[9] and high-performance materials.[1]

On the other hand, proteins are much more heterogeneous in their chemical nature, and display a range of functional groups that allow for versatile chemical derivatisation.[10] Since the advent of proteomics, they have been extensively studied in biochemistry to gain further understanding of cellular processes that underpin both physiological and pathological events. Besides, they have found ample scope of application in the fields of biotechnology[11] and sensing,[12] thanks to their rapid flexibility in adopting complex 3D structures with function of exquisite specificity. Their ability to rapidly respond to changes in the local environment is a fascinating subject of study, as they associate into complexes, catalyse molecular transformations, trigger and modulate cellular cascades through rapid chemical changes (*e.g.*, *via* acquisition and loss of phosphate groups).

It is thus not surprising that the encounter between CNTs and proteins is a matter of prolific research, since it has the potential to combine very different properties and chemistries. The coupling of CNT resilience and electronic function with proteins' biological activity is already pushing the frontiers of sensing, and the topic has been extensively reviewed elsewhere.[6,13–15] Here, we report on advances in protein encapsulation in CNTs and, on the other hand, CNT coating by protein structures. A brief discussion will then follow on the nature of protein–CNT interactions, and how they can be exploited to interface the two components. Proteins can be coupled to CNTs both covalently and non-covalently. The first method offers the advantage of achieving chemically stable products at the expense of potentially

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altering electronic features and chemical activity of both CNTs and proteins. Conversely, the latter has the potential to preserve the intrinsic properties of both components, although usually it leads to products with instability issues over time or environmental conditions. Often, a combination of both methods is used to leverage benefits, while minimising weaknesses of each. We will look at all approaches with an eye for the physical and chemical interactions between the two, and their effect on their respective functions. We will not review CNT interaction with native proteins *in vivo*, for instance following CNT administration for medicinal or diagnostic purposes, since this complex topic deserves a separate discussion, as numerous other factors come into play.[2] Finally, we will give a general overview of the, at times, surprisingly imaginative potential applications of CNT–protein conjugates, to anticipate the future direction and scope of this multidisciplinary research area.

2. Protein encapsulation in CNTs

The hollow nature of CNTs may lead to immediately consider them as potential tubular vectors to be filled with biomolecules; upon application of a specific trigger, the load could be delivered to the desired site of action. As a matter of fact, protein smart encapsulation and preservation in CNTs is not at all straightforward, as complex factors come into play at the nanoscale. From an experimental viewpoint, the concept is more challenging to realise than one might think. As a piece of evidence, only very few experimental studies exist on the topic, although the issue has been widely investigated *in silico*.

An early study on open-ended CNTs with an internal diameter of 3–5 nm suggested that CNTs are able to encapsulate a number of different proteins with firm binding. However, the protein was so strongly kept inside the CNTs that this was actually an obstacle for subsequent protein release. In fact, it was not possible to remove the encapsulated proteins with buffer washes under various conditions of pH and ionic strength. However, a benefit of great potential was the realisation that encapsulation protected the proteins from radiation damage (during TEM analysis). As a result, although some perturbation in protein shape was observed, at least some enzyme activity was retained.[16] It is thus tempting to imagine future applications in smart devices. Radiation could be employed to engineer a certain CNT response, such as damage of surrounding tissue at a disease site (*i.e.* by thermal ablation) and concomitant delivery of the preserved protein cargo.

Protein stability is indeed a key factor for the application of CNT encapsulation; molecular dynamics offer useful tools to study the effect of confinement inside narrow SWNTs on protein stability. Simulations have been used to analyse the confined fate of specific types of peptide conformation. In one study on alpha-helix secondary structures, solvent entropy emerged as a key factor affecting stability. Water drives hydrogen bonding, and its exclusion from hydrophobic surfaces maintains their correct association in folded structures in a hydrophilic environment. The marked decrease of water entropy inside the narrow CNT surface has thus obvious implications negatively affecting protein secondary structures. This ''hydrophilic'' effect was held responsible for the general destabilising nature of confining spaces encountered by proteins.[17] In line with these findings, a separate study on beta-sheets led to the conclusion that they are more prone to deformation relative to alpha helices. Interestingly, such deformation occurred as globular proteins *spontaneously* entered CNTs of sufficient diameter, and underwent a step-wise conformational change to maximise interactions with the surface of the tubes.[18] Clearly, the extent of translation of these destabilising effects to wider CNTs or large proteins is a different matter that requires separate studies. From an energetic point of view, it is important to note that the enthalpy decrease and entropy increase associated with protein encapsulation suggested that, under appropriate conditions, protein entrance is energetically favoured. Indeed, the overall free energy of the system was notably reduced upon encapsulation.[18] Obviously, for each protein exists an appropriate CNT diameter to ensure encapsulation in a spontaneous process; CNTs that are too large offer insufficient initial van der Waals attraction to the protein, for it to move inside the nanotubes.[19] An additional consideration to make is that the major contributions to the overall free energy changes to the system are given by changes in the water molecules. Such changes occur as they move out of the nanotubes while the protein gets in, and as they change the intermolecular hydrogen bonding network structure. As a result, one has to remember that the nature of the solvent is another key factor in achieving protein encapsulation.[19]

On the bright side, there are encouraging molecular dynamics studies showing that proteins, irrespective of their secondary structure, interact favourably with SWNTs, and the adsorption strength of oligopeptides is greater within the concave inner surface of the tube, relative to the convex outer surface; this is especially true when applied to hairpin conformations that maximize surface contacts.[20]

In conclusion, given that appropriate system conditions are identified, it appears that protein encapsulation in CNTs is likely to occur, its preservation is possible to some extent, while its smart release is still a matter that awaits new solutions.

3. CNT embedding within protein structures

What if we switch the roles of CNTs and proteins in the encapsulation process? Imaginative chemists have indeed engineered systems where CNTs are effectively coated all over by a protein armour structure, as if it were encapsulated within the biomolecules.

3.1 CNTs coated by protein cages

Streptavidin is a protein known to have hydrophobic domains that allow for purification on resins and for formation of 2D crystals on lipid films. It was shown to be able to form a single-protein layered coating of MWNTs with helically-ordered protein crystals that occurred stochastically (Fig. 1).

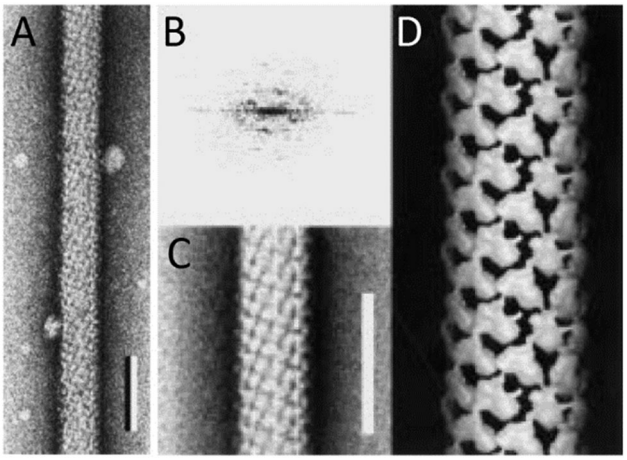


Fig. 1 Helical organization of streptavidin-coated MWNTs. Scale bars = 50 nm. (A) Negative-stained TEM image. (B) Optical diffraction pattern from X-ray analysis. (C) Noise-free average view obtained from correlation of X-ray analysis data. (D) 3D-model calculated by retroprojecting the helical repeat shown in (C). Reproduced with permission from ref. 21, Copyright © 1999 WILEY-VCH Verlag GmbH, Weinheim, Fed. Rep. of Germany.

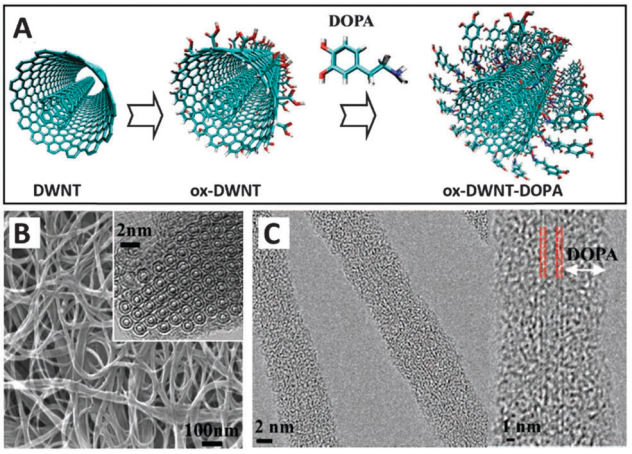


Fig. 2 (A) Schematic illustration of the formation of a covalent bond between the carboxylic acid of oxidised DWNTs and the amine of dopamine (DOPA). (B) Pristine DWNTs form thick bundles as seen by SEM and TEM (inset). (C) DOPA-DWNTs are well dispersed in individual tubes, and display a uniform coating, as seen by TEM; the double walls of a DWNT are highlighted in red. Adapted with permission from ref. 22, Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

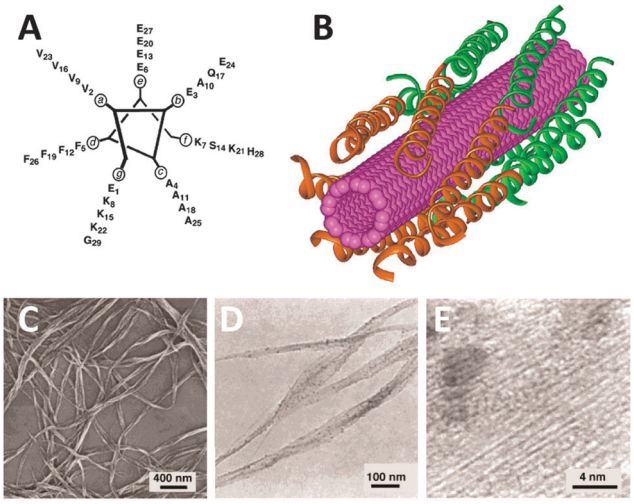


Fig. 3 (A) Amphiphilic peptide sequence with aligned amino acids along the helix. (B) Two rows (orange and green) of aligned peptide hexamers along the CNT (pink). (C) SEM image of hybrid fibers. (D and E) TEM images of the hybrid fibers, at low (D) and high (E) magnification, revealing aligned CNTs. Adapted with permission from ref. 24, Copyright (2003) American Chemical Society.

Remarkably, the binding was stable since the protein did not desorb in the presence of Tween 20, and the authors suggested that these new nanostructures could find application in industrial processes, by facilitating CNT manipulation.[21]

Since pristine CNTs have the big drawback of forming thick bundles that limit their dispersibility and processability, it is easy to envisage use for protein durable coatings that may improve CNT affinity for water or polar solvents. Indeed, cross-linked protein coatings of the CNTs were engineered for this purpose, exploiting functional groups naturally occurring in the macromolecule (*i.e.*, Ar–OH or SH).[22,23]

In one example, direct covalent attachment to oxidised CNTs was applied to recombinant adhesive mussel protein, which can easily be crosslinked *via* its dopamine (DOPA) residues (Fig. 2A). As a result, while the pristine DWNTs tended to aggregate into thick bundles (Fig. 2B), their DOPA-coated derivatives yielded highly stable water-dispersions of uniformly-coated, luminescent, individual nanotubes (Fig. 2C). Such material could, in principle, be easily processed into films or electrospun fibers for further use.[22]

Alternatively, a virus-like coating of the CNTs was achieved by cross-linking a sulfhydryl-rich protein. At low pH, beta-lactoglobulin adsorbed on MWNTs; thanks to the repulsion between the charged protein molecules, its addition favoured debundling and dispersion of CNTs in water. Upon heating, numerous disulphide bridges were formed, effectively cross-linking the protein into a uniform, thick cage coating the tubes. Importantly, the structures were responsive to NIR irradiation and showed reduced cytotoxicity *in vitro*, giving scope for their use in the thermal ablation of diseased tissue in cancer therapy.[23]

Proteins can also be exploited to impart hierarchical order in self-assembled structures for controlled geometry at the nanoscale. For instance, appropriately designed amphiphilic alpha-helical peptides effectively coated CNTs and yielded fibers of controlled size and geometry (Fig. 3). Hierarchical order was imparted by the design of an alpha helix with specific amino acids (*e.g.*, hydrophobic, charged, *etc.*) aligning along its surface, which displayed an amphiphilic nature (Fig. 3A). In this way, the hydrophobic side of the helices could interact with the CNT surface, while the hydrophilic side would be exposed to the surrounding aqueous medium. As a result, alignment was observed both for the peptide molecules along the CNT surface (Fig. 3B), and for the CNT structures along the hybrid fibers (Fig. 3E).[24] However, as expected for this kind of interactions, the system was not stable to dilution, and to overcome this

issue the authors cross-linked the peptides. As a result, the dispersing capacity of the peptide was increased, and the stable structures could self-assemble into aligned fibers upon drying, suggesting a templating ability conferred by the peptide–peptide crosslinks that allow SWNT self-organisation in space.[25]

3.2 Use of protein cages to template AuNP-assembly on CNTs

When hierarchical order also involves gold nanoparticles (AuNPs), an additional scope is attained. Either peptides able to self-organise into virus-like cages[26] or small amphiphilic adhesion proteins[27] were used to direct the ordered self-assembly of AuNPs along the CNTs. One of the advantages of these techniques is the bottom-up arrangement of AuNPs into ordered arrays, which otherwise can be obtained through much more laborious top-down methods.[27]

In one case, helical peptides were designed to display, on one side, a surface reminiscent of an alanine-coil-like structure, and on the other, a surface reminiscent of a leucine-zipper. In this way, peptides could interlock into an antiparallel hexamer around a SWNT of appropriate dimensions, resulting in a structure quite similar to that shown in Fig. 3B. Upon insertion of an additional cysteine at the *N*-terminal, convergent gold-binding sites would occur in pairs along the hexamer surface at the peptide ends. Subsequent reduction of Au(III) resulted in the formation of 2–4 nm-wide AuNP arrays, as seen by TEM imaging, where order was imparted by the underlying CNT–peptide hybrid.[26]

Simpler alternatives to achieve the same scope of ordered AuNP arrays are also possible, by substituting designed peptides with proteins of known amphiphilic surface, to achieve self-assembly. If the protein (Fig. 4A) has further interesting features, such as the ability to form elastic films at the air/water interface (Fig. 4B), then, additional opportunities arise to achieve versatile nanocomposite materials. The protein was engineered to contain a cysteine for covalent binding of monomaleimido–AuNPs (Fig. 4A). The protein effectively coated CNTs with aligned AuNP-arrays into hybrids of regular self-assembled structures as observed by TEM analysis (Fig. 4C and D). Additionally, by changing the conditions for self-assembly of the system, composite elastic films were observed, where the CNTs were embedded in a protein matrix.[27]

These are just a few examples, some elaborate, others simple, of protein–CNT assemblies of hierarchically ordered nanostructure.

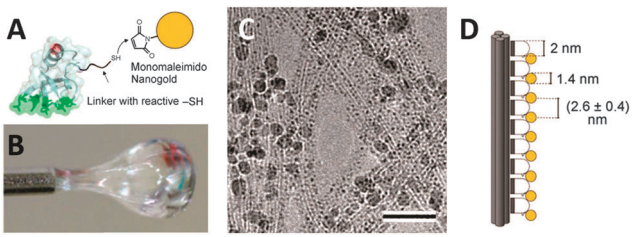


Fig. 4 (A) Illustration of the protein engineered to bind AuNPs (hydrophobic region in green). (B) The protein forms elastic films at the air–water interface. (C) TEM image of CNT–protein–AuNPs hybrids. (D) Illustration of the hybrid structure. Adapted with permission from ref. 27, Copyright © 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

They clearly illustrate how innovative nanomaterials of unprecedented properties (*e.g.* optical, biological, catalytic and electronic) are rendered accessible by the synergistic combination of CNTs and proteins.

4. Nature of CNT–protein interactions

4.1 Intermolecular forces

Although the realms of CNTs and proteins have been traditionally quite far apart from each other, their encounter has already thrilled a number of scientists. The first question to answer when looking at such adventurous union is how to manage their mutual interaction for it to be long-lasting and mutually-favourable.

The less imaginative chemist will typically account for van der Waals and hydrophobic interactions as a key driver to unite the two.[28–36] These forces *per se* are weak, however, the tendency of hydrophobic groups to cluster together in water, to avoid exposure to the hydrophilic medium, leads to a large number of potential contacts between the surfaces of adsorbed proteins and CNTs. The resulting additive effect makes these interactions dominant; this is especially true for protein complexes or polymers, where several proteins cluster together *via* association of hydrophobic regions (*e.g.*, in filamentous actin).[35]

Due to the aromatic nature of CNTs, it is rather common that protein–CNT interactions are rationalised in terms of π–π stacking with aromatic amino acids.[29,31,32] In particular, histidine and tryptophan appear to have the highest affinity for CNTs,[28,29] and this was rationalised in terms of a HOMO–LUMO interaction between the orbitals of tryptophan and CNT, respectively.[37]

However, peptide sequences with high affinity for CNT binding and devoid of aromatic residues have also been identified, suggesting that other factors come into play.[30] Amphiphilicity is indeed another key property that appears to promote interaction of peptides and proteins with CNTs in aqueous media.[24,27,30] This is not surprising since the proposed binding mechanism is similar to that of surfactants, which wrap around the tubes with their hydrophobic regions, while exposing hydrophilic or charged residues to the external media.[24,27] The same principle can be extended to individual amino acids bearing an aliphatic chain with terminal charged groups.[31] In line with these findings, lung surfactant proteins were shown to bind to DWNT bundles in a calcium-dependent manner, and their structure and function appeared to be preserved.[38]

Electrostatic interactions are generally considered to play a minor role in protein–CNT binding, although they have been reported in various studies, often taking into consideration the relation between protein charge and pH.[36,39] Charged amino acids, such as lysine and arginine, are thought to establish cation–π interactions with the tubes.[40–43] Charge transfer can occur even in the absence of a net charge, for instance during physisorption of aminic and amidic residues.[41] The effect is more pronounced for metallic tubes[40] or when considering the gating effect of protein binding onto semiconducting CNTs.[43] Such electrostatic interactions can dramatically affect

the electronic properties of CNTs, thus, should not be neglected. This is especially true if the intended use of CNTs would benefit from their preservation (*e.g.* in sensors).[41,42] On the bright side, appropriately designed charge transfer can be exploited to fine-tune CNT electronic properties according to the desired use; the net effect is the generation of n-doped or p-doped CNTs, upon introduction of electron-donor or -acceptor functional groups, respectively.[42] Alternatively, protein gating of semiconducting CNTs can be used to detect the protein in solution with direct electronic readout.[41]

4.2 Importance of primary, secondary, and tertiary protein structures

Having established the molecular basis for CNT–protein interaction, can we rationally design *de novo* amino acid sequences with high affinity and selectivity for CNTs? The literature is rich with studies that attempt to satisfy this question with credible answers. The identification, or even the design, of peptide motifs and/or single amino acid residues with high CNT affinity has thus been given ample investigation.[26,30,44] However, it was shown that proteins with similar content of individual amino acids bind CNTs to a different extent.[28–30,45] Similarly, also proteins with a primary sequence made of different amino acids but equal amounts of aromatic, apolar and polar character, bind CNTs to various extents.[32,45] This is not surprising if we consider that amino acids can be arranged differently in space, and it is the resulting polypeptide surface, rather than its primary sequence, that will establish CNT-contact.

In this respect, both secondary and tertiary protein structure play an important role. The protein surface exposed to CNTs may be determined also by partial unfolding, which will effectively disrupt the protein tertiary structure while leaving single domains or local secondary structures unaltered. In fact, changes in solvent accessible protein surfaces can be used as a tool to evaluate protein–CNT binding.[31,32] Shape complementarity between proteins and CNTs (*e.g.* their curvature, thus their diameter) was also suggested as a key factor for their interaction.[20] Molecular dynamics has shown how SWNTs, depending on their dimensions, interfere with peptide backbone hydrogen bonding to induce a certain degree of curvature in the biomolecules. As a result, eventually the peptides tend to wrap around the nanotube.[33,34] Additionally, SWNTs (rather than MWNTs), due to their small dimensions, can directly interfere with protein function. It has been suggested that they can fit into ion channel pores and hinder either ion passage or conformational change required for proper channel function.[46] SWNTs may bind to specific hydrophobic pockets onto the protein surface, thus interfering with folding and natural protein–ligand interaction.[36]

Due to similar shape, dimensions, and hydrophobic character shared by CNTs with certain proteins, CNTs can also interfere with biological protein polymerisation required for proper cell function. They can associate to filamentous actin and interfere with the generation of traction forces through the cytoskeleton, thus affecting cell motion.[35] They can also interfere with microtubule dynamics, leading to anomalies in cell division and subsequent apoptosis.[47]

In conclusion, protein shape and surface properties, which are not predictable simply by their amino acid content, are key factors for CNT-interaction. Although aberrant or reduced protein function can be a negative outcome of such interaction, it can also be exploited in therapy by appropriate design (see Applications).

5. Protein–CNT non-covalent binding

5.1 Scope for non-covalent binding

The choice of this approach is typically made with the rationale that it leads to the least possible perturbation of both structures, and thus to the preservation of their function too. Nevertheless, this is not always the case, since protein partial unfolding may occur,[36,48,49] as discussed in the section below.

A large scope of this method is found in the field of biosensors, where the CNT sp^2 graphitic sidewall integrity is needed for optimal electrical conductivity. When this is achieved, CNTs can be exploited as transducers, for instance for the catalytic electrochemical detection of small molecules (*e.g.*, glucose) that are substrates of enzymes bound onto the CNTs without their activity being compromised (*e.g.*, glucose oxidase).[14] Protein adsorption on CNTs has also been proposed as a way to disperse CNTs in aqueous environments, with lysozyme and albumin among the most popular proteins used.[23,36,40,45,49–52] In some cases, protein-aided dispersibility proved to be effective especially at pH values far away from the protein isoelectric point,[36,50,53] suggesting a role played by electrostatic interactions, as supported also by concentration-dependent effects induced by the addition of guanidinium chloride.[36] The dispersibility was further improved also by the addition of fluoroalcohols (*i.e.* 1,1,1,3,3,3-hexafluoroisopropanol and 2,2,2-trifluoroethanol), which did not significantly alter protein conformation, while markedly reducing inter-tube hydrophobic interactions that mediate CNT bundling.[53] Nevertheless, one shortcoming of protein (or surfactant polymer) passive adsorption to disperse CNTs is the typical precipitation observed upon dilution for biological use. This is why crosslinking between protein (or peptide) molecules, or even between the protein and CNTs is advisable to overcome the issue.[25]

5.2 Protein (partial) unfolding

In general, as a protein comes into contact with the inner or outer curved CNT surface, hydrophobic regions normally buried within its folds may be exposed, driven by van der Waals or other hydrophobic interactions. We have already discussed instances for this to happen (Sections 2 and 4.2). This is particularly relevant for proteins that self-associate into oligomers to gain function, since their hydrophobic interactions can be heavily perturbed by the presence of the hydrophobic CNTs.[54] However, this effect may be mitigated by appropriate formulation design, for instance by using reverse micelles where the enzyme is located at the water–oil interface, sometimes resulting even in increased enzyme activity relative to aqueous environments. The authors suggested that the effect is due to increased

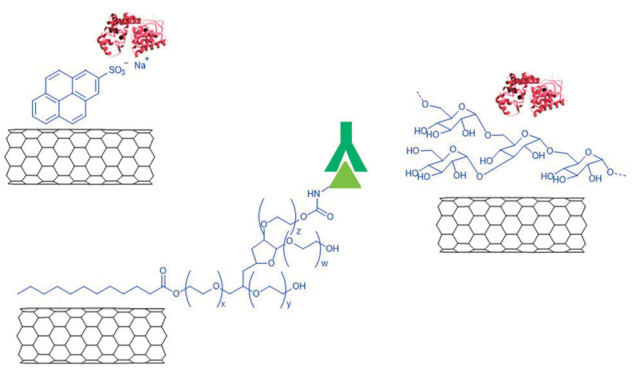
interfacial area and increased local concentration of the enzyme substrate.[49]

Another interesting concept is the ordering of water molecules onto the CNT surface, and the study of its effect on the adsorption of proteins onto the tubes. It has been suggested that an ordered shell of hydration of the tubes can have a positive effect, such as the preservation of protein conformation (by preventing a tighter protein adsorption onto the CNT with partial protein unfolding)[55] when it occurs on the outer surface of the tubes. In contrast, a negative effect of reduced water entropy on protein conformation stability is expected in the case of proteins inside the CNTs (as discussed in Section 2).[17]

5.3 CNT–proteins with or without a linker

Various approaches can be used to achieve non-covalent binding of proteins onto CNTs, namely intercalation of a small molecule linker,[56] polymer- or surfactant-mediated attachment,[57] or simply physical adsorption of the protein directly onto the CNT hydrophobic surface (Scheme 1).[14,49,58] A typical method to intercalate a linker between CNTs and proteins is to exploit a polyaromatic unit to bind the nanotubes, such as a pyrene moiety, bearing functional groups for protein binding (*e.g.*, sulfonic acid moieties to electrostatically bind the positively charged surface of a protein).[56] Alternatively, long aliphatic chains of surfactants such as Tween 20 can coat the tubes *via* van der Waals interactions, while their –OH groups from the hydrophilic heads can be activated (*e.g.* with carbonyldiimidazole) and functionalised with protein-binding ligands (*e.g.*, biotin or antigens).[57]

Direct protein adsorption on CNTs is probably the simplest method to achieve non-covalent binding, although it is not devoid of drawbacks. First of all, lack of specificity, since it is known that a large number of proteins will adhere on the tubes, for instance by simple means of van der Waals and hydrophobic interactions.[2] Second, it is rather difficult to control the stability of the conjugates, as we can imagine proteins will slowly desorb from the nanocarbons, even more so when local conditions are changed (*e.g.*, upon exposition to dilution or surfactants).[25,29,52] Third, proteins that non-covalently adsorb onto CNTs may partially unfold and lose their biological function as discussed above.[49]



Scheme 1 Examples of CNT–protein non-covalent attachment mediated by linkers (in blue). Proteins are represented by long pink ribbons. Antigen–antibody interaction is represented in green.

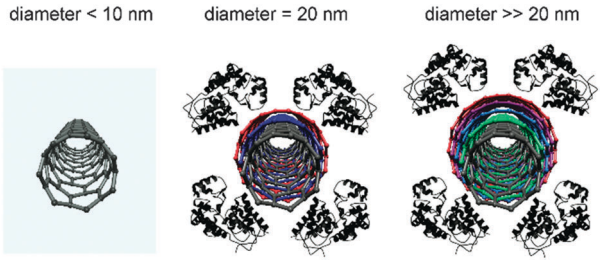


Fig. 5 Protein adsorption on CNTs is affected by the tube diameter. Nanotubes narrower than 10 nm (left) virtually bind no proteins on their surface, while for tubes with a diameter equal to or larger than 20 nm (centre and right respectively) formation of a protein corona is independent from the tubes width.[61]

5.4 Importance of CNT diameter

When long-lasting direct protein adsorption on CNTs is desired, one important parameter to consider is the CNT diameter, which should ideally be larger than 10 nm (Fig. 5), as suggested by a growing body of work. An antifullerene IgG antibody was shown to specifically recognize and bind to SWNT ropes of diameter $>$ 10 nm, thanks to the presence of a cluster of hydrophobic amino acids in a cavity.[58] Surfactant-stable MWNT coating by proteins was achieved with ordered protein arrangement, as long as CNTs of diameter larger than 12 nm were used.[21] The relation between CNT diameter and protein attachment was also confirmed in another work, where stronger protein binding occurred for 40 nm-wide MWNTs relative to 10 nm-wide tubes.[39] In another work, components of the complement system bound significantly more onto MWNTs (diameter $>$ 25 nm) relative to DWNTs (diameter $\sim$ 5 nm) or SWNTs (diameter $\sim$ 1 nm).[51] However, examples do exist on proteins adsorbing onto SWNT bundles of diameter less than 10 nm or single SWNTs, although in this case there is limited information on the stability and specificity of the binding, since experiments were carried out with purified protein solutions and washes did not contain any detergent.[48,52,59] In fact, dialysis led to protein desorption from the SWNTs, suggesting the presence of an equilibrium between bound protein and protein in solution.[52] The concept that proteins may bind more strongly to SWNT bundles relative to single tubes is not new.[52] In fact, it was shown that protein binding to SWNTs may be weaker compared to bundles.[60]

Additionally, the CNT surface curvature also has other effects on adsorbed proteins. It is noteworthy that CNT surface suppresses unfavourable protein–protein lateral interactions, thus it can be exploited to increase protein stability in harsh environments,[62] such as high temperature, high salt, or organic solvent exposure. Such an effect appears to be related more to the physical curvature rather than the chemical nature of CNTs, since it is also shared by other curved nanomaterials (*e.g.* gold NPs) to a greater extent relative to flat supports of more similar chemical structure (*e.g.*, graphite flakes).[59]

6. Protein–CNT covalent binding

Covalent protein attachment to CNTs is not the most popular approach, since usually proteins are rather adsorbed either

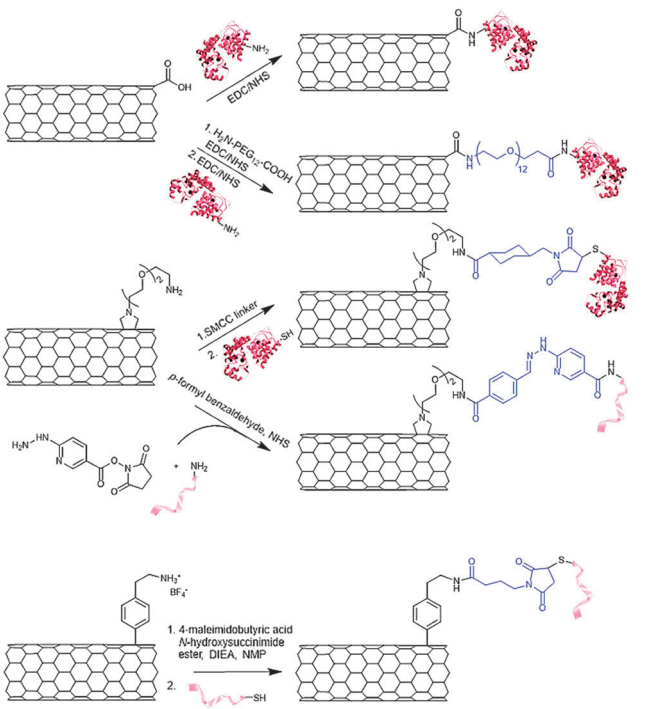
directly onto the tubes, or indirectly through the use of linkers attached to the tubes (see Section 7). The main rationale is that covalent modification of both the CNTs and the protein will (1) alter the structure, and to some extent, possibly also the function of both, and (2) induce a level of rigidity that may not be suitable for many applications. Nevertheless, one can find ample examples in the literature to achieve covalent linkages between CNTs and proteins, usually revolving around amine and/or thiol chemistry (Scheme 2).

The typical covalent linkage exploits the free protein amines by coupling to carbodiimide-activated COOH groups of oxidised CNTs to form a stable amide bond (Scheme 2, top).[62–67] However, the direct attachment of a bioactive protein to CNTs, without the use of a flexible linker, usually affects the protein ability to adopt different conformations, and, thus, reduces its function. This effect was seen for covalently-coupled luciferase luminescence[63] and other enzymes, for which the activity was improved when a PEG_{12} linker was used.[66,67] The latter approach is preferable especially for large proteins where monomer association is required for function; typically, protein oligomers are held together *via* hydrophobic-surface interactions which can easily be otherwise engaged in non-specific binding to CNTs, thus impeding enzyme activity.[67] The use of a short flexible linker is indeed an appropriate method to preserve the bioactivity of molecules that are to be covalently attached, and so was the case for the proteins or peptides used to target specific cells.[68]

Alternatively, protein-derived minimalist bioactive motifs are used. A popular example is the cell-adhesion promoter integrin-binding RGD motif,[69] which is often used as a cyclic derivative for prolonged lifetime in biological environments. The peptide is typically derivatised with a cysteine to exploit thiol-maleimide chemistry for covalent attachment. A popular linker is the heterobifunctional succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxy-(6-amidocaproate) with a maleimide group on one end, and an amine reactive NHS ester on the other (Scheme 2, centre). For instance, the RGD-linker moiety can be appended to SWNTs functionalised with an amino-terminated triethyleneglycol (TEG) linker, introduced *via* the popular 1,3-dipolar cycloaddition of azomethine ylides.[68] The same approach can be used for thiol-containing mAbs[64,70] or other peptides.[71,72]

Another viable option is the selective reaction of a peptide *N*-terminus (avoiding coupling of the lysine amino side chain) with succinimidyl hydrazino nicotinamide at neutral pH, thus exploiting the different pK_as of the α- and ε-amines. The aromatic hydrazine can then be transformed into hydrazone by reaction with the versatile TEG linker, previously reacted with activated p-formyl benzaldehyde (Scheme 2, centre).[73]

CNT chemistry is rather versatile; aryl diazo coupling is yet another approach that has been gaining ample popularity, since it has the great advantage to occur in water. Similarly to what was described above, this chemistry can also be used to attach maleimido-terminated linkers to covalently bind protein thiols (Scheme 2, bottom).[74]

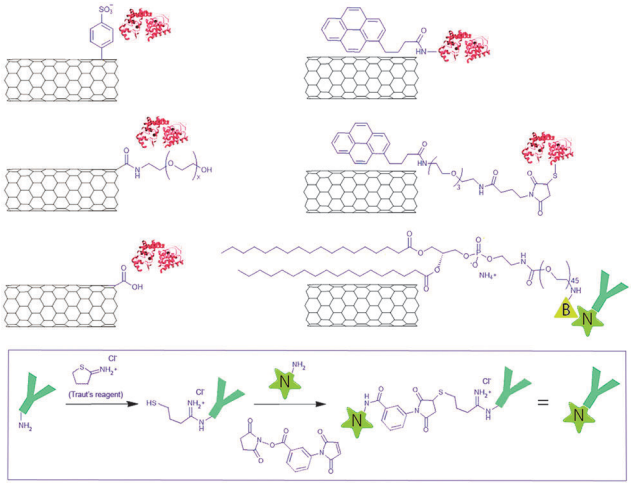


Scheme 2 Examples of CNT–protein covalent attachment. Proteins and peptides are represented by long or short pink ribbons, respectively. Linkers are in blue.

7. Protein–CNT conjugation based on a combination of covalent and non-covalent approaches

Combined approaches are employed when only one of either protein or CNT is covalently functionalised, while the other is bound through non-covalent interaction (Scheme 3). However, often authors refer to this approach simplistically as either covalent or non-covalent, simply considering the transformations that occur only to one of the two components (*i.e.*, either the CNT or the protein). It is actually preferable to be precise and not to neglect chemical perturbations that affect either of the two components. Otherwise it is not possible to have an accurate understanding of the system, with its capabilities and limitations.

In biological applications where the electronic properties of CNTs are not relevant, CNTs can be covalently functionalised without affecting the desired performance of the system. For instance, enzyme inhibition can be achieved,[75] even with active site-specificity, given that the protein structure is well characterised and the CNTs are properly derivatised. For instance, generation of a diverse library of functional groups appended onto oxidised MWNTs is a useful way to proceed.[76] Indeed, the use of oxidation to generate COOH groups onto the CNTs, onto which one can append a variety of small or large molecules, is very popular.[75,77] Polyethylene glycol (*i.e.*, PEG) chains can be attached *via* this method, and have been used to target the nucleus of cancer cells and inhibit telomerase activity.[75] Alternatively, CNT oxidation can be used simply to favour protein adsorption,[63,78]



Scheme 3 Examples of CNT–protein combined attachment where either the CNTs (examples on the left) or the proteins (examples on the right) are covalently functionalised. Proteins are represented by long pink ribbons. In green, biotin (B)–neutravidin (N) binding, with neutravidin coupled to an antibody (Y), through use of the Traut's reagent and a heterobifunctional linker (bottom).

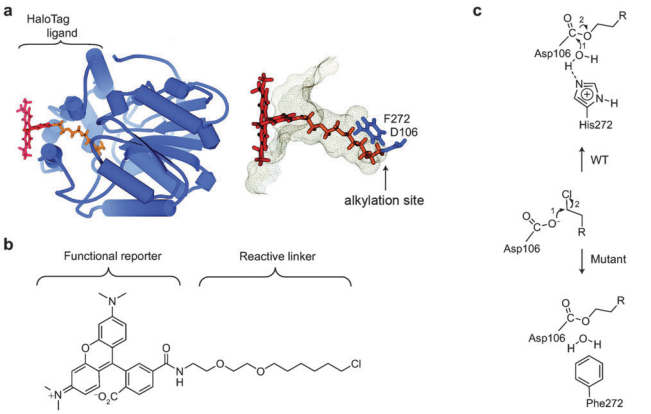


Fig. 6 (a) The Halotag ligand (red/orange in the cartoon) fits in a binding pocket of the Halotag protein. (b) The ligand has a fluorescent part (reporter) and a reactive ligand with a terminal chloride. (c) Protein alkylation occurs when the chloride on the ligand gets displaced, upon nucleophilic attack by the carboxyl group of an aspartic acid (D106) side chain. As a result, the Halotag protein is covalently linked to the fluorescent reporter, and this is possible thanks to a protein mutation (phenylalanine F272 in blue, instead of a histidine in the wild type protein). Adapted with permission from ref. 82. Copyright © 2008, American Chemical Society.

since it decreases the hydrophobicity of the tubes, and thus it increases the stability of non-covalent protein wraps.[50,79]

Electrostatic interactions can be exploited too. A straightforward method for covalent CNT functionalisation is the diazo coupling, which can be used to introduce a variety of functional groups anchored onto an aromatic moiety; if sulfonic acid groups are present, they can interact with the positive charges on the protein surface.[56] The popular EDC-mediated cross-linking was applied to an amphiphilic alpha helix peptide, thus conferring stability against dilution to the peptide oligomers that wrap around SWNTs, as discussed previously in Section 3.2.[25]

As described above for the non-covalent methods, this kind of approach is employed especially in the assembly of sensors, where the least perturbation of the CNT electronic structure is desired, and this can effectively be achieved for instance by functionalisation through π–π stacking with pyrene-based linkers that do not alter the CNT conductivity and that are resistant against desorption. The other end of the linker may display a succinimidyl ester for covalent attachment of the protein amines, or a maleimide unit to bind thiol groups, to achieve robust enzyme immobilisation.[74] A similar approach can be extended also to other amphiphilic linkers; biotinylated polar lipids for instance yield stable CNT dispersions, and can be selectively and avidly bound by neutravidin–mAbs for cancer cell targeting.[80] In this case, the mAbs have been linked to neutravidin by first introducing reactive thiol groups on the proteins by using the Traut's reagent (*i.e.*, 2-iminothiolane), and then by coupling to the amines of neutravidin *via* a bivalent crosslinker (*i.e.*, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester), having an NHS moiety to react with the amines, and a maleimide to react with the sulfhydryl groups (Scheme 3, bottom).[80]

Examples also exist where it is the protein that is covalently modified, while the tube chemistry is preserved for biosensor applications. For instance, surfactants can be adsorbed *via* van der Waals interactions on the tubes, while the functional groups on their hydrophilic heads can be covalently linked to proteins, *e.g.*, by reacting the protein surface amines with carbonyl-diimidazole activated hydroxyl groups present on the polymer.[57]

Another interesting property of CNTs that can be used is SWNT luminescence. This has recently been exploited to track live-cell motion dynamics. The nanotube structure was preserved by DNA-wrapping as a non-covalent method to disperse short (100–300 nm) tubes. The oligonucleotides exhibited a terminal amine, used to covalently bind the DNA–CNT complex to a reactive Halotag® ligand *via* classical succinimidyl ester chemistry. Protein conjugation was then achieved by exploiting the modern Halotag® reporter technology, which involves expression in the target cell to study a recombinant protein of interest with a Halotag®; the latter is a smaller protein that displays a specific binding pocket for the Halotag® ligand (Fig. 6). The Halotag® protein active site is situated at the inner end of the binding pocket. There, the carboxyl group of an aspartate residue displaces the terminal chloride of the ligand, thus generating a stable covalent bond between the ligand (attached to the DNA–CNT complex, in this case) and the protein. This approach proved to be effective to track microtubule dynamics within mammalian cells by attaching the DNA–CNT complex to a kinesin motor protein.[81]

8. Applications

There are numerous applications for CNT–protein systems. We will now provide an overview of potential uses encompassing very different areas of science, as would be applied in industry, clinics, or academic research.

Despite the intense scientific effort in this area, most of the intended uses of CNT–protein systems are still at an early stage

of "proof of concept". This is likely the result of the fact that early reports of CNT toxicity upon inhalation effectively acted as a handbrake on the development of CNT-based products.[82,83] However, there is ample evidence that CNT toxicity and fate in the environment are heavily dependent on their functionalisation, on their physical properties (*e.g.*, length, number of walls), and on the route of contact (*e.g.*, inhalation, skin contact, *etc.*).[84–88] Remarkably, in 2013 it was shown that asbestos-like pathogenicity of pristine CNTs can be completely alleviated upon chemical functionalisation.[3] Nevertheless, the current lack of internationally harmonised guidelines for nanomaterials is limiting their adoption in marketed products, and is a growing concern in the public. This is an issue[89] that must be addressed with urgency to allow the full realisation of the high potential of CNTs, as recognised by the establishment of relevant initiatives, such as the Nanosafety Cluster by the EU.[90]

8.1 Biosensing

The most widely known use for CNT–protein systems is biosensing, for the quantification of a target molecule (*e.g.*, food toxin[70]) or of a biomarker (*e.g.*, a protein) in diagnostics. To this end, a selected protein (that binds or transforms the target compound to detect) is bound to CNTs. Upon binding with the target, the protein then undergoes a certain change (*e.g.* conformational, or of surface charge due to redox reactions or pH changes) which can be transduced to the CNT. The nanotube response will then be transferred to a detector and depending on its type, we will have electrochemical,[70,91] electrical,[92] or field-effect-transistor (FET)[15] based sensors. More recent devices that exploit CNT optical properties such as near-infrared (NIR) fluorescence have the advantage of allowing for contactless signal transduction and multiplexing of complex samples.[93] Additionally, integration of photoresponsive proteins (*e.g.*, photosystem II) with CNTs can be used for photobioelectrochemical cells and photobiofuel cells for solar energy conversion.[94,95]

8.2 Enzyme support

The high surface area offered by CNTs is another interesting property that comes useful for enzyme support, for instance for industrial biocatalysis. Advantages include increased enzyme stability and possibility to recycle the enzymes.[48,59,62] As with any other solid support, higher enzyme activity is obviously achieved with proper spatial control of protein attachment onto the CNT surface, ensuring small molecule accessibility to the active site. Conversely, if the enzyme catalytic site is involved in CNT interaction, there will be a dramatic or even total loss of substrate conversion into product.[48]

Protein–CNT composites have been achieved with anti-fouling,[96] sporicidal,[67] and antimicrobial[66] properties, for use in the fields of self-cleaning paints and active coatings. In these composites, enzymes have the active role of catalysis and as a CNT dispersing agent, while CNTs are enzyme supports and confer useful mechanical properties to the final material. In addition, anti-fouling activity is key also to reduce biofilm formation and extend the work-life of CNT-containing materials, such as membranes for waste water decontamination or active paints to preserve ship bottoms in marine waters.

8.3 Biomaterials

CNT–protein composites are also interesting biomaterials for tissue engineering, where proteins interface CNTs with cells and CNTs provide structural support and add new properties, such as electric conductivity to extracellular matrix (ECM) protein hydrogels.[79,97] Alternatively, ECM proteins, such as collagen type 1 and fibronectin, can be presented onto CNT platforms to successfully achieve cell adhesion and growth.[98,99] The carbon nanostructure component can be used to monitor tissue integration by means of optical, magnetic, photoacoustic detection, or to alter intra- and inter-cellular processes.[7,61] CNTs can be also exploited to guide and sense cell growth by chemical or electrical means, and this is especially relevant for conductive tissue, such as cardiac and nerve tissues.[97,98] Laminin-functionalised CNTs favour neural adhesion and growth, and support both neurite elongation and formation of neural networks (Fig. 7).[100,101] Importantly, they can direct axonal growth also on flexible polyimide polymers, and thus have great potential for the development of nerve conduits in neural implants and nerve tissue engineering.[101] It has been proposed that the neural differentiation-promoting effect exerted by CNTs onto mesenchymal stem cells is also mediated by neural growth factors adsorbing onto the tubes, highlighting once again the underlying importance of protein–CNT interactions in tissue engineering applications.[102] Also non-conductive tissues can benefit from the use of CNTs. Fibroblast growth factor (FGF)-conjugated CNTs promoted the proliferation of bone marrow stromal cells; when used as coating of a collagen sponge for bone regeneration, after two weeks of implantation *in vivo*, the biomaterials were integrated with newly formed bone tissue.[65]

8.4 Drug delivery

In nanomedicine, CNTs have great potential for drug delivery, thanks also to their unusual ability to pierce cell membranes and translocate inside cells to deliver their cargo.[103] Proteins of therapeutic interest can be used as cargo for CNTs, either by internal loading or by external adsorption[104] onto the tubes. Alternatively, protein or protein-derived bioactive peptide motif CNT decoration can be used to achieve targeted therapy,[64,68,71,80,105] or to allow for imaging (*e.g.* by using luminescent proteins).[63] mAbs–CNT conjugates that selectively target cancer cells can be used to achieve thermal ablation upon NIR light exposure.[80] Particularly promising appears the use of functionalised MWNTs

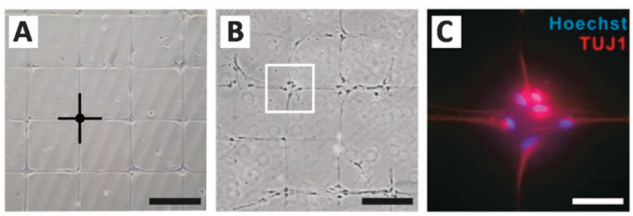


Fig. 7 Selective growth and differentiation of human neural stem cells into neural networks on laminin-coated CNT micropatterns. (A and B) Phase contrast images, scale bar = 200 μm. (C) Fluorescence microscopy image, scale bar = 50 μm. Adapted with permission from ref. 92, Copyright © 2011, American Chemical Society.

(for instance, relative to functionalised DWNTs or pristine CNTs), which yield stable water dispersions.[64]

8.5 Cancer therapy

In cancer therapy, an emerging paradigm is the use of self-assembly to integrate foreign structures with cell components to direct biological activity. Remarkably, MWNTs can assemble with tubulin to generate hybrid microtubule structures that lead to aberrant cell division and cell death.[47] The formation of hybrid structures is possible in light of the similar size and geometry of tubulin protofilaments and CNTs; thanks to their interaction with protein hydrophobic surfaces,[35] CNTs may promote self-assembly and stabilise microtubules, thus impairing their dynamics.[47] Considering that cancer therapy often relies on drugs that target microtubule dynamics (*e.g.* taxol), we can see how powerful CNTs could be as nanomedicines based on this mode of action.[47]

Efforts have also been made toward the use of CNTs to interfere with the self-assembly process of peptides and proteins involved in pathological disorders other than cancer, such as Abeta oligomers in Alzheimer's disease.[34,106,107]

8.6 Immunisation

Another useful therapeutic application is immunisation, where macromolecular carriers are needed for antigenic motifs to elicit proper immune response.[54,78] Antigenic peptides covalently bound on CNTs are presented correctly for the specific binding of antibodies, while CNTs boost immunogenic reactivity without affecting specificity.[72,73,108] In other words, CNTs can act as vaccine carriers as alternative to proteins, which have the disadvantage of being immunogenic *per se* and thus of lowering vaccine specificity.

8.7 CNT sorting

Peptides and proteins have also been shown to be useful tools in CNT sorting based on electronic conductivity, diameter, or even chirality. For instance, they have a preference for metallic CNTs, possibly due to the preferential binding of charged residues onto metals.[40,41] Cyclic peptides of selected sequence length can be used to sort CNTs based on their diameter.[109] Similarly, lysozyme was shown to bind preferentially to larger diameter tubes, and thus to be a useful means of purifying DWNTs from SWNT contaminations.[48] Peptides selected by phage display (Fig. 8) were shown to be able to preferentially disperse CNTs based on their chirality.[28]

8.8 Protein separation

In biotechnology, CNTs have also been proposed as nucleating agents for protein crystallisation studies, and remarkable examples do exist of protein crystallisation onto CNTs.[21,51,110] The non-specific binding of proteins onto CNTs has also been proposed as a method to increase the resolution of electrophoretic protein separation in SDS-PAGE gels, and both SWNTs and MWNTs proved fit for purpose in composite acrylamide gels (Fig. 9).[111,112] These ideas are interesting, although it is difficult to imagine that they will be embraced in molecular biology laboratories, considering that

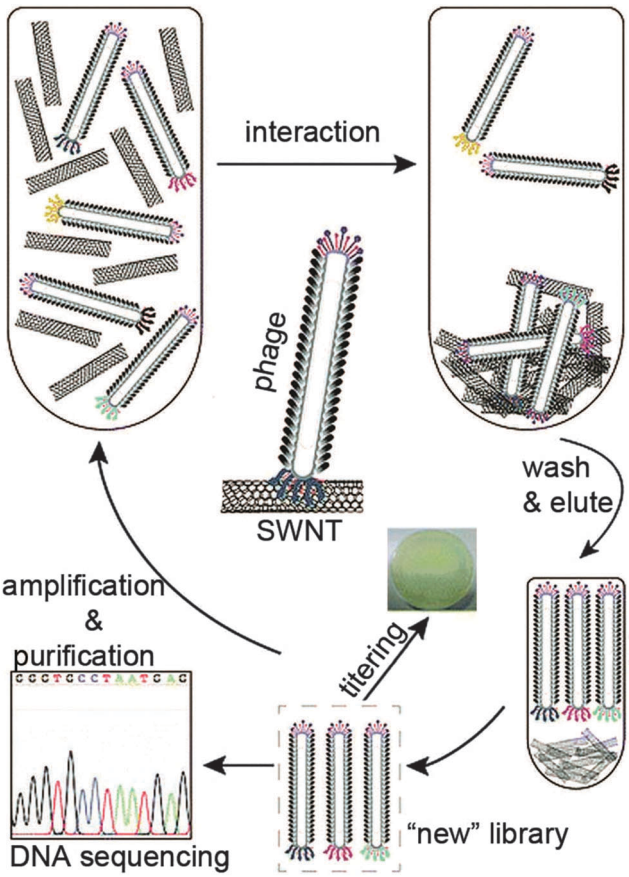


Fig. 8 Schematic illustration of a biopanning experiment. Peptides are selected by phage display for high affinity for (7,6) SWNTs. Adapted from ref. 28 with permission from The Royal Society of Chemistry.

the required adoption of safety measures for correct handling and disposal of nanomaterials is likely a barrier that is too high for the benefit achieved. In addition, other practical limitations lie in the fact that incorporation of CNTs reduces the ability to visualise proteins by the optical means typically used in molecular biology.

9. Conclusions and perspectives

The point of contact between the realms of carbon nanotubes and proteins is a fascinating area of research, where interesting proof-of-concept studies for high-tech applications continuously emerge. Scientific efforts have shown how such combined systems are characterised by a fine complexity of multiple key factors of physical, chemical, and biochemical nature. We now know that hydrophobic interactions tend to be dominant due to their additive effect. Electrostatics are also very important, especially in light of applications that exploit electronic CNT and/or protein behaviour (*e.g.* in sensing). By contrast, the impact of π–π stacking has been somehow reappraised, with the amino acids tryptophan and histidine emerging as key players over phenylalanine. The quest towards an amino acid sequence capable of specific CNT binding has identified some potential candidates,

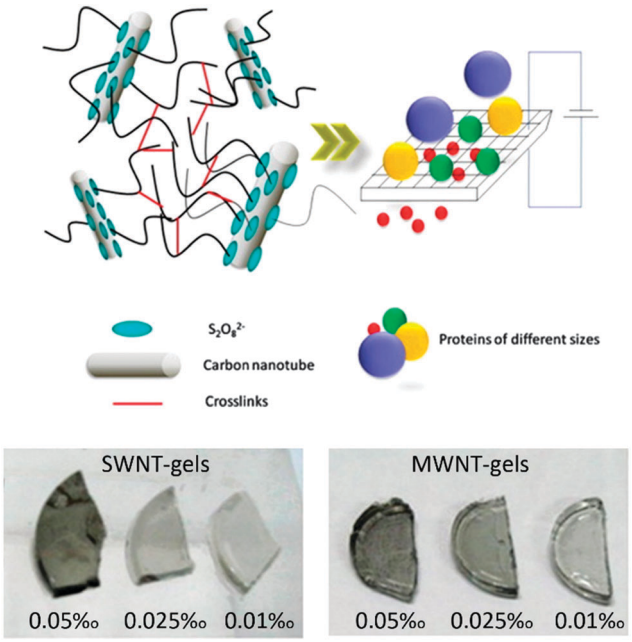


Fig. 9 Schematic illustration of the cross-linked CNT–polyacrylamide gels for protein electrophoretic separation (top) and photographs of gels obtained with different levels of SWNTs or MWNTs (bottom). Adapted from ref. 102, Copyright (2011) with permission from Elsevier.

but its relevance has also been reconsidered, in light of the importance revealed by other aspects, such as geometry and surface. For instance, CNT diameter and chemical functionalisation have emerged as key players for the fine-tuning of protein interaction. On one hand, MWNTs of larger diameter appear more suitable for durable protein binding in complex environments (for instance in tissue engineering) and are also more promising candidates for industrial applications due to their generally lower cost, higher purity, and higher ease of handling relative to SWNTs. On the other hand, the smaller sizes of SWNTs allow them to fit into protein hydrophobic pockets, thus leading to interference with protein–ligand interaction; proteins can alter their conformation and wrap around the SWNTs, giving scope for hierarchical self-assembly of hybrid superstructures for use also in sensors. The interaction between CNTs and proteins can favourably be mediated by small molecules and/or polymers and detergents, and we have attempted here to describe the numerous studies that exist on the topic and to highlight the key parameters affecting both CNT and protein properties and activities.

In terms of applications, systemic drug delivery is still at its infancy due to the complexity of factors related to the circulation and metabolism of CNTs in the whole organism, while more rapid progress has been made in local administration of agents for cancer theranostics (*i.e.*, therapy and high resolution imaging for diagnostics). In addition, two areas that hold great potential for high impact technological advancement are conductive tissue engineering (*i.e.*, implants for the heart and the brain) and chip electrodes for biosensing as personal point-of-care devices. Despite the requirement of more efforts to fine-tune CNT–protein hybrid systems, we believe their high potential will lead to important technological progress for the benefit of society.

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Notes and references

1 M. F. L. De Volder, S. H. Tawfick, R. H. Baughman and A. J. Hart, *Science*, 2013, **339**, 535–539.
2 S. Marchesan, K. Kostarelos, A. Bianco and M. Prato, *Mater. Today*, 2015, **18**, 12–19.
3 H. Ali-Boucetta, A. Nunes, R. Sainz, M. A. Herrero, B. Tian, M. Prato, A. Bianco and K. Kostarelos, *Angew. Chem., Int. Ed.*, 2013, **52**, 2274–2278.
4 A. Bianco, K. Kostarelos and M. Prato, *Chem. Commun.*, 2011, **47**, 10182–10188.
5 M. Melchionna and M. Prato, *ECS J. Solid State Sci. Technol.*, 2013, **2**, M3040–M3045.
6 I. Willner and B. Willner, *Nano Lett.*, 2010, **10**, 3805–3815.
7 S. Marchesan and M. Prato, *ACS Med. Chem. Lett.*, 2013, **4**, 147–149.
8 S. Park, M. Vosguerichian and Z. Bao, *Nanoscale*, 2013, **5**, 1727–1752.
9 M. Melchionna, M. Bonchio, F. Paolucci, M. Prato and P. Fornasiero, in *Making and Exploiting Fullerenes, Graphene, and Carbon Nanotubes*, ed. M. Marcaccio and F. Paolucci, Springer, Berlin Heidelberg, 2014, vol. 348, pp. 139–180.
10 D. Schumacher and C. P. R. Hackenberger, *Curr. Opin. Chem. Biol.*, 2014, **22**, 62–69.
11 G. Walsh, *Proteins: Biochemistry and Biotechnology*, John Wiley & Sons, UK, 2nd edn, 2014.
12 S. Cao, J. Chen, X. Jin, W. Wu and Z. Zhao, *Biosensor Nanomaterials*, Wiley-VCH Verlag GmbH & Co. KGaA, 2011, pp. 95–115.
13 B. L. Allen, P. D. Kichambare and A. Star, *Adv. Mater.*, 2007, **19**, 1439–1451.
14 J. Wang, *Electroanalysis*, 2005, **17**, 7–14.
15 A. S. D. R. Kauffman, *Chem. Soc. Rev.*, 2008, **37**, 1197–1206.
16 J. J. Davis, M. L. H. Green, H. Allen O. Hill, Y. C. Leung, P. J. Sadler, J. Sloan, A. V. Xavier and S. Chi Tsang, *Inorg. Chim. Acta*, 1998, **272**, 261–266.
17 E. J. Sorin and V. S. Pande, *J. Am. Chem. Soc.*, 2006, **128**, 6316–6317.
18 Y. Kang, Y.-C. Liu, Q. Wang, J.-W. Shen, T. Wu and W.-J. Guan, *Biomaterials*, 2009, **30**, 2807–2815.
19 Y. Kang, Q. Wang, Y.-C. Liu, J.-W. Shen and T. Wu, *J. Phys. Chem. B*, 2010, **114**, 2869–2875.
20 G. Raffaini and F. Ganazzoli, *Langmuir*, 2013, **29**, 4883–4893.
21 F. S. Balavoine, P. Schultz, C. Richard, V. Mallouh, T. W. Ebbesen and C. Mioskowski, *Angew. Chem., Int. Ed.*, 1999, **38**, 1912–1915.
22 Y. C. Jung, H. Muramatsu, K. Fujisawa, J. H. Kim, T. Hayashi, Y. A. Kim, M. Endo, M. Terrones and M. S. Dresselhaus, *Small*, 2011, **7**, 3292–3297.
23 C. X. Li, S. Bolisetty, K. Chaitanya, J. Adamcik and R. Mezzenga, *Adv. Mater.*, 2013, **25**, 1010–1015.
24 G. R. Dieckmann, A. B. Dalton, P. A. Johnson, J. Razal, J. Chen, G. M. Giordano, E. Munoz, I. H. Musselman, R. H. Baughman and R. K. Draper, *J. Am. Chem. Soc.*, 2003, **125**, 1770–1777.
25 H. Xie, A. Ortiz-Acevedo, V. Zorbas, R. H. Baughman, R. K. Draper, I. H. Musselman, A. B. Dalton and G. R. Dieckmann, *J. Mater. Chem.*, 2005, **15**, 1734–1741.
26 G. Grigoryan, Y. H. Kim, R. Acharya, K. Axelrod, R. M. Jain, L. Willis, M. Drndic, J. M. Kikkawa and W. F. DeGrado, *Science*, 2011, **332**, 1071–1076.
27 K. Kurppa, H. Jiang, G. R. Szilvay, A. G. Nasibulin, E. I. Kauppinen and M. B. Linder, *Angew. Chem., Int. Ed.*, 2007, **46**, 6446–6449.

28 T. Yu, Y. Gong, T. Lu, L. Wei, Y. Li, Y. Mu, Y. Chen and K. Liao, *RSC Adv.*, 2012, **2**, 1466–1476.
29 S. Wang, E. S. Humphreys, S. Y. Chung, D. F. Delduco, S. R. Lustig, H. Wang, K. N. Parker, N. W. Rizzo, S. Subramoney, Y. M. Chiang and A. Jagota, *Nat. Mater.*, 2003, **2**, 196–200.
30 S. Brown, T. S. Jespersen and J. Nygard, *Small*, 2008, **4**, 416–420.
31 G. H. Zuo, S. G. Kang, P. Xiu, Y. L. Zhao and R. H. Zhou, *Small*, 2013, **9**, 1546–1556.
32 C. Ge, J. Du, L. Zhao, L. Wang, Y. Liu, D. Li, Y. Yang, R. Zhou, Y. Zhao, Z. Chai and C. Chen, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 16968–16973.
33 C. C. Chiu, G. R. Dieckmann and S. O. Nielsen, *J. Phys. Chem. B*, 2008, **112**, 16326–16333.
34 Z. M. Fu, Y. Luo, P. Derreumaux and G. H. Wei, *Biophys. J.*, 2009, **97**, 1795–1803.
35 H. Shams, B. D. Holt, S. H. Mahboobi, Z. Jahed, M. F. Islam, K. N. Dahl and M. R. Mofrad, *ACS Nano*, 2014, **8**, 188–197.
36 A. Hirano, Y. Maeda, X. F. Yuan, R. Ueki, Y. Miyazawa, J. Fujita, T. Akasaka and K. Shiraki, *Chem. – Eur. J.*, 2010, **16**, 12221–12228.
37 Z. Su, K. Mui, E. Daub, T. Leung and J. Honek, *J. Phys. Chem. B*, 2007, **111**, 14411–14417.
38 C. Salvador-Morales, P. Townsend, E. Flahaut, C. Vénien-Bryan, A. Vlandas, M. L. H. Green and R. B. Sim, *Carbon*, 2007, **45**, 607–617.
39 Q. X. Mu, W. Liu, Y. H. Xing, H. Y. Zhou, Z. W. Li, Y. Zhang, L. H. Ji, F. Wang, Z. K. Si, B. Zhang and B. Yan, *J. Phys. Chem. C*, 2008, **112**, 3300–3307.
40 D. Nepal and K. E. Geckeler, *Small*, 2007, **3**, 1259–1265.
41 K. Bradley, M. Briman, A. Star and G. Gruner, *Nano Lett.*, 2004, **4**, 253–256.
42 D. R. Samarajeewa, G. R. Dieckmann, S. O. Nielsen and I. H. Musselman, *Nanoscale*, 2012, **4**, 4544–4554.
43 A. Star, J. C. P. Gabriel, K. Bradley and G. Gruner, *Nano Lett.*, 2003, **3**, 459–463.
44 M. S. Deshpande and S. Mazumdar, *Biomacromolecules*, 2012, **13**, 1410–1419.
45 K. Matsuura, T. Saito, T. Okazaki, S. Ohshima, M. Yumura and S. Iijima, *Chem. Phys. Lett.*, 2006, **429**, 497.
46 K. H. Park, M. Chhowalla, Z. Iqbal and F. Sesti, *J. Biol. Chem.*, 2003, **278**, 50212–50216.
47 L. Rodriguez-Fernandez, R. Valiente, J. Gonzalez, J. C. Villegas and M. L. Fanarraga, *ACS Nano*, 2012, **6**, 6614–6625.
48 S. S. Karajanagi, A. A. Vertegel, R. S. Kane and J. S. Dordick, *Langmuir*, 2004, **20**, 11594–11599.
49 D. Das and P. K. Das, *Langmuir*, 2009, **25**, 4421–4428.
50 H. S. Kim, S. H. Yoon, S. M. Kwon and H. J. Jin, *Biomacromolecules*, 2009, **10**, 82–86.
51 W. L. Ling, A. Biro, I. Bally, P. Tacnet, A. Deniaud, E. Doris, P. Frachet, G. Schoehn, E. Pebay-Peyroula and G. J. Arlaud, *ACS Nano*, 2011, **5**, 730–737.
52 S. S. Karajanagi, H. C. Yang, P. Asuri, E. Sellitto, J. S. Dordick and R. S. Kane, *Langmuir*, 2006, **22**, 1392–1395.
53 A. Hirano, Y. Maeda, T. Akasaka and K. Shiraki, *Chem. – Eur. J.*, 2009, **15**, 9905–9910.
54 T. R. Fadel, N. Li, S. Shah, M. Look, L. D. Pfefferle, G. L. Haller, S. Justesen, C. J. Wilson and T. M. Fahmy, *Small*, 2013, **9**, 666–672.
55 J. W. Shen, T. Wu, Q. Wang, Y. Kang and X. Chen, *ChemPhysChem*, 2009, **10**, 1260–1269.
56 C. Li and R. Mezzenga, *Langmuir*, 2012, **28**, 10142–10146.
57 R. J. Chen, S. Bangsaruntip, K. A. Drouvalakis, N. W. Kam, M. Shim, Y. Li, W. Kim, P. J. Utz and H. Dai, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 4984–4989.
58 B. F. Erlanger, B.-X. Chen, M. Zhu and L. Brus, *Nano Lett.*, 2001, **1**, 465–467.
59 P. Asuri, S. S. Karajanagi, H. C. Yang, T. J. Yim, R. S. Kane and J. S. Dordick, *Langmuir*, 2006, **22**, 5833–5836.
60 M. Shim, N. W. S. Kam, R. J. Chen, Y. M. Li and H. J. Dai, *Nano Lett.*, 2002, **2**, 285–288.
61 S. Marchesan, M. Melchionna and M. Prato, *Fullerenes, Nanotubes, Carbon Nanostruct.*, 2013, **22**, 190–195.
62 P. Asuri, S. S. Bale, R. C. Pangule, D. A. Shah, R. S. Kane and J. S. Dordick, *Langmuir*, 2007, **23**, 12318–12321.
63 R. El-Sayed, M. Eita, A. Barrefelt, F. Ye, H. Jain, M. Fares, A. Lundin, M. Crona, K. Abu-Salah, M. Muhammed and M. Hassan, *Nano Lett.*, 2013, **13**, 1393–1398.
64 E. Venturelli, C. Fabbro, O. Chaloin, C. Menard-Moyon, C. R. Smulski, T. Da Ros, K. Kostarelos, M. Prato and A. Bianco, *Small*, 2011, **7**, 2179–2187.
65 E. Hirata, C. Menard-Moyon, E. Venturelli, H. Takita, F. Watari, A. Bianco and A. Yokoyama, *Nanotechnology*, 2013, **24**, 435101.
66 R. C. Pangule, S. J. Brooks, C. Z. Dinu, S. S. Bale, S. L. Salmon, G. Zhu, D. W. Metzger, R. S. Kane and J. S. Dordick, *ACS Nano*, 2010, **4**, 3993–4000.
67 C. Z. Dinu, G. Zhu, S. S. Bale, G. Anand, P. J. Reeder, K. Sanford, G. Whited, R. S. Kane and J. S. Dordick, *Adv. Funct. Mater.*, 2010, **20**, 392–398.
68 C. H. Villa, M. R. McDevitt, F. E. Escorcia, D. A. Rey, M. Bergkvist, C. A. Batt and D. A. Scheinberg, *Nano Lett.*, 2008, **8**, 4221–4228.
69 C. G. Gahmberg, S. C. Fagerholm, S. M. Nurmi, T. Chavakis, S. Marchesan and M. Gronholm, *Biochim. Biophys. Acta*, 2009, **1790**, 431–444.
70 V. A. Zamolo, G. Valenti, E. Venturelli, O. Chaloin, M. Marcaccio, S. Boscolo, V. Castagnola, S. Sosa, F. Berti, G. Fontanive, M. Poli, A. Tubaro, A. Bianco, F. Paolucci and M. Prato, *ACS Nano*, 2012, **6**, 7989–7997.
71 A. Battigelli, J. Russier, E. Venturelli, C. Fabbro, V. Petronilli, P. Bernardi, T. Da Ros, M. Prato and A. Bianco, *Nanoscale*, 2013, **5**, 9110–9117.
72 D. Pantarotto, C. D. Partidos, R. Graff, J. Hoebeke, J. P. Briand, M. Prato and A. Bianco, *J. Am. Chem. Soc.*, 2003, **125**, 6160–6164.
73 C. H. Villa, T. Dao, I. Ahearn, N. Fehrenbacher, E. Casey, D. A. Rey, T. Korontsvit, V. Zakhaleva, C. A. Batt, M. R. Philips and D. A. Scheinberg, *ACS Nano*, 2011, **5**, 5300–5311.
74 A. Knyazev, L. Louise, M. Veber, D. Langevin, A. Filoramo, A. Prina-Mello and S. Campidelli, *Chem. – Eur. J.*, 2011, **17**, 14663–14671.
75 Y. Chen, K. G. Qu, C. Q. Zhao, L. Wu, J. S. Ren, J. S. Wang and X. G. Qu, *Nat. Commun.*, 2012, **3**, 1074–1087.
76 B. Zhang, Y. H. Xing, Z. W. Li, H. Y. Zhou, Q. X. Mu and B. Yan, *Nano Lett.*, 2009, **9**, 2280–2284.
77 H. Y. Zhou, Q. X. Mu, N. N. Gao, A. F. Liu, Y. H. Xing, S. L. Gao, Q. Zhang, G. B. Qu, Y. Y. Chen, G. Liu, B. Zhang and B. Yan, *Nano Lett.*, 2008, **8**, 859–865.
78 T. R. Fadel, E. R. Steenblock, E. Stern, N. Li, X. Wang, G. L. Haller, L. D. Pfefferle and T. M. Fahmy, *Nano Lett.*, 2008, **8**, 2070–2076.
79 C. M. Voge, J. Johns, M. Raghavan, M. D. Morris and J. P. Stegemann, *J. Biomed. Mater. Res., Part A*, 2013, **101**, 231–238.
80 P. Chakravarty, R. Marches, N. S. Zimmerman, A. D. Swafford, P. Bajaj, I. H. Musselman, P. Pantano, R. K. Draper and E. S. Vitetta, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 8697–8702.
81 N. Fakhri, A. D. Wessel, C. Willms, M. Pasquali, D. R. Klopfenstein, F. C. MacKintosh and C. F. Schmidt, *Science*, 2014, **344**, 1031–1035.
82 J. G. Li, Q. N. Li, J. Y. Xu, X. Q. Cai, R. L. Liu, Y. J. Li, J. F. Ma and W. X. Li, *J. Nanosci. Nanotechnol.*, 2009, **9**, 1384–1387.
83 K. Donaldson and C. A. Poland, *Nat. Nanotechnol.*, 2009, **4**, 708–710.
84 V. E. Kagan, N. V. Konduru, W. Feng, B. L. Allen, J. Conroy, Y. Volkov, I. I. Vlasova, N. A. Belikova, N. Yanamala, A. Kapralov, Y. Y. Tyurina, J. Shi, E. R. Kisin, A. R. Murray, J. Franks, D. Stolz, P. Gou, J. Klein-Seetharaman, B. Fadeel, A. Star and A. A. Shvedova, *Nat. Nanotechnol.*, 2010, **5**, 354–359.
85 C. A. Poland, R. Duffin, I. Kinloch, A. Maynard, W. A. H. Wallace, A. Seaton, V. Stone, S. Brown, W. MacNee and K. Donaldson, *Nat. Nanotechnol.*, 2008, **3**, 423–428.
86 K. Kostarelos, A. Bianco and M. Prato, *Nat. Nanotechnol.*, 2009, **4**, 627.
87 H. Nagai, Y. Okazaki, S. H. Chew, N. Misawa, Y. Yamashita, S. Akatsuka, T. Ishihara, K. Yamashita, Y. Yoshikawa, H. Yasui, L. Jiang, H. Ohara, T. Takahashi, G. Ichihara, K. Kostarelos, Y. Miyata, H. Shinohara and S. Toyokuni, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, E1330–E1338.
88 G. P. Kotchey, Y. Zhao, V. E. Kagan and A. Star, *Adv. Drug Delivery Rev.*, 2013, **65**, 1921–1932.
89 http://ec.europa.eu/research/industrial_technologies/policy_en.html.
90 http://www.nanosafetycluster.eu/.
91 Z. G. C. Gao, J. H. Liu and X. J. Huang, *Nanoscale*, 2012, **4**, 1948–1963.
92 K. M. K. Mahehashi, *Sensors*, 2009, **9**, 5368–5378.
93 S. Kruss, A. J. Hilmer, J. Zhang, N. F. Reuel, B. Mu and M. S. Strano, *Adv. Drug Delivery Rev.*, 2013, **65**, 1933–1950.
94 F. Wang, X. Liu and I. Willner, *Adv. Mater.*, 2013, **25**, 349–377.

95 O. Yehezkeli, R. Tel-Vered, J. Wasserman, A. Trifonov, D. Michaeli, R. Nechushtai and I. Willner, *Nat. Commun.*, 2012, **3**, 742.
96 P. Asuri, S. S. Karajanagi, R. S. Kane and J. S. Dordick, *Small*, 2007, **3**, 50–53.
97 V. Martinelli, G. Cellot, A. Fabbro, S. Bosi, L. Mestroni and L. Ballerini, *Front. Physiol.*, 2013, **4**, 239.
98 N. Cai, C. C. Wong, Y. X. Gong, S. C. W. Tan, V. Chan and K. Liao, *ACS Appl. Mater. Interfaces*, 2010, **2**, 1038–1047.
99 S. Namgung, T. Kim, K. Y. Baik, M. Lee, J. M. Nam and S. Hong, *Small*, 2011, **7**, 56–61.
100 A. Fabbro, S. Bosi, L. Ballerini and M. Prato, *ACS Chem. Neurosci.*, 2012, **3**, 611.
101 S. Y. Park, D. S. Choi, H. J. Jin, J. Park, K. E. Byun, K. B. Lee and S. Hong, *ACS Nano*, 2011, **5**, 4704–4711.
102 Y. S. Chen and G. H. Hsiue, *Biomaterials*, 2013, **34**, 4936–4944.
103 L. Lacerda, H. Ali-Boucetta, S. Kraszewski, M. Tarek, M. Prato, C. Ramseyer, K. Kostarelos and A. Bianco, *Nanoscale*, 2013, **5**, 10242–10250.
104 N. W. Kam and H. Dai, *J. Am. Chem. Soc.*, 2005, **127**, 6021–6026.
105 M. R. McDevitt, D. Chattopadhyay, B. J. Kappel, J. S. Jaggi, S. R. Schiffman, C. Antczak, J. T. Njardarson, R. Brentjens and D. A. Scheinberg, *J. Nucl. Med.*, 2007, **48**, 1180–1189.
106 A. K. Jana and N. Sengupta, *Biophys. J.*, 2012, **102**, 1889–1896.
107 A. K. Jana, J. C. Jose and N. Sengupta, *Phys. Chem. Chem. Phys.*, 2013, **15**, 837–844.
108 D. Pantarotto, C. D. Partidos, J. Hoebeke, F. Brown, E. Kramer, J. P. Briand, S. Muller, M. Prato and A. Bianco, *Chem. Biol.*, 2003, **10**, 961–966.
109 A. Ortiz-Acevedo, H. Xie, V. Zorbas, W. M. Sampson, A. B. Dalton, R. H. Baughman, R. K. Draper, I. H. Musselman and G. R. Dieckmann, *J. Am. Chem. Soc.*, 2005, **127**, 9512–9517.
110 P. Asanithi, E. Saridakis, L. Govada, I. Jurewicz, E. W. Brunner, R. Ponnusamy, J. A. S. Cleaver, A. B. Dalton, N. E. Chayen and R. P. Sear, *ACS Appl. Mater. Interfaces*, 2009, **1**, 1203–1210.
111 M. Parthasarathy, J. Debgupta, B. Kakade, A. A. Ansary, M. I. Khan and V. K. Pillai, *Anal. Biochem.*, 2011, **409**, 230–235.
112 G. Huang, Y. Zhang, J. Ouyang, W. R. G. Baeyens and J. R. Delanghe, *Anal. Chim. Acta*, 2006, **557**, 137–145.