**Introduction**

Cancer is fundamentally characterized by the irregular wild proliferation of abnormal cells with aggressiveness to invade and metastasize. Since cancers are viewed as complex systems wherein a variety of cells are involved, its concurrent early detection and simultaneous therapy are of necessary steps for success of treatment modalities.\(^1\) The malignant cells, in comparison with normal cells, show some important genomic and/or epigenetic alterations (e.g., DNA mutations, DNA methylation status and overexpression of some genes and literally proteins) prior to macroscopic phenotypic changes. Such inadvertent alterations have led classification of various cancer marker molecules (CMMs) such as plasma membrane integrated proteins (the-so-called cell surface receptors) or intracellular biomolecules involved in cell signaling. The nature, specificity and level of expression of CMMs are largely dependent upon type of cancers, wherein their early detection is increasingly becoming important and useful steps in terms of diagnosis and prognosis of malignancies. For instance, the gastric cancer is one of the most devastating malignancies with high mortality rate worldwide, hence the only chance to reach better outcomes largely lays on an early-stage diagnosis and simultaneous therapy.\(^2\) In fact, the treatability of other types of solid tumors (e.g., pancreas, ovarian, bladder, colorectal, thyroid, and breast cancers) are almost similar. While monitoring of the level of the expressed CMMs can result in improvement of treatment strategies and detection of cancer recurrence, they possess intrinsic potential to be exploited as targets for early detection of tumor and simultaneous therapy. As a result, having exploited CMMs, several monoclonal antibodies (mAbs) and their fragments have been developed and translated into clinical applications.\(^3\) Further, there exist compelling evidences that most of the solid tumors are immunogenic tumors, therefore immunotherapy modalities can be pursued for effective therapy of these diseases.\(^4\) Nevertheless, similar to chemotherapy alone, immunotherapy appears not to be effective enough when used alone.\(^5\) Although cancer chemotherapy has been accepted as an effective treatment modality for various malignancies, this approach is often associated with inadvertent intrinsic side effects mainly because of cytotoxic nature of the most anticancer agents. To tackle this problem, multifunctional nanomedicines and theranostics have been engineered to improve pharmacokinetic and pharmacodynamics impacts because they are able (a) to target cancer cells specifically through homing devices, (b) to monitor the disease status through imaging device, ...
and (c) to deliver the anticancer agent(s) actively to the target site. However, engineering of these long circulating smart “bioshuttles” demands several steps of synthesis, formulation and bioconjugation processes. In the current study, we will review the advanced materials used for engineering of surface modified multifunctional nanomedicines and theranostics as well as the commonly used conjugation materials and techniques.

Multifunctional nanomedicines and theranostics
From translational standpoint, it is the treatment strategy (e.g., cancer type, biological architecture at cellular/molecular dimension, and disease/patient conditions) that bestows the directionality and endpoint objectives of the seamless coordinated diagnostics and therapeutics of a single multifunctional nanomedicine (the-so-called “theranostics”). However, of enormous investigations towards development and advancement of multifunctional NSs, a very minor percentile studies represent the medical applications and schematic architectures of different types of multifunctional NSs. Of these NSs, the macromolecules with globular structures (e.g., liposomes, micelles and dendrimers) can entrap/encapsulate the diagnostic and therapeutic agent(s) and improve both the solubility and the blood circulation period, while protecting them from quick elimination and/or biodegradations. As shown in Fig. 1, multimodal NSs may harbor the entrapped anticancer agents such as doxorubicin (DOX) and paclitaxel (PTX), which are also conjugated with homing devices such as antibody (Ab) or aptamer (Ap) and imaging devices such as gold NPs (AuNPs) and quantum dots (QDs). Such bioshuttle can result in increased accumulation of drug in tumor tissue, the so called enhanced permeation and retention (EPR) effect, as a result of the leaky vasculature surrounding rapidly growing neoplasm. To be maximally effective, the surface of NSs need to be modified with hydrophilic materials such as poly(ethylene glycol) (PEG), a process the so-called PEGylation, and conjugated with homing and imaging devices. Fig. 2 represents a simple conjugation scheme for PEGylation and Ab bioconjugation of NPs functionalized with carboxylic groups such as acid terminated poly(lactic-co-glycolic acid) (PLGA) NPs. These NPs can be activated using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS), which can be then PEGylated and conjugated with Ab through a one-/two-step processes.

Impacts of advanced nanomaterials as imaging devices
A prerequisite for simultaneous imaging and therapy of cancerous cells using theranostics is implantation of photo-acoustic nanomaterials with desirable characteristics. So far, anticancer chemotherapies, Abs or Aps conjugated with AuNPs, magnetic nanoparticles (MNPs), QDs and carbon nanotubes (CNTs) have been used for the engineering of multifunctional NSs since simultaneous pinpointing of cancerous cells by such NSs can even impart existence of a single cancerous cell at the course period of treatment. It is clear now that the real time

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**Fig. 1.** Schematic representation of various multifunctional nanosystems (NS) used as theranostics. For engineering multimodal nano-systems, various moieties (e.g., anticancer agent(s), antisense, siRNA, Aptamer, imaging agents, antibody fragments, targeting agents) are generally entrapped, encapsulated or conjugated with different delivery systems such as polymers/lipids. Therapeutics and homing devices can be conjugated to magnetic nanoparticles (MNPs) and quantum dots (QDs) for simultaneous detection and therapy. Note: not drawn to scale and not shown the actual mechanism of conjugation.
optical monitoring of diseased cells at molecular/cellular levels can significantly favor the targeted therapy – an approach that is largely dependent upon the exquisite sensitivity and versatility of optical technologies.

Quantum Dot
The quantum dot semiconductors are the most studied nanocrystals used as an imaging agent in formulation of cancer nanomedicine and theranostics because they display superior fluorescent properties as compared with the conventional chromophores and contrast agents. When excited with laser beam, the QDs can emit fluorescent light based on their size, from the blue region to the red region of the optical spectrum. Of these inorganic fluorophores, semiconductor nanocrystals are typically composed of atoms from groups II-VI elements (e.g., CdSe, CdS, CdTe, ZnSe), III-V (InP and InAs) and IV-VI (PbSe). Among various QDs preparation methods, a common method to produce bulk quantities of QD nanocrystals is to use high-temperature conditions. In a study, the routine method to produce bulk quantities of QD nanocrystals is to use high-temperature conditions. These researchers showed profoundly longer photo-stability of QDs bioconjugates in comparison with Alexa 488 bioconjugates of anti-Her2 IgG-QD, showing superiority of these engineered QD imaging agents for the early diagnosis of epithelial tumors. Antibody-cytotoxic drug conjugate can be used in assays involving fluorescence resonance energy transfer (FRET), or bioluminescence resonance energy transfer (BRET). The most important pitfall of QDs for most biomedical applications, similar to various advanced materials such as cationic lipids and polymers used as DDSs or gene delivery systems (GDSs), is their intrinsic toxicity which is yet to be fully understood. For example, in human umbilical vein

### Table 1. Selected examples of multifunctional nanomedicines and theranostics

| Nanosystems | Size (nm) | Therapeutic/imaging agents | Application |
|-------------|----------|----------------------------|-------------|
| Liposomal nanoparticles (NPs) | 30-300 | Maghemite nanocrystals | MR imaging and cancer therapy<sup>20</sup> |
| Cisplatin | | Cisplatin nanoparticles<sup>21</sup> |
| Herceptin | | Antibody-labeled PEGylated liposomes<sup>12</sup> |
| Micellar NPs | 20-200 | Anticancer drugs, antibodies, genes | Dendrimeric theranostics nanocomposites<sup>14</sup> |
| Solid lipid NPs | 50-500 | Small anticancer drugs, antibodies, genes | Increased specificity of gold nanoshells for HER2+ breast cancer<sup>20</sup> |
| Dendrimeric NPs | <100 | - | Cancer cell imaging and PTT/PDT<sup>14</sup> |
| Gold NPs | <50 | - | Cancer cell imaging and PTT/PDT<sup>14</sup> |
| Magnetic NPs (MNPs) | <50 | Small anticancer drugs, antibodies, genes | Multifunctional porous silica NPs as DDS<sup>16,19</sup> |
| Silica NPs | 20-300 | Trastuzumab | Immunotargeted nanoshells for NIR photothermal therapy using anti-HER2 antibody<sup>20</sup> |
| Nanoshell | <100 | Anti-HER2 antibody | - |
| Fullerenes | <50 | Small anticancer drugs, antibodies, genes | Non-invasive cancer imaging and therapy<sup>21</sup> |
| Carbon nanotubes (CNTs) | <100 | Small anticancer drugs and antibodies | Cancer cell targeting and photoacoustic therapy by CNTs as nanobombs<sup>23</sup> |
| Nanorod | <100 | Photosensitizer | ZnO nanorods for treatment of single cancer cells<sup>24</sup> |
| Quantum dots (QDs) | <10 | - | Cancer cells imaging and PTT/PDT<sup>21</sup> |
| Bioconjugated MNPs | 50-200 | PAION-Ab | HER2/neu antibody conjugated SPIONs for breast cancer MRI<sup>25</sup> |
| Bioconjugated QDs | 20-100 | Cetuximab | Cetuximab-QDs bioconjugate targeting EGFR positive cancer cells<sup>24</sup> |
| Bioconjugated aptamer | 50-200 | Small anticancer drugs, antibodies, genes | Aptamer-antibody sandwich ELISA for the early diagnosis of epithelial tumors<sup>27</sup> |
| Trastuzumab and Maytansinoid | | Antibody-cytotoxic drug conjugate<sup>28</sup> |
| Bioconjugated antibody | 50-200 | BRCA1 antibody | BRCA1-NPs for in vivo targeting of gastric cancer<sup>29</sup> |
| Anti-EGFR antibodies | | Anti-EGFR antibody conjugated gold NPs for cancer diagnostics<sup>29</sup> |
| Bioconjugated CNTs | <200 | Small anticancer drugs, antibodies, genes | Targeted killing of cancer cells in vivo and in vitro with EGF-directed carbon nanotube-based drug delivery<sup>41</sup> |

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ECs (HUVECs), QDs (10µg/mL of CdTe QDs) were shown to elicit significant oxidative stress, mitochondrial network fragmentation as well as disruption of mitochondrial membrane potential, leading to apoptosis through upregulation of Bax, downregulation of Bcl-2, release of mitochondrial cytochrome c and cleavage of caspase-9/caspase-3. However, surface modification is deemed to alter the toxicity of QDs because it has been shown that the QD-capping material, rather than the core metalloid complex, is responsible for the majority of their toxicity and biological activity. Therefore, unlike molecules covered with a toxic agent that display cytotoxicity, the surface-modified QDs conjugated with biomolecules seem to retain the biological effects of the conjugate.

**AuNPs**

In addition to QDs, other types of inorganic nanomaterials (e.g., AuNPs, gold nanoshells, AuroShell and ferrofluid, silica NPs) have successfully been exploited for sensing and/or therapy of cancers resistant to immunotherapy or chemotherapy. In 2011, Carpin et al. reported successful targeting and ablation of trastuzumab-resistant cells using anti-HER2-conjugated silica-gold nanoshells and a near-infrared laser. As the main concept for enhancing thermal ablation of cancer by AuNPs, bioconjugation of CMMs targeting mAbs/aptamers with AuNPs appears to provide a useful platform for AuNP-
based photothermal therapy (PTT) and imaging of cancer.\textsuperscript{56} AuNPs can simply be conjugated through ionic interactions, hydrophobic interactions, or dative binding (e.g., thiolation) using an appropriate linker such as N-hydroxysuccinimimidyl (NHS) ester that is mainly used for engineering of immunosensors and bioships. Technically, the electrolyte-mediated coagulation phenomenon is the basis of formation of gold-mAbs bioconjugates, in which if mAbs are present in the colloidal suspension, adsorption of mAbs can occur as the electrolyte concentration (NaCl or buffer salts) is raised to surpass the negative repulsion effects. It should be noted that spontaneous adsorption of protein on the surface of AuNPs happens because of electrostatic, hydrophobic, and Van der Waals interactions between AuNPs and mAbs.

**Silica NPs**

Mesoporous silica nanoparticles (MSNPs) have great potential to be used as multimodal drug delivery system (DDS). The mesoporous structures of these biodegradable ceramic based matrices appear to provide a shelter for incorporation of various agents (e.g., drugs, proteins, imaging agents, photosensitizers), while the outer surface can simply be modified and functionalized.\textsuperscript{61} For example, multimodal silica NPs (7 nm), which have recently been approved for clinical trial, were used as imaging agents.\textsuperscript{57} The MSNPs displayed high potential for dye-encapsulating, surface functionalization with cyclic arginine-glycine-aspartic acid peptide ligands and radiodioxide as well as safe kidney clearance. In fact, the high binding affinity of these NSs makes them tumor-selective NPs as reported in serial in vivo positron emission tomography (PET) imaging of tumor-selective targeting and nodal mapping through multi-scale near infrared (NIR) optical fluorescence imaging.\textsuperscript{57} Further, to circumvent the P-gp mediated efflux, endosomal pH-sensitive MSNPs have successfully been used to control the release of DOX in vitro and in vivo, which resulted in profound induction of apoptosis through upregulation of caspase-3.\textsuperscript{58} Silica NPs (SNPs) show ability to entrap a large number of fluorescent dye molecules and the resultant fluorescence SNPs (FSNPs) with bright optical properties can be further modified for specific targeting of CMMs.\textsuperscript{60}

**Carbon nanotubes**

As another advanced DDSs, CNTs have been shown to display high potential of photothermal (PT) and photoacoustic (PA) properties, which make them very suitable NSs for imaging and treating tumors.\textsuperscript{7,52,63} CNTs are able to absorb NIR radiation (700 and 1100 nm), in which body tissues are most transparent, and transform the adsorbed NIR energy into PT and/or PA signals. As a result, they can be used as an imaging agent more deeply within tissues than other optical modalities can offer, resulting in an efficient heating within the surrounding environment.\textsuperscript{53} In addition to being highly mechanically flexible, the small size and high surface area make CNTs very attractive nanomaterials for development of seamless multifunctional NSs for simultaneous diagnosis and therapy of cancer.\textsuperscript{60} CNTs can be functionalized with targeting device (e.g., Abs, Fabs, scFvs, Aps), magnetic nanoparticles (MNPs) and also cytotoxic agent (e.g., DOX, PTX) mainly via molecular adsorption or chemical conjugation methods (e.g., cleavable ester bond, amide bond).\textsuperscript{64} It has been reported that the growth head and neck squamous carcinoma cells, which overexpress the epidermal growth factor receptor (EGFR), can significantly be inhibited by CNTs loaded with cisplatin (CP) and armed with EGF (CNT-CP-EGF). These NSs were shown to be highly selectively taken up by cancerous cells and hence result in profound inhibition of malignancies.\textsuperscript{7} The distribution and clearance study of PEGylated CNTs carrying CP molecules (PEG-CNT-CP) in mice have revealed that the PEG-CNT-CP were highly dispersed in aqueous medium, and upon conjugation with EGF, they were able to efficiently inhibit the growth of squamous cell tumors, in large part due to better cellular internalization.\textsuperscript{8} Besides, single walled CNTs (SWCNTs) were shown to be heated up under a radiofrequency (RF) field—a de novo safe method for selective elimination of malignant cells. Hence, application of 13.56-megahertz RF field had a heating impacts on injected functionalized SWCNTs in the hepatic VX2 tumors in rabbits, so that at 48 hours, all treated tumors displayed complete necrosis.\textsuperscript{75} Taken all, it seems that such promising PT and PA properties of CNTs can be used for selective destruction of cancer cells and may change the directionality of the cancer diagnosis and therapy in the near future.

**Magnetic nanoparticles**

The other important group of inorganic NSs are MNPs and superparamagnetic iron oxide NPs (SPIONs), which are deemed to provide a robust platform for cancer targeting and imaging. These NPs may be categorized as (a) ultra-small superparamagnetic iron oxide (IO) NPs (USPIONs) with 10-50 nm in diameter, (b) small superparamagnetic IO NPs (SPIONs) with 50-150 nm in diameter, and (c) monocrystalline IO NPs (MI-ONs) with 100-200 nm in diameter.\textsuperscript{74} They are superior to traditional gadolinium-based magnetic resonance (MR) contrast agents mainly because of lower toxicity and stronger enhancement of proton relaxation resultant in lower detection limit.\textsuperscript{75} MNPs have increasingly been used for clinical applications such as magnetic resonance imaging (MRI), drug delivery and magnetic fluid hyperthermia. The MNPs-based thermal therapy has been examined in prostate cancer, showing good tolerability.\textsuperscript{7,72} Further, SPIONs-enhanced MRI (Ferumoxtran-10) has successfully been used for diagnosis of nodal staging in patients with head and neck cancer. From a total of 63 nodes studied (36 nonmetastatic, 25 metastatic, and 2 inflammatory), SPIONs-enhanced MRI resulted in diagnosis of 24 metastatic and 30 nonmetastatic nodes, i.e. yielding a sensitivity of 96%, a specificity of 78.9%, a positive predictive value of 75%, and a negative predictive value of 96.8%, while the overall accuracy of the technique was about 85.7%.\textsuperscript{75} SPIONs with diameter around 30 nm are currently under clinical trials for prostate cancer imaging and thermal therapy.\textsuperscript{7,75} Functionalized MNPs have also shown great potential as theranostics.\textsuperscript{76} For example, using selected surface modification methods, we have recently engineered PEGylated MNPs functionalized with folic acid (FA) and loaded with either mitoxantrone (MTX) or tamoxifen (TMX) to target the folate receptor (FR) overexpressing cancer cells for specific delivery of the anticancer agents.\textsuperscript{77,79} Based on our findings, we proposed both MTX- and TMX-loaded FA-armed PEGylated MNPs as novel multifunctional theranostics for concurrent targeting, imaging and therapy of the FR-positive cancer cells, which can be translated into clinical applications with high efficacy and safety. In a study, MNPs were coated with oleic acid (OA) and PEG to form water-dispersible NSs which were then exploited to adsorb DOX onto the OA layer. Such coated MNPs conjugated to anti-HER2 mAb (~184 nm diameter with ~8 nm iron-oxide core) were successfully
used for active targeting of the human MCF-7 breast cancer cells.77 Taken all, although the MNPs need further characterization and optimization prior to their applications in clinic to ensure upon their early/late biologic impacts, they provide promising platform for advancement of multimodal NSs.

**Biocompatibility of polymers and lipids for development of multifunctional NSs**

A large variety of natural, semisynthetic (modified natural polymers), synthetic polymers (linear, branched and dendritic architectures) and lipids have so far been examined for their safety and potential as DDSs or gene delivery systems (GDSs). However, unfortunately, very few polymers have successfully been translated into clinical applications. In fact, many of these materials (e.g., cationic polymers, dendrimers and lipids) were shown to elicit intrinsic cytotoxicity and toxicogenomics.44-53,80

There exist several important biodegradable and natural polymers that possess promising characteristics and suitability for further development towards clinical uses. Issues relating to the suitability of polymers and/or their conjugates for development towards clinical uses have previously been well reviewed.46-50 Pivotal parameters for an ideal polymer/lipid based DDSs/GDSs for clinical applications include (a) maximal drug delivery capacity, (b) minimal toxicity following acute or chronic uses by the NS or its metabolite(s), (c) reproducibility in manufacturing, (d) appropriateness for pharmaceutical formulation, (e) acceptable stability (both physicochemical and biological), (f) suitable *in vitro* (cellular) and *in vivo* (whole body) pharmacokinetics properties, and (g) the cost-effectiveness for the large scale production.41

**Surface modification and bioconjugation paradigms**

Technically, the development of NSs demands surface modification and conjugation steps to some extent. In fact, in the most cases, there exists a need for alteration of the native structure of a biomacromolecule to provide functional groups on their surface.44 For example, a simple polymeric NS may have a tripartite structure including (a) the backbone polymer, (b) the linker molecule and (c) the payload molecule(s) such as small drugs, peptides, or proteins. In the case of multimodal theranostics, some other moieties such as targeting and/or imaging agents are also linked to the NSs using cross-linking agents. In the following section, we will briefly provide an overview on different types of cross-linkers.

**Cross-linking agents**

The smallest available reagent systems (the-so-called zero-length cross-linkers) are routinely used for bioconjugation, in which they mediate the conjugation of two molecules through formation of a bond containing no additional atoms. The widely used zero-length cross-linkers are carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide (CMC), dicyclohexyl carbodiimide (DCC), disopropyl carbodiimide (DIC), Woodward's reagent K (N-ethyl-3-phenylisoxazolium-3'-sulfonate), N,N'-carbonyldiimidazole (CDI). Further, both homofunctional and heterofunctional agents have also been successfully utilized as cross-linkers for modification and conjugation of macromolecules.46

Fig. 5 represents molecular structures of some selected zero-length cross-linkers (panel A) and homobifunctional cross-linkers (panel B).

It should be pointed out that, the use of homobifunctional reagents may generate a broad range of undesired conjugates. For example, conjugation of two different scFv Ab fragments may result in formation of scaffolds with the same type of scFvs instead of two different scFvs. However, heterobifunctional systems provide greater control on bioconjugation process, wherein one scFv Ab fragment can be modified through the cross-linker’s most reactive or most labile end and purified from excess reagents (using gel filtration or rapid dialysis) and then conjugated with the second scFv Ab fragment. In fact, most heterobifunctional cross-linkers contain at least one reactive group with good stability in aqueous settings, and hence providing possibility towards extensive purification of the intermediate scaffold prior to conjugation of the second moiety. The NHS ester–maleimide heterobifunctional epitomizes such reactivity through its NHS ester end with the amine groups of excess reagents (using gel filtration or rapid dialysis) and then conjugated with the second scFv Ab fragment. However, heterobifunctional cross-linkers contain at least one reactive group with good stability in aqueous settings, and hence providing possibility towards extensive purification of the intermediate scaffold prior to conjugation of the second moiety. The NHS ester–maleimide heterobifunctional epitomizes such reactivity through its NHS ester end with the amine groups of excess reagents (using gel filtration or rapid dialysis) and then conjugated with the second scFv Ab fragment. In fact, most heterobifunctional cross-linkers contain at least one reactive group with good stability in aqueous settings, and hence providing possibility towards extensive purification of the intermediate scaffold prior to conjugation of the second moiety. The NHS ester–maleimide heterobifunctional epitomizes such reactivity through its NHS ester end with the amine groups of excess reagents (using gel filtration or rapid dialysis) and then conjugated with the second scFv Ab fragment.
also be performed using water-soluble Traut’s reagent (2-iminothiolane), while a versatile reagent for introducing sulfhydryl groups onto target particle (e.g., proteins such as mAbs and NPs) appears to be the heterobifunctional cross-linkers such as N-succinimidyl S-acetylthioacetate (SATA)/sulfo-SMCC (Fig. 6A). Note: not drawn to scale.

Further, similar to SATA (Fig. 7A), SPDP can react with amine-containing molecules through its NHS ester end to form amide bonds. The pyridyl disulfide group then can be cleaved with an excess of hydroxylamine. SATA: N-succinimidyl S-acetylthioacetate; SPDP: N-succinimidyl 3-(2-pyridyldithio)propionate; NHS: N-hydroxysuccinimide (NHS). Note: not drawn to scale.

For detailed information, reader is referred to the textbook of "Bioconjugate Techniques".84 Note: not drawn to scale.

Fig. 6. Molecular structures of heterobifunctional cross-linkers (A) and nanoparticles bioconjugations process by SATA (B). To generate sulfhydryl groups, these researchers conjugated SATA to both mAb and IA by undertaking stepwise reactions using S-acetylthioacetate (ATA)-mAb or ATA-IA for generation of IA-(A-SH) to mAb-(A-SH) and homobifunctional cross-linker, 1,8-bis(maleimido)diethyleneglycol (BM[PEO])

They showed that monomeric mAb-(A-S-S-[PEO]$_2$S-S-A-IA)$_n$(mAb-IA)$_n$ radiolabeled with $^{111}$In by 2-(p-isoiodoacetobenzyl)cyclohexyl-DTPA and with $^{125}$I by iodogen method showed over 70% bindability to the integrin αvβ3 receptor (0.4 µM). Upon intravenous injection to nude mice with the receptor-positive M21 tumor, the mAb-IA$_n$ radiolabeled with both $^{111}$In and $^{125}$I accumulated rapidly and retained in the tumor for a period of 44 h, while the radioactivity cleared quickly from the blood, thereby resulted in increased tumor-to-blood ratios over the time. As a proof of concept, the fluorescence microscopic revealed a rapid blood clearance, a short peak tumor uptake time, and a low peak tumor uptake value with prolonged tumor retention for mAb-IA$_n$. It was shown that mAb-IA$_n$ can primarily bind to the integrin αvβ3 receptors on angiogenic vessels, but not on the tumor.85 SATA has also been used for preparation of multimodal proteins, or proteins labeled with both fluorescent and magnetic reporter groups, which can be used in a wide range of in vitro and in vivo imaging such as FACS flow cytometry, fluorescence microscopy, MRI and/or NIR optical imaging as well as fractionation of cells by magnetic cell sorting.86 To avoid problems such as loss of bioactive sites due to modification points during preparation of multimodal proteins, Schellenberger et al. (2004) reported the synthesis of a magneto/optical form of annexin V, which was performed by reacting the amino-CLIO NPs with Cy5.5 and N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) to produce a fluorescent, sulfhydryl reactive NPs. To pursue such aim, these researchers added a single reactive sulfhydryl group to annexin V using SATA cross-linking, by which they were enabled to preserve the protein’s ability to bind apoptotic Jurkat T cells. Then, reacting SATAlabeled annexin V with an SPDP activated NP yielded Anx-CLIO-Cy5.5 (i.e., a magneto/optical form of annexin V). Having showed high specific binding of Anx-CLIO-Cy5.5 to apoptotic Jurkat T, they proposed such conjugate to preserve the strength of the interaction between annexin V and apoptotic cells, with capability to develop NPs including colloidal QDs and AuNPs.85 Further, similar to SATA (Fig. 7A), SPDP can react with amine-containing molecules through its NHS ester end to form amide bonds. The pyridyl disulfide group then can be then coupled to a sulfhydryl-containing molecule to create a cleavable disulfide bond (Fig. 7B). This cross-linker agent is

Fig. 7. Schematic representation of functionalization of monoclonal antibody (mAb) and conjugation of single chain fragment variable (scFv) antibody fragments. A) SATA-based conjugation of a model mAb. B) SPDP-based conjugation of amine group with sulfhydryl group in two different scFvs. The modified protein (mAb) with a protected sulfhydryl end using SATA can be stored without degradation and subsequently deprotected with an excess of hydroxylamine. SATA: N-succinimidyl S-acetylthioacetate; SPDP: N-succinimidyl 3-(2-pyridyldithio)propionate; NHS: N-hydroxysuccinimide (NHS). Note: not drawn to scale.
technically extensively used to conjugate proteins such as Ab scaffolds (e.g., mAb, Fab, scFv) to form multispecific systems and also immunotoxin) that can be used for in vivo applications. succinimidyl-4- (N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) is a heterobifunctional reagent with significant utility in crosslinking proteins, particularly in the preparation of Ab – enzyme. For example, for the tumor-specific imaging through targeting EGFR using QD-cetuximab conjugates, Lee et al. (2010) reported three different conjugation strategies. Successful conjugation of cetuximab to QDs was reported upon exploitation of PEG conjugated polymer-coated QDs and two long-chain heterobifunctional linkers (i.e., sulfu-LC-SPDP and sulfu-SMCC) with dissociation constant of the QD-cetuximab conjugates to EGFR of 0.61 +/- 0.28 nM and efficient internalization. Since the cellular imaging experiments using the QD-cetuximab conjugates resulted in a clear endocytosis and colocalization of the QD-cetuximab conjugates with dye-labeled transferrin, the QD-cetuximab conjugates were suggested to be used as an imaging modality for EGFR overexpressing cancer cells. In another study, for the characterization of QDs and their conjugates to biological molecules by capillary electrophoresis coupled with laser-induced fluorescence, non-selective and selective methods were used for preparation of QDs conjugated to some biomolecules. For the non-selective approach, 1-ethyl-3- [3-dimethylaminopropyl] carbo diimide hydrochloride (EDC)/sulfo-NHS was used for the conjugation of BSA and myoglobin to carboxylic acid-functionalized QDs. For the selective approach, heterobifunctional cross-linker sulfo-SMCC was utilized for the conjugation of partially reduced IgG to amine-functionalized QDs and the conjugation of periodate-oxidized IgGs to hydrazide-functionalized QDs. In general, there are different approaches for surface modification and bioconjugation of NPs, including: (a) use of a bifunctional ligand such as mercaptoproic acid, (b) trioc tyolphosphate/trioc tyolphosphate oxide (TOP/TOPO)-capped NPs bound to a modified acrylic acid polymer through hydrophobic forces, (c) NPs solubilization and bioconjugation using a mercaptosilane compound, (d) positively charged biomolecules linked to negatively charged NPs by electrostatic attraction, and (e) incorporation of NPs into microbeads and nanobeads.

For example, immunoQDs (i.e., Ab-QD bio- conjugates) can be produced through different methods, including (a) QDs conjugation to Ab fragments via disulphide reduction and sulphhydryl-amine coupling, (b) covalent coupling between carboxylic acid (-COOH) coated QDs and primary amines (-NH2) on intact Abs using EDC or EDC/NHS chemistry, (c) site-directed conjugation via oxidized carbohydrate groups on the Ab Fc portion and covalent reactions with hydrazide-modified NPs, (d) conjugation of histidine-tagged peptides or Abs to Ni-NTA modified QDs, and (e) noncovalent conjugation of streptavidin-coated QDs to biotinylated Abs.

Fig. 8 exemplifies different bioconjugations processes including thiolation of an amine-containing scFv Ab fragment with methyl 3-mercaptopropionimidate (panel A) and conjugation of two scFv Ab fragments with carboxylic acid group and amine group through 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (panel B).

It should be also stated that different cross-linkers have been exploited for surface modification of QDs, including (a) bifunctional linkage (via homo/heterobifunctional cross-linkers), (b) hydrophobic attraction (TOPO-capped QDs bound to a modified acrylic acid polymer), (c) silanization, and (d) electrostatic attraction. Technically, most QDs’ surfaces for biological applications contain negatively charged carboxylates for conjugation with amine-containing molecules via a carbodiimide reaction with EDC and sulfo-NHS. The QDs have been also exploited for live cell imaging, nonetheless these NSs showed cytotoxic effects to some extent. Accordingly, recent developments in silicon QDs, non-blinking QDs, and QDs with reduced-size and controlled-valence further make these QDs bioanalytically attractive because of their low toxicity, biocompatibility, high quantum yields, and diverse surface modification flexibility. The potential of multiplexed...
sensing using QDs with different wavelengths of emission is promising for simultaneous detection of multiple biomarkers of disease.\textsuperscript{97,98} It should be stated that various types of NPs (e.g., polymeric/lipidic NPs, QDs, MNPs and AuNPs) can simply be PEGylated and conjugated using NHS-PEG-maleimide (Fig. 9), which has been widely used for production of multifunctional nanomedicines and theranostics.\textsuperscript{99-102} For example, in 2013, Chan et al. have exploited NHS-PEG-maleimide for development of PEGylated fluorescent polystyrene NPs conjugated with anti-EGFR M225 Abs, which were successfully used for optical molecular imaging in human epidermoid carcinoma A431 cells and lung squamous cell carcinoma NCI-H520 cells as well as human esophageal tissue.

**Biological implications of surface modified nanomedicines**

The foremost biological barrier against injected NSs is reticuloendothelial system (RES) that can predominantly limit the clinical efficacy of nanomedicines and theranostics. In general, the anatomy and size of NPs play a key role in terms of RES function, that is, NPs >250 nm can be physically trapped by the fenestrations in the spleen while NPs <70 nm can be accumulated in liver. Thus, NPs in a range of 70–200 nm are able to stay in blood stream for a longer period of time.\textsuperscript{103} However, these NPs are also subjected to opsonization, which is a process that can lead the foreign particulate invaders to be covered by opsonins and subsequently seen by phagocytic cells that are responsible for sequestration and immune clearance of the invading NPs.\textsuperscript{104,105} Hence, blocking the electrostatic and hydrophobic interactive surface of NPs by means of surface adsorbed or grafted shielding groups (e.g., long hydrophilic polymer chains and non-ionic surfactants) can help to circumvent the opsonization. Hydrophilic materials (e.g., polysaccharides, polycryliclamide, poly(vinyl alcohol), poly(N-vinyl-2-pyrrolidone), PEG, and PEG-containing copolymers poloxamers, poloxamines, polysorbates and PEG copolymers) have successfully been exploited.\textsuperscript{106} Of these, PEGylation is the most widely used method for making stealth NPs. However, regardless of being PEGylated, a 250 nm PEGylated NP can be cleared from the blood stream much quicker than a 70 nm PEGylated NP.

Further, consequences for activation of the complements by NPs may be the reactogenicity functions such as hypersensitivity reactions as reported for liposomal drugs (e.g., Doxil®). To understand the mechanism of such adverse immune reaction, the-so-called C activation-related pseudoallergy (CARPA), Szebeni et al.\textsuperscript{2011} analyzed the relationship among liposomes’ features, C activation in human serum in vitro, and liposome-induced cardiovascular distress in a pig model for human CARPA. These researchers found that among the structural variables (e.g., surface charge, presence of saturated/unsaturated moieties, PEGylation, and use of CP/DOX in liposomal formulations), high negative surface charge and the presence of DOX were the significant contributors in terms of the reactogenicity both in vitro and in vivo, where the effect of DOX appeared to be indirect perhaps through distorting the morphology of liposomes.\textsuperscript{107} Doxil® mediated complement opsonic fragments was shown to elicit C3b deposition and degradation (65 and 40/43 kDa fragments) that can reach the plateau within 5 min, followed by generation of high molecular weight C3b- and iC3b-containing complexes (C3-X).\textsuperscript{107} Complement activation by Doxil® has also been reported in cancer patients through significant elevation of SC5b-9 (the terminal complex activation marker of complement system) levels in plasma within 10–30 min of infusion.\textsuperscript{108} In addition to reactogenicity, multifunctional NPs may act as immunomodulators, activating immune responses where needed. Recently, polysaccharide-based pH-sensitive NSs have been engineered to target mannose-ligands based cell-surface receptors which was able to enhance internalization and activation of antigen presenting cells (APCs).\textsuperscript{109} This may lead us towards tunable modulation of immune responses. Cui et al. showed that the mannosylated NPs exhibited enhanced antigen presentation in the context of major histocompatibility complex (MHC) class I molecules in dendritic cells (DCs). Such functionalized pH-sensitive NSs seem to open new avenue for vaccine development, in which the conjugation of cell-surface receptor ligands can deliver antigens to specific intracellular pathways and accordingly provide a tool for better controlling the antigen presentation to T cells, or even produce specific signals to manipulate the cytokine production and activation of APCs. Regarding clinical impacts, specific/nonspecific effects of multifunctional nanomedicines have yet to be fully understood. In general, it seems that the clinical impacts of multifunctional nanomedicines and theranostics, in most of the cases, are largely dependent upon their ability to cross biological membranes and barriers efficiently, to target the desired cells specifically and to interact with/to internalize into the target cells. Upon interaction of nanomedicines with the target CMMs, they are mostly prone to endocytosis through fluid-phase or receptor-mediated endocytosis.\textsuperscript{31} Various cell surface receptors have so far been reported to be involved in endocytosis phenomenon, including: clathrin coated pits, caveolin proteins, transferrin, EGFR. For example, in ovarian cancer cells, cisplatin (CP) nanocapsules endocytosis and toxicity was shown to be cell-dependent and high cytotoxicity of CP nanocapsules appeared to be largely dependent on expression of caveolin-1 endocytosis followed by release of the drug from a late endosomal/lysosomal compartment and CP-DNA-adduct formation. Thus, cells with higher expression of caveolin-1 (e.g., Igrov-1 cells) shows higher responsiveness to CP nanocapsules compared to those with lower/no expression of caveolin-1 (e.g., Ovcar-3 cells).\textsuperscript{110} This concept should be taken into account for development of anticancer nanomedicines.

**Final remarks**

Of various advancements for improved targeted therapy of cancer, seamless multifunctional nanomedicines and theranostics appear to hold great promises. These NSs can be used for simultaneous imagining (optical/non-optical) and therapy of cancerous cells. Ideally, they should represent some important physicochemical and biological features such as (a) long blood circulation time, (b) high tumor-accumulation through passive targeting (EPR effect), (c) specific interaction with cancer cells through active targeting by homing devices, (d) high drug-loading capacity, (e) no/low toxicity, (f) low polydispersity index, and finally (g) simple method of formulation. Formulation of these NSs demand several steps of surface modifications such as PEGylation and conjugation with targeting and imaging devices, which demands integration of several domains for successful engineering of smart and safe seamless NSs. Further, such smart multifunctional NSs must be equipped with suitable stimuli to be able to trigger the liberation of drugs on demand during monitoring of the status of patients with malignancies. Taken all, smart multifunctional

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NSs provide new promising premises for simultaneous diagnosis and therapy of cancer, and to be much more efficient, they need to be designed based on disease condition leading to personalized targeted therapy of cancer.

**Ethical issues**
The authors declare no ethical issues.

**Competing interests**
The authors declare no conflict of interests.

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