Expert Opinion

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Advanced methodologies to formulate nanotheragnostic agents for combined drug delivery and imaging

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Introduction: Recent advances in nanoparticle synthesis engineering have made it possible to combine disease diagnosis and therapy. This progress could help to open the door to 'personalized' medicines.
Areas covered: This review highlights the significant applications of theragnostic nanoparticles in therapy. The basic elements to be included in the formulation of theragnostic nanotools are briefly compiled and explained. Special attention is given to the analysis of current formulation strategies from case studies in the literature published after 2000 for simultaneous selective disease imaging and efficient image-guided drug (gene) delivery. This contribution provides a systematic overview of important features in the formulation of theragnostic nanoparticulate systems. Special insight is given to the introduction of passive and active targeting concepts in the engineering of such multifunctional nanoplatforms to gain control of their biological fate.
Expert opinion: Theragnostic nanotechnologies will optimize the way of delivering therapeutic and imaging molecules to disease sites; as a consequence, combined selective diagnosis and effective pharmacotherapy could be used in unison to combat severe diseases. Nanotoxicity investigations, which illustrate the risks of toxicity/immunogenicity associated with the use of such nanoplatforms, will determine their introduction into the clinic.

Keywords: contrast agent, drug therapy, enhanced permeation and retention effect, gene therapy, ligand-receptor targeting, nanomedicine, nanotoxicity, personalized medicine, signal emitter, stimuli-sensitive nanomaterial, theragnosis

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1. Introduction

Chemical synthesis of novel therapeutic molecules and development of combined treatment schedules have tried to improve current pharmacotherapy. However, the clinical effectiveness of conventional active agents is frequently hampered by their inappropriate physicochemical properties (e.g., hydrophobicity and high surface electrical charge, typically leading to a nonspecific biodistribution and reduce accumulation into the disease site), pharmacokinetic profile (usually, rapid metabolism and plasma clearance), and the induction of tissular and/or cellular drug resistances. As a result, many drug molecules showing promising possibilities *in vitro* could even be toxic *in vivo* [1,2]. These problems are also frequently faced by signal emitters or contrast agents, for example, MRI probes, luminophores and radionuclides. As a consequence, such molecules would exhibit an extensive biodistribution, and an almost negligible specificity for the disease region which could determine a limited efficacy in the diagnosis and follow-up of diseases [3].

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Article highlights.

- Preclinical investigations have shown that the combination of imaging agents and drugs (or genes) in multifunctional nanoparticles improves non-healthy tissue targeting efficiency.
- Nanotheragnostic technologies can lead to more accurate disease detection and better therapeutic efficacy, probably enabling the first steps towards the development of 'personalized' medicines.
- Theragnostic nanoparticles are made of a signal emitter, a therapeutic molecule and a biocompatible material.
- Advantageously, such nanomedicines can consist of a multimodality imaging technique combined to a multidrug delivery system plus other treatment mechanisms (i.e., photodynamic therapy, photothermal therapy, hyperthermia).
- The introduction of passive and active delivery strategies in the formulation of theragnostic nanoparticles will allow controlling their biological fate.
- The introduction of theragnostic nanotools into the clinic will strongly depend on both the complete integration in preclinical research of characterizing structure–toxicity relationships and the minimization of safety problems.

This box summarizes key points contained in the article.

Thanks to nanotechnology approaches, the delivery of therapeutic and diagnostic molecules to non-healthy organs, tissues and/or cells has become more selective and specific, permitting the emergence of novel treatments with enhanced efficacy. In fact, nanotechnology has been described to open very significant possibilities in biomedicine, that is, pharmacotherapy (drug–gene delivery), diagnosis, tissue engineering, immunoassay, hyperthermia, cell separation or detoxification of biological fluids to cite just a few [2-6]. For instance, properly synthesized nanoplatforms can be exploited to administer active agents in a controlled manner, maximizing their accumulation into targeted cells and, more interestingly, overcoming cell resistance problems [7-10]. Regarding tissue and molecular imaging, biodegradable nanoparticles have been proposed to deliver imaging probes to non-healthy sites [3,6]. This is an interesting approach to the frequently encountered problems of little sensitivity of current imaging techniques, and difficult early disease diagnosis. It is expected that the signal emitter loaded to the nanoplatform will exhibit an extended $t_{1/2}$ for selective contrast enhanced imaging [3,11,12]. For instance, recent preclinical investigations have established that the efficient delivery of MRI contrast agents (e.g., gadolinium, europium, superparamagnetic iron oxides (SPION)) can result in optimum signal detection [13-16].

The number of published investigations on the use of biocompatible nanoplatforms in drug (gene) delivery and biomedical imaging has grown exponentially during the last decade. However, lot of research is still needed before their complete introduction into the clinic could be considered possible. For instance, the problem of the toxicity of the nanoparticulate system is not entirely defined: until now, very little has been done to elucidate the toxic response, and predictive models are required for toxicity evaluations [17,18]. The number of marketed nanomedicines is also very limited by important limitations that could be associated with their engineering (**Table 1**) [2,18]. Thus, the finest therapeutic and diagnostic outcomes can be only possible if new ideas arise to revolutionize the current way of delivering therapeutic molecules and contrast agents.

The development of nanotechnologies for combined therapeutic and imaging capabilities ('nanotheragnostics') is expected to overcome the problems associated with current pharmacotherapy and disease diagnosis. Such improved nanomedicines could assure monitoring of disease location, image-guided drug delivery, non-healthy tissue targeting levels and controllable drug release kinetics. The present work is focused on the analysis of the possibilities, applications and engineering aspects of theragnostic nanomedicines.

2. Theragnosis: the move towards simultaneous diagnosis and therapy of diseases

The term theragnosis was introduced a decade ago in the biomedical arena. This concept initially described diagnostic tests intended to detect disease biomarkers and signals to be used to personalize pharmacotherapy. Recently, the word theragnosis has been applied to conceptualize the use of multifunctional nanomaterials for combined diagnosis and therapy of severe diseases. In fact, it is accepted that a theragnostic nanomedicine is an integrated nanoparticulate system which can be simultaneously used to diagnose diseases, deliver targeted therapy and monitor response to therapy [15,19-24]. The last potential use of theragnostic nanotools is expected to improve conventional pharmacotherapy in individual patients: the real-time monitoring of the *in vivo* fate of the nanoplatform would provide enough information for its appropriate engineering. As a result, personalized nanomedicine will make possible the best therapeutic effect.

At the moment, it is very difficult to completely understand the possibilities arising from the development of theragnostic nanomedicines (**Table 2** tries to define its potential relevance to the clinic). Theragnosis not only will be of great help in the development of novel drug molecules, but also in the clarification of the effectiveness of a pharmacotherapy approach in a particular patient. With the help of the theragnostic nanoparticle, the clinician would detect the localization of the disease, use specific stimulus (i.e., magnetic gradients, ultrasounds, light) to control the release of the pharmacotherapy agent from the nanoplatform (thus, activating targeted pharmacotherapy) and monitor the response of the patient. The last aspect will permit the clinician to discard patients for whom the selected therapy is not appropriate, decide the re-initiation of the treatment or, alternatively,

Table 1. Major limitations to the introduction in the clinic of nanoplatforms loaded with drug–gene and imaging molecules.

Practical limitation	Clinical consequence
Reduced vehiculization of the signal emitter or active agent	Subtherapeutic concentrations into the disease site, although an effective delivery occurs Toxicity associated with the high dose of nanomaterial needed to obtain a therapeutic effect, or disease signaling
Burst release in plasma of the loaded active molecule	Poor activity and severe systemic toxicity
Drug delivery cannot be monitored	Unfeasible evaluation of drug (gene) targeting efficiency, intra-site localization and release kinetics
Complex definition of the optimum formulation conditions	Synthesis procedure cannot be scaled up in the pharmaceutical industry

Table 2. Biomedical significance of theragnostic nanomedicines.

Biomedical advance	Clinical application
Non-invasive and real-time visualization of the biological fate of the nanoplatform	Prediction/analysis of the pharmacokinetics, biodistribution and *in vivo* efficacy of the nanomedicine. This information will facilitate an improved (personalized) formulation of the multifunctional nanocarrier for more effective and less toxic pharmacotherapy regimens
Monitor, quantify and facilitate triggered drug release	Drug release with controlled temporal and spatial specificity (exclusively into the non-healthy site)
Forecast drug responses	Detection of disease biomarkers and signals for the choice of therapy Identification of the patients that will respond to the nanomedicine Optimization and individualization of treatment protocols
Longitudinal evaluation of the pharmacotherapy effect	Real-time investigation of disease progression and treatment outcome Characterization of the evolution of the targeted non-healthy site in response to therapy. This is particularly interesting in cancer treatment when tumor cells develop multi-drug resistances

define a follow-up program when sufficient regression or cure takes place [25,26].

Nanotheragnosis has entered the preclinical stage only 2 years ago, and very interesting *in vitro* and *in vivo* research reports have highlighted its potential clinical use. Maybe we could think that the introduction of diagnosis and therapeutic functionalities into the same nanoplatform will definitively bridge the gap between disease diagnosis and therapy. Under these bases, it could be hypothesized as a revolution in disease managing, but unfortunately precautions should be taken until important questions related to the *in vivo* behavior and toxicity associated with such nanoparticles are completely defined.

Ideally, theragnostic nanoparticles would be based on a multimodality imaging technique combined to a multidrug delivery system, plus complementary treatment mechanisms (e.g., hyperthermia, photothermal therapy, photodynamic therapy) [15,23]. However, an analysis of the design of these nanotools easily emphasizes that multimodality imaging could be not so significant for disease diagnosis, if the selective delivery of an imaging probe assures the enhancement of the diagnostic imaging technique. What definitively will be more important is to combine such molecules to a multidrug delivery system implemented with other treatment mechanisms (as mentioned above). Under these conditions, non-healthy cells will be treated by complementary/simultaneous strategies, along with a real-time monitoring of the response to therapy. Another aspect that should be always considered when theragnostic nanoparticles are engineered is that not all the drugs need theragnosis. Theragnosis found an important use in situations when the drug molecule must target specific cells, tissues and/or organs. Only in such a case it could be considered that the multifunctional nanoplatform would be of great help to satisfactorily monitor drug delivery and precisely control drug release into the site of action.

Perhaps oncology is the most promising area of knowledge in which theragnosis is expected to transform disease diagnosis and pharmacotherapy. Numerous *in vitro* and *in vivo* investigations have pointed out this possibility. To cite just a representative example, a recent investigation has demonstrated that gold-based theragnostic nanoparticles could be of great help in metastatic cancer cell imaging and apoptosis. In this *in vitro* study, gold nanoparticles were surface decorated with

fluorescent dye labeled heparin molecules to detect metastatic tumor cells overexpressing heparin-degrading enzymes (e.g., heparanase) [27]. Compared to conventional cancer cells, the release of dye labeled heparin molecules from gold nanoparticles specifically took place under the heparin cleavage enzymatic conditions typical of tumor metastasis. Subsequently, the heparin fragments enabled emission of clear fluorescence signals, given the fact that the conjugated fluorescent dyes in the released heparin chain are not quenched by gold nanoparticles. Further, surface functionalization of the nanoparticles with cell adhesive arginine-glycine-aspartic acid (RGD) peptides assured the greater intracellular uptake of the theragnostic nanoagent and, as a consequence, highly specific apoptotic activities for cancer cells overexpressing RGD receptors (**Figure 1**). This investigation concluded that the antitumor effect of the nanomedicine was due to an apoptotic event triggered by the internalized heparin macromolecules.

Additional reasons that justify the significance of theragnosis in cancer are that such multifunctional nanoplatforms may facilitate the identification of primary tumors, the localization of metastatic malignancies, and the evaluation/follow-up of cancer progression and the effects of surgery, radio(nuclide) therapy and/or chemotherapy [22]. Preliminary results have emphasized the capability of theragnostic nanoparticles for the detection of a malignant cell by optical scattering, plus immediate induction of selective cell ablation (confirmed by this scattering technique) [28]. In this respect, it will be very important to define the real *in vivo* applicability of certain experimental methodologies that have been proposed for theragnostic purposes. For example, UV absorbance and scattering intensity are not easily measured *in vivo*.

Theragnostic nanotechnologies could further improve alternative treatment approaches against cancer, that is, photodynamic therapy, photothermal therapy and hyperthermia. These multifunctional nanomaterials have been investigated for image-guided photodynamic therapy of tumor cells. Theragnostic nanoparticles could facilitate the precise localization of the malignant tissue by diagnostic imaging (photosensitizer molecules are inherently fluorescent), providing accurate guidance for light irradiation of the targeted site to activate the photosensitizer [3,25]. For instance, a recently published investigation has postulated the potential use of MRI-guided photodynamic therapy against cancer. In this study [29], PEGylated poly-(L-glutamic acid) nanoparticles were loaded with the photosensitizer mesochlorin e6 and the MRI contrast agent gadolinium^{3+}-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid. Image-guided photodynamic therapy was possible thanks to the selective tumor uptake of the nanoplatform. The extended $t_{1/2}$ of the PEGylated nanoparticles led to a negligible liver accumulation, and tumor uptake occurred by the enhanced permeation and retention (EPR) effect characteristic of malignant tissues (see Section 4.1 for further details). As a consequence, a significant tumor contrast enhancement was identified, leading to an efficient image-guided therapy and optimized anticancer efficacy.

Photothermal therapy of cancer could also take advantage of theragnostic nanotechnologies. Several research reports have demonstrated that image-guided irradiation of tumor tissues with electromagnetic radiation can very efficiently induce thermal damage to cancer cells [25,30,31]. Theragnosis could permit cancer imaging and localization (i.e., by MRI or X-ray CT), thus making feasible the precise localization of malignant cells, and providing accurate guidance for tumor radiation with visible-near infrared light to significantly enhance local tumor temperature (**Figure 2**). The multifunctional nanoparticles would further allow immediate evaluation of spatial and temporal changes in tissue morphology, tumor temperature during the therapeutic procedure and tumor response to therapy [30]. Recently, an *in vitro* investigation concluded that gold-based nanoparticles could be used for the selective and efficient photothermal therapy of human EGFR-2 (HER-2)-overexpressing and drug-resistant ovarian cancer OVCAR3 cells. These theragnostic nanoplatforms made possible the image-guided irradiation of the targeted tumor cells, thanks to their fluorescence optical and MRI properties [32]. Finally, it is of interest to highlight that photodynamic and photothermal therapies based on theragnosis could find interesting applications in the treatment of infectious diseases [25].

Very promising results have been further obtained when theragnosis is applied to cancer hyperthermia. As an example, bovine serum albumin-based magnetic nanoparticles were developed for combined MRI diagnosis and hyperthermia [33]. In this investigation, tissue heating (the consequence of hyperthermia) was described to be extremely cytotoxic to tumor cells, and it was additionally used to trigger drug release from these thermosensitive nanoplatforms. Interestingly, hyperthermia induced greater tissue blood flow and vessel permeability when the targeted site was heated to $\approx 42^{\circ}C$. As a consequence of the hyperthermic effect, an enhanced extravasation of the theragnostic nanoparticles into the tumor tissue was observed, with a simultaneous improvement of drug toxicity in multidrug resistant cancer cells [25].

3. Key aspects in the formulation of theragnostic nanoparticulate systems

Multifunctional nanoplatforms must be properly tailored for combined medical imaging and drug (gene) delivery. The biological fate of the theragnostic particle is strongly influenced by the physical chemistry of the nanomaterial. This fact could be advantageously used to target non-healthy cells, tissues and/or organs. Thus, the design of the nanoparticle involves a complex combination of physicochemical factors and formulation possibilities (**Table 3**). When these parameters are properly 'cooked', an adequate *in vitro* and *in vivo* behavior of the theragnostic nanotool could be expected [4,13,15,16].

Taking into account the literature on the development of theragnostic nanoparticles, we can establish that the basic design of such nanomedicines involves the use of a

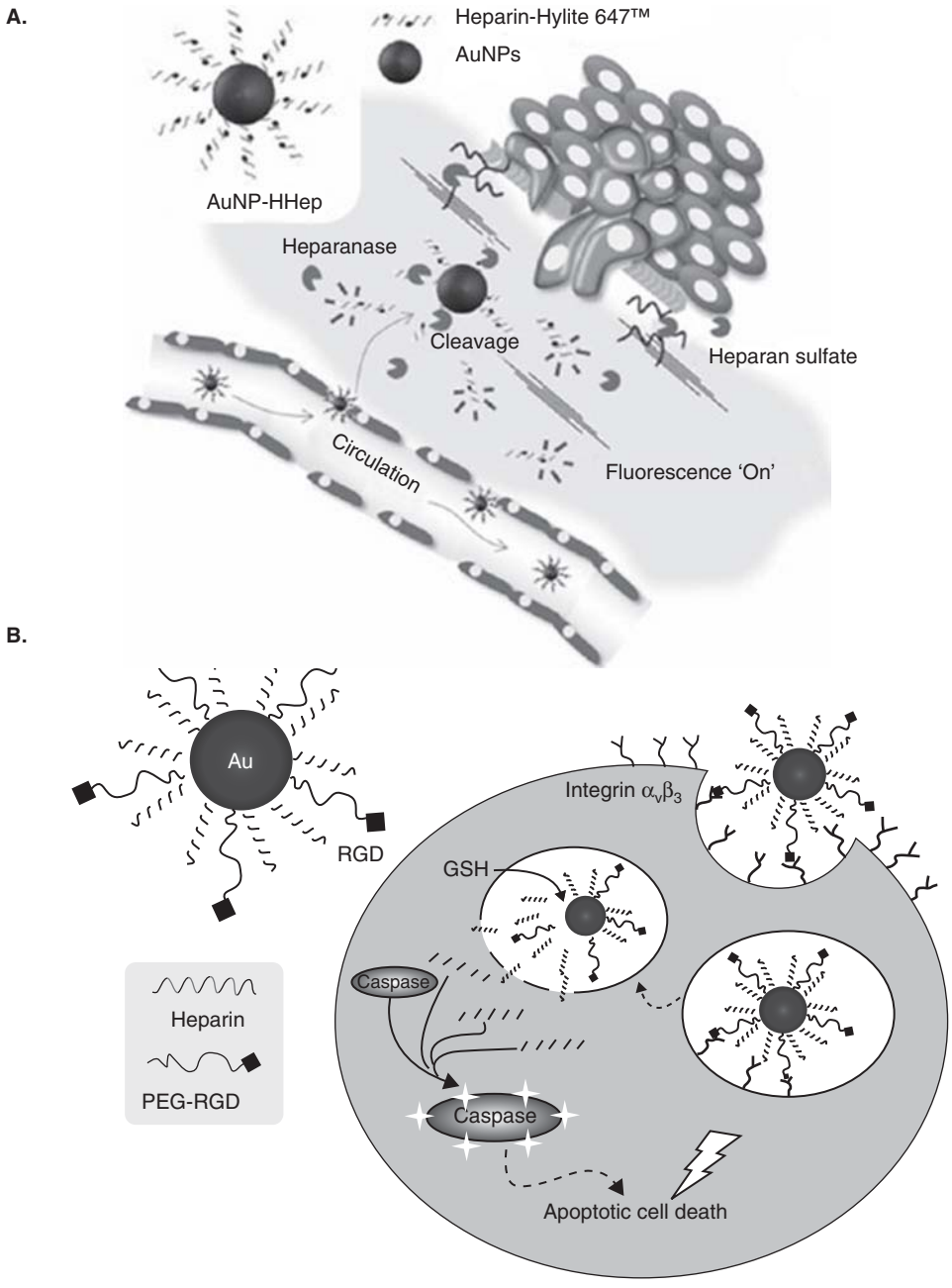

Figure 1. Metastatic cancer cell imaging and apoptosis by AuNP-HHep. A. The nanoplatforms can produce fluorescence signals, thanks to the NIRF dye HiLyte-Fluor™ 647-amine, under the heparin cleavage enzymatic conditions typical of tumor metastasis. **B**. Additional conjugation of RGD peptides, by using PEG chains as spacers, onto the nanoparticle surface (AuNP-HHep–PEGRGD) facilitated the apoptotic effect in $\alpha_v\beta_3$-integrin overexpressing tumor cells.

Adapted with permission from Elsevier [27].

AuNP-HHep: Heparin-immobilized gold nanoparticle; NIRF: Near-infrared fluorescence; RGD: Arginine-glycine-aspartic acid.

stimuli-sensitive biodegradable nanomaterial surface functionalized with hydrophilic polymer chains and ligand moieties (**Figure 3**). The latter functionalization could be directly conjugated to the particle surface and/or covalently bonded to the hydrophilic polymer chains. Thanks to this structure, the nanoparticle will both passively and actively target non-healthy sites (see Section 4 for further details). Finally, to that nanoplatform, at least an imaging agent (e.g., MRI probe, luminophore and/or radionuclide) and a drug molecule (and/or gene) must be loaded. These molecules can be either incorporated onto the nanoparticle surface and/or embedded into the particle matrix. Additional engineering

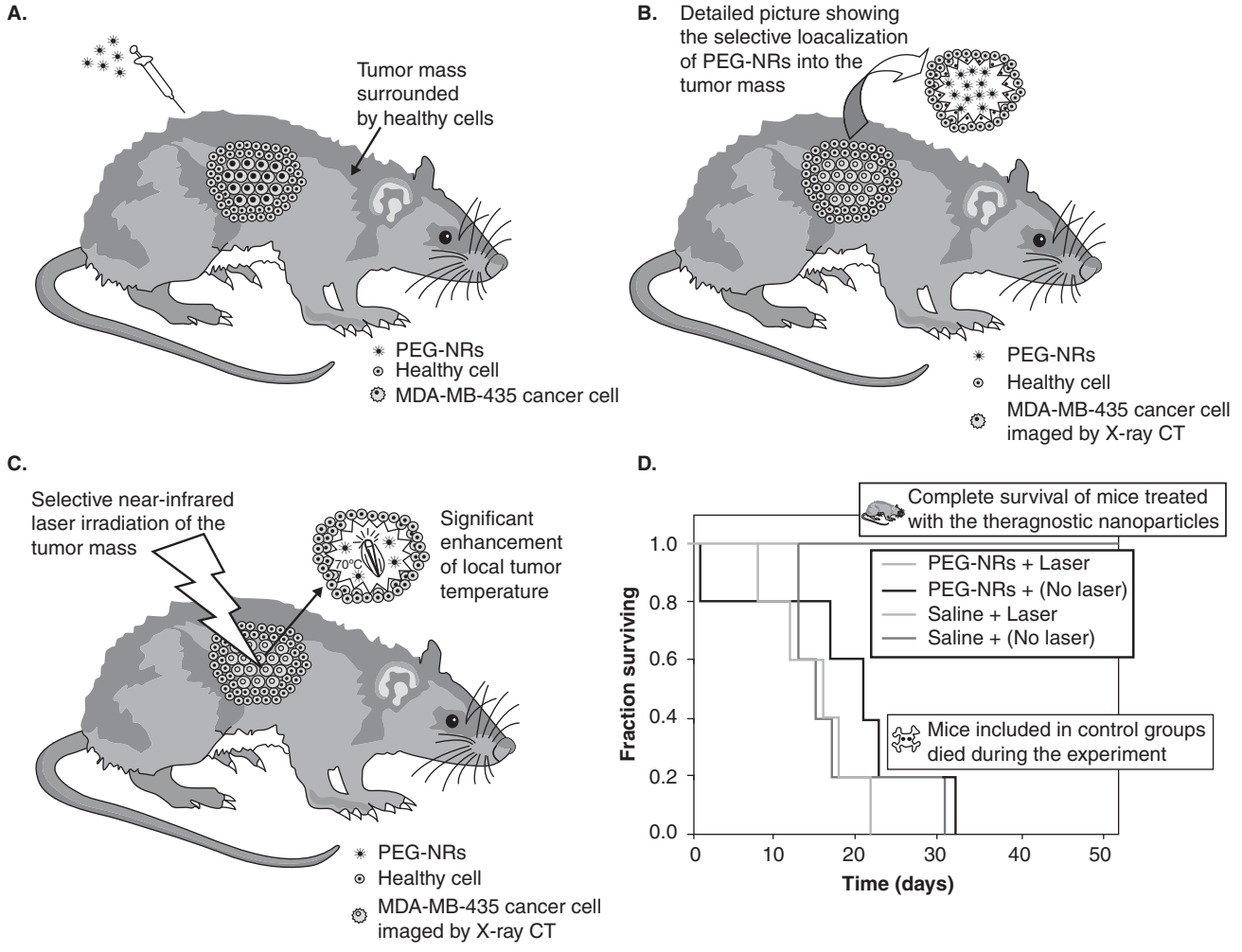

Figure 2. Exemplification of image-guided photothermal therapy against cancer. The *in vivo* study analyzed the possibility of implementing combined X-ray CT imaging and photothermal therapy by the administration of PEG-NRs (mean size ≈ 13 × 47 nm; dose: 20 mg/kg) to mice bearing subcutaneous MDA-MB-435 tumor (breast adenocarcinoma cell line) [30]. **A**. Intravenous administration of PEG-NRs. **B**. The tumor mass was precisely localized by using the long-circulating nanoplatform ($t_{1/2}$ ≈ 17 h) as an X-ray CT contrast agent, thanks to a selective accumulation by the EPR effect into the malignant tissue. **C**. These nanoparticles were simultaneously used as efficient photothermal agents to cause thermal damage when exposed to near-infrared laser irradiation (2 W/cm^2 at 810 nm wavelength). Accurate image-guided irradiation of the tumor mass avoided significant damage to the surrounding healthy tissue. **D**. As a result of this targeted therapy, greater survival of mice treated with the theragnostic nanoparticles was described in comparison to controls.

Adapted with permission from Elsevier [25].

EPR: Enhanced permeability and retention; PEG-NR: PEGylated gold nanorod.

elements for complementary therapy (e.g., hyperthermia, photodynamic therapy and/or photothermal therapy) can be further introduced in the nanoformulation.

3.1 The imaging agent

Since the beginning of the investigations in the area of drug delivery to the disease site, the problem of monitoring the biodistribution of the colloid on administration was described. In fact, the clear need for the real-time analysis of the efficiency of the drug targeting strategy by a non-invasive procedure has classically limited the optimization of drug transport to targeted cells, tissues and/or organs [2]. Fortunately, numerous investigations subsequently described the enhanced imaging sensitivity provided by nanoparticles loaded with contrast agents [11,34-37]. Thereafter, the logic conceptualization of drug-loaded nanoparticulate systems functionalized with imaging molecules naturally occurred [2,4]. This approach has enabled remarkable possibilities for new improvements in nanoparticle engineering based on data coming from image-assisted biodistribution characterizations.

Numerous non-invasive imaging techniques have been investigated for real-time visualization of the *in vivo* fate of

Table 3. Important requisites to be fulfilled by a theragnostic nanoplatform for an efficient combined disease diagnosis and therapy.

Formulation requisite	Influence on the biological fate
Small size (below 100 nm)	Extended biodistribution: the nanoparticle will easily reach very small capillaries Uniform perfusion into the targeted tissue through very small gaps between endothelial cells of the blood vessels
Negligible surface electrical charge and hydrophilicity	Delayed plasma clearance resulting from particle opsonization and recognition by macrophages
In vitro and *in vivo* physical stability	Absence of particle aggregation/precipitation leading to embolization problems and inappropriate biodistribution profiles
Ability to transport the proper dose of drug–gene and imaging agent into the nanoparticle matrix	The body will not be overloaded with foreign material These active molecules will not be exposed to enzymatic degradation
Controlled (or prolonged) release of the drug–gene and imaging molecules	Negligible burst release of these molecules immediately on administration Sustained therapeutic (and diagnostic) effect: the consequence of an extended contact time between the drug–gene (and signal emitter) and the targeted cell
Maximum biocompatibility and biodegradability, and minimal antigenicity	Null toxicity
Proper functionalization of the nanostructure, taking advantage on both passive and active targeting strategies	Targetable pharmacokinetic and biodistribution profile Enhanced residence time of the drug–gene and signal emitter in plasma, and improved intracellular loading Minimization of the toxicity associated with a wide biodistribution of such molecules

theragnostic nanoparticulate systems and image-guided drug delivery, for instance, MRI, ultrasound, optical imaging (bioluminescence and near-infrared fluorescence; NIRF), radionuclide-based imaging (positron emission tomography (PET) and single photon emission CT), fluorescence-mediated tomography and photoacoustic tomography. However, significant limitations were associated with these individual imaging modalities, for example, limited sensitivity, resolution and anatomical information.

As an alternative, multimodality imaging techniques (i.e., MRI-optical, PET-CT and MRI-PET) have been satisfactorily introduced in theragnostic arena [15,16]. The development of PET/NIRF/MRI triple functional oleate-coated SPION could be considered as a representative example of multiple disease imaging by nanoparticulate platforms [38]. In detail, the multifunctional nanoparticles consist of magnetite-based nanoplatforms encapsulated into a human serum albumin matrix and labeled with ^{64}Cu-1,4,7,10-tetraazadodecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid and the NIRF dye cyanine 5.5 (Cy5.5). *In vivo* efficacy of the triple functional imaging nanoplatform was investigated in a subcutaneous U87MG xenograft mouse model. The composite nanoparticles were intravenously injected (dose: 10 mg Fe/kg) and PET/NIRF images were acquired at prefixed times (1, 4 and 18 h post-injection). Regarding the NIRF results, a clear tumor delineation was observed at the 1 h time point, the contrast improved over time and the tumor:muscle ratio increased from ≈ 2 at 1 h to ≈ 3.1 at 18 h. A similar tumor homing trend was observed by PET: the results proved a gradually elevated tumor uptake. Compared with NIRF results, PET imaging showed much higher tumor:muscle ratios (e.g., ≈ 8.3 at 18 h time point), thanks to the cleaner background of PET. Finally, MRI scans performed pre-injection and 18 h post-injection found a signal drop of ≈ 30 % at the tumor sites. Interestingly, the authors concluded that the integration of the three imaging modalities could lead to synergistic benefits. Concretely, MRI could offer a high spatial resolution and a better description of the particle distribution pattern than either PET or NIRF. On the contrary, to compensate the limited sensitivity of MRI, PET and NIRF functionalities were introduced into the nanoparticle structure. It was demonstrated that PET provided a better signal:noise ratio, while NIRF permitted the visualization of the nanoformulation both *in vivo* and *ex vivo*, thus bridging the *in vivo* and histological observations. All the information obtained from the imaging and histological examinations demonstrated the high extravasation and retention of the nanomedicine into the tumor tissue.

Last but not least, the characteristics of the imaging agent must be also taken into consideration to assure the best image signal. Independent of the imaging technique, any given signal emitter or imaging agent must present unique optical, magnetic or radioactive properties. These characteristics will determine the physical and/or chemical modifications that the molecule undergoes on exposure to the external stimulus that is directed to the disease site. The most widely utilized strategies to induce changes in the emitted signals are relaxivity changes (magnetic switch), quenching (pairing–unpairing of deactivator and fluorophores), chemical exchange saturation transfer (CEST) and Förster resonance energy transfer [15]. Then, these variations in the physical chemistry of the signal emitter will define the changes in the amplitude

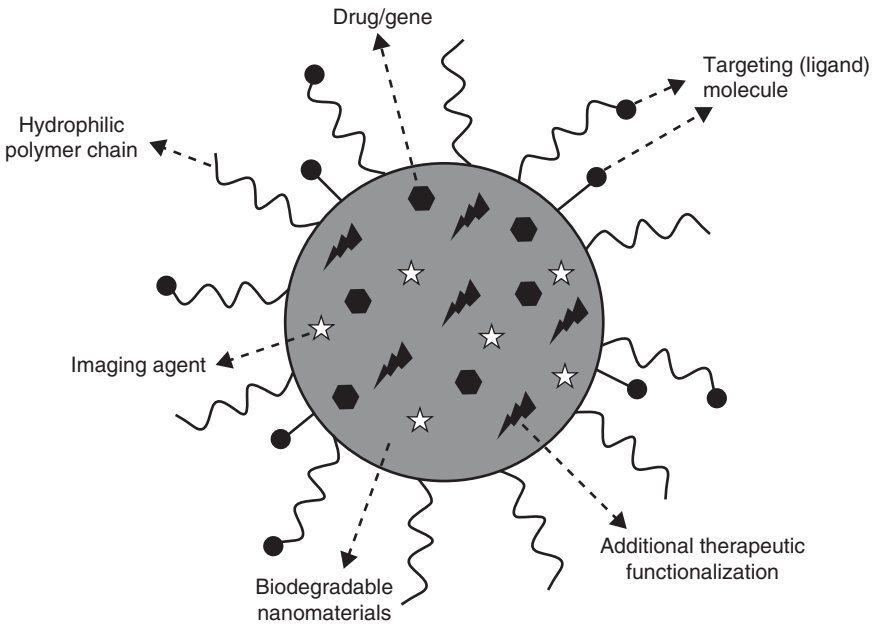

Figure 3. Basic structure of a theragnostic nanoparticulate system. Three basic elements must be included in the formulation of such a multifunctional platform: the imaging agent or signal emitter, the therapeutic molecule and the biodegradable nanocarrier. The last component should be properly engineered to assure both an efficient delivery of the active molecules to the targeted site and a null toxicity.

or composition of the emitted signal that will be subsequently detected by an external receiver and reconstructed to form images. Thus, it is easy to understand that the own issues of each imaging molecule (sensitivity, depth penetration, resolution, etc.) must be carefully considered when engineering the theragnostic nanoplatform. If possible, the theragnostic nanotool should be loaded with more than one signal emitter to obtain synergistic benefits that can assure the effective *in vivo* detection of the nanomedicine. We can cite as interesting examples the previously described PET/NIRF/MRI triple functional imaging nanoplatform [38], and the nanoformulation based on the conjugation of SPION with a fluorescent dye for combined MRI and optical imaging [39,40].

3.2 The therapeutic molecule

The best pharmacotherapy response is exclusively obtained if it is guaranteed the complete accumulation of the dose of active agent (a drug or a gene) into the site of action. Unfortunately, on conventional administration of a drug–gene, an extensive biodistribution is described which habitually leads to subtherapeutic concentrations into the targeted non-healthy cell, tissue and/or organ, and systemic toxicity. Drug–gene incorporation to nanocarriers is intended to focus all the molecules of active agent into the desired site [10,18]. As previously indicated, the analysis of the *in vivo* fate of the nanomedicine could be of great help in the formulation of the nanocarriers that very efficiently improved the concentration of the therapeutic agent into the site of action.

Drug molecules included into theragnostic nanosystems principally belong to pharmacotherapeutic groups for the treatment of cardiovascular diseases (e.g., atherosclerosis and thrombosis) and cancer. As well, theragnostic nanoplatforms can be designed with drugs used in the treatment of arthritic diseases (i.e., rheumatoid arthritis), neurodegenerative diseases (e.g., Parkinson's disease), age-related macular degeneration and psoriasis, to cite just a few [13,25,41-47]. Image-guided drug delivery is possible by the introduction of signal emitters into the theragnostic agent structure. For example, a recent research report has described the potential application of such multifunctional nanoplatforms in the diagnosis of atherosclerotic lesions with combined drug delivery to this disease site [47]. In detail, this investigation described the preparation of perfluorocarbon nanoparticles loaded with paramagnetic gadolinium (an MRI contrast agent) and the antiproliferative agent rapamycin. The non-invasive assessment of the nanoplatform and the inhibition of stenosis were possible when the nanoparticles were further functionalized with the peptidomimetic vitronectin antagonist to target $\alpha_v\beta_3$-integrin (an upregulated cell surface receptor in these lesions). Another study reported the successful use of self-assembled manganese^{2+}-labeled nanoparticles for combined pharmacotherapy and MRI of fibrin in thrombus [44]. Cancer treatment can also take advantage of image-guided drug delivery. For instance, MRI-guided chemotherapy has been shown to be possible by the formulation of polymeric micelles loaded with gadolinium and platinum antitumor drugs [48].

As an alternative to the vehiculization of drug molecules into theragnostic nanoparticles, gene delivery (transfection) can also take advantage of theragnostic nanotools [3,4]. The use of molecular imaging strategies to monitor the delivery of genes and the therapeutic response at the targeted site are expected to immensely contribute to the optimization of gene delivery [13]. An interesting example on this matter has been recently published [14]. In this work, positively-charged polymeric nanoplatforms were functionalized with compact plasmid DNA and lanthanide chelates (paramagnetic gadolinium or luminescent europium). It was determined that these macromolecules were efficiently protected in the nanoparticle matrix against degradation processes. As a result, MRI and fluorescence techniques satisfactorily monitored the transfection efficiency on the tissue scale [14].

When the theragnostic nanoparticle is prepared, the loading of the therapeutic molecule can be made possible by covalent links with chemical groups of the nanomaterial. Alternatively, the incorporation of nucleic acids and hydrophobic drugs to such nanoparticles can be also done by non-covalent linkages involving ionic and hydrophobic interactions, respectively [15]. However, the best loading results and the best controlled release properties are mostly obtained when the active agents are incorporated into the nanoparticle matrix [2,5]. Ideally, the multifunctional nanoplatforms should include more than one pharmacotherapy agent to induce complementary therapeutic activities. As an illustrative example, theragnostic nanotools based on *N*-(2-hydroxypropyl) methacrylamide copolymers loaded with both doxorubicin (DOX) and gemcitabine were tested in a radio- and chemoresistant Dunning AT1 rat prostate carcinoma model. The investigation proved the potential of the nanomedicine to interact synergistically with radiotherapy [1]. Finally, an additional important aspect in the development of theragnostic nanoparticles is the need for an adequately controlled release of the therapeutic agent from the nanoplatform. In this way, external (alternating magnetic gradients, light excitation) or environmental (enzymes, pH, temperature degradation) stimulus could be used to specifically trigger drug (or, even, gene) release from the theragnostic nanostructure into the targeted non-healthy tissue (see Section 4.2. for detailed comments).

3.3 The nanoplatform

Since the beginning of last century when Dr Paul Ehrlich introduced the concept of magic bullet in pharmacotherapy, the number of investigations devoted to the development of colloids for drug delivery to disease sites has grown exponentially [2,5]. Thereafter, the use of nanoparticulate materials for biomedical applications permitted significant advances in diagnosis, tissue engineering and drug delivery, to cite the most important examples [49]. In the last case, the very promising preclinical results resulted in the introduction of these nanoformulations into the clinic. We can easily think of some examples of marketed nanomedicines: Lipoplatin®, Depocyte®, Myocet®, DaunoXome® and Doxil®. In fact, nanoparticulate systems have improved the current way of administering drugs and biomacromolecules to patients, that is, through the percutaneous route [50].

Not considering subsequent engineering and functionalization approaches to the nanoplatform (to be described in Section 4), the nature and physical chemistry of the nanomaterial clearly influence the *in vitro* and *in vivo* behavior of the theragnostic particle [5,6]. Thus, a successful theragnostic effect relies on the structure and formulation of the multifunctional nanoplatform. In this way, significant characteristics of the nanomaterial that facilitate an optimum theragnostic activity (with minimized toxicity) are the geometry (very small size, $\leq$ 100 nm and spherical shape), null or almost negligible surface electrical charge, hydrophilic character, biocompatibility and controlled biodegradability. A proper nanostructure could even be of great help in the delivery and follow-up of drug molecules and contrast agents through the BBB [2,5]. To this concrete aim, the nanoplatform should be designed with a very small size, a slightly positive electrical charge and, if possible, surface functionalized with adequate ligands that could facilitate nanoparticle endocytosis by ligand–receptor interactions [2,51].

With respect to the chemical composition, the nanoparticulate material can be based on inorganic material or organic matrix [13,52,53]. Inorganic theragnostic nanotools are commonly based on quantum dots (semiconductor nanocrystals composed of cadmium selenide which exhibit significant fluorescence imaging properties) [54], metals (e.g., silver, gold, dielectric silica cores coated by an ultrathin gold layer) [55] and SPION [56]. By themselves, they have been classically used as signal emitters/contrast agents in MRI [57], optical coherence tomography [58], confocal imaging [59], photoacoustic tomography [60] and/or near-infrared tomography [61].

Nanotheragnostic agents can be also formulated by using organic materials, mainly biodegradable polymers (e.g., poli (D,L-lactide), poly(*N*-isopropylacrylamide), poly(*N*-(2-hydroxypropyl) methacrylamide) and copolymers, to cite just a few) [62-64], and lipid-based nanoparticles (liposomes and niosomes) [45,65]. Finally, organic–inorganic nanohybrids, that is, based on an inorganic core embedded into an organic matrix (such as, SPION/biodegradable polymer (core/shell) nanostructures) are of greater interest due to their multimodality imaging possibilities, their capability for multidrug delivery, plus additional treatment options (i.e., hyperthermia, photodynamic therapy, photothermal therapy) [13,51,52,66]. In the ideal case, more than one signal emitter, photosensitizer agent and drug molecule (or gene) could be incorporated into the organic matrix, while the inorganic core could also play a key role as contrast agent and/or as a significant functionalization structure for active targeting to non-healthy sites; for example, SPION have been demonstrated to be efficient contrast agents for MRI, drug nanocarriers and hyperthermic particles [67].

Taking into account all the information given in this section, it is easily ascertained that currently used nanomaterials for theragnosis could be non-biodegradable, for example, semiconductors containing selenium and cadmium, but more

advantageously biodegradable. Independent of their chemistry, the use of biodegradable nanomaterials will facilitate the evolution of the concept of nanotheragnosis towards the clinical stage. In fact, biodegradability is a key property to assure both targetable drug–gene delivery properties (when it can be easily controlled) and a negligible toxicity [3,4]. In the latter case, if nanoparticles undergo rapid metabolism and clearance when their *in vivo* role is ended, the organism would never be overloaded with foreign material. This is particularly interesting when the theragnostic treatment must be administered to the patient during long periods of time and overloading of the body with foreign material would result in toxicity.

4. Engineering strategies for a targetable biological fate

Conventional formulation of multifunctional nanoparticulate platforms does not completely assure its proper concentration into the site of action. Despite *in vitro* investigations, hypothesizing an adequate theragnostic activity, preclinical evaluations of these nanostructures have pointed out an insufficient effect. It has been described that this could be the consequence of the interaction of the nanoparticles with the reticuloendothelial system (RES), enzymatic systems, vasculature walls and so on [2,4-8]. Fortunately, the latest advances in nanoparticle engineering would allow the control of the *in vivo* behavior of theragnostic nanoparticles. The introduction into their formulation procedure of passive and active targeting strategies could permit the control (and optimization) of the biological fate (and efficacy) of such multifunctional nanodevices [9,18].

The former strategy (passive targeting) is based on the EPR effect frequently exhibited by disease sites (i.e., tumor interstitium and inflammatory tissues), and involves the design of long-circulating nanotheragnostic agents [68]. On the contrary, active targeting strategies are related to the surface functionalization of the theragnostic nanotool with biomacromolecules for ligand- or receptor-mediated delivery [69] and/or to the synthesis of the nanotheragnostic agent with stimuli-sensitive materials [70]. Nevertheless, both engineering approaches are needed to be included in the formulation of these multifunctional nanoplatforms to guarantee the combined therapeutic and diagnostic activities [15,16,23,71]. In fact, it is hypothesized that theragnostic nanoparticles properly synthesized for simultaneous passive and active targeting to the disease site would be able to evade the RES, reaching the targeted non-healthy cells, tissues and/or organs. Under these advantageous conditions, the dose of drug molecule (or gene) and imaging agent will be very efficiently concentrated into the site of action [4,6,8].

4.1 Passive targeting

On administration to a patient, any given nanomaterial will undergo an intense interaction with the RES that habitually leads to rapid plasma clearance ($t_{1/2}$ < 5 min). Before elimination, the nanoparticulate system can be localized in Küpffer cells and related organs of the RES (liver, spleen, bone marrow, lungs, etc.). Thus, if these sites are the main target for the combined diagnostic and therapeutic effect, this typical body clearance could be an interesting advantage: the theragnostic nanoparticle will naturally accumulate into such organs to display the targeted simultaneous activities. On the contrary, this biological fate will be a dramatic disadvantage when other organs or tissues are the target: the nanotheragnostic agent will never be able to accumulate into the site of action due to its intense and very rapid clearance [18,72].

As an alternative to the problem, passive targeting of theragnostic nanoparticles will be facilitated by the enhanced capillary permeability of non-healthy targeted tissues (mainly tumors, inflammatory tissues (e.g., arthritic joints), and infectious sites) [4,72]. However, the multifunctional nanoparticle must exhibit an extended biodistribution and prolonged $t_{1/2}$ to take advantage of the characteristic physiology of such disease sites. The reduction in plasma clearance has been described to be possible if the engineering parameters introduced in the formulation of theragnostic nanomedicines assure the control of their intrinsic properties. These are: very small size (< 100 nm), spherical shape, hydrophilicity, null or almost negligible surface electrical charge, and, very importantly, introduction onto the nanoparticle surface of hydrophilic moieties (i.e., PEG, poloxamers, poloxamines or polysaccharides) by either chemical conjugation or physical adsorption. Even if particle uptake by the RES could take place when the nanotool is characterized by a mean diameter between 50 and 100 nm, it is the conjunction of all the previously mentioned characteristics which significantly limited the interaction with the RES [73]. It has been demonstrated that the shell of hydrophilic chains onto the particle surface will slow down the *in vivo* recognition (by opsonization) and plasma clearance of the nanoparticulate system [10,18,68,72]. Long-circulating multifunctional nanoplatforms will then be able to exploit the structural abnormalities of the vasculature of non-healthy tissues, consequently undergoing a specific extravasation and accumulation [10,71,72].

Recently published preclinical investigations can be commented to illustrate the advantages of passive targeting strategies. For instance, a recent study evaluated the biological fate of PEGylated quantum dots/liposome (core/shell) nanocomposites in B16F10 melanoma tumor bearing C57BL6 mice. It was determined that in contrast to non-sterically stabilized nanostructures, the incorporation of the hydrophilic PEG chains onto the particle surface very significantly prolonged their $t_{1/2}$ on systemic administration. This effect led to enhanced and prolonged tumor accumulation [66]. A very interesting example of the possibilities arising from the introduction of passive targeting strategies in the formulation of theragnostic nanotools for gene therapy involved the formulation of PEGylated liposomes loaded with anti-survivin siRNA and labeled with a fluorophore (Alexa Fluor 488) and an MRI agent (gadolinium^{3+} 2-(4,7-bis-carboxymethyl-10-((*N*,*N*-distearylamidomethyl-*N*α-amido-methyl)-1,4,7,10-tetraazacyclododec-1-yl)-acetic acid). In this research report [45], it was demonstrated that the nanomedicine very efficiently

delivered functional anti-survivin siRNA to OVCAR-3 tumor bearing mice, leading to a significant reduction in both survivin expression and tumor growth when compared to controls. To completely understand the significance of these results, it must be considered that the apoptotic gene survivin is upregulated in many cancers, but not expressed in healthy tissues. The theragnostic nanoplatforms were shown to accumulate into the tumors by MRI and fluorescence microscopy 24 h post-administration. Thus, the nanomedicine was able to elicit a very important anticancer activity, and allowed for real-time monitoring of the gene delivery by MRI in combination with fluorescence.

Finally, we can also comment on a promising investigation on the development of a long-circulating theragnostic nanoparticle against cancer. In this study [46], spherical theragnostic glycol chitosan-based nanoparticles (average diameter ≈ 250 nm) were formulated to accumulate into the tumor mass by taking advantage of the enhanced capillary permeability of this non-healthy tissue. The nanotool was loaded with a NIRF dye (Cy5.5) and the chemotherapy drug paclitaxel (PTX) for combined medical imaging and drug delivery to cancer. NIRF label allowed non-invasive monitoring of both the *in vivo* fate of the nanoparticles and their real-time therapeutic efficacy. *In vivo* efficacy studies were conducted in C57BL/6 male mice which subcutaneous dorsa were inoculated with murine squamous carcinoma cells (SCC7, 3×10^6 cells). Compared to controls (i.e., water-soluble polymer and polymeric beads), non-invasive optical fluorescence imaging demonstrated the rapid and excellent concentration of the theragnostic nanoplatforms into the tumor mass, with low nonspecific uptake by other tissues. NIRF signal started at 1 day post-injection and tumors maintained the maximal NIRF intensity for 3 days, but the signal persisted for up to 10 days post-injection, with a gradual decrease in the tumor. On the contrary, negligible NIRF signal was observed in normal tissues. Therefore, even if the nanoparticulate system exhibits an extensive biodistribution, its accumulation exclusively occurred into the tumor mass. As a consequence of this superior tumor specificity, the therapeutic PTX concentration into tumor tissues was dramatically greater than healthy tissues, thus leading to an optimized anticancer effect (**Figure 4**).

4.2 Active targeting

Targeted drug delivery based on long-circulating nanoparticles does not always result in an efficient uptake of the active agent by non-healthy cells. Despite an increase in drug (gene) concentration is assumed to occur in the tissue interstitium, the homogeneous distribution and effective internalization of these molecules by the whole population of targeted cells are not always possible. Additionally, drug release from long-circulating nanoplatforms cannot be at all times properly triggered to take place with the desired selectivity. Thus, under these conditions the inefficacy of pharmacotherapy is expected to occur. In order to solve the problem, active (or specific) targeting strategies were developed to focus the drug (gene) dose in the non-healthy site [2,18].

Theragnostic nanoparticles could take advantage of active drug targeting approaches to assure the efficacy of the combined diagnostic and therapeutic activities. This is expected to be principally the result of the: i) selective and homogeneous accumulation of the drug (or gene) and signal emitter molecules into the site of action and ii) controlled activation of the signal emitter and drug (gene) release. However, from a practical point of view, an optimized loading of the therapeutic molecules (and/or imaging agent) into non-healthy cells can exclusively be possible by the combination of this strategy to passive targeting [2,4,18].

The strategies included under the concept of specific targeting of the theragnostic activity are based on the selective concentration of the nanoparticulate system through a specific recognition mechanism (ligand- or receptor-mediated targeting) and/or on the use of stimuli-sensitive materials in the design of the nanoplatforms (**Figure 5**).

4.2.1 Theragnostic nanoparticles engineered for ligand-mediated targeting

This approach is based on the functionalization of the surface of a properly nanosized (< 100 nm) theragnostic agent with targeting molecules (ligands) that can specifically recognize and bind receptors that are unique to non-healthy cells. Ligand-mediated targeting is based on molecular recognition mechanisms habitually inducing receptor-mediated cell internalization of the theragnostic nanoplatform which will lead to the accumulation of the nanoparticle within the site of action [4]. Examples of targeting moieties that can be conjugated onto the nanotheragnostic platform are mAbs (e.g., the anti-HER-2 MAb trastuzumab), peptides, folic acid, transferrin and aptamers [15,74]. Cancer imaging and therapy is an exciting example of the possibilities of this delivery strategy. In this case, theragnostic nanoparticles are surface engineered to interact with tumor cells (and/or tumor endothelium) that express onto their surface particular biomacromolecules in a much greater proportion than healthy cells, for example, toll-like receptor 9 (on neuroblastoma cells), steroid receptors, EGFRs, integrins, folate receptors, somatostatin receptors, glucose transporters and so on [22,75-78].

An interesting exemplification of this strategy can be found in a recently published investigation that described the formulation of poly(*N*-isopropylacrylamide)-*co*-poly(*N*-acryloxysuccinimide)-*co*-poly(fluorescein *O*-methacrylate) nanocomposites surface decorated with H-glycine-arginine-glycine-aspartate-serine-NH_2 peptides as targeting moieties [63]. The nanoplatform was loaded with the imaging agent gadolinium and the antitumor drug methotrexate. MRI determinations clearly demonstrated the efficient delivery of the anticancer molecule to the tumor cells. Another interesting example described the synthesis of magnetic micelles containing SPION as MRI agents and the chemotherapy molecule DOX, and surface engineered

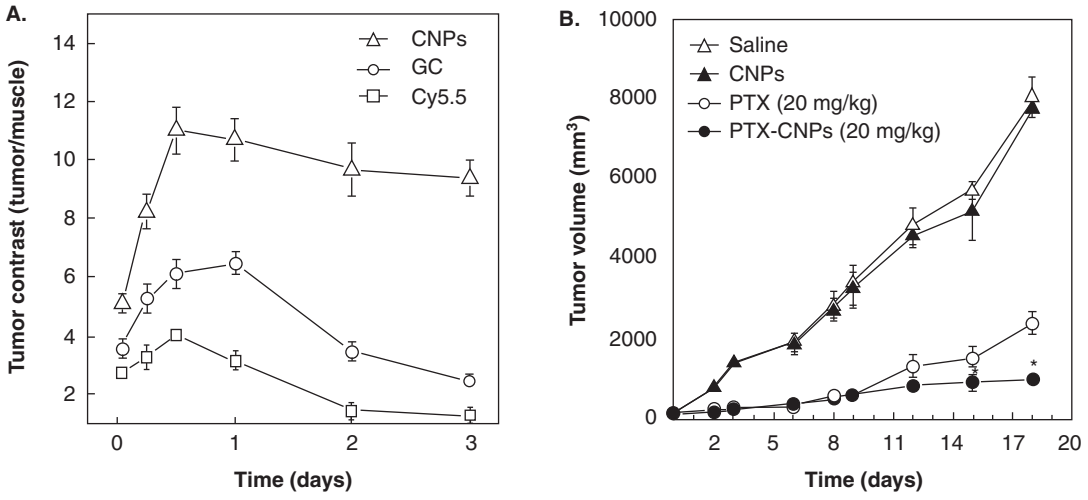

Figure 4. A. Evolution of tumor to background (muscle) contrast after administration of Cy5.5, Cy5.5-labeled water-soluble GC and Cy5.5-labeled CNPs in SCC7 tumor-bearing mice. **B**. Antitumor effect of PTX–CNPs in SCC7 tumor-bearing C57BL/6 mice (n = 10, initial tumor diameter ≈ 8 mm).

Adapted with permission from Elsevier [46].

*Statistically significant differences for the PTX–CNP treated group compared to controls (ANOVA, CI: 95 %). All data represent mean ± s.e.

CNP: Chitosan-based nanoparticle; Cy5.5: Cyanine 5.5; GC: Glycol chitosan; PTX: Paclitaxel.

with a lung cancer targeting peptide for increased $\alpha_v\beta_6$-dependent H2009 lung cancer cell targeting [79]. It was determined in this *in vitro* study that, compared to controls, cell uptake of such multifunctional magnetic micelles was significantly enhanced (threefold greater). T_2-weighted MRI showed clear contrast differences between H2009 cells incubated with the peptide-encoded theragnostic nanoparticles compared to non-functionalized nanoparticles. Finally, an ATP activity assay demonstrated the superior cytotoxicity of the peptide-encoded nanoparticles over controls (IC_{50} = 28.3 ± 6.4 and 73.6 ± 6.3 nM, respectively).

4.2.2 *Theragnostic nanoparticles engineered to be externally controlled*

As an interesting possibility for active targeting, externally controlled theragnostic nanomaterials are formulated to be disrupted and/or controlled under exposure to a specific external stimulus. The stimulus (which could be image-guided) is intended to determine (control) the activation of the theragnostic nanoparticle by inducing the release of the therapeutic agent and imaging probe into the non-healthy site (**Figure 5**). To understand the significance of this mechanism of action, it must be kept in mind that the therapeutic effect will only occur when the drug molecule (or gene) is out of the nanoparticle matrix [2,18]. Regarding the imaging agent, it could already be active when it is embedded into the particle matrix (i.e., theragnostic nanoplatforms containing SPION as contrast agents for MRI) or, on the contrary, once it is released from the nanoplatform. The applied stimulus could even determine the activation of the signal emitter, indirectly facilitating the quantification of drug delivery [62]. Additionally, the stimuli (light) that activates photodynamic and photothermal therapies could be easily guided to the non-healthy site by visualizing the imaging molecules included into the multifunctional nanoparticles [4].

The stimuli responsiveness of certain nanomaterials can be used to trigger drug (or gene) release and activate the signal emitter exclusively into the non-healthy tissues (e.g., ultrasound-, acid-, thermosensitive-, light- or enzyme-triggered release/activation) and/or accumulate these molecules into the targeted site before allowing its release (e.g., magnetically responsive nanocarriers) [2,4,10,18]. Critical reviews on the possibilities of this active targeting strategy have highlighted the potential problems arising from the localization of the stimulus deep into the body. In fact, numerous investigations have tried to define the possibilities of localizing the external stimulus into deeper non-healthy sites. Even though more research is still needed, preliminary studies have shown that this is possible. For instance, controlled delivery of magnetic colloids to deeper cancer sites can be assured by magnetic implants located into the tumor mass [80].

Light-triggered theragnosis typically combine imaging and photoactivation of therapeutic molecules [4]. Recent investigations have been devoted to the development of theragnostic nanoplatforms for simultaneous cancer diagnosis and photo-triggered chemotherapy. Such multifunctional nanoparticles will be disrupted under exposure to the electromagnetic radiation exclusively localized at the targeted site. These materials are irreversible damaged by a single light dose or, more interestingly, can behave as multi-switchable nanoplatforms for pulsatile drug release by undergoing reversible structural changes when cycles of light/dark are applied. Furthermore,

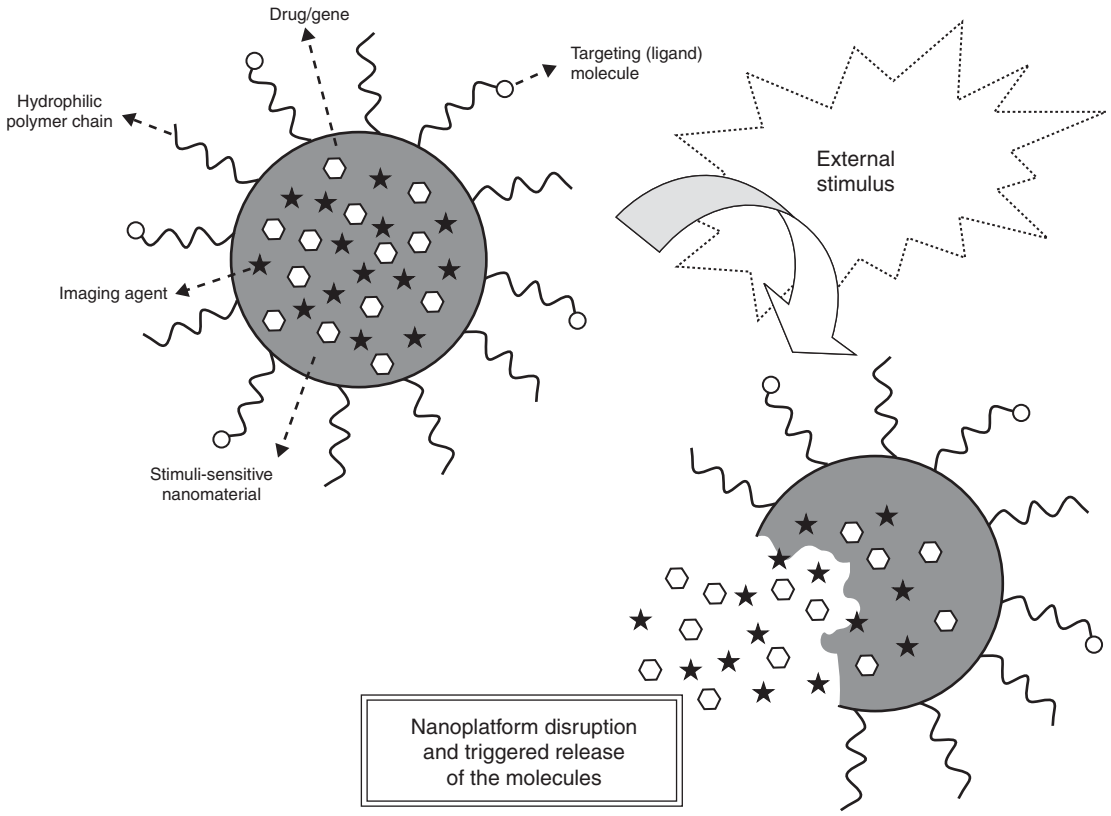

Figure 5. Stimuli-sensitive nanoparticulate system containing imaging agents to visualize and quantify drug (gene) delivery. The targeting molecules incorporated onto the nanomedicine surface will lead to a specific recognition of the particle by the non-healthy cell which generally induces an endocytic process. The stimuli-sensitive nanomaterial will facilitate the triggered drug (gene) release into the site of action by using an external stimulus.

light could be used to release endocytosed macromolecules into the cytosol and activate cytotoxic molecules inside targeted cells. Light-based theragnosis has been further investigated for combined selective disease imaging and efficient image-guided drug delivery to infectious diseases [25] and inflammatory atherosclerosis [47]. In the former case, image-guided photodynamic therapy and image-guided photothermal therapy could be of great help to achieve antimicrobial diagnostics to overcome resistances against antibiotics, and in the treatment of leishmaniasis, fungal (i.e., *Trichophyton rubrum*) and virus infections (e.g., herpes virus) [25,81-83]. For instance, this targeting strategy has demonstrated great specificity for ampicillin-resistant bacteria, and the potential advantage to distinguish between human and microbial cells (i.e., human foreskin fibroblasts, and *Staphylococcus aureus* strain 8179, respectively) [84]. Regarding the use of light-based theragnosis against inflammatory atherosclerosis, the accumulation of the nanomedicine into the macrophage rich atherosclerotic lesions can be monitored thanks to near-infrared fluorophores loaded into the nanoparticles, while light activation of the drug molecules can be obtained at 650 nm. As a result, the atherosclerotic lesion was stabilized due to the eradication of inflammatory macrophages [85].

pH-sensitive theragnostic nanotools are designed to be exclusively degraded under exposure to acidic environments being stable at the physiological pH 7.4. In this case, particle engineering involves the introduction into the nanoplatform structure of pH-sensitive functional groups (e.g., sulfonamide) to facilitate the acid-triggered disruption. pH-sensitive fluorescence probes (i.e., borondipyrromethene fluorophore and derivatives) should be also incorporated into the nanostructure to obtain pH-activatable images [25]. A recent publication has described the development of theragnostic nanoparticles for MRI and fluorescence imaging of cancer cells, with simultaneous intracellular acid-triggered DOX release [86]. The study reported the preparation of PEGylated SPION (mean size ≈ 45 nm) loaded with DOX (loading capacity ≈ 3.5 %), and surface decorated with the fluorescent dye 5-carboxyfluorescein and the mAb HuCC49ΔC_H2 (a humanized C_H2 domain-deleted anti-TAG-72 MAb). PEGylation of the magnetic nanoparticles was responsible for their greater accumulation into the tumor tissue (passive targeting thanks to the EPR effect). Meanwhile, the incorporation of the mAb onto the particle surface allowed receptor-mediated nanoparticle internalization into cancer cells overexpressing the tumor-associated glycoprotein

72 (a human mucin like glycoprotein complex) (active targeting). A very efficient cancer targeting was monitored by MRI and fluorescent microscopy in LS174T colon cancer cells. The magnetic core (mean diameter ≈ 10 nm) was used as an MRI contrast agent, inducing a shorter T_2 relaxation (transverse or spin–spin relaxation) which decreased signal intensity on a T_2-weighted image from ≈ 120 to ≈ 55 ms in LS174T cells. pH-dependent drug release was investigated *in vitro* by fixing the following pH values (with HOAc/NH_4OAc/NH_4OH buffers): 3.21, 4.19, 4.95, 5.66, 6.65 and 7.21. Additionally, the intracellular pH-dependent DOX release in endosomes/lysosomes was visualized by tracking the characteristic fluorescence of the drug. It was concluded that the anticancer molecules were exclusively released into the acidic lysosomes, and diffused into cytosol and nuclei. This interesting result could be due to the protonation of the primary amine of the drug molecule which dramatically increased drug hydrosolubility. As a result, lower IC_{50} values than DOX-loaded nonspecific SPION were measured (1.44 vs 0.44 μM).

pH-sensitive theragnostic nanoparticles have been also postulated for image-guided gene delivery. For example, a recent *in vitro* investigation explored the possibility of combined stimuli-enhanced gene silencing and stimuli–responsive multimodal optical imaging. In this investigation [87], siRNA was encapsulated into polyplexes (polymeric gene carriers) surface coated with small gold nanoparticles via acid-cleavable linkages. It was demonstrated that under acid tumor microenvironments, gold nanoparticles were dissociated from the polyplexes and, as a consequence, optical signal changed (diminished scattering intensity, increased variance of Doppler frequency and blue-shifted UV absorbance). The dissociation of gold nanoparticles from the polymeric structure additionally exposes the siRNA-loaded polyplexes for enhanced cellular uptake and transfection.

Theragnostic nanomedicines based on thermosensitive biodegradable materials (i.e., poly(*N*-isopropylacrylamide) and derivatives or copolymers) could help in controlling the delivery of therapeutic molecules and imaging agents to non-healthy tissues. In fact, on heating, an interesting correlation can be established between the activated drug release and the observable contrast coming from a change in the local environment [62]. A recent research report on the formulation of amphiphilic and thermosensitive copolymeric nanoparticles (mean diameter ≈ 200 nm) detailed the incorporation of hydrophobic (oleic acid coated) SPION into a PEG-*b*-poly(*N*-(2-hydroxypropyl) methacrylamide dilactate) matrix. The investigation demonstrated that the nanoparticles exhibited high T_2 and T_2^* relaxivities and interesting drug loading capabilities [64]. Finally, multifunctional temperature-sensitive liposomes have been formulated to allow drug carrier localization (by ^{1}H-CEST) and simultaneous quantification of drug release in response to a localized temperature increase (by ^{19}F-MRI) [65].

Ultrasound-mediated delivery of theragnostic particles is based on the selective application of a given frequency of ultrasounds to targeted tissues which leads to: i) enhanced extravasation and cellular uptake of drug molecules, contrast agents and/or theragnostic particles, thanks to the alteration of the cell membrane permeability and ii) nanocarrier disruption and specific release of the drug and the imaging agent into the targeted site. These positive consequences are generally due to the *in vivo* effects of ultrasounds: cavitation, local tissue heating and radiation force. The oscillating ultrasound pressure waves and the local tissue heating are responsible for the disruption of nanoplatforms sensitive to mechanical forces and temperature, respectively. Preclinical studies have highlighted the possibility of combined MRI and ultrasound imaging in bringing to clinic ultrasound-triggered drug (and gene) delivery [62,88,89]. Maybe, it can be hypothesized that the localization of the ultrasounds deep into the body would be the major limitation to the final introduction of such multifunctional nanoparticles into the clinic. Additionally, the toxicity arising from the application of ultrasounds to patients is not completely characterized.

Last but not least, theragnostic nanoparticles based on magnetic colloids are engineered to be easily guided by an applied magnetic gradient to non-healthy cells. Then, the magnetically responsive nanoparticles can be kept there until the drug (gene) dose is entirely released (magnetic targeting) [6]. Combined passive and active targeting strategies can be further adapted to the formulation of such magnetic multifunctional nanoparticles. The magnetic colloid would be preferably based on SPION which by themselves are very efficient MRI contrast agents with further functionalization capabilities, that is, with NIRF dyes for complementary optical imaging [47]. A recent study has confirmed that on parenteral administration of such nanomedicines to mice bearing xenograft breast tumors, optical imaging can help in the qualitative evaluation of the magnetic nanoparticle targeting to the tumor mass [56]. Thanks to the magnetic responsiveness of the nanoplatform, a maximum accumulation of the nanotheragnostic agent into the tumor tissue was obtained 48 h post-administration, before slowly declining over the next 11 days. Unfortunately, the authors did not evaluate the antitumor activity of the nanoformulations. More recently, a research report described the formulation of a theragnostic nanomedicine which combines the ability to magnetically target a prodrug of gemcitabine to an experimental solid tumor, with the imaging of the targeted tumoral nodule [90]. The structure of the theragnostic nanoplatform consisted of an iron oxide nuclei embedded into self-assembling molecules of a squalenoyl gemcitabine bioconjugate. On injection to the L1210 subcutaneous mice tumor model, these nanocomposites were magnetically guided, and they displayed considerably greater anticancer activity than control treatments (concretely, the nanomedicine non-magnetically guided, the pure gemcitabine bioconjugate and the antitumor drug free in solution). The superior therapeutic activity and enhanced tumor accumulation of the nanoplatforms were successfully visualized using T_2-weighted imaging in MRI.

4.2.3 *Theragnostic nanoparticles engineered for combined active and passive targeting functionalities*

Ideally, passive and active targeting strategies must be introduced in the formulation of theragnostic nanoparticles to significantly improve simultaneous disease diagnosis and therapy [4,6,86]. The combination of both strategies into a theragnostic nanosystem leads to a very rapid accumulation of the nanoparticles into the site of action which avoids the delayed imaging (therapy) from injection. For instance, an interesting work has proved the very rapid accumulation of the SPION MRI contrast agents in the cancer tissue [90]. In this preclinical study, a successful visualization of the malignant mass in a L1210 subcutaneous mice tumor model was demonstrated by using T_2-weighted imaging in MRI, only 2 h after intravenous injection.

As an example of the importance of the arrangement of both strategies into the same nanoparticle, PEGylated phospholipid micelles have been loaded with quantum dots and antitumor drugs for *in vivo* tumor vasculature imaging and chemotherapy [91]. This nanoparticulate platform was surface decorated with an RGD peptide which targets $\alpha_v\beta_3$-integrins overexpressed in angiogenic tumor vasculatures. Near-infrared optical imaging of mice bearing pancreatic cancer xenografts (implanted both subcutaneously and orthotopically) demonstrated (compare to controls) that the nanomedicine very efficiently label the tumors with very high signal: noise ratio. Similar results were also reported when $\alpha_v\beta_3$-targeted or $\alpha_5\beta_1$-targeted paramagnetic nanoparticles were used in neovasculature MRI and antiangiogenic therapy of MDA-MB-45 tumors [74].

Another interesting exemplification of the benefits coming from combined passive and active targeting strategies in theragnosis can be found in the use of polyacrylamide-based theragnostic nanoplatforms (containing SPION as contrast agents and photofrin as photosensitizer) for *in vivo* MRI enhancement and photodynamic therapy of brain cancer. The nanomedicine was surface functionalized with PEG chains and the F3 peptide, which binds to nucleolin expressed on tumor endothelium and cancer cells. Alexa Fluor 594 was also labeled to the nanoplatform for supplementary optical imaging. *In vitro* results in MDA-MB-435 human breast cancer cells showed that F3-targeted nanoparticles were internalized and concentrated within tumor cell nuclei. Even more, *in vivo* studies demonstrated that photodynamic therapy based on F3-targeted photofrin-containing nanoparticles significantly improved the treatment outcome compared to controls [92].

5. Nanotoxicity, economics and regulatory aspects in the development of theragnostic nanoparticles

On administration, the interaction of nanoparticulate systems with biological components leads to unique metabolism, clearance, biodistribution and immune response consequences. This interaction is facilitated by the longer circulation times and great capability to penetrate basic biological structures (cells and cellular organelles) exhibited by the (very small) multifunctional nanoplatforms. A typical consequence of this *in vivo* behavior is the disruption of the normal function of such biological structures. Examples of direct nanoparticle toxicity/immunogenicity include tissue inflammation and altered cellular redox balance towards oxidation, causing abnormal function or cell death [93]. Of course, as these nanoplatforms are mainly recognized by immune cells, particular attention should be given to nano–immuno interactions inducing toxicity [94]. The incidence and severity of the toxic side effects have been found to depend largely on the physical chemistry of the nanomaterial and biodegradation products (chemical composition, geometry (principally, the surface area) and structure, surface chemistry (including reactivity and hydrophilic character), surface charge, purity and solubility), dose of exposure (mass administered), delivered dose (mass per cell or cm^3), cellular dose (internalized mass), method of administration, biocompatibility, biodegradability, pharmacokinetics and biodistribution [17,95]. However, even though recently published investigations have tried to throw some light on the problem of the safety of theragnostic particles (i.e., by exploring the toxicity of nanomaterials related to granuloma formation, oxidative stress and enzyme function), the very active research on nanomaterials for biomedical purposes has been not counterbalanced by an adequate knowledge of their pharmacokinetics and toxicity.

The emergence of all data coming from physicochemical, preclinical and clinical investigations on theragnostic nanoplatforms must be used to develop better and safer multifunctional nanomedicines as well as the approaches for achieving such safeguards. In fact, recently published investigations have emphasized the need for predictive models to analyze the experimental data and elucidate/postulate the toxic response to nanotheragnostics [18]. Additional improvements in nanoparticle engineering should come from image-assisted biodistribution characterizations. Very importantly, the information coming from the investigations of the *in vivo* fate and toxicity of the nanoparticles must be used to properly modify the quantitative and qualitative composition of the theragnostic nanotool. Only under this rational design of the theragnostic nanoagent, the best combined diagnostic and therapeutic effect and toxicity profile will be guaranteed.

During the last decade, numerous international agencies started to focus their attention on the safety of nanotechnology-based medicines. This interest in the field has determined that the final introduction of theragnostic nanoparticles in the clinic strongly depends on the complete integration in preclinical research of characterizing structure–toxicity relationships and limiting safety problems [96]. However, international regulatory agencies do not currently possess effective standardized approaches to predict and monitor the risk associated with nanoparticle exposure (i.e., monitoring protocols, predictive models for toxicity

evaluations), and do not already specify distinct safety regulations/requirements that must be met by nanoparticle manufacturers [97]. Therefore, very little is known about the toxicity of nanoparticulate systems and contributing to the problem is the lack of enough funding [98]. Thus, actually it is recommended to treat these nanoparticulate systems as additives with potential side effects [99].

Finally, we would like to highlight that novel methodologies are under development to fill gaps in the knowledge of interactions between these nanoplatforms and biosystems. For example, synchrotron radiation-based analytical techniques have been postulated to provide a potent means for characterizing the toxic or *in vivo* behaviors of nanoparticles in biological systems [100]. It is expected that these advances could lead to an optimized characterization of nanomaterials in biological samples, quantification in living systems, and investigation of their biodistribution, location and chemical status *in vitro* and *in vivo* and so on.

6. Conclusions

Novel nanoengineering approaches have been claimed to play a key role in the advance of the simultaneous diagnosis and treatment of diseases. In the near future, the proper combination of a pharmacologically active molecule, a signal emitter, a biodegradable material and special targeting strategies in the same nanoplatform will definitively allow to predict and improve the therapeutic intervention, and to visualize and better understand important aspects of drug delivery. The optimization of this revolutionary concept and its transformation into marketed nanomedicines will be only possible if more research efforts are concentrated on the formulation, physicochemical characterization, and preclinical and clinical evaluation of the multifunctional nanomedicines. Additional key aspects to a definitive introduction of theragnostic nanoparticles into the clinic are the prediction and monitoring of particle exposure risks, and the analysis of the costs of creating these complex structures according to good manufacturing practices standards.

7. Expert opinion

The efficient management of severe diseases (those characterized by great morbidity and mortality, e.g., cancer) has become a real problem in patient's healthcare. Nanotechnology has tried to maximize the localization of therapeutic agents (and signal emitters) within the non-healthy site to improve the therapeutic effect (and the selective disease imaging). Unfortunately, nanoparticulate delivery systems loaded with these agents can fail in the early disease diagnosis and/or in obtaining an adequate therapeutic outcome. Important reasons can be given to justify such failure, that is, the reduced selectivity of the nanocarrier for non-healthy cells, tissues and/or organs, and the drug resistance mechanisms expressed by malignant cells.

With the aim of meeting these challenges, theragnostic nanotechnologies have been proposed for combined disease diagnosis and therapy activities. Current state of the art has created great expectations to definitively achieve an efficient and integrative management of the disease. In fact, the rise of literature in the field has hypothesized that in the near future, the quest for the real introduction of 'personalized' medicines into the clinic would reach a satisfactory end. However, this will only be possible if multifunctional nanoplatforms can substantially contribute to define: i) the real potential of therapeutic interventions; ii) treatment regimens able to enhance the therapeutic effect; iii) an optimized therapy for individual patients; and iv) a real-time feedback of the biological fate of the nanomedicines to clarify engineering aspects for an efficient disease targeting. This will only be possible if a better knowledge of the biological disorders causing disease is gained and novel molecular targets are identified, further biocompatible materials and functionalization approaches (e.g., enzyme-triggered release) are introduced as safe products in the formulation of theragnostic nanoparticles, and a complete *in vivo* evaluation of the nanoplatforms is done. In the last case, mathematical models could be of great help as they can represent the *in vivo* conditions that will face the nanomedicine on administration, correlating *in vitro* characteristics of the nanoplatforms to their *in vivo* capabilities. Maybe, the analysis of all of these investigations will reveal that a lot of work is still to be done to clarify the *in vivo* fate of the nanoparticulate systems when the theragnostic activity is finished and elucidate their biological clearance mechanisms.

Very importantly, the combination of passive and active targeting strategies in the design of these nanomedicines is needed to assure an optimum control of their *in vivo* fate and efficacy, significantly contributing to the improvement of their biomedical applications. It is expected that theragnostic nanoparticles engineered under these premises could safely reach the targeted site. Both strategies can never be compared as both events must happen together to assure that the dose of drug molecule (or gene) and imaging agent will be completely accumulated into the targeted site.

Last but not least, a rapid move towards *in vivo* studies is urgently needed to bring to light very important aspects of such multifunctional nanotools determining their therapeutic potential, for example, biological fate (biodistribution, plasma clearance, metabolism and immune response), biocompatibility, optimal drug–contrast agent vehiculization and controlled release, mechanisms of theragnosis activity, and nanotoxicity. In this way, the clear need for a deep elucidation of the relationship between the physicochemistry of nanomedicines and its *in vivo* behavior cannot be ignored. Understanding these basic aspects will offer guidance for future nanomaterial engineering and development. Of course, significant research efforts should be concentrated on the development of theragnostic agents to be taken orally by the patients with very few administrations in order to facilitate dosing and improve patient's compliance.

The final introduction of theragnostic nanoparticles in the clinic will strongly depend on the complete integration in preclinical research of characterizing structure–toxicity relationships. International regulatory agencies must develop standardized approaches to predict and monitor the risk associated with nanoparticle exposure and establish safety regulations/requirements to be met by nanoparticle manufacturers. Therefore, more cautious optimism is encouraged until the day arrives when there is widespread use of theragnostic agents in the clinic. We are merely at the beginning of the development of an exciting biomedical field, and the clinical possibilities arising from theragnostic nanotechnologies are more than fascinating.

Declaration of interest

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