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Nanoparticles for Radiooncology: Mission, Vision, Challenges

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Abstract

Cancer is one of the leading non-communicable diseases with highest mortality rates worldwide. About half of all cancer patients receive radiation treatment in the course of their disease. However, treatment outcome and curative potential of radiotherapy is often impeded by genetically and/or environmentally driven mechanisms of tumor radio-resistance and normal tissue radio-toxicity. While nanomedicine-based tools for imaging, dosimetry and treatment are potential keys to the improvement of therapeutic efficacy and reducing side effects, radiotherapy is an established technique to eradicate the tumor cells. In order to progress the introduction of nanoparticles in radiooncology, due to the highly interdisciplinary nature, expertise in chemistry, radiobiology and translational research is needed. In this report recent insights and promising policies to design nanotechnology-based therapeutics for tumor radio-sensitization will be discussed. An attempt is made to cover the entire field from preclinical development to clinical studies. Hence, this report illustrates (1) radio- and tumor biological rationale for combining nanostructures with radiotherapy, (2) tumor-site targeting strategies and mechanisms of cellular uptake, (3) biological response hypotheses for new nanomaterials of interest, and (4) challenges to translate the research findings into clinical trials.

Contents

Introduction

According to the world health organization (WHO), the number of cancer-related mortalities per year is projected to increase by 45 % from 2007 to 2030, influenced in part by an increasing global aging population. In today's society, the costs of cancer care are enormous, where the EU spends annually ~ 126 billion €. More than 14 million new cases and >8 million cancer deaths were reported worldwide during 2012 to 2013, with an elevating trend described in GLOBOCAN by the International Agency for Research on Cancer (IARC) and the Institute for Health Metrics and Evaluation (IHME) [1, 2]. These data underline the urgent need for a re-evaluation and prioritization of new approaches to complement and improve current diagnostic tools and treatment methods. The latter comprises the three main pillars of cancer treatment, namely surgery, radio and chemotherapy, which were over the past decade extended by a range of novel chemotherapeutic as well as individually applicable targeted therapeutics and immunotherapies. Patients with specific malignant diseases clearly benefit from the reasonable progress in surgical and chemotherapeutic treatment. However, only marginal improvement in overall clinical management of cancers patients could be achieved, with some malignant diseases such as pancreatic cancers and glioblastomas, as well as most advanced stage cancers, remaining an unsolved therapeutic challenge. Here, the most prominent limitations of currently available treatment options such as dose-limiting toxicity, lack of specificity, selectivity, bioavailability of drug candidates or local distribution, and morbidity become particularly apparent. Novel strategies that are generally applicable, have high (local) efficacy and are cost-efficient, and of utmost urgency [3]. A great hope lies in the field of nanomedicine, where nanoparticles (NPs) can be specifically designed using advanced engineering tools to treat and visualize tumors. Several nanoparticles (NP)-based formulations are undergoing clinical trials, or are even already used in clinics [4, 5]. Most applications however merely utilize NPs as drug delivery vehicles for or as mediators in physical anticancer methods, such as heating of tumor cells. In particular the delivery vehicle aspect has been critically discussed recently [6]. These methods suffer from several drawbacks, such as the need for advanced NP surface chemistry, specialized equipment, lack of specificity, low efficiency in drug release rates, and undesired NP toxicity [7-11]. For imaging applications, NPs either contain intrinsic contrast (*e.g.* FeO_x cores for magnetic resonance imaging (MRI)), or are further functionalized through chemical means (*e.g.*

fluorescence or radiolabeled probes) [12]. These functionalities enable follow-up of the NPs' location after administration, but do not give any direct information on the ongoing therapy.

Photodynamic therapy (PDT) consists of light, photosensitizer and oxygen during treatment. The mechanism of the photodynamic involves activating photosensitizers *via* certain wavelength of light followed by emission (recombination). The whole excitation-emission process is accompanied with the energy release that is transferred to the near surface oxygen generating ROS (singlet oxygen, free radicals and/or superoxide) [13]. The chemical reactions take place during PDT is (1) the direct interaction of excited photosensitizers with the cell membrane or the cellular components transferring H atom to form potentially hazardous radicals (2) direct energy transfer from the excited photosensitizers to surface oxygen generating singlet oxygen ($^1O_2$) and/or highly oxidizing superoxide [13]. Hence, for the progress in the cancer therapy especially phototherapeutic technology, the development of new light sensitive photosensitizers is necessary. During treatment, these efficient photosensitizers are expected to be cleared from the body faster and absorb light at higher wavelengths leading to a limited period of photosensitivity in the targeted area [14-19]. Designing such sensitizers (altered or mixed to target specific cell abnormalities) targeting various organs and parts of the cell such as membrane and lysosomes are very promising in treatment of tumors. Although Photodynamic therapy is one of the effective techniques to treat cancer, it has a serious drawback. These effective photosensitizers with reduced duration of light irradiation have intense and prolonged chemical reactions post treatment [20]. Hence, in the future, the photodynamic therapy (PDT) in combination with the surgery and radiotheraphy could be uniquely tailored to treat cancers [21]. The treatment includes the development of new photosentizers, using optimal photodynamic therapy protocols (light fraction and/or drug dose) [22, 23]. Furthermore, the clinical trials involving selective and friendly sensitizers with low energetic light irradiation may improve the photodynamic therapy technique in cancer treatment [24, 25].

The use of NPs in the context of radiotherapy is a particular issue that has been challenging in the past. Radiotherapy as one of the key modalities to treat solid cancers is the major treatment option beyond surgery with high curative potential. Today, about 50-60 % of the cancer patients receive radiotherapy, most frequently in entity-specific combinatorial radio/chemotherapeutic approaches [26, 27]. The success rate and outcome of patients is still limited by normal tissue toxicities and the development of individual, highly variable intrinsic as well as

microenvironmentally-driven tumor therapy resistances that require improvement and optimization of the current treatment policies [27-31]. Here development of novel strategies and types of nanoparticles and -materials, in particular to ameliorate the cancer-specific efficacy of radiotherapy would be highly helpful. It is recognized that some materials might be considered as dosimetric *in -vivo* nanosensors to monitor therapeutic levels of ionizing radiation as recently shown for C12 TAB-templated gold NPs exhibiting unique spectral profiles under ionizing radiation [3]. However, in this report focus will be given rather describing a vision of NPs for radio-sensitization based on the cellular irradiation effects and tumor biological rationales, as depicted in the following. Therapeutic challenges will be highlighted and some specific examples of interest are given.

Cellular irradiation effects and tumor biological rationales

Radiotherapy may eradicate cancer cells through a set of physical and chemical changes induced in the tumor tissues *via* transmitted energy. Many different types of ionizing radiation have been employed for medical diagnostic and therapeutic applications including photons (X-rays, gamma rays), leptons (electrons), hadrons (negative pi-mesons, neutrons, protons) and heavier ions (carbon, silicon, neon, helium). The major considerations for selections of the certain type of ionizing radiation for medical use include its controllability within an atomic site, inherent pattern of ionizing density defined by the linear energy transfer (LET), and relative biological effectiveness (RBE), attributed to the relative biological effects per unit energy [32]. Up to date, X-rays (photons) remain the most common type of radiation therapy due to its low production cost [33]. State-of-the-art photon radiotherapy is based on continuous technological progress over the past decades that led to an advanced 3D conformal treatment, and includes the use of intensity-modulated radiation therapy (IMRT) techniques with in-room image guidance (image-guided radiation therapy). Particle therapy with protons or heavier ions, such as carbons have the potential for higher dose conformity compared with photon beams, due to a reverse depth dose profile, *i.e*. particle beams can be directed more precisely as they deposit most of their energy over a narrow range (Bragg peak) [34-39]. The energy of the beam defines the depth of the Bragg peak in tissue and can be modulated to achieve maximum ionization within the tumor site and spare organs of risk to minimize normal tissue injury. Although high equipment and facility costs are the major obstacle for wider applications, proton and carbon ion therapy has been shown to be

an efficient treatment modalities for different types of malignancies, including head and neck squamous cell carcinoma (HNSCC), prostate, brain, and pediatric cancers [40-42]. More details on the technical improvements in photon and particle therapy have been discussed in a recent report highlighting the efforts in biology-driven precision radiation oncology [27]. Despite improved precision of radiotherapy delivery, treatment-related toxicities often show late effects. The intrinsic and environmentally-driven tumor radio-resistance, tumor metastasis, poor disease-free and overall survival of cancer patients, remains a clinical and scientific challenge [29, 31].

The curative potential of irradiation mainly depends on its ability to induce non-repairable DNA damage in tumor cells, either by direct ionization of the DNA molecules, or by generation of free radicals, including oxygen-derived chemically reactive products [26, 43]. Tolerance to DNA damage-induced cell death *via* activation of pro-survival signaling cascades (*e.g.* phosphatidylinositol-3 kinases (PI3K/AKT), nuclear factor κB (NF-κB), and mitogen-activated protein kinase (MAPK)) are distinctive feature of cancer cells that might reduce the efficacy of radiotherapy (Figure 1) [28]. Beside the intrinsic mechanisms affecting tumor response to radiation, micro-environmental constraints such, as the intra-tumoral oxygen level, also play an important role for tumor radio-curability. The oxygen distribution in solid malignant tissues is inhomogenous, due to a pathological capillary network in the growing tumor mass which is unorganized, leaky, fragile, and shows perfusion malfunctions. This goes along with non-(patho)-physiological and steep spatiotemporal and micro-regional oxygen gradients resulting in chronic, diffusion-limited as well as acute, perfusion-limited, and intermittent oxygen deficiencies (hypoxia) [44, 45]. Cancer cells residing in hypoxic areas can be more shielded from radiation-induced DNA damage due to reduced ROS generation and activation of pro-survival signaling pathways, *e.g. via* the regulation of hypoxia-inducible factor (HIF)/HIF-1α–dependent transcriptional control [28, 46].

Combination treatment for improvement of radiotherapy efficacy

The results of preclinical and clinical studies (combination of radiotherapy and chemotherapy) hinted that the judicial selection of the drug combinations might enhance tumor sensitivity to radiotherapy, thus allowing lower total irradiation doses and/or shorter exposure times [47]. One option is to combine radiotherapy with cytotoxic and/or target-specific drugs. Here, selection of the most promising agents for combination is critical to guarantee a reasonable therapeutic

window and to avoid severe side effects, as shown recently for example for some, but not all inhibitors of epidermal growth factor receptor (EGFR) [48]. Today, it is well known, that enhanced radio-response might in principle be achieved by various drug treatment strategies. These treatments include (1) chemicals that mimic the radio-sensitizing effect of oxygen by metabolic interference, *e.g.* by impacting oxidative phosphorylation and/or reducing local oxygen deficiencies (acting *via* different mechanisms as functional oxygen mimetics), (2) inhibitors directed against DNA damage response (DDR) molecules and DNA binding molecules such as specific PARP (Poly-ADP-ribose polymerase) or HDAC (histone deacetylases) family members, as well as (3) antibodies and inhibitors targeting receptor tyrosine kinases (RTK) such as EGFR and/or the respective signaling pathways [49, 50]. Accordingly, a broad range of clinical studies to combine radiotherapy with novel drugs and targeted therapies has been initiated over the past decade, as exemplified and functionally classified in Table 1.

Table 1. Selected tumor radio-sensitizer currently used in clinical trials (selected).

Tumor Entity; Molecular Mechanism and Drug	**Clinical trial phase, Primary endpoint and Gov. Identifier**	**Ref.**
Inhibitor Class 1 (DDR Inhibitor)		
Laryngeal, rectal, breast cancer, HNSCC; PARP inhibitor; **Drug: Olaparib**	**Phase I:** Dose limiting toxicities NCT02229656, NCT01589419, NCT01477489	[51, 52]
Solid tumors refractory to conventional treatment; ATM/ATR inhibitor; **Drug: AZD6738**	**Phase I:** Maximum tolerated dose NCT02223923	[53]
SCLC, rectal cancer; Topoisomerase inhibitor; **Drug: Topoteca**	**Phase I, II:** Safety, efficacy NCT00043862, NCT00158886, NCT00215956	[54]
Inhibitor Class 2 (Kinase/RTK inhibitor)		
CRC, HNSCC; EGFR targeting antibody **Drug: Cetuximab**	**Phase III:** FDA approved; Overall survival NCT00673738, NCT00815308 NCT00343083, NCT00124618	[55-60]
CRC, EGFR targeting antibody; **Drug: Panitumumab**	**Phase II:** FDA approved, Overall survival, loco-regional control NCT00798655, NCT00547157	[61, 62]
Esophageal Squamous Cell Carcinoma, EGFR targeting antibody; **Drug: Nimotuzumab**	**Phase II/III:** Disease free survival**,** Overall survival	[63-65]

	NCT02272699, NCT01232374	
Pancreatic, rectal, esophageal cancer, metastatic NSCLC, GBM, HNSCC, EGFR small molecule inhibitor; **Drug: Erlotinib**	**Phase I/II:** Toxicity, Progression-free survival**,** Overall survival NCT00096265, NCT00766636 NCT00410826	[66-69]
Soft tissue sarcoma, prostate cancer, VEGF inhibitor; **Drug: SU5416**	**Phase I, II/III:** Safety and tolerability**,** Side effects NCT00023738, NCT00026377	[70-72]
Prostate cancer, glioblastoma, HNSCC, Multi-targeted RTK (*e.g.* PDGF, VEGF, c-Kit, FLT, CSF, RET); **Drug: Sunitinib**	**Phase I, II:** Maximum tolerated dose, safety and tolerability objective response rate NCT00631527, NCT01100177 NCT00437372	[73-77]
HNSCC, NSCLC, prostate cancer, GBM mTOR inhibitor; **Drug: Everolimus**	**Phase I, Phase I/II:** Toxicity NCT01217177, NCT00943956 NCT00943956	[78-81]
Pancreatic cancer, Malignant Glioma, SCC, Rectal cancer, VEGF inhibitor **Drug: Bevacizumab**	**Phase II:** Toxicity, safety, efficacy, local tumor response NCT00305877, NCT00782756, NCT00408694 NCT00113230	[82-85]
Liver, prostate cancer, Multi-targeted RTK (*e.g.* Raf, VEGFR); **Drug: Sorafenib**	**Phase I/II, III:** Safety and tolerability, overall survival NCT01730937, NCT00924807	[86-88]
GBM Src/Abl kinase inhibitor **Drug: Dasatinib**	**Phase II:** Overall survival NCT02661113	[89]
Lung, rectal, pancreas cancer, GBM, HIV protease inhibitor, PI3K/AKT inhibitor; **Drug: Nelfinavir**	**Phase I, II:** Dose Escalation NCT01447589	[90-93]
GMB, NSCLC PKC inhibitor; **Drug: Enzastaurin**	**Phase I:** Dose-limiting toxicity NCT00509821, NCT00415363	[94-96]
Inhibitor Class 3 (Functional Oxygen Mimetics)		
HNSCC, Fixation of free radicals **Drug: Nimorazole**	**Phase II:** Locoregional control, nodal control, disease free survival, metastasis NCT01880359 NCT01507467	[97-99]
GBM, Increased Cerebral Oxygen Tension; **Drug: NVX-108**	**Phase I:** Safety and tolerability NCT02189109	[100]
High grade Glioma; Oxygen delivery **Drug: Trans sodium crocetinate**	**Phase I:** Safety, and Pharmacokinetic NCT00826930	[101, 102]
HNSCC, Dual CAIX Inhibitor **Drug: DTP348**	**Phase I:** Dose-escalation study NCT02216669	[103-105]
Recurrent breast Cancer, Hydrogen peroxide (0.5%), Induction of oxidative stress;	**Phase I, II:** Intratumoral pain, tumor response	[106]

Drug: Oxydol (KORTUC)	NCT02757651	
Inhibitor Class 4 (Other mechanism)		
NSCLC, Cox2-inhibitor; **Drug: Celecoxib**	**Phase II:** Tumor response rate NCT00181532	[107, 108]
Brain tumors, HDAC inhibitor; **Drug: Panobinostat**	**Phase I:** Maximum tolerated dose NCT01324635	[109, 110]
Brain tumors, HDAC inhibitor; **Drug: Valproic acid**	**Phase II:** Safety, efficacy, median progression free survival NCT00302159	[111, 112]
Pancreatic cancer, GBM Inhibitor of thioredoxin reductase and ribonucleotide reductase, ROS production **Drug: Motexafin, Gadolinium**	**Phase I:** Dose escalation and pharmacokinetic, toxicity, maximum-tolerated dose NCT00003411, NCT00032097, NCT00006452, NCT00004262	[113-115]
SCLC, GBM Anti-autophagy; **Drug: Chloroquine**	**Phase I:** Safety Study NCT01575782	[116, 117]
Prostate cancer; Androgen-deprivation **Drug: Goserelin**	**Phase III:** Improved 5-year survival rate NCT00423475	[118]
Pancreatic cancer, HNSCC PD-1/PDL-1 inhibitor, immune checkpoint inhibition; **Drug:Pembrolizumab/Tremelimumab**	**Phase I, II:** Safety, tolerability NCT02311361, NCT02587455 NCT02775812, NCT02735239	[119, 120]
Melanoma, NSCLC, Cervical cancer Anti-cytotoxic T lymphocyte antigen (CTLA-4); **Drug: Ipilimumab**	**Phase II:** Response rates NCT01689974, NCT01557114 NCT01711515, NCT02221739	[121, 122]
HNSCC, NSCLC, Rectal cancer Proteasome inhibitor; **Drug: Bortezomib**	**Phase I:** Dose-limiting toxicity, maximum tolerated dose; NCT00629226, NCT00093756, NCT01445405, NCT00280176	[123-126]

Pre-clinical (with promising results): - Chk1/2 inhibitor**;** - DNA-PK inhibitor

Some of the radio-sensitizers work as synthetically lethal combination when administered with irradiation, such as modulators of the DNA damage response [28, 102, 127]. However, despite enormous efforts, many new drugs may (as in the past) fail to improve patient survival due to pharmacokinetic limitations, undesired side effects, and biological toxicities. Furthermore, the increasing knowledge and insights in many radio-sensitizing mechanisms derived from knock-down experiments cannot yet be sufficiently translated into medical approaches because of lack of functional inhibitors. Therapeutic application of small inhibitory RNAs, which are not useful as such *in vivo*, due to restricted life-time and distribution characteristics, is therefore of great

interest. The emergence of nanotechnology especially for medical applications offers an avenue towards the use of such novel classes of therapeutics based on small RNA molecules, underlining the rational for NPs in the delivery of radio-sensitizing moieties.

Nanoparticle delivery of siRNA for therapeutic applications

In the last 15-20 years, the role of certain small RNA molecules in modulating/inhibiting the expression of their target genes has been well established. More specifically, the mechanism of RNA interference (RNAi) in the nematode worm *Caenorhabditis elegans* was reported in 1998 to specifically silence genes by exogenous double-stranded RNA. Soon after, RNAi was shown to be a highly conserved mechanism in most eukaryotic cells that can be triggered by small interfering RNA [128]. These siRNAs, 21-23 base pairs in length and containing 2-3 nucleotides 3' overhangs, interact with the multifunctional enzyme Argonaute-2 (Ago 2) and are incorporated into the RNA-induced silencing complex (RISC). The activated RNA-induced silencing complex with the intact siRNA guide strand (RISC*) can bind to its target mRNA sequence, and finally cleaves the mRNA into smaller fragments, which are subsequently degraded due to their unprotected terminations. An outstanding property of the RNA interference, in contrast to classic antisense technologies, is the catalytic activity of the activated RISC, while antisense oligonucleotides (AON) inhibit the translational processing by complementary target mRNA binding in a stoichiometric ratio. The silencing effect can last for up to 7 days in fast growing cells, and many weeks in weakly dividing cells [129]. Since all other components of the RNAi machinery are provided by the cell, only the delivery of the siRNA is necessary and sufficient for the knock down of a given gene. In the process, siRNA will be responsible and determine the target gene specificity to inhibit any genes of interest. While in vitro (tissue culture) RNAi is well-established as a tool e.g. in functional (onco-)gene analyses (see below and Fig. 2C, here exemplified by the knockdown of the reporter gene EGFP), the in vivo application leads to novel therapeutic approaches when targeting oncogenes which are rate-limiting for tumor growth (see below and Fig. 2D).

In the context of radio-sensitization, siRNAs have been extensively explored in tissue culture. RNAi screening using pools of different siRNA strands were exploited to identify key components of DNA repair, after using ionizing radiation therapy [130]. During the RNAi screening process, some of the genes associated in DNA repair such as BRCA1 and BRCA2

and/or POLQ (expressed differentially in cancer tissue), could be detected for their involvement in radio-resistance [131, 132]. Therapeutically targeting BRCA2 *via* RNA interference was one of the first successful approaches to radio-sensitize cancer cells *in vitro* [61]. In another study, the effectiveness of siRNA in radio-sensitization targeted to Ku80 protein (part of the DNA-PK complex) was shown [133]. Very recent studies also demonstrated the importance of RNAi in the radio-sensitization of head and neck squamous carcinoma by using a lentiviral siRNA approach, [134] and Mcl1 protein targeted by siRNA to radio-sensitize pancreatic ductal adenocarcinoma [2]. Additionally, it was clear that Neuropilin-2 and VEGF-C were observed to be potential radio-sensitizers [135, 136]. The impact of the Neuropilin-interacting protein GIPC1 was tested by siRNA preclinically [137]. Non-coding RNAs could be targets of short interfering RNAs [137].

Number of studies has shown the role of long non-coding RNAs (lncRNAs) in radio-resistance of cancer. Wang *et al*. demonstrated the role of different lncRNAs in the radio-resistance of nasopharyngeal carcinoma [138]. In contrast, there are significantly more studies investigating the role of microRNAs (miRNAs) in radio-resistance. It has already been shown that the DNA repair machinery is also regulated by miRNAs, and that targeting miRNAs could be a viable therapeutic approach [78]. Further studies have confirmed the role of different other miRNAs in radio-resistance [73, 139]. While RNAi offers broad applications for treating undruggable diseases or to specifically target pathologically relevant (overexpressed) genes, a major hurdle is still the delivery of siRNAs into the particular tissue. The large polyanionic molecules are not actively internalized by the cells and are not able to freely cross the cell membrane. Moreover, rapid degradation by serum nucleases and renal clearance further impede organ delivery and cellular uptake. Once taken up, the nucleic acids are prone to the endosomal/lysosomal degradation processes. Furthermore, siRNA molecules can induce an innate immune response in size- and sequence-specific manner by activating toll-like receptors. For addressing these problems, various strategies have been explored. Chemical modifications of the ribose backbone, the introduction of novel nucleotide modifications, and the rational design of the chosen siRNA sequence, have been widely investigated to enhance stability, specificity, improve silencing and mitigate immune reactions [140-142]. In light of concerns associated with the use of viral vectors, various non-viral strategies have been investigated for siRNA delivery [see Figure 2 (A), Figure 3]. These include siRNA conjugation to entities such as lipids, cell penetrating peptides, proteins/antibodies and polymers, acting as self-delivery devices [143-145] *N*-acetylgalactosamine (GalNAc)-siRNAs have already entered several clinical trials. [146]

However, appropriate systems are only available for certain target organs, with liver hepatocytes being the best example. Alternatively, NP systems made from diverse natural or synthetic materials including polymers, lipids and inorganic materials have been explored [147, 148]. Inorganic nanoparticles (metal, silica) can adsorb siRNAs on their surface or incorporate them in pores (see [149] for review). Liposomes are self-assembled, usually phospholipid-based vesicles that separate an inner aqueous core from surrounding aqueous compartment, and are thus highly attractive delivery systems for a wide range of applications. The amphiphilic character of phospholipids allows for the encapsulation of small lipophilic and hydrophilic drugs as well as large biomolecules in the inner core, in order to improve the drug stability, bioavailability and to minimize side effects [150, 151]. Particularly efficient for nucleic acid delivery are synthetic cationic lipids, which are able to spontaneously form positively charged lipoplexes in the presence of nucleic acids like siRNAs. In the recent years, many synthetic lipids with improved head groups and linker moieties have been introduced and are commercially available for *in vitro* applications [152, 153].

While major issues for their therapeutic application include instability (aggregation, premature release), rapid clearance, immune stimulation (interferon response, inflammation reactions), and probably genotoxicity (as shown for DOTAP) [144, 154-157], these are mainly caused by the cationic charge and can be minimized by the incorporation of polyethylene glycol (PEG)-lipids [158, 159]. These “stealth liposomes” avoid detection by the immune system due to PEG (polyethylene glycol) covering the outer surface, and also allow for prolonged circulation half lives. “Solid-lipid nanoparticles” are used for siRNA delivery (see [160] for review) and SNALPs (stable nucleic acid lipid nanoparticles) are especially designed for the delivery of siRNAs, combining the key features of classical cationic lipids and conventional liposomes. In several pre-clinical and first clinical studies, these neutrally charged NPs have shown to be well tolerated with significant target gene silencing [138, 161, 162]. Polymeric micelles are self-assembling nano-constructs of amphiphilic block copolymers that form nanoscopic core/shell structures which are used for various applications including gene delivery. On the other hand, cationic polymers are able to electrostatically interact with siRNAs forming nanoparticles [See Figure 2 (B), (C)]. These polymeric nanoparticles can be chemically modified for example with PEG for reduced surface charge and/or with ligands for specific ligand-mediated binding to and uptake into target cells [See Figure 2 (B)]. The combination of polymeric, e.g., PEI-based nanoparticles with liposomes is possible as well. These lipopolyplexes have been explored for

siRNA delivery in vitro [see Figure 2 (C)] and in vivo [see Figure 2 (D)]. The interaction of siRNAs with several classes of polymers including poly-L-lysine (PLL), polyamidoamine dendrimers (PAMAM), polypropylenimine (PPI) dendrimers and polyethylenimine (PEI) have been studied. In addition, the interaction of specifically designed polymers such as poly(β-amino esters), cationized cyclodextrins, the biodegradable polymer poly(lactic-co-glycolic) acid (PLGA), combinations between the various polymers prepared by polymer-grafting, or sequence-defined oligomers have also been investigated [163, 164]. From all these classes of polymers, polyethylenimine (existing in branched and linear structures with different molecular weights), a promising candidate for therapeutic siRNA delivery, is one of the most studied cationic polymers. The most prominent commercially available linear PEI is the 22 kDa jetPEI® used as transfection reagent and GMP product available for for clinical studies. The outstanding property of PEI is the high density of which ~ 20 % that are protonated at physiological pH, allowing efficient complexation of negatively charged nucleic acids at optimal ratios (the so-called nitrogen (in PEI) / phosphate (in RNA) (N/P) ratio) [165, 166]. The nanoscale PEI polyplexes are able to interact with negatively charged components on the cell membrane leading to nanoparticle internalization *via* various endocytosis pathways [167]. The "proton sponge effect" of PEI is defined as the capability of PEI to absorb protons during endosomal acidification, resulting in endosome swelling and eventually rupture. This may be a key for the polyplexes to escape the endo-/lysosomal system [168, 169]. During the process, transfection efficacy and biocompatibility depends on the molecular properties and complex preparation conditions. The most suitable PEIs are in the range of 5-25 kD, because (1) PEIs with higher molecular weights induce severe cytotoxicity [170], and (2) lower molecular weight PEIs are biologically inactive [165, 171]. This trend is generally valid for both, linear and branched polymers, despite enhanced tolerance for linear PEIs and better knockdown results with siRNA for branched PEI [172]. Furthermore, several aspects of the preparation conditions may influence the physicochemical and biological properties. Various chemical modifications have been introduced to further improve efficacy and biocompatibility. These include polymer grafting with fatty acids [173, 174], PEG [175-178], amino acids [179-182], and carbohydrates [183, 184], as well as strategies towards targeted delivery by introducing binding ligands to the NP surface. Several modified and non-modified PEIs have been employed in preclinical studies *in vivo* [183].

Recently, the star polymers for siRNA delivery were designed. These delivery materials contained different lengths of cationic poly(dimethylaminoethyl methacrylate) (PDMAEMA)

side-arms and varied amounts of poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA). They demonstrated that star-POEGMA polymers could readily self-assemble with siRNA to form nanoparticles and deliver siRNA with high efficiency to pancreatic cancer cells [Figure 4 (A)-(B)] [185].

As of 2015, more than 50 clinical trials on RNAi have been conducted or are under way, involving at least 26 different siRNAs. Promising results have been obtained especially for lipid-based siRNA carriers. A cationic lipid formulated with two helper lipids was able to achieve disease stabilization in 52 % of patients with solid tumors [186, 187]. Among the most successful systems "stable nucleic acid lipid particles" (SNALPs), ionizable DlinDMA-lipids were found to be most effective. SNALPs were able to reduce the expression of target genes in hepatocellular carcinoma and metabolic diseases (hypercholesterolemia) [162, 188]. An alternative approach includes the self-delivery target such as *N*-acetylgalactosamine-conjugated siRNAs (GalNAc-siRNA) injected subcutaneously. Phase III trials based on GalNAc-siRNA or siRNA formulated in SNALPs are under way for the familial amyloid polyneuropathy (FAP) and familial amyloid cardiomyopathy (FAC) treatment caused by transthyretin (TTR) mutations leading to TTR misfolding and aggregation. In phase I / phase II, a sustained > 80% knock down of serum TTR has been observed [189]. The other additional systems include siRNA trial using a targeted four-component polymer NP (CALAA-01) for melanoma cancer therapy using a cationized cyclodextrin, adamantane-PEG, adamantane-PEG-transferrin targeted delivery, and the siRNA delivery system. The results of the clinical trials showed that for the patients with solid tumors who were intravenously treated with the NPs, a significant reduction of mRNA and protein levels were achieved. The treatment was first well tolerated but severe adverse effects occurred post 1 year period. NP dissociation /dissolution would have been the main reason upon storage for this adverse effect [188, 190]. While so far no clinical studies on siRNA-mediated radio-sensitization have been reported, this approach appears clearly feasible based on the preclinical siRNA studies detailed above and the increasing availability of siRNA delivery strategies that can also be employed in clinical studies. Still, efficient and non-toxic siRNA delivery remains a major issue, probably requiring further developments in the NP field.

Structure meets function: carbon nanostructure-based drug delivery

Carbon Nanostructures including fullerenes, carbon nanotubes (CNTs), carbon nanohorns, nanoribbons, nano-diamonds, and graphene, with various shapes and sizes (*e.g.* sheets, spheres, ellipsoids, or tubules) [see Figure 5(A)] can have acute biological response *via* deliberate or undeliberate exposure to the living system [191]. The peculiar physicochemical properties (distinctly different for each structure) play a key role in different research fields including cancer therapy [192, 193]. Among different carbon nanostructures, carbon nanotubes (CNTs) and graphene (GP) derivatives [Figure 5(A)] are known for their use in biomedicine [194-197]. They possess an ordered structure with high aspect ratio, ultralight weight, high mechanical strength, high electrical conductivity, high thermal conductivity, metallic or semi-metallic behavior, and high surface area [198]. Their unique shape allows them to be internalized into cells by penetrating the phospholipid membrane *via* a ‘‘snaking effect’’ (spiraling or winding motion) and through transient pores or by active endocytosis ending up in endosomes [199]. These properties make them unique in biomedicines for cancer treatment [200-204]. When using them as drug delivery vehicles, CNT-drug interactions can be mediated by three different mechanisms [205] (1) drug filling inside the CNT channels, (2) absorption of the drug onto the CNT surface and (3) covalent linkage of the drug to the exterior walls [as an example see Figure 5 (B) and (C)]. While non-covalent CNT-drug interaction takes place *via* π–π stacking, different pathways have been described for covalent attachment of bioactive molecules including acylation and 1,3-dipolar cycloaddition of azomethine ylides reactions [206-210]. As for many other materials, the suitability of CNTs as drug carriers for cancer therapy critically depends on their pharmacokinetic profile goverened by a sufficient transportation to the relevant sites *via* the blood (intravenous) or lymphatic (subcutaneous and abdominal) circulation. Existing data are not yet conclusive to prove efficacy [209]. Indeed, it is already known that CNTs quickly disperse throughout the body, regardless of the administration site, preferentially accumulate in kidney, stomach and bones with 94% clearance from the urine and 6% from biliary pathways, showing potential for carrying drugs and radiotherapeutics to different organ sites and a vital 100% particle and excess drug clearance [211].

The establishment of CNTs and C-based nanostructures in oncology and radiotherapy requires in-depth *in vitro* and *in vivo* biological response assessment [212]. Uncertainty of the biological outcomes (positive and negative toxicological effects) for CNT-based materials suggests that using them in drug delivery systems is far reached [213-215]. Furthermore, systemic toxic effects of CNTs such as inflammation, fibrosis, granulomas, and necrosis *via* strong interference with the

cellular redox couples also demonstrate the biological hazards post exposure conditions [216]. However, convincing literature data indicate that shorter and thicker CNTs are safer than the longer and thinner ones [217-219]. The application of CNTs for drug delivery and radiotherapy will also require surfacemodifications (material engineering) to improve the hydrophilic behavior and biocompatibility of the material for a changing cellular redox state [220, 221]. Several covalent or non-covalent functionalization approaches are known to optimize biocompatibility of CNTs using natural or chemically synthesized polymeric materials. These materials are active elements for effective delivery vehicles for chemotherapeutics and radio-sensitizing agents [222-224]. The manipulation of ROS level by redox modulation *via* functionalization has been suggested to be feasible for selectively killing cancer cells without any effects to the normal cells [220]. Hence, a key challenge in radiooncology is the development of effective delivery systems for chemotherapeutics, bioactive molecules for ROS generation, or engineering novel materials that reduce the antioxidant defense in the cancer cells. We hypothesize that, amongst others, CNTs allowing controlled release of reactive oxygen to the cancer cell components followed by local irradiation can enhance tumour cell death and treatment outcome [225]. In any case, material engineering and extensive suitability studies are key issues for progress.

Like CNTs, graphene and/or graphene oxide exhibit distinct properties (Figure 5A) [60, 226-229]. Graphene is a promising material in medicinal research due to its high cellular interaction and efficient uptake by endocytosis; although a clathrin-dependent endocytosis process has been documented recently for graphene oxide internalization [230], a detailed overview of mechanistic material-cell interactions is not yet possible [231, 232]. Nonetheless, extensive studies focussing on graphene fabrication and its use in tissue scaffolding, bio-imaging, photothermal ablation of malignant cells, and targeted drug delivery have been performed [233, 234]. The physical and chemical interactions such as van der Waals forces and hydrogen bonds are known to be the driving forces for the drug loading in the graphene network [235].

Like CNTs, graphene also has bio-response issues during exposure and might significantly affect normal cells when it is used as drug delivery vehicle. The sources of such responses include graphene edge defects or internal defects of graphene oxide sheets for the generation of massive intracellular ROS [236, 237]. Other cytotoxicity mechanisms involve direct cell membrane damage, depletion of micronutrients, adsorption of nucleic acids, and the DNA intercalation through coordination with chelating ions [238-241]. Pharmacokinetics data show

graphene/graphene oxide sheets get accumulated in the lungs, liver and spleen, with a much longer retention time (even up to one month) in the lungs, where they induce strong dose-dependent inflammatory cell infiltration, pulmonary edema, and granuloma formation [242, 243]. This observation is strictly related to the size, where micro-sized graphene/graphene oxide causes severe inflammation response as compared to 100 - 350 nm sheets. In addition, while the graphene/graphene oxides are quickly cleared through the renal routes, the micro-sized particles are preferentially expelled by liver secretion into the biliary tract [60, 244]. It is interesting to note that the graphene surface is immediately covered by biomolecules (proteins, lipids, enzymes) in the cellular interior thus acquiring a new "biological identity" with a dramatic reduction in bio-response profile both *in vivo* and *in vitro* [245, 246]. Functionalization of graphene/graphene oxide sheets is performed *via* strong chemical reactions (nucleophilic and electrophilic substitutions, condensation and addition reaction) between the functional groups (carboxylic, epoxy and/or hydroxyl) and graphene edges, defects, and the basal plane [243, 247]. While covalent and non-covalent graphene systems showed promising drug delivery characteristics, the integration of this research domain with radio-sensitizers for cancer therapy might significantly contribute to the existing state of the art in this field [248, 249].

In summary, CNTs and graphene are promising materials for the use as carriers for chemo- and radio-therapeutics. The intrinsic properties of the carbon surface play a synergistic role in determining biological efficacy and tumor remission. It is clear that the precise synthesis and functionalization of these classes of materials are essential pre-requisites for future studies. Hence, there is a need for fabricating suitable carbon based vehicles possessing high biocompatibility *via* functionalization with suitable polymeric materials before using them for chemotherapeutic and/or radio-sensitizer delivery in combination therapies.

Polymer functionalization for therapeutics

In general, the literature shows massive development of nanomaterials and biodegradable delivery systems for improving the efficacy of cancer therapy. Such systems are known for increasing drug solubility in aqueous solution, optimizing pharmacokinetic properties and enhancing intratumoral drug concentration [250]. Other materials recently developed include carbon based materials (discussed earlier) and polymeric materials, protein NPs, organic liposomes, micelles and dendrimers [250, 251]. These nanostructures have been demonstrated as

delivery vehicles to transport different anticancer agents (chemical drugs, nucleic acids, antibodies and simultaneous multiple drug delivery) to the tumor sites [250, 252]. While the systematic delivery of the drugs to the target sites are now possible, low tumor-to-blood concentration ratio prevents selectivity and triggers severe toxicity in the cells. In contrast, NPs are able to accumulate within the tumor *via* passive and/or active targeting providing a very high local concentration of the drugs in the tumor tissues. The passive targeting of such NPs relies on the dwelling of these particles within the tumor tissues giving rise to enhanced permeability and retention (EPR) effect (unique feature of the tumor vasculature) [253, 254]. After particle entry and extravasation from the hyperpermeable tumor vessels to the tissue compartment, NPs can increase the local concentration of anticancer drugs by controlled release in the tumor cells. Depending on their sizes, NPs can be internalized by tumor cells through endocytosis mechanisms and passive diffusion across cellular membranes [255-259]. It should be noted that passive tumor targeting might lead to dramatic accumulation of the drugs in tumor sites as high as 5-10 times compared to the free drugs.[260] Furthermore, nanocarrier-based agents have a prolonged half-life compared to the free drugs while simultaneously decreasing systemic toxicity [255, 261, 262]. The representative examples of such delivery systems approved by the FDA (US food and Drug administration) for cancer therapy and the treatment of metastatic breast cancer patients in the European Union include albumin coated NPs for paclitaxel delivery, Abraxane®, poly(ethylene glycol) (PEG) - coated doxorubicin filled liposomes, Doxil® and non-pegylated liposomal doxorubicin Myocet® [255, 263]. The EPR-dependent delivery of nanocarriers to the tumor sites suffer from several limitations (1) irregular tumor vascularization and poor blood flow inside hypoxic tumors (2) high tumor interstitial fluid pressure impeding the uptake of the particles by tumor cells [264] (3) limitation of the NP internalization due to lack of specific binding motifs to the tumor cells (4) inhomogeneous drug distribution, adsorption and metabolism within the tumor tissues resulting into drug resistance. Hence, the strategic development to overcome these limitations of drug delivery systems would be to efficiently bind NPs to the tumor-specific antibodies or ligands. These targets then bind to the tumor surface receptor inducing receptor-mediated endocytosis releasing the drugs inside the cells. This approach provides high target specificity as well as drug delivery efficiency avoiding drug resistance mechanisms as shown in recent preclinical studies in different types of cancers using these nanocarriers [265-268]. Hence, to overcome the limitations specific functionalized NPs will be designed and will be used in various nano-sensitizers for cancer therapy. The use of

biocompatible NPs able to deliver nanomedicines at the target cancer sites without affecting normal cells would be an alternative for reducing side effects and biological toxicities. In oncology, polymer therapeutics consist of an anticancer drug covalently bound to a water-soluble macromolecular system from both, natural (*e.g.* polysaccharides, proteins, peptides) and synthetic polymers (polyethylene glycol, *N*-(2-hydroxypropyl) methacrylamide-HPMA, polyethyleneimine – PEI, poly(L-lysine) – PLL) [269-271]. In principle, the polymer-drug conjugation involves using a biodegradable linker to obtain polymeric prodrug and/or formation of non-cleavable chemical bonds (for examples please see Figure 5C and Figure 6) [272, 273].

To date, different polymer therapeutics are in clinical trial aiming at effective anticancer activity and demonstrating the importance of polymeric moieties and their chemical functionalities [274-276]. FDA (US food and Drug administration) approval was obtained for the anti-tumor protein neocarzinostatin conjugated to styrene maleic anhydride (SMANCS), and Pegaspargase or Oncaspar (PEG–L-asparaginase) for the treatment of acute lymphoblastic leukemia [277-280]. The clinical trials also involve polymer conjugates of traditional cytotoxic drugs such as platinates, camptothecin and analogs, paclitaxel/docetaxel, irinotecan, methotrexate, and gemcitabine [132, 281-295]. The concept of water-soluble polymeric-drug conjugates was first proposed by Ringsdorf and hypothesizes about the possibility to modulate either pharmacokinetic profiles of the linked drug, or the site-specificity for the insertion of homing moieties (Figure 5 B and Figure 6, see covalent and non-covalent linkage) [296, 297]. The conjugated system consists of three different units, (1) a region-device unit for controlling physicochemical properties (2) a drug-linking unit, and (3) an active targeting unit (*e.g.* monoclonal antibody) allowing for site-specificity at the cellular level [298, 299].

Conventional anticancer agents suffer from a relatively low therapeutic index and toxic side effects. Hence, due to their low molecular weight, these anticancer agents allow fast clearance from the circulation *via* renal filtration [283, 300]. Their conjugation to macromolecular systems with improved pharmacokinetic and pharmacodynamics properties [301] can be described as (1) increased solubility in biological fluids [302], (2) increased circulation time in blood [303, 304], as a function of the polymer size [305], (3) decreased toxicity [306], (4) ability to overpass drug resistant mechanism [307], (5) ability to elicit immunostimulatory effects [308, 309], and (6) the possibility to confer active targeting behavior [310, 311]. In addition, polymer therapeutics offer the possibility to combine synergistically radiotherapy and drug targeting [312]. The radio-

sensitizing effect of a chemotherapeutic agent can be further enhanced by delivering an optimal concentration of the drug maintained in the tumor for a prolonged period [313]. Some key examples of this rationale are poly (L-glutamic acid)-paclitaxel (Xyotax) and albumin-paclitaxel (Abraxane) conjugates. The results demonstrated that the Xyotax was able to reduce the original dose from 53.9 to 7.5 Gy, resulting into 50 % tumor cure in a mouse model. The combination of polymer therapeutics with single dose radiotherapy on ovarian carcinomas showed a dose reduction from 66.6 to 7.9 Gy [314].

In a phase I clinical trial involving 12 patients with localized esophageal and gastric cancer, the same conjugate (6 doses; weekly) in combination with fractionated radiotherapy (28 cycles; 1.8 Gy daily), allowed four complete responses, and an additional seven partial responses (with reductions in tumor size of more than 50 %) [315]. In another study, Abraxane (radio-sensitizing agent) exposed to ovarian or mammary carcinoma mouse models showed a reduction of the required dose producing 50% tumor cures from 54.3 to 35.2 Gy, where the increase in the normal tissue radio-toxicity was not observed [316]. The functionalization and/or coating of polymers on the NP surface allows for coupling of the NP properties with the polymer [317], giving rise to engineered nanohybrids with increased performance for therapeutics oncology or radio-oncology. The interaction of functional nanohybrids (coupling pharmacokinetics and polymer therapeutics) with different human tumors exhibiting various properties including heterogeneity, less pronounced enhanced permeations and retention (EPR) effect, and high propensity of developing resistance to therapies, might increase the performance and reduce the gap between preclinical and clinical human trial data [318], Preliminary data in this direction involve testing of the chemo- and radio-therapeutic efficiency of functional nanohybrids composed of three components comprising (1) polyphenol groups (biologically active component) (2) suitable polymeric materials (biocompatible and stabilizing counterpart), and (3) CNT (cell-interacting element). Results showed that nanohybrid materials with polyphenol groups (bioactive component) suffer from unfavorable pharmacokinetic profiles with low stability in serum and dramatic reduction of *in vivo* efficiency, despite promising observation *in vitro* [319, 320].

In a second investigation, polymer-flavonoid conjugates and in particular a dextran-catechin conjugate obtained from enzyme catalysis *via* free radical reaction was used as therapeutics [321]. Results showed remarkable pharmacokinetic properties, enhanced anticancer activity in pancreatic cancer and neuroblastoma cells, as compared to free flavonoid and superparamagnetic

NPs (SPIONs) [322-324]. Similarly, the insertion of quercetin into an acrylate polymer chain resulted in a polymeric material with high chemical stability. This therapeutics was used as a component of CNT nanohybrids with high anticancer efficiency and synergistic effect in combination therapy with cisplatin [325-327]. In a pilot study, also anticancer or the radio-sensitizing effect of polyphenols was demonstrated, that can be significantly enhanced after incorporation into gelatin-CNT nanohybrids, opening a new research domain in radiooncology [319, 328]. In the latter case it was shown that combination of irradiation and Catechin-nanohybrids can potentially be used for radio-sensitization and eradication of prostate cancer stem cells. A combination of X-ray and treatment with the nanohybrids caused a decrease in the protein level of stem cell-related transcription factors and regulators including Nanog, Oct4, and β-catenin leading to an increase of cancer cell radio-sensitivity. One may hypothesize about a multifactorial combination therapy involving functional nanohybrids, where the efficiency of suitable chemotherapeutic/radio-sensitizing agents is modulated by means of chemistry, formulations, pharmacokinetics, and biomedicine, to overcome the possible drawback and toxicity concerns enhancing the therapeutic efficiency and safety to reach higher tumor remission rates.

Tumor targeting with nanomaterials

The literature shows massive development of nanomaterials and biodegradable delivery systems for improving the efficacy of cancer therapy. Such systems are known for increasing drug solubility in aqueous solution, optimizing pharmacokinetic properties and enhancing intratumoral drug concentration [250]. To name few of those material systems recently developed include carbon based materials, polymeric materials, protein NPs, organic liposomes, micelles and dendrimers [250, 251, 329]. These nanostructures have been demonstrated as delivery vehicles to transport different anticancer agents (chemical drugs, nucleic acids, antibodies and simultaneous multiple drug delivery) to the tumor sites [250, 252]. While the systematic delivery of the drugs to the target sites are now possible, low tumor-to-blood concentration ratio prevents selectivity and triggers severe toxicity in the cells. In contrast, NPs are able to accumulate within the tumor *via* passive and/or active targeting providing a very high local concentration of the drugs in the tumor tissues. The passive targeting of such NPs relies on the dwelling of these NPs within the tumor tissues, tissues giving rise to enhanced permeability and retention (EPR) effect (unique

feature of the tumor vasculature) [253, 254, 330, 331]. After NP entry and extravasation from the hyperpermeable tumor vessels to the tissue compartment, NPs can increase the local concentration of anticancer drugs by controlled release in the tumor cells. Depending on their sizes, NPs can be internalized by tumor cells through endocytosis mechanisms and passive diffusion across cellular membranes [255-259]. It should be noted that passive tumor targeting might lead to dramatic accumulation of the drugs in tumor sites as high as 5-10 times compared to the free drugs [260]. Very recently, it was shown that after initial near-infrared photoimmunotherapy of tumors, the EPR effect is drastically enhanced. This phenomenon is termed super EPR (SUPR) effect [332-334]. Furthermore, nanocarrier-based agents have a prolonged half-life compared to the free drugs while simultaneously decreasing systemic toxicity [255, 261, 262]. The representative examples of such delivery systems approved by the FDA for cancer therapy and the treatment of metastatic breast cancer patients in the European Union include albumin coated NPs for paclitaxel delivery, Abraxane®, poly(ethylene glycol) (PEG) - coated doxorubicin filled liposomes, Doxil® and non-pegylated liposomal doxorubicin Myocet® [255, 263]. The EPR-dependent delivery of nanocarriers to the tumor sites suffer from several limitations as (1) irregular tumor vascularization and poor blood flow inside hypoxic tumors, (2) high tumor interstitial fluid pressure impeding the uptake of the particles by tumor cells [264], (3) limitation of the NP internalization due to lack of specific binding motifs to the tumor cells, and (4) inhomogeneous drug distribution, adsorption and metabolism within the tumor tissues resulting into drug resistance. Hence, the strategic development to overcome these limitations of drug delivery systems would be to efficiently bind NPs to the tumor-specific antibodies or ligands. These targets then bind to the tumor surface receptor inducing receptor-mediated endocytosis releasing the drugs inside the cells. This approach provides high target specificity as well as drug delivery efficiency avoiding drug resistance mechanisms as shown in recent preclinical studies in different types of cancers using these nanocarriers [265-268]. Again, one needs to take care that the ligand actually survive until the NP has reached the tumor site [335], and that they do not increase the NP size too much. Hence, to overcome the limitations one should design specific functionalized NPs and use them in various nano-sensitizers for cancer therapy. The use of biocompatible NPs able to deliver nanomedicines at the target cancer sites without affecting normal cells would be an alternative for reducing side effects and biological toxicities. It is worth mentioning that the intratumoral application of radiolabeled particles is a

very efficient strategy to destroy cancerous tissues. This is for example demonstrated by the treatment of cancer patients with FDA-approved ^{90}Y-containing glass microspheres [336-338].

Promising yet challenging performance: magnetic delivery strategies

Targeted enrichment of drug-carrying magnetic NPs by the means of internally or externally applied magnetic fields is a promising technique for selective tumor therapy. The basic underlying physical principle hereby is that a magnetic dipole will experience a force in a magnetic field gradient. In this way, magnetic NPs can be directed with magnetic fields [339, 340]. Various magnetic materials on the nanoscale have been developed and applied in different systems. Magnetic implants are mostly deployed, with few exceptions, through blood vessels to guide magnetic drug delivery vehicles. One important approach is intrathecal administration of NPs post deposition of magnetic implants in the subarachnoid space predicted to treat tumors more efficiently *via* bypasses blood brain barrier [341]. The technique of magnetic implant assisted intravenous application of magnetic NPs offers possibilities for further improvements. The locally generated magnetic field could be enhanced by applying an additional external field to previously implanted micro-ferromagnetic wires. The subsequent NPs administration would lead to enrichments in the targeted area. However, magnetic deposition largely depends on the distance of the applied magnetic field, and therefore it is difficult to be achieved in inner body organs. Currently, some ingenious concepts were introduced to overcome these limitations; nevertheless they are still far reached from realistic clinical application [342, 343]. This is also true for systems which do not rely on magnetic attraction, but on the directional magnetotaxis of drug loaded self-propelling bacteria [344]. The intra-arterial application of FeO_x NPs guided by an external electron magnet could be a reasonable route towards clinical application [345]. Those who are developing targeting strategies aiming at clinical translation should follow quality controlled environmental (GXP) protocols, clinically approved technical equipment and pharmaceutically recommended nanoparticles.

Conventional chemotherapy, radiotherapy, or combinations thereof, lack sufficient treatment selectivity in the tumor area, and cause negative effects. Severe short- and long-term side effects can occur including haematologic toxicities, i.e. suppression of bone marrow, as well as non-hematologic adverse effects. This range from dysfunction of liver and kidneys, loss of hair, diarrhea, nausea to reversible but also irreversible skin reactions to lymphedema, tissue fibrosis and even induction of secondary cancers (see for example:[346-351]). One of the future

therapeutic challenges is thus the development of directed therapy approaches, addressing the tumor more specifically, while sparing the remaining tissues, and increasing the efficiency of the conventional chemotherapeutics or further active ingredients. In this context, nanomedicine in common offers a promising platform for directed applications, capable for reducing the negative side effects of conventional tumor therapy. Regarding the therapeutic applications, drug transportation in the NP-bound form makes even less soluble components available to reach tumor cells [352-354]. A multitude of antitumor drugs, radiotherapeutic nuclides, genetic material, and antibodies can use the NP delivery platform for an improved localized enrichment [355]. Among the materials most commonly used for drug-delivery systems are the NPs or nano shells made of natural or synthetic polymers, as well as metal or metal oxide NPs, such as superparamagnetic magnetic iron oxide NPs (SPIONs).The latter consist of iron oxide cores, often coated with organic materials such as fatty acids, polysaccharides, or polymers [356], to improve colloidal stability and to prevent separation into particles and carrier medium [357, 358]. The magnetic properties of SPIONs allow remote control of their accumulation by means of external magnetic fields. Conjugation of SPIONs with drugs, in combination with an external magnetic field gradient to target the NPs (so called “Magnetic Drug Targeting”, MDT), has additionally emerged as a promising strategy of drug delivery. For the concept of MDT with intraarterial administration of magnetic NPs, an appropriate NP size (80-150 nm) is important to attract them by means of an external magnet [359]. As demonstrated by *in-vivo* studies, MDT using mitoxantrone-carrying SPIONs result in increased drug payloads in the target tissue, followed by reducing their systemic dose and toxicity, leading to complete tumor remissions without side effects [345, 360, 361]. Prior to MDT treatments the localization and vascularization of the target tumor is visualized by cone beam flat panel angiography. Beyond that, functional imaging is an attractive tool to recognize target tumor cells in advance. Among the multitude of different possibilities it is worthwhile to especially mention optical coherence tomography (OCT) as useful device for the detection of cancer tissue [362]. Utilizing SPIONs as contrast agents there is another imaging modality called magneto-motive optical Doppler tomography (MM-ODT) which enables the in-vivo control tumor tissue which is labelled with SPIONs [363]. This would leverage the MDT application since the realtime control of the enrichment of previously applied particles is possible.

The underlying principle of MDT is illustrated in (Figure 7). Biocompatible-coated SPIONs functionalized with cytostatic agents are administered intraarterially into tumor-supplying vessels, avoiding major capture by the mononuclear phagocyte system (MPS). More precisely, the particles consist of super paramagnetic iron oxide cores, a primery layer of fatty acids as a linker to the second layer albumine, which is the coating shell in which the chemotherapeutic drug mitoxantrone is bound electrostatically. For the direction of the NPs, an electromagnet, with a magnetic field gradient of 72 T/m directly at the tip of the pole shoe, was installed [364]. The injection of NPs into the tumor-supplying vessel (*arteria femoralis*), and simultaneous magnetic field application over a VX2-squamous cell carcinoma placed at the hind limb of the rabbit led to convincing results. An enrichment of SPIONs in the tumor tissue was demonstrated using histology, MRI, and computed-micro- tomography (µCT) [365-367]. Further morphological investigations showed no pathological alterations in liver, kidneys, spleen, lung and brain [364]. The fact that SPIONs in common were usually applied for cancer imaging in liver using MRI, underlines their non-harmfulness. Latest investigations concerning toxicity of SPIONs showed significant effect via surface coating of the nanoparticles. Higher toxicity of SPIONs is related with stronger in situ degradation of the particles and therefore more release of iron ions [368]. In reverse, stabile nanoparticles, like those which applied for MDT, are considered to be safe.

Metabolic decomposition of the SPIONs normally occurs in liver and spleen, in analogy to the physiological iron metabolism. In these organs, iron oxide NPs could detect histologically for three months after application. Radiotracer studies using ^{59}Fe provided evidence that iron from the SPIONs was utilized into the hemoglobin biosynthesis [369] and the cellular uptake of iron oxide [364]. Electron microscopy verified by energy dispersive X-ray analysis visualized the iron oxide NPs inside tumor cells. The therapeutic application of drug-carrying SPIONs (5 %-10 %) for the treatment of tumors in rabbits showed complete tumor remissions [360]. This promising outcome has been further confirmed in a large scale study on the application of magnetic NPs in tumor bearing rabbits. The distribution profile after MDT displayed 57.2 % of the total recovery of administered drug, with 66.3 % of the NPs localized in the tumor region, as compared to less than 1 % of drug and NPs reaching the tumor region during conventional intravenous application without magnetic targeting [345]. Angiographic imaging clearly demonstrated that a single MDT application lead to complete and permanent tumor remission.

MDT could promote the development of super selective and highly efficient tumor therapy approaches. The basic idea of the attempted NP-supported delivery of radio-sensitizers and the respective subsequent irradiation is shown in Figure 8. Each of the applied systems acts on a certain body compartment over a certain time span and only marginally affects the respective region by causing *per se* just negligible effects in the tissues. Only at the intersection of both spheres, a tremendous increase of cell damage efficacy occurs, inducing tumor destruction. The surface of magnetic NPs can be modified and therefore numerous different radio-sensitizers may be at disposal for MDT. Keeping in mind, NP syntheses should be as complex as necessary but also as simple as possible. This is especially true for the implementation of quality controlled manufacturing concerning current good manufacturing (GMP) guidelines. The stability of the currently deployed SPION carrier has been already proven over time, and a suitable purification strategy is already available. These are preconditions for the overall implementation of a highly localized therapy with minimized side effects [370, 371].

Renal clearable nanomaterials with tumor specific binding as putative radio-sensitizers

Rapid elimination of nanomaterials containing heavy or toxic metals from the body *via* urinary system is the desired route for their clearance for minimizing potential health risks originating from nonspecific accumulation and long-term metabolic decomposition in the body. The material properties such as size, surface charge, shape and composition influences the pharmacokinetics of nanomaterials as well as their glomerular filtration [59, 372]. The bio distribution as well as blood residence time of circulating NPs depends fundamentally on their *in vivo* hydrodynamic diameter that can be substantially larger than their effective *in vitro* diameter [373]. This is a consequence of unspecific surface adsorption of serum components such as proteins and lipids [374], and in particular agglomeration, due to the presence of high ionic strength [375]. The formation of a biomolecular corona often results in trapping by the MPS and frequently requires adequate surface modifications to prevent this phenomenon [376, 377]. PEGylation for example reduced protein adsorption and thus increases retention times [378, 379]. For a complete analysis, NPs always have to be seen as hybrid systems, involving the actual NP core, a surface coating, and the biomolecular corona [380]. Consequently also the fate of the different NP compounds may vary with time. Retention of NPs including their byproducts in the MPS may trigger an immunological or an inflammatory response, *e.g.* by intracellular enzymatic breakdown leading

to the disruption of the protein corona, the surface coating and release of toxic metal ions [335, 381-383]. Many renal clearable nanoparticles with different compositions have been reported and proposed for medicinal applications [383, 384]. In addition to their applications as nanodiagnostics, renal clearable nanomaterials [385], have been discussed as nanotherapeutics for the treatment of diseases, primarily cancer, through photothermal and photodynamic therapy as well as NP-enabled radio-sensitization [383]. Regarding the latter, renal clearable nanomaterials containing chemical elements with a high atomic number (high Z elements) such as gold [386, 387] or gadolinium have been investigated in detail [388-397]. The chelates of such heavy metals, *e.g.* Gd, functionalized with polysiloxane network have been proposed as theranostic radio-sensitizers due to their (1) facile elimination through the kidneys following intravenous injection in non-tumor bearing mice, and (2) the fact that they accumulate passively in tumors of gliosarcoma bearing rats [252, 392-394, 396, 398]. Irradiation experiments using an orthotopic brain tumor model revealed significant increase in median survival time as compared with untreated animals due to the presence of the gadolinium-based nanomaterials in the brain glioma [393, 394]. It is important to note that the radiation resistance of head and neck squamous cell carcinoma was overcome by the combinational treatment of tumor-bearing mice with these gadolinium containing polysiloxane materials and photon irradiation (Figure 9) [395].

Glutathione-coated gold nanoclusters with core sizes below 2 nm possess attractive features for clinical use as next generation radio-sensitizers, such as substantial passive tumor accumulation and strong enhancement of external radiotherapy, combined with effective renal elimination and no significant liver or kidney toxicity. Upon intraperitoneal injection into tumor bearing mice, these materials predominantly distribute to tumor and kidneys within 24 h with low absorption in MPS. The gamma-ray irradiation on the NP- exposed tumors resulted in a substantial decrease of tumor volume and weight [388, 389]. In all these reports, the radio-sensitizing NPs accumulate passively in the malignant tissues, depending on the pathophysiological vascular architecture of fast growing tumors. In contrast to long circulating NPs, renal clearable agents with short blood retention time are less prone to passive tumor targeting, as they quickly diffuse back to the vasculature and re-enter the systemic circulation. This rapid efflux of passively accumulated NPs from the tumor results only in transient intratumoral presence without substantial retention. The efflux can be diminished by increasing the interactions between NPs and tumor cells and through improvement of cellular uptake. Active or ligand-mediated targeting represents a strategy to

enable selective recognition of certain membrane receptors or antigens on cancer cells, and to facilitate cellular internalization of NPs through specific interactions such as receptor-mediated endocytosis. A multitude of targeting agents including small molecules, peptides, nucleic acids, as well as proteins, antibodies and their fragments have been investigated for active nanomaterial targeting [383, 399, 400]. These materials have been identified as potential targeting agents for the epidermal growth factor receptor, representing tyrosine kinase overexpressed and/or deregulated in a variety of solid tumors [243, 401-406]. However, the covalent functionalization of nanomaterials with targeting agents is often accompanied by an increase of size and an alteration of surface characteristics [407]. Although extremely exciting and sophisticated ideas have been conceived concerning the application of renal clearable materials in the field of nanomedicine, their clinical translation often proves to be difficult. In order to attain this objective, reproducible and scalable synthesis procedures to obtain precise highly monodisperse and uniform nanomaterials are required [408, 409]. This issue is of fundamental importance for nanomaterials of all sizes as differences in size and shape have a substantial influence on their blood retention time, biodistribution and elimination [383, 410].

Hypothesis-driven development of new NPs for cancer therapy

Apart from drug delivering nanovehicles, noble metal-based NPs also show potential to increase drug release in the target cell due to enhanced DNA damage and tumor cell death mediated *via* ROS generation [411-414]. Recently, NPs such as ZnO and CuO obtained *via* flame aerosol technology (FSP) [415-422]. showed striking observations that they ionize in the cellular system, thus posing immediate biohazard (*via* ROS generation) in the living system (Figure 10 and 11). Dissolution has also been observed for ZnO NPs synthesized with wet-chemistry approaches [423]. The generation of NPs with controllable dissolution kinetics might be a useful therapeutic anticancer agent and under precise conditions of ionic release might selectively kill cancer cells (ongoing work, data not shown). The use of this robust and generic strategy, fine-tuning of the NPs' chemical composition, along with an engineered surface, would enable targeted approaches through intravital administration [424]. Although toxic effects for certain types of NPs have recently been reported, there is still a lack of knowledge for fully understanding their long-term effects in biological systems [425, 426]. The precise designing of engineered NPs (by either reengineering and/or by doping/functionalizing) could have significant impact in the cancer

therapy [427, 428]. The demonstration of physicochemical properties contributing to hazardous interactions at the nano/bio interface requires advanced techniques to meet these challenges [234, 429-431]. The cellular injury that may be resulting at the membranes, proteins, DNA, organelles, the circulation, and a variety of tissues and organs may show adverse bio-impacts at various oxidative stress levels [414, 432-434]. One approach for countering such impacts for cancer treatment would be to probe such NPs and their wide range of properties using a high-throughput screening platform (a mechanism based approach for screening engineered NPs *in vitro* for injury) to study their relationship to specific injury responses [435]. The validation of this information at the *in vitro* level to biological injury *in vivo* for developing a predictive toxicological paradigm at the biomolecular levels is essential for specific cancer treatments [436-438]. The unique properties of engineered NPs warrant safe implementation going beyond traditional hazard, exposure, NP impacts, and risk assessment models to during cancer treatments [439, 440]. It is known that ZnO NPs dissolve in the cellular interior and Zn^{2+} ions make their way to the different organs disrupting the cellular metabolism [see Figure 10 (A)] [409, 430, 441-445]. Hence, controlled release of Zn^{2+} (the concentration needed to chelate the cancer specific components) in cancer cells combined with radio-sensitizers (surface functionalization) might be the unique pathway for cancer treatment. Similarly, a new material based on rare earth metal oxide (REO) also showed significant dissolution profile and triggered cytotoxicity in the cellular environment. The uptake of the NPs in the lysosomes (using macrophage cell-lines) showed pH-dependent particle dissolution [Figure 10 (B)]. The released heavy lanthanide ions were chelated by the phosphates from the lysosomes giving rise to deposition of the urchin-shaped crystalline $LaPO_4$. After depleting the phosphate groups of the lysosome, the excess La^{3+} extracting the phosphate groups of the lysosome membrane leads to cascades of cellular responses such as organelle damage, cathepsin B release, NLRP3 inflammasome activation, and IL-1β release. IL-1β is responsible in progressive events including the generation of pro-fibrogenic growth factors by epithelial cells, resulting in pulmonary fibrosis. Hence it is clear that the NPs entering lysosomes or the uptake of the NPs in the cellular interior are prone to dissolution and induction of several biological pathways for hazard generation [431]. The central idea of using this material in cancer treatment is by monitoring of the ionic release of the radio-sensitizer functionalized NPs in the specific cancer cells to increase the reaction kinetics of $(PO_4)^{3-}$ depletion from the cancer cells without affecting the normal cells (*via* controlled NP delivery). It is known that the photo-toxicity paradigm (with Fe doped TiO_2 NPs) is based on the electrons that are excited to

the conduction band of TiO_2 creating a hole in the valence band *via* UV light irradiation [446]. The materials that are capable of separating e^-/h^+ pair in the electronic bands are technologically important, but are critically hazardous to the environment. The e^-/h^+ pair could then interact with surrounding H_2O and molecular oxygen to generate ROS ($HO^\bullet$ radical and/or superoxide) [429]. The high energetic UV light responsible for such charge separation is a regular obstacle for acquiring knowledge on biological effects through photo activation. Hence, a library of Fe doped TiO_2 was developed using flame spray pyrolysis which allowed electronic excitation at lower energy wavelength increasing the cellular apoptosis [429]. The flame aerosol synthesis is a cost-efficient route to new and functional NPs. The scope of the NPs that can be produced using FSP is much larger due to its utilization of liquid precursors that are directly atomized and ignited forming a spray flame [417, 418, 447, 448]. The liquid precursor, a mixture of organic solvents and metal organic precursors, carries all the energy into the flame. During combustion, nanoparticles grow at very high flame temperatures after the nucleation, surface growth, coagulation and coalescence [422]. The flame aerosol stream is quenched to room temperature with the cold gas [415, 417, 419, 420, 434, 449]. The availability of the different metal precursors makes FSP an attractive technique for the synthesis of single and mixed metal oxide particles and their functionalization with noble metals [450, 451]. Using this synthetic knowledge, *in-situ* mixing of photoluminescence functionalized Er^{3+}/Yb^{3+} doped La_2O_3 NPs with Fe doped TiO_2 can easily be produced and allows easy down conversion emission through functionalized rare earth doped La_2O_3 with wavelengths exactly equal to the band gap of Fe doped TiO_2 NPs [452]. The emitted light (in the near visible range, and thus harmless to tissue) will excite electrons in the Fe doped TiO_2 NPs and interact with the cancer cells. These combined effects can be exploited to trace the particles in the cells (due to its violet colour emission) at a specific location for possible interaction with the cancer cells to produce ROS species (Figure 12). The paradigm described here might have significant development towards cancer therapy but requires precise materials designing and characterization.

Demanding Intermediate: Preclinical Models for Nanoparticle Evaluation

In recent years, nano-modified drugs have shown improved material chemistry such as solubility, pharmacokinetics, and bio-distribution compared to small molecules [453]. NP based drugs can

be effective at lower doses making cancer treatment more economic and minimizing side effects. However, the design of such effective NP based drugs for translation into the clinics requires extensive and careful *in vivo* evaluation. The use of appropriate preclinical models of human cancers is key to this process. Models of choice should (1) resemble the pathophysiological and microenvironmental characteristics of the malignant disease of interest for target identification and/or validation, (2) allow to study pharmacodynamic/pharmacokinetic properties of novel NPs of interest, and (3) be suitable for exploring the utility of functionalized NPs to synergize with radiotherapeutic treatment.

Various animal models are available and should be carefully considered for NP testing based on their specific properties and the analytical endpoint(s) of interest. These include:

- Ectopic xenograft models – human cancer cells (primary or established cell lines) or tumor biopsy material injected or implanted (subcutaneously, intraperitoneally, intravenously, and/or intramuscularly) into syngeneic or immune-suppressed hosts.
- Orthotopic xenograft models - human cancer cells (primary or established cell lines) transplanted into the host organ that corresponds to their tissue of origin
- Carcinogen-induced models – animal tumors developing spontaneously after exposure to chemical or radiation stimuli
- Germ-line transgenic and conditional transgenic models (GEMMs) – animal tumors developing upon tissue- and temporal-specific regulation of specific (human-relevant) oncogenes or tumor suppressor genes

Further models of interest based on the above but with particular characteristics are:

- Primary human tumor grafts (implanted ectopically or orthotopically) – xenograft is expected to have the identical genotypic profile as the primary human tumor
- Humanized xenograft models – animal for human cancer cell engraftment (ectopically or orthotopically) is manipulated to develop human-like immune responses.

Ectopic xenografts are a valuable tool for the assessment of nano-drugs, radiotherapy, and other therapeutic components exploited for specific cancer treatments. In principle, these models have been used to study and quantify dose responses along with tumor pharmacodynamics [454]. Subcutaneous cancer cell implantation for example, allows to monitor tumor growth via simple calliper measurements and are easy-handling and efficient for demonstrating biodistribution and treatment response. So far, subcutaneous xenografts are the only feasible model for the assessment of the radiotherapeutically-relevant curative analytical endpoint tumor control dose

50 as the radiation dose required to cure 50% of tumor-bearing mice. This value should be lowered upon combination with radiosensitizing (drug) moieties [455]. Nonetheless, despite promising results in the literature, ectopic models have several disadvantages such as (1) irregular tumor growth in the host and loss of heterogeneity [456], (2) limited reflection of primary genetic profiles, clinical outcome and pathophysiological characteristics, [457], and (3) difficulty in studying angiogenesis induction and metastasis.

In orthotopic cancer models, explants of the primary tumor cell are injected to the tissue of the malignancy origin. Results from this model are realistic and show enhanced metastatic rates of human cancer progression [458]. It is important to note that immune-compromised animals or humanized mice are absolutely essential for the injection and engraftment of human cancer cells [459].Comparing orthotopic with ectopic xenograft models, the former have the possibility to develop an organotypic microenvironment, recapitulate the local milieu, and study the role of organ-specific cell-stromal interactions on tumor growth and metastasis [138]. These models have been successfully applied in preclinical trials to evaluate drug-dose combinations and animal survival [460]. Major drawbacks are the high variation in the tumor development (due to animal morbidities during orthotopic surgical implantation) and the requirement of sophisticated imaging for tumor detection and monitoring.

Animal tumors (Carcinogen-induced models and GEMMs) are highly relevant for mechanistic studies and immune response monitoring. Examples of such well known carcinogen-induced pre-clinical models in immune-competent mice and rat include: NMU (*N*-nitroso-*N*-methylurea)-induced mammary carcinoma, DEN (diethylnitrosamine)-induced hepatocellular carcinoma, and NMU- and MNNG (*N*-methyl-*N*-nitro-*N*-nitrosoguanidine)-induced gastric carcinoma models [461-463]. These models are key in determining the progression and stage during treatment and evaluation of suitable preventative interventions with therapeutic agents. However, latest improvements in genetic engineering have particularly extended the panel of transgenic (genetically engineered mouse tumor) models and broadened their use for demonstration of complex biological processes and therapy testing in the presence of a mature immune system. Transgenic and genetically engineered mouse tumor models directly relate to the dysregulation of oncogenes and tumor suppressor genes. They are histologically and genetically comparable or even similar to human cancers but tumors often develop asynchronously which can be problematic for standardization of treatment testing. Most of the models show low penetrance and/or latency in tumor development and growth suffering from the strict requirement of

exclusive tissue-specific promoters regulating the transgene expression [464]. Notably, novel approaches for the development of conditionally regulated transgenic models with high penetrance properties and clinical relevance have recently been described [465]. As for orthotopic models, all of these organ-specific animal tumors require sophisticated imaging technology, and application of clinically relevant fractionated radiotherapy regimes for combination treatment testing will not be possible without an advanced 3-D animal treatment planning strategy.

Patient-derived tumor xenografts (PDTX) are innovative fast emerging models for preclinical trials in cancer treatment. PDTX develop from primary human tumor material excised within a few hours of surgery and grafted into immune-deficient mice preserving the genotypic and phenotypic features of the original tumor. Literature data imply a 30-40% failure rate for engraftment of implants, and successfully implanted material requires several months (6–8) for *in vivo* propagation via serial transplantation [466]. While this model looks particularly promising with respect to physiological and clinical relevance for therapy response prediction, quality assessment of the freshly excised primary tumor tissue is difficult, estimation of engraftment success is thus not possible and handling remains expensive and time-consuming. In any case, PDTX are delicate models for systematic and extended treatment test programs and also restrict the study of immunotherapies, i.e. anti-cancer agents that target components of the immune system cannot be studied. In this context, humanized xenograft models might be the next generation model.

Humanized xenograft models are obtained *via* co-engrafting tumor fragments with human peripheral blood/bone marrow cells into NOD/SCID mice and allowing reconstitution of the murine immune system. This approach enables to (1) resemble human immune responses in a mouse model, (2) monitor the impact of human immune cells on tumor progression and metastasis and (3) study the impact of immune modulators and drugs directed against or stimulating the human immune system [467].

Different models might be exploited to verify the potential use of nanomaterials for anti-cancer treatment. Indeed, due to the limitations and advantages of each model, exposure of the same nano-drug in several animal models might be required to evaluate its *in-vivo* activity, pharmacokinetics and bio-responses alone and in combination with irradiation However, as stated by Ruggeri et al. in their highly informative review on pre-clinical animal models of cancer [456]: “ultimate proof of concept for efficacy and safety of novel oncology therapeutics lies in

humans" This is off course true for any nano-radiosensitizer approaches and the concepts highlighted herein.

Conclusion

While the standard radio/chemotherapy can kill the bulk tumor cells, even a small population of surviving malignant cells (radio-resistant and putatively multi-drug resistant cancer cells) can result in tumor recurrence and metastasis. Hence, the use of specially-designed, reengineered NPs for radiooncology is proposed to critically contribute to the sensitization of all tumor cells, including the disastrous radio-resistant population, thereby improving the cure of cancer by biologically individualized radiotherapy [Figure 13]. For future use of NPs in radiooncology a potential comprehensive strategy as discussed in this report would focus on (1) precise NP synthesis for radiooncology and cancer therapy, (2) functionalization of these NPs using radio-sensitizers, (3) biological response assessment using conventional and sophisticated *in-vitro* and *in-vivo* models, and (4) pre-clinical and clinical testing for treatment outcome. Future objectives in developing NPs for cancer treatment should lie on the clinical utilization of radio-sensitizing strategies based on (1) the optimized delivery of known and novel radio-sensitizing drugs, in particular therapeutic small RNA molecules for radio-sensitization (no report yet), and (2) manufacturing of nanomaterials that can be activated during state-of-the-art individualized radiotherapy [Figure 13 (A) and (B)].

The multidisciplinary expertise will allow for exploring a multifactorial combination therapy involving functional nanohybrids, where the efficiency of suitable therapeutic, radio-sensitizing agents is modulated by means of chemical formulations, pharmacokinetics, and biomedicine to overcome the possible drawback and toxicity concerns enhancing the therapeutic efficiency and safety to reach high tumor remission rate. The combination of NP research, their functionalization towards radiooncology and clinical trials is one of the potential research domains and directions for curing cancer in the 21st century.

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Figure Captions

Figure 1. Schematic illustration of key intracellular tumor radioresistance mechanism and possible therapeutic intervention for radio-sensitization. Ionizing radiation is inducing single (SSB) and double strand breaks (DSB) within the DNA by direct or indirect effects via the generation of free radicals. These DNA damages are recognized by DNA repair machinery proteins, e.g. phosphorylated histone H2 isoform AX (γH2AX), protein kinase ataxia-telangiectasia mutated (ATM), ataxia telangiectasia, Rad3-related serine/threonin protein kinase (ATR), and DNA-dependent protein kinase (DNA-PK). High numbers of unrepaired residual DNA breaks lead to tumor cell death. The efficacy of radiotherapy is affected by three main tumor radio-resistance mechanisms (1) enhanced DNA repair capacity, (2) tumor hypoxia (low levels of oxygen (O_2) accompanied by reduced reactive oxygen species (ROS) due to the pathophysiological vascular network in the tumor tissue,, and (3) hyperactivation of cell survival signaling such as the phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathways and/or inactivation of the p53 tumor suppressor gene. Therapeutic interventions interfering with these mechanisms as exemplified herein can induce tumor radio-sensitization and may enhance the curative potential of radiotherapy upon combinatorial treatment..

Figure 2: (A) Overview of various (nanoparticle) systems for the delivery of RNAi-inducing agents [468]. For details, see text. (B) The complex nanoparticle systems rely on chemical surface modifications, aiming at reduced non-specific interactions with blood components or non-target tissues, improved pharmacokinetics (biodistribution, clearance/excretion, and

circulation halflife), enhanced biocompatibility and, in case of a coupling ligand, and targeted delivery. Beyond siRNAs, other chemically modified or non-modified RNA molecules, or other oligonucleotides, can be delivered as well. (C) Scheme of PEI-based nanoparticle (polyplex) formation, based on the electrostatic interaction between PEI and siRNAs. By combining the polyplexes with liposomes, lipopolyplexes are formed. Upon their endocytosis, the polyplexes or lipopolyplexes are released from the endosomal/lysosomal system due to the so-called proton-sponge effect, prior to release of the siRNA from the nanoparticle and the siRNA-induced target gene knockdown (for details, see text). Right panel: silencing of EGFP in stable reporter cell lines upon PEI-mediated delivery of siEGFP (lower panel) vs. negative control siRNA (upper panel). (D) (Lipo-) polyplexes have also been explored for therapeutic siRNA delivery in vivo. Upon systemic injection of lipopolyplexes, intact siRNAs are delivered into various organs including tumor xenografts, as indicated by the bands in gel electrophoresis/autoradiograhy representing full-length, [32] P-labeled siRNAs (upper right). In a subcutaneous tumor xenograft model (lower left), treatment with siRNAs targeting the anti-apoptotic proto-oncogene survivin (siSurv) leads to marked tumor-inhibitory effects, as compared to the negative control treatment group (siCtrl). This inhibition of tumor growth is based on target gene knockdown, as determined from the survivin levels in the tumors upon termination of the experiment (lower right). (A) Adapted with permission, copyright (2015) Elsevier Science. (D) Adapted with permission, copyright (2016) Elsevier Science.

Figure 3: Examples of liposomal or polymeric systems suitable for siRNA delivery. Polymers like PEI are available as branched or linear molecules (upper panels), and branched polymer structures also include dendrimers (upper right). DOTMA and DOTAP represent examples of older lipids, while SNALPs, comprising of the components shown in the lower right, have later on been developed for siRNA delivery in vivo.

Figure 4. High power magnified cation confocal microscopic images of MiaPaCa-2 cells transfected with star 3-siRNA showing nternalized siRNA had no or less colocalization (A) Systemic administration of star-POEGMA-siRNA gave rise to enhanced siRNA accumulation in pancreatic tumors in mice. siRNA binding efficiency and cell uptake of star-POEGMA-siRNA complexes in pancreatic cancer cells (B) Confocal microscope images demonstrating cell uptake of fluorescently labeled-siRNA (green shown by arrow) [185]. Adapted with permission, copyright (2008) American Chemical Society

Figure 5 (A) Representation of carbon nanostructures. [242] (B) Single-walled carbon nanotubes (SWNTs) may present antibodies with a higher local density to stimulate T cells to release Interleukin-2 (IL-2). The schematic representation (not to the scale) shows the anti-CD3-adsorbed CNT inducing T-cell (B3Z cells) stimulation. (C) Representation of CNTs used as a drug carrier. Cisplatin is covalently ligated to surface-oxidized CNTs as an effective anti-tumor agent, and a folic acid molecule is further coupled to the cisplatin as a targeting molecule. The large surface area of CNTs makes it possible to carry more cisplatins into tumor cells. Adapted with permission, copyright (2016) Elsevier, copyright (2008) American Chemical Society [242, 469, 470].

Figure 6. Putative chemical modifications of nanoscale materials (carbon nanotubes are shown as example) result in functionalized moieties that can interact with the surrounding cellular

components. Upper panel: Aromatic molecules functionalized on the particle surface via π–π interactions (non-covalent interaction). Middle panel: Polymers are wrapped around the carbon based NPs by non-covalent interactions (including π–π, van der Waals forces and charge-transfer interactions. Lower panel: Chemical groups that covalently bind to the surface react with styrene monomers to form polystyrene chains. Adapted with permission from nature publishing group copyright (2007) [471].

Figure 7: Principle of Magnetic Drug Targeting (MDT). Left side: Drug-carrying magnetic nanoparticles are injected intra-arterially close to the tumor. An electromagnet positioned in the tumor area directs and attracts the iron oxide particles in the tumor region. Right side: the particles themselves consist of the super-paramagnetic iron oxide core, a primary layer of fatty acids as biocompatible spacer and a second layer of albumin. The chemotherapeutic agent mitoxantrone is bound electrostatically inside the albumine layer.

Figure 8. A recognized tumor will be treated by Magnetic Drug Targeting in order to enrich radio-sensitizers which are enriched by NPs in the tumor area, followed by irradiating to induce neutron capture to destroy cells only in the region of boron or gadolinium accumulation.

Figure 9: Simplified scheme comparing the biodistribution of NPs. Renal clearable nanomaterials (blue) and their larger counterparts (green) differ substantially in blood retention time and route of excretion. The former can be eliminated rapidly from the circulatory system via bladder and urine after passing the glomerular filtration. Clearance of nanomaterials bypassing the renal filtration occurs mainly in the liver, where their metabolic decomposition and biodegradation take place. Both subsets of NPs can passively or actively target malignant tissues, whereas the later approach requires their functionalization with appropriate targeting moieties.

Figure 10. Particle dissolution mechanism in the cells (A) ZnO dissolution in the material-biocomponent interface and lysosome generates cellular toxicity through the release of Zn^{2+} ions inducing a cascades of harmful cellular events such as lysosomal damage, mitochondrial perturbation, ROS production, excitation of pro-inflammatory cytokine and chemokine production. (B) Cellular mechanisms showing pro-fibrogenic effects via rare earth oxides exposure. The uptake of RE oxides by macrophages and lysosomes damage cell organelles lead to IL-1β production causing pulmonary fibrosis. The lower cellular model shows molecular mechanism where phospholipids are dephosphorylated causing crystalline $REPO_4$ to precipitate on the surface. Adapted with permission from nature publishing group copyright (2009) and ACS copyright (2014) (ACS Editors' Choice article) [409, 431].

Figure 11. Flames spray pyrolysis (FSP) technique for NP synthesis. Left panel: The schematic diagram of the FSP reactor. Right panel: a photograph of a roaring flame during the NP production.

Figure 12. The combination of down conversion emission followed by near visible excitation for the e^-/h^+ pair separation using CNT non/functionalized Er^{3+}/Yb^{3+} doped La_2O_3 in-situ mixed with Fe doped TiO_2 an application which could be effectively tested in the cells.

Figure 13 (A) Schematic illustration of NP-based mechanisms for tumor radio-sensitization. Fast growing tumors depend on constant supply of nutrients and hence are able to induce vascular

sprouting, leading to an irregular vascular network inside the tumor (neoangiogenesis). In these tumors, the vascular network efficiently captures NPs, resulting into an accumulation in the tumor by several uptake mechanisms such as phagocytosis, pinocytosis and receptor/clathrin-mediated endocytosis. After internalization, endosomal escape may take place, followed by nuclear transport to release drugs (B) Schematic illustration of NP-based treatment modalities for tumor radio--sensitization. Standard cancer therapy is including a combination of radio- and chemotherapy. This treatment is reducing the tumor volume by killing tumor bulk cells, whereas radio-resistant tumor cells cells survive, regrow the tumor, leading to tumor relapse. The combination of radio/chemotherapy with NP-based systems including radio-sensitizing agents has the potential to sensitize the radio-resistant population and eradicate the tumor bulk cells together with the radio-resistant cells. Tumor control and patient cure can only be achieved when all tumor cells are killed.

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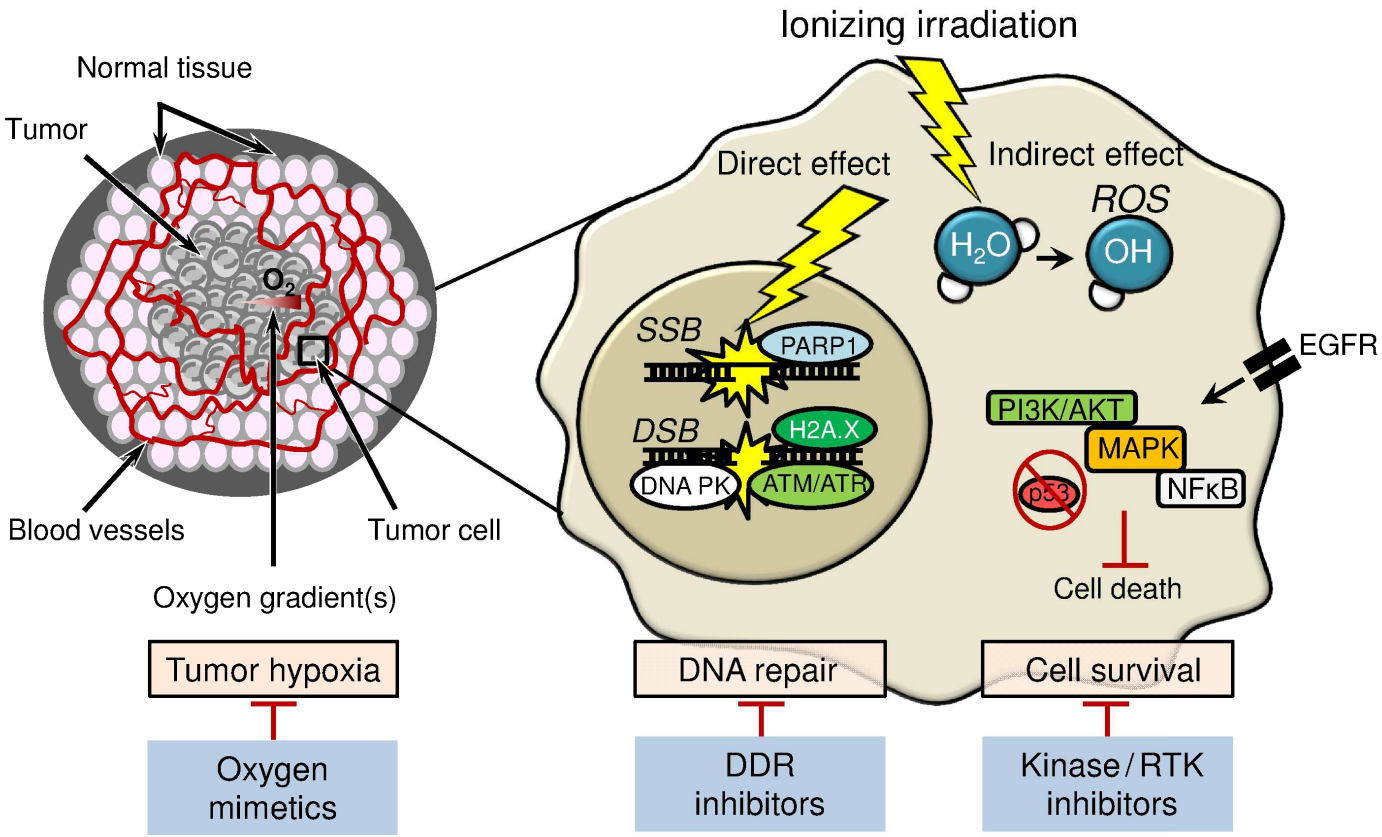
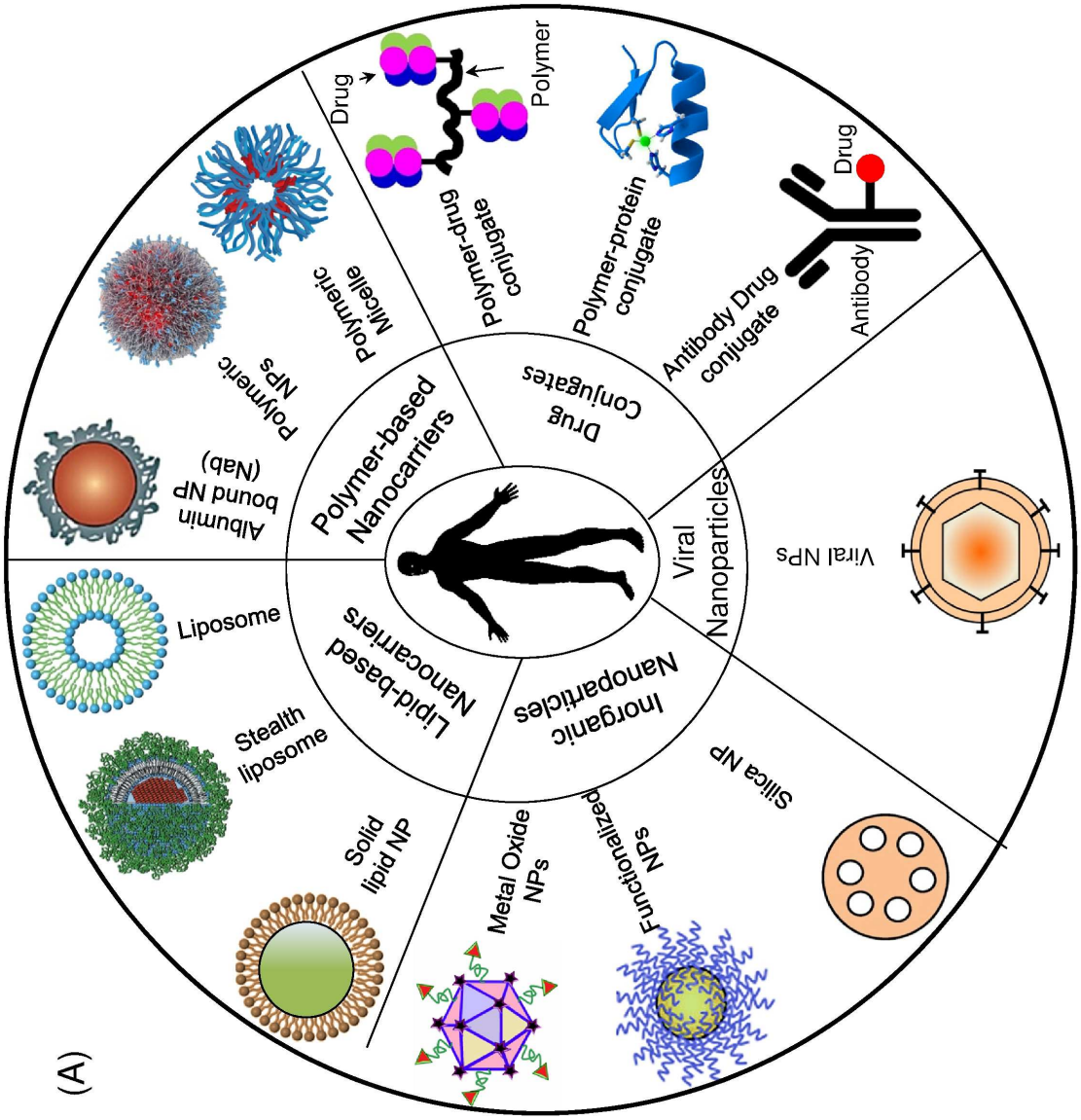
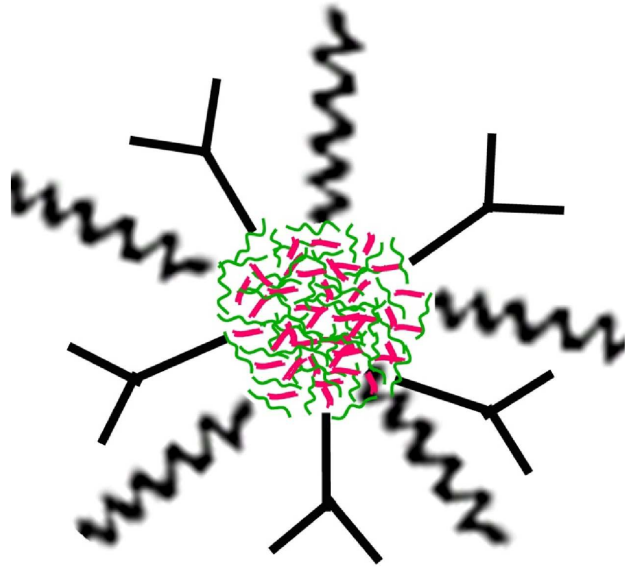



Polymer-based Nanocarriers
Drug Conjugates
Viral Nanoparticles
Inorganic Nanoparticles
Lipid-based Nanocarriers
Polymer-drug conjugate
Polymer-protein conjugate
Antibody Drug conjugate
Viral NPs
Silica NP
Functionalized NPs
Metal Oxide NPs
Solid lipid NP
Stealth liposome
Liposome
Albumin bound NP (Nab)
Polymeric NPs
Polymeric Micelle
Drug
Polymer
Antibody
Drug

(A)

(B)

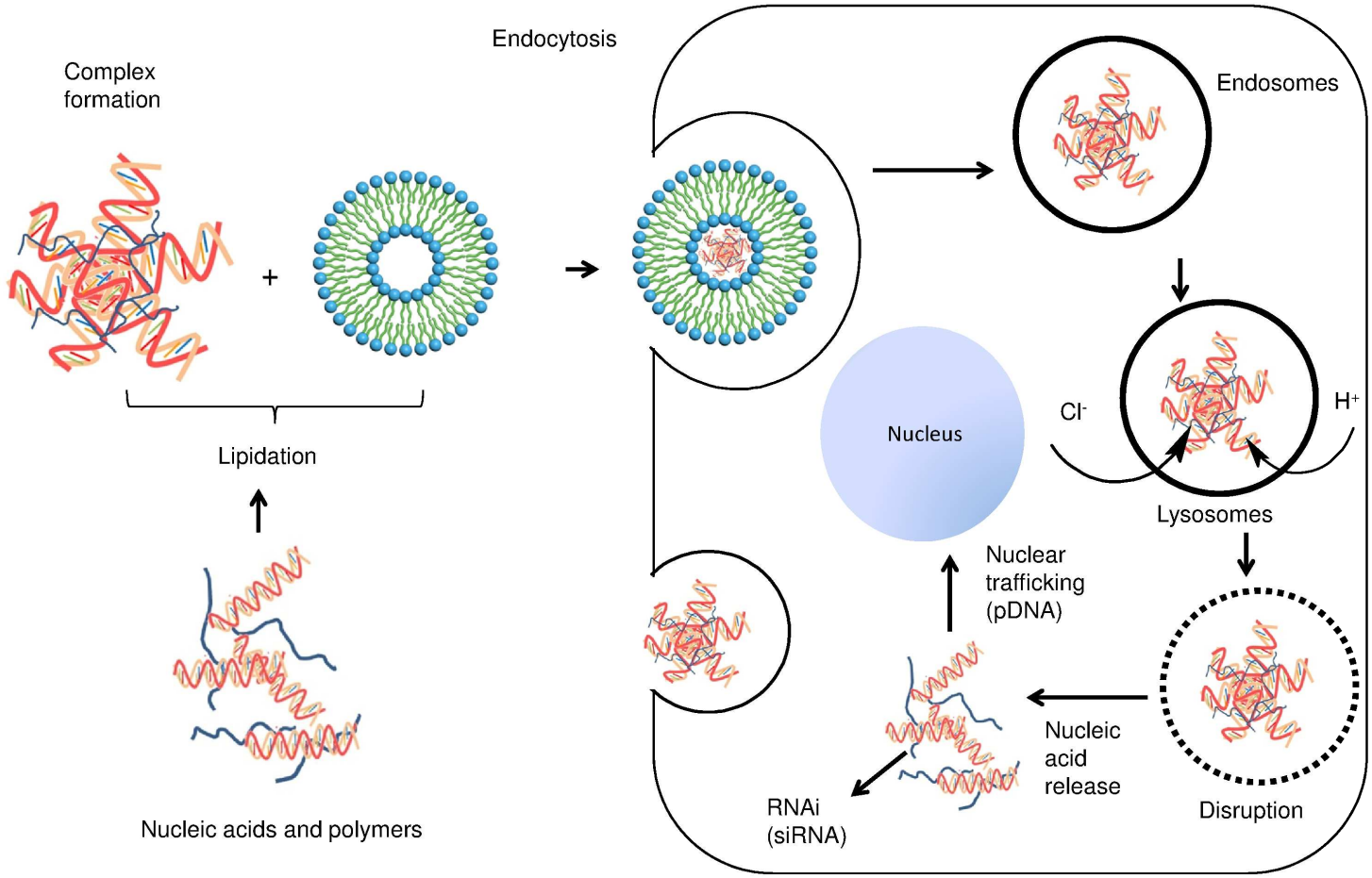


siRNAs, miRNAs
Novel therapeutic drugs

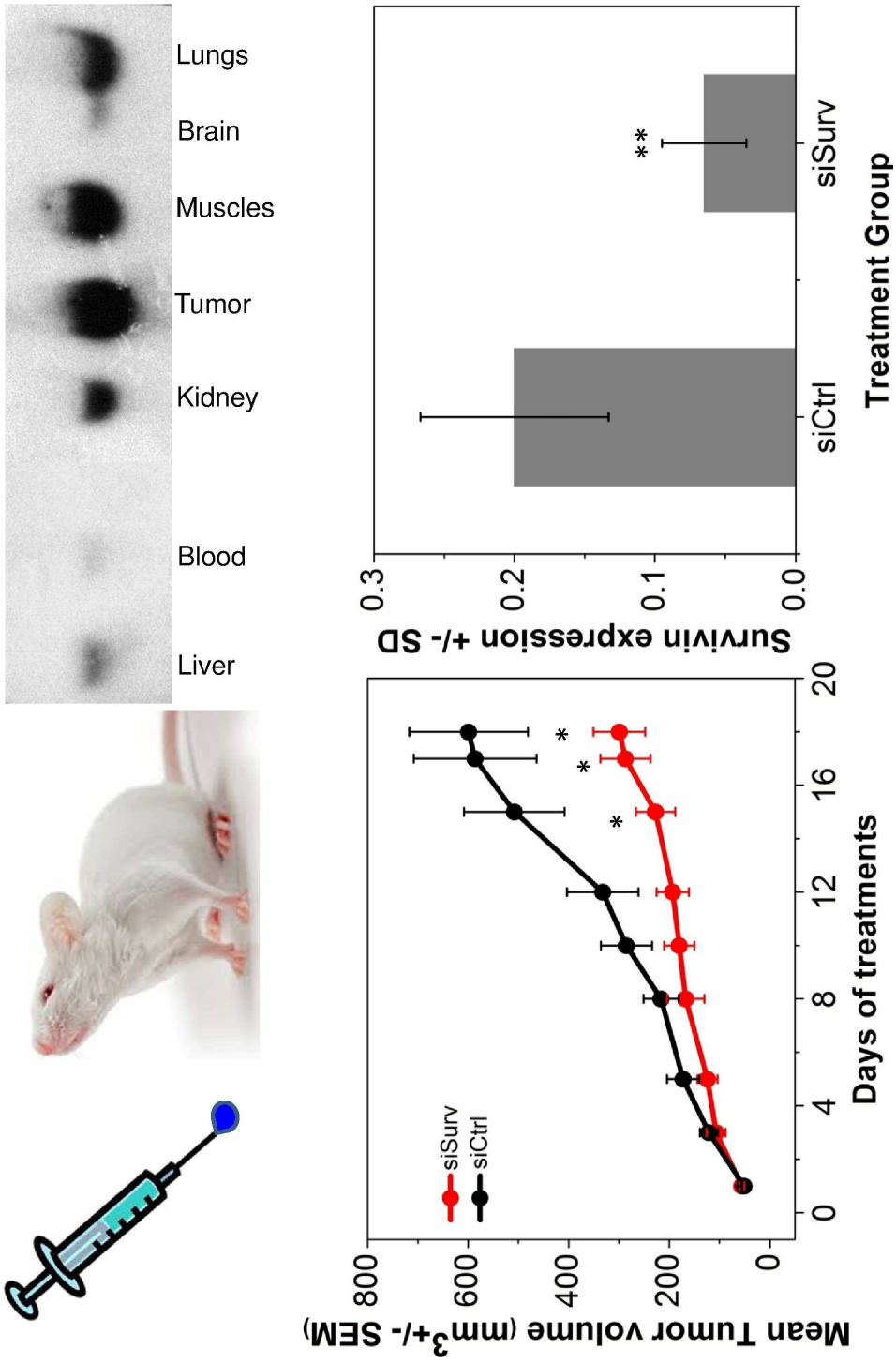
Chemical Modification
Reduced non-specific Interactions (1) modified pharmacokinetics (2) enhanced biocompatibility

Ligand coupling
Enhanced target cell / target tissue specificity and uptake

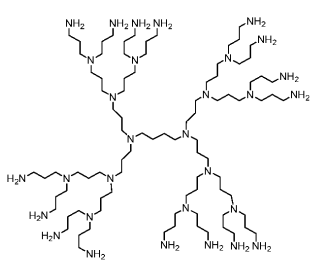
(C)



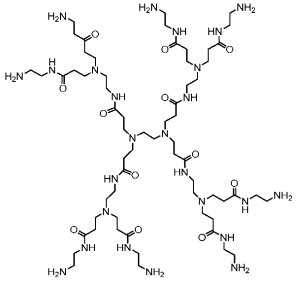
(D)


Liver
Blood
Kidney
Tumor
Muscles
Brain
Lungs
Mean Tumor volume (mm3+/- SEM)
800
600
400
200
0
siSurv
siCtrl
0
4
8
12
16
20
Days of treatments
Survivin expression +/- SD
0.3
0.2
0.1
0.0
**
siCtrl
siSurv
Treatment Group

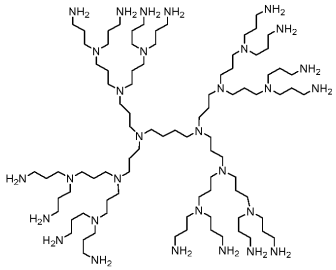
Cationic polymers



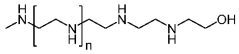
Branched PEI



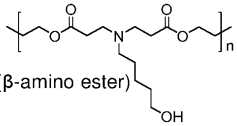
PPI dendrimer



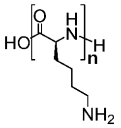
PAMAM dendrimer



Linear PEI

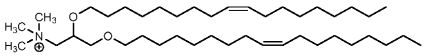


Poly(β-amino ester)

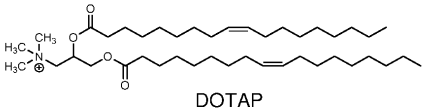


Poly-*L*-lysine (PLL)

Cationic lipids

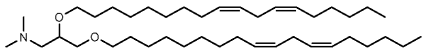


DOTMA (Lipofectin®)
First cationic lipid

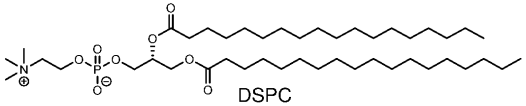


DOTAP

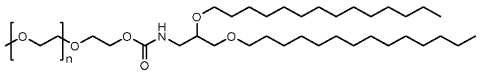
SNALP platform



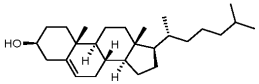
DlinDMA (ionizable lipid)



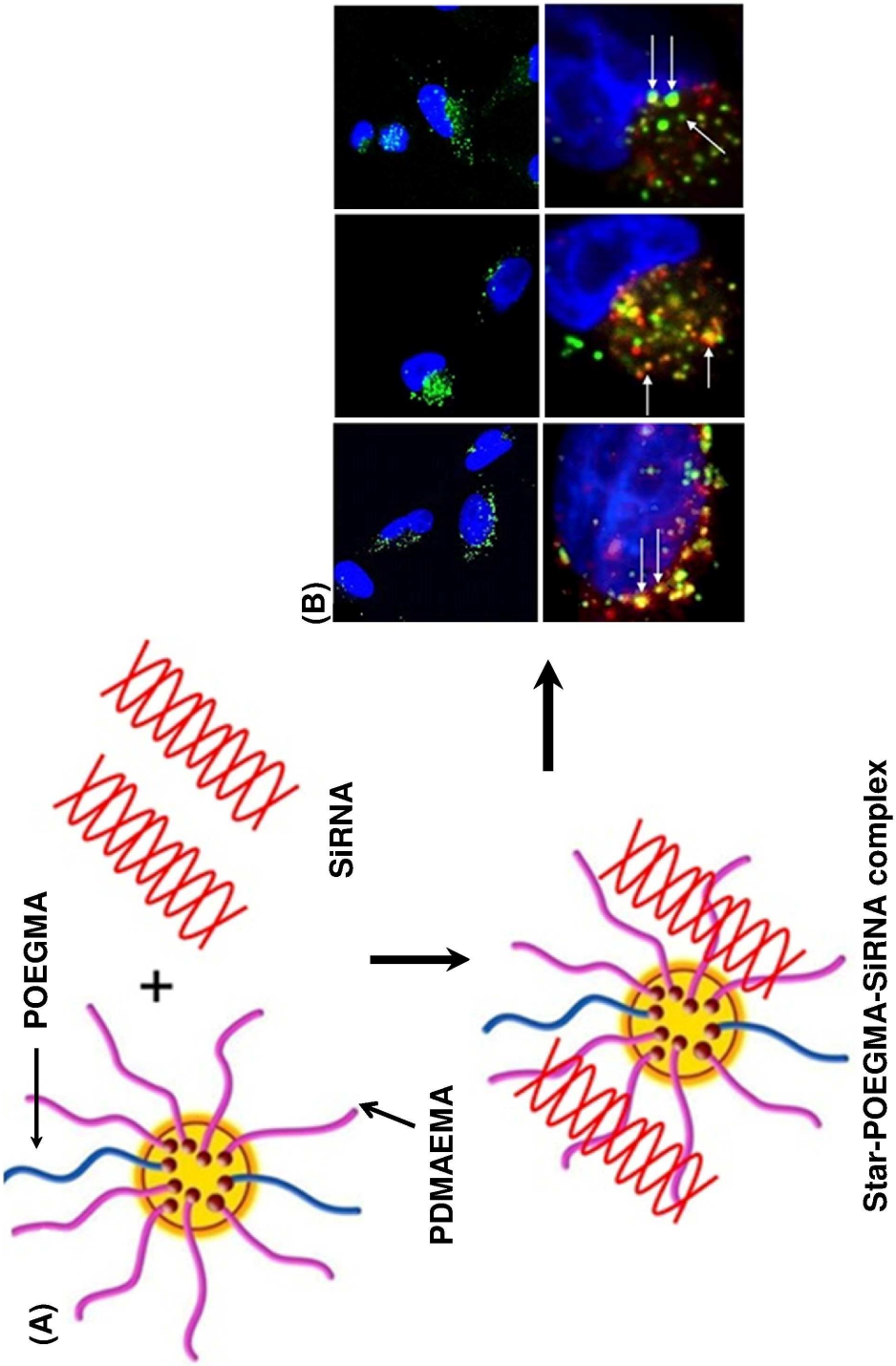
DSPC

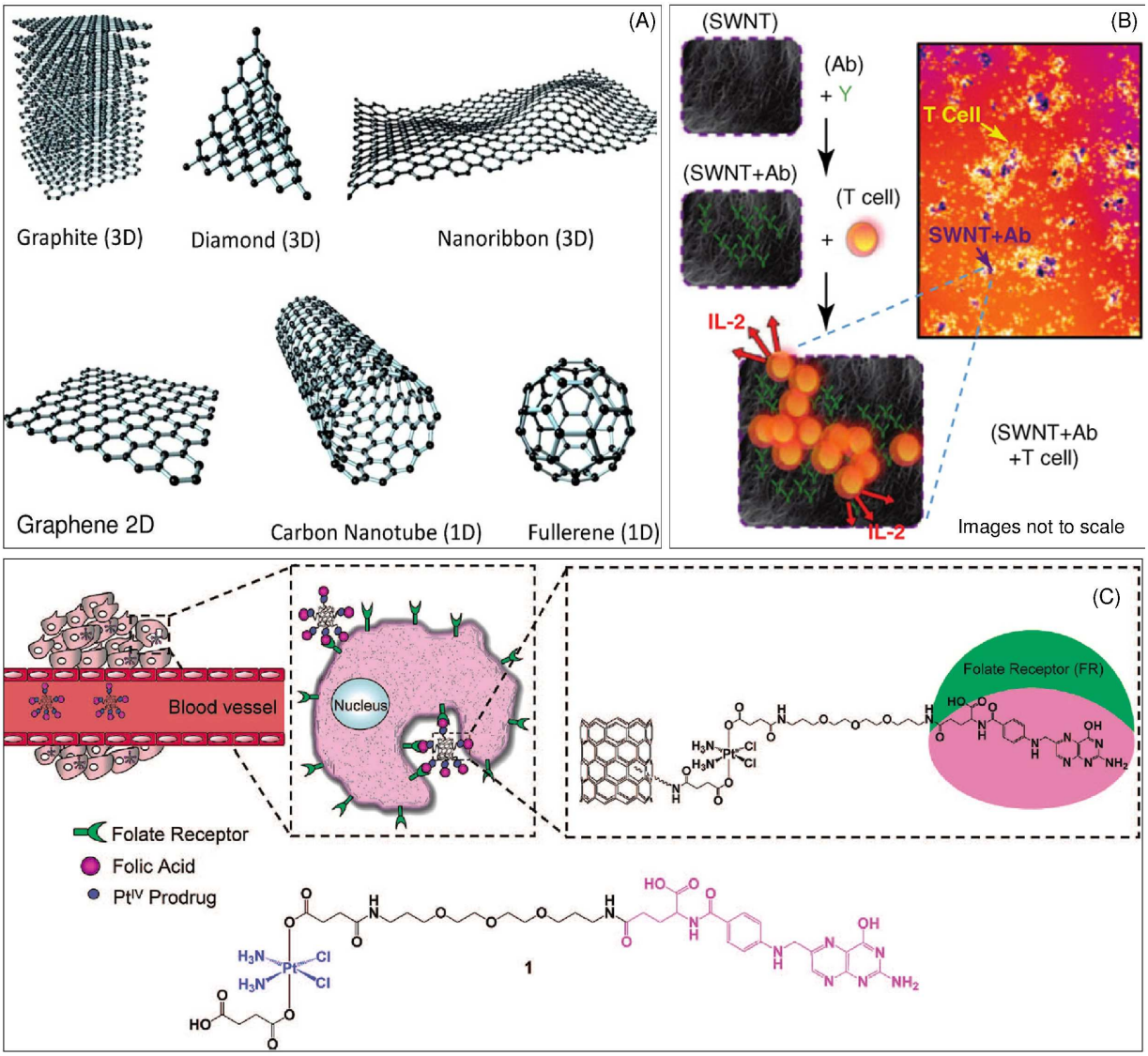


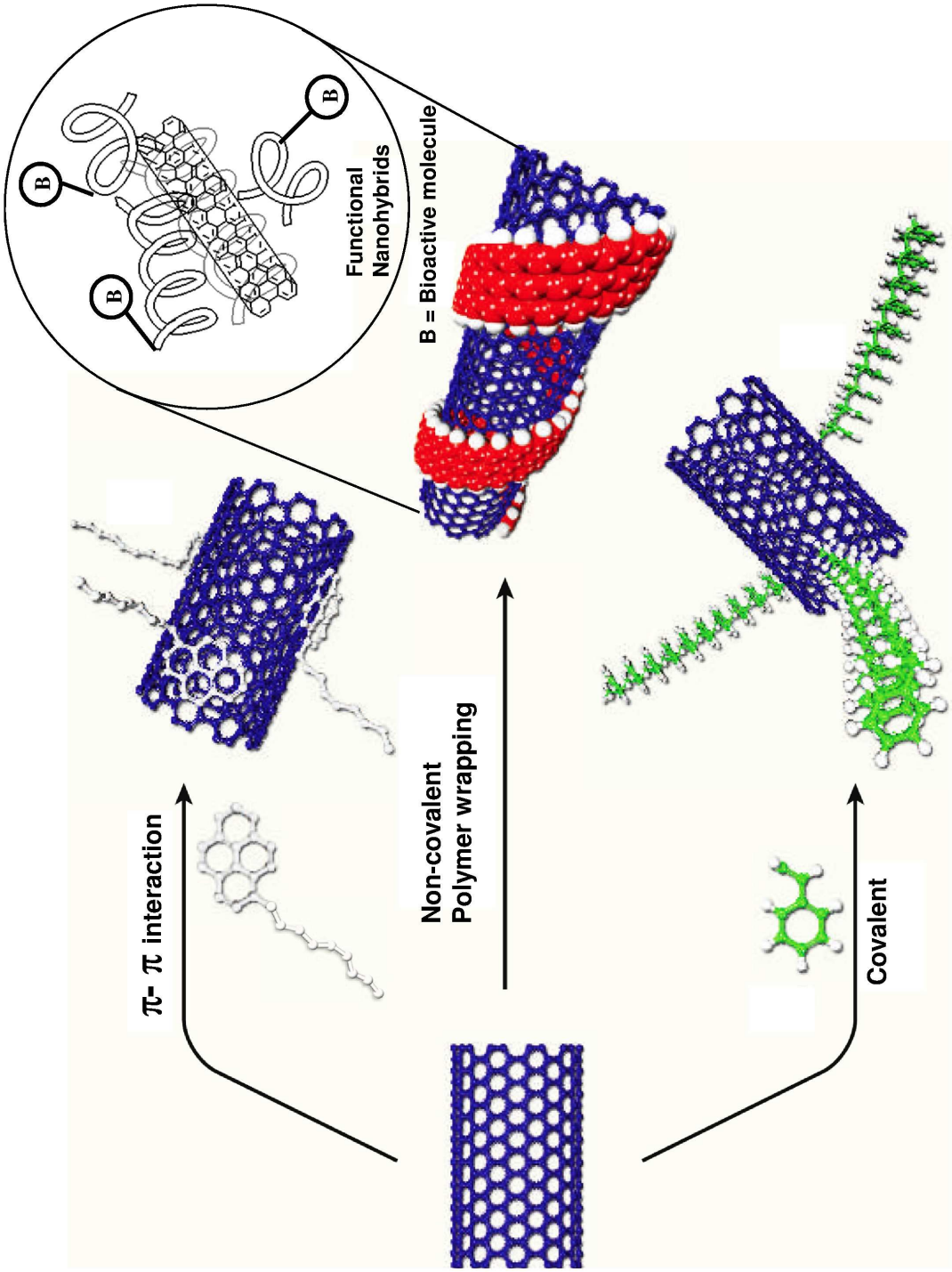
PEG-C-DMA

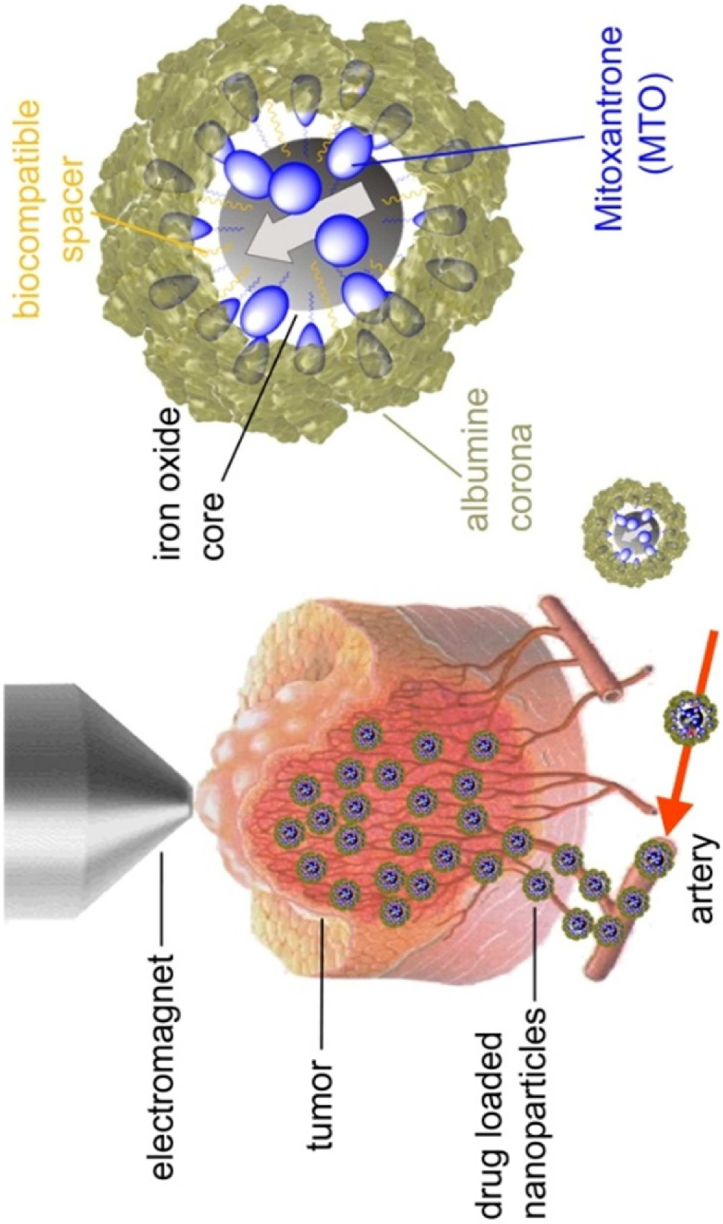


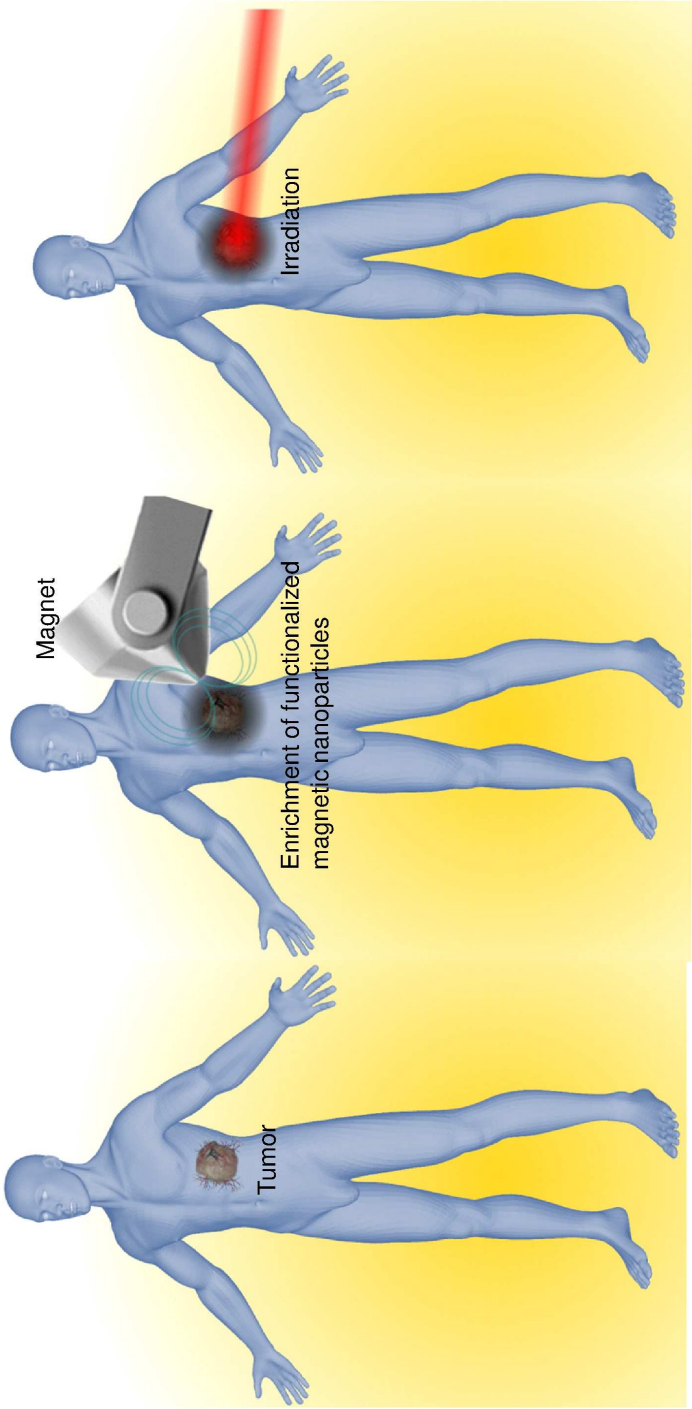
Cholesterol

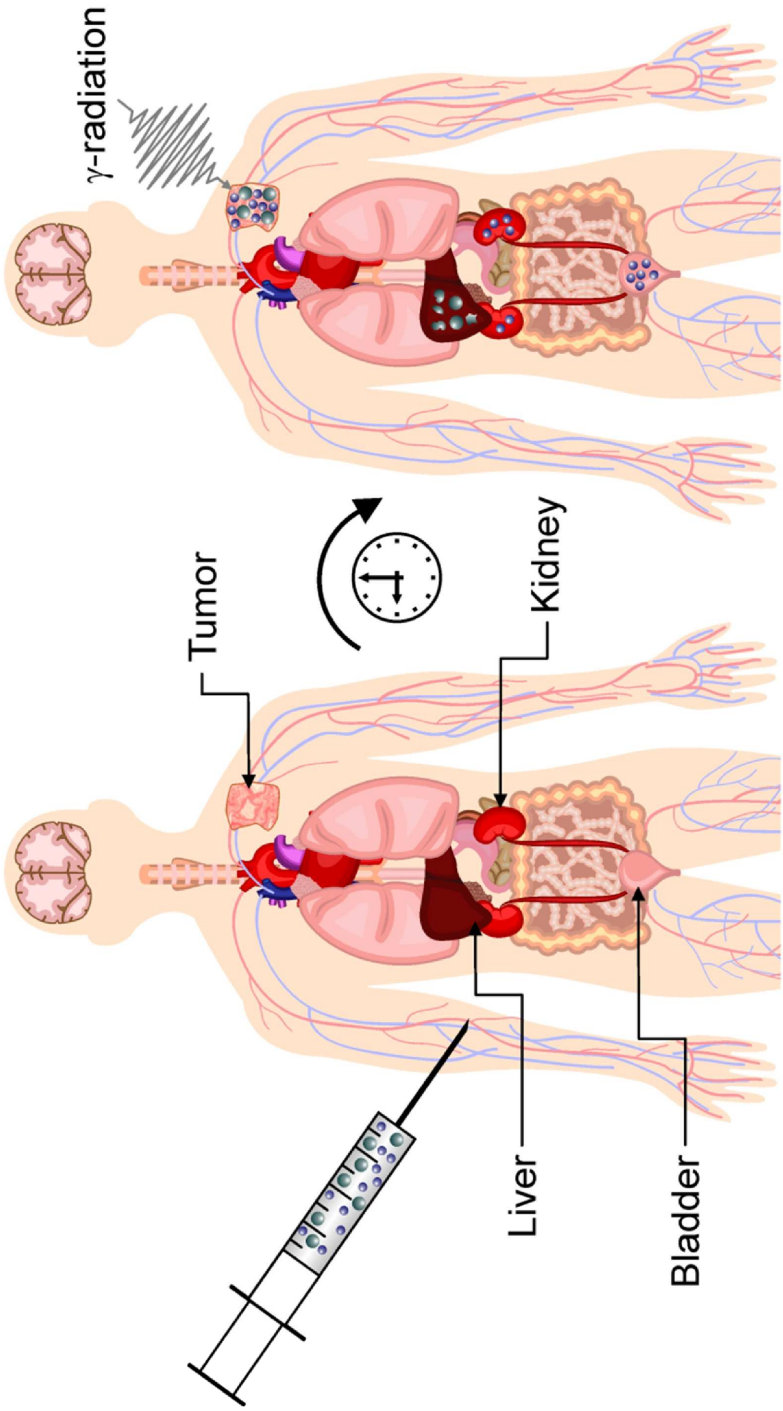

(A)
POEGMA
+
SiRNA
PDMAEMA
Star-POEGMA-SiRNA complex
(B)

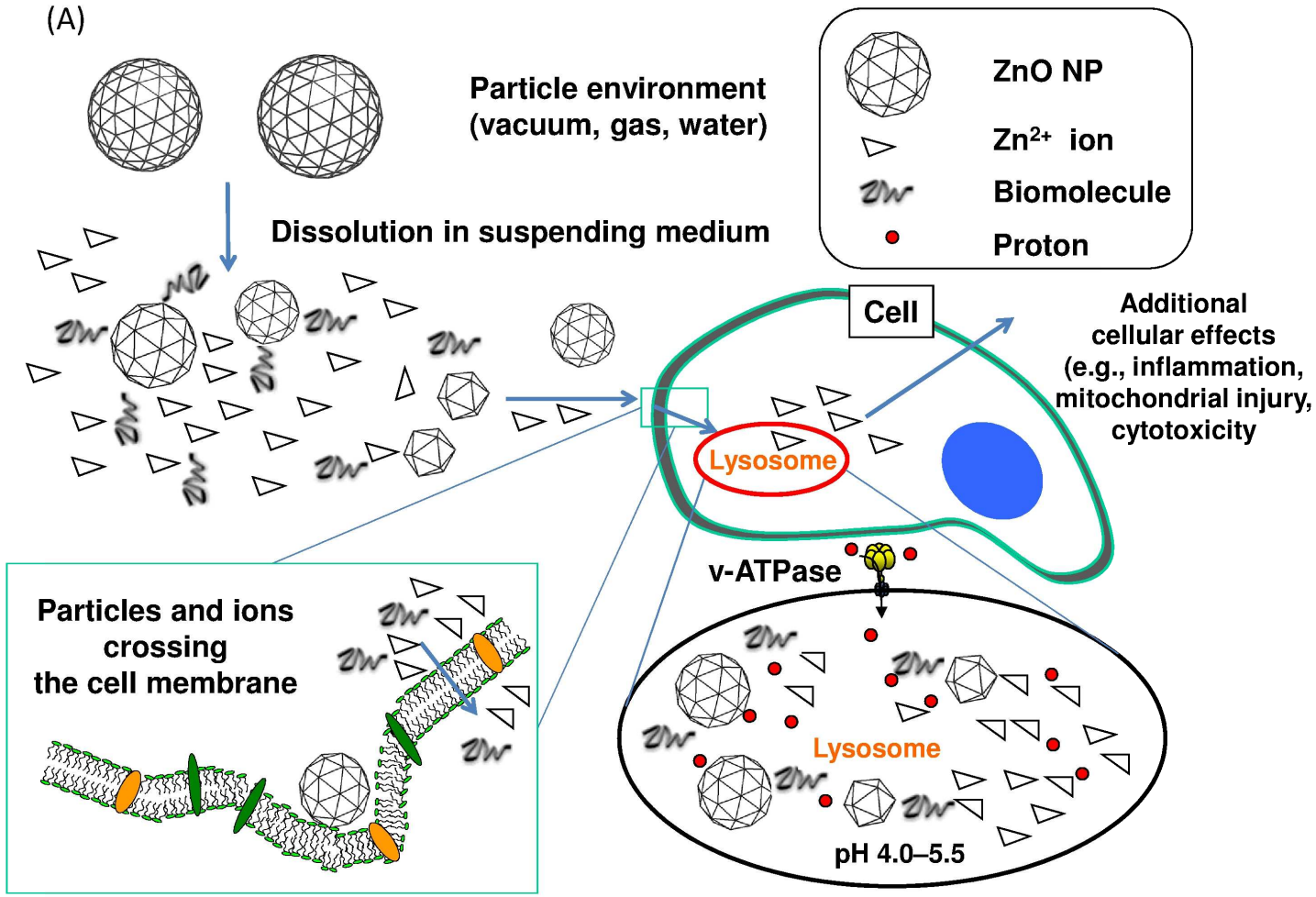

(A)
Graphite (3D)
Diamond (3D)
Nanoribbon (3D)
Graphene 2D
Carbon Nanotube (1D)
Fullerene (1D)
(B)
(SWNT)
(Ab)
+ Y
(SWNT+Ab)
(T cell)
+
IL-2
IL-2
T Cell
SWNT+Ab
(SWNT+Ab
+T cell)
Images not to scale
(C)
Blood vessel
Nucleus
Folate Receptor (FR)
Folate Receptor
Folic Acid
Pt^IV Prodrug
1

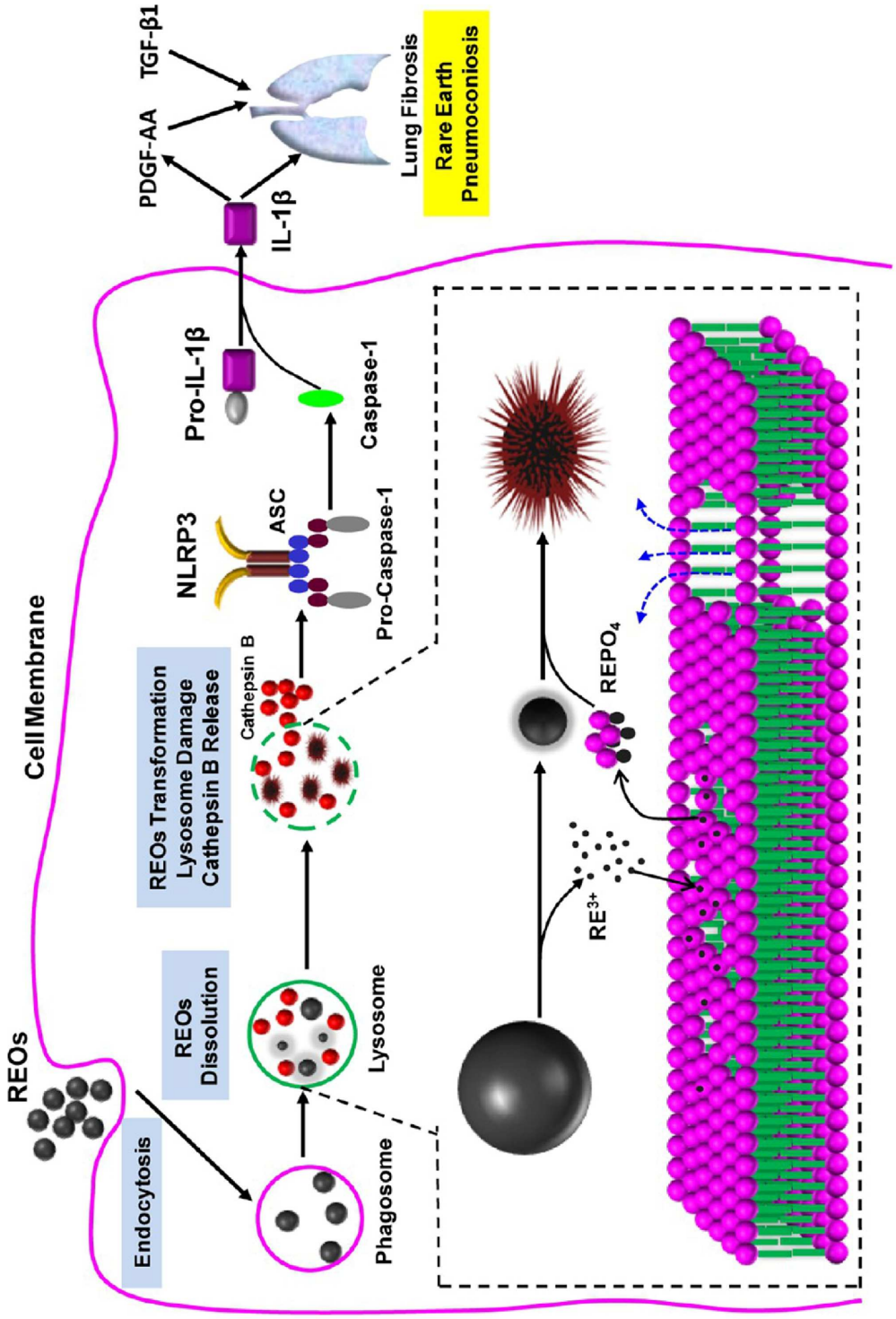

π- π interaction
Non-covalent
Polymer wrapping
Covalent
B = Bioactive molecule
Functional
Nanohybrids
B
B
B

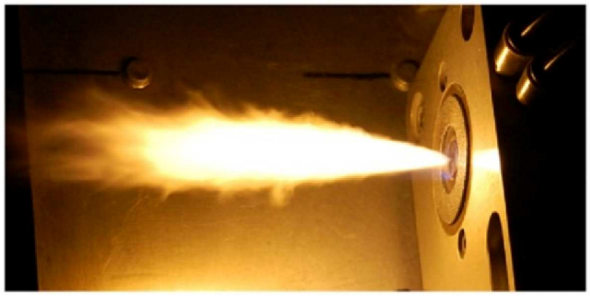

biocompatible
spacer
iron oxide
core
albumine
corona
Mitoxantrone
(MTO)
electromagnet
tumor
drug loaded
nanoparticles
artery

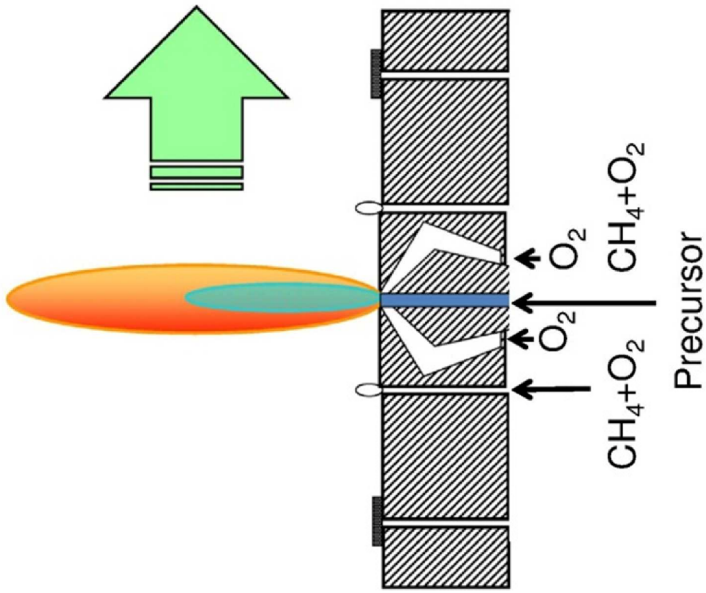

Tumor
Magnet
Enrichment of functionalized
magnetic nanoparticles
Irradiation

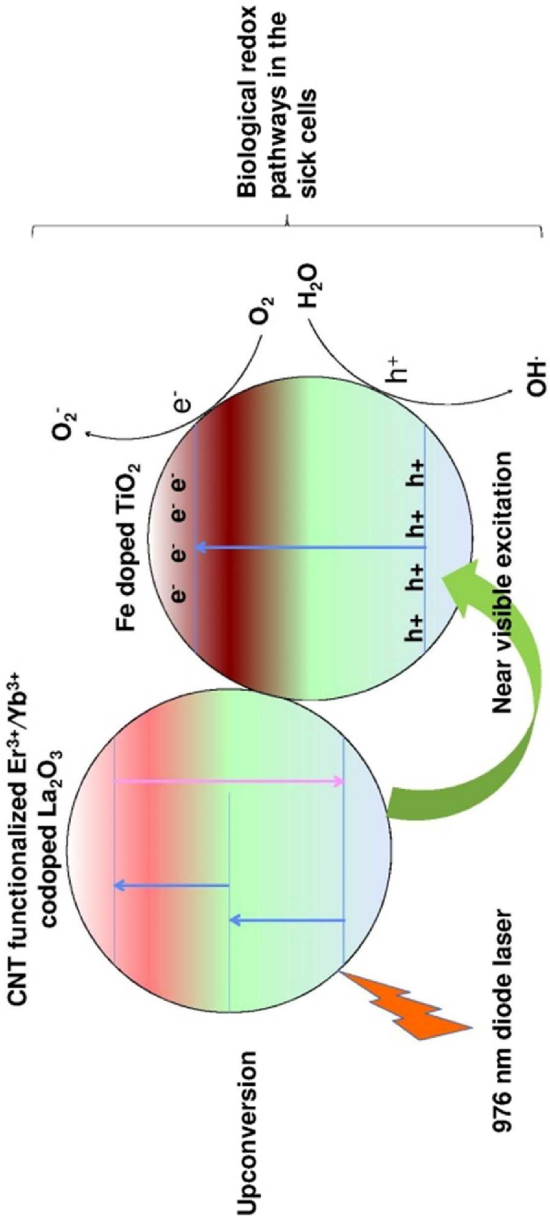

γ-radiation
Tumor
Kidney
Liver
Bladder

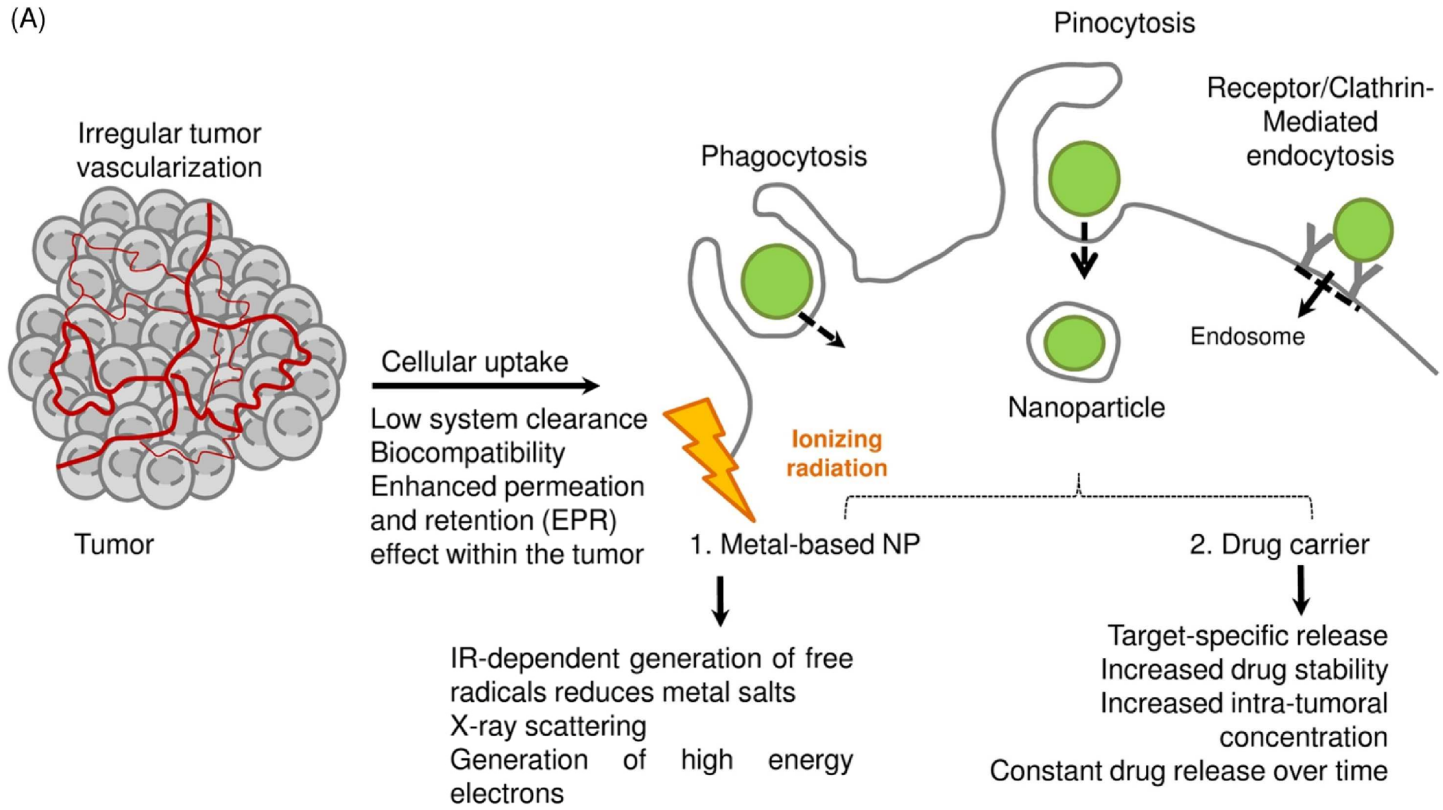


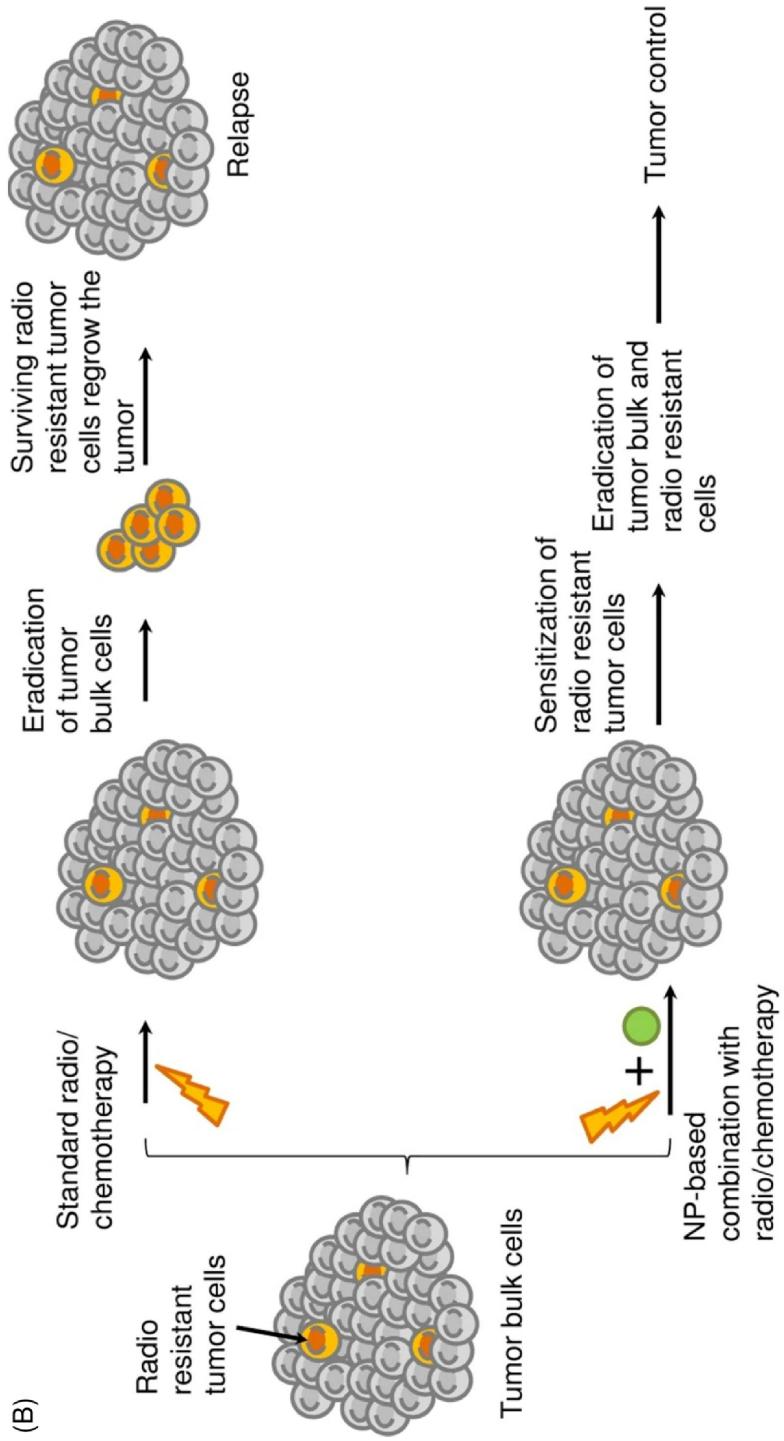

Cell Membrane
REOs
Endocytosis
REOs Dissolution
Phagosome
Lysosome
REOs Transformation
Lysosome Damage
Cathepsin B Release
Cathepsin B
NLRP3
ASC
Pro-Caspase-1
Caspase-1
Pro-IL-1β
IL-1β
PDGF-AA
TGF-β1
Lung Fibrosis
Rare Earth
Pneumoconiosis
RE^{3+}
$REPO_4$




O_2
CH_4+O_2
O_2
Precursor
CH_4+O_2


Biological redox pathways in the sick cells
O_2^-
e^-
O_2
H_2O
h^+
OH^-
Fe doped TiO_2
e^- e^- e^- e^-
h+ h+ h+ h+
Near visible excitation
CNT functionalized Er^{3+}/Yb^{3+} codoped La_2O_3
Upconversion
976 nm diode laser




(B)
Radio resistant tumor cells
Tumor bulk cells
Standard radio/ chemotherapy
Eradication of tumor bulk cells
Surviving radio resistant tumor cells regrow the tumor
Relapse
NP-based combination with radio/chemotherapy
Sensitization of radio resistant tumor cells
Eradication of tumor bulk and radio resistant cells
Tumor control