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Biomimetic gold nanocomplexes for gene knockdown: Will gold deliver dividends for small interfering RNA nanomedicines?

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Abstract

RNA interference (RNAi) effectors such as siRNA and miRNA can selectively downregulate any gene implicated in the pathology of a disease. Hence RNAi-based therapies present immense potential for the treatment of a wide range of diseases. However, pharmacokinetic and pharmacodynamic studies have revealed that these therapeutic agents have poor bioactivity due to a number of factors including insufficient plasma drug levels, short plasma half-lives, renal clearance and hepatic metabolism. Non-viral delivery may facilitate the clinical application of siRNA-based therapeutics by helping to overcome these barriers. Recently, the potential of gold nanoparticles (AuNPs) as multifunctional carriers for transporting drugs, proteins and genetic materials has been demonstrated. In this review, some of the key properties of AuNPs relevant to siRNA delivery such as physical properties and surface chemistry are described. In addition, we provide examples of AuNP-based formulation strategies, which have resulted in successful siRNA delivery. Finally, we evaluate the current limitations of siRNA nanomedicines such as delivery barriers and nanotoxicity and discuss potential methods to overcome these barriers achieving translational development of AuNPs for siRNA delivery.

1. Introduction

RNA interference (RNAi) is an endogenous mechanism of gene regulation that occurs post-transcriptionally and that inhibits gene expression in a sequence-dependent manner. This occurs via either of two methods; firstly by causing degradation of corresponding mRNA or secondly by blocking its translation into protein [1]. RNAi is mediated by double-stranded RNA (dsRNA) molecules, including non-coding micro RNA (miRNA) and small interfering RNA (siRNA) [2]. As a high-throughput screening tool, RNAi is a powerful strategy that has facilitated both the discovery of disease mechanisms and the identification of potential therapeutic targets [3] [4]. Due to the fact that siRNA is able to effectively and specifically downregulate the expression of any gene, this technique has been under investigation for the treatment of various diseases [5] [6] [7]. However, many challenges associated with the use of siRNA have to be addressed before the clinical application of siRNA-based therapeutics can be fully realised. These challenges include insufficient plasma drug levels, short plasma half-life, renal clearance and hepatic metabolism [8].

Viral particles were traditionally used for RNAi delivery as they are known to generate long-term gene silencing by integrating small hairpin RNA (shRNA) expression cassettes into the cell genome and can be manipulated for specific tissue tropism [9]. However their use has been limited by the potential for insertional mutagenesis leading to dysregulated gene expression in the target cell [10]. In addition, viral vectors can be removed from the bloodstream by pre-existing antibodies and tend to trigger complement and coagulation factor activation, leading to severe immunogenicity problems [11]. Furthermore, the clinical use of viral delivery vectors has been impeded by difficulties with large-scale production and cost-inefficiency. As a result, clinical trials involving viral-vector based gene therapies have steadily declined over the past decade (comprising only 19.7% of trials in 2012 compared to 22.8% in 2007 and 28% in 2004), while the number of non-viral vector trials has grown over the same period [12]. Indeed, various non-viral delivery constructs have been investigated in recent years and offer great potential for facilitating the development of successful siRNA nanomedicines [5] [6] [7].

Gold possesses a number of attractive properties as a bulk metal, including high electrical conductivity, reflectivity, malleability and resistance to corrosion and oxidation [13]. Recently, this metal has demonstrated various promising properties when it is finely tuned into the nanoscale size range (1~100 nm). For instance, because of their unique physical,

chemical and optical properties, gold nanoparticles (AuNPs) have been widely utilised in biomedical applications such as clinical chemistry, immunoassays, optical imaging, monitoring of cells and tissues and as biosensors [14-17]. The gold core of these nanoparticles (NPs) is generally inert, non-toxic and biocompatible and thus is considered a favourable starting material for carrier construction. Indeed, bioengineered multifunctional AuNPs demonstrate potential for transporting a variety of therapeutic cargos (i.e. drugs, proteins, genetic materials and small molecules) to their sites of action without showing significant adverse effects [18] [19] [20] [21].

In this review, our focus will be directed towards AuNPs used as siRNA nanomedicines. In this regard, we highlight some of the key features of AuNPs for siRNA delivery, such as size and shape dependent physical and chemical properties. In addition, we provide examples of AuNP-based vectors and describe formulation strategies that have achieved successful siRNA delivery. Challenges associated with siRNA nanomedicines including delivery barriers and nanotoxicity will be evaluated and approaches to overcome these hurdles using AuNPs will be discussed. Finally, we outline an 'ideal' multifunctional AuNP for siRNA nanomedicines, based on a critical analysis of the approaches discussed in the review.

2. Development of gold nanoparticles for siRNA delivery

2.1 Key properties of gold nanoparticles

AuNPs have recently been utilised for diagnosis and drug delivery due to their favourable pharmaceutical properties which include relatively bio-inert surfaces, easily modified surface chemistry, and the high degree of control over size and shape offered during synthesis [13] [22]. Key properties of AuNPs for siRNA delivery, namely physical properties (i.e. optics and structures) and surface chemistry (i.e. functional moieties and targeting ligands conjugation), will now be further discussed (Fig. 1).

Figure 1.

2.1.1 Physical properties

Gold has been used since the 17th century as a ruby pigment for stained glass. In 1857 Michael Faraday demonstrated that the red colour was due to metallic gold in colloidal form,

a discovery that was later followed in 1908 by the explanation of such optical properties by Gustav Mie [13]. The unique optical properties of AuNPs are now well known to be due to their Surface Plasmon Resonance (SPR), a phenomenon associated with coherent oscillations of conduction-band electrons on nanoparticle surfaces in the three dimensions of space, giving rise to localised surface plasmon resonance (LSPR) upon interaction with light [23-25]. LSPR generates light absorption, scattering phenomena and fluorescence (Fig. 1). The application of these major optical processes has been discussed at length by Huang et al [26]. In general, the SPR depends on particle morphology (i.e. size and shape), the surrounding environment and the aggregation state of the NP [27-31].

Morphology: Recent advances in AuNP synthesis have improved size distribution and facilitated particle size control within the range of 9-200 nm during synthesis in aqueous media [31-36]. It was reported that when the size of AuNPs was over 15-20 nm the plasmon was shifted to a higher wavelength, which resulted in a change of colour of the colloidal solution from red to red-grey [24, 37]. In contrast, both a blue-shift and a red-shift have been observed by decreasing the particle size [24, 32-35, 37]. The technique of UV-visible spectrophotometry has been used to assess the particle size difference in relation to modification (i.e. PEGylation, stabilising ligands, silica encapsulation and targeting ligands) of AuNPs which are designed to achieve a multifunctional carrier for siRNA delivery.

In addition, SPR is also sensitive to changes in the shape of particles [38, 39]. Geometric changes cause a variation in density of the electric field on the AuNP surface, leading to alterations in the oscillation frequency of the electrons, thereby producing different optical characteristics, such as absorption and scattering [28, 40-43]. For example, the plasmon absorption of gold nanorods is divided into two bands corresponding to the oscillation of free electrons along axes perpendicular and transverse to the axis of nanorods [44, 45]. Therefore the spectrum of nanorods in solution shows a transverse mode resonance at about 520 nm, which coincides with that of the plasmon band of spherical particles, while the resonance of the longitudinal mode is shifted to the red, strongly depending on the aspect ratio 'AR' (defined as the length divided by the width of the nanorods) [46]. Particle shape of AuNPs is now known to play an important role in biological stability and cellular uptake, which can significantly influence siRNA delivery (see reviews in section of 3.1.2).

Moreover, gold nanoshells and nanocages are known to absorb light in the Near-Infra Red (NIR), making them interesting materials as contrast agents for imaging probes in the NIR

and as photothermal therapy for cancer treatment [47-50]. Recently, NIR has been utilised to assist escape of AuNP-based siRNA delivery systems from endosomes/lysosomes, one of critical barriers for successful siRNA delivery (see reviews in section of 3.1.2).

Environments and aggregation state: The dielectric constant of the surrounding environment plays a key role in determining both the position of the plasmon maximum and its intensity. For instance, this occurs during transfer of AuNPs from water to a transparent oxide matrix [51]. Mulvaney et al. showed that a plasmon band red-shift was observed for silica-coated AuNPs due to an increase in thickness of the silica shell that altered the dielectric constant locally around the particles [52]. Moreover, Underwood showed that the colour of a colloidal solution of AuNPs synthesised in water (n = 1.336) and in a mixture of butyl acetate (n = 1.38) and carbon disulphide (n = 1.62) changed from pale red to purple due to a displacement of the plasmon band of about 23 nm towards higher wavelength [53].

In addition, the interparticle distance and aggregation state of NPs strongly affect the SPR band. Ung et al. synthesised composite of homogeneous monolayer films from silica-coating AuNPs with a control of the silica shell thickness and the AuNPs interparticle distance [54]. The distance between the particles is controlled directly by altering the thickness of the silica layer and it has been shown that the observed colour of AuNPs varies greatly with the interparticle distance. Enlargement of the plasmon and a red shift were seen due to coupling between the AuNPs cores, and when the distance was shorter, the resonance frequency became lower [54]. In addition, Maye and colleagues [55] used multidentate thioether ligands as molecular mediators to form spherical assemblies of controllable size (20 to 300 nm in diameter) with small diameter AuNPs (5 nm) stabilised by tetraalkylammonium. The UV-visible spectrum showed a change in absorbance or wavelength of the plasmon band in response to the addition of various thioether/AuNPs ratios to NPs. This change provided a measure of particle assemblies' size, shape and aggregation or elongation state.

Due to these aforementioned SPR properties the techniques of UV-visible spectrophotometry inductively coupled plasma atomic emission spectroscopy (ICP-AES), light-scattering system (DLS), transmission electron microscopy (TEM), and bright-field and dark-field microscopy, can be used to determine the size, shape and polydispersity of AuNP-based siRNA delivery systems and monitor their tissue distribution, cellular internalisation and intracellular trafficking [56] [57], all of which play critical roles in controlling AuNP pharmacokinetics

(PK) and pharmacodynamics (PD) for successful systemic delivery of siRNA (see reviews in section 3.1).

2.1.2 Surface chemistry

The inherent surface chemistry of gold contributes to making AuNPs a promising platform for biomedical applications. There are a number of methods available for the chemical synthesis of AuNPs (i.e. Brust-Schiffrin method, Turkevich-Frens approach and Hussain method) [58]. AuNP synthesis is typically started using the commercially available agent $HAuCl_4$ which acts as a gold precursor [19]. The gold precursor is reduced by the addition of a reducing agent which results in the nucleation of Au ions into AuNPs [14]. In addition, a stabilising agent is used that is either adsorbed or chemically bound to the surface of the AuNP [19]. A typical reducing agent often used is trisodium citrate (sometimes referred to simply as sodium citrate) that acts as both a reducing and stabilising agent. As trisodium citrate is typically charged, the equally charged AuNPs repel each other, thereby providing the final AuNP with electrostatic colloidal stability in aqueous media [59, 60]. A number of reducing and stabilising agents that are commonly used for AuNP production in both water and organic solvent are further discussed by Rana et al. [61]. In addition, it is interesting to note that the application of biological routes (i.e. the green nanotechnology) has been recently developed to facilitate the formation of AuNPs [62] [63] [64], which can achieve tuneable sizes and shapes of gold nanostructures instead of chemical synthetic routes based on conditions involving toxic chemicals. The synthesis of various gold nanostructures has been described by reducing Au salts with light and natural source extracts, such as plant extracts or extracts from fungi or microorganisms [65] [66] [67] [68].

Although AuNPs can be modified by a variety of surface stabilising agents including ligands, surfactants, polymers, dendrimers and biomolecules [14, 35, 69], the most popular method was demonstrated by Brust and colleagues [70]. This group used the high affinity of thiol (-SH) to gold to produce AuNP-thiolates (Au-S). This was achieved using $HAuCl_4$, thiol, tetraoctylammonium bromide and $NaBH_4$ in water-toluene, and was stabilised via Au-S-stabiliser bonds [70]. However, these NPs are dispersed in organic solvent and require further phase transfer or ligand exchange to pass them into water, as well as an essential purification step to remove impurities for use in biological application (i.e. siRNA delivery) [23, 71]. In addition, ligands containing phosphine and amine groups which similarly have high affinity to the surface of gold have been used as efficient stabilising agents [71].

These thiol, amine and phosphine groups have also been utilised to achieve AuNP functionalisation to anchor stabilising ligands to the surface of gold [72, 73] (Fig. 1). For example, PEG-SH has been used to increase stability, minimise non-specific interactions of serum proteins with AuNPs, and improve platelet biocompatibility [74-76]. In addition, other biomolecules including proteins, peptides, polysaccharides and nucleic acids, have been used to stabilise AuNPs using similar strategies with different linkers [77] [78] [79]. For example, Helmut et al. conjugated AuNPs with Trypsin-NH$_2$ via immobilisation through EDC/NHS bioconjugation onto the carboxylic functionalised AuNPs (AuNP-S-Spacer-COO$^-$) achieving AuNPs-trypsin with an amide linkage in between [80]. In addition, Tao et al. synthesised M2e-conjugated AuNPs through thiol-gold interactions, which could be lyophilised and stably re-suspended in water [81]. When mice were immunised with M2e-AuNPs (M2e, the highly conserved extracellular region of the matrix 2 protein of influenza A virus) in combination with soluble CpG (cytosine-guanine rich oligonucleotide, as an adjuvant), animals were fully protected from challenge with lethal PR8-H1N1 influenza virus.

In addition to the stabilising ligands that are grafted onto AuNP surfaces to maintain colloidal stability, AuNPs surface may also be modified with bioactive targeting ligands in order to achieve site-specific delivery of therapeutic cargos into a defined organ or, more specifically, a particular cell type [82]. Novel targeting moieties including small molecules (i.e. folic acid) [83], proteins/peptides [84] [85], nucleic acids [86] and antibodies [87], have been utilised for AuNPs to influence the tissue distribution, improve PK profile and minimise side effects to healthy cells [56]. Furthermore, these moieties are used to facilitate receptor mediated endocytosis of siRNA by cells of interest (see reviews in section 3.1.2). For example, Shiao et al. recently reported novel AuNPs that were synthesised via a thiol-Au linkage grafting a double stranded DNA [termed ds(AS1411) (a type of aptamer)] onto the surface of AuNPs, that can specifically target cancer cells that overexpress nucleolin [88]. These targeted AuNPs were then used as effective carriers for the delivery of anticancer drugs (doxorubicin (Dox)) and a photosensitizer (TMPyP4) to HeLa cells (a nucleolin positive cancerous cell line) [88]. Moreover, Arosio et al. demonstrated novel AuNPs capped with α-fucosyl-β-alanyl amide that can effectively bind cellular DC-SIGN, one of the best characterised carbohydrate-specific receptors in dendritic cells (DCs) [89]. These AuNPs with different sugar densities (15%, 30% and 50% of fucosylamide) demonstrated high water solubility and excellent dispersion; when AuNPs bound to DC-SIGN expressing cells they induced DC-SIGN internalisation without inducing DC maturation or elevating anti-inflammatory

cytokine IL-10, implying a possible application as imaging tools and antigen delivery devices [89]. However, it is worth noting that the targeted delivery strategy is highly dependent on the ratio of overexpression of the targeting receptor on the desired cells compared to other tissues [90] [91].

2.2 Formulation of gold nanoparticles - siRNA delivery strategies

As aforementioned, AuNPs can be functionalised by grafting biocompatible polymers and natural or synthetic biomolecules onto the surface of gold in order to achieve a multifunctional carrier. In this section, selected examples of AuNP-based vectors are provided and their formulation strategies, including chemisorption, electrostatic interaction and layer-by-layer self-assembly (Fig. 1), are described in order to facilitate an siRNA delivery system and also to allow for site-specific administration and controlled release (Table I).

Table I. [69, 92-122]

2.2.1 Chemisorption

Due to the high affinity of thiol (-SH) to gold to produce AuNP-thiolates (Au-S), thiol-functionalised siRNA (siRNA-SH) has been used to attach onto AuNPs thus forming a complex. Patel and co-workers evaluated the structural requirements of siRNA for Dicer recognition and serum stability when prepared with AuNPs [101]. In this study, 26 to 28 base-pair pre-siRNA with a variety of modifications were chemisorbed onto citrate-stabilised AuNPs (13 ± 1 nm) via the thiol group on the sense of pre-siRNA. The siRNA modifications included a 2-base overhang, a blunt A-U end, a blunt G-C end, pentose sugar modification with LNAs and modification of the phosphate backbone using a phosphorothioate (P=S) link. The results showed that by altering nucleic acids to contain certain features such as changing the 3'-end overhang, making a blunt A-U end or incorporating LNA and P=S, the siRNA was preferentially recognised by Dicer to generate functional siRNA. However, these modifications of siRNA also caused unwanted dramatic serum degradation of pre-siRNA. In contrast, pre-siRNA modified with a blunt G-C end was significantly resistant to serum degradation without compromising Dicer recognition. More importantly, AuNPs containing pre-siRNA with a blunt G-C end demonstrated increased cellular uptake and improved Green Fluorescent Protein (GFP) gene knockdown in comparison to other siRNA modifications. These results suggest that minor changes in nucleic acid structural properties can dramatically

influence Dicer processing, serum stability, cellular uptake and gene knockdown of siRNA-AuNPs [101].

Novel spherical nucleic acid nanoparticle conjugates (SNA-NCs) were developed by Zheng and co-workers. These NPs possess a gold core surrounded by a dense shell of highly oriented, covalently immobilised thiolated siRNA [104]. SNA-NCs containing siRNA against EGFR achieved improved gene silencing compared to siRNA delivered with commercial lipid agents in cultured keratinocytes. In addition, topical delivery of 50 nM SNA-NCs (1.5 µM EGFR siRNA) for 3 weeks to hairless mouse skin dramatically reduced EGFR expression, suppressed downstream ERK phosphorylation, and decreased epidermal thickness by ~40%, without showing clinical or histological evidence of toxicity. These results suggested that topical delivery of SNA-NCs may be promising for cutaneous tumours, skin inflammation, and dominant negative genetic skin disorders.

Furthermore, it was reported that folate receptor-targeted hollow gold nanospheres carrying siRNA via thiol groups against the NF-kappaB p65 subunit could achieve downregulation of the targeted gene when induced by NIR light [106]. The NF-kappaB transcription factor itself is known to regulate the expression of genes involved in tumor formation and progression. The results of micro-positron emission tomography/computed tomography imaging showed that the targeted NPs generated higher tumor uptake than non-targeted counterparts following i.v. administration to mice xenografted with cervical cancer cells. When combined with NIR light irradiation, the targeted NPs demonstrated efficient and controllable intracellular trafficking of siRNA. It is worth noting that efficient silencing of NF-kappaB p65 was achieved only in tumours irradiated with NIR light but not in non-irradiated tumours grown in the same mice [106]. In addition, no significant adverse effect was found on major organs including the liver, spleen, kidney and lung. These data suggest that AuNP-based RNAi therapeutics may benefit from NIR light that can penetrate the skin and be delivered with high spatiotemporal control, while avoiding unwanted side effect.

2.2.2 Electrostatic interaction

In addition to attachment of siRNA onto gold via thiol groups, negatively charged siRNA can also be condensed or complexed by AuNPs with positively charged groups via electrostatic interaction. It is known that electrostatically stabilised citrate-AuNPs have very limited applications due to their fast aggregation in complex media with high ionic strengths, i.e. in

biological fluid [35]. Therefore, the dispersion of AuNPs must be controlled to provide a better platform for the advancement of these materials in catalytic and biological applications. Recently, it has been reported that the synthesis of well dispersed AuNPs (5 to 60 nm) was achieved in water and under physiological conditions (0.15 M NaCl). This was achieved by attaching PEG-thiol (mPEG-SH) as a stabilising ligand to the surfaces of AuNPs making them suitable for application in biology and catalysis [69]. The results showed the PEG-AuNPs with diameters <30 nm were useful as catalysts in the homocoupling of arylboronic acids in water; whereas citrate-AuNPs (5, 15, 30 and 60 nm) had minimal catalytic efficiency due to their aggregation in the reaction media. siRNA was then condensed with protamine (a small, arginine-rich, nuclear protein that has been used for enhancing nucleic acid transfection) to achieve cationic 'protamine.siRNA' and complexed with the partially negatively charged PEG-AuNPs, with the final formulation demonstrating favourable cell viability, effective cellular uptake and successful *in vitro* luciferase gene knockdown [69].

In addition, synthesis of PEI-coated AuNPs using catechol-conjugated PEI (PEI-C) was developed for siRNA delivery [110]. Since the conjugated catechol groups are reductive and moderately hydrophobic, PEI-C formed spherical multi-cored micelles in aqueous solution and served as reductive templates for the growth and synthesis of spherical AuNPs with tunable sizes and surface charges. PEI-C was stably anchored on the surface of growing crystal gold seeds, resulting in robust cationic AuNPs to effectively complex siRNA. The siRNA-PEI-C-AuNP complexes with an average diameter of 15.3 nm and a surface zeta-potential value of +5 mV showed significantly higher GFP suppression than counterparts that have larger diameters and higher surface charge values, indicating that particle size and surface charge had a great influence on gene silencing efficiency. Interestingly, the PEI-capped AuNPs exhibited an extremely low cytotoxicity, most likely due to the reduced density of primary amine groups and the absence of free PEI in aqueous solution [110].

Recently, Kong and co-workers developed cationic lipid-coated AuNPs (L-AuNPs) for efficient intracellular delivery of therapeutic siRNA [111]. In this study, hydrophobic dodecanethiol-capped AuNPs with an average size of 5 nm were first produced and then solubilised in an organic solvent with three different lipid components (3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol), L-α-dioleoyl phosphatidylethanolamine (DOPE), and cholesterol). The subsequent emulsification and solvent evaporation processes resulted in the swift assembly of the amphiphilic lipid building

blocks around the AuNPs via hydrophobic interactions, achieving a final L-AuNP with an outer cationic lipid shell and an inner AuNP core allowing them to be dispersible in water. L-AuNPs were shown to effectively condense siRNA and demonstrated efficient cellular uptake and gene knockdown (i.e. GFP, VEGF, Ubiquitin B, and Hepatitis B Virus Surface Antigen) in a variety of cell lines including MDA-MB-435 (human melanoma cells), A549 (human lung carcinoma cells), PC3 (human prostate carcinoma cells), HepG2 (human Hepatocellular carcinoma cells), and HepG2.2.15 (Hepatitis B virus (HBV)-producing hepatocellular carcinoma cells). In addition, L-AuNPs did not induce any significant cytotoxicity. These results suggest that L-AuNPs are an efficient and safe siRNA delivery construct which could potentially be used for the treatment of a variety of diseases.

2.2.3   Layer-by-layer self-assembly

Layer-by-layer (LbL) assembly is a prominent method of AuNP formulation for siRNA delivery in which multi-layered films with tailored properties can be fabricated on substrates [123]. This method has been used to prepare AuNPs with multi-layered shells through particle templating [124]. For instance, Elbakry et al. reported an LbL strategy whereby citrate-AuNPs were modified with mercaptoundecanoic acid (MUA), resulting in a more stable negatively charged MUA-AuNPs [119]. The MUA-AuNPs were electrostatically interacted with PEI (PEI-MUA-AuNPs) which formed a cationic outer layer that efficiently complexed siRNA (siRNA-PEI-MUA-AuNPs) resulting in AuNPs with an anionic outer layer. Finally, siRNA-PEI-MUA-AuNPs were coated by PEI to form PEI-siRNA-PEI-MUA-AuNPs in which siRNA is sandwiched between two layers of PEI polymer and a cationic outer surface. This final formulation demonstrated efficient endosomal escape and effective Enhanced Green Fluorescence Protein (EGFP) gene silencing in CHO-K1 cells stably expressing EGFP. More importantly, this LbL approach to preparing AuNPs is an excellent tool to study how the size and the surface properties of delivery systems influence siRNA in terms of their cellular uptake, endosomal escape and release into cytoplasm [119].

Another LbL approach was demonstrated by Han and co-workers using chitosan (CS), poly(allylamine hydrochloride)-citraconic anhydride (PAH-Cit) and PEI [158]. In this work, AuNPs were reduced and stabilised by CS, resulting in a cationic AuNP-CS. This was then used to condense PAH-Cit at pH 7.4 (PHA-Cit is a pH-responsive charge-reversible polymer, being anionic at physiological pH but cationic at acidic pH). This formed PAH-Cit-CS-AuNPs with a negatively charged outer layer. Subsequently, PEI was deposited onto PAH-

Cit-CS-AuNPs achieving PEI-PAH-Cit-CS-AuNPs which could bind siRNA through electrostatic interaction between the cationic PEI and anionic siRNA. A subsequent *in vitro* release study demonstrated efficient siRNA release from siRNA-PEI-PAH-Cit-CS-AuNPs at pH 5.5, potentially facilitated by the pH-induced charge-reversing feature of PAH-Cit. Moreover, this formulation, when prepared with siRNA against Multidrug Resistance Protein 1 (MDR1, the gene encoding the drug exporter P-gp), exhibited effective gene knockdown in drug-resistant MCF-7 cells, thereby promoting the uptake of doxorubicin [118].

Although PEI has been widely used for siRNA delivery to improve cell internalisation and facilitate endosomal escape, it causes unfavourable cytotoxicity, particularly when high molecular weight varieties are used and may not be biocompatible for clinical applications [125]. As an alternative, poly(allylamine hydrochloride) (PAH) has been used for different types of siRNA delivery constructs to overcome these barriers [35, 124]. Recently, a LbL strategy was reported by Zhao et al [115]. In this method bovine serum albumin (BSA) was first reduced by $NaBH_4$ which resulted in denatured BSA (dBSA). This dBSA carried 35 thiol groups used for capping AuNPs [115]. Due to the presence of numerous carboxyl groups on dBSA, dBSA-AuNPs were electrostatically coated with PAH to form a cationic layer (PAH-dBSA-AuNPs) which could efficiently bind siRNA (siRNA-PAH-dBSA-AuNPs). Subsequently, the siRNA layer was electrostatically coated with extra PAH to form the outermost cationic layer (PAH-siRNA-PAH-dBSA-AuNPs) which facilitated cellular uptake and intracellular trafficking. As a result, PAH-AuNPs containing anti-EGFR siRNA showed improved downregulation of EGFR in human breast cancer MCF-7 cells, in comparison with the commercially available vector Lipofectamine 2000®. Although PAH-AuNPs demonstrated similar siRNA release and gene silencing efficacy compared to PEI-AuNPs, PAH-AuNPs demonstrated significantly higher cell viability, making it a more suitable alternative for efficient *in vitro* and *in vivo* gene delivery.

3. *Challenges for gold nanoparticles in translational development of siRNA nanomedicines*

RNAi is maintained by small non-coding RNAs that mainly include siRNA and micro RNA (miRNA) (numerous review articles regarding RNAi have recently been published [126-129]). It is possible to activate the siRNA-based RNAi pathway in either of two ways [130]:

(A) plasmid-, bacterial- or viral-based vectors delivering therapeutic DNA to the nucleus result in the transcription of shRNA. This is exported to the cytoplasm where it is recognised by Dicer. Dicer is an enzyme that cleaves dsRNA and pre-micro RNA (pre-miRNA) into siRNA and miRNA, respectively. Thus shRNA is processed into siRNA [~19-23 base pairs (bps) with 2-nucleotide (nt) 3' overhangs] for subsequent RISC loading. (B) Synthetic siRNA is introduced into the cytoplasm, bypassing the nucleus transcription of DNA step, to directly allow RISC loading. When siRNA is loaded into the RNA-induced silencing complex (RISC, a multiprotein complex) the sense strand is removed and the antisense strand retained to produce antisense-RISC. This complex can achieve sequence-specific cleavage of perfect (or nearly perfect) matching messenger RNA (mRNA) targets. In contrast, miRNA primarily binds to the 3' un-translated regions (UTRs) of imperfectly complementary mRNA and induces translational suppression or transcript deprivation via RISC [127].

Over the past decade, RNAi has been widely used for both *in vitro* and *in vivo* high-throughput screening, facilitating the discovery of disease mechanisms and the identification of potential therapeutic targets [3] [4]. Recent advances in our knowledge of molecular genetic data, facilitated by sequencing the genome of malignant cells, has resulted in significant changes in our understanding of the molecular pathogenesis of many diseases (i.e. many types of cancer, infection diseases and genetic disorders) [131-133] and has facilitated the identification of a number of molecular targets against which siRNA and miRNA have been developed [134-137].

Although a number of AuNP-based formulation strategies for siRNA delivery have been reviewed in Table 1, there are still many challenges for translational development of AuNPs to achieve siRNA nanomedicines. New AuNP-based delivery vectors which are safe, efficient and effective must be developed in order to overcome the various barriers associated with siRNA use, administration barriers (i.e. short plasma half-lives, non-specific biodistribution, renal clearance and hepatic metabolism) and nanotoxicity.

3.1 Delivery barriers

3.1.1   Local administration

When designing a carrier construct for siRNA delivery one of the first decisions is whether to develop the formulation for local or systemic administration. Local administration involves delivery of siRNA therapeutics directly into target tissues. This delivery method is known to

offer several advantages over systemic delivery, including lower doses, ease of access, facilitation of site-specific delivery and fewer adverse effects [138]. Indeed, successful local delivery of siRNA into several tissues, such as the foot, skin, eye, mucus membranes and tumours, has been demonstrated as shown in [138]. Recently, multifunctional AuNPs for local administration have been designed based on changes in the local environment [56]. For instance, Cole et al. developed bisphosphonate-functionalised AuNPs (BP-AuNPs) that facilitate improved sensitivity and specificity for the detection of microcalcifications which are deposits of hydroxyapatite (HA) mineral within breast tissue and the most common abnormality detected by mammography for breast cancer compared to other surrounding tissues [139]. After intra-mammary administration in a mouse breast tumour model, BP-AuNPs provided enhanced contrast for the detection of microcalcifications that were otherwise below the computed tomography (CT) detection limit.

Physical approaches including microneedle, electroporation, ultrasound and magnetic stimulation may improve local administration of therapeutic cargos [140-143]. For instance, it has been reported that the transfection efficiency of AuNPs carrying a DNA plasmid was improved ~1.5−2 fold when combined with electroporation without inducing any toxicity [109]. In addition, Wang et al. improved the delivery of gold nanorods by combining three mechanisms: gold nanorods encapsulated in protein-shell microbubbles (AuMBs), molecular targeting (anti-VEGF receptor 2), and sonoporation employing acoustic cavitation of microbubbles (MBs) [142]. Following intravenous injections in mouse xenograft models, targeting AuMBs could bind to the angiogenesis marker and stay at the tumour site over a longer period of time compared to nontargeting counterpart; when tumour areas were treated with ultrasound, MBs were destroyed to release gold nanorods that could subsequently enter into cells due to acoustic cavitation induced during MB destruction by sonoporation (i.e. increase in transient cellular permeability) [142]. These studies imply that the combination of a physical and chemical delivery concept may facilitate local administration of AuNP-based siRNA delivery systems.

3.1.2   Systemic circulation

Although local siRNA delivery has presented therapeutic potential for the treatment of disease in several instances, many diseases (e.g. metastatic cancers) can only be treated via systemic administration of therapeutic agents into the bloodstream [2, 138]. Here, we discuss AuNP-based approaches to overcome problems associated with systemic siRNA delivery

including serum degradation, instability in the circulation, targeting to specific cells of interest, activation in response to the local environment and endosomal escape (Fig. 2).

Figure 2.

Serum degradation: Unprotected siRNA is rapidly degraded in the plasma/serum due to the susceptibility of nucleic acids to degradation by serum nucleases. As described in section 2.2, the formulation of siRNA with AuNPs can be achieved using different methods and may offer the benefit of providing steric protection of siRNA from serum nucleases [130].

However, it is important to note that tight binding or condensation of siRNA may also potentially impair gene silencing efficacy, mostly like due to inefficient or incomplete release of siRNA from NPs that are too tightly associated [144]. Therefore, efforts must be made to balance the binding strength between the two, so that AuNPs will allow both protection of siRNA from serum degradation in the initial stages after administration, while also allowing the release of siRNA into the cytoplasm where it can exert its therapeutic effect.

Instability in the circulation: Considering the high surface to volume ratio of AuNPs, systemic circulation of AuNPs in bloodstream may induce non-specific binding of plasma proteins causing large particles and therefore, may be taken up by the reticuloendothelial system (RES) [also known as mononuclear phagocyte system (MPS)] in a process known as opsonisation [2]. This phenomenon leads to recognition and removal of NPs from the blood by circulating phagocytes and tissue macrophages (mainly the hepatic Kupffer cells and the marginal zone and red-pulp macrophages in the spleen) [145, 146], which can eventually result in large particles trapped in the liver and lung and, to a lesser extent, in the kidneys and spleen [147].

It is interesting to note that particle shape of AuNPs may also play an important role in protein adsorption, therefore controlling their PK profiles. AuNPs are easily tailored into various structures, such as gold nanospheres, gold nanorods, gold nanostars, gold non-pyramids and gold nanocages. Among them, Au nanospheres and Au nanorods have been substantially studied for biomedical application, especially in imaging and drug delivery [148]. Recently, direct interaction of bovine serum albumin (BSA) with gold nanospheres and nanorods was investigated to confirm the interaction of protein with AuNPs [149]. In this study, the binding constants for BSA to 10 nm anionic citrate-capped gold nanospheres and cationic CTAB-conjugated gold nanorods (aspect ratio of 2.3) were 2.34 x 10^{11} M^{-1} and 5 x

10^4 M^{-1}, respectively. The same approach was also used to calculate the binding constants of BSA to 14 nm citrate-capped gold nanospheres (1.7 x 10^8 M^{-1}) and CTAB-capped gold nanorods of aspect ratio 3 (1.3 x 10^7 M^{-1}) and 8 (2.7 x 10^7 M^{-1}) [150], suggesting that the interaction of proteins onto various AuNPs with a variety of shapes, sizes and surface functionalities need to be substantially studied. Due to the physical characteristics of AuNPs (reviewed in section 2.1.1), spectroscopic techniques that follow the shifts in the LSPR bands have been utilised to determine the interaction of different proteins with AuNPs [151]. Other analysis methods including isothermal titration calorimetry (ITC), quartz crystal microbalance (QCM), circular dichroism (CD), dynamic light scattering (DLS), gel electrophoresis and mass spectrometry, have also been used to study the protein-NP interactions [152].

One of the most widely characterised methods to avoid MPS uptake is the addition of neutral stabilising components to the surface of NPs [e.g. PEG (Polyethylene glycol), block copolymers and hyaluronic acids] to form so called 'stealth' particles [153]. Such modifications stabilise complexes against salt-, protein- and complement-induced inactivation by steric effects as well as by shielding surface charge. Indeed, PEGylation of non-viral vectors has been used to improve stability and prolong blood circulation time of siRNA delivery systems [154-156].

Although the hydrophilic PEG chain is considered to be biocompatible, non-toxic, non-immunogenic and non-antigenic and has been approved for use by the Food and Drug Administration (FDA), it is important to note that PEG has also presented unexpected toxicity and immunostimulation *in vivo* [157, 158]. For example, it has been recently reported that PEGylated AuNPs caused an unexpected effect on the primary function of erythrocytes [159]. In this study, the deformability and oxygen-delivering ability of erythrocytes were decreased due to the interaction between PEGylated AuNPs and erythrocyte membranes, implying how a careful design is required for PEG-functionalised AuNPs for biomedical applications.

In addition, it is of interest to note that certain surface-adsorbed proteins (e.g. opsonic proteins, antibodies in the blood), rather than increasing clearance from the circulation, might in fact prolong nanoparticle half-life by virtue of the spatial arrangement of these non-specific proteins (conformation, spacing and surface density) (these have been reviewed in [57] [160]).

Targeting and Environmental activation: The blood vessel walls of solid tumours are typically 'leakier' that those found in healthy tissues. This process, termed angiogenesis, is a critical step in the transformation of tumours from a benign to a malignant state [161]. These leaky vasculature and endothelial junctions provide what is known as an enhanced permeation and retention (EPR) effect which facilitates the entry of small particles (normally with diameter less than 500 nm) into sites of solid tumours (Fig. 2). Due to unique physicochemical features as described in section 2.1 AuNP-based siRNA delivery systems may present favourable size, shape and polydispersity, compliant with the requirements for tissue distribution through EPR effect [56].

In addition, it has been reported by Chithrani and co-workers that cellular uptake of AuNPs is size- and shape-dependent [162]. In this study, the uptake half-life was 2.10, 1.90 and 2.24 h at a rate of 622, 1294 and 417 particles per hour for the 14, 50 and 74 nm spherical AuNPs. In addition, data showed that cells took up 375 % and 500 % more 14 and 74 nm gold nanospheres than 74 x 14 nm gold nanorods, indicating that shape is also one reason for the difference in uptake [162]. Although cellular uptake of these AuNPs mainly results from non-specific binding of serum proteins, the size and shape of AuNPs appear to matter in the uptake, highlighting the importance of proper design of AuNPs for internalisation of siRNA.

In order to achieve cell-specific delivery of siRNA one strategy is the conjugation of targeting ligands to the distal site of PEG chain to direct AuNPs to specific cell-surface receptors overexpressed on malignant tissues, whereupon they enter cells via receptor-mediated endocytosis [130]. For instance, Suresh et al. synthesised and characterised a group of Au nanocages that were capped with bombesin (BBN) peptide toward gastrin releasing peptide receptors (GRPR, aberrantly expressed in numerous cancers) [163]. In this study, competitive cell binding and TEM results demonstrated that internalisation of Au nanocage-BBN in prostate cancer cells results from clathrin mediated pathways, suggesting that peptide ligands conjugated to the surface of AuNCs maintain their target specificity.

The specialised tumour microenvironment (TME) is adapted specifically to support the growth, invasion and metastasis of cancer cells [161]. For instance, physiological pH of blood vessels near the extracellular area of solid tumours is reduced to a slightly acidic condition (from pH 7.4 to ~pH 6.5-7.2) [164]. The low oxygen and acidic environment of such tumours are capable of triggering cancer cells with sustained tumourigenesis and can also impair anti-cancer drug therapies which cannot function in this acidic environment [165]. Moreover,

extensive tumour tissue remodelling is attributed to the overexpression of many extracellular matrix (ECM) components (i.e. matrix metalloproteinases, MMPs) and their secretion into ECM [161]. Recently, a novel angiopep-2 conjugated AuNP delivery system was developed to respond TME and release doxorubicin (DOX) to glioma [166]. This system, namely An-PEG-DOX-AuNPs, was associated with DOX through hydrazone (an acid-responsive linker) and was functionalised with angiopep-2 [a specific ligand of low density lipoprotein receptor-related protein-1 (LRP1); it may facilitate this system to penetrate the blood brain barrier and target to glioma cells]. At TME pH values (i.e. 5.0 and 6.0), release of DOX was more efficient than that at pH 6.8 and 7.4; in a glioma mouse model, intravenous administration of An-PEG-DOX-AuNPs facilitated the distribution into tumour areas at a higher intensity and achieved longer median survival time in comparison with nontargeting PEG-DOX-AuNPs and free DOX, without causing significant toxicity [166].

Furthermore, many central nervous system (CNS) disorders (e.g. Alzheimer's disease, Huntington's disease, Parkinson's disease and brain tumours) are extremely difficult to treat, largely due to the significant obstacle presented by the blood-brain barrier (BBB) (Fig. 2). The BBB (substantially reviewed in references [167, 168]) is a highly regulated and efficient barrier that limits the access of large molecules to the CNS. Recently, multifunctional NPs have been developed to facilitate delivery of therapeutic agents across the BBB. This has been achieved by modification of the delivery material with the attachment of targeting ligands for receptors found in neurons and the capillary endothelial cells of the BBB, such as the transferrin receptor, lactoferrin receptor, insulin receptor and acetylcholine receptor [85, 168-170]. For instance, a peptide sequence THRPPMWSPVWP was conjugated onto the AuNPs, and this peptide sequence could interact with the transferrin receptor present in the micro-vascular endothelial cells of the BBB, thus causing an increase in the permeability of the conjugate in brain, as demonstrated by experiments *in vitro* and *in vivo* [85]. In addition, external stimulation factors such as light, temperature, radiofrequency, magnetic field and ultrasound, may also facilitate delivery of NPs across to the BBB [171], which may provide additive or synergistic therapeutic efficacy in combination with AuNP-based delivery systems.

The potential targeting ligands that can recognise cell- or tissue-specific antigens/receptors in a variety of disorders, have been substantially described in [127] [85, 168-170] [172] [173],

which may provide great opportunities for achieving 'smart' bioactive AuNPs for siRNA delivery.

Intracellular trafficking: NPs bind to the cell surface through electrostatic interaction and are generally internalised by adsorptive pinocytosis, a process whereby extracellular fluid together with its contents are taken into the cell to form intracellular vesicles. Alternatively, NPs designed with targeting ligands bind to a specific cell-surface receptor and enter the cell by receptor-mediated endocytosis [130]. Following cellular uptake by either pathway, NPs are generally entrapped inside endosomal/lysosomal vesicles that experience a drop in pH during vesicle progression (from pH ~5-6 in endosomes to ~4.5 in later stage lysosomes) which can lead to drug and nucleic acid inactivation due to the acidic conditions and the presence of harmful hydrolytic enzymes in such vesicles. For siRNA to reach the cytoplasm and exert its effect, efficient escape from these hostile environments is critical.

AuNPs can be specifically designed to facilitate and improve endosomal/lysosomal escape in several ways; (A) functionalisation of AuNPs is generated with functional groups that can assist the release of siRNA from the endosomal/lysosomal vesicles. For example, a novel AuNP (GNR-DSPEI-PEG-GRD) was prepared by conjugating gold nanorods with multiple disulphide cross-linked short polyethyleneimine (PEI, the structure of which can be linear, whereby it possesses secondary amines only, or branched, which contains primary, secondary and tertiary amines), PEG and a terminal RGD peptide for specific targeting of integrin $\alpha_v\beta_3$-overexpressed cancer cells [174]. The GNR-DSPEI-PEG-RGD was able to enter into cells via the receptor-mediated pathway and subsequently escape from endosomal/lysosomal compartments due to PEI-induced 'proton sponge effect' (a phenomena that can enhance endosomal Cl$^-$ accumulation and give rise to osmotic swelling and endosomal rupture) [174]. Other functional moieties including membrane-disruptive materials, fusogenic peptides, glutathione (GSH)- and pH-sensitive linkers that can facilitate release of NPs from endosomal membranes, have been studied in [175-177] [178], which may be conjugated to AuNPs, achieving bio-responsive delivery of siRNA. (B) when following excitation the plasmons on AuNP surfaces can decay by either radiative damping or energetic relaxation, which may create nonequilibrium 'hot' electron-hole pairs [179]. For instance, the aforementioned GNR-DSPEI-PEG-RGD has an aspect ratio of gold nanorods compliant with NIR regions; when triggered by NIR laser release of GNR-DSPEI-PEG-RGD from

endosomal/lysosomal vesicles was much higher than that GNR-DSPEI-PEG-RGD without NIR treatment [174].

It is worth noting that intracellular trafficking studies are mainly dependent on qualitative comparison of immunofluorescence images and colocalisation of fluorescently labelled siRNA or NPs with different organelles. Alternatively, AuNPs are easily visualised by TEM, and bright-field and dark-field microscopy without functionalisation of fluorochrome, which enable quantitative characterisation of the intracellular trafficking steps and allow a more thorough understanding of this process [162] [174] [180].

3.2 Nanotoxicity

NPs are now known to induce multiple biological and genomic effects when in contact with living systems [181]. With the growing use of nanomaterials aiming to achieve clinically relevant siRNA delivery, considerable attention has been focused on understanding the potential side effects and toxicity of these NPs [182].

Although bulk gold is considered bio-inert, gold exhibits different properties at nanoscale size during syntheses and applications. The aforementioned physicochemical characteristics of AuNPs (i.e. particle size, surface charge, morphology, surface coating and particulate aggregation) are key properties that must be tuned to facilitate efficient delivery to target tissues and cells, which may cause possible AuNP toxicity from their interaction with specific cell receptors, cell membranes or intracellular organelles. Although interaction mechanisms between AuNPs and biological systems are not yet fully delineated, toxicity of AuNPs may be investigated for any of the following concerns: (A) synthesis of gold nanostructures [65] [66] [67] [68]; (B) particle size, morphology and surface charge of gold nanomaterials [183] [184]; (C) surface conjugates and ligands onto AuNPs [185] [186]; (D) administration routes and doses of AuNPs [187] [188]; (E) other effects from interfering with the specific cell types (i.e. immune cells) and biological organelles (i.e. cell membrane, lysosomes, mitochondria, Golgi apparatus and nucleus) [189] [190] [191]. Numerous toxicological studies have been conducted to investigate how these factors influence AuNP toxicity, with the aim of ensuring any undesirable effects are avoided (see reviews in [192] [193] [194] [195] [196]).

To date it is difficult to substantially evaluate nanotoxicity due to the lack of standardised approaches, inadequate models and the limitations of classical toxicological endpoints [197]. Nevertheless, the methods of assessing toxicity associated with bioavailability, PK,

persistency in the body, by-products of metabolism, cell internalisation, intercellular trafficking and cell interaction, are critical for both researchers and regulatory authorities alike. In addition, appropriate toxicity models must be carefully selected; for example, the use of *in vitro* models alone are not sufficient to measure and predict these toxicological effects, and further research using *in vivo* models for the assessment of AuNP toxicity is urgently required.

4 Conclusions and future perspectives

siRNA has attracted great attention in recent years owing to its capacity for highly sequence-specific gene downregulation and almost unlimited therapeutic target choice, especially those considered as 'undruggable'. To overcome the major delivery obstacles associated with siRNA, a variety of delivery approaches have been reported using non-viral vectors. Although several siRNA-based therapeutics have already entered clinical trials and are in the developmental pipeline, most of them are not administered intravenously but rather into the target tissues directly. In 2005, Kostarelos and Miller [198] reported a rational strategy to formulation design by classification of the formulation components in an 'ABCD' system (Fig. 2), in which 'A' represents the nucleic acid cargos (i.e. siRNA), 'B' is a complexation/encapsulation component, 'C' refers to a biological stabilising group (i.e. PEG) and 'D' represents a bioactive targeting ligand to achieve selectivity. The development of safe and effective 'ABCD' delivery systems presents great promise for siRNA administration for the treatment of diseases for which local delivery is not an option and that require systemic administration [e.g. metastatic cancers, leukaemia and immunodeficiency syndrome (AIDS)].

Successful AuNPs for diagnosis and drug delivery have been reported. Such AuNPs have been possible due to the unique optical properties and surface chemistry of gold (Fig. 1). As discussed in this review, bioengineered multifunctional AuNPs with different size, shape, chemistry and synthesis strategies have been designed and offer great promise in facilitating siRNA delivery *in vitro* and *in vivo* (Table I). However, siRNA delivery using AuNPs is still very much in its infancy and further investigation achieving the 'ABCD' nanoparticle is required before its widespread application can be fully realised. With advancements in the

design of multifunctional delivery technologies, AuNPs will consequently lead siRNA-based nanomedicines and the translation of gene therapy successfully from bench to bedside.

Acknowledgements

Table I. A summary of studies on the *in vitro* and *in vivo* delivery of antisense-oligonucleotide (AS-ODN), miRNA and siRNA using AuNP-based strategies, including: formulations, effectors, targeted receptors, targeted genes, *in vitro* models, *in vivo* models (the route of administration) and comments. (N/A = not applicable)

Figure 1. Development of gold nanoparticles for siRNA delivery; **(1)** Surface Plasmon Resonance (SPR) is an important feature of gold nanoparticles that results from the collective oscillation of delocalised electrons in response to an external electric field from light radiation. When AuNPs are exposed to light, many processes may occur, including re-emission of fluorescence, Mie scattering, surface-enhanced Raman scattering and the photothermal effect. Since the SPR is sensitive to particle size, shape, environment and aggregation state, it can be used to assess the size, shape and polydispersity of AuNP-based siRNA delivery systems and monitor their tissue distribution, cellular internalisation and intracellular trafficking. **(2)** Commonly studied AuNPs can be categorised depending on their morphology, such as gold nanospheres, gold nanorods, gold nanoshells and gold nanocages. **(3)** Gold nanoparticles can be conjugated with various functional moieties, including therapeutic cargos (e.g. drugs, proteins, peptides or nucleic acids), charged materials, biological stabilising groups (e.g. PEG), 'smart' bio-responsive linkers, and bioactive targeting ligands. **(4)** siRNA delivery strategies using gold nanoparticles include (A) chemisorption (thiolated siRNA is conjugated onto the surface of AuNPs via stable Au linker-S bonds), (B) electrostatic interaction (AuNPs are modified with cationic moieties, which in turn complex siRNA via electrostatic interaction) and (C) layer-by-layer (LbL) self-

assembly (siRNA is formulated with AuNPs which have multi-layered films with tailored properties).

Figure 2. Systemic delivery of siRNA using multifunctional AuNP-based vectors: from administration to site of action. (**1**) Typical composition of multifunctional AuNPs consisting of (A) siRNA which can be associated with (B) AuNPs. This AB layer, can be modified by (C) biological stability-enhancing groups (e.g. PEG) and (D) distal targeting ligands (i.e. monoclonal antibodies). (**2**) Barriers to systemic delivery of siRNA (A) degradation by blood nucleases, (B) rapid renal filtration, (C) binding of unmodified AuNPs to serum proteins and (D) particle aggregation and opsonisation. Such barriers are overcome by utilising shielding materials (e.g. PEG) with/without a targeting ligand to facilitate prolonged systemic circulation. (**3**) Enhanced Permeation and Retention Effect (EPR) uptake into tumours. Following systemic circulation, siRNA formulations with optimum size (normally < 500 nm) are able to penetrate through the leaky endothelium of the tumour via the EPR effect. (**4**) siRNA uptake into the brain. siRNA formulations may cross the Blood Brain Barrier (BBB) via (A) receptor-mediated transport via transferrin, lactoferrin and insulin receptors, i.e. a molecular Trojan horse approach to induce receptor-mediated transcytosis, (B) osmotic disruption of tight junctions or (C) via cell-penetrating peptides and magnetic force. (**5**) 'Smart' targeting for RNAi. (A) AuNPs can utilise either cell-specific receptors or extracellular environments (i.e. pH and enzymes) to enhance cellular uptake. The siRNA formulations are normally localised inside endosomal/lysosomal vesicles. (B) External stimuli such as light, temperature, electroporation, ultrasound and magnetic stimulation, may facilitate local administration of AuNP-based siRNA delivery systems and induce direct localisation of siRNA formulations inside cytoplasm. (C) Several materials such as cell penetrating peptides are capable of delivering siRNA into cells without entrapment inside endosomes. However, in most cases, AuNPs must facilitate efficient endosomal escape of siRNA to prevent its subsequent degradation in the late endosome/lysosome. (D) Following dissociation from AuNPs, siRNA in the cytoplasm is loaded into RISC which can initiate the RNAi pathway.

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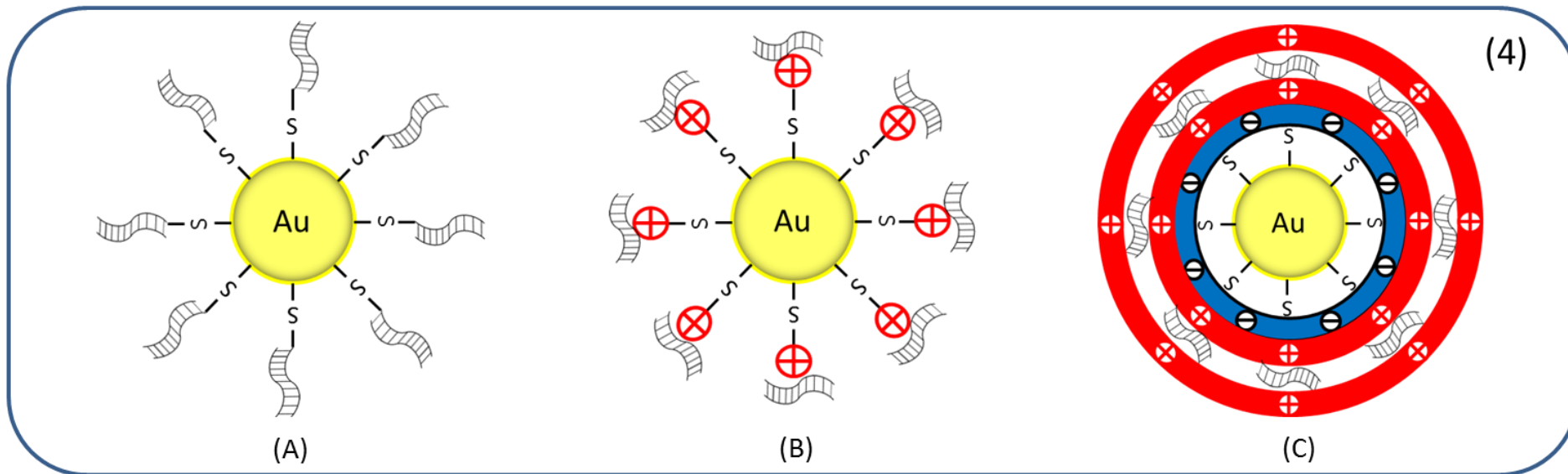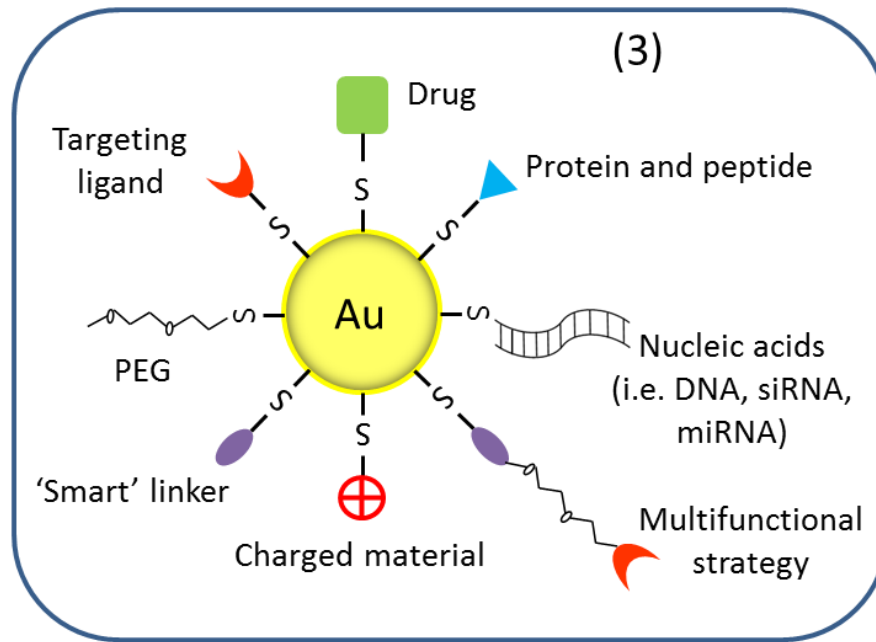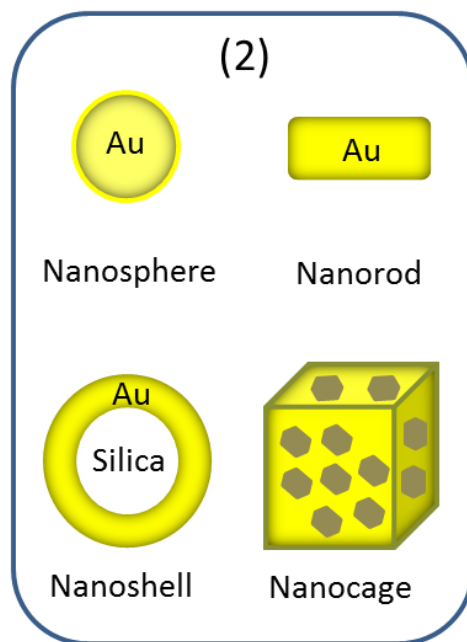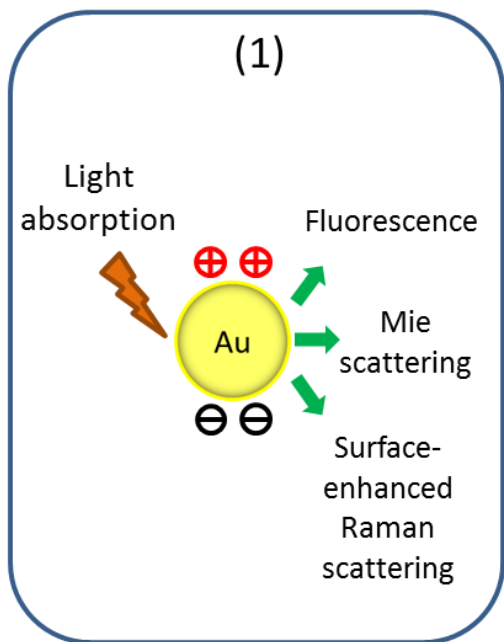
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(1)
Light absorption

Fluorescence

Mie scattering

Surface-enhanced Raman scattering

Au

(2)
Au — Nanosphere

Au — Nanorod

Au / Silica — Nanoshell

Nanocage

(3)
Drug

Targeting ligand

Protein and peptide

PEG

Au

Nucleic acids (i.e. DNA, siRNA, miRNA)

'Smart' linker

Charged material

Multifunctional strategy

(4)

(A)

(B)

(C)

(1)

A = siRNA
B = gold-related nanosystems
C = biological stability groups
D = distal targeting ligands

(2)

Blood

Avoid
- A. Nuclease degradation
- B. Renal filtration
- C. Serum protein binding
- D. Uptake of MPS

Prolonged systemic circulation

(3) Tumour tissue

Blood

EPR effect

Extracellular area of tumour

(4) Blood-brain barrier

Blood

Tight junction

Brain

A   B   C

(5) 'Smart' targeting for RNAi

A. Bioactive and bioresponsive targeting

B. External stimuli

Cytoplasm

C. Endosomal release

D. Release of siRNA

RISC

Cap   Poly (A)

Nucleus

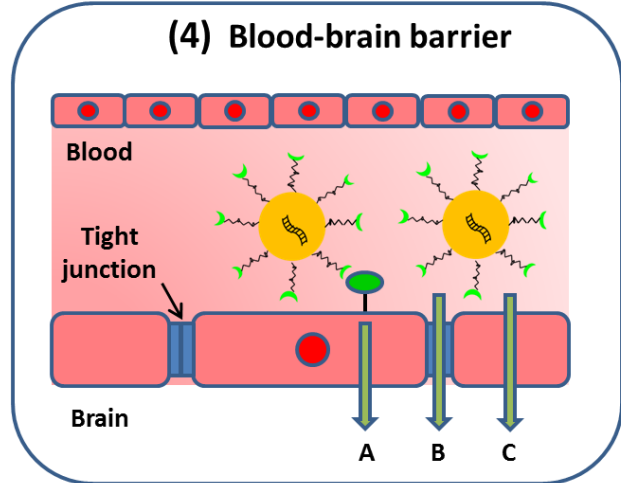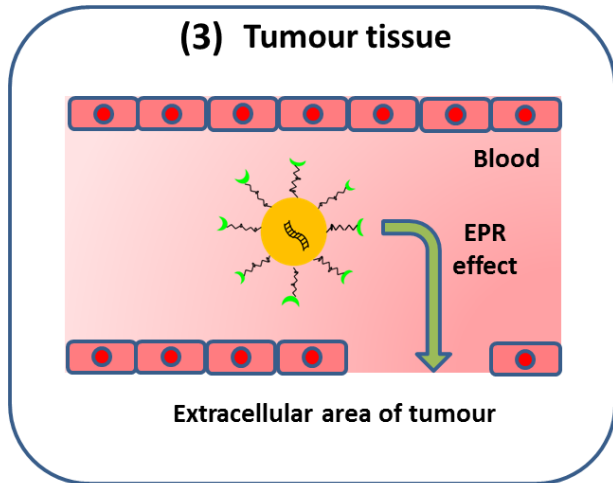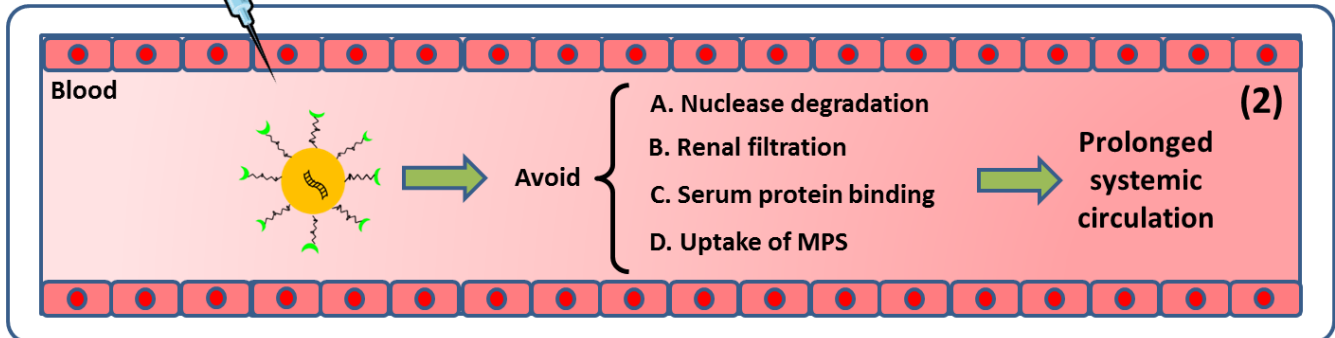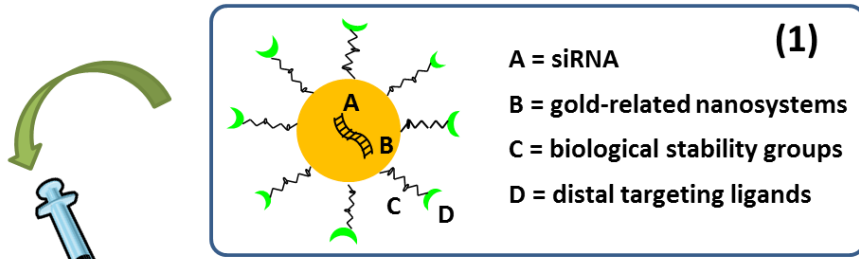Table II. A summary of studies on the *in vitro* and *in vivo* delivery of antisense-oligonucleotide (AS-ODN), miRNA and siRNA using AuNP-based strategies, including: formulations, effectors, targeted receptors, targeted genes, *in vitro* models, *in vivo* models (the route of administration) and comments. (N/A = not applicable)

Formulations	Effectors	Targeted receptors	Targeted genes	*In vitro* models	*In vivo* models	Comments	Refs.
Chemisorption							
AS-ODNs modified with either monothiol or tetrathiol were chemisorbed onto citrate-stabilised AuNPs	AS-ODN	N/A	EGFP	C166 EGFP, mouse endothelial cells	N/A	When compared with Lipofectamine® 2000 and Cytofectin, AS-ODN-AuNPs demonstrated higher EGFP silencing and less cytotoxicity.	[92]
PEGylated AuNPs were produced via functionalisation of citrate-AuNPs using PEG chains and thiolated siRNAs were then conjugated onto PEG-AuNPs via thiol-gold recognition.	AS-ODN	N/A	EGFP	HCT-116, colorectal cancer cells	N/A	siRNA-PEG-AuNPs were able to achieve successful gene silencing without showing toxicity proving more successful than Lipofectamine® which did not achieve any knockdown	[93]
Citrate-AuNPs were functionalised with cargo DNA conjugates that were hybridised by AS-ODN, forming AS-ODN-AuNPs.	AS-ODN	N/A	miRNA-29 miRNA-21	HeLa, human cervical carcinoma cells KGN, human granulose cell-derived cells 293T, human embryonic kidney cells	N/A	The resulting constructs demonstrated effective targeted miRNA regulation in all cell lines	[94]
Citrate-capped AuNPs were first treated with diethyl pyrocarbonate followed by autoclaving treatment. The thiolated miRNA duplexes were added to the post-treatment AuNPs, followed by addition of oligoethylene glycol (OEG) thiol.	miRNA	N/A	Luciferase	MM.1S, human multiple myeloma cells	N/A	Cell transfection and functional luciferase assays confirmed the delivery of miRNA into cells.	[95]

AuNPs were anchored by either of two miRNAs (miR-205 and miR-20a) thiolated at the 3' end of the sense strand, forming miR-205- and miR-20a-AuNPs.	miRNA	N/A	E2F1 PTEN	PC-3, human prostate cancer cells	N/A	These constructs downregulated gene expression up to three fold more effectively than analogous molecular miRNAs delivered using a commercial carrier DharmaFECT™.	[96]
Au Nanobeacons were conjugated with PEG stabilising groups which were then further modified by attachment of thiolated siRNA or miRNA	siRNA miRNA	N/A	EGFP	HCT-116, colorectal carcinoma cells	N/A	Both NP formulations (siRNA/miRNA) achieved successful EGFP fluorescence downregulation with similar expression levels achieved by both	[97]
Phosphine-capped AuNPs were immobilised with either single-stranded antisense siRNA or single-stranded sense siRNA. These NPs were subsequently crosslinked and annealed due to their complementarity and condensed with linear PEI	siRNA	N/A	GFP VEGF	MDA-MB-435, human melanoma cells PC3, prostate cancer cells	N/A	The siRNA-AuNP-PEI NP resulted in greater gene silencing efficacy than naked siRNA-PEI alone for both GFP in MDA-MB-435 cells and VEGF in PC3 cells. The NPs could also be visualised by micro-CT imaging.	[98]
Sense strands of siRNA with an ethylene glycol spacer and alkylthiol were first hybridised with antisense strands of siRNA to achieve duplex siRNA. The resultant siRNA was then chemisorbed onto the surface of AuNPs via thiol-gold bond, which was followed by the passivation with a biological stabilising group (oligoethylene glycol-thiol).	siRNA	N/A	Luciferase	HeLa, human cervical carcinoma cells	N/A	The final formulation demonstrated improved stability under cell culture conditions and enhanced efficacy of the bound siRNA, while retaining the ability to effectively knockdown the firefly luciferase gene.	[99]
The Au-S surface was modified with HS-PEG1000-NH$_2$ to generate NH$_2$-PEG-AuNPs. These were further conjugated with a disulphide linker allowing the chemisorption of thiolated siRNA to produce siRNA-PEG-AuNPs. NPs were then coated with a variety of end-modified poly(β-amino esters) (PBAEs) prior to transfection	siRNA	N/A	Luciferase	HeLa, human cervical carcinoma cells	N/A	For optimised formulations siRNA-PEG-AuNP-PBAEs showed significantly higher firefly luciferase gene knockdown than the commercially available lipid reagent, Lipofectamine 2000®.	[100]
Sense strands of siRNA with an ethylene glycol spacer and alkylthiol were first hybridised with antisense strands of siRNA to achieve duplex siRNA. The resultant siRNA was then chemisorbed onto the	siRNA	N/A	eGFP	C166-GFP, mouse endothelial cells	N/A	AuNPs functionalised with a blunt G-C ended siRNA had increased stability, significantly higher cellular uptake and improved gene knockdown relative to	[101]

Description	Cargo	Target	Gene	Cell line	Animal model	Outcome	Ref
surface of AuNPs via thiol-gold bond, which was followed by the passivation with a biological stabilising group (oligoethylene glycol-thiol).						other siRNA modifications	
An i-motif-based DNA AuNP was synthesized that utilised pH-responsive dynamic motion of the i-motif DNA strands and aggregational behaviour of AuNPs to elicit programmed delivery of therapeutic siRNA.	siRNA	N/A	Luciferase	NIH3T3	N/A	The i-motif DNA formed an interstrand tetraplex, which could induce cluster formation of AuNPs resulting in endosomal escape and higher gene silencing efficiency. Furthermore, the cluster formation of AuNPs accelerated photothermal ablation of cells when irradiated with laser.	[102]
Spherical nucleic acid nanoparticle conjugates (SNA-NCs) were synthesized by the attachment of thiolated siRNA to citrate-stabilised AuNPs creating a densely packed NP with siRNA surrounding an inorganic gold core.	siRNA	N/A	Bcl2Like12	Glioma cells	Orthotopic glioma mouse xenograft (intravenous injection)	AuNPs successfully reduced Bcl2L12 expression both *in vitro* and *in vivo* following systemic IV injection. In addition SNA-NCs increased tumoural apoptosis, reduced tumour burden and decreased tumour progression in xenografted mice without adverse side effects.	[103]
SNA-NCs were synthesized via thiol-gold chemistry between hybridized siRNA duplexes and citrate-stabilized gold colloids. Salt was added to shield repulsive charges and resulted in densely functionalized nanoconjugates.	siRNA	N/A	EGFR	hKC, primary human keratinocyte cells	C57BL/6J hair-bearing mouse SKH1-E hairless mouse	SNA-NCs delivered *in vitro* achieved more persistent EGFR knockdown than the commercial vector DharmaFECT1 and achieved similar levels of knockdown with 100-fold less siRNA. In both mouse strains, SNA-NCs penetrated through the stratum corneum as little as 3 hours after application. Topical delivery of EGFR siRNA for 3 weeks to hairless mouse skin significantly reduced EGFR expression, without toxicity.	[104]
Citrate-stabilised AuNPs were conjugated with a 25% PEG surface layer. The Au surface of PEG-AuNPs was then functionalised with RGD peptide for targeting delivery followed by the conjugation of	siRNA	Integrins	C-myc	LA-4, epithelial type II like adenocarcinoma cells	Orthotopic lung mouse xenograft C57BL/6 mice induced with	The targeted AuNP formulation mediated successful integrin binding, cellular uptake and C-myc downregulation *in vitro*. Intratracheal instillation significantly reduced c-myc	[105]

thiolated siRNA					LA-4 adenocarcinoma cells (Intratracheal delivery)	expression and inhibited tumour growth with resulting significantly increased survival.	
Core/shell-structured hollow gold nanospheres (HAuNS) sensitive to near-IR (NIR) light-induced siRNA release were conjugated by thiolation with an siRNA duplex (HAuNS-siRNA). A folate targeting ligand was then added using folate-targeted PEG-thioctic acid (F-PEG- HAuNS -siRNA).	siRNA	Folate receptors	NF- κB p65	HeLa, human cervical carcinoma cells	Subcutaneous cervical cancer mouse xenograft (intravenous injection)	*In vitro,* NIR radiation resulted in the loss of HAuNS structural integrity and subsequent siRNA dissociation leading to significantly reduced p65 expression. Targeted NPs exhibited significantly higher tumour uptake in xenografted mice compared to non-targeted counterparts following intravenous injection. Irradiation with NIR light achieved downregulation of NF-κB p65. In contract, no downregulation was attained in non-irradiated tumours grown in the same mice.	[106]
Electrostatic interaction							
Cationic cysteamine-AuNPs were used to electrostatically bind miRNA, followed by the addition of thiolated-PEG chains to cover the cysteamine-free surface.	miRNA	N/A	miRNA-31 miRNA-1323	NGP neuroblastoma cells (MYCN amplified) HEYA8, wild-type ovarian cancer cells	N/A	PEG-AuNPs-miRNA was non-cytotoxic and demonstrated effective cellular uptake and miRNA release, which specifically regulated target gene expression and cellular phenotype.	[107]
PEI-capped AuNPs (manufactured using PEI as the reductant and stabiliser) complexed with siRNA duplexes via electrostatic interaction.	siRNA	N/A	GFP Polo-like kinase 1 (PLK1)	MDA-MB-435s, metastatic cancer cells	N/A	PEI-AuNP-siRNA successfully reduced GFP expression and NPs containing antiPLK1 siRNA showed significant PLK1 protein reduction. In addition, the NP was less cytotoxic relative to the PEI counterpart.	[108]

AuNPs were synthesised by attaching PEG-thiol as a stabilising ligand to the surfaces of AuNPs. siRNA was condensed with protamine to achieve 'protamine.siRNA' which was further complexed with negatively charged PEG-AuNPs.	siRNA	N/A	Luciferase	CT26, murine colon carcinoma cells	N/A	This formulation demonstrated effective cellular uptake and successful luciferase gene knockdown.	[69]
PEI-stabilised AuNPs were used to complex siRNA via electrostatic interaction.	siRNA	N/A	GFP Luciferase	NIH 3T3 K562, myeloid leukaemia cells	N/A	siRNA-PEG-AuNPs were introduced into cells by electroporation. Gene silencing using siRNA was significantly enhanced without showing significant cytotoxicity.	[109]
AuNPs were anchored by catechol-conjugated PEI (PEI-C) resulting in robust cationic PEI-C-AuNPs which were further modified with a small amount of thiolated PEG. PEI-C-AuNPs formed stable complexes with siRNA via electrostatic interaction.	siRNA	N/A	GFP	MDA-MB435, human breast cancer cells	N/A	siRNA-PEI-C-AuNP complexes showed effective cellular uptake, endosomal release and GFP suppression, without showing significant cytotoxicity.	[110]
Cationic lipid-coated AuNPs (L-AuNPs) were formed by co-dissolving hydrophobic dodecanethiol-capped AuNPs with three lipid components (DC-chol, DOPE and cholesterol) in organic solvent and using an emulsification/solvent evaporation process to form LNPs with an outer cationic lipid shell and an inner gold NP cluster core. These cationic L-AuNPs could interact with siRNA to form polyelectrolyte complexes.	siRNA	N/A	GFP VEGF UBB HBsAg	MDA-MB-435, human melanoma cells (GFP) A549, human lung carcinoma cells (UBB) PC-3, human prostate cancer (VEGF) HepG2.2.15, Hepatitis B virus-producing hepatoma (HBsAg)	N/A	L-AuNPs were able to bind siRNA to form polyelectrolyte complexes which efficiently delivered siRNA into cancer cells and significantly suppressed target gene expression. In addition these L-AuNPs did not show severe cytotoxicity when compared to PEI.	[111]

Amine-functionalized gold nanoparticles (AF-AuNPs) were synthesized through chemical reduction of gold precursor anions in the presence of cysteamine hydrochloride on addition of sodium borohydride. siRNA-PEG was synthesized with a disulphide link capable of being cleaved in the reductive environment of the cytoplasm. This was electrostatically interacted with AF-AuNPs to form PEG-siRNA/AF-AuNP complexes	siRNA	N/A	GFP	PC-3, prostate cancer cells	N/A	The PEG chain improved the stability of the complexes by protecting them from aggregation. PEG-siRNA/AF-AuNPs had higher cell internalisation than siRNA/AF-AuNPs and significantly suppressed GFP expression. It achieved gene knockdown levels comparable to PEI but without the severe cytotoxicity associated with this vector.	[112]
Dendronised AuNPs were synthesized to create ligands with biodegradable glutamic acids scaffolds and cationic TETA moieties that were able to effectively condense siRNA via electrostatic interaction.	siRNA	N/A	β-galactosidase	SVR-bag4, endothelial cells	N/A	Complexation of siRNA with dendronised AuNPs produced efficient gene silencing (∼50%) comparable to Lipofectamine® while maintaining cell viability	[113]
PEI-capped AuNPs were conjugated with EpCAM monoclonal antibody for targeting the Epithelial Cell Adhesion Molecule (EpCAM) on the surface of cells. The resulting antibody-targeted PEGI-AuNPs were able to bind siRNA via electrostatic interaction.	siRNA	EpCAM	EpCAM	Y79, retinoblastoma cells	N/A	EpCAM antibody conjugated PEI-AuNPs loaded with siRNA showed greater uptake and enhanced gene silencing efficacy when compared to siRNA-PEI-AuNPs synthesized without the targeting ligand antibody conjugation.	[114]
Layer-by-layer self-assembly							
Denatured BSA (dBSA) was first coated onto the surface of AuNPs creating negatively charged dBSA-AuNPs. These were electrostatically coated with a cationic polymer (either PAH, PEI or PDDA) to form a positive layer which could be used to bind siRNA. Finally, the siRNA layer was electrostatically coated with the same cationic polymer again to form a positive layer to improve cellular uptake and intracellular trafficking.	siRNA	N/A	EGFR	MCF-7, breast cancer cells	N/A	Confocal microscopy studies indicated PEI-AuNPs and PAH-AuNPs can induce better siRNA release than PDDA-AuNPs. In addition, PEI and PAH-AuNPs demonstrated gene knockdown levels significantly better than both PDDA-AuNPs and Lipofectamine 2000.	[115]

The surface of citrate-AuNPs was modified by mercaptoundecanoic acid (MUA) resulting in negatively charged MUA-AuNPs. The resulting MUA-AuNPs were electrostatically interacted with PEI (PEI/MUA-AuNPs) which formed a cationic layer which was coated by PAH-Cit (PAH/PEI/MUA-AuNPs). PAH/PEI/MUA-AuNPs were electrostatically coated with PEI (PEI/PAH/PEI/MUA-AuNPs) which resulted in positively charged particles that could electrostatically bind siRNA (siRNA/PEI/PAH/PEI/MUA-AuNPs).	siRNA	N/A	Lamin A/C	HeLa, human cervical carcinoma cells	N/A	PAH-Cit, a pH-dependent charge-reversal polymer, remains negative in physiological pH but changes to cationic at pH 5-6. Confocal microscopy studies implied that PEI-PAH-PEI-MUA-AuNPs released more siRNA into the cytoplasm compared to other controls. This formulation demonstrated improved gene knockdown compared to Lipofectamine 2000.	[116]
Negatively charged AuNPs were coated with PLL to generate a cationic surface that was used to complex siRNA forming siRNA/PLL/AuNPs. The coating procedure was repeated numerous times and resulted in a final formulation with 4 layers of PLL and 3 layers of siRNA surrounding the AuNP core.	siRNA	N/A	Luciferase	MDA-MB231-luc2 LNCaP-luc2	N/A	siRNA was released gradually from PLL-AuNPs and showed extended gene silencing effect, which is most likely due to slow degradation of PLL. Importantly, the inhibition effect in cells was found to correlate with the number of siRNA layers. The NP was more effective than Lipofectamine but with less toxicity.	[117]
AuNPs were reduced and stabilised by chitosan (CS), forming a positively charged CS-AuNP core which was coated by PAH-Cit (PAH/CS-AuNPs). This formed NPs with an anionic layer which were electrostatically coated by PEI, producing cationic PEI/PAH/CS-AuNPs. This was subsequently used to complex siRNA (siRNA/PEI/PAH/CS-AuNPs).	siRNA	N/A	MDR1	MCF-7R, docetaxel-resistant breast cancer cells	N/A	Specific silencing of MDR1, a gene encoding the drug exporter P-gp, was achieved using the resulting formulation, which promoted the uptake of doxorubicin. This is an anticancer agent known to be effluxed from cells by P-gp resulting in treatment failure.	[118]
Citrate-AuNPs were modified by mercaptoundecanoic acid (MUA) resulting in negatively charged MUA-AuNPs. The resulting MUA-AuNPs were electrostatically interacted with PEI (PEI/MUA-AuNPs) forming a cationic layer which was used to complex siRNA (siRNA/PEI/MUA-AuNPs). Finally, these NPs were	siRNA	N/A	EGFP	CHO-K1, hamster ovary cells	N/A	These layer-by-layer formulations provided an excellent tool to study how the size and the surface properties influence the portal of entry into cells and direct distinct particles to the correct site of activity. The NPs were able to mediate significant gene knockdown	[119]

coated by PEI forming PEI/siRNA/PEI/MUA-AuNPs.						without affecting cell viability.	
A positively charged CTAB-modified gold nanorod was coated with two layers of polyelectrolytes, negatively charged PEDT/PSS and positively charged PDDAC.	siRNA	N/A	DARPP-32	Dopaminergic neuronal (DAN) cells *In vitro* BBB models	N/A	This multilayer process generated positively AuNPs that 'masked' the CTAB layer which is notorious for its cytotoxicity. The resulting siRNA complexes achieved target gene knockdown in DAN cells without significant cytotoxicity. More importantly, these complexes demonstrated successful transmigration across an *in vitro* model of the blood-brain barrier (BBB).	[120]
A positively charged CTAB-modified gold nanorod was coated with two layers of polyelectrolytes, negatively charged PEDT/PSS and positively charged PDDAC.	siRNA	N/A	GAPDH	Embryonic dorsal root ganglion (DRG) neurons	Sprague-Dawley rats (hippocampal injection)	Nanoplexes formed by electrostatic binding between siRNA and positively charged GNRs silenced the expression of the GAPDH housekeeping gene (70% GAPDH silencing, >10 days post-injection), within the CA1 hippocampal region of the rat brain, without showing cytotoxicity.	[122]
Cationic cysteamine-modified AuNPs were coated by siRNA (siRNA/CM-AuNP), followed by addition of PEI forming PEI/siRNA/CM-AuNPs. Positively charged PEI/siRNA/CM-AuNPs were further coated with negatively charged hyaluronic acid (HA), producing HA/PEI/siRNA/CM-AuNPs.	siRNA	HA receptors	Luciferase VEGF ApoB	B16F1, mouse melanoma cells	Balb/c mice (tail vein injection)	The resulting formulation selectively delivered anti-VEGF siRNA into HA receptor-positive cells and resulted in reduced VEGF expression. In addition, the NPs mediated significant Luciferase gene knockdown and systemic administration containing anti-ApoB siRNA effectively decreased expression of the targeted gene in the liver of mice.	[123]