3

Conjugation of Gold nanoparticles and liposomes for combined vehicles of drug delivery in cancer

Sara Figueiredo[1,†], Rita Cabral[1,†], Daniel Luís[2], Alexandra R. Fernandes[2] and Pedro V. Baptista[1,*]

[1] CIGMH, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal

[2] Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal.

[†] These authors contributed equally to the Chapter

* Corresponding Author

Outline:

Strategic targets for cancer therapeutics

In our current understanding, cancer is the result of a multi-step process of sequential somatic alterations in several oncogenes, tumour-suppressor genes or micro RNA (miRNA) genes that regulate cell growth and differentiation. These genetic alterations include base substitutions, insertions and deletions (indels), DNA rearrangements, copy number variations and even epigenetic changes [1]. They are random, unpredictable and cumulative, and some of them establish the basis for gene deregulation, which underlies the uncontrolled growth or increased survival of cancer cells [2]. The evolutionary progression of cancer depends upon a growth advantage within the micro-environmental constraints, including resource limitations, presented by the surrounding tissues [3]. Typically, cancer therapy is based on surgery, radiation therapy, and chemotherapy with wide-range cell killing chemicals. Maybe due to the adaptive evolution of cancer cells, most conventional therapies fail as tumour heterogeneity within the same diagnostic type results in variable clinical behaviour and response/sensitivity to treatment [2]. Also, these chemical therapeutic agents subject cancer cells to new potent form of selective pressure, favouring the survival and proliferation of cells or clones with growth advantages, which become resistant to treatment [4].

To overcome the limited therapeutic response of some cancers, alternative strategies have been proposed, where nanomedicine has been playing a pivotal role. Firstly, personalised specific therapies may be required, bearing in mind that the emergence of genetically favoured resistant clones is always a possibility. Second, the therapeutic focus may be directed towards understanding and controlling the evolutionary process of tumours in its early stage, including the genetic principles and the impact of combinations of mutations on cancer phenotypes [4, 5]. Third, it might be more effective to focus on combinatorial therapies, designed to the individual cancer genome and simultaneously targeting more than one component of networked signalling pathways, hitting the primary target as well as the compensatory mechanisms to overcome resistance to therapy [4]. Other suggested targets include: (1) components of the self-renewing programme of cancer stem cells regardless of the specific mutant genotype, especially in cases where these can be distinguished from normal adult stem cells; (2) the micro-environment surrounding cancer cells, aiming at angiogenesis or inflammation, for instance, or exploring hypoxic conditions; (3) cell division in general, with the use of cytostatic drugs to control the cancer and convert it into a chronic disease, by delaying both progression and mortality [4].

Most of these suggestive treatments are still in research and slowly translating into the clinics. Due to their high versatility, nanotechnology based platforms have been pushing forward novel therapeutic approaches, mainly by combining the steady advances in the molecular targeting of events in cancer and the optimised delivery of anti-cancer drugs. Here we shall be focusing on the developments of combined therapy for cancer using gold nanoparticles and liposomes towards delivery of RNA interference (RNAi) effector molecules and chemotherapy. Optimisation of these combined strategies, mainly by introduction of active targeting moieties and increased selectivity of chemical agents against cancer cells, holds great promise for the introduction of highly effective drug delivery systems for cancer therapeutics while overcoming drug resistance.

Here we shall discuss trends on the use of gold nanoparticles for vectorisation of anti-cancer drugs, in particular the possibility of directed targeting and siRNA technologies. Then we will focus on the use of liposomes for selective drug delivery in cancer. Finally, the conjugation of AuNPs and liposomes for combined therapeutic strategies against cancer will be discussed.

RNAi-based cancer therapy – going nano

RNAi is a conserved biological process, thought to have evolved as an innate defence mechanism against foreign genetic material [6], that plays a crucial role in transcriptional and post-transcriptional gene regulation [7, 8]. RNAi relies on short antisense RNAs (siRNA) to repress translation or to degrade cytoplasmic messenger RNA (mRNA) of homologous sequence by post-transcriptional gene silencing triggered by double–stranded RNAs (dsRNAs) within the cell [7, 9]. Perfect complementarity to the target mRNA induces cleavage of this transcript at a very precise location [10], followed by its degradation by cellular exonucleases [11]. Chemically synthesised siRNAs can be introduced into the cytoplasm of cultured mammalian cells as a means to manipulate gene expression, sometimes able to bypass the initiation phase and transiently trigger the RNAi biological response, leading to strong and sequence-specific silencing of gene expression [12]. There are currently two ways to 'artificially' trigger the natural RNAi process in mammalian cells: either by applying siRNAs directly to cells or by engineering small hairpin RNA (shRNAs) structures and inducing their expression in the cells, where they get processed into siRNAs.

RNAi-induced gene silencing mirrors the inhibitory effects of protein-based drugs (e.g. antibodies and vaccines) and small molecules that block the function of their targets, with several additional advantages. First, it can overcome the problem of "non-druggable" disease targets, such as non-enzymatic molecules or proteins which conformation is not amenable to conventional drugs or small molecule compounds [13-15]. It can also limit resistance mechanisms such as those caused by anti-angiogenic molecules [16]. Second, because a single mRNA molecule translates into many protein copies, targeting the mRNA is more efficient than blocking the function of its products [15]. Many siRNAs can dampen target gene expression while simultaneously stimulating the immune response in a sequence-independent manner, stimulating dendritic cells to respond immunologically to cancer cells, while reducing blood and lymphatic vessel growth, thus inhibiting angiogenesis [17-20]. Despite these advantages offered by RNAi-based systems, one of their major challenges stems from the need for intracellular delivery, unlike some of the protein-based and small molecule drugs.

Besides ensuring the chemical and biological stability of siRNAs, one needs to carefully consider their effective entry into the target cells and the associated biological processes, as the extent of RNAi-mediated gene downregulation largely depends upon this step [21]. In addition, naked siRNAs delivered systemically are quickly cleared by the kidney and eliminated, or degraded by serum exonucleases [22]. These obstacles have prompted the development of a wide array of gene/RNAi delivery vehicles and their application through two major delivery technologies: (1) Transfection systems (siRNAs and non-viral expression vectors), which involve complexing siRNAs or RNAi trigger-expressing plasmids with a carrier, most often lipid-based vesicles or cholesterol, to allow them to transpass the cell membrane while protecting siRNA against degradation; (2) Transduction of viral vectors [20]. A more detailed summary of the various approaches for delivering siRNA*in vitro* and *in vivo* can be found in some recent reviews [23-28]. Among the more traditional delivery systems, cationic liposomes and viral vectors are the most commonly used, some of which have reached clinical trials. However, all of them present safety concerns and problems both *in vitro* and *in vivo*, such as: (1) immune recognition for most viral systems; (2) mutagenic integration for some viral systems; (3) inflammatory toxicity, rapid clearance and low efficiency in primary and non-dividing cells for liposomes [22]; (4) lack of a cell specific targeting mechanism for all traditional delivery systems; (5) limited endosomal/lysosomal escape of RNAi-effector molecules for non-viral systems; (6) limited release of RNAi-effector molecules from the non-viral carrier leading to the need to administer large amounts of siRNA for efficient gene silencing [29-31]. Nanotechnology has allowed to overcome some

of these obstacles while providing for effective RNAi delivery to target cells with extremely promising clinical efficacy.

Gold nanoparticles: powerful vehicles for RNAi delivery

Gold nanoparticles (AuNPs) have emerged as versatile, selective and highly multifunctional anti-cancer therapeutics due to their unique properties, and represent a powerful alternative to the conventional RNAi delivery systems [32]. AuNPs can be easily synthesised in a wide range of sizes (e.g. 1 to 100 nm) and shapes (e.g. nanospheres, nanoshells, nanorods), and their surface functionalised with several biomolecules, providing specific functions, targeting selectivity and stability in biological environments [33, 34]. In addition, AuNPs present particular optical and electronic properties arising from their reduced size, which we can take advantage of for both therapeutic and bioimaging purposes [32, 34]. AuNP conjugates can also be delivered systemically, with long circulatory half-lives while eliciting low immunogenic responses [32]. In the following sections we will further discuss some of the properties AuNPs offer, with special emphasis on their application as RNAi delivery systems for improved cancer therapeutics.

AuNP-siRNA conjugation

When conjugated to AuNPs, siRNAs have been shown to exhibit increased stability, cellular uptake and efficacy in physiological conditions, retaining the ability to act through the RNAi pathway [26, 35]. The first demonstration that DNA-AuNP conjugates could be easily internalised into cells, without the need for transfection agents, and induced gene silencing by an antisense mechanism was reported by Rosi and co-workers in 2006 [36]. This remarkable study prompted others to use AuNPs as siRNA delivery systems and contributed to the development of many strategies to improve intracellular siRNA delivery *in vitro* and *in vivo*. These strategies can be grouped into two major categories that are currently used for tethering siRNAs to AuNPs, namely (1) the gold-thiol bond and (2) electrostatic interactions. Both categories involve, in some way, the use of poly(ethylene glycol (PEG) or other passivating agents (see 2.2) for stabilisation and to promote endosomal escape of the AuNP conjugates into the cell cytoplasm [28].

Gold-thiol bond

The gold-sulphur interaction is a strong but dynamic, quasi-covalent bond that can be dissociated under reductive conditions, such as in the presence of high glutathione concentrations in the cell cytoplasm, due to oxidation of thiol groups [28]. However, gold-thiol bonds are formed easily and its dynamic structure has been exploited for the formation of AuNP-siRNA conjugates [28]. Oishi and co-workers were the first to report the conjugation of thiolated siRNAs (SH-siRNA) with AuNPs for gene silencing [37]. The authors decorated 15 nm AuNPs with a thiolated poly(ethylene glycol)-*block*-poly(2-(*N*,*N*-dimethylamino)ethyl methacrylate) copolymer ($SH\text{-}PEG_{5000}$–$PAMA_{7500}$), followed by immobilisation of thiolated siRNAs (SH-siRNA) directly onto the AuNPs. With these conjugates, where the size of siRNAs (~14 kDa) and the co-loaded PEG-PAMA (~12.5 kDa) were equivalent, they reported ~45 siRNA molecules per AuNP. Following 24h incubation with 100 nM of siRNA targeting the firefly luciferase gene, they observed a 65% downregulation of luciferase expression in HUH-7 hepatocarcinoma cells [37]. Giljohann and co-workers later reported a slightly different approach, where the sense strands of the siRNA duplexes contained an ethylene glycol spacer and an alkylthiol group ($SH\text{-}PEG_{400}$-siRNA), the latter used to tether the siRNAs onto 13 nm AuNPs. The remaining AuNP

surface was further covered with PEG_{400} molecules for additional stability. The siRNA sequences were designed to target the firefly luciferase gene and labelling of antisense strands with Cyanine 3 (Cy3) fluorescent dye was used to determine siRNA loading. In this system, PEG molecules are significantly smaller that the siRNAs, however the authors report a loading of ~33 siRNAs per AuNP, which is similar to that of the previous study. A 70% luciferase knockdown was observed following a 4 day-incubation with 100 nM of siRNA in HeLa cells [35]. These authors made a few other interesting observations: (1) internalisation of AuNP conjugates was detected 6h after incubation, as revealed by fluorescence imaging, and confirmed in over 99% of the population by flow cytometry; (2) in stability experiments, AuNP-siRNA conjugates revealed a 6 times greater half-life than molecular siRNA duplexes in 10% serum (816 ± 59 min vs. 133 ± 30 min), as determined by fluorescence measurements, indicating a protective effect on siRNAs owing to conjugation with AuNPs; (3) transfection of the same number of luciferase siRNA duplexes (100 nM) using the commercial agent Lipofectamine 2000 showed a less efficient luciferase knockdown compared to delivery of AuNP-siRNA conjugates, 4 days after treatment (33 ± 2% for lipofectamine-siRNA vs. 73 ± 7% for AuNP-siRNA) [35].

Others have reported comparable silencing of luciferase by 13 nm PEGylated AuNPs functionalised with thiolated siRNAs and Lipofectamine complexed siRNA, but AuNPs-siRNA elicit lower innate immune response (about one third less) [38]. IFN-β is a cytokine produced by host cells in response to foreign nucleic acids or microorganisms that contributes to activation of the innate immune system. Activation of the innate immune system can result in adverse stress toxicity by triggering signalling events that induce cell death, sequester immune cells and activate the adaptive immune response. These events can challenge siRNA therapy, in which cases they should be avoided. In this study, IFN-β levels inversely correlated with the nucleic acid density at the surface of AuNPs, suggesting that tightly packed siRNAs on AuNP surfaces can lessen the immune response [38]. Interestingly, increased packing of siRNAs onto AuNPs also contributes to increased stability and subsequent increased cellular internalisation of the conjugates [39, 40]. Mirkin and co-workers have used a pharmacological approach to show that, unlike the common belief that serum proteins aid in the cellular uptake of oligonucleotide-functionalised AuNPs, their entry is actually mediated by scavenger receptors on the cell surface and increases proportionally to the density of oligonucleotides on the AuNP surface [40].

In a similar approach, Lee and co-workers have exploited the photothermal properties of near infrared (NIR) absorbing Au nanorods (AuNRs) for a facilitated and controlled intracellular delivery of AuNP-siRNA conjugates and knockdown of oncogenic genes [41]. Here, antisense oligonucleotides targeting the breast cancer biomarker *HER2* gene were hybridised with thiol-modified sense oligonucleotides previously linked to AuNRs. When irradiated with NIR light of appropriate wavelength, AuNP photothermal heat causes denaturation of the double-stranded oligonucleotides, releasing the antisense strands. The system was optimised for minimal cellular photodamage while allowing spatial and temporal control of the effector gene silencing oligonucleotides. Using this approach, they observed ~10% increase in HER2 downregulation in breast carcinoma cells (BT474) after treatment and irradiation of AuNRs compared to controls (no AuNRs, no NIR or scrambled sequences) [41]. Braun et al. employed 40 nm Au nanoshells to improve endosomal escape and achieve spaciotemporal silencing of a reporter gene (*GFP*) using a similar NIR photothermal approach, but this time Au nanoshell-siRNA conjugates were further coated with a TAT lipid cell internalizing peptide (derived from HIV-1 trans-activator peptide) [42]. In contrast with the previous study, these authors observed laser cleavage of the Au-sulphur bond with concomitant siRNA release which, however, remained in endosomes unless these were ruptured by more disruptive nanoshell heating (higher laser power). Upon nanoshell surface release and endosomal escape, *GFP*-targeting siRNAs enabled reporter gene silencing comparable to Lipofectamine transfection [42].

Another way to covalently link siRNAs to AuNPs is by first loading AuNPs with a SH-PEG-NH_2 and then conjugating the siRNAs to the terminal amine of the PEG using a disulphide crosslinker, N-succinimidyl 3-[2-pyridyldithio]-propionate] (SPDP). Using this approach, Lee and co-workers reported a loading of ~30 siRNAs per AuNP (15 nm), in concordance with the previous studies, which were then coated with the positively charged, terminally-modified poly(beta-amino ester)s (PBAEs), previously shown to facilitate intracellular DNA delivery. Two out of 14 screened PBAE coatings showed efficient *in vitro* delivery of the corresponding AuNP conjugates, comparable to that of the commercial Lipofectamine. A 120nM siRNA dose for 24h resulted in over 90% downregulation of luciferase expression in HeLa cells for AuNP conjugates bearing these PBAE coatings, but AuNP-siRNAs without PBAEs did not reveal any silencing capability [43]. This was probably due to a less efficient cellular internalisation of the AuNP conjugates lacking PBAE and shows that for each system, the AuNP surface properties need to be carefully studied and controlled to ensure efficient cellular uptake and subsequent gene silencing.

Electrostatic interactions

As an alternative to the covalent Au-thiol conjugation, siRNAs can be linked to the AuNP surface by electrostatic interactions. Generally, AuNPs are synthesised via citrate reduction, which makes their surface negatively charged due to citrate adsorption. Given that siRNA is also negatively charged, several approaches have used in a layer-by-layer format, incorporating a positively charged polymer, e.g, poly(ethylene)amine (PEI), between the Au-citrate core and the siRNA. AuNPs within this system can have either siRNAs or PEI exposed in the terminal layer, with final sizes ranging between 20-25 nm. Their loading capacity can be ~780 siRNAs per AuNP, which is much higher than that of AuNPs bearing thiolated siRNAs [44]. While AuNPs bearing siRNAs in the final exposed layer appear to have increased cellular uptake, they were shown by transmission electron microscopy (TEM) to be trapped within the endosome and, presumably for that reason, resulted in no reporter gene silencing, even when considerable amounts of siRNA (~288 nM) were used. AuNPs containing an external PEI layer resulted in 70% reporter gene downregulation 48h post-treatment [44]. A pH sensitive anionic charge-reversal polyelectrolyte layer has also been incorporated between PEI layers, allowing disassembly of the complex upon acidification inside endosomes. This strategy has been shown to improve endosomal escape and to result in knockdown efficiencies comparable to those of Lipofectamine siRNA transfections [45].
Other strategies involve changes to the traditional citrate reduction synthesis method, such as: (1) replacing the citrate by PEI (as the reducing and stabilizing agent) during AuNP synthesis, yielding PEI-capped AuNPs with a positively charged surface that can be electrostatically covered with siRNAs. Using this strategy, Song et al have demonstrated efficient knockdown of the oncogene *polo-like kinase 1* (*PLK1*) followed by induction of apoptosis in MDA-MB-435s breast cancer cells [46]; (2) incorporating a stabilizing cationic polymer, poly(N-2-hydroxypropyl methacrylamide(70)-block-N-[3-(dimethylamino)propyl] methacrylamide(24) [P(HPMA(70)-b-DMAPMA(24))], which conveys a non-immunogenic, hydrophilic and sterically stabilizing shell (similar to the role of PEG in the previously discussed particles) and a positively charged surface, amenable to siRNA linkage [47]; (3) incorporating cysteamine hydrochloride to produce amine-functionalised AuNPs to which siRNA-PEG moieties can be added by electrostatic interactions between siRNAs and the positive AuNPs [41].

Biocompatibility, biodistribution and targeting of AuNPs

Non-functionalised AuNPs are prone to non-specific protein adsorption, to degradation and to aggregation in biological media [48]. Surface conjugation with chemical functional groups or

biomolecules serves multiple purposes and confers to AuNP conjugates three major properties that are crucial for biomedical applications: biocompatibility (and colloidal stability), targeting specificity and functionality. Au surfaces have affinity for a number of organic molecules (e.g. thiolates, dithiolates, amines, carboxylates, cyanides, isothiocyanates, phosphines), which self-assemble in high coverage monolayers through chemical bonds that, in most cases, form rapidly and spontaneously at room temperature ([32, 48].Proper surface functionalisation of AuNPs determines their interaction with the environment and can be tailored for specific applications [49].

The biodistribution and pharmacokinetics of AuNPs depend greatly upon their physicochemical properties, including size, shape, charge and surface coating. Physiological environments, including cell culture media, can compromise the stabilising capacity of many AuNP ligands (compared to water and other low ionic strength media) because of their high salt and serum content, where attractive forces between AuNPs often overrule electrostatic repulsions leading to aggregation [32]. Biocompatibility and stabilisation of AuNPs in biological fluids is thus achieved by decorating AuNPs with hydrophilic polymers, such as PEG, polylysine (PLL), polystyrene sulfonate (PSS), starches and polyvinylpyrrolidone (PVP) [32]. The modification of AuNP surfaces with PEG moieties, or PEGylation, is the most commonly used approach in biomedical applications, and has been shown to increase AuNP stability in high salt concentrations and in biological environments [50]. Thiol groups bind with great affinity to noble metal surfaces, in particular to gold (bond strength ~44 Kcal/mol; [51], and therefore PEG molecules bearing terminal thiol groups are often used.

When delivered systemically, AuNPs must travel in the blood stream, undetected by the immune system, long enough to reach the target tumour tissue, before they can extravasate into the tumour interstitial space. Circulating plasma proteins, known as opsonins, can bind to NPs or other foreign small molecules and microorganisms and remove them from circulation through the mononuclear phagocyte system (MPS), composed mainly of monocytes and macrophages. Besides conferring stability to AuNPs in physiological environments, PEGylation (or functionalisation with other hydrophilic polymers) results in their increased half-life blood circulation and subsequent tumour accumulation, by preventing adsorption of proteins and molecules involved in the phagocytic uptake [52-57].

As discussed above, tumours develop by accumulating genetic mutations that confer growth advantages to certain populations of cancer cells, which progress to divide rapidly and uncontrollably. In order to sustain this uncontrolled growth, tumours require oxygen and nutrients, which they acquire by recruiting new blood vessels in a process known as angiogenesis. These new blood vessels are formed rapidly upon tumour demand and as a consequence exhibit a disorganised, defective and leaky endothelium with large gaps or fenestrations (600-800 nm) into the tumour interstitial space. In addition, the growing tumour mass exerts damaging pressure onto the lymphatic system, which collapses and fails to efficiently drain fluids out of the tumour. These unique characteristics of most solid tumours contribute to what is known as the enhanced permeability and retention (EPR) effect – see Figure 3.1. The EPR effect sets the stage for the so called *passive targeting* of AuNPs and other nanosized circulating molecules, which gain access to the tumour by passively diffusing through the large fenestrations on angiogenic vessels and remain at the tumour site due to its defective lymphatic drainage [34, 58, 59]. Passive targeting via the EPR effect of tumours has been shown *in vivo* for a number of nanostructures, including micelles, liposomes, metal NPs and quantum dots, as well as for several protein/DNA/polymer complexes, and preferential tumour accumulation is expected for particles with hydrodynamic diameter above the renal clearance threshold and up to two micrometres [32, 60]. Very small particles tend to easily extravasate through the leaky capillary walls but are often pushed out of the tumour by blood, thus showing good permeability but poor tumour retention. Conversely, tumour access of large particles is limited by the pore cut-off size of tumour blood vessels,

which can be as small as 7-100 nm for tumours such as gliomas and ovarian cancer [61, 62]. The type of cancer, stage and location also influence the optimal size for EPR of AuNP conjugates [32].
Other biological barriers need to be addressed for AuNPs to reach the target site, e.g. the liver, kidney and lymphoid organs. They must be small enough (≤ 100 nm) and possess negative or neutral surface charge to avoid uptake by the MPS, but large enough (≥ 6 nm) and hydrophilic or neutral to thwart rapid renal clearance. AuNPs bearing positively charged coatings tend to stick non-specifically to cells, and therefore neutral AuNPs are preferred for extended blood circulation time [63]. Upon intravenous injection into rats, spherical AuNPs of sizes between 10 nm and 250 nm have been shown to accumulate preferentially in the liver and spleen, whereas AuNPs smaller than 10 nm were more broadly distributed [64]. A strong negative charge on the particle surface also leads to accumulation in the liver due to phagocytic uptake [63].

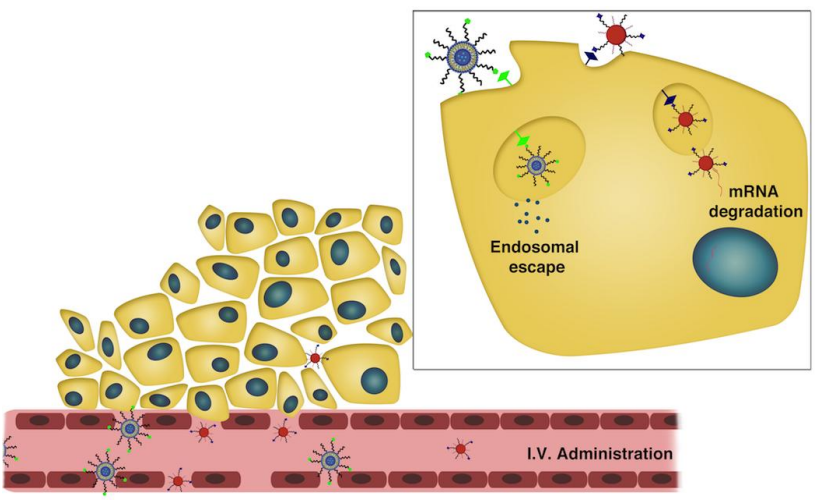

FIGURE 3.1
The potential of combined therapy mediated by AuNPs and liposomes capable of passive and active targeting.

When administered intravenously, both AuNPs and liposomes tend to extravasate through the tumour defective endothelium into the tumour interstitial space. With the aid of active targeting moieties at the surface of both nanoparticles, they specifically bind to the surface of tumour cells and enter them mostly by receptor-mediated endocytosis. Once inside tumour cells, different strategies can be used to promote endosomal escape and delivery of effector molecules to the cytosol, where they perform their therapeutic function.

Cellular internalisation and active targeting

Experimental studies reveal that many macromolecules and nanomaterials are actively internalised into cells via endocytosis, a vesicular transport mechanism that embraces different pathways, collectively known as phagocytosis and pinocytosis [65]. Clatherin mediated endocytosis (CME) is the best-characterised mechanism and, when receptor mediated, it results in the internalisation of several ligands, such as low-density lipoprotein (LDL), transferrin, growth factors and insulin [66]. Caveolin-mediated endocytosis (CvME) is the most common clatherin-independent uptake pathway and is involved in the uptake of smaller molecules. Both CME and CvME pathways have been shown to

mediate cellular internalisation of AuNPs [67, 68]. However, the route and degree of cellular internalisation depends on the size [69, 70], shape [69], surface charge [71] and functionalisation [68] of the AuNP conjugates, as well as on the temperature [67, 72] and cell type [68], among other factors. One of the major advantages AuNPs offer is the opportunity for *active targeting*. Taking advantage of tumour molecular markers as docking sites to concentrate the therapeutic effect at tumours, it is possible to increase therapeutic efficacy while reducing systemic exposure and off-target effects. Several of these tumour molecular markers are surface proteins/receptors present in cancer cells and in tumour vasculature that are not expressed or are expressed at much lower levels in normal cells, thereby distinguishing tumour masses from the surrounding normal tissues [73]. The surface of AuNPs can be modified to accommodate targeting biomolecules that allow specific biochemical interactions with surface proteins/receptors highly expressed on target cells. Tumour-targeting biomolecules currently in use include monoclonal antibodies, peptides/proteins [e.g. transferrin, epidermal growth factor (EGF)], folic acid, carbohydrates and DNA/RNA [33, 49, 59]. For example, the anti-epithelial growth factor receptor (EGFR) monoclonal antibody has been used as an active targeting agent, since EGFR and its ligands are commonly overexpressed in a variety of solid tumours [74-76]. The targeting of tumour angiogenic vessels is also gaining increased attention, as it may improve therapeutic efficiency in cases where tumour cells are less accessible [73]. In combination with passive targeting strategies, active targeting may enhance receptor-mediated endocytosis of the AuNP conjugates in target cells [69, 77]. The intracellular localisation of effector molecules can be further directed with the use of cell-penetrating peptides, which are short peptides that facilitate the delivery of various cargoes to cells, or with nuclear localisation sequences, which direct cargos to the nucleus [78-80]. Decorating AuNPs with proton sponge groups or using photothermal heating can further assist escape from endosomal sequestration/degradation [81]. Active targeting by AuNPs [82-84] has been shown to result in greater tumour accumulation than passive targeting, when AuNPs are administered systemically *in vivo* (6-13% versus 2-5%) [82, 83].

AuNP-RNAi conjugates for cancer therapy: in vitro and in vivo studies

Over the past several years, many studies have demonstrated promising proofs of concept for therapeutic applications of AuNPs. Specifically, numerous studies have demonstrated the potential of AuNPs to deliver RNAi effector molecules specifically to target cancer cells, with subsequent knockdown of tumorigenic proteins [43, 85-87]. However, *in vivo* studies using this system are still scarce, alerting us for the need to overcome remaining barriers that prevent its translation into the clinics. In this section, we summarise some of the milestone studies that hopefully can lead us to improved therapeutic approaches against cancer.

Zhang and co-workers have developed an anti-metastasis therapy consisting of AuNRs conjugated electrostatically with siRNAs, which targeted the *protease-activated receptor 1* (*PAR-1*). These conjugates were then delivered to highly metastatic human breast cancer cells. The authors observed efficient downregulation of PAR-1 mRNA and protein levels and decreased metastatic ability of the cancer cells [88]. Another interesting study showed the development of single-stranded DNA-functionalised AuNPs (via a thiol-Au bond) as a general gene delivery system (AuNP-GDS) for loading and delivering DNA oligonucleotides and shRNAs [36]. This platform allows any short nucleic acid to be hybridised to the cargo DNA covalently linked to the AuNP, the former which can be designed for a specific purpose, such as gene knockdown, redirection of alternative splicing, and modulation of signal transduction pathways. Using this system, the authors delivered shRNAs targeting the *Mcl-1L* mRNA to a xenograft tumour in a mouse model, and showed a ~26% reduction in protein expression which was sufficient to induce apoptosis of the xenograft tumour cells [89]. These studies did not include a

targeting strategy because they were performed either *in vitro* or *in vivo* by directly injecting the conjugates into tumours. However, for systemic delivery, an additional targeting moiety is generally required to improve treatment efficacy and reduce off-target effects. Lu and co-workers used Au nanocages targeted to folate receptors (overexpressed in many types of cancer) and carrying a siRNA against the *NFkBp65*, which encodes a transcription factor highly involved in tumour formation and progression. They injected these constructs intravenously into in nude mice bearing HeLa cervical cancer xenografts and observed a significantly higher tumour uptake of the targeted conjugates compared to the non-targeted ones. They additionally took advantage of the photothermal properties of the Au nanocages to achieve a controlled cytoplasmic delivery of siRNA upon NIR light irradiation and observed efficient NF-kappaB p65 downregulation only when tumours were irradiated with NIR light [81]. Conde et al have recently demonstrated the efficiency of multifunctionalised AuNPs for the specific silencing of the *c-myc* proto-oncogene in three biological systems of increasing complexity, namely *in vitro* cultured human cancer cells (HeLa), *in vivo* invertebrate (freshwater polyp, Hydra) and vertebrate (mouse, C57BL/6j) models. Their AuNPs were functionalised with PEG, cell penetration (TAT) and cell adhesion peptides (RGD, which binds to the integrin αVβ3 receptor family; [90], and *c-myc* targeting-siRNAs [91]. They have also shown in a more recent study that these same nanoconjugates are capable of targeting tumour cells in a lung cancer murine model and of inducing significant downregulation of the *c-myc* oncogene, followed by tumour growth inhibition and prolonged survival of lung tumour bearing mice [92].

Toxicity evaluation of AuNPs for therapy

As the utility of AuNPs largely depends on the degree of inherent toxicity, studies on the toxicological profile of AuNPs are of utmost relevance before they can be used for cancer treatment and management. AuNPs have been considered safe for long due to the biocompatibility and general inertness of gold in its bulk form [93]. Indeed, a few studies support the safety of these nanocarriers *in vitro* and *in vivo* using animal models [94-97]. However, as more studies on the use of AuNPs for gene delivery have been reported, evidence of cytotoxicity has increased dramatically [98-101]. On their review about the toxicity and cell uptake of AuNPs, Alkilany and Murphy addressed a fundamental issue: the effect of the supernatant in cell viability should always be assessed in order to conclude that the observed toxicity is really due to the nanoparticles and do not result from any toxic component used for their preparation [102]. Also, because toxicity experiments typically rely on the use of colorimetric techniques to measure cell viability, care should be taken to ensure that the dye is neither being adsorbed on nanoparticles nor reacting with them and that the photochemical properties of the AuNPs are compatible with the selected methodology, therefore being advisable to compare the results obtained from multiple methods [103, 104].

Toxicity of AuNPs is generally accepted to be dependent on particle size, shape, and surface charge and chemistry [105-108]. For instance, very small particles (1.4 and 5 nm in diameter) seem to be capable to enter the nucleus, where they can interact with DNA and cause molecular disturbance [109, 110]. Larger particles (16 and 33 nm) are retained in endosomes and accumulate in the periphery of the nuclear region [111, 112]. At least three different studies reported that cellular uptake of AuNPs reach maximum levels for a particle size of about 50 nm [67, 72, 113]. Also, surface functionalisation seem to be capable of inducing higher level of apoptotic cell death, probably related to increased cell uptake when compared to unmodified 40 nm AuNPs [72]. According to data from *in vitro* studies, AuNP toxicity is believed to result mainly from the induction of oxidative stress [95, 98, 101]. Indeed, upregulation of stress related genes was found to result from cell exposure to AuNPs, which also promoted the downregulation of cell cycle related genes [101, 114, 115]. Nevertheless, most of these

studies paid little attention to genome damage, such as DNA strand breaks and nuclear abnormalities, or characterization of protein markers for toxicity. An integrated toxicology evaluation encompassing DNA damage, stress related enzymes and a proteome profiling approach showed no significant cytotoxicity of PEGylated AuNPs and no upregulation of proteins related to oxidative damage [97].
Also, both positive and negatively charged AuNPs were found to be similarly more cytotoxic against human keratinocytes (HaCaT cells) when compared to neutral AuNPs, with LD_{50} values of roughly half of those determined for the latter [116]. Despite the disruption in cell morphology and the dose-dependent toxicity observed for all three types of AuNPs, both anionic and cationic AuNPs induce mitochondrial stress and apoptosis in opposition to the necrotic cell death caused by neutral particles [116]. Another *in vitro* study comparing positive and negatively charged AuNPs reported that cationic NPs were far more toxic to Cos-1 cells, human red blood cells and *E. coli* than anionic NPs, possibly as a result of cell lysis, as shown by a dye leakage technique [107]. However, Alkilany and co-workers clearly showed that serum proteins become readily adsorbed to the surface of charged NPs, inducing an inversion of surface charge in particles that were originally cationic [117]. This would reduce electrostatic interaction between the original positive NPs and the negative cell membrane, the first step towards cell lysis mediated toxicity of cationic NPs [107].
Regarding *in vivo* experiments, several studies have demonstrated that AuNPs of 50 nm and larger were non-toxic to mice, conversely to what has been observed for AuNPs <40 nm [108, 118]. In fact, there are concordant data from different studies on the biodistribution and accumulation of AuNPs in mice showing that most of the intravenously injected nanoparticles are retained in the liver, regardless of their size [64, 119, 120]. There is also an agreement in that AuNPs have the ability to transpose the blood-brain barrier and thus reach the brain, with a cut-off limit in diameter of around 20 nm [121], and that smaller particles have the most widespread organ distribution [64, 119, 120]. Organ distribution seems to be ruled by a more or less complex relationship with nanoparticle size. For instance, it is known that renal excretion of AuNPs is maximized for a narrow size range of 6-8 nm, resulting in an accelerated clearance rate [122]. Despite the valuable use of animal models, the effect of size on the toxicity of AuNPs in humans is difficult to predict since the size of endothelial cells' fenestrae is highly variable between individuals, thereby affecting nanoparticle clearance [123]. Therefore, more consistent data on the toxicological profile of AuNPs *in vivo* is necessary. For a more complete review on biodistribution, encompassing earlier studies and administration routes other than intravenous injection, see Khlebtsov and Dykman [121]. Furthermore, core size, charge and surface chemistry of AuNPs seems to correlate to toxicity on the development of zebrafish embryos, with positive and negatively charged AuNPs causing mortality and malfunctions to the embryos, respectively [100]. Adverse effects were also found in the model system *Drosophila melanogaster* after exposure to citrate-capped AuNPs, which were shown to reduce fertility in a dose-dependent manner and also the life span [98, 99].
Nonetheless, long-term studies in higher organisms are necessary to further characterise the safety of AuNPs as therapeutic agents, so they can be safely administrated to humans without concerns about late toxicity symptoms.

Liposomes for drug delivery - Great things sometimes come in small packages

Conventional cancer chemotherapy deals with poor therapeutic efficacy and increased toxicity to normal tissues, due to reduced drug selectivity. Nanomedicine has provided valuable concepts to

develop systems that could unambiguously increase drug concentration at the target site and, thus, increase the therapeutic index of drugs [124]. Nanoparticles have the ability to carry large payloads, to protect the agent of interest and to improve the bioavailability of water-insoluble drugs. In addition, incorporating imaging agents into nanoparticles allows the *in vivo* visualisation of biological processes occurring at the cellular and/or molecular level, revealing the outcome of a personalised therapeutic. Lipid base nanoparticles (e.g., liposomes, solid lipid nanoparticles, oily suspensions, lipid microbubbles, lipid microspheres) [125] have attracted much attention, and nanosystems are currently being introduced into clinical use and several more under clinical trials or pre-clinical development (Table 3.1) [126-128].These self-assembling lipid-based nanovesicles can be tailored to meet the individual biological characteristics of the target and are especially used as drug-delivery carriers, but recently there has been a growing interest in protocols for image-guided drug delivery [129-131]. Liposomes are considered highly attractive particles, as they portray low toxicity while permitting controlled drug release and targeting. Moreover, liposomes present both an aqueous cavity and hydrophobic membrane, allowing incorporation of both lipophilic and hydrophilic drugs. Furthermore, in a pharmaceutical perspective, large-scale production of liposomes is feasible.

The use of modified phospholipids allows the introduction of functional groups, such as maleimide, that prompt conjugation to antibodies or other ligands. Liposomes can be loaded with a variety of water-soluble and insoluble drugs [132] and the entrapment of the chosen drug can be achieved through several different processes, including (i) encapsulatation of drugs into the aqueous cavity of the vesicle, (ii) incorporation of lipophilic drugs in the membrane bilayer, (iii) active entrapment methods such as pH gradient protocols and (iv) electrostatic interactions between drugs and the liposome membrane. The characteristics of liposomes can be tuned by changing lipid composition, pulling off apposite physico-chemical properties, such as size, zeta potential (surface charge), permeability and stability. Liposomes can be classified according to the number of lamellae (uni, oligo-, and multi-lamellar vesicles), size (small, large or giant), and net charge (cationic, anionic and neutral nanoparticles) [126, 133].

TABLE 3.1
Selected Liposomal formulations clinically approved or under clinical trials.

Composition	Trade name	Indication	Administration
Liposome formulations under clinical trials			
Liposomal annamycin	L-Annamycin	Phase I – Acute lymphocytic leukaemia, acute myeloid leukaemia	I.V.
Liposomal cisplatin	SLIT Cisplatin	Phase II - Progressive osteogenic sarcoma metastatic to the lung	Aerosol
Liposomal doxorubicin	Sarcodoxome	Phase I/II – Soft tissue sarcoma	I.V.
Liposomal fentanyl	AeroLEF	Phase II – Postoperative analgesic	Aerosol
Liposomal Iurtotecan	OSI-211	Phase II - Ovarian cancer	I.V.
Liposomal Vincristine	Onco TCS	Non-Hodgkin's lymphoma	I.V.
Liposomal Paclitaxel	LEP-ETU	Phase I - Advanced Cancer	I.V.
Liposomal doxorubicin/vincristine	CPX-351	Phase II - acute myeloid leukaemia	I.V.
Liposomal irinotecan /floxuridine	CPX-1	Phase II - Colorectal cancer treatment	I.V.
Clinically approved liposomal formulations			
Liposomal amphotericin B	Abelcet and Ambisome	Fungal Infections	I.V
Liposomal cytarabine	Depocyt	Malignant lymphomatous meningitis	I.T
Liposomal daunorubicin	Daunoxome	HIV-related Kaposi's Sarcoma	I.V
Liposomal doxorubicin	Myocet	Metastatic Breast cancer	I.V.
Liposome-PEG doxorubicin	Doxil/Caelyx	HIV-related Kaposi's Sarcoma, Metastatic breast and ovarian cancer	I.V.
Liposomal IRIV vaccine	Inflexal V/Epaxal	Influenza/Hepatitis A	I.M.
Liposomal morphine	DepoDur	Postsurgical analgesia	Epidural
Micellular estradiol	Estrasorb	Menopausal therapy	Topical

Note: I.V.- intravenous; I.M.- intramuscular; I.T.- intrathecal.

Towards optimised properties - size and surface charge

Liposomes are considered suspensions that can be used in several therapeutic platforms, such as aerosol and lotion, but intravenous administration is the most common route for drug administration. As already discussed, *in vivo* circulation and biodistribution of nanoparticles depends on several physicochemical parameters, and it is clear that the size and surface charge play a major role in determining their fate, where small vesicles can be rapidly cleared by the kidneys and larger nanoparticles may be captured by the MPS [134, 135]. Also, following systemic administration, nanosystems accumulate differently along the organism – lung tends to capture micron-sized particles, the MPS engulfs medium sized ones and smaller particles are able to extravasate and accumulate via the EPR effect and/or cleared by the kidney.

Once in circulation, larger nanoparticles are rapidly opsonised, recognised and captured by the immune system. Fang and co-workers showed that protein adsorption on smaller particles is much lower than on larger particles, which correlated with a higher uptake of larger nanoparticle by macrophages [136]. Dexi Liu and co-workers in the early 90's showed that liposomes' surface could become highly hydrophilic via covalent attachment of polymers (such as PEG), helping these vesicles to overcome MPS, thus increasing their blood circulation half-life [137]. It was demonstrated that 4h after injection of stealth-liposomes (functionalised with PEG), those smaller than 70 nm tended to accumulate in the liver, while the larger ones were essentially gathered in the spleen. Additionally, as already discussed, particle size may affect tumour uptake and also influence the mechanism of cellular internalisation - clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis, or phagocytosis in immune cells -which have tremendous influence on the fate of the particle and the outcome of the drug [138].

Concerning charge, liposomes can be characterised as cationic, neutral or anionic. Due to the natural propensity to aggregate, bare neutral lipid-based nanoparticles are generally not used for drug delivery. Instead, positively or negatively charged liposomes have been continuously used as vectors for therapeutic proposes. Cationic liposomes enter cells by endocytosis induced by ionic attractions, which are able to stabilize electrostatic interactions with the negatively charged cell membranes. Krasnici and colleagues assessed the distribution of neutral, anionic and cationic liposomes and revealed that cationic liposomes accumulate on the vascular endothelium while neutral and anionic are promptly cleared after intravenous injection [139]. Hence, cationic liposomes are an efficient tool for intracellular delivery but care must be taken to prevent non-specific internalisation [140]. The positive charge of the liposome is often due to incorporation of cationic lipids, such as DOTAP (1,2-dioleoyl-3-trimethylammonium propane), DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) or DODAP (1,2-dioleyl-3- dimethylammonium-propane), and tent to be used in association with nucleic acids (forming lipoplexes). A major drawback of cationic liposomes is the observed correlation between their positive charge and high toxicity, an aspect that will be further discussed. As a consequence, neutral and anionic liposomes become the natural alternatives and it has been established that neutral particles suffer less opsonisation than charged particles. Another rather remarkable study was performed by Lee and Huang [141], where folate-targeted pH-sensitive anionic liposomes were prepared, to which DNA complexed to polylysine was added. It was detected that at low lipid to DNA ratios, the overall charge of the particles waspositive and the cellular uptake was independent of the folate receptor. On the other hand, using high lipid to DNA ratios (negatively charged particles) the uptake was folate receptor-dependent. Hence, DNA delivery systems based on anionic lipids are a possible alternative to cationic lipids with the major advantage of reduced cytotoxicity (see section 'Toxicity and bioavailability') [142].

Passively targeted vs. ligand-targeted liposomes

To prevent detection by the immune system and increase their circulation half-life, "stealth liposomes" were developed, in which the liposomal membrane could be functionalised with sialic acid derivatives (monosialoganglioside (GM1)) that mimic the erythrocyte membrane. Another approach consisted in shielding the membrane with a hydrophilic coating, generally with PEG, since it is used in several approved formulations and is FDA approved, and because it prevents interactions with plasma proteins, probably due to steric hindrance [143-145]. However, although it was thought that PEG was not immunogenic, soon it was shown that the administration of particles functionalised with PEG caused IgM production and accelerated blood clearance in a second dose, an effect that will be latter discussed [146]. What is even more critical, some reports have evidenced that PEG impairs the uptake of liposomes by the tumour cells, an issue that could compromise the effect of the drug [147]. An approach used to tackle this issue consists in using "detachable" PEG conjugates (DSPE-S-S-PEG_{5000}) [148] or PEG-derivatised phospholipids with lengths different than those of the anchor molecules that are effortlessly uncorked from the liposomal membrane. Although stealth liposomes have favourable characteristics for clinical applications, there is still plenty to be done before their full potential can be ascertained.

In order to be internalised by tumour cells, liposomes need to find and bind cancer cells, and active targeting could enhance this association. In theory, active targeting would improve the therapeutic index of the drug and would also minimize side effects to healthy cells. Targeting moieties include antibodies, peptides, glycoproteins, carbohydrates, or specific receptor ligands [149] – see Table 3.2. Liposomes that have the aptitude to target tumour cells but they still need to escape from the blood stream and, as already mentioned, extravasation is still the limiting step. For instance, Park and co-workers showed that liposomes conjugated to monoclonal antibody fragments specific for the HER2 receptor are taken up much more efficiently than are untargeted liposomes [150]. Presence of targeting ligands on the liposomes' surface clearly improves internalisation but success is highly dependent on the proper choice of the ligand. Coupling of the targeting moiety to the liposomes can be undertaken by covalently attaching the ligands to liposomes, functionalised with PEG-derivatised phospholipids. Another method consists in the transference of the targeting moiety from a PEG-derivatised phospholipid micelle to the liposome [151]. A major drawback of adding targeting moieties to the surface of these nanocarriers is that it often leads to an increased immunogenicity, as peptides and antibody fragments can be considered by the host immune system as antigens [152].

TABLE 3.2
Some functionalisation moieties for liposome and AuNP active targeting.

Targeting Moiety	Nomenclature	Target	Ref.
Antibody	Anti-EGFR (cetuximab)	Epidermal growth factor receptor (EGFR)	[153-156]
	Anti- HER2 (Trastuzumab-Herceptin)	Epidermal growth factor receptor type 2	[157-159]
	Anti-VEGF (bevacizumab)	Vascular endothelial growth factor (VEGF)	[160, 161]
	anti-CD19	CD19-expressing B-cell lymphoma	[151, 162, 163]
	Anti-CD20 Ab (rituximab)	B-lymphocyte antigen CD20	[164, 165]
Peptides/ Proteins	Arg–Gly–Asp-based (RGD)	αvβ3 integrin receptor	[166, 167]
	Arg-Lys-Lys-His (RKKH)	α2β1 integrin receptor	[168]
	Transferrin	Transferrin receptor	[83, 169, 170]
	VEGF peptide	VEGF receptor	[171-173]
Sugars	Lactose	asialoglycoprotein (ASGP) receptors	[174]
	Galactose	asialoglycoprotein (ASGP) receptors	[175, 176]
	Mannose	Mannose receptor (scavenger receptors) in Tumour associated macrophages	[177]
Small Molecules	Folic acid	Folate receptor	[178-181]
	Hyaluronic acid	CD44 receptor	[182-184]

Toxicity and bioavailability

Liposomes are designed to improve pharmacokinetics and efficacy of bioactive agents, while reducing systemic toxicity. Despite being generally considered as nontoxic, biodegradable, and biocompatible, early release of the drug may not only increase undesirable toxicity against healthy cells but also lower the amount of drug that will be available at the target site and thus promote the acquisition of resistance arising from exposure to sub-lethal doses [185]. Drug release is dependent on the chemical properties of the drug itself and on the liposome lipid composition. The lipid bilayers present low permeability to hydrophilic molecules that are enclosed in the aqueous core, but are highly permeable to hydrophobic drugs, such as paclitaxel, thus reducing their appropriate retention [186].
Besides the drug cytotoxicity concerns, several other liposome related characteristics, such as vesicle size, surface charge, lipid composition, drug-to-lipid ratio and liposome functionalisation may potentiate toxicity effects [187-190]. Some authors have reported that smaller liposomes are more damaging to cell physiology *in vitro*, which may result from the higher curvature of the smaller particles

[191]. These data have been contradicted by *in vivo* studies reporting that reduction of liposome size is accompanied by a decrease in toxicity [192-195].

Conventional first generation liposomes negatively or positively charged have been reported to be toxic, rapidly removed from the circulation and too leaky to ensure appropriate stability *in vivo*. As a result, the fluidity of those vesicles had to be subsequently reduced by incorporating cholesterol or sphingomyelin [186, 194]. In particular, positively charged liposomes, such as those used to deliver nucleic acids for gene therapy, were shown to be highly toxic to macrophages, especially when associated with the fusogenic lipid DOPE [196]. Moreover, cationic DOTAP nanoparticles were shown to promote hepatotoxicity, as revealed by the release of the liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), and associated body weight loss in mice [197]. Also, cationic DOTAP nanoparticles, unlike their uncharged and anionic counterparts, are able to enhance cytokine and interferon response genes in T-cells, B-cells and monocytes [197]. As such, cationic liposomes are even more likely to potentiate adverse immune reactions as well as interact with serum proteins that are negatively charged [198]. Even though an increasing number of cationic liposomes are used, studies have demonstrated that besides the formation of aggregates in blood, and the tendency to induce inflammatory response, these liposomes still cause significant toxicity, such as cell contraction and mitotic inhibition [196, 199, 200]. Cytotoxicity of cationic liposomes arises from the inhibition of protein kinase C (PKC) activity by their tertiary or quaternary amines, and from the interaction with other enzymes. Particularly, lipids bearing tertiary amines have been shown to result in lower cytotoxicity when compared to those with quaternary ammonium groups, such as DOTAP and DSTAP [201]. Nevertheless, induction of reactive oxygen species (ROS) was observed in the presence of liposomes bearing either DOTAP or DSTAP, with the amount of generated ROS correlating with the levels of cationic lipid present [191, 202, 203]. This has prompted researchers to design new liposomal structures with modified cationic liposome structure [204]. For instance, lipids whose hydrophilic groups contain heterocyclic rings are associated with lower cytotoxicity and also higher transfection efficiency by allowing the delocalization of the positive charge [205]. Other strategies include the transformation of the cationic group into a neutral or negative group by covalent modification and the use of titratable lipids to formulate liposomes with pH-dependent surface charge [206]. As such, these pH-sensitive liposomes can be designed to remain neutral at physiological pH, only becoming positively charged when they reach acidic environments, such as those found in solid tumours, hence preventing the negative effects of the cationic surface charge on systemic toxicity. In a study where the *in vivo* toxicity (in mice) of pH-sensitive liposomes loaded with cisplatin was assessed, the liposomal formulation was able to increase the LD_{50} by 3-fold compared to free drug and no alterations were found after haematology and histopathology analysis [207]. Also, no clinical symptoms were detected at doses lower than the LD_{50}, in contrast with the symptoms observed for the free drug even at low doses, such as diarrhoea, ataxia, and weakness. Changes in drug-to-lipid ratio also markedly affect the acute toxicity of encapsulated doxorubicin and liposomal amphotericin B [189, 208].

As discussed above, stealth liposome technology was developed to overcome most of the challenges encountered by conventional liposome technology namely the toxicity due to charged liposomes [209, 210]. PEGylated liposomal doxorubicin, Doxil, represents a step forward in cancer therapy by reducing the dose limiting cardiotoxicity associated with the free drug [211]. Nevertheless, this formulation is not completely devoid of undesirable side effects, causing cutaneous and mucosal toxicity [212, 213]. Indeed, the most common side effect associated with Doxil treatment, the hand-foot syndrome, is dose-limiting [213] and may compromise the efficacy of chemotherapy.

Toxicity of stealth liposomes is likely to be affected by the accelerated rate of blood clearance that results from repeated administration. This effect increases the accumulation of liposomes in the liver

and spleen and thus may promote severe hepato- and splenotoxicity when highly toxic drugs are being transported [191, 214, 215]. Therefore stealth liposomes also induce an immunogenic response through complement activation that affects their pharmacokinetic behaviour and raises concerns about safety. Several authors have also observed an increased accumulation of doxorubicin in mice liver, spleen and lugs for both PEG, lactoferrin- or transferrin-PEG liposomes compared to the free drug [216-218]. This accumulation, related to the rich blood supply of these organs [219], decreases the amount of drug loaded liposomes that is available to reach the target tissue. This is particularly relevant when the delivery of the nanocarrier relies on passive targeting, namely on the EPR effect found in solid tumours. Furthermore, since the liver and spleen are major organs inthe MPS, the high uptake of liposomes by the macrophages in these tissues may lead to a depletion of these phagocytic cells and therefore cause a negative impact onthe immune system [219].
Most of the *in vitro* and *in vivo* toxicity studies involve survival and tissue distribution studies, serum enzyme analysis and ROS assessment [191, 196-200, 202-204, 207]. Thus far, little attention has been directed to the detection of genome damage, such as DNA strand breaks and formation of nuclear abnormalities.

Combined therapy

Cancer therapeutics is not only being hampered by barriers to drug delivery (anatomical, chemical and clinical) but also by tumour heterogeneity and adaptive resistance to treatment, which together lead cancer cells to evade therapy. The development of a multimodal treatment strategy, through the use of combined therapy with different mechanisms of action, will likely increase the probability of eradicating all cells within a tumour. Hence, joint therapies can be achieved through the combination of the following approaches: i) chemotherapy; ii) immunotherapy; iii) hormonal therapy and iv) gene therapy. On this basis, multifunctional particles could surmount the greatest challenges and potentiate new treatments and an ideal platform should be able to encapsulate both conventional anticancer drugs with the new genetic drugs and present outstanding properties, such as proper retention of drugs, outstanding targeting efficiency and long circulation lifetime. Similarly, the combination of distinct types of nanoparticles, including AuNPs, liposomes, polymeric micelles, dendrimers, carbon nanotubes or quantum dots, could result in new platforms with application in a variety of areas. We believe that combinatorial drug delivery technology offers the potential to improve the efficacy of single agents and the hope is that by increasing the amount of research in this field, one can contribute to the translation of combined technologies into the clinics.
Although combination therapy may seem an expensive therapeutic approach, it could provide greater savings by decreasing the amount of drug needed per treatment (thus lowering the rate of treatment failure). Indeed, the use of nanomedicines for combined therapy could even improve the balance between the efficacy and the toxicity of the used drugs. Co-loading of multiple drugs can be achieved by i) co-encapsulation of hydrophilic drugs in the inner core of the liposome; ii) encapsulation of hydrophilic drugs and simultaneous incorporation of lipophilic drugs within the membrane; iii) co-encapsulation of hydrophilic drugs with gene-therapy based drugs (such as siRNA). Hence, for instance, combined therapies designed to simultaneously target more than one signalling pathway could lead to decreased drug resistance and favour better outcome.
Abraham and co-workers anticipated that the therapeutic potential of drugs could be increased by co-encapsulating doxorubicin and vincristine in liposomes. Indeed, these drugs have been used in several combination regimens due to their different intracellular mechanisms (doxorubicin (DOX)– intercalation into DNA and generation of free radicals; vincristine – vinca alkaloid that binds to tubulin,

inhibiting proper assembly cell cytoskeleton) and several individual liposomal formulations have been continuously evaluated [220]. The co-encapsulation method itself is challenging and depends on the complexation of DOX and also on the addition of an electroneutralionophore that creates a pH gradient which facilitates the accumulation of vincristine into liposomes pre-loaded with DOX. The efficacy of the formulation was assessed in mice bearing human breast cancer cells, however, they noticed that the co-encapsulation of these two drugs did not result in a synergistic effect, concluding that the appropriate ratio of drugs must be taken in account [221]. This knowledge led to the proposal of CPX-351 (cytarabine/daunorubicin at a 5:1 molar ratio) [222] and CPX-1 (irinotecan/floxuridine at a 1:1 molar ratio) [223], formulations, which are currently in phase II clinical trials.

As already mentioned, multidrug resistance (MDR) is probably the utmost hindrance to successful cancer chemotherapy and is associated with overexpression of a glycoprotein (Pgp) that acts as an efflux pump that reduces the intracellular concentration of the drug. Verapamil has been shown to have Pgp inhibitory activity and additionally, it may induce cardiotoxicity, since it is a known calcium channel inhibitor. Hence, liposomal delivery of Verapamil may reduce its cardiotoxicity while contributing to a milder MDR. Wu and co-workers prepared a transferrin-conjugated liposome co-encapsulating DOX and verapamil and assessed its efficiency in DOX-resistant cells, presenting a promising approach to reverse cancer drug resistance [224].

The interaction between the tumour and its microenvironment is one of the hallmarks of cancer. Tumour microenvironment is an array of extracellular matrix components and cell types that together determine tumour progression. Aiming at the microenvironment is also an interesting approach to fight cancer. For example, tumours need to generate their own new blood vessels to support their growth and several studies proposed liposomes as anti-angiogenic therapeutic platforms [225-227]. Of particular interest are those strategies that use a single liposomal formulation that can target both endothelial and cancer cells. Moura and co-workers designed sterically stabilised, pH-sensitive liposomes entrapping DOX targeting the nucleolin receptor, present in both cell types. Mice treated with this formulation showed a positive outcome, with significant decrease of the tumour and microvascular density [228]. Luo and co-workers showed similar results using paclitaxel-loaded liposomes targeting aminopeptidase N receptors, which are expressed both in the tumour endothelium and on tumour cells [229]. However, the neovascularisation tends to create disorganised and leaky vessels, which enhances the leakage of nanoparticles to the tumour area by EPR, and therefore treatment with drugs that inhibit angiogenesis could reduce the actual targeting of the tumour. Actually, studies performed with an anti-angiogenic drug, sunitinib (VEGFR/PDGFR kinase inhibitor), have shown that by inhibiting primary tumour growth, tumour invasion and metastasis is promoted, an effect considered outrageous in the fight against cancer [230].

Co-loading of drug and RNAi effector molecules

Despite all the progresses in the development of new chemotherapeutic agents, conventional cancer therapy often fails with drug resistance being considered the primary hindrance towards the cure. Cutting edge multifunctional nanoparticles can provide simultaneous targeted delivery of chemotherapeutics and gene silencing agents, with the rationale behind the use of combined treatment being based on the heterogeneity of tumour cells. Drugs and genetic material can be delivered into the target through the mechanisms already described and this dual therapeutic platform is a promising approach for the treatment of neoplastic diseases.

Combining strategies that induce increased drug sensitivity could ideally decrease the number of cells that are unaffected by drug (through evasion or acquired drug resistance). A common example consists of combined strategies that target the oncogene *BCR-ABL* (for gene silencing) with widespread

chemotherapeutic agents (as for example imatinib), that once entrapped in a nanoparticle, induce pre-sensitisation of tumour cells, followed by gene silencing, as presented by Mendonça and co-workers [231]. In this report, several formulations encapsulating siRNA and imatinib at different ratios were prepared and their anti-leukemic activity was also assessed in both imatinib resistant and sensitive leukaemia cells. The data showed that the liposomal formulation with higher amount of anti-BCR-ABL siRNA led to higher reduction of imatinib IC_{50}. Furthermore, the researchers assessed the effect of pre-sensitisation of cells using an administration schedule, in which cancer cells were incubated during 48h with the pre-sensitisation stimuli, followed by 48h of treatment with the complementary component, and showed that pre- sensitisation with siRNA followed by treatment with imatinib resulted in higher therapeutic index, an expected observation as the BCR-ABL oncoprotein is known to be involved in imatinib-resistance [232]. However, the efficiency of liposomes is still reduced, with low transfection efficiency being their major drawback [233].
Hence, proper combinatorial regimens should be tested based on the assumption that the pre-sensitisation of cells with a first therapeutic approach enhances the response to the second drug, therefore decreasing the probability of drug resistance and therapy failure. Another possibility is to take advantage of the ability of AuNPs to be readily taken up by cells, combined with the ability of liposomes to efficiently reach the tumour microenvironment, and to design formulations where liposomes work as carriers for AuNPs.

Nanoparticle cocktail

New-generation nanoparticles are considered effective systems for cancer treatment and are an assembly of biomaterials, with improved properties, ideally, with effective dual functions, through the incorporation of both diagnostic and therapeutic agents. These nanostructures are usually referred to as hybrid nanoparticles, and combine lipid-based nanoparticles with polymers or AuNPs, with the main goal of achieving a biocompatible nanostructure which effect is superior to the one obtained from the mixture of the individual components [234].

Conclusion

Tumours are complex tissues that recruit cells and nutrients in order to enhance their survival and proliferation. Cancer biology is described through its six hallmarks, that consist of i) immortality; ii) proliferative signalling - growth factors from oncogenes that stimulate their own growth; iii) evading growth suppressors - override 'stop' signals - anti-growth signals from tumour suppressor genes; iv) resisting cell death and apoptosis; v) inducing new-angiogenesis; and vi) contact inhibition and evasion. Tumour complexity arises also from their individual specialised cell types – tumour microenvironment - that can be either tumour-promoting or tumour-killing agents [235, 236].These hallmarks are potential targets for drug therapy and have been widely explored over the past few years. However, the therapeutic doses used in the clinics are also lethal to normal cells, leading to undesirable side effects and systemic toxicity [237]. Nanomedicine can provide the necessary tools for tackling these issues, such as the use of nanoparticles as drug delivery agents, minimising side effects and toxicity of the drugs. Furthermore, the selective targeting of cancer cells or tumour vessels by nanoparticles containing anticancer drugs and/or therapeutic genetic material are now a recognised strategy for improving the therapeutic efficacy of conventional cancer treatment and have shown promise in preclinical models. Some recent advances in ligand-targeted liposomes have started to demonstrate their promised improvement in cancer therapy but many tumours become resistant to drugs, making it

challenging to develop drug targeting vehicles that deliver high concentrations of combinatorial therapeutics. Various attractive approaches have been explored to maximise dual-drug transportation to the tumour that can be performed by association of lipid-based nanoparticles with hybrid polymers, systems that tend to have high loading capacity and to be biologically compatible.

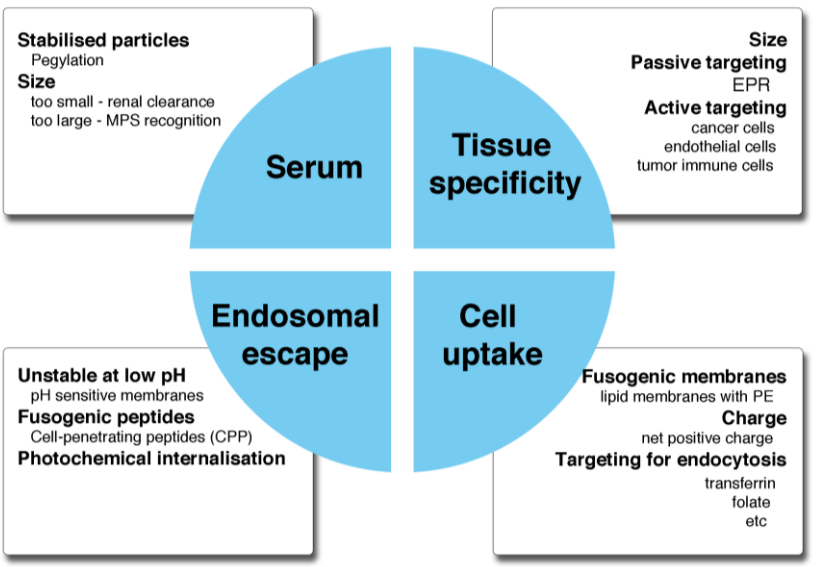

FIGURE 3.2
The fate of therapeutic nanoparticles and their characteristics.

The use of multiple nanoparticles that can be used together may overcome current limitations of each individual nanoformulation alone. For example, AuNPs have proven to be outstanding vectorisation systems for gene delivery and can be used to target molecular pathways, including those involved in drug resistance and in survival of cancer cells. Acting together with drug loaded liposomes, suitable for increased drug delivery with targeting capability, may decrease the surge of drug resistance while enhancing the chances of a successful clinical outcome. Nanoparticles can be engineered to be identified after *in vivo* delivery, working as imaging agents. Furthermore, new developments with AuNPs have elucidated their ability to transduce optical energy into thermal energy, which can be employed in thermal ablation, to destroy tumour cells. Here, we have discussed nanomedicine platforms with enhanced properties, prompt to detect or treat cancer, where new polymeric modalities have been envisioned, with improved biocompatibility, circulation and therapeutic response. Despite these improvements and advantages, AuNP based systems are still in the research phase and are expected to reach pre-clinical and Phase I trials within the next 2-3 years. Liposomal formulations have been going into the clinics for the last decade and have made an impact on the delivery of standard chemotherapeutic drugs against cancer. Combination of these two platforms, alone or conjugated, is still inside the research lab and ought to take at least 5 years before we can see an impact.

Acknowledgements

The authors would like to thank Fundação para a Ciência e Tecnologia (MEC) for financial support (PEst-OE/SAU/UI0009).

References

1. Stratton MR. Exploring the genomes of cancer cells: progress and promise. Science 2011;331(6024) 1553-8.
2. Croce CM. Oncogenes and cancer. New England Journal of Medicine 2008;358(5) 502-11.
3. Greaves M, Maley CC. Clonal evolution in cancer. Nature 2012;481(7381) 306-13.
4. Ashworth A, Lord CJ, Reis-Filho JS. Genetic interactions in cancer progression and treatment. Cell 2011;145(1) 30-8.
5. Nowell PC. The clonal evolution of tumor cell populations. Science 1976;194(4260) 23-8.
6. Koppers-Lalic D, Hogenboom MM, Middeldorp JM, Pegtel DM. Virus-modified exosomes for targeted RNA delivery; a new approach in nanomedicine. Advanced Drug Delivery Reviews 2013;65(3) 348-56.
7. Castel SE, Martienssen RA. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. Nature Reviews: Genetics 2013;14(2) 100-12.
8. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998;391(6669) 806-11.
9. Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. Nature Reviews: Genetics 2009;10(2) 94-108.
10. Tomari Y, Zamore PD. Perspective: machines for RNAi. Genes & Development 2005;19(5) 517-29.
11. Orban TI, Izaurralde E. Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. RNA 2005;11(4) 459-69.
12. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001;411(6836) 494-8.
13. Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, Harborth J, Heyes JA, Jeffs LB, John M, Judge AD, Lam K, McClintock K, Nechev LV, Palmer LR, Racie T, Rohl I, Seiffert S, Shanmugam S, Sood V, Soutschek J, Toudjarska I, Wheat AJ, Yaworski E, Zedalis W, Koteliansky V, Manoharan M, Vornlocher HP, MacLachlan I. RNAi-mediated gene silencing in non-human primates. Nature 2006;441(7089) 111-4.
14. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Rohl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliansky V, Limmer S, Manoharan M, Vornlocher HP. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 2004;432(7014) 173-8.
15. Daka A, Peer D. RNAi-based nanomedicines for targeted personalized therapy. Adv Drug Deliv Rev 2012;64(13) 1508-21.
16. Filleur S, Courtin A, Ait-Si-Ali S, Guglielmi J, Merle C, Harel-Bellan A, Clezardin P, Cabon F. SiRNA-mediated inhibition of vascular endothelial growth factor severely limits tumor

resistance to antiangiogenic thrombospondin-1 and slows tumor vascularization and growth. Cancer Research 2003;63(14) 3919-22.
17. Robbins M, Judge A, MacLachlan I. siRNA and innate immunity. Oligonucleotides 2009;19(2) 89-102.
18. Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, Baffi JZ, Albuquerque RJ, Yamasaki S, Itaya M, Pan Y, Appukuttan B, Gibbs D, Yang Z, Kariko K, Ambati BK, Wilgus TA, DiPietro LA, Sakurai E, Zhang K, Smith JR, Taylor EW, Ambati J. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 2008;452(7187) 591-7.
19. Cho WG, Albuquerque RJ, Kleinman ME, Tarallo V, Greco A, Nozaki M, Green MG, Baffi JZ, Ambati BK, De Falco M, Alexander JS, Brunetti A, De Falco S, Ambati J. Small interfering RNA-induced TLR3 activation inhibits blood and lymphatic vessel growth. Proceedings of the National Academy of Sciences of the United States of America 2009;106(17) 7137-42.
20. Davidson BL, McCray PB, Jr. Current prospects for RNA interference-based therapies. Nature Reviews: Genetics 2011;12(5) 329-40.
21. Aigner A. Delivery systems for the direct application of siRNAs to induce RNA interference (RNAi) in vivo. Journal of Biomedicine and Biotechnology 2006;2006(4) 71659.
22. Seow Y, Wood MJ. Biological gene delivery vehicles: beyond viral vectors. Molecular Therapy 2009;17(5) 767-77.
23. Higuchi Y, Kawakami S, Hashida M. Strategies for in vivo delivery of siRNAs: recent progress. Biodrugs 2010;24(3) 195-205.
24. Khurana B, Goyal AK, Budhiraja A, Arora D, Vyas SP. siRNA delivery using nanocarriers - an efficient tool for gene silencing. Current Gene Therapy 2010;10(2) 139-55.
25. Guo P. The emerging field of RNA nanotechnology. Nat Nanotechnol 2010;5(12) 833-42.
26. Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. Nature Reviews: Drug Discovery 2009;8(2) 129-38.
27. Pecot CV, Calin GA, Coleman RL, Lopez-Berestein G, Sood AK. RNA interference in the clinic: challenges and future directions. Nature Reviews: Cancer 2011;11(1) 59-67.
28. Lytton-Jean AK, Langer R, Anderson DG. Five years of siRNA delivery: spotlight on gold nanoparticles. Small 2011;7(14) 1932-7.
29. Zhao E, Zhao Z, Wang J, Yang C, Chen C, Gao L, Feng Q, Hou W, Gao M, Zhang Q. Surface engineering of gold nanoparticles for in vitro siRNA delivery. Nanoscale 2012;4(16) 5102-9.
30. Lee JM, Yoon TJ, Cho YS. Recent developments in nanoparticle-based siRNA delivery for cancer therapy. Biomed Res Int 2013;2013 782041.
31. Dominska M, Dykxhoorn DM. Breaking down the barriers: siRNA delivery and endosome escape. Journal of Cell Science 2010;123(Pt 8) 1183-9.
32. Dreaden EC, Mackey MA, Huang X, Kang B, El-Sayed MA. Beating cancer in multiple ways using nanogold. Chemical Society Reviews 2011;40(7) 3391-404.
33. Conde J, Doria G, Baptista P. Noble metal nanoparticles applications in cancer. J Drug Deliv 2012;2012 751075.
34. Cabral RM, Baptista PV. The Chemistry and Biology of Gold Nanoparticle-mediated Photothermal Therapy: Promises and Challenges. Nano LIFE 2013;03(03) 1330001.
35. Giljohann DA, Seferos DS, Prigodich AE, Patel PC, Mirkin CA. Gene regulation with polyvalent siRNA-nanoparticle conjugates. Journal of the American Chemical Society 2009;131(6) 2072-3.
36. Rosi NL, Giljohann DA, Thaxton CS, Lytton-Jean AK, Han MS, Mirkin CA. Oligonucleotide-modified gold nanoparticles for intracellular gene regulation. Science 2006;312(5776) 1027-30.

37. Oishi M, Nakaogami J, Ishii T, Nagasaki Y. Smart PEGylated Gold Nanoparticles for the Cytoplasmic Delivery of siRNA to Induce Enhanced Gene Silencing. Chemistry Letters 2006;35(9) 1046-7.
38. Massich MD, Giljohann DA, Seferos DS, Ludlow LE, Horvath CM, Mirkin CA. Regulating Immune Response Using Polyvalent Nucleic Acid−Gold Nanoparticle Conjugates. Molecular Pharmaceutics 2009;6(6) 1934-40.
39. Giljohann DA, Seferos DS, Patel PC, Millstone JE, Rosi NL, Mirkin CA. Oligonucleotide loading determines cellular uptake of DNA-modified gold nanoparticles. Nano Letters 2007;7(12) 3818-21.
40. Patel PC, Giljohann DA, Daniel WL, Zheng D, Prigodich AE, Mirkin CA. Scavenger receptors mediate cellular uptake of polyvalent oligonucleotide-functionalized gold nanoparticles. Bioconjugate Chemistry 2010;21(12) 2250-6.
41. Lee SH, Bae KH, Kim SH, Lee KR, Park TG. Amine-functionalized gold nanoparticles as non-cytotoxic and efficient intracellular siRNA delivery carriers. International Journal of Pharmaceutics 2008;364(1) 94-101.
42. Braun GB, Pallaoro A, Wu G, Missirlis D, Zasadzinski JA, Tirrell M, Reich NO. Laser-Activated Gene Silencing via Gold Nanoshell-siRNA Conjugates. ACS Nano 2009;3(7) 2007-15.
43. Lee JS, Green JJ, Love KT, Sunshine J, Langer R, Anderson DG. Gold, poly(beta-amino ester) nanoparticles for small interfering RNA delivery. Nano Letters 2009;9(6) 2402-6.
44. Elbakry A, Zaky A, Liebl R, Rachel R, Goepferich A, Breunig M. Layer-by-layer assembled gold nanoparticles for siRNA delivery. Nano Letters 2009;9(5) 2059-64.
45. Guo S, Huang Y, Jiang Q, Sun Y, Deng L, Liang Z, Du Q, Xing J, Zhao Y, Wang PC, Dong A, Liang XJ. Enhanced gene delivery and siRNA silencing by gold nanoparticles coated with charge-reversal polyelectrolyte. ACS Nano 2010;4(9) 5505-11.
46. Song WJ, Du JZ, Sun TM, Zhang PZ, Wang J. Gold nanoparticles capped with polyethyleneimine for enhanced siRNA delivery. Small 2010;6(2) 239-46.
47. Kirkland-York S, Zhang Y, Smith AE, York AW, Huang F, McCormick CL. Tailored design of Au nanoparticle-siRNA carriers utilizing reversible addition-fragmentation chain transfer polymers. Biomacromolecules 2010;11(4) 1052-9.
48. Love JC, Estroff LA, Kriebel JK, Nuzzo RG, Whitesides GM. Self-Assembled Monolayers of Thiolates on Metals as a Form of Nanotechnology. Chemical Reviews 2005;105(4) 1103-70.
49. Sperling RA, Parak WJ. Surface modification, functionalization and bioconjugation of colloidal inorganic nanoparticles. Philos Trans A Math Phys Eng Sci 2010;368(1915) 1333-83.
50. Liu Y, Shipton MK, Ryan J, Kaufman ED, Franzen S, Feldheim DL. Synthesis, stability, and cellular internalization of gold nanoparticles containing mixed peptide-poly(ethylene glycol) monolayers. Analytical Chemistry 2007;79(6) 2221-9.
51. Dubois LH, Nuzzo RG. Synthesis, Structure, and Properties of Model Organic Surfaces. Annual Review of Physical Chemistry 1992;43(1) 437-63.
52. Otsuka H, Nagasaki Y, Kataoka K. PEGylated nanoparticles for biological and pharmaceutical applications. Adv Drug Deliv Rev 2003;55(3) 403-19.
53. Niidome T, Yamagata M, Okamoto Y, Akiyama Y, Takahashi H, Kawano T, Katayama Y, Niidome Y. PEG-modified gold nanorods with a stealth character for in vivo applications. Journal of Controlled Release 2006;114(3) 343-7.
54. Paciotti GF, Myer L, Weinreich D, Goia D, Pavel N, McLaughlin RE, Tamarkin L. Colloidal gold: a novel nanoparticle vector for tumor directed drug delivery. Drug Delivery 2004;11(3) 169-83.
55. Gref R, Luck M, Quellec P, Marchand M, Dellacherie E, Harnisch S, Blunk T, Muller RH. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the

corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloids and Surfaces B: Biointerfaces 2000;18(3-4) 301-13.
56. Owens DE, 3rd, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. International Journal of Pharmaceutics 2006;307(1) 93-102.
57. van Vlerken LE, Vyas TK, Amiji MM. Poly(ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery. Pharmaceutical Research 2007;24(8) 1405-14.
58. Maeda H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. Advances in Enzyme Regulation 2001;41 189-207.
59. Anajwala CC, Jani GK, Swamy SMV. Current Trends of Nanotechnology for Cancer Therapy. Internatinal Journal of Pharmaceutical Sciences and Nanotechnology 2010;3(3) 1043-56.
60. Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Itty Ipe B, Bawendi MG, Frangioni JV. Renal clearance of quantum dots. Nature Biotechnology 2007;25(10) 1165-70.
61. Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, Jain RK. Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. Proceedings of the National Academy of Sciences of the United States of America 1998;95(8) 4607-12.
62. Kong G, Braun RD, Dewhirst MW. Hyperthermia enables tumor-specific nanoparticle delivery: effect of particle size. Cancer Research 2000;60(16) 4440-5.
63. Melancon M, Lu W, Li C. Gold-Based Magneto/Optical Nanostructures: Challenges for In Vivo Applications in Cancer Diagnostics and Therapy. Materials Research Bulletin 2009;34(6) 415-21.
64. De Jong WH, Hagens WI, Krystek P, Burger MC, Sips AJ, Geertsma RE. Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials 2008;29(12) 1912-9.
65. Kettiger H, Schipanski A, Wick P, Huwyler J. Engineered nanomaterial uptake and tissue distribution: from cell to organism. Int J Nanomedicine 2013;8 3255-69.
66. Lesniak A, Campbell A, Monopoli MP, Lynch I, Salvati A, Dawson KA. Serum heat inactivation affects protein corona composition and nanoparticle uptake. Biomaterials 2010;31(36) 9511-8.
67. Chithrani BD, Ghazani AA, Chan WC. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Letters 2006;6(4) 662-8.
68. Saha K, Kim ST, Yan B, Miranda OR, Alfonso FS, Shlosman D, Rotello VM. Surface functionality of nanoparticles determines cellular uptake mechanisms in mammalian cells. Small 2013;9(2) 300-5.
69. Chithrani BD, Chan WC. Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. Nano Letters 2007;7(6) 1542-50.
70. Walkey CD, Olsen JB, Guo H, Emili A, Chan WC. Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. Journal of the American Chemical Society 2012;134(4) 2139-47.
71. Cho EC, Xie J, Wurm PA, Xia Y. Understanding the role of surface charges in cellular adsorption versus internalization by selectively removing gold nanoparticles on the cell surface with a I2/KI etchant. Nano Letters 2009;9(3) 1080-4.
72. Jiang W, Kim BY, Rutka JT, Chan WC. Nanoparticle-mediated cellular response is size-dependent. Nat Nanotechnol 2008;3(3) 145-50.
73. Ruoslahti E, Bhatia SN, Sailor MJ. Targeting of drugs and nanoparticles to tumors. Journal of Cell Biology 2010;188(6) 759-68.

74. Yasui W, Sumiyoshi H, Hata J, Kameda T, Ochiai A, Ito H, Tahara E. Expression of epidermal growth factor receptor in human gastric and colonic carcinomas. Cancer Research 1988;48(1) 137-41.
75. Costa S, Stamm H, Almendral A, Ludwig H, Wyss R, Fabbro D, Ernst A, Takahashi A, Eppenberger U. Predictive value of EGF receptor in breast cancer. Lancet 1988;2(8622) 1258.
76. Ang KK, Berkey BA, Tu X, Zhang HZ, Katz R, Hammond EH, Fu KK, Milas L. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. Cancer Research 2002;62(24) 7350-6.
77. Dreaden EC, Mwakwari SC, Sodji QH, Oyelere AK, El-Sayed MA. Tamoxifen-poly(ethylene glycol)-thiol gold nanoparticle conjugates: enhanced potency and selective delivery for breast cancer treatment. Bioconjugate Chemistry 2009;20(12) 2247-53.
78. Kang B, Mackey MA, El-Sayed MA. Nuclear targeting of gold nanoparticles in cancer cells induces DNA damage, causing cytokinesis arrest and apoptosis. Journal of the American Chemical Society 2010;132(5) 1517-9.
79. de la Fuente JM, Berry CC. Tat Peptide as an Efficient Molecule To Translocate Gold Nanoparticles into the Cell Nucleus. Bioconjugate Chemistry 2005;16(5) 1176-80.
80. Krpetic Z, Saleemi S, Prior IA, See V, Qureshi R, Brust M. Negotiation of intracellular membrane barriers by TAT-modified gold nanoparticles. ACS Nano 2011;5(6) 5195-201.
81. Lu W, Zhang G, Zhang R, Flores LG, 2nd, Huang Q, Gelovani JG, Li C. Tumor site-specific silencing of NF-kappaB p65 by targeted hollow gold nanosphere-mediated photothermal transfection. Cancer Research 2010;70(8) 3177-88.
82. Lu W, Xiong C, Zhang G, Huang Q, Zhang R, Zhang JZ, Li C. Targeted photothermal ablation of murine melanomas with melanocyte-stimulating hormone analog-conjugated hollow gold nanospheres. Clinical Cancer Research 2009;15(3) 876-86.
83. Choi CH, Alabi CA, Webster P, Davis ME. Mechanism of active targeting in solid tumors with transferrin-containing gold nanoparticles. Proceedings of the National Academy of Sciences of the United States of America 2010;107(3) 1235-40.
84. Melancon MP, Lu W, Yang Z, Zhang R, Cheng Z, Elliot AM, Stafford J, Olson T, Zhang JZ, Li C. In vitro and in vivo targeting of hollow gold nanoshells directed at epidermal growth factor receptor for photothermal ablation therapy. Molecular Cancer Therapeutics 2008;7(6) 1730-9.
85. Wadhwani AR, Klein WL, Lacor PN. Efficient Gene Silencing in Neural Cells by Functionalized Gold Nanoparticles. Nanoscape 2010;7(1) 6-10.
86. Heinemann D, Schomaker M, Kalies S, Schieck M, Carlson R, Murua Escobar H, Ripken T, Meyer H, Heisterkamp A. Gold nanoparticle mediated laser transfection for efficient siRNA mediated gene knock down. PloS One 2013;8(3) e58604.
87. Ghosh R, Singh LC, Shohet JM, Gunaratne PH. A gold nanoparticle platform for the delivery of functional microRNAs into cancer cells. Biomaterials 2013;34(3) 807-16.
88. Zhang W, Meng J, Ji Y, Li X, Kong H, Wu X, Xu H. Inhibiting metastasis of breast cancer cells in vitro using gold nanorod-siRNA delivery system. Nanoscale 2011;3(9) 3923-32.
89. Ryou SM, Park M, Kim JM, Jeon CO, Yun CH, Han SH, Kim SW, Lee Y, Kim S, Han MS, Bae J, Lee K. Inhibition of xenograft tumor growth in mice by gold nanoparticle-assisted delivery of short hairpin RNAs against Mcl-1L. Journal of Biotechnology 2011;156(2) 89-94.
90. Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, Cheresh DA. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 1994;79(7) 1157-64.

91. Conde J, Ambrosone A, Sanz V, Hernandez Y, Marchesano V, Tian F, Child H, Berry CC, Ibarra MR, Baptista PV, Tortiglione C, de la Fuente JM. Design of multifunctional gold nanoparticles for in vitro and in vivo gene silencing. ACS Nano 2012;6(9) 8316-24.
92. Conde J, Tian F, Hernandez Y, Bao C, Cui D, Janssen KP, Ibarra MR, Baptista PV, Stoeger T, de la Fuente JM. In vivo tumor targeting via nanoparticle-mediated therapeutic siRNA coupled to inflammatory response in lung cancer mouse models. Biomaterials 2013;34(31) 7744-53.
93. Powell AC, Paciotti GF, Libutti SK. Colloidal gold: a novel nanoparticle for targeted cancer therapeutics. Methods in Molecular Biology 2010;624 375-84.
94. Connor EE, Mwamuka J, Gole A, Murphy CJ, Wyatt MD. Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity. Small 2005;1(3) 325-7.
95. Hauck TS, Ghazani AA, Chan WC. Assessing the effect of surface chemistry on gold nanorod uptake, toxicity, and gene expression in mammalian cells. Small 2008;4(1) 153-9.
96. Browning LM, Lee KJ, Huang T, Nallathamby PD, Lowman JE, Xu XH. Random walk of single gold nanoparticles in zebrafish embryos leading to stochastic toxic effects on embryonic developments. Nanoscale 2009;1(1) 138-52.
97. Conde J, Larguinho M, Cordeiro A, Raposo LR, Costa PM, Santos S, Diniz MS, Fernandes AR, Baptista PV. Gold-nanobeacons for gene therapy: evaluation of genotoxicity, cell toxicity and proteome profiling analysis. Nanotoxicology 2013.
98. Sabella S, Brunetti V, Vecchio G, Galeone A, Maiorano G, Cingolani R, Pompa P. Toxicity of citrate-capped AuNPs: an in vitro and in vivo assessment. Journal of Nanoparticle Research 2011;13(12) 6821-35.
99. Pompa P, Vecchio G, Galeone A, Brunetti V, Sabella S, Maiorano G, Falqui A, Bertoni G, Cingolani R. In Vivo toxicity assessment of gold nanoparticles in Drosophila melanogaster. Nano Research 2011;4(4) 405-13.
100. Harper SL, Carriere JL, Miller JM, Hutchison JE, Maddux BL, Tanguay RL. Systematic evaluation of nanomaterial toxicity: utility of standardized materials and rapid assays. ACS Nano 2011;5(6) 4688-97.
101. Pan Y, Leifert A, Ruau D, Neuss S, Bornemann J, Schmid G, Brandau W, Simon U, Jahnen-Dechent W. Gold nanoparticles of diameter 1.4 nm trigger necrosis by oxidative stress and mitochondrial damage. Small 2009;5(18) 2067-76.
102. Alkilany AM, Murphy CJ. Toxicity and cellular uptake of gold nanoparticles: what we have learned so far? Journal of Nanoparticle Research 2010;12(7) 2313-33.
103. Lewinski N, Colvin V, Drezek R. Cytotoxicity of nanoparticles. Small 2008;4(1) 26-49.
104. Dreaden EC, Alkilany AM, Huang X, Murphy CJ, El-Sayed MA. The golden age: gold nanoparticles for biomedicine. Chemical Society Reviews 2012;41(7) 2740-79.
105. Pan Y, Neuss S, Leifert A, Fischler M, Wen F, Simon U, Schmid G, Brandau W, Jahnen-Dechent W. Size-dependent cytotoxicity of gold nanoparticles. Small 2007;3(11) 1941-9.
106. Yildirimer L, Thanh NTK, Loizidou M, Seifalian AM. Toxicology and clinical potential of nanoparticles. Nano Today 2011;6(6) 585-607.
107. Goodman CM, McCusker CD, Yilmaz T, Rotello VM. Toxicity of gold nanoparticles functionalized with cationic and anionic side chains. Bioconjugate Chemistry 2004;15(4) 897-900.
108. Chen YS, Hung YC, Liau I, Huang GS. Assessment of the In Vivo Toxicity of Gold Nanoparticles. Nanoscale Res Lett 2009;4(8) 858-64.
109. Tsoli M, Kuhn H, Brandau W, Esche H, Schmid G. Cellular uptake and toxicity of Au55 clusters. Small 2005;1(8-9) 841-4.

110. Ryan JA, Overton KW, Speight ME, Oldenburg CN, Loo L, Robarge W, Franzen S, Feldheim DL. Cellular uptake of gold nanoparticles passivated with BSA-SV40 large T antigen conjugates. Analytical Chemistry 2007;79(23) 9150-9.
111. Nativo P, Prior IA, Brust M. Uptake and intracellular fate of surface-modified gold nanoparticles. ACS Nano 2008;2(8) 1639-44.
112. Patra HK, Banerjee S, Chaudhuri U, Lahiri P, Dasgupta AK. Cell selective response to gold nanoparticles. Nanomedicine: Nanotechnology, Biology, and Medicine 2007;3(2) 111-9.
113. Arnida, Malugin A, Ghandehari H. Cellular uptake and toxicity of gold nanoparticles in prostate cancer cells: a comparative study of rods and spheres. Journal of Applied Toxicology 2010;30(3) 212-7.
114. Li JJ, Hartono D, Ong CN, Bay BH, Yung LY. Autophagy and oxidative stress associated with gold nanoparticles. Biomaterials 2010;31(23) 5996-6003.
115. Li JJ, Zou L, Hartono D, Ong CN, Bay BH, Lanry Yung LY. Gold Nanoparticles Induce Oxidative Damage in Lung Fibroblasts In Vitro. Advanced Materials 2008;20(1) 138-42.
116. Schaeublin NM, Braydich-Stolle LK, Schrand AM, Miller JM, Hutchison J, Schlager JJ, Hussain SM. Surface charge of gold nanoparticles mediates mechanism of toxicity. Nanoscale 2011;3(2) 410-20.
117. Alkilany AM, Nagaria PK, Hexel CR, Shaw TJ, Murphy CJ, Wyatt MD. Cellular uptake and cytotoxicity of gold nanorods: molecular origin of cytotoxicity and surface effects. Small 2009;5(6) 701-8.
118. Cho WS, Cho M, Jeong J, Choi M, Cho HY, Han BS, Kim SH, Kim HO, Lim YT, Chung BH, Jeong J. Acute toxicity and pharmacokinetics of 13 nm-sized PEG-coated gold nanoparticles. Toxicology and Applied Pharmacology 2009;236(1) 16-24.
119. Sonavane G, Tomoda K, Makino K. Biodistribution of colloidal gold nanoparticles after intravenous administration: effect of particle size. Colloids and Surfaces B: Biointerfaces 2008;66(2) 274-80.
120. Hirn S, Semmler-Behnke M, Schleh C, Wenk A, Lipka J, Schaffler M, Takenaka S, Moller W, Schmid G, Simon U, Kreyling WG. Particle size-dependent and surface charge-dependent biodistribution of gold nanoparticles after intravenous administration. European Journal of Pharmaceutics and Biopharmaceutics 2011;77(3) 407-16.
121. Khlebtsov N, Dykman L. Biodistribution and toxicity of engineered gold nanoparticles: a review of in vitro and in vivo studies. Chemical Society Reviews 2011;40(3) 1647-71.
122. Longmire M, Choyke PL, Kobayashi H. Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats. Nanomedicine (Lond) 2008;3(5) 703-17.
123. Lim ZZ, Li JE, Ng CT, Yung LY, Bay BH. Gold nanoparticles in cancer therapy. Acta Pharmacologica Sinica 2011;32(8) 983-90.
124. Couvreur P. Nanoparticles in drug delivery: past, present and future. Adv Drug Deliv Rev 2013;65(1) 21-3.
125. Lim SB, Banerjee A, Önyüksel H. Improvement of drug safety by the use of lipid-based nanocarriers. Journal of Controlled Release 2012;163(1) 34-45.
126. Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC. Nanoparticles in medicine: therapeutic applications and developments. Clinical Pharmacology and Therapeutics 2008;83(5) 761-9.
127. Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. Int J Nanomedicine 2006;1(3) 297-315.

128. Duncan R, Gaspar R. Nanomedicine(s) under the microscope. Molecular Pharmaceutics 2011;8(6) 2101-41.
129. Lammers T, Kiessling F, Hennink WE, Storm G. Nanotheranostics and image-guided drug delivery: current concepts and future directions. Molecular Pharmaceutics 2010;7(6) 1899-912.
130. Shubayev VI, Pisanic TR, 2nd, Jin S. Magnetic nanoparticles for theragnostics. Adv Drug Deliv Rev 2009;61(6) 467-77.
131. Hynynen K. MRI-guided focused ultrasound treatments. Ultrasonics 2010;50(2) 221-9.
132. Mulder WJ, Strijkers GJ, van Tilborg GA, Griffioen AW, Nicolay K. Lipid-based nanoparticles for contrast-enhanced MRI and molecular imaging. NMR in Biomedicine 2006;19(1) 142-64.
133. Rawat M, Singh D, Saraf S, Saraf S. Lipid carriers: a versatile delivery vehicle for proteins and peptides. Yakugaku Zasshi. Journal of the Pharmaceutical Society of Japan 2008;128(2) 269-80.
134. Petros RA, DeSimone JM. Strategies in the design of nanoparticles for therapeutic applications. Nature Reviews: Drug Discovery 2010;9(8) 615-27.
135. Alexis F, Pridgen E, Molnar LK, Farokhzad OC. Factors affecting the clearance and biodistribution of polymeric nanoparticles. Molecular Pharmaceutics 2008;5(4) 505-15.
136. Fang C, Shi B, Pei YY, Hong MH, Wu J, Chen HZ. In vivo tumor targeting of tumor necrosis factor-alpha-loaded stealth nanoparticles: effect of MePEG molecular weight and particle size. European Journal of Pharmaceutical Sciences 2006;27(1) 27-36.
137. Liu D, Mori A, Huang L. Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1992;1104(1) 95-101.
138. Panariti A, Miserocchi G, Rivolta I. The effect of nanoparticle uptake on cellular behavior: disrupting or enabling functions? Nanotechnology, Science and Applications 2012;5 87-100.
139. Krasnici S, Werner A, Eichhorn ME, Schmitt-Sody M, Pahernik SA, Sauer B, Schulze B, Teifel M, Michaelis U, Naujoks K, Dellian M. Effect of the surface charge of liposomes on their uptake by angiogenic tumor vessels. International Journal of Cancer 2003;105(4) 561-7.
140. Khalil IA, Kogure K, Akita H, Harashima H. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. Pharmacological Reviews 2006;58(1) 32-45.
141. Lee RJ, Huang L. Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. Journal of Biological Chemistry 1996;271(14) 8481-7.
142. Patil SD, Rhodes DG, Burgess DJ. Anionic liposomal delivery system for DNA transfection. AAPS J 2004;6(4) e29.
143. Drulis-Kawa Z, Dorotkiewicz-Jach A. Liposomes as delivery systems for antibiotics. International Journal of Pharmaceutics 2010;387(1-2) 187-98.
144. Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proceedings of the National Academy of Sciences of the United States of America 1988;85(18) 6949-53.
145. Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1991;1066(1) 29-36.
146. Tagami T, Nakamura K, Shimizu T, Ishida T, Kiwada H. Effect of siRNA in PEG-coated siRNA-lipoplex on anti-PEG IgM production. Journal of Controlled Release 2009;137(3) 234-40.
147. Song LY, Ahkong QF, Rong Q, Wang Z, Ansell S, Hope MJ, Mui B. Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense

DNA mediated by cationic lipid liposomes. Biochimica et Biophysica Acta (BBA) - Bioenergetics 2002;1558(1) 1-13.
148. Zalipsky S, Qazen M, Walker JA, 2nd, Mullah N, Quinn YP, Huang SK. New detachable poly(ethylene glycol) conjugates: cysteine-cleavable lipopolymers regenerating natural phospholipid, diacyl phosphatidylethanolamine. Bioconjugate Chemistry 1999;10(5) 703-7.
149. Willis M, Forssen E. Ligand-targeted liposomes. Adv Drug Deliv Rev 1998;29(3) 249-71.
150. Kirpotin DB, Drummond DC, Shao Y, Shalaby MR, Hong K, Nielsen UB, Marks JD, Benz CC, Park JW. Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. Cancer Research 2006;66(13) 6732-40.
151. Iden DL, Allen TM. In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. Biochimica et Biophysica Acta (BBA) - Bioenergetics 2001;1513(2) 207-16.
152. Desai N. Challenges in development of nanoparticle-based therapeutics. AAPS J 2012;14(2) 282-95.
153. Lehtinen J, Raki M, Bergstrom KA, Uutela P, Lehtinen K, Hiltunen A, Pikkarainen J, Liang H, Pitkanen S, Maatta AM, Ketola RA, Yliperttula M, Wirth T, Urtti A. Pre-targeting and direct immunotargeting of liposomal drug carriers to ovarian carcinoma. PloS One 2012;7(7) e41410.
154. Pan X, Wu G, Yang W, Barth RF, Tjarks W, Lee RJ. Synthesis of cetuximab-immunoliposomes via a cholesterol-based membrane anchor for targeting of EGFR. Bioconjugate Chemistry 2007;18(1) 101-8.
155. Oliveira S, Schiffelers RM, van der Veeken J, van der Meel R, Vongpromek R, van Bergen En Henegouwen PM, Storm G, Roovers RC. Downregulation of EGFR by a novel multivalent nanobody-liposome platform. Journal of Controlled Release 2010;145(2) 165-75.
156. Kao H-W, Lin Y-Y, Chen C-C, Chi K-H, Tien D-C, Hsia C-C, Lin M-H, Wang H-E. Evaluation of EGFR-targeted radioimmuno-gold-nanoparticles as a theranostic agent in a tumor animal model. Bioorganic & Medicinal Chemistry Letters 2013;23(11) 3180-5.
157. Raju A, Muthu MS, Feng SS. Trastuzumab-conjugated vitamin E TPGS liposomes for sustained and targeted delivery of docetaxel. Expert Opin Drug Deliv 2013;10(6) 747-60.
158. Yang T, Choi MK, Cui FD, Kim JS, Chung SJ, Shim CK, Kim DD. Preparation and evaluation of paclitaxel-loaded PEGylated immunoliposome. Journal of Controlled Release 2007;120(3) 169-77.
159. Hainfeld JF, O'Connor MJ, Dilmanian FA, Slatkin DN, Adams DJ, Smilowitz HM. Micro-CT enables microlocalisation and quantification of Her2-targeted gold nanoparticles within tumour regions. British Journal of Radiology 2011;84(1002) 526-33.
160. Kuesters GM, Campbell RB. Conjugation of bevacizumab to cationic liposomes enhances their tumor-targeting potential. Nanomedicine: Nanotechnology, Biology, and Medicine 2010;5(2) 181.
161. Ambasta RK, Sharma A, Kumar P. Nanoparticle mediated targeting of VEGFR and cancer stem cells for cancer therapy. Vasc Cell 2011;3 26.
162. Allen TM, Sapra P, Moase E, Moreira J, Iden D. Adventures in targeting. Journal of Liposome Research 2002;12(1-2) 5-12.
163. Nguyen CT, Nguyen JT, Rutledge S, Zhang J, Wang C, Walker GC. Detection of chronic lymphocytic leukemia cell surface markers using surface enhanced Raman scattering gold nanoparticles. Cancer Letters 2010;292(1) 91-7.
164. Yu B, Mao Y, Bai LY, Herman SE, Wang X, Ramanunni A, Jin Y, Mo X, Cheney C, Chan KK, Jarjoura D, Marcucci G, Lee RJ, Byrd JC, Lee LJ, Muthusamy N. Targeted nanoparticle delivery

overcomes off-target immunostimulatory effects of oligonucleotides and improves therapeutic efficacy in chronic lymphocytic leukemia. Blood 2013;121(1) 136-47.
165. Bisker G, Yeheskely-Hayon D, Minai L, Yelin D. Controlled release of Rituximab from gold nanoparticles for phototherapy of malignant cells. Journal of Controlled Release 2012;162(2) 303-9.
166. Kibria G, Hatakeyama H, Ohga N, Hida K, Harashima H. The effect of liposomal size on the targeted delivery of doxorubicin to Integrin alphavbeta3-expressing tumor endothelial cells. Biomaterials 2013;34(22) 5617-27.
167. Arosio D, Manzoni L, Araldi EM, Scolastico C. Cyclic RGD functionalized gold nanoparticles for tumor targeting. Bioconjugate Chemistry 2011;22(4) 664-72.
168. Knudsen NO, Schiffelers RM, Jorgensen L, Hansen J, Frokjaer S, Foged C. Design of cyclic RKKH peptide-conjugated PEG liposomes targeting the integrin alpha(2)beta(1) receptor. International Journal of Pharmaceutics 2012;428(1-2) 171-7.
169. Gaspar MM, Radomska A, Gobbo OL, Bakowsky U, Radomski MW, Ehrhardt C. Targeted delivery of transferrin-conjugated liposomes to an orthotopic model of lung cancer in nude rats. Journal of Aerosol Medicine and Pulmonary Drug Delivery 2012;25(6) 310-8.
170. Vaidya B, Vyas SP. Transferrin coupled vesicular system for intracellular drug delivery for the treatment of cancer: development and characterization. Journal of Drug Targeting 2012;20(4) 372-80.
171. Bartczak D, Muskens OL, Sanchez-Elsner T, Kanaras AG, Millar TM. Manipulation of in Vitro Angiogenesis Using Peptide-Coated Gold Nanoparticles. ACS Nano 2013;7(6) 5628-36.
172. Herringson TP, Altin JG. Effective tumor targeting and enhanced anti-tumor effect of liposomes engrafted with peptides specific for tumor lymphatics and vasculature. International Journal of Pharmaceutics 2011;411(1-2) 206-14.
173. Sugiyama T, Asai T, Nedachi YM, Katanasaka Y, Shimizu K, Maeda N, Oku N. Enhanced active targeting via cooperative binding of ligands on liposomes to target receptors. PloS One 2013;8(6) e67550.
174. Zhou X, Zhang M, Yung B, Li H, Zhou C, Lee LJ, Lee RJ. Lactosylated liposomes for targeted delivery of doxorubicin to hepatocellular carcinoma. Int J Nanomedicine 2012;7 5465-74.
175. Guo B, Cheng Y, Li N, Li X, Jin M, Li T, Li J. In vitro and in vivo studies of galactose-modified liver-targeting liposomes. Journal of Drug Targeting 2012.
176. Dorasamy S, Narainpersad N, Singh M, Ariatti M. Novel targeted liposomes deliver sirna to hepatocellular carcinoma cells in vitro. Chemical Biology & Drug Design 2012;80(5) 647-56.
177. Locke LW, Mayo MW, Yoo AD, Williams MB, Berr SS. PET imaging of tumor associated macrophages using mannose coated 64Cu liposomes. Biomaterials 2012;33(31) 7785-93.
178. Lee RJ, Low PS. Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. Journal of Biological Chemistry 1994;269(5) 3198-204.
179. Yang C, Ding N, Xu Y, Qu X, Zhang J, Zhao C, Hong L, Lu Y, Xiang G. Folate receptor-targeted quantum dot liposomes as fluorescence probes. Journal of Drug Targeting 2009;17(7) 502-11.
180. Zhao X, Li H, Lee RJ. Targeted drug delivery via folate receptors. Expert Opin Drug Deliv 2008;5(3) 309-19.
181. Mansoori GA, Brandenburg KS, Shakeri-Zadeh A. A Comparative Study of Two Folate-Conjugated Gold Nanoparticles for Cancer Nanotechnology Applications. Cancers 2010;2(4) 1911-28.
182. Esposito G, Geninatti Crich S, Aime S. Efficient cellular labeling by CD44 receptor-mediated uptake of cationic liposomes functionalized with hyaluronic acid and loaded with MRI contrast agents. ChemMedChem 2008;3(12) 1858-62.

183. Arpicco S, Lerda C, Dalla Pozza E, Costanzo C, Tsapis N, Stella B, Donadelli M, Dando I, Fattal E, Cattel L, Palmieri M. Hyaluronic acid-coated liposomes for active targeting of gemcitabine. European Journal of Pharmaceutics and Biopharmaceutics 2013.
184. Dong J, Jiang D, Wang Z, Wu G, Miao L, Huang L. Intra-articular delivery of liposomal celecoxib-hyaluronate combination for the treatment of osteoarthritis in rabbit model. International Journal of Pharmaceutics 2013;441(1-2) 285-90.
185. Drummond DC, Meyer O, Hong K, Kirpotin DB, Papahadjopoulos D. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacological Reviews 1999;51(4) 691-743.
186. Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. Advanced Drug Delivery Reviews 2013;65(1) 36-48.
187. Olson F, Mayhew E, Maslow D, Rustum Y, Szoka F. Characterization, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes. European Journal of Cancer and Clinical Oncology 1982;18(2) 167-76.
188. Storm G, Roerdink FH, Steerenberg PA, de Jong WH, Crommelin DJ. Influence of lipid composition on the antitumor activity exerted by doxorubicin-containing liposomes in a rat solid tumor model. Cancer Research 1987;47(13) 3366-72.
189. Mayer LD, Tai LC, Ko DS, Masin D, Ginsberg RS, Cullis PR, Bally MB. Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Research 1989;49(21) 5922-30.
190. Mufamadi MS, Pillay V, Choonara YE, Du Toit LC, Modi G, Naidoo D, Ndesendo VM. A review on composite liposomal technologies for specialized drug delivery. J Drug Deliv 2011;2011 939851.
191. Soenen SJ, Brisson AR, De Cuyper M. Addressing the problem of cationic lipid-mediated toxicity: the magnetoliposome model. Biomaterials 2009;30(22) 3691-701.
192. Gabizon AA. Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. Cancer Research 1992;52(4) 891-6.
193. Nagayasu A, Shimooka T, Kinouchi Y, Uchiyama K, Takeichi Y, Kiwada H. Effects of fluidity and vesicle size on antitumor activity and myelosuppressive activity of liposomes loaded with daunorubicin. Biological & Pharmaceutical Bulletin 1994;17(7) 935-9.
194. Zou Y, Ling YH, Reddy S, Priebe W, Perez-Soler R. Effect of vesicle size and lipid composition on the in vivo tumor selectivity and toxicity of the non-cross-resistant anthracycline annamycin incorporated in liposomes. International Journal of Cancer 1995;61(5) 666-71.
195. Bally MB, Nayar R, Masin D, Cullis PR, Mayer LD. Studies on the myelosuppressive activity of doxorubicin entrapped in liposomes. Cancer Chemotherapy and Pharmacology 1990;27(1) 13-9.
196. Filion MC, Phillips NC. Major limitations in the use of cationic liposomes for DNA delivery. International Journal of Pharmaceutics 1998;162(1-2) 159-70.
197. Kedmi R, Ben-Arie N, Peer D. The systemic toxicity of positively charged lipid nanoparticles and the role of Toll-like receptor 4 in immune activation. Biomaterials 2010;31(26) 6867-75.
198. Tran MA, Watts RJ, Robertson GP. Use of liposomes as drug delivery vehicles for treatment of melanoma. Pigment Cell & Melanoma Research 2009;22(4) 388-99.
199. Sakurai H, Kawabata K, Sakurai F, Nakagawa S, Mizuguchi H. Innate immune response induced by gene delivery vectors. International Journal of Pharmaceutics 2008;354(1-2) 9-15.
200. Stewart MJ, Plautz GE, Del Buono L, Yang ZY, Xu L, Gao X, Huang L, Nabel EG, Nabel GJ. Gene transfer in vivo with DNA-liposome complexes: safety and acute toxicity in mice. Human Gene Therapy 1992;3(3) 267-75.

201. Lv H, Zhang S, Wang B, Cui S, Yan J. Toxicity of cationic lipids and cationic polymers in gene delivery. Journal of Controlled Release 2006;114(1) 100-9.
202. Yan W, Chen W, Huang L. Reactive oxygen species play a central role in the activity of cationic liposome based cancer vaccine. Journal of Controlled Release 2008;130(1) 22-8.
203. Chen W, Yan W, Huang L. A simple but effective cancer vaccine consisting of an antigen and a cationic lipid. Cancer Immunology, Immunotherapy 2008;57(4) 517-30.
204. Aberle AM, Tablin F, Zhu J, Walker NJ, Gruenert DC, Nantz MH. A novel tetraester construct that reduces cationic lipid-associated cytotoxicity. Implications for the onset of cytotoxicity. Biochemistry 1998;37(18) 6533-40.
205. Ilies MA, Seitz WA, Caproiu MT, Wentz M, Garfield RE, Balaban AT. Pyridinium-based cationic lipids as gene-transfer agents. European Journal of Organic Chemistry 2003(14) 2645-55.
206. Li WJ, Szoka FC. Lipid-based nanoparticles for nucleic acid delivery. Pharmaceutical Research 2007;24(3) 438-49.
207. Leite EA, Giuberti Cdos S, Wainstein AJ, Wainstein AP, Coelho LG, Lana AM, Savassi-Rocha PR, De Oliveira MC. Acute toxicity of long-circulating and pH-sensitive liposomes containing cisplatin in mice after intraperitoneal administration. Life Sciences 2009;84(19-20) 641-9.
208. Janoff AS, Boni LT, Popescu MC, Minchey SR, Cullis PR, Madden TD, Taraschi T, Gruner SM, Shyamsunder E, Tate MW, et al. Unusual lipid structures selectively reduce the toxicity of amphotericin B. Proceedings of the National Academy of Sciences of the United States of America 1988;85(16) 6122-6.
209. Cattel L, Ceruti M, Dosio F. From conventional to stealth liposomes: a new Frontier in cancer chemotherapy. Journal of Chemotherapy 2004;16 Suppl 4 94-7.
210. Li S-D, Huang L. Stealth nanoparticles: High density but sheddable PEG is a key for tumor targeting. Journal of Controlled Release 2010;145(3) 178-81.
211. Ewer MS, Martin FJ, Henderson C, Shapiro CL, Benjamin RS, Gabizon AA. Cardiac safety of liposomal anthracyclines. Seminars in Oncology 2004;31(6 Suppl 13) 161-81.
212. Recchia F, Saggio G, Amiconi G, Di Blasio A, Cesta A, Candeloro G, Carta G, Necozione S, Mantovani G, Rea S. A multicenter phase II study of pegylated liposomal doxorubicin and oxaliplatin in recurrent ovarian cancer. Gynecologic Oncology 2007;106(1) 164-9.
213. von Moos R, Thuerlimann BJ, Aapro M, Rayson D, Harrold K, Sehouli J, Scotte F, Lorusso D, Dummer R, Lacouture ME, Lademann J, Hauschild A. Pegylated liposomal doxorubicin-associated hand-foot syndrome: recommendations of an international panel of experts. European Journal of Cancer 2008;44(6) 781-90.
214. Ishida T, Ichihara M, Wang X, Yamamoto K, Kimura J, Majima E, Kiwada H. Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. Journal of Controlled Release 2006;112(1) 15-25.
215. Wang X, Ishida T, Kiwada H. Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. Journal of Controlled Release 2007;119(2) 236-44.
216. Li XM, Ding LY, Xu YL, Wang YL, Ping QN. Targeted delivery of doxorubicin using stealth liposomes modified with transferrin. International Journal of Pharmaceutics 2009;373(1-2) 116-23.
217. Wei M, Xu Y, Zou Q, Tu L, Tang C, Xu T, Deng L, Wu C. Hepatocellular carcinoma targeting effect of PEGylated liposomes modified with lactoferrin. European Journal of Pharmaceutical Sciences 2012;46(3) 131-41.

218. Han HD, Lee A, Hwang T, Song CK, Seong H, Hyun J, Shin BC. Enhanced circulation time and antitumor activity of doxorubicin by comblike polymer-incorporated liposomes. Journal of Controlled Release 2007;120(3) 161-8.
219. Maruyama K. Intracellular targeting delivery of liposomal drugs to solid tumors based on EPR effects. Advanced Drug Delivery Reviews 2011;63(3) 161-9.
220. Abraham SA, McKenzie C, Masin D, Ng R, Harasym TO, Mayer LD, Bally MB. In vitro and in vivo characterization of doxorubicin and vincristine coencapsulated within liposomes through use of transition metal ion complexation and pH gradient loading. Clinical Cancer Research 2004;10(2) 728-38.
221. Mayer LD, Harasym TO, Tardi PG, Harasym NL, Shew CR, Johnstone SA, Ramsay EC, Bally MB, Janoff AS. Ratiometric dosing of anticancer drug combinations: controlling drug ratios after systemic administration regulates therapeutic activity in tumor-bearing mice. Molecular Cancer Therapeutics 2006;5(7) 1854-63.
222. Bayne WF, Mayer LD, Swenson CE. Pharmacokinetics of CPX-351 (cytarabine/daunorubicin HCl) liposome injection in the mouse. Journal of Pharmaceutical Sciences 2009;98(7) 2540-8.
223. Batist G, Gelmon KA, Chi KN, Miller WH, Jr., Chia SK, Mayer LD, Swenson CE, Janoff AS, Louie AC. Safety, pharmacokinetics, and efficacy of CPX-1 liposome injection in patients with advanced solid tumors. Clinical Cancer Research 2009;15(2) 692-700.
224. Wu J, Lu Y, Lee A, Pan X, Yang X, Zhao X, Lee RJ. Reversal of multidrug resistance by transferrin-conjugated liposomes co-encapsulating doxorubicin and verapamil. Journal of Pharmacy & Pharmaceutical Sciences 2007;10(3) 350-7.
225. Tagami T, Suzuki T, Matsunaga M, Nakamura K, Moriyoshi N, Ishida T, Kiwada H. Anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery. International Journal of Pharmaceutics 2012;422(1-2) 280-9.
226. Banciu M, Metselaar JM, Schiffelers RM, Storm G. Liposomal glucocorticoids as tumor-targeted anti-angiogenic nanomedicine in B16 melanoma-bearing mice. The Journal of Steroid Biochemistry and Molecular Biology 2008;111(1–2) 101-10.
227. Wu HC, Chang DK. Peptide-mediated liposomal drug delivery system targeting tumor blood vessels in anticancer therapy. Journal of Oncology 2010;2010 723798.
228. Moura V, Lacerda M, Figueiredo P, Corvo ML, Cruz ME, Soares R, de Lima MC, Simoes S, Moreira JN. Targeted and intracellular triggered delivery of therapeutics to cancer cells and the tumor microenvironment: impact on the treatment of breast cancer. Breast Cancer Research and Treatment 2012;133(1) 61-73.
229. Luo LM, Huang Y, Zhao BX, Zhao X, Duan Y, Du R, Yu KF, Song P, Zhao Y, Zhang X, Zhang Q. Anti-tumor and anti-angiogenic effect of metronomic cyclic NGR-modified liposomes containing paclitaxel. Biomaterials 2013;34(4) 1102-14.
230. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell 2009;15(3) 232-9.
231. Mendonca LS, Moreira JN, de Lima MC, Simoes S. Co-encapsulation of anti-BCR-ABL siRNA and imatinib mesylate in transferrin receptor-targeted sterically stabilized liposomes for chronic myeloid leukemia treatment. Biotechnology and Bioengineering 2010;107(5) 884-93.
232. Glowacki S, Synowiec E, Blasiak J. The Role of Mitochondrial DNA Damage and Repair in the Resistance of BCR/ABL-Expressing Cells to Tyrosine Kinase Inhibitors. Int J Mol Sci 2013;14(8) 16348-64.
233. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nature Reviews: Genetics 2003;4(5) 346-58.

234. Sailor MJ, Park JH. Hybrid nanoparticles for detection and treatment of cancer. Advanced Materials 2012;24(28) 3779-802.
235. Hanahan D, Weinberg R. The hallmarks of cancer. Cell 2000;100(1) 57 - 70.
236. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144(5) 646-74.
237. Braun MS, Seymour MT. Balancing the efficacy and toxicity of chemotherapy in colorectal cancer. Therapeutic Advances in Medical Oncology 2011;3(1) 43-52.