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

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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-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-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].
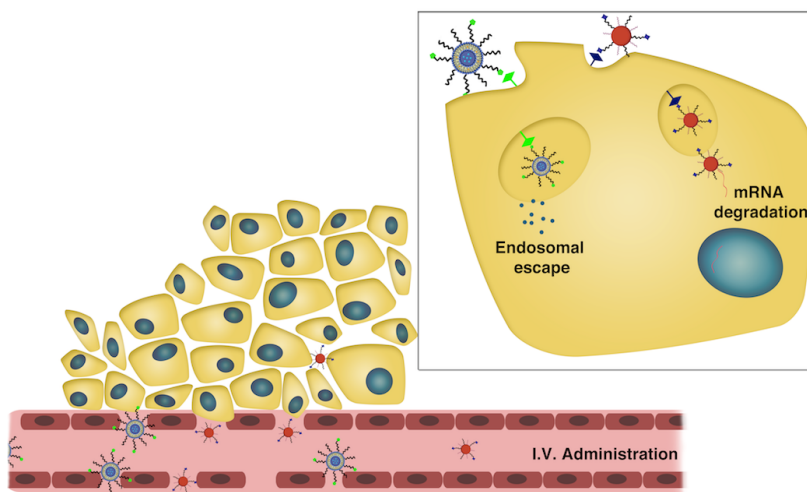

FIGURE 3.1
The potential of combined therapy mediated by AuNPs and liposomes capable of passive and active targeting

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 administered 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 lurtotecan	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)	$\alpha v\beta 3$ integrin receptor	[166, 167]
	Arg-Lys-Lys-His (RKKH)	$\alpha 2\beta 1$ 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 in the 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 on the 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 electroneutral ionophore 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.
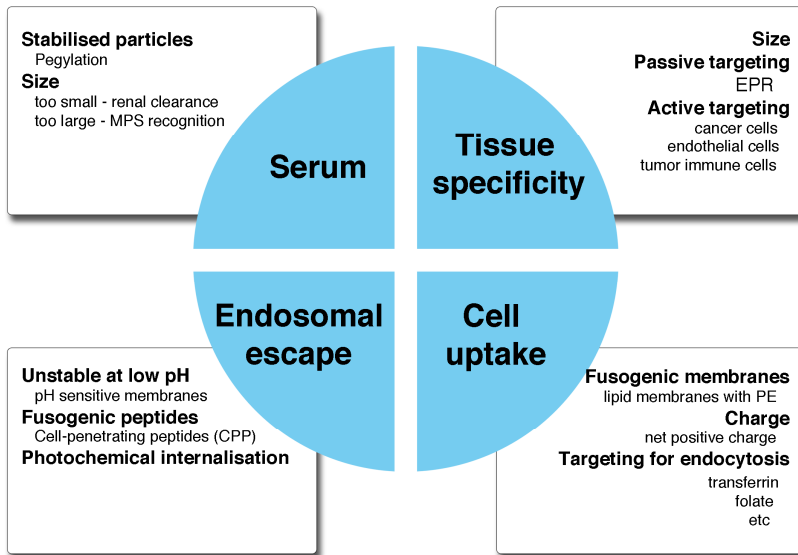

FIGURE 3.2

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

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