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Multi-Functionality in Theranostic Nanoparticles: is more Always Better?

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Since the launch of the National Nanotechnology Initiative (NNI) in 2000, the pace of innovation in nanotechnology related areas worldwide has reached unprecedented levels. In the US alone the NNI umbrella covers the individual and/or cooperative nanotechnology centered activities of 26 different Federal Agencies. The 2013 federal budget for NNI is reported to be \$1.8 billion [1].

With the advent of the ability to “construct” nanoparticles/nanocrystals (NCs) with atomic level precision, the terms “nanomaterials” and “nanotechnology” have realized their true meaning [1–13]. The diverse variety of research and application possibilities soared. Today we cannot imagine our lives without the direct and/or indirect benefits of one or another “nanomaterial” product. They have become an integral part of our daily lives, and the field is still in an expansion mode.

During this time, building from bottom-up has been a key, attractive, and successful avenue for generating functionally-diverse and molecularly-precise constructions, with high fidelity and better control of desired properties. The journey began with modestly functionalized “nanoplatfroms” with only one or two functionalities and today we are talking about “theranostics”, where the number and type of the functionalities have multiplied as well as diversified [14–19], Figure 1 illustrates just a few of the components that have been attached to theranostic nanoparticles. A more comprehensive list is given in Table 1.

Photodynamic Therapy (PDT) is a long-established approach to generate Reactive Oxygen Species (ROS) using photosensitizers (PS) and visible light. To circumvent some issues related with PDT (such as limited light penetration, lack of high enough target selectivity for

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PS molecules, and problematic skin phototoxicity), “dynamic” nanoplatform constructs have been developed. These new PDT paradigms can include infrared active dyes or very short-pulsed infrared lasers combined with two-photon absorbing PS and/or prodrug molecules. They may also include tumor-site recognition functionalities providing the ability to concentrate better in tumors and they are likely to also possess tumor-imaging capabilities. Progressively, these precision engineered constructs have become sophisticated built from bottom-up “dynamic” nanoplatforms Figure 2.

These nanoconstructs have evolved to an extent that they can be injected into the blood stream, circulate harmlessly, and upon encountering the targets they recognize have the capability to zoom in and accumulate in the “problematic area” as shown in Figure 3. All this is achievable through selective molecular-targeting and recognition capabilities, and finally, when appropriate laser irradiation activates the chromophores, they deliver the designated application benefit(s) by killing cancer cells with locally generated ROS. Figure 4 shows how the wide variety of nanoparticles may assist PDT to move from “bench to bedside” or in this case “from mouse to man”.

Over recent years, the great variety of nanoparticles as drug delivery vehicles in anticancer therapeutics has been gaining speed. Due to the facts that they exhibit unique pharmacokinetics (including minimal renal filtration, which in turn extends circulation time in the blood, depending on the surface functionalization characteristics [20]), they have high surface to volume ratios (that enables modification with various functional groups), and possess the possibility to attach tailored functionalities together have made these nanoparticles enormously attractive for medical applications. Nanoparticle-constructs with capabilities to, simultaneously, target, allow real time imaging, and deliver therapeutics has become a reality, with plenty of room for further developments. The ultimate goal is to have the abilities of imaging and monitoring the diseased tissue; delivering prodrugs/drugs with enhanced spatial specificity; and a long-term hope of gaining the ability to finely tune the therapeutic time and dosage, and thus individualize medicine, as opposed to adopting a ‘one size fits all’ approach. This ultimate goal trajectory in itself moves the field of medicine toward an era of more effective and personalized treatment approaches.

To produce theranostic nanoparticles, various therapeutic strategies, such as nucleic acid delivery, chemotherapy, hyperthermia (photothermal ablation), photodynamic, and radiation therapy have been combined with one or more imaging functionalities in both *in vitro* and *in vivo* studies. Therapeutic delivery vehicles can be decorated with different imaging probes, such as MRI contrast agents (T1 and T2 agents), fluorescent markers (organic dyes and inorganic quantum dots), and nuclear imaging agents (PET/SPECT/CT agents) in order to facilitate their imaging and, in doing so, we gain information about the trafficking pathway, kinetics of delivery, and therapeutic efficacy. Thus, creative approaches have being developed for the two major classes in clinical treatment of a disease: diagnosis and therapy via advancements and possibilities offered by nanotechnology.

At the same tune, in recent years many researchers have been focused on development of new synthetic-nonviral vehicles as potentially nontoxic and less immunostimulatory carriers [21,22]. These vehicles can be based on synthetic polymers, dendrimers, liposomes, cell-penetrating peptides, and inorganic nanoparticles. Moreover, developments in generating successful delivery vehicle combined with specific DNA labeling capabilities allowed the use of more sophisticated imaging techniques, such as time-lapse microscopy, Fluorescence Resonance Energy Transfer (FRET), Fluorescence Correlation Spectroscopy (FCS) [23–25], and single-molecule fluorescence spectroscopy [25]; techniques that can provide more precise information regarding polyplex trafficking, detachment, and thus, leading to development of better imaging and delivery systems. More importantly, with advancements

in our imaging capabilities, researchers have realized that labeling polymers with “classical” fluorescent types of labels can change the physiochemical properties of these polymers. With the help of FCS it was found that the cationic diblock polyethyleneglycol–polyethylenimine (PEG-PEI) copolymers lose their ability to bind to nucleic acids when labeled with a fluorescent cyanine dye Cy5 [26].

With the achievements in synthesizing quantum size confined nanoparticles known as quantum dots (QDs) [2,4–7,10,11] the nanoplatfrom concept has gained new meaning and span. QDs paired with fluorescent organic dyes (as FRET pairs) have been used in monitoring polyplex trafficking *in vitro* [15]. The intracellular dissociation of polymer–pDNA complexes was monitored via QD-FRET in HEK293 cells to monitor the unpackaging of chitosan–pDNA complexes. In the study streptavidin-functionalized, Cy5-labeled chitosan was conjugated to QD-labeled pDNA via biotin–streptavidin binding [27]. To address possible toxicity issues regarding the use of QDs for medical applications an *in vivo* study with murine animals was performed. In the study InAs/InP/ZnSe QDs were injected intravenously and showed significant renal clearance; confirmed both by Near-Infrared (NIR) fluorescence imaging and urine sample assessments [28]. Nie and colleagues reported an *in vivo* cancer targeting and imaging with semiconductor QDs where a triblock copolymer of co-polybutylacrylate–polyethylacrylate–polymethacrylic acid with hydrophobic hydrocarbon side chains was used to modify QDs and to which a Prostate-Specific Membrane Antigen (PSMA) targeting antibody was conjugated as well. When administered to prostate cancer bearing mice they showed that the QDs accumulated in the tumor area. This accumulation was attributed to both Enhanced Permeability and Retention (EPR) effect and specific antibody–antigen interaction specific to the tumor site [29].

In our study we used engineered nanoplatforms built from bottom-up with CdSe/ZnS core/shell QDs and double fuctionalization: PEGylation for the EPR effect and cyclic RGD-peptide functionalization for $\alpha_v\beta_3$ integrin specific recognition ($\alpha_v\beta_3$ integrin being overexpressed during angiogenesis). As a negative control for $\alpha_v\beta_3$ integrin specificity, cyclic RAD-peptide functionalization was employed. *In-vitro* studies were done with human umbilical vein endothelial cells (HUVECs), while the *in-vivo* studies were performed with male C57/BL6 mice carrying syngeneic murine melanoma cells. As seen in Figure 5a RGD functionalized nanoplatforms were target specific for the $\alpha_v\beta_3$ integrins and homed to the tumor site (24 hrs after being injected at the mice tail) whereas the RAD functionalized nanoplatforms did not show that effect. Confocal images in y–z axis (Figure 5b) clearly show the uptake of the RGD-functionalized nanoplatforms by the HUVECs (lower panel), whereas the RAD-functionalized nanoplatforms do not show that effect.

Along the same lines, to achieve simultaneously imaging, site-specific homing, and therapeutics delivery effects, a nanoplatform with the QDs, aptamers (Apt), and doxorubicin (DOX) elements was prepared in the study by Bagalkot et al. [30], where they used 10-base RNA aptamer coupled to the QDs as a bio-vector- targeting Prostate-Specific Membrane Antigen (PSMA). After homing to the tumor site DOX payload gradually got released from the nanoplatform, thus initiating the therapeutic functions and recovering the QDs fluorescence [30].

In various studies QDs were used either as photosensitizers themselves or as carriers of drug/prodrug moieties [31–33]. QDs photosensitizer activity can happen, since the particles can be activated by light, and then, can transfer the triplet state energy to the nearby oxygen molecules, causing cell damage [34]. Tsay et al. [35] showed the generation of Reactive Oxygen Intermediates (ROI), through Nitroblue Tetrazolium (NBT) assay in QDs modified with streptavidin and conjugated with biotinylated pDNA. When these modified QDs were photoactivated, it was shown that they can generate ROI through, thus eliciting damage to

purine and pyrimidine bases [35]. Samia et al. [34] have studied the interaction between CdSe QDs with a silicon phthalocyanine (Pc4) PDT photosensitizer. They found that the QDs could be used to sensitize the PDT agent through (FRET) mechanism, or interact directly with molecular oxygen via a Triplet Energy-Transfer process (TET), thus generating reactive singlet oxygen species that can be used for PDT cancer therapy [34]. Rakovich et al. [36], studied photodynamic properties of CdTe QDs and methylene blue hybrid photosensitizer. *In vitro* studies on HepG2 and HeLa cancerous cells showed effective cell kill by this hybrid system [36]. Thus, it is safe to say that QDs have proven to have a wide range of biomedical applications.

In case of the gold nanoparticles, with their strong surface plasmon absorption properties, it is a completely different ball game. Coupled with their stability, biosafety, and easy tunability, the unique opportunities that gold nanoparticles (Au NPs) offer in the medical imaging areas (such as computed tomography (CT), photoacoustics and Surface-Enhanced Raman Spectroscopy (SERS)) can easily be appreciated. Just changing the shape of Au NPs, from spherical to rod-like, can push the absorption wavelength to the near-IR (NIR) region (650–900 nm). This suggests specific roles for Au NPs as probes in photoacoustic imaging or as mediators in photothermal therapeutics [37,38]. In order to achieve desirable NIR-active capability, attainable by playing with the shape of pure gold NPs, researchers developed a nanoparticle with cheaper silica core coated with a gold shell layer [39]. In various studies Au NPs were functionalized to carry various drug (paclitaxel (PTX) [40,41]) and cytokine (tumor necrosis factor (TNF) [42,43]) molecules. In various studies many different ways of anchoring pharmaceuticals, other than the well known thiol–Au association, have been investigated; high PDT efficient Pc4 was adsorbed on PEGylated Au NPs and similarly on Zn-Pc [44]. Further functionalization, using amphiphilic-block-copolymer-coated Au NP, for tumor targeting and drug delivery were formulated [45]. Not only pure gold NPs, but also metallic core/shell NPs with iron oxide cores and gold shells, have been explored to provide combined benefits of imaging and therapeutic delivery. For instance, Au NPs with iron oxide cores (magnetite (Fe₂O₄) or maghemite (γ -Fe₂O₄)) are been used for imaging via MRI [15].

Active investigation of iron oxide nanoparticles (IONPs) as nanoplatfroms on which active construct probes containing multiple imaging motifs are the ambitious goal to load drugs into these nanosystems to achieve all-in-one theranostic agents that possess capabilities, including diagnostic imaging, drug delivery, and therapeutic monitoring are been the thrust in the biomedical research [46,47]. IONPs having magnetic properties allow them to accumulate together up on an external magnetic field. This property has made it utilize as a targeting mechanism to improve its drug delivery efficacy. One such study, when patients with metastatic breast cancer were infused with epirubicin-loaded IONPs and after that a magnetic field was established around the tumor. The magnetic field successfully directed ferrofluid to the tumor to induce tumor regression [19]. Recently feridex particles are been approved by FDA for the detection of liver and spleen lesions, and also the analog Combidex has entered into phase III clinical trials for lymph node imaging [19]. Further due to high temperature decomposition, IONPs can itself play dual role for imaging/therapy, because of its potential in hyperthermia it has emerged as a useful strategy in nanoparticle preparation for theranostics application. Besides its wide application as a theranostics material, still more work needs to be done to expound the interaction of such combinational therapy in both *in vitro* and *in vivo* applications.

A class of nanostructures that has attracted interest in the past two decades for biomedical applications is the nano-carbon allotropes: including fullerenes, carbon nanoparticles, Carbon Nanotubes (CNTs), graphene, and nano-diamonds [48–50]. Nano-carbons are classified into sp² and sp³-carbon nanomaterials based on their bonding structures and as

such they possess inherent optical properties such as fluorescence and photoacoustic emission that makes them a useful contrast agent in optical imaging and sensing [51,52]. PEGylation of CNTs by both covalent and non-covalent methods are much less toxic *in vitro* and *in vivo*, which make them to be preferred for biomedical application [53].

Fullerenes have shown potential application in several different cancer therapeutic approaches such as photodynamic therapy, photothermal treatment, radiotherapy and chemotherapy. Fullerenes can also acts as novel contrast agents in Magnetic Resonance (MR) imaging [54]. Similarly graphene has risen considerable interest for biomedical applications [55]. Unique physical and surface features are a key player in the field of nanomedicine. Besides loading drug molecules, nanoparticles such as IONPs or AuNPs can be easily bound to CNTs to further enrich the nanoplatform. But still their non-biodegradability and toxicity remains to be a great concern in the area of biomedical applications [56].

With the modern trend of steadily increasing complexity and a race to produce the theranostic agent with the greatest number of different functionalities, we must ask ourselves if there is a practical (as well as a theoretical) limit? As the constructs getting more and more sophisticated, and the number of functionalities increases, we must realize that there must be a limit set before they start cross-talking, quenching each other, interfering with each other in some other fashion, or impeding the expected/sought after effects. Thus, at what point will the “law of diminishing returns” take over and become a dominant factor? These are some of the questions that must be answered by the proponents of the mantra “the more, the merrier” with regard to theranostic multi-functionalization.

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Figure 1. Nanoplatfroms with theranostic functionalities: Fe_2O_3 , magnetic NPs, photosensitizer, appropriate polymeric coating, surface-charge tuning groups, optical imaging dyes, targeting agents, adsorbed/bonded drugs, sensitive coverage, DNA and other nucleic acids, polyethylene glycol, proteins, MRI active agents, etc.

Figure 2. Dynamic nanoplateforms for PDT functionalized with molecular targeting moieties, two-photon chromophores, and sensing molecules all engineered into a core matrix.

Figure 3.

A wide range of lipid, polymer, ceramic, magnetic and other functionalized nanoparticles have been used in PDT that can improve photosensitizer delivery, together with fullerenes, carbon nanotubes and quantum dots, and raises the question of whether nanotechnology will truly improve the translation of PDT from mouse to man.

Figure 4.
The concept of long-circulating nanoparticles with tumor localizing ligands attached being injected and left to selectively accumulate in tumors by a combination of active and passive (enhanced permeability and retention effect) targeting.

Figure 5.

a) Epifluorescence imaging of doubly functionalized RGD-PEGylation-QDs (showing $\alpha_v\beta_3$ integrin targeting due to RGD peptide) and RAD-PEGylation-QD nanoplatfoms (lacking the integrin targeting due to the scrambled RAD peptide)

b) Confocal images in the y-z axis, showing uptake of RGD-PEGylated-QDs nanoplatfoms by the HUVECs (the lower panel), and the lack of uptake in the case of RAD-PEGylated-QD nanoplatfoms (upper panel). The yellow color (as a pseudocolor) shows the propidium iodide labeled nuclei and the green color is due to QDs fluorescence.

Table 1

List of functionalities used in theranostic nanoparticles.

Therapeutics	Imaging
PDT[57,58]	Fluorescence [59,60]
Photothermal (plasmonic) [37,39]	Phosphorescence [61,62]
Alternating magnetic [63,64]	Photoacoustic [65,66]
Static magnetic (rheological) [77]	Radiolabeled [67,68]
Radioactive [69]	PET [70,71]
X-ray activated [72]	SPECT [71,73]
	Magnetic [74,75]
	MRI relaxation (Gd) [76]