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The use of covalent reactions to improve the biomedical applications of carbon nanomaterials

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Carbon nanomaterials (CNMs), including fullerenes, carbon nanotubes, graphene, nanodiamonds and their derivatives, have been extensively used for biomedical applications, especially for cancer diagnosis and treatment. Most of these applications require covalent reactions to give the CNMs a high dispersibility in physiological environments, new biological functions for diagnosis and treatment, and tailored interactions with biological systems for efficient therapy. In this review, we summarize: the covalent bonding approaches to functionalize CNMs with polar surface groups, hydrophilic polymer layers, and bioactive molecules, and the specific surface chemistry that can prevent the interactions of CNMs with plasma proteins to suppress non-specific uptake and to enhance targeted uptake, aiming to advance the design of CNM-based therapeutics for precise theranostics.

KEYWORDS : Graphene, Diamond, Surface functionalization, Protein corona, Non-specific interactions, Targeted uptake

1. Introduction

Nanomaterial-based therapeutics have demonstrated notable performance in various biological applications, especially in cancer theranostics, as they can selectively detect and destroy tumor cells to make treatment more accurate and more efficient, overcoming the limitations of traditional radio- and chemo-therapy [1]. Carbon nanomaterials including fullerenes, carbon nanotubes (CNTs), graphene, nanodiamonds (NDs), and their derivatives, have gathered considerable attention in this regard, owing to their excellent biocompatibility, large surface area, unique optical, electronic and photothermal properties [2]. As a platform for constructing drug delivery systems, 2D graphene has shown extraordinary performances, benefited from its high capacity to load drugs on double sides of the basal plane [3]. Fullerenes is also attractive because both the inner space and outer surface can be used to transport therapeutic agents [4, 5]. Aside from drug delivery, single-walled CNTs (SWCNTs) that possesses intrinsic fluorescence emission in the second near-infrared window are very useful for deep tissue fluorescence imaging [6]. NDs containing nitrogen-vacancy centers (NV centers) render imaging background-free fluorescent by using a time-gating technique to eliminate the auto-

fluorescence from biological samples [7]. In addition, the sensitivity of spin state of NV centers to the subtle changes of chemicals and temperature in the surrounding environment can be explored for early disease diagnosis based on optically detected magnetic resonance techniques [8].

In spite of all the advantages that CNMs offer, most of them are naturally hydrophobic due to the presence of a large number of sp^2 carbon atoms [9], which limits their practical application in nanomedicine. Noncovalent binding of surfactants (*e.g.*, sodium dodecyl sulfonate and Triton X-100) and macromolecules (*e.g.*, phospholipid-polyethylene glycol and DNA) to CNMs [10], mainly through electrostatic, hydrophobic, van der Waals and supramolecular interactions, improves the dispersibility of CNMs in aqueous solutions. However, this approach is not appropriate for *in vivo* applications, because the adsorbed functionalities may detach from the materials or can be replaced by other biomolecules with high affinity to CNMs during the blood circulation, thus changing their behavior and functions [11]. Covalent chemistry, in contrast, introduces polar groups and hydrophilic polymers onto the surface of CNMs via stable chemical bonding [12], which are robust enough to hold grafted polymers onto the surface of CNMs, even in physiological environments, mak-

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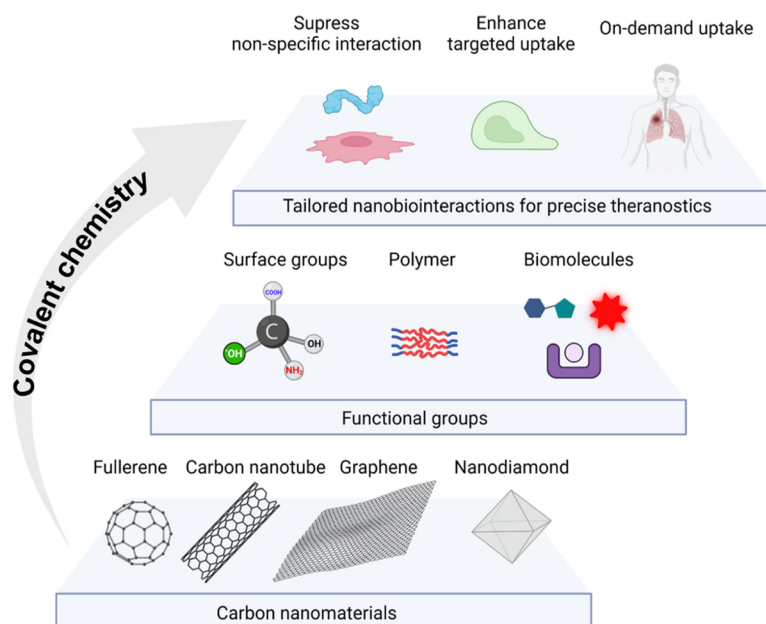


Fig. 1 Schematic representation of covalent chemistry approach to enhance theranostics using different types of CNMs.

ing them suitable for *in vivo* applications. Covalent chemistry has also the advantage for further incorporation of targeting ligands and bioactive molecules onto CNMs, which will facilitate their interaction with targeted cells and the subsequent translocation into desired subcellular destinations, enhancing the precision of therapy [13].

Nevertheless, in biofluids, CNMs tend to adsorb proteins to form a coating layer known as protein corona [14]. It is well known that the proteins in the corona layer will initiate the non-specific uptake to accelerate the clearance of CNMs from blood circulation, thus, reducing the amount of CNMs reaching to tumor sites [15]. Moreover, protein corona has been reported to disable the function of targeting moieties such as transferrin and arginine-glycine-aspartic acid (RGD) peptide [16], lowering targeting efficacy and specificity. Therefore, exploring effective solutions that can prevent protein corona formation to suppress off-target uptake during the circulation and to preserve the function of immobilized ligands to enhance targeted uptake, has become an important pursuit for researchers in this field. As the interaction of CNMs with proteins is largely dependent on their physicochemical properties, particularly the hydrophilicity and surface charge [17], covalent chemistry that can shape the surface properties of CNMs has in turn been considered as an effective tool for tailoring the interactions at the nanobiointerface.

In order to provide insights for developing CNMs with high targeting efficacy, in this review, we summarize the covalent chemistry for CNM functionalization and highlight the strategies that are critical for modulating their uptake behavior (**Fig. 1**). First of all, we describe various functionalities including surface groups, hydrophilic polymers, and biomolecules, that can be introduced onto CNMs via covalent chemistry approaches. Then, we briefly introduce the roles of surface functional groups at the nanobiointerface, with a particu-

lar focus on those with targeting ability. Followed by this, we show examples how protein corona alters the targeting ability of CNMs and discuss the surface chemistry that can be employed to suppress non-specific interactions and to enhance targeted uptake. At the end, we look forward to the future directions and propose promising approaches useful for activating on-demand uptake of CNMs.

2. Functional Groups Introduced by Covalent Chemistry

In this section we will discuss the covalent chemistry approaches for functionalizing CNMs with polar surface groups, hydrophilic polymers, and biomolecules (**Fig. 2**). Through appropriate modification methods, CNMs will acquire high dispersibility in various media including physiological buffers, and will be equipped with biological functions such as targeting, imaging, and therapy, which are essential for their application for cancer theranostics.

2.1 Surface groups

Oxygen-containing functions including carboxyl, carbonyl, epoxy, and hydroxyl groups are usually generated by treating CNMs with mixtures of strong oxidants like HNO_3 , H_2SO_4 , KMnO_4 [18]. As the main product of an acid treatment, carboxyl groups are mainly located on the ends of CNTs and at the edges of graphene oxide (GO) [19]. However, they can uniformly cover the surface of fullerene and NDs [20]. The ionized carboxylates normally decrease the zeta potentials of CNMs to -30 to -50 mV and will attract counter ions from the surrounding environment to form an electric double layer, stabilizing CNMs in aqueous solution through electrostatic repulsion [21]. The epoxy and hydroxyl groups are usually lying on the side-wall of CNTs and on the basal plane of GO, further enhancing their hydrophilicity.

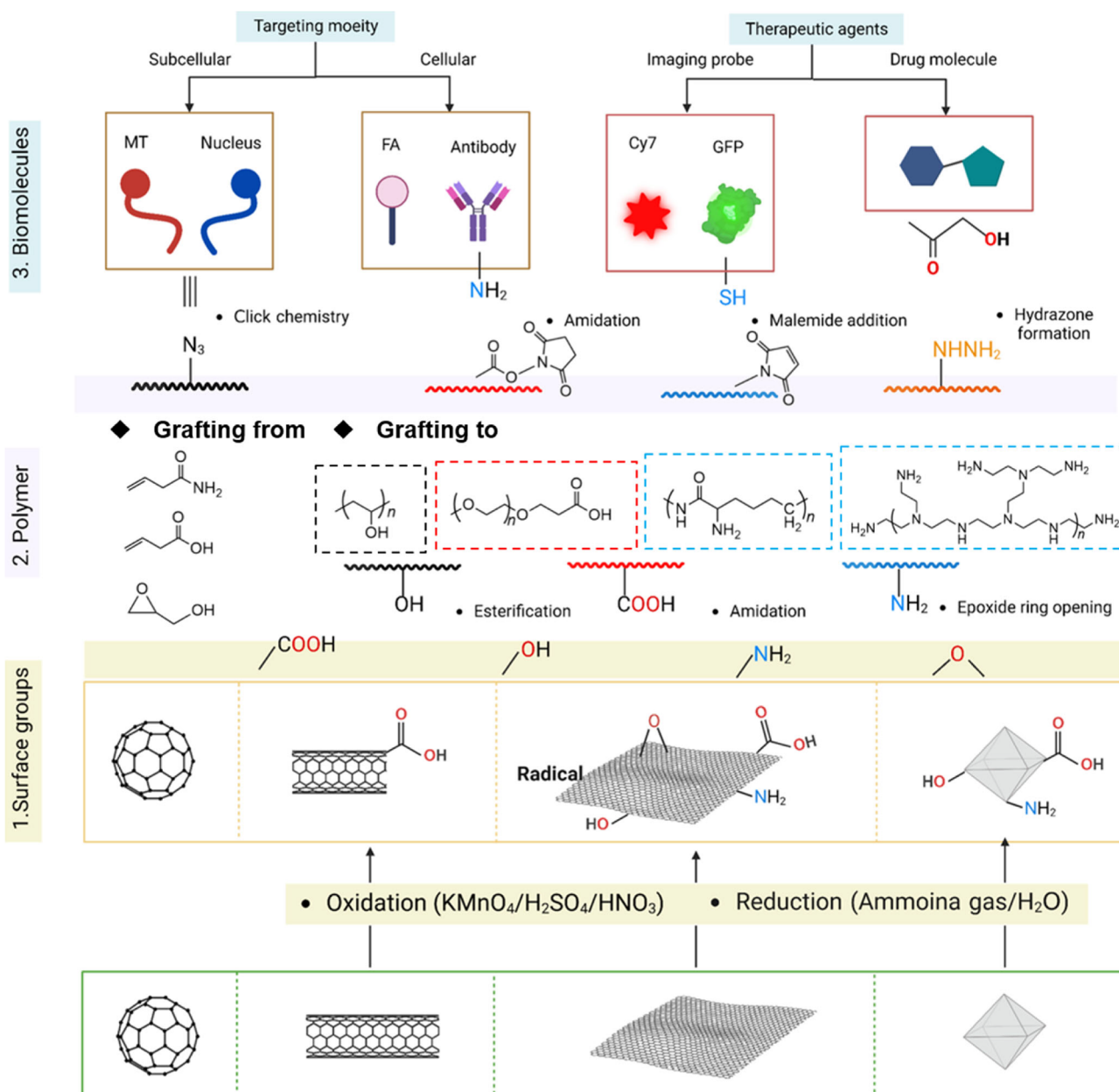


Fig. 2 Covalent chemistry approaches to functionalize CNMs with polar surface groups, hydrophilic polymers, and biomolecules. “Grafting to” and “grafting from” refer to the conjugation of pre-synthesized polymer chains to CNMs and *in situ* growing of polymer chains on the surface of CNMs, respectively. MT: mitochondria; FA: folic acid; Cy7: cyanine dye; GFP: green fluorescent protein.

Functionalization of CNMs with nitrogen-containing groups is also attractive since they can improve the stability of CNMs in water solutions by impacting a positive zeta potential, as opposite to that of carboxyl groups. Till now, the direct amination of CNMs is mainly achieved through dry methods, for example, treating CNMs with ammonia gas [22] and/or plasma irradiation [23]. Despite widespread application, dry methods often lead to a heterogeneous distribution of amino groups and requires specific apparatus to proceed the reaction, hindering its further development [24]. Wet chemistry, on the other hand, is drawing growing interests as the intrinsic limitations including multiple-step reactions and low yields are gradually tackled in recent approaches. For instance, an electroless method was developed

by Simon *et al.* [25] to directly functionalize diamond with amino groups in a liquid ammonia solution containing persulfate as oxidizer. Based on Leuckart reaction, a more facile amine functionalization of GO was carried out by Bolados *et al.* [26]. The carbonyl groups on the GO were efficiently reduced to amino groups in the presence of ammonium formate, affording an amination degree up to 3.2 at%.

Note that the contents or the ratios of these polar surface groups are tunable, depending on the species or amounts of reactants used. For instance, high content of oxygen-containing groups can be obtained by increasing the amounts of oxidants in the reaction system [27] or augmenting the reaction temperature [28]. Carboxyl and carbonyl groups can be partially converted to hydroxyl groups by the

addition of LiAlH_4 , NaBH_4 and $\text{BH}_3\text{-THF}$ as reducing agents [29], and can be further reduced to amine groups with the aid of hydrazine and its derivatives [30]. Quite recently, Morimoto *et al.* [31] have set a progressive control of the oxidation degree of graphene-like materials, *i.e.*, stepwise increasing the oxygen content of graphite from 18% to 59% and decreasing it back to 12% by adjusting the ratios of KMnO_4 to graphene and hydrazine to GO, respectively, providing an excellent example for producing tailor-made graphitic materials toward specific applications.

After the functionalization of heteroatom-containing polar groups, CNMs can disperse well in aqueous solutions, but they tend to aggregate in buffer solutions due to the compression of electric double layer. However, these surface groups, along with the carbon radicals generated from the cleavage of C–O bonds [32], offer convenient starting points for further functionalization of CNMs with polymers and biomolecules.

2.2 Polymer layers

Covalent functionalization of CNMs with hydrophilic polymers is an effective strategy to enhance their dispersibility in various aqueous solutions including physiological buffers. Depending on the functionalization methods, the polymer layer can be attached onto CNMs via either “grafting to” or “grafting from” methods.

In the “grafting to” method, pre-synthesized polymer chains that have reactive species such as hydroxyls, carboxyl, and amines can be covalently conjugated to CNMs via coupling reactions, mostly through the esterification and amidation reactions [33]. For example, the hydroxyl groups from poly(vinyl alcohol) [34] can react with the carboxylic groups on CNMs to form ester bonds in the presence of carbodiimides and appropriate bases. Poly(ethylene glycol) (PEG) terminating with a carboxyl group can easily react with amines on CNMs to form amide bonds, after its activation with a combination of carbodiimide and N-hydroxysuccinimide [35]. This reaction can be carried out in mild conditions (*e.g.*, H_2O or buffer as solvent, and room temperature), and the as-formed amide bonds are more stable than ester bonds. In addition, poly(lysine) [36] and poly(ethylenimine) [37] bearing numerous amino groups can form crosslinker with more reactive epoxy groups abundantly present onto GO via epoxide ring opening reactions, to afford a dense coverage.

Different from coupling reactions, cycloaddition reactions allow attaching polymers through the formation of fused rings between dienes (*e.g.*, carbenes, nitrenes, and their derivatives) at the terminal part of polymer chains and alkene bonds on the surface of CNMs. In a modified Bingel reaction, bis(polyamide)-malonate dendrimer was added to [60]fullerene through nucleophilic cyclopropanation in the presence of diazabicyclo(5.4.0)undec-7-ene and CBr_4 . The derivatized fullerene carried 18 carboxylic groups at the periphery of the dendrimeric layer (Fig. 3a), giving a water solubility of 34 mg/mL at pH 7.4 [38]. Similar to nitrene cycloaddition, azomethine ylides,

generated by thermal condensation of an α -amino acid and an aldehyde, were first developed by the group of Prato [39] to incorporate a triethylene glycol moiety onto CNTs via 1,3-dipolar cycloaddition reaction. Owing to uniform distribution of attached short polymer and a large number of amino groups at the terminal of glycol unit (Fig. 3b), the as-functionalized CNTs exhibited a remarkably high dispersibility in water (>20 mg/mL). As the reactive amino groups are amenable for further chemical modifications with peptides and fluorescent probes, this method has also opened up the applications of CNTs in immunology [40, 41] as well as aided in elucidating the cellular internalization mechanisms of CNTs [42]. In a more recent example, commercially available 2,4,6-trichloro-1,3,5-triazine was reacted with sodium azide at low temperature to yield 2-azido-4,6-dichloro-1,3,5-triazine, which was subsequently introduced onto the surface of reduced GO (rGO) through nitrene [2+1] cycloaddition reaction [43]. Thanks to the controllable functionalization degree of the triazine and the selective reactivity of dichlorotriazine towards amines and other nucleophiles, high molecular weight polymers with different functions could be grafted onto rGO in a stepwise manner (Fig. 3c), enhancing the dispersibility of rGO as well as its practical applications in nanomedicine.

The “grafting from” method refers to the *in situ* growing of polymers onto the surface of CNMs. It involves the processes of: 1) incorporation of polymer precursors to the initiating sites, and 2) elongation of polymer chain in the presence of monomeric species. Free radical polymerization is a versatile method for grafting vinyl monomers onto the surface of CNMs. For instance, carbon radicals on the basal plane of GO can initially react with acrylate and acrylamide [44, 45], and can be subsequently transferred to carbon-carbon double bonds in the monomeric unit for further polymerization. Nevertheless, the polymerization degree using this approach is limited due to the short life time of the radicals. Alternatively, atomic transfer radical polymerization permits a higher degree of polymerization due to the stability of the initiators. In this approach, the initiators are pre-immobilized on CNMs and are then transferred to the terminal group of grafted units after the initial reaction, inducing the successive addition of monomeric units. Based on this principle, CNTs was first functionalized with a 2-bromo-2-methylpropionyl function, and the introduced bromide moiety initiated the polymerization of hydrophobic methyl methacrylate to yield CNT-PMMA (polymethyl methacrylate) [46]. Since the activity of bromides was maintained in CNT-PMMA and their amounts remain constant after the purification, hydrophilic hydroxyethyl methacrylate can be further polymerized onto the surface of CNT-PMMA to generate amphiphilic copolymer brushes.

The radicals can also initiate the ring-opening polymerization of cyclic monomers on the surface of CNMs. Nevertheless, this type of reaction often requires the usage of metallic catalysts, which may potentially alter the intrinsic property of CNMs, such as damaging the

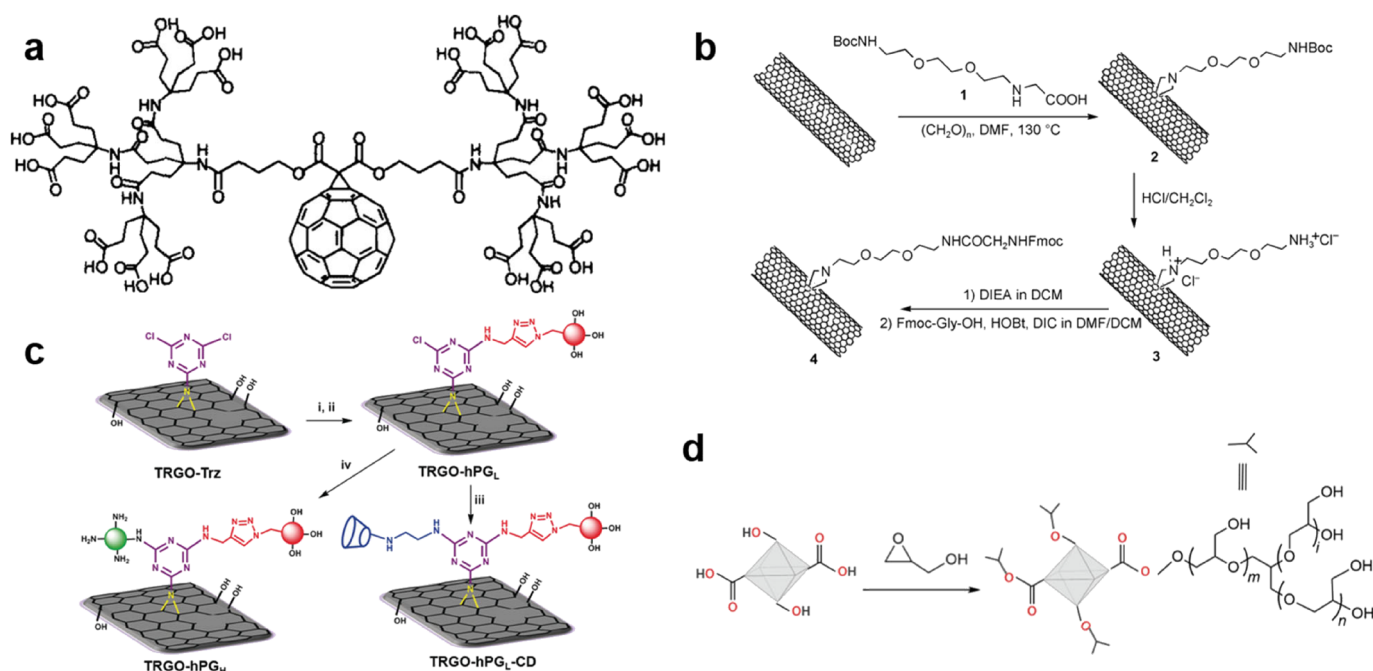


Fig. 3 Typical examples of functionalized CNMs with polymers. (a–c) Cycloaddition reactions to link dendrimers to fullerene (a), amino acids to CNTs (b), high molecular weight polymers to graphene (c), and (d) to graft polyglycerol to NDs. Panels (a), (b) and (c) were adapted with permission from Ref [38], [39] and [42], respectively. Copyright 1998, Elsevier Ltd., Copyright 2002, Royal Society of Chemistry, and Copyright 2020, Wiley-VCH.

fluorescent performance of SWCNT [47]. As a result, the carboxyl and hydroxyl groups on the surface of CNMs turn out to be better initiating sites for the polymerization of a variety of cyclic monomers including epoxides [48], lactones [49], and lactides [50]. One unique character of this approach is the possibility to generate hyperbranched polymer layer that can cover CNMs more densely. In this context, the polyglycerol (PG) grafting developed by Komatsu and co-workers [13] has demonstrated its effectiveness in improving the dispersibility of carbonaceous materials. A large number of hydroxyl groups are not only introduced at the periphery of hyperbranched PG layer but also buried inside the polymer brush, making CNMs highly dispersible in a physiological buffer. Considering NDs of 30 nm size as example, the PG raises its dispersibility up to 16 mg/mL in phosphate-buffered saline, which is 400 times higher than the one functionalized with PEG, a mostly widely used polymer for imparting hydrophilicity [51]. The hydrophilic nature of PG is also beneficial for preventing the non-specific interactions between NDs and proteins (see section 3.3). Moreover, the hydroxyl groups can be easily converted into more reactive functional groups (*e.g.*, carboxylic, azido, and amino groups) for further bioconjugation and drug loading [52, 53]. Since the PG functionalization is a facile process that can be carried out by reacting NDs in glycidol with no need of solvent or additive (**Fig. 3d**), and it is applicable to other types of CNMs bearing hydroxyls and/or carboxyl groups [54], this functionalization method is expected to bring more convenient designs for CNMs to advance their biomedical applications.

2.3 Biomolecules for therapeutic purpose

In order to impart CNMs with biological functions, *e.g.*, disease diagnosis and treatment at the desired site, it is necessary to incorporate biomolecules such as targeting moieties and therapeutic agents onto CNMs. Sensitive molecules including antibodies, short peptides, cyanine-based dyes and fluorescent proteins are often conjugated onto CNMs via mild reactions, *e.g.*, click chemistry [55], hydrazone bond formation [53], and maleimide addition [40], along with aforementioned amidation. The chemically stable molecules can be also introduced onto CNMs under relatively harsh reaction conditions, *e.g.*, N-methylation [56] and metal coordination [57].

Although most of cancers are treatable by the nano-formulations furnished with a specific function, the complexity and heterogeneity of tumor microenvironment urge for the development of multifunctional drug delivery platforms that can simultaneously implement the functions of targeting, imaging, and therapy for efficient treatment [58]. The diversity of covalent chemistry approaches for CNMs offers advantage in this aspect, as multiple functional molecules can be introduced either at the terminal of a polymer layer or directly onto the surface of CNMs. However, to better exert the activity of functional molecules, the order and the way of CNM functionalization need to be rationally arranged. Quite recently, Lucherelli *et al* [59], have presented an elegant strategy for stepwise deprotection of amino groups on the surface of graphene, which renders sequential functionalization with imaging probe, targeting moiety, and anticancer agents to obtain multifunctional graphene (mfG) (**Fig. 4a**). In this approach

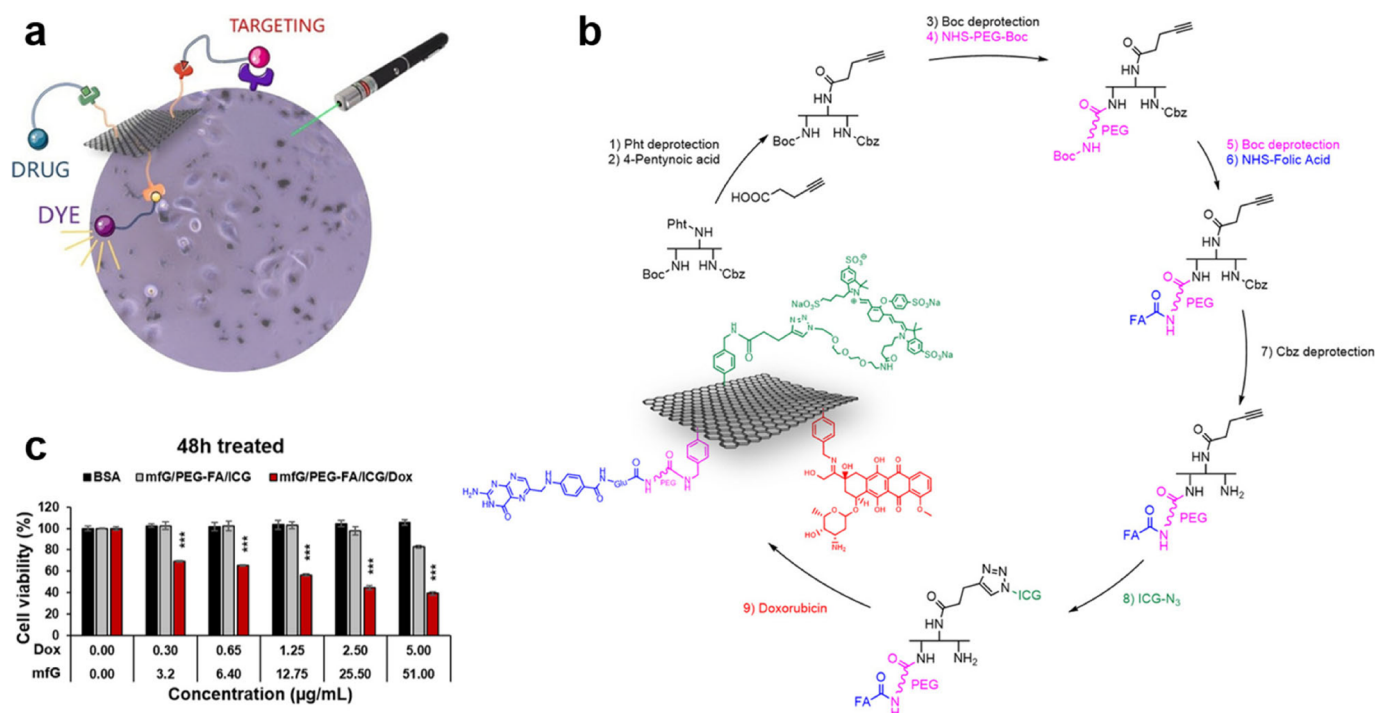


Fig. 4 Synthesis of mfG/PEG-FA/ICG/Dox. (a) Schematic representation of mfG integrating an imaging probe (ICG), a targeting moiety (FA), and an anticancer agent (Dox) into one single platform. (b) Synthetic routes for the stepwise deprotection of the amino groups on the surface of graphene and sequential functionalization with PEG, FA, ICG and Dox to obtain mfG/PEG-FA/ICG/Dox. (c) Cell viability assay of HeLa cells treated with bovine serum albumin (BSA), mfG/PEG-FA/ICG and mfG/PEG-FA/ICG/Dox for 48 h. Adapted with permission from Ref [59], Copyright 2020, Wiley-VCH.

(Fig. 4b), the pristine graphene was first functionalized with benzylamine derivatives bearing three different quasi-orthogonal protecting groups, namely, phthalimide (Pht), *tert*-butyloxycarbonyl (Boc) and benzyloxycarbonyl (Cbz). The Pht was initially removed and the released amines were reacted with pentynoic acid to introduce an alkyne moiety. The click reaction of alkyne moieties with indocyanine green (ICG) was performed after the introduction of folic acid (FA). The linkage of FA with graphene was bridged by a PEG chain that pre-reacted with the amines liberated from Boc deprotection. Lastly, Cbz was removed, and doxorubicin (Dox) was reacted with released amines with via the formation of an imine bond to yield mfG/PEG-FA/ICG/Dox. Thanks to the precise design, the as-prepared mfG/PEG-FA/ICG/Dox demonstrated remarkable targeting ability as well as the controlled drug release behavior, leading to efficient killing of cancer cells (Fig. 4c).

3. Surface Chemistry Tailoring Nanobiointeractions for Precise Theranostics

3.1 The roles of surface chemistry at the nanobiointerface

After modifying CNMs with various functional moieties, these related materials are ready for disease treatment and diagnosis. However, before moving to practical applications, it is necessary to unveil the roles of surface chemistry of CNMs at the nanobiointerface, *i.e.*, how chemical modifications mediate the interactions of CNMs with

the cells and the subcellular organelles, as these results will in turn give feedback to the rational design of CNM-based drug delivery platforms for enhanced targeting efficacy. Here we summarize a portion of representative examples closely related to the cellular uptake and subcellular localization of CNMs.

In biological systems, the cellular membrane is the primary biological barrier that CNMs needs to crossover. Due to the presence of negatively charged glycans and proteins on the cell surface, the positively charged CNMs are generally holding a high affinity toward cell membranes thus inducing a more efficient uptake. For instance, ethylenediamine functionalized fullerenes [60], amine-modified [61] and dendritic amine-modified [62] GO, and lysine functionalized NDs [63] have all demonstrated higher uptake in various cell lines including HUVEC cells, MCF7 cells, and HeLa cells, as compared to their analogs bearing negatively charged or neutral functionalities. Particularly, owing to the high density of cationic charge, the polyethyleneimine-modified SWCNTs were efficiently diffused into the xenograft of pancreatic BxPC3 cells [64], an intractable cancer that was rarely penetrable by other types of systems.

Despite the preferential uptake of positively charged CNMs, the lack of specificity prevents to discriminate normal from cancer cells, making them useless for targeting therapy. Instead, targeting moieties, such as aptamers, antibodies, and peptides, that can recognize certain ligands specifically or that are over-expressed on tumor cells,

are more effective. The RGD peptide has been reported to enhance the internalization of SWCNTs [6], GO [65] and NDs [53] into tumor cells overexpressing the $\alpha v\beta 3$ integrin receptor. The pioneering work from Dai's group demonstrated that, antibody Rituxan facilitated the interaction of nanosized GO with tumor cells specifically, rendering the clear visualization of targeted tumor cells and tissues [66]. Other types of ligands such as N-formyl-methionyl-leucyl-phenylalanine peptide that can interact with formyl peptide receptor also contributed to the active targeting of CNMs [67].

After being internalized into targeted cells, CNMs may need to escape the endosome trapping to reach their final subcellular destination, such as mitochondria or nucleus, for subcellular targeted therapy. Since mitochondria are organelles encapsulated by a bilayer-membrane bearing negative potentials at inner membranes, lipophilic cations such as triphenylphosphorus (TPP) and dequalinium are extensively utilized to direct CNMs into mitochondria [68]. Cell nucleus is also targetable, if the CNMs are modified with cell-penetrating peptides that consists of large amounts of arginine residues. However, the nuclear pore complexes on the nuclear envelope prevent CNMs from entering into the nucleus. The diameters of nuclear pores are generally in the range of 55–70 nm [69], therefore, the CNMs with size above this range, *i.e.*, GO with microns or larger lateral size, are unable to pass through these tiny pores to get into the inner sites of nucleus. To circumvent this problem, Tu *et al.* [70] reduced the size of GO down to 15–30 nm. Subsequently, the nano GO was covered with hydrophilic polymer PG and further functionalized with a cyclic R10 peptide, a nuclear targeting peptide, fulfilling the task of delivery anticancer drug Dox into cell nucleus for combating multidrug resistance.

3.2 Protein corona altering the surface chemistry of CNMs

On the basis of the excellent targeting efficacy offered by the surface chemistry of CNMs at the nanobiointerface, it is reasonable to anticipate that CNM-based drug delivery platforms will promote the targeted theranostics through their practical applications. However, upon contact with biofluids, CNMs tend to adsorb a variety of proteins [71] including serum albumin, immunoglobulin, coagulation proteins, lipoproteins, etc., to form a coating layer known as protein corona (**Fig. 5a**). It is well known that the proteins in this layer alter the surface chemistry of CNMs and form a new interface between CNMs and cells and tissues, thereby influencing their bioactivity and their interactions with the living body, which in turn determine the biological fate and therapeutic outcomes [15, 72]. Here, we mainly discuss the negative effects of protein corona on the targeting efficiency, for other biological effects of protein corona formation such as transcellular transportation and endothelial leakiness, please refer to a recent review written by Ren *et al.* [73].

The rapid removal of nanomaterials from the blood circulation (one of the most frustrating consequences of protein corona formation on

targeted delivery) is facilitated by the interactions between opsonin and complement proteins from the corona layer and macrophages abundantly circulating in the reticuloendothelial system (RES) organs (*e.g.*, liver, spleen and lung) [74]. In an early work pioneered by Chen's group [75], pristine CNTs were demonstrated to have a high binding capacity toward opsonins such as fibrinogen and gamma globulins via π - π stacking and hydrophobic interactions. In the cellular response assays, the adsorbed proteins were found to affect the association of CNTs to human acute monocytic leukemia cell line to alter their cytotoxicity. These important findings have become imperatives for the exploration of the influence of corona proteins in the interaction of CNMs with immune cells from blood stream. In a related study, Lu *et al.* [17] revealed that the complex of GO-IgE, either formed in the blood stream *in situ* or preformed in a test tube, were quickly cleared from blood circulation, shortening the half-life to less than 4 h. The findings from above two studies also correlate well with the high accumulation of pristine CNTs and GO in the RES organs, as determined by a label-free laser desorption/ionization mass spectrometry imaging technique [76]. Similarly, the ^{14}C -labeled [60]fullerene was reported to be cleared from the circulation within 1 min [77], and $\text{C}_{60}\text{-COOH}$ and $\text{C}_{60}\text{-OH}$ were found to be mainly distributed in the liver, bone, muscle and skin after their intravenous injection [78].

Aside from diminishing the fraction of administered CNMs to target sites via trapping them in the RES organs, compromising the ability of targeting ligands is another disadvantage of protein corona on the targeting efficacy. As we mentioned above, the active targeting strategy relies on the efficient pairing of targeting ligands with specific receptors on tumor cells. However, one can envision that, after the formation of protein corona, most of the ligands are buried inside the corona layer and cannot get access to the receptors on cell surface freely, thereby leading to complete or partial reduced targeting efficacy. In 2013, Salvati *et al.* [79] have first experimentally proved that transferrin functionalized nanoparticles (NPs) lost their targeting specificity to corresponding receptor in a protein rich medium. After that, the shielding effect of protein corona on the targeting ability of antiCD63 antibody [80], and aptamers, such as RGD peptide [81], was gradually disclosed. Actually, the accumulation of RGD functionalized-SWCNT in a glioblastoma U87MG tumor-bearing mice ($\alpha v\beta 3$ integrin positive tumor) was around 10–15% injected dose (ID)/g, which is only 3–4% ID/g higher than the one without RGD modification [6]. The moderate enhancement in accumulation of SWCNT-RGD should be arisen from the passivation effect of protein corona on the activity of the peptide.

3.3 Suppression of non-specific interactions and enhancement of targeted uptake

As protein corona presents an intrinsic hindrance limiting the targeting efficacy of CNMs, mitigating the non-specific interactions of CNMs with blood proteins has become an important issue to solve in

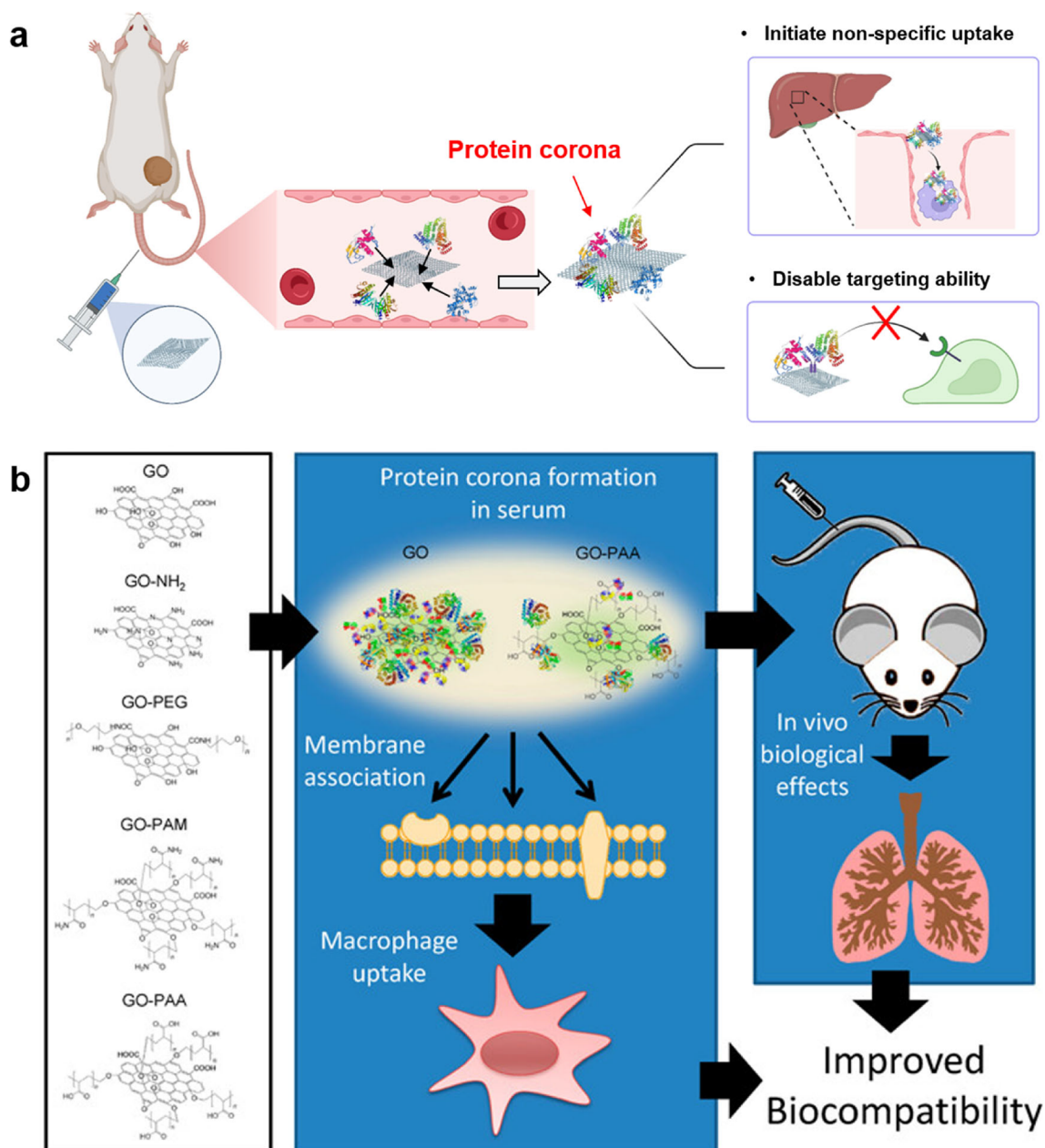


Fig. 5 Protein corona formation on CNMs and its prevention by specific surface modification. (a) Schematic representation of protein corona formation on CNMs and its consequent effects on non-specific uptake of RES organs and targeting ability of functionalized ligands. (b) Comparison study on protein affinity, macrophage uptake and *in vivo* distribution of GO and GO functionalized with PAM, PAA and PEG. Adapted with permission from Ref [89], Copyright 2016, American Chemical Society.

order to enhance the accumulation of CNMs at the tumor sites. Since the hydrophobic and electrostatic interactions are the driving forces for the adsorption of proteins onto CNMs, modifying CNMs with hydrophilic and neutral polymers has turned out to be an effective strategy to counteract protein adsorption, and consequently, to discourage the recognition of CNMs by macrophages for prolonged circulation.

Among various functionalization methods, PEGylation is the most widely used one [82], as PEG brushes immobilized on the surface of CNMs can build up a strong steric hindrance toward proteins to prevent them from approaching onto CNMs. However, in consideration of the different protein adsorption capacity of the different CNMs,

the properties of used PEG including chain length, conformation, and surface density, need to be optimized for each type of CNMs, to achieve the desired effect. While linear PEG of 2 kDa can efficiently concentrate a conjugate of C60 and iron oxide nanoparticles at the tumor site for magnetic resonance imaging [4], the efficient transportation of SWCNTs into tumor sites requires PEG with larger molecular weight (7 kDa) [83]. Owing to the large specific surface area and many defects in the basal plane, GO was documented to possess a higher protein affinity than other types of CNMs [84]. As a result, branched PEG that can afford a denser coating is more preferable for GO functionalization. Indeed, 4-arm PEG-modified GO was able to

co-deliver anticancer drugs cisplatin and Dox into tumor site for efficient cancer eradication [85], while the carrier modified with linear PEG was mainly accumulated in the liver and spleen after intravenous injection into BALB/c mice [86].

In spite of the effectiveness of PEG in suppressing non-specific interactions, drawbacks of PEG have also been recognized. For example, PEG was reported to have the propensity to provoke blood clotting and to develop PEG antibodies [87], which may accelerate the blood clearance of modified CNMs. Therefore, a growing number of research teams have begun to search alternatives for PEG. Zwitterionic molecules such as polybetaines or polysaccharides [88] that can generate hydrophilic shells when coupled to NPs have been discussed and investigated. However, the dextran functionalized GO was found to distribute mainly in liver, spleen and lung, resulting in a slightly faster clearance than that of GO-PEG [62]. In an attempt to offer a better solution, Xu *et al.* [89] have functionalized GO with polyacrylamide (PAM) and polyacrylic acid (PAA), and systematically studied their affinity to plasma proteins, uptake by J774A.1 macrophage and *in vivo* distribution, and compared the outcomes to those of PEG-functionalized GO. Following these investigations, the authors clearly concluded that PAA outperformed PEG, as GO-PAA adsorbed much less amount of immunoprotein IgG than GO-PEG to induce a lower macrophage uptake and lower accumulation in RES organs (Fig. 5b).

Although better solutions are continuously emerging, none of current strategies have realized complete suppression of non-specific uptake. Under such circumstances, we seek to explore the potential of PG for antifouling, since ND-PG was reported to have very high hydrophilicity [51] and very low non-specific uptake [53]. To this end, NDs of 30, 50, and 100 nm were grafted with PG at varying densities, and their ability to reduce protein corona formation and U973 macrophage uptake were examined and compared to that of PEG functionalization [90]. We found that, at similar grafting densities, PG shielded protein adsorption and avoided macrophage uptake more efficiently than did PEG (Fig. 6a). In particular, NDs coated with PG at 30 wt%, a coating content that cannot be accomplished by PEGylation, were found to prevent corona formation almost completely, regardless of ND core sizes, leading to the complete evasion of macrophage uptake (Fig. 6b). Since the hyperbranched PG can cover NDs more densely introducing a large number of hydroxyl groups at the backbone of ethylene glycol units (Fig. 3d), the stealth effect of PG is assumed to arise from its high hydrophilicity, binding water molecules more stably through hydrogen bonding to hinder the protein corona formation more efficiently than PEG containing ether groups. Inspired by the unprecedented stealth effect offered by PG, we designed PG functionalized NPs and applied them for *in vivo* cancer therapy and tumor imaging. ^{10}B enriched boron carbide (B₄C) NPs were functionalized with PG to prolong its circulation *in vivo*.

A pharmacokinetic study revealed that the concentration of ^{10}B in tumor tissue was larger than 20 ppm and the ratio of tumor/blood was higher than 3 (Fig. 6c). As a result, the tumor growth was significantly suppressed without inducing any obvious side effects to normal tissues [91] (Fig. 6d). Similarly, an extreme long half-life of 58 h was achieved for PG coated NDs. By attaching a fluorescent probe to ND-PG, the tumor lesions were clearly imaged even 8 days after injection [92] (Fig. 6e).

In addition to enhancing the accumulation of CNMs at the tumor sites, the protein-resistance property of PG functionalization also provides a background-free interface for quantitative estimation of the interactions of functional molecules with proteins, which is fundamental to realize their intended functions. By introducing carboxyl and amino groups at the surface of ND-PG at the densities of low (*l*), medium (*m*), high (*h*) and extra-high (*xh*) [93], we revealed that the carboxylate ($-\text{COO}^-$) and ammonium ($-\text{NH}_3^+$) groups interact exclusively with positively charged lysozyme (LYS) and negatively charged BSA, respectively, and that their interaction strength becomes stronger as the functional group density increases (Fig. 7a). In particular, the association constants (*K*) between BSA and ND-PG- NH_3^+ correlates linearly with the density of ammonium groups (Fig. 7b). In a more recent work from our group, we grafted TPP, a lipophilic cation for mitochondria targeting, onto the surface of ND-PG at low (*l*) and high (*h*) densities, and studied their targeting ability in response to protein corona formation [94]. Similar to that of charged groups, the TPP moiety was found to induce protein corona formation in a density-dependent manner in both 10% FBS and 55% human plasma (HP) solutions (Fig. 7c). Consequently, we found that the ND-PG-TPP-*l* adsorbed negligible amounts of proteins preserved their targeting efficacy and specificity to mitochondria (Fig. 7d), whereas ND-PG-TPP-*h* with high protein affinity were restricted in endosomal and lysosomal compartments (Fig. 7e). These fresh findings are inspiring for designing CNMs with enhanced targeting ability, as the activity of the ligands can be maintained by adjusting their density on a protein-free interface.

Aside from the prevention of protein corona, using covalent chemistry to remodel the protein corona composition on the surface of CNMs to enhance their therapeutic efficacy is another effective strategy toward successful biomedical applications. Guo *et al.* [95] have recently demonstrated that graphdiyne oxide (GDYO) nanosheets have such potential. GDYO is a derivative of graphene that bears diacetylenic bonds (made of single- and triple-bonds) at the junctions of hexagonal aromatic rings, and well-arranged carboxyl and hydroxyl groups at the intervals of acetylenic bonds or at the edges of aromatic rings. In this study, GDYO was demonstrated to preferentially adsorb signal transducer and activator of transcription 3 (STAT3). Since the distance between any two adjacent C=O and/or C–OH groups in GDYO (5.5 Å) is almost similar to the vertical dis-

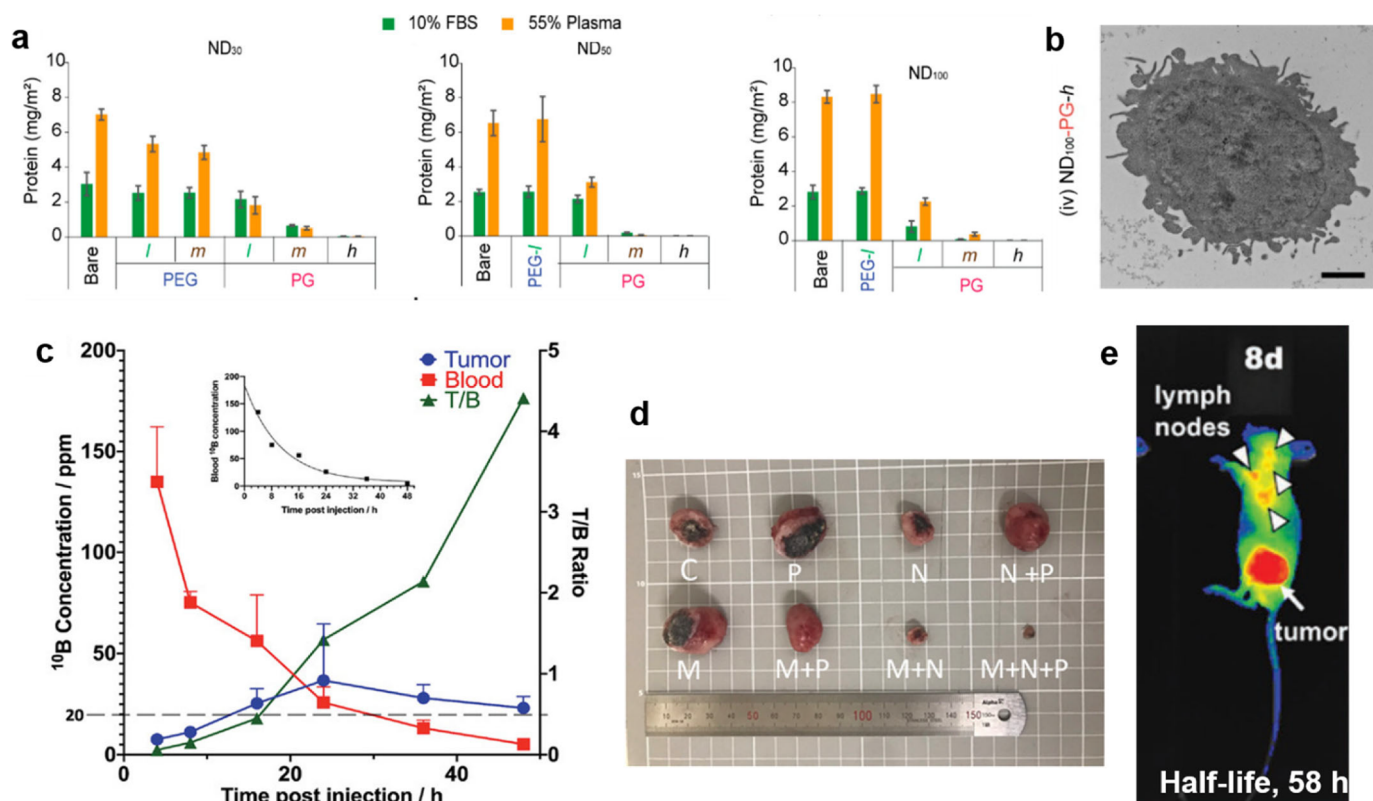


Fig. 6 The effects of PG functionalization on protein corona formation and cancer theranostics. (a) The amounts of proteins adsorbed on ND, ND-PEG and ND-PG after their incubation in 10% fetal bovine serum (FBS) and 55% plasma solutions. (b) U937 macrophage uptake of ND-PG. Scale bar, 2 μm . (c–d) Blood circulation (c) and therapeutic effect (d) of B4C-PG. (e) *In vivo* tumor imaging of ND-PG-Cy7. Panels (a–b), (c–d) and (e) were adapted with permission from Ref [90], [91] and [92], respectively. Copyright 2020, American Chemical Society, Copyright 2019, Wiley-VCH, and Copyright 2021, Wiley-VCH.

tance between consecutive turns of the α -helix pitch at the N-terminal domain of STAT3 (5.4 \AA), the high affinity of GDYO toward STAT3 is proposed to be driven by their structural matching. In addition, the formation of hydrogen bonds and salt bridges between the C=O and C–OH groups in GDYO and the α -helical N-domain of the protein also strengthens their interactions. STAT3 is an intracellular protein that can stimulate the generation of M2 protumor macrophages, however, this biological function was lost after its interaction with GDYO, thus resulting in an enhanced production of M1 antitumor macrophage in the tumor microenvironment. The immune modulation ability of GDYO was further explored for boosting the therapeutic outcomes of anti-PDL1 monoclonal antibodies *in vivo*.

4. Conclusions and Perspectives

A comprehensive understanding on the roles of the surface chemistry of CNMs at the nanobiointerface and the exploration of effective approaches to modulate their performance in biological system are critically important for the biomedical applications of CNMs, and their future translation in clinics. In this review, we have shown various covalent chemistry approaches that can be used to improve the dispersibility of CNMs and to impart them with biological functions such as active targeting and theranostics features. We also

emphasized the necessity of using appropriate covalent chemistry approaches to suppress non-specific interactions of CNMs with plasma proteins to escape RES organ trapping and to maintain their synthetic targeting ability. To better improve the therapeutic outcomes of CNM-based drug delivery platforms, we encourage the researchers from this community to use PG functionalization, as it satisfies all the challenging requirements for the biomedical applications of CNMs: 1) high hydrophilicity to disperse CNMs in a physiological environment, 2) unprecedented stealth effect to avoid CNMs from uptake by macrophage of RES organs, and 3) abundant hydroxyl groups to integrate a variety of biological functionalities onto CNMs.

As pushing the limit of targeting precision and efficacy is an important goal for future research in this field, the ideal CNM-based therapeutics should concurrently implement the functions of suppressing non-specific uptake during the circulation and inducing sufficient targeted uptake at tumor sites [96]. Unfortunately, active targeting ligands cannot be used for such purpose. Due to their hydrophobic and electropositive nature, they tend to adsorb proteins during the circulation, and the amounts of adsorbed proteins usually increases as the density of grafted ligands increases as in the case of TPP we mentioned above. Since large amounts of adsorbed proteins are known to diminish targeting ability of ligands, they cannot achieve

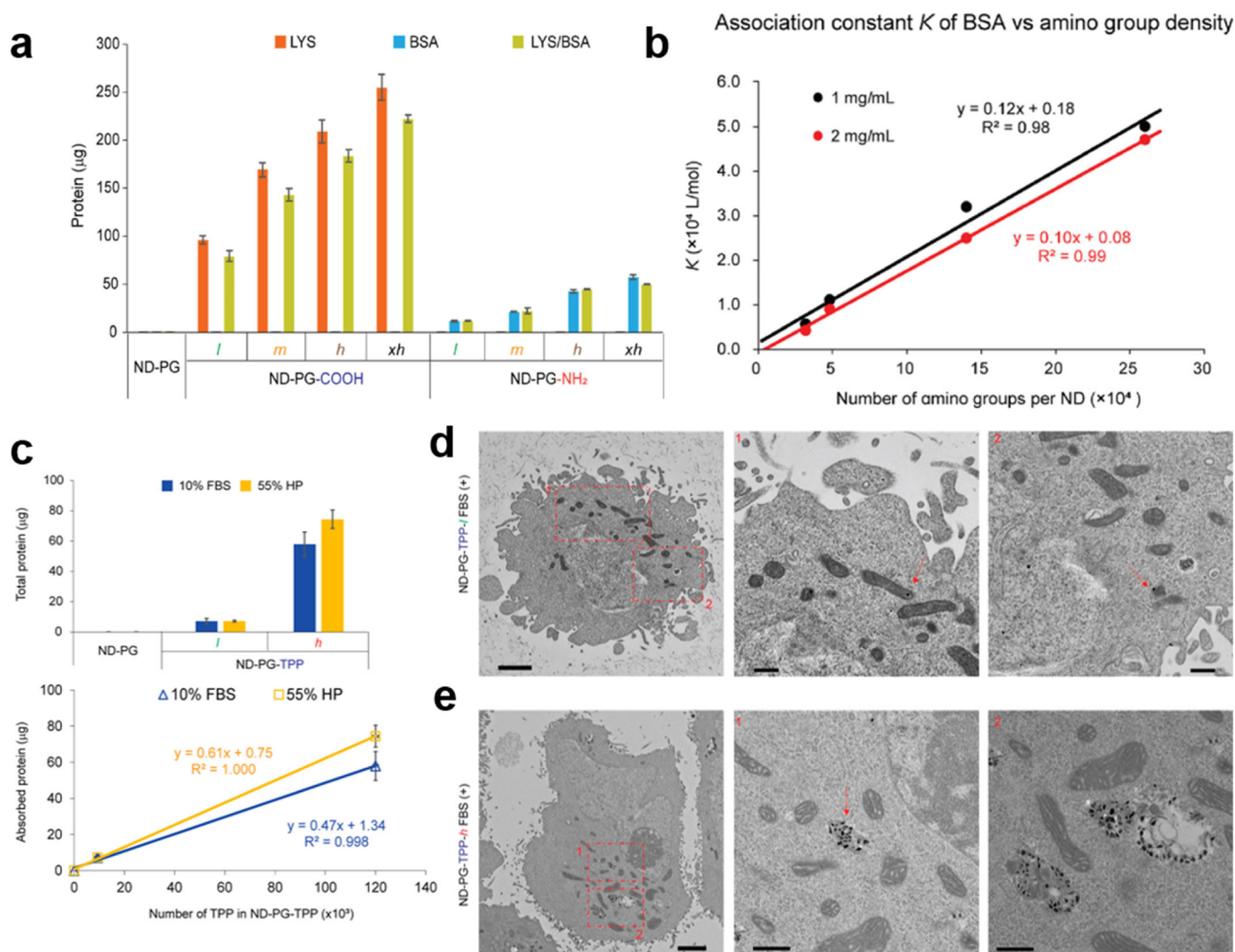


Fig. 7 Quantitative estimation of the bioactivities of the functional groups on ND-PG. (a) Amounts of LYS and BSA adsorbed on ND-PG bearing carboxylate ($-\text{COO}^-$) and ammonium ($-\text{NH}_3^+$) groups of *l*, *m*, *h* and *xh* densities, and (b) linear relationship of K between BSA and ND-PG-NH₃⁺ against the density of ammonium groups (b). (c) Amount of FBS and HP proteins adsorbed on ND-TPP-*l* and -*h* and their correlation with TPP density. (d) Subcellular localization of ND-TPP-*l*, and (e) ND-TPP-*h* in the presence of FBS. Scale bars of low and high magnification images are 2 μm and 500 nm, respectively. Panels (a–b) and (c–e) were adapted with permission from Ref [93] and [94], respectively. Copyright 2020, Elsevier and Copyright 2021, American Chemical Society.

targeting ability and high uptake at the same time. Instead, to meet such requirement, the concept of stimuli-responsive surfaces that can “turn off” the internalization ability of nanomaterials to normal cells during circulation and “turn on” their internalization ability to tumor cells in response to the stimulants at tumor microenvironment (e.g., low pH, redox potential, and overexpressed enzymes) [97] has been proposed. For example, positively charged nanomaterials can be protected by an inert surface through sensitive chemical bonds to shield their high uptake ability during the circulation, and upon arriving the target site, the cleavage of sensitive chemical bonds will result in the detachment of protecting surface and the exposure of positive charged surface to activate on-demand uptake. Although several successful examples have been demonstrated in polymeric NPs [98], this is actually one area lacking sufficient research for CNMs. Looking forward, we are awaiting appropriate covalent chemistry approaches

that can be explored for constructing such smart interfaces for CNMs to fully realize the objective of precise theranostics.

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