Current status and future perspectives of gold nanoparticle vectors for siRNA delivery

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Discovering the vast therapeutic potential of siRNA opened up new clinical research areas focussing on a number of diseases and applications; however significant problems with siRNA stability and delivery has hindered its clinical applicability. As a result, interest in the development of practical siRNA delivery systems has grown in recent years. Of the numerous siRNA delivery strategies currently on offer, gold nanoparticles (AuNPs) stand out thanks to their biocompatibility and capacity to protect siRNA against degradation; not to mention the versatility offered by their tuneable shape, size and optical properties. Herein this review provides a complete summary of the methodologies for functionalizing AuNPs with siRNA, paying singular attention to the AuNP shape, size and surface coating, since these key factors heavily influence cellular interaction, internalization and, ultimately, the efficacy of the hybrid particle. The most noteworthy hybridization strategies have been highlighted along with the most innovative and outstanding *in vivo* studies with a view to increasing clinical interest in the use of AuNPs as siRNA nanocarriers.

Background

Since Fire, Mello and colleagues discovered the ability of RNA to silence gene expression in *Caenorhabditis elegans* in 1998,¹ the interest in RNA interference (RNAi) as a tool for treating a wide-range of diseases has increased exponentially. Although RNA interference is a regulatory mechanism naturally present in a variety of eukaryotic organisms including fungi, plants and animals, the therapeutic importance of RNAi was best demonstrated in 2001 when Elbashir *et al* utilized synthetic small interfering RNAs (siRNAs) to achieve gene-specific knockdown in a mammalian cell line.² The following year marked another milestone when the first *in vivo* study applying RNAi was reported. In this investigation, the research group of Mark A. Kay showed that transgene luciferase expression could be suppressed in adult mice by synthetic small interfering RNA (siRNA) and short hairpin RNAs (shRNA).³ These pioneering results showed how the robust silencing effect of RNAi represented an extremely valuable research tool that was able to inhibit the messenger RNA (mRNA) translation process. Moreover, they also illustrated how a hitherto unknown therapeutic agent could be used against various disease targets, including (but not limited to) viral infections,⁴ hereditary disorders⁵ and cancer.⁶

These landmark achievements in therapeutic RNAi research would not have been possible without a fundamental understanding of RNAi itself. The biochemical pathways for RNAi activity are varied and wide-ranging and several types of RNAs have been connected to the interference pathway: including highly conserved endogenous non-coding microRNA (miRNA), shRNA normally encoded in an exogenous expression vector and siRNA.⁷ Although the initial steps of the RNAi mechanism differ strongly from one type of RNA to another (shRNA, siRNA or miRNA), all involve the interaction of RNA with a protein complex that specifically recognizes and cleaves a

messenger RNA (mRNA). While various exogenous RNA can be used for therapeutic purposes, the scientific literature has tended to focus on siRNA due to its simplicity and the ease by which it can be conjugated to nanostructured delivery systems. For these reasons, this text is based solely on the use of siRNA and does not cover the use of other RNAi molecules. Synthetic siRNA are usually produced as double-stranded RNA of 21-to-23 nucleotides long with two nucleotides overhanging on the 3' ends, showing a similar structure to the natural products of cleaved double-stranded RNA by the Dicer enzyme that takes part in the intrinsic regulatory mechanism.⁸

Figure 1. Overview of siRNA delivery problems that must be overcome, accompanied by a simplified cytoplasmic molecular scheme of siRNA biochemical mechanism. This figure was produced using elements from the Servier Medical Art PowerPoint image bank.

Importantly, siRNA should be delivered to the cell cytoplasm where the gene-silencing action takes place. There, siRNA interacts with a protein complex called the RNA-induced silencing complex (RISC) producing the RNA strands separation,⁹ as seen in Figure 1. While the passenger strand is degraded, the RISC complex - still joined to the guide strand - recognizes the messenger RNA (mRNA) complementary to the guide strand and catalyzes its cleavage.¹⁰ This activated RISC complex will continue degrading the specific mRNA, causing a transient descent on protein expression. Studies have shown how the duration of the interference effect varies markedly from one type of cells to another: the duration of gene silencing in rapidly growing cell lines lasts a few days while this increases to approximately three weeks in non-dividing cells.¹¹ This difference is produced in part due to dilution of siRNA inside the cytoplasm as a result of cell division; however, the interference effect can be prolonged by administration of several siRNA doses.¹¹

The route of administration is a critically important factor that should be considered when applying siRNA¹² since each delivery route poses a variety of barriers and problems before the siRNA can reach the cell cytoplasm to perform its function. This is particularly important in the case of systemic administration, which shows a specific complexity due to the numerous factors that compose the biological response to exogenous siRNA¹³ (refer to Figure 1 for details). Systemic administration introduces siRNA molecules directly into the blood torrent, where they can be subjected to hydrolysis by various serum nucleases¹⁴ and the innate immune system,

which in some cases can recognize them in a sequence-dependent manner.¹⁵ Naked siRNA is also rapidly eliminated by renal clearance, showing a serum half-life of less than five minutes,⁴ and it can be also excreted *via* the intestine by the hepatobiliary pathway.¹⁶ To stand any chance of performing their desired function at the target cells, siRNA must first cross the vascular endothelium.¹⁷ Once at the target site, siRNA molecules are too large and hydrophilic to diffuse across the cytoplasmic membrane,¹⁸ meaning that internalization most often occurs *via* endocytosis, which occurs through a host of different mechanisms such as phagocytosis, macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis depending on the cell type.¹³ In an ideal scenario, endocytic internalization concludes with the siRNAs contained within cytoplasmic vesicles such as the endosomes, although siRNAs must then escape from these intracellular vesicles into the cell cytosol to participate in the cellular RNAi machinery to carry out their function.

The incredible potential of siRNA for medical applications became evident in 2006 when Fire and Mello won the Nobel Prize in Physiology or Medicine for their discovery of RNA interference. This discovery has since then stemmed thousands of scientific publications about siRNAs and their delivery and, in parallel, thousands of related patents have been registered concerning the future drug technology. More precisely, by the year 2007 more than 2000 RNAi patent applications had been filled in the US Patent and Trademark Office.¹⁹ After seven years, in 2014, the company Alnylam Pharmaceuticals possessed a predominant position on RNAi market, performing

six different clinical trials, one of them in Phase III.²⁰ Recently Alnylam shares of 48 %, and increasing doubts about RNAi future in real clinical treatment. however, in October 2016, Alnylam Pharmaceuticals had to put an abrupt end to the Phase III clinical trial of one of their two core products due to 18 patient deaths provoking a fall in

Table 1. Summary of the most recently published and promising clinical trials on gene silencing using siRNA. The table includes information about the type of siRNA carrier, the target protein, the patients treated, the administration route and a summary of the principle result of the study.

Registration Code	Year of publication	Target	Patients	Administration	Vehicle	Results summary
NCT00363714 ²¹	2010	Vascular endothelial growth factor receptor-1	26 patients with neovascular age-related macular degeneration (AMD)	Intravitreal injection	Chemically modified siRNA	Improvement in visual acuity and foveal thickness
NCT00689065 ²²	2010	Ribonucleoside-diphosphate reductase subunit M2 (RRM2)	3 patients with solid cancers refractory to standard-of-care therapies	Intravenous infusion	Targeted Protein-Polymeric-siRNA Nanoparticle	Decrease in RRM2 expression
NCT00716014 ²³	2010	Keratin 6 with an specific mutation	A patient with inherited skin disorder Pachyonychia congenital	Intralesional injection	Unmodified siRNA	Healthy skin in administration area
NCT00658086 ²⁴	2011	RSV N protein	24 lung transplanted patients infected with Respiratory Syncytial Virus (RSV)	Respiratory system nebulization	Unmodified siRNA	Symptoms decrease
NCT00672542 ²⁵	2013	3 inducible immunoproteasome subunits	12 patients with metastatic melanoma	Intradermal injections of dendritic cells externally electroporated with unmodified siRNA		Circulating melanoma cell levels fell
NCT00938574 ²⁶	2014	Protein kinase N3	34 patients with advanced solid tumors	Intravenous infusions	Liposomal particles	Disease stabilization in some patients
NCT01437059 ²⁷	2014	Proprotein convertase subtilisin/kexin type 9 (PCSK9)	32 healthy adult volunteers with elevated LDL-cholesterol	Intravenous administration	Lipid nanoparticle	Reduction in PCSK9 plasma protein and LDL cholesterol
NCT01188785 ²⁸	2015	Mutated KRAS(G12D)	15 patients with non-operable Locally Advanced Pancreatic Cancer (LAPC)	Tumor insertion	Biodegradable polymer implant	Decrease in tumor marker CA19-9
NCT01438281 ²⁹	2016	Transient receptor potential cation channel subfamily V member 1 (TRPV1)	156 healthy subjects and patients with Dry Eye Disease (DED)	Topical administration	Unmodified siRNA	Safe treatment and Visual Analogue Scale (VAS) improvement

Surprisingly, despite the large amount of siRNA patents registered, only 63 clinical trials concerning siRNA have been registered between the U.S. National Institutes of Health and the EU Clinical Trials Register and only one siRNA-based therapeutic drug has been recently approved in August 2018. This drug, Onpattro (patisiran), is a lipid complex to delivery siRNA to treat polyneuropathy caused by an illness called hereditary transthyretin-mediated amyloidosis (hATTR amyloidosis).

The relatively low number of clinical trials carried out to completion provides an idea of just how little is currently known about siRNA delivery and the aforementioned problems, and therefore illustrates that a significant amount of studies should be performed to apply this technology effectively for any of the many potential therapeutic purposes. Despite the extensive bibliography described for siRNA delivery, a wide part of it has studied the RNA interference of artificial reporter genes, such as luciferase gene and green fluorescent protein (GFP) gene. Although the employment of cell cultures provide an easy way to evaluate their efficiency in preliminary studies, animal cases present complexities that these models do not mimic. For this reason, more abundant efforts should be performed to study the siRNA delivery in real therapeutic genes and pre-clinical animal models in order to allow rapid, cost- and time-effective screenings.³⁰

1. siRNA delivery strategies

Due to these siRNA delivery problems in real applications, recently researchers have focused their efforts to develop different strategies to improve pharmacokinetic profiles in order to achieve gene silencing by RNAi in medical treatments. Some of the best options for therapeutic clinical usage involve chemically modified siRNAs or the use of delivery systems as siRNA vehicles, for example using polymeric, lipidic or inorganic nanoparticles, to transport the siRNA through the body and direct it to the site of action while simultaneously protecting the siRNA against degradation. The most promising siRNA clinical trials that have shown favorable results are detailed in Table 1, where the majority of these examples have employed strategies such as modified-siRNA or the use of siRNA delivery carriers. These trials are described briefly in the following pages.

As seen in Table 1, unmodified siRNA can be used in some specific applications when the administration route allows direct contact between the siRNA and the desired tissue where there is an absence of barriers or delivery problems to overcome. For example, naked siRNA has proven useful for treating skin disorders where it can be injected directly in the skin²³ and also when it is nebulized for treatment against viruses of the respiratory system.²⁴ Another interesting application of this naked siRNA appears when the target cells can be extracted from the patient and the siRNA can be artificially introduced into their cytoplasm, as for the case of dendritic cells.²⁵ This approach solves most of the siRNA delivery problems and allows a more effective administration, however it can only be

employed in a few of the potential siRNA applications and complicates considerably the treatment process.

The range of applications of unmodified siRNA can be amplified introducing chemical modifications. For instance, modification of the ribose 2'-OH position (particularly by 2'-O-methyl or 2'-fluoro) provides improved plasma stability against nucleophilic attack of 2'-OH by several nucleases³¹ and allows a reduction in siRNA immunogenicity.³² The most frequently employed chemical modifications of siRNA are 2'-O-methyl, locked nucleic acid, 2'-deoxy and 2'-fluoro.³³ Although chemically modified siRNAs can improve the applicability of siRNA and they have been employed successfully for local administration such as intravitreal injection in clinical trial,²¹ they do not overcome other delivery problems such as renal clearance.

Another proposed siRNA delivery method consists of the employment of viral delivery systems, using for example adenovirus, lentivirus and retrovirus vectors. Proof-of-concept studies of viral delivery systems have been demonstrated in animal models of neurodegenerative diseases, retinal diseases, cardiac disease, viral infections, cancer, and many other diseases.³⁴ Nevertheless, viral vectors present several drawbacks as immunogenicity, carcinogenicity, and inflammation, limiting their application in clinical treatment.³⁵ Consequently, researchers have searched for improved siRNA delivery systems and the use of nanomaterials as siRNA carriers has emerged as a suitable solution with multiple advantages.

Nanoparticles have demonstrated a broad utility and unique versatility for drug delivery and their use in clinical applications is certain to increase exponentially in the years to come. Nanoparticles are showing promising results as drug carriers even although a low amount of the administered dose is typically found in the desired tissue.³⁶ It therefore stands true that their efficiency will be enhanced - and their side effects will be decrease - if their targeting efficiency can be improved.

The association of siRNA to different nanocarriers can be carried out through three different strategies: adsorption, covalent binding or encapsulation inside the material. The selection of the most appropriate nanomaterial depends on the final application and the corresponding route of administration and for the resulting material to be effective, both should be designed in concordance. As an illustrative example, a nanomaterial carrying RNA for systemic administration should be engineered to provide stability against plasma nucleases, evasion of the immune system, avoidance of non-specific interactions with plasma proteins and non-target cells, prevention of renal clearance, exit from blood vessels to reach target tissues, cell entry and incorporation into the RNAi machinery.¹⁸ Much of the research and most important results concerning siRNA delivery have been obtained using lipidic and polymeric nanomaterials, as previously described lipidic Onpattro (patisiran), the only registered drug that employs siRNA. Liposomal nanoparticles have been successfully employed for intravenous administration of siRNA in patients with solid tumors²⁶ and elevated cholesterol,²⁷ and they are

currently being evaluated for efficacy in patients from Sierra Leona who have been infected with the Ebola virus, following initial proof of their effectiveness in infected rhesus monkeys.³⁷

One of the main advantages of using nanomaterials for siRNA is that they can be used as multifunctional tools, carrying not only the siRNA but also other molecules of interest to increase siRNA efficiency such as other drugs, antibodies, internalization peptides and enzymes (See 3.1 *Targeted delivery* and 3.2 *Co-delivery of other drugs* Sections). One such example of a multifunctional nanocarrier is the CALAA-01 nanoparticle that has been tested clinically in patients with solid tumors. CALAA-01 is a polymeric nanoparticle composed of multiple components including a linear cyclodextrin-based polymer, a ligand targeting human transferrin protein to engage transferrin receptors on the surface of the cancer cells, a hydrophilic polymer (polyethylene glycol (PEG) used to promote nanoparticle stability in biological fluids), and siRNA included specifically to reduce the expression of the ribonucleoside-diphosphate reductase subunit M2 (RRM2).²² Several other classes of nanomaterials have been employed for siRNA delivery: mesoporous silica nanoparticles,³⁸ magnetic nanoparticles,³⁹ carbon nanotubes,⁴⁰ graphene,⁴¹ quantum dots,⁴² calcium phosphate nanoparticles,⁴³ gold nanoparticles⁴⁴ and oligonucleotide nanoparticles,⁴⁵ although generally there are fewer of these examples in the literature.

From all of the aforementioned siRNA delivery systems, gold nanoparticles (AuNPs) stand out due to their unique optical properties and their relative inertness and corresponding biocompatibility coupled with the ability to control their size, shape and surface functionalization on the nano and molecular level, **in contrast to other inorganic carriers such as silica nanoparticles, iron oxide nanoparticles and quantum dots.** Not to mention their capacity to protect siRNA against **RNase degradation**, probably provoking steric hindrance between the **RNases** and the siRNA and preventing physical contact with the catalytic site of the enzyme. Although there is an extensive amount of literature on RNA-conjugated AuNPs as nanocarriers, a concise summary of the most relevant studies is lacking. This review focuses on summarizing the available siRNA-AuNP functionalization methodologies in order to maximize RNA interference, paying special attention to the importance of AuNP shape, size and surface coating. The most innovative and applicable approaches for increasing siRNA efficacy have been included and we have endeavored to gather the most noteworthy studies from the field to discuss the potential of these hybrid nanomaterials with a view towards their application in a clinical setting.

2. Designing the most appropriate gold nanoparticle (AuNP) as a carrier for siRNA therapeutics

Gold nanoparticles (herein AuNPs) have been shown genuinely useful for nucleic acid delivery;⁴⁶ however, if anything can be derived from the vast amount of literature on the subject it is that there is no universal AuNP for siRNA delivery. In contrast,

one of the principle advantages of using AuNPs is that they can be engineered specifically for the desired application and route of administration by the design of the most appropriate size, shape, surface coating and functionalization strategies for each purpose. These are the primary considerations that should be taken into account for AuNP-mediated delivery of siRNA, the details of which are discussed in the following paragraphs.

2.1 Choosing the most appropriate AuNP size and shape

Although Faraday carried out the first documented studies on gold colloid synthesis in 1857,⁴⁷ the previous two decades have witnessed an exponential increase in the number of publications and nowadays thousands of scientific papers concerning gold nanoparticle synthesis are published each year. A number of different synthetic routes can be used to produce AuNPs of different sizes and morphologies, both of which represent critical factors for determining their own individual optical properties, cellular interactions and biodistribution properties. One further critical factor worth considering is the avoidance of toxic compounds in the AuNP synthesis, since residual traces often remain associated to the surface of the nanoparticle: impurities which can also undermine their applicability in biological studies.

Selecting the correct type of AuNP may seem like a trivial parameter, but key factors such as the yield and scalability of the nanoparticle synthesis acquires critical significance to be employed in a real world clinical setting. Almost any kind of AuNP shape can be obtained through numerous conventional synthetic routes (Figure 2), and in the majority of cases the size of the AuNP can be controlled with ease by changing one of a few key experimental parameters during their synthesis, e.g. reagent concentration, reaction time, solution pH, reduction potential, ionic strength, and so forth.^{48–50}

The size of AuNPs strongly influences the cellular interaction and internalization pathways, uptake efficiency and cytotoxicity. Despite efforts in AuNP cellular internalization studies, the huge amounts of sizes and shapes and the dependence of internalization rates on cell type make internalization processes of AuNPs difficult to predict. In addition, the surface coating of AuNPs strongly modifies these processes. It is generally accepted that AuNPs are internalized by different uptake pathways in a size-dependant manner⁵¹ and, in general terms, smaller AuNPs have a higher probability to be passively internalized and usually cause higher toxicity than their larger counterparts (particles of same shape but different size).⁵² When AuNPs are administered intravenously, they must remain in the bloodstream in order to reach their site of action or desired target avoiding renal clearance and immune system elimination. The pore size of glomerulus (located in the kidneys) is *ca.* 8 nm, and for this reason nanomaterials used for drug delivery purposes tend to be larger than 20 nm in diameter in order to avoid renal clearance.¹⁸ This concept has been verified by Hirn *et al.*, showing that spherical AuNPs of 1.4, 2.8 and 5 nm were found in mice urine but not the 18, 80 and 200 nm variants.⁵³ In a similar experiment, De Jong and co-workers

administered spherical AuNPs of 10, 50, 100 and 250 nm intravenously and the highest amounts of gold were subsequently detected in the blood, liver and spleen.⁵⁴ Of these four AuNP sizes, the 10 nm AuNPs accumulated in the different organs more than any other size and the highest amount of Au was found in the liver.

Figure 2. A summary of some of the many gold nanoparticle (AuNP) morphologies that can be accessed via conventional synthetic methodologies: small (a) and large (b) nanospheres, (c) nanorods, (d) sharpened nanorods, (e) nanoshells, (f) nanocages/frames, (g) hollow nanospheres, (h) tetrahedra/octahedra/cubes/icosahedra, (i) rhombic dodecahedra, (j) octahedra, (k) concave nanocubes, (l) tetrahexahedra, (m) rhombic dodecahedra, (n) obtuse triangular bipyramids, (o) trisoctahedra, and (p) nanoprisms. Reproduced with permission from ¹⁸⁷.

We have already mentioned that a wide range of AuNPs shapes and sizes are accessible for siRNA delivery (Figure 2); however, the choice of the AuNP shape should not be taken lightly, because properties such as cellular internalization, biodistribution, siRNA loading capacity and optical properties can all vary markedly. For example, a comparison between gold nanorods and nanoprisms of similar size and LSPR band for photothermal therapy showed almost no cellular internalization in the case of gold nanorods while comparable gold nanoprisms with a similar surface coverage displayed a high internalization.⁵⁵ Another interesting comparison was performed by Wang and collaborators, where they showed how gold nanorods internalize less than gold nanocages and nanohexapods in the breast cancer cell line MDA-MB-435.⁵⁶ In this same report, they studied the biodistribution of these nanoparticles after intravenous injection in athymic nude mice with induced tumors and discovered that gold nanorods and nanohexapods accumulated in the tumors more efficiently than nanocages, making them more adequate candidates for anticancer treatment. Although shape and size of AuNP are important factors influencing cellular and tissue interactions,

both factors can be altered after siRNA functionalization. For these reason, these studies comparing different AuNPs should be performed after siRNA functionalization like the one carried out recently by Yue *et al.*⁵⁷ In this study, they observed much higher cellular uptake efficiency in U87 cells for 50 nm gold nanospheres and 40 nm nanostars compared to 13 nm spheres. Surprisingly, after 24 hours the larger particles (40 nm stars and 50 nm spheres) had escaped from endosomes and were distributed throughout the cytoplasm while the 13 nm spheres remained in the endosomes, suggesting that larger nanoparticles were better candidates for siRNA delivery.

As previously mentioned, one of the main advantages of AuNPs as siRNA delivery agents is their unique optical properties. These optical properties result from coherent oscillations of conduction electrons on the metal surface due to excitation by electromagnetic radiation at a metal-dielectric interface, called surface plasmon resonance (SPR) effect.⁵⁸ The frequency of the surface plasmon resonance depends on the metal, the refractive index of the media and the presence of other molecules in the surrounding environment. These surface plasmon resonance effect is called localized surface plasmon resonance effect (LSPR) when the electromagnetic wavelength is confined on a limited nanostructure.⁵⁹ When the size of spherical AuNP changes, their LSPR bands displace slightly. However, in the case of anisotropic AuNPs such as nanorods, nanocages or nanoprisms, their optical properties change very dramatically depending on the aspect ratio (proportion between their different dimensions).⁶⁰ This property can be harnessed for siRNA delivery applications using photothermal therapy, photothermal drug delivery or optoacoustic imaging, as described in section 3.3.

Although the synthesis of AuNPs of different shapes is now incredibly well documented in the literature (Figure 2), their use for siRNA delivery is surprisingly scarce. For *in vivo* siRNA delivery, gold nanospheres⁶¹ or nanorods⁶² are the most commonly employed variants, although recently there have been some interesting studies employing gold nanostars⁶³ and gold nanoshells⁶⁴ in mice. In the case of *in vitro* siRNA delivery studies, gold nanospheres,⁶⁵ nanorods⁶⁶ and nanoshells⁶⁷ are the most frequently studied; although gold nanoclusters,⁶⁸ dendritic nanoparticles,⁶⁹ and nanoflowers⁷⁰ have also been documented. All this information is summarized in **Figure 3**.

Another attractive approach is to use mixed metal nanoparticles made of gold and other hard materials in order to take advantage of the properties of both materials. By this way, siRNA has been delivered in different cells employing different engineered hybrid nanomaterials: magnetic-gold nanoparticles in order to confer magnetic properties and used also as magnetic resonance imaging contrast agents;⁷¹ silica-gold nanorods with higher siRNA loading capacity than bare gold nanorods⁷², **core-shell bismuth-selenide nanoparticles to confer appropriate properties for Surface Enhanced Raman Spectroscopy (SERS)**⁷³ and gold-nickel nanorods that possessed

two different material surfaces for controlled functionalization of different molecules by different strategies.⁷⁴

Figure 3. Frequency of utilization of different AuNP shapes employed for siRNA delivery from 2012 to present for *in vitro* (left) or *in vivo* (right) studies according to the search “gold nanoparticle siRNA” in Scopus and Web of Science databases, and verifying that the AuNPs have been employed for siRNA delivery. This figure was produced using some elements from Servier Medical Art PowerPoint image bank.

2.2 Gold nanoparticle surface coatings

It is clear that selecting the most appropriate AuNP is an important consideration for the final application, but by the same token, it is the chemical surface of the AuNP what determines many of the biointeractions and subsequent properties of the particles in cells and living systems and is therefore another crucial factor that must be considered. For this reason, AuNPs are usually coated with molecules that confer colloidal stability and improve the bioavailability of the particle. The nature and extent of AuNPs surface coating controls the interaction with all the components of the physiological medium, such as different proteins, ions, hydrophilic or hydrophobic molecules as well as with the other nanoparticles. The AuNP-medium interface determines NP stability and governs the properties of the particle, affecting different phenomena such as immune response, extravasation, cellular internalization or accumulation in a cell-type dependent manner. As an illustrative example, Liu *et al.* proved that AuNPs of different sizes possessing differently charged end groups in their surface internalized similarly in phagocytic RAW 264.7 cells, but positively charged nanoparticle uptake was increased in the case of non-phagocytic HepG2 cells, independently of their size.⁷⁵

It is noteworthy that surface chemistry of nanomaterials not only influences their internalization, but can also affect their intracellular location and elimination. For these reasons a proper surface coverage is crucial for any AuNP biological use. In a biological medium, biomolecules (particularly proteins and lipids) tend to adsorb on the nanoparticle surface, forming an encapsulating complex around the nanoparticle most often referred to as the protein corona.^{76,77} The composition of protein corona varies greatly depending on the AuNP surface,⁷⁸ although it can be also influenced by their size⁵¹ and shape.⁷⁷ Differences in the protein corona have been shown to alter the

cellular internalization pathways in a cell-type dependent manner. As an example, protein corona hampers the recognition of AuNPs by scavenger receptors but promotes the clathrin receptor-mediated recognition.⁵¹ Advances on the *in situ* analysis of protein corona interacting with nanoparticles will help in the near future to design surface coating on demand for each application.⁷⁹

In order to minimize any non-desired effect of protein corona formation, such as changes in internalization pathways, aggregation or macrophages recognition, and to maximize the nanoparticle stability in biological media, different AuNP coating strategies can be employed. Although some approaches use the reducing agent required for the AuNP synthesis as a final stabilizer (e.g., citrate), the most interesting results are obtained when the coating is designed specifically for the desired application, especially when it can be used for subsequent functionalization with other molecules of interest, such as siRNA or targeting ligands. Most frequently, AuNP surfaces are stabilized with coordinating thiol groups where the most common coating agents include different types of polymers and surfactants, e.g. thiolated poly(ethylene glycol) (PEG), thiolated end-capped polystyrene (PS), poly(ethylenimine) (PEI), thiolate poly(vinyl pyridine) (PVP), polyamidoamine (PAMAM) dendrimers and cetyltrimethylammonium bromide (CTAB), among others.⁸⁰ In the case of *in vivo* applications, the coating determines the blood half-life, the reticuloendothelial system avoidance and the delivery in the desired tissue. The length of the PEG chain has been shown to drastically affect and change AuNP blood half-life.⁸¹ Silica shell coatings represent another interesting kind of coating recurrent in AuNP bibliography and one that confers high stability to AuNPs when they are irradiated with a pulsed laser for optical applications.⁸² It can be employed to carry different drugs in cellular studies, providing a high internalization and cell viability;⁸³ moreover, silica coated AuNPs were used in the only successful clinical trial carried out in humans using AuNPs.⁸⁴

In the case of siRNA delivery, different coatings have been successfully employed, the most common being thiol-terminated polymers,⁸⁵ layer-by-layer coatings made of different polymer layers⁸⁶ or silica.⁶⁶ For siRNA delivery the surface coating must not only provide an increased cellular internalization but also help the endosomal escape of the nanoparticle to the cytoplasm, where the siRNA action takes place. pH-sensitive siRNA vehicles were employed by Liang and co-workers to liberate siRNA in the cytoplasm thanks to charge-reversal action of different coating layers.⁸⁷ Another common strategy consists of taking advantage of the ‘proton-sponge effect’ caused by cationic polymers that cause the disruption of endosomes due to osmotic swelling, as in the case of poly(allylamine hydrochloride) and siRNA multi-layered AuNPs.⁸⁸ Other approaches include the use of amphiphilic bifurcated ligands to facilitate the permeation of AuNPs to the cytoplasm⁸⁹ or the use of pH-responsive DNA-conjugated-AuNPs carrying siRNA that aggregate inside endosomes helping their cytoplasmic release, as shown by TEM imaging.⁹⁰

Despite the numerous studies on AuNPs for siRNA delivery, just a few have focused on the importance of the AuNP coating. In this respect, an interesting work was developed by Garg and co-workers, comparing three different polyelectrolytes (polyethyleneimine, citraconic anhydride modified poly (allylamine) hydrochloride and poly L-arginine) for layer-by-layer coating of AuNPs for siRNA delivery in BACE1 neuronal cells, demonstrating that the most efficient silencing was obtained with polyethyleneimine due to a higher transfection and loading capacity.⁹¹

Reich and co-workers employed another noteworthy coating strategy, where thiolated siRNA was employed as the AuNP coating but using different ratios of thiol-siRNA and biotin-functionalized thiol-siRNA. After that, the nanoparticles were incubated with streptavidin that bound specifically to biotin group and was used as a scaffold for subsequent linkage of biotin-TAT peptide.⁹² Dark Field imaging was used to show that the internalization of the AuNPs increased clearly with the fraction of thiol-siRNA-biotin employed (Figure 4).

Since the first AuNP-mediated siRNA delivery was performed in 2006,⁹⁴ a long list of techniques have been employed to “functionalize” (i.e. associate or conjugate) AuNPs with siRNA. However, due to large variety of AuNP shapes, functionalization strategies, siRNA-targeted genes, cell lines and analytical techniques used to detect the siRNA activity, finding a general conclusion concerning the most suitable siRNA functionalization technique for any given application poses a significant challenge. What follows represents a summary of the most common siRNA functionalization techniques (Figure 5), paying special attention to their potential use in a clinical setting.

RNA monolayers can be created by linking siRNAs directly to the AuNP surface *via* Au-thiol coordination bonds (Figure 5A) or to the polymeric surface coating of the AuNP through covalent bonds (Figure 5B), e.g. amide, by means of a crosslinker. The use of electrostatic interactions (Figure 5C) represents a less specific yet highly effective means of functionalizing AuNPs with siRNA. A different approach utilizes AuNP functionalized with DNA to provide ssDNA-specific sequence hybridization with ssRNA (Figure 5D) by hydrogen bonding. In the case of the direct Au-thiol coordination bonding mode (Figure 5A), synthetic thiol-terminated siRNAs are employed to interact directly with the gold surface, producing a stable Au-S bond at physiological conditions. This technique was first reported in 1996 by Mirkin *et al.* to conjugate DNA to AuNPs.⁹⁵ When thiol capped siRNAs are employed for AuNPs functionalization they can saturate the entire AuNP surface forming an RNA monolayer surrounding the nanoparticle⁶⁷ (see Figure 5A.1). This functionalization technique was employed to evaluate RNA interference by AuNP delivery of siRNA, single-stranded RNA (ssRNA) and single-stranded DNA, providing similar successful results in the three cases.⁹⁶ However, a better control of nanoparticle surface is obtained and a decrease in non-specific protein adsorption is observed when a mixed monolayer is obtained using a combination of thiol-capped siRNA and thiol-capped polymers (Figure 5A.2), for example PEG.⁹⁷ In addition, when the number of ssRNA per nanoparticle can be controlled, two complementary ssRNAs can be linked to different nanoparticles and hybridized as building blocks to form nano-assemblies with controlled geometries.⁹⁸ However, some limitations should also be considered, for example Barnaby *et al.* demonstrated how serum nucleases degraded siRNA bound to the nanoparticle in a sequence-dependent manner.⁹⁹ In the same study, the authors showed how nucleases splice the siRNA in a different position depending on whether they are linked to the nanoparticle or are free, highlighting the influence of the AuNP when choosing the siRNA sequence. Thus, the scientific literature can be used as a guideline for selecting the most active siRNA sequences and some algorithms have been developed to predict the hybridization thermodynamics of siRNA to a complementary target mRNA,¹⁰⁰ but the selection of the siRNA should be made carefully.

Figure 4. Example of the importance of AuNP coating in siRNA delivery. Hollow gold nanoshells were coated with increasing levels of biotin-modified RNAs to provide enhanced cellular internalization. A) Streptavidin interaction is strongly related with the ratio between siRNA-biotin and siRNA (levels I-IV) employed in the RNA assembly to the AuNP surface. B) Dark-field images of AuNPs (gold spots) with different levels of streptavidin coating internalized around nuclei (blue zones by Hoechst staining) in HeLa cells (top) and hESC H9 cells (bottom). Reproduced with permission from ⁹²

It should be noted that a proper surface coating not only provide the advantages listed for the siRNA delivery but also may permit the administration of the particle through new administration routes. For example, recently AuNPs carrying siRNA against Akt have been covered with glycol chitosan-taurocholic acid in order to protect the siRNA from gastrointestinal degradation when administered orally.⁹³ This coating also facilitated active transport through enterocytes and enhanced selective accumulation in colorectal liver metastasis in animal models, where the siRNA action took place causing cancer apoptosis and displayed enhanced tumor reduction with longer survival time for the mice.

2.3 Gold nanoparticle siRNA functionalization strategies

Figure 5. Most common siRNA functionalization strategies for AuNPs based on electrostatic association, covalent/coordination bonding and hydrogen bonding. siRNA can be directly linked to the gold surface by thiol-bonding (A) to form a RNA monolayer (A.1) or a mixed monolayer with other thiolated molecules (A.2). Covalent bonding between siRNA and AuNP coating can be obtained by the employment of a crosslinker (B). Electrostatic interaction (C) takes advantage of negative charge of siRNA to interact with different positively charged parts of the AuNPs, including the surface of AuNP coating (C.1), the inner part of block copolymers in the AuNP coating (C.2) or different positive layers surrounding the nanoparticle in a layer-by-layer assembly (C.3). Specific sequence hybridization can be obtained using ssDNA linked to AuNP complementary to ssRNA by hydrogen bonding (D). This figure was produced using some elements from Servier Medical Art PowerPoint image bank.

Another often reported siRNA functionalization technique consists of simple electrostatic interactions, which use the intrinsic negative charge of siRNA to attach to a positively charged molecule (Figure 5C). As a result, different positively charged molecules can be anchored to the nanoparticle, including poly(L-lysine),¹⁰¹ chitosan,¹⁰² poly(diallyldimethylammoniumchloride),¹⁰³ poly(2-(dimethylamino) ethyl methacrylate-2-hydroxyethyl methacrylate),¹⁰⁴ polyethyleneimine,¹⁰⁵ protamine,⁶⁵ (16-mercaptohexadecyl) trimethylammonium bromide,⁸⁹ polyamine,¹⁰⁶ cationic phospholipids,¹⁰⁷ poly(allylamine hydrochloride),¹⁰⁸ bovine serum albumin⁷⁰ or even metallic cations,¹⁰⁹ among others. Although electrostatic binding of siRNA can be performed using AuNPs with a positively charged surface coating¹¹⁰ (Figure 5C.1), using this approach the siRNA remains exposed in the surface and cationic nanoparticles have demonstrated serious toxicity problems in some cases.¹¹¹ To overcome this problem, different strategies have been investigated, such as the use of AuNPs covered with block copolymers with a positively charged inner part that interacts with the siRNA and an external part with low toxicity (Figure 5C.2). Some studies have included poly(ethylene glycol)-b-poly(L-lysine)¹¹² and poly(ethylene glycol)-polyarginine as block copolymers.¹¹³

Another widely utilized strategy consists of a layer-by-layer deposition surrounding the AuNP (Figure 5C.3). The layer-by-

layer fabrication method utilizes the interaction of oppositely charged polyelectrolytes to generate multiple thin films.¹¹⁴ In the case of siRNA functionalized AuNPs, by this method multiple layers of RNA can be sandwiched between biodegradable polymers,¹¹⁵ allowing a sequential release of the genetic materials. Such nanoparticles have demonstrated their resistance to nuclease degradation¹¹⁶ and have been employed for siRNA delivery *in vivo* where a significant interference effect was maintained over a period of 20 days.¹¹⁷

A direct comparison of covalent vs. electrostatic siRNA functionalization was performed by M. de la Fuente and co-workers.⁶¹ In this work AuNPs with different PEG coatings were analysed as nanocarriers for c-myc siRNA whereby the siRNA was linked to the AuNP either by thiol-Au siRNA functionalization or electrostatic interaction using an azide-terminated PEG. Further, a pyramidal functional screening strategy was employed to probe the nine types of different AuNPs for RNA interference, whereby the nanoparticle variants were first tested in a mammalian cell model, before proceeding to two separate *in vivo* models: first in *Hydra vulgaris* and finally in mice. At each stage of the process the most effective nanoparticles were selected for testing in the higher order model (Figure 6), demonstrating that thiol-bonding of siRNA worked better than ionic interaction in the studied system. Using three biological models of increasing complexity the

authors reduced time and costs, avoiding unnecessary vertebrate testing.

Other functionalization techniques that did not imply thiol bonds or electrostatic interactions have also been performed. For example, Altinkaya and co-workers utilized PEG-coated

Recently, a similar approach has been reported using siRNA specific binding proteins anchored to the AuNP.¹²¹

There have also been some recent examples of siRNAs co-encapsulated with AuNPs in different types of nano- and microcapsules.^{122–124} It should be noted that molecular

Figure 6. *In vitro* and *in vivo* gene silencing using covalent or ionic siRNA functionalization approaches based on multi-functional AuNPs covered with PEG, cell penetration peptides and adhesion peptides. The study employed a hierarchical scheme using three biological systems of increasing complexity: cultured cells (HeLa), freshwater polyp (*Hydra vulgaris*), and mouse (C57BL/6j) to select the most effective engineered AuNP and test it in the higher order level. a) RNAi efficiency against the fusion protein Renilla luciferase-myc in HeLa cells. Data marked with asterisks are statistically significant relative to the corresponding NP-ion/cov with control siRNA. Sample t test (**, $P \leq 0.0001$; *, $P \leq 0.001$). b) Hymc RNAi in *Hydra vulgaris* study by qRT-PCR. Sample t test (*, $P \leq 0.005$). c) c-myc RNAi in mouse, analyzing RNA extracted from lung tissue by qRT-PCR. Sample t test (*, $P \leq 0.001$; **, $P \leq 0.01$). Adapted with permission from ⁶¹.

nanoparticles with covalently attached siRNA using a cross-linker (Figure 5B) (concretely N-succinimidyl-3-(2-pyridyldithio)-propionate). Thereafter the siRNA@AuNP core was covered by a pentablock copolymer via electrostatic interactions. Importantly, the degradation by nucleases was highly dependent on the copolymer composition.¹¹⁸

Another approach is the specific hybridization of the ssRNA with complementary ssDNA molecules through hydrogen bonding (Figure 5D). By this method, several siRNAs can be attached to a DNA molecule in a specific manner¹¹⁹ or a complex DNA assembling can be used to control the siRNA release.¹²⁰

dynamics simulations are doing a great deal to assist in siRNA functionalization by simulating size and charge-dependent siRNA complexation with gold nanoclusters¹²⁵ and assembly and release of siRNA from polymer/AuNPs polyplexes.¹²⁶ Theoretical studies such as these could help to design siRNA delivery vehicles in the near future.

Once the functionalization has been performed it is of course essential to evaluate the amount and functionality of siRNA that has been conjugated to the AuNP. Several different strategies can be employed to quantify the siRNA that has been linked to the particle. In cases where siRNA can be easily

released from the nanoparticle (e.g. using DTT for thiol-bond reduction),¹¹⁸ it can then be quantified by agarose gel or spectroscopic absorbance measurement.⁸⁷ Other methods include the labeling of siRNA with a fluorophore before the release and their subsequent quantification using a fluorimeter⁸⁹ or the use of commercial detection kits.¹¹⁸ In other cases, these same measurements can be performed with the siRNA collected from the reaction supernatant solutions (i.e. siRNA not linked during the conjugation process) inferring that any siRNA that is not identified in the supernatant has been linked to the AuNP. To analyze the cellular internalization and endosomal escape some authors employ fluorophore-labelled siRNA.^{87,108}

In order to evaluate the functionality many studies employ siRNA targeting a reporter gene, such as GFP¹²⁷ or luciferase.¹¹⁸ siRNA functionality for other genes can be measured at the mRNA level by RT-PCR or at the protein level using western-blot when the corresponding antibody is available¹²² or ELISA.¹⁰⁸

3. Approaches to improve siRNA delivery efficacy and clinical interest

To date, the most widespread use of AuNPs carrying siRNA in the literature has been as a tool for targeted siRNA release in anticancer applications. Concretely, these organic-inorganic hybrid nanoparticles have demonstrated an ability to reduce tumor volume,¹²⁸ to silence chemotherapeutic resistance,^{87,124} to improve radiosensitivity,¹⁰³ to decrease cell migration and cell invasion,¹⁰⁸ often in combination with photothermal therapy.¹²⁹ However, siRNA-AuNP complexes have also been utilized, to a lesser extent, in the study of opiate addiction,¹³⁰ the study of topical delivery,¹³¹ as an antiviral for Dengue virus infection treatment,¹¹⁶ for gene expression analysis,¹³² as transmission electron microscopy markers,¹²³ for X-ray contrast agents,⁷⁰ in HIV infection studies,¹³³ for psoriasis treatment,¹³⁴ for stem cell differentiation,¹³⁵ to control photonic gene circuits¹⁰⁷ as well as for chronic pancreatitis,¹³⁶ to improve wound healing in diabetic mice¹³⁷ and to evaluate efficacy of non-classic siRNA variants.¹³⁸

Despite the vast amount of biotechnological applications that have been explored using AuNPs they also present several drawbacks that should be considered before continuing developing their use as siRNA carriers; in particular when compared with liposomes, which are currently the most advanced and safest carrier for siRNA. Liposomes and lipid nanoparticles have shown promising results in this field¹³⁹ and continue to undergo development for new siRNA delivery applications.^{140–142} Their physicochemical properties readily lend them to siRNA delivery and show high efficacy, safety at the studied doses and limited or no side-effects, as

demonstrated by the high number liposomal delivery systems in preclinical trials and in particular the recent approval of lipid-based medicine Patisiran (see Section 1). By comparison, AuNPs present several limitations that should be overcome before their clinical employment. The main problem of AuNPs and other metal-based nanoparticles is of course their long-term accumulation *in vivo* as a result of their low biodegradability.¹⁴³ It has been described how the polymeric coating of AuNPs can facilitate a more rapid degradation, earlier than the bare metallic core, which will tend to accumulate in organs instead of being excreted.¹⁴⁴ This is particularly problematic in the case of larger AuNPs⁵³. Although several studies show accumulation of AuNPs in tissues and organs,^{54,145} it is not clear if the long-term effect could produce a real danger to the health of the patient. For this reason, a conscientious effort should be made by researchers working with AuNPs for biomedical applications to address this uncertainty. Other problems related with AuNPs are immunogenicity,⁴⁶ instability and toxicity¹⁴⁶, associated with ROS generation, formation of apoptotic body and impairment of mitochondrial activity¹⁴⁷. However these issues can certainly be reduced - as seen in Section 2.2 - by a chemical control of the surface coating of the AuNPs, correct for the desired application or route of administration.

Regardless of the end application, the aforementioned drawbacks of AuNPs can be improved using several strategies, namely targeted delivery of the material, co-delivery of other drugs, harnessing the optical properties of the AuNPs and by encapsulating the AuNP with the siRNA. In addition these strategies can also be employed to increase the efficacy, specificity and clinical interest of AuNP-mediated siRNA delivery. The following sections of this review offer an overview of these key strategies by providing details of the most recent reports from the literature.

3.1 Targeted delivery

The objective of targeted delivery consists of the predominant accumulation of the desired drug at the zone of interest. When talking about systemic delivery, targeted delivery includes reaching the desired tissue and entering inside the cytoplasm of the target cell.¹⁴⁸ In many anticancer applications nanomaterials reach the tumor due to the enhanced permeability and retention (EPR) effect, which consists of the accumulation of blood circulating nanoparticles in malignant tissue penetrating the highly permeable discontinuous endothelia.¹⁸ However, this approach commonly leads to the accumulation of the nanomaterials in the cells of the reticuloendothelial system (RES) before they can reach their

Table 2. Summary of examples employing targeted delivery of siRNA using AuNPs. The table includes information about the ligand molecule employed with the AuNP, the target receptor and other drugs co-delivered with the siRNA.

Authors	Year of publication	Targeted delivery molecule linked to the AuNP	Targeted receptor of the delivery	Co-delivery of other drugs
J. Conde <i>et al.</i> ⁶¹	2012	TAT peptide	Not clear (different mechanisms have been proposed)	
M. Mitra <i>et al.</i> ¹⁴⁹	2013	EpCAM monoclonal antibody	EpCAM (epithelial cell adhesion molecule)	
X. Huang <i>et al.</i> ⁶⁷	2014	TAT peptide	Not clear (different mechanisms have been proposed)	
S. Acharya <i>et al.</i> ¹⁵⁰	2014	KDEL (Lys-Asp-Glu-Leu) peptide	KDEL (KDEL protein retention receptor)	
J.H. Choi <i>et al.</i> ¹²²	2015	Antibody against HER-2	HER-2	
P. Zhang <i>et al.</i> ¹²⁰	2016	RGD peptide (arginylglycylaspartic acid)	Integrins	Doxorubicin
J. Conde <i>et al.</i> ¹²⁹	2016	Influenza haemagglutinin (HA1) Peptide	HA receptor in endosomal membranes	Avastin (VEGF inhibitor)
L. Gamrad <i>et al.</i> ¹⁵¹	2016	Nuclear Localization Signal (NLS) peptide	Importin α	
K.A. Fitzgerald <i>et al.</i> ¹⁵²	2016	Anisamide	Sigma receptor	
L. Fan <i>et al.</i> ¹²⁸	2016	Monoclonal antibody against FAT1	Protocadherin Fat 1	HCPT and Doxorubicin
S. Wang <i>et al.</i> ⁶³	2016	Hyaluronic acid	CD44 receptor	
N. Li <i>et al.</i> ¹⁵³	2017	Nuclear Localization Signal (NLS) peptide	Importin α	
H. Zhu <i>et al.</i> ¹⁵⁴	2017	2-deoxyglucose	Glucose transporter 1 (GLUT1)	

target. To improve the targeting effect, an active targeting approach involving the conjugation of targeting ligands to the nanoparticle that interacts specifically with molecules in the target cells can be employed. In the case of AuNPs, the conjugation of different cell penetrating peptides to the particle can facilitate cytoplasmic entry in the desired tissues. These cell penetrating peptides are small sequences of amino acids derived from different natural proteins, formed by fusion of other cell-penetrating peptides or completely synthetic. The exact entry mechanism by which AuNP are internalized in the cell and released to the cytoplasm thanks to this peptides is not clear, although different endocytosis and endosomal escape models have been proposed.¹⁵⁵ For example Kannan and co-workers showed how AuNPs functionalized with bombesin peptide for promoting interaction with gastrin-releasing peptide receptor exhibited a completely different biodistribution from their non-functionalized counterparts after intraperitoneal injection. Liver accumulation was decreased more than twice but the accumulation in organs that express the receptor was increased.¹⁵⁶

Different cell penetrating peptides have also been utilized for siRNA-functionalized AuNP delivery, including peptides with the motif arginylglycylaspartic acid (RGD) that is recognized by integrins,¹²⁰ T-complex protein 1 subunit alpha (TCP-1) peptide that targets colorectal cancer cells and influenza haemagglutinin (HA1) peptide (a fusogenic peptide that destabilizes the endosomal membrane),¹²⁹ the transactivator of transcription (TAT) peptide that promotes endocytosis,⁶⁷ peptides with the sequence Lys-Asp-Glu-Leu (KDEL) that promotes localization in the endoplasmic reticulum¹⁵⁰ or peptides with the nuclear localization signal (NLS) to be imported into the cell nucleus.¹⁵¹ The importance of employing RGD in AuNP-mediated siRNA delivery against c-myc by intratracheal administration has been demonstrated to cause a 65 % downregulation of c-myc observed by qRT-PCR, as opposed to no significant interference recorded for similar NPs without RGD.⁶¹ Another surprising work has demonstrated that when AuNPs with siRNA were functionalized with NLS peptide they were translocated to the nucleus and maintained their silencing effect in three different cell lines for long-term periods of more than 30 days instead of 2-3 days for their counterparts without NLS peptide.¹⁵³

Other ligand molecules can be used to target AuNP to cells that express determined surface receptors, such as small molecules as folic acid or galactose, hormones and antibodies.¹⁵⁷ In this line, cell studies have been performed using siRNA-conjugated AuNPs with anisamide to target sigma receptor¹⁵² or with 2-deoxyglucose,¹⁵⁴ a glucose analogue that is recognized by glucose transporter 1 (GLUT1), a vital prognostic indicator of tumorigenesis. Antibodies have also been recurrently employed for siRNA-AuNPs targeting and several studies have been published employing epithelial cell adhesion molecule monoclonal antibody,¹⁴⁹ FAT1 antigen antibody¹²⁸ and anti-HER-2 antibodies.¹²² An interesting report was published recently by Lu and co-workers, whereby AuNPs functionalized with siRNA *via* a layer-by-layer method with an

external layer of hyaluronic acid improved their CD44-targeting ability and was verified using flow cytometry, confocal microscopy imaging and competitive binding analysis in different cell lines. Further, these particles were used to selectively sensitize tumors expressing CD44 receptor to photothermal treatment in an *in vivo* mouse model.⁶³ Other innovative strategies have been developed to increase cellular internalization, for example Chen and colleagues employed ultrasound-propelled siRNA-DNA-gold nanowires to facilitate the cell entry in different cell lines,¹¹⁹ showing that they can dramatically accelerate the mRNA silencing compared with static nanowires. This strategy could be interesting in the future to increase local internalization in *in vivo* delivery and also when the target cells can be extracted from the body, as long as they resist the treatment. Another interesting approach using iontophoretic topical administration of AuNS-siRNA by electric field application was employed recently for melanoma cancer suppression showing similar results to intratumoral administration.¹⁵⁸

3.2 Co-delivery of other molecules

One of the main advantages of nanomaterial-based delivery vectors is that they offer the possibility to direct different drugs and biomolecules using the same carrier, a strategy usually called co-delivery. AuNPs act as scaffolds and can be functionalized with almost any desired compound, including small molecules such as drugs, vitamins, lipids, sugars, peptides, fluorophores as well as larger biomolecules like nucleic acids (DNA and/or RNA), antibodies and cell signaling proteins.¹⁵⁹ Co-delivery strategies pave the way for a wider application of siRNA in future clinical treatments due to the sheer number of compounds that can be delivered together with siRNA in order to produce a synergistic effect.

The engineering of AuNPs to obtain multi-functional carriers requires different functionalization techniques in order to control the interaction of each molecule through step-wise processes. For example, AuNPs can be coated with a polymer with different chemical end-groups that permit the functionalization of each of these groups with different molecules *via* different chemical conjugation steps.⁶¹ Another strategy is the use of different surface coating molecules; where each one is functionalized previously with a different compound in order to form a complete coating with all the molecules together, for example using a block copolymer as coating agent with an inner part that interacts with siRNA protecting it and an outer part where the targeting ligand (octreotide) is linked and a methyl thioglycolate as the other coating agent to provide a pH-labile hydrazone linkage of doxorubicin to enable pH-controlled drug release.¹¹³ A recurrent alternative is the use of layer-by-layer functionalization technique, to locate different compounds in each layer.^{136,160} However, one highly versatile approach was proposed by Oh and co-workers.⁷⁴ In this work, the authors employed a synthetic route for a two-component nanorod complex by electro-deposition, obtaining rods clearly characterized half of Ni and half of Au. The presence of different

metal surfaces permit the union of a variety of molecules - in this case siRNA and a fluorophore - using different functionalization techniques for each metal surface.

Different types of nanocarrier, each one functionalized with different (bio)molecules of interest can be also applied together to obtain a synergistic effect. One nice example of this approach was employed by Conde *et al.*, using a hydrogel patch to deliver gold nanorods functionalized with avastin (a Vascular Endothelial Growth Factor (VEGF) inhibitor) and spherical gold nanoparticles with siRNA.¹²⁹ Nevertheless, probably the most complex and advanced work of co-delivery concerning AuNPs with siRNA has been carried out by Jin and colleagues.¹²⁸ In this paper, SiO₂-Au nanoparticles are used as a complex vehicle for sequential drug delivery of several chemotherapeutics and siRNA with antitumor efficacy (**Figure 7**). This system is composed by an inner SiO₂ nanoparticle used for encapsulating 10-hydroxycamptothecin (a DNA topoisomerase I inhibitor) and for adsorbing doxorubicin. The inner SiO₂ nanoparticle is covered by several small AuNPs linked by electrostatic interactions forming a complex system of 50 nm diameter. These AuNPs also are functionalized with siRNA against B-cell lymphoma 2 protein (Bcl-2), an important anti-apoptotic protein in cancer, and antibodies against FAT1 antigen, a protein expressed in the cell surface of the target cells. After demonstrating that the system interacted with cells expressing FAT1 antigen, its cellular internalization and its endosomal escape, the authors studied the GSH-mediated siRNA release that occurs quite fast in less than 2 hours. They also showed how doxorubicin release in the cytoplasm took around 15 hours while 10-hydroxycamptothecin took around 30 hours. Finally, they studied the efficacy of SiO₂-Au sequential drug delivery system by intravenous injection in a xenograft mouse model, showing a remarkable anticancer effect by delaying tumor growth that in the case of the most complex nanoparticles produced the tumor weight decreased 400-fold. They did not report any noteworthy decrease in the weight of the mice, suggesting that this nanocomplex did not display a significant toxicity.

Despite being applicable to a variety of diseases and conditions, until now most of the research on AuNP-based siRNA co-delivery has been directed to cancer treatment. Doxorubicin is one of the most often employed antitumor agents that has been co-delivered with siRNA.^{66,120} Other studies include the use of multilamellar gold niosomes encapsulating thymoquinone and siRNA to overcome Akt induced resistance (one of the major mechanisms of chemotherapeutic resistance in cancer cells due to the key role of protein Akt in cellular survival pathways) in breast cancer,¹²⁴ Rap2b siRNA in order to improve the efficiency of the anticancer drug adriamycin,¹⁶¹ imatinib mesylate in order to improve STAT3 siRNA activity against melanoma,¹⁵⁸ captopril and siRNA in antiangiogenesis breast cancer therapy¹⁶² or docetaxel and siRNA AuNP co-delivery for metastatic breast cancer.¹⁶³ Another application of co-delivery is devoted to improve siRNA interference efficiency mediated by AuNPs, as it is the case of hydroxychloroquine that has been employed to enhance

endosomal escape of gold nanoparticles carrying siRNA.¹⁶⁴ DNA is one of the biomolecules that has also been co-delivered with siRNA.¹⁶⁵ In this way, siRNA can be used to decrease the expression of specific proteins while plasmid DNA can be used to overexpress both exogenous and endogenous proteins.¹⁶⁶ This approach has also been studied using Interleukin 10 (IL-10)

Figure 7. a) Illustrative scheme representing the synthesis of the silica and AuNP delivery system and the drugs and siRNAs sequential release in the cancer cell cytoplasm. The morphology and size of NPs were characterized by TEM imaging of (b) silica nanoparticles with HCPT and doxorubicin (c) AuNPs carrying siRNA and (d) silica and AuNP complete delivery system. e) Antitumor effect of each of the sequential components employed in silica and AuNP siRNA delivery on nude mice bearing Colo-205 cells subcutaneously, showing dissected tumor tissues from the nude mice. Reproduced with permission from ¹²⁸

plasmid DNA and Transforming Growth Factor β (TGF- β) siRNA AuNPs to treat chronic pancreatitis.¹³⁶

3.3 Capitalizing on the optical properties of gold nanoparticles

The unique optical properties of AuNPs depend mainly from the LSPR effect (see section 2.1) and by harnessing these optical properties, AuNPs have been studied for a wide variety of applications from drug delivery¹⁶⁷ and biosensing^{168,169} to optoacoustic imaging¹⁷⁰ and photothermal therapy.¹⁷¹ In fact, the only completed clinical trial using AuNPs registered in the U.S. National Institutes of Health employed photothermal treatment applying laser at near-infra-red (NIR) wavelength in order to produce a temperature increase due to the interaction between laser light and AuNPs to reduce atheroma volume in coronary artery disease patients.⁸⁴ In this clinical trial, involving 180 patients, silica-gold nanoparticles were administrated using stem cells in a surgically implanted patch or by intracoronary infusion of stem cells carrying iron-bearing silica-gold nanoparticles. After that the nanoparticles were irradiated by percutaneous transcatheter intravascular or out-of-body transcutaneous intercostal NIR laser irradiation, achieving a total atheroma volume reduction of 38%. Although AuNPs have also been employed for laser-induced transient membrane perforation that facilitates siRNA cellular internalization,^{172,173} the most interesting results are obtained when the irradiation is performed using AuNP functionalized with siRNA since the AuNP can be used as a dual-active delivery and release carrier.

Laser-triggered release of siRNA from AuNPs has been shown to increase RNA interference.¹¹⁰ In order to understand the mechanism by which AuNPs release siRNA under laser irradiation, an innovative study was carried out by Day and co-workers showing that pulsed laser irradiation release is a temperature independent process more efficient than continuous wave irradiation¹⁷⁴. Laser-controlled siRNA release from AuNPs also shows potential as a precise strategy for controlled release. In this line, Lee and colleagues employed siRNA-conjugated Au nanorods ('nanoantennas') to obtain precise optical control for the reconfiguration of gene circuits in living cells.¹⁰⁷ In this study, two different Au nanorods with aspect ratio of 2.5 and 4.0 were used for the controlled release of siRNA when irradiated with 660 nm and 785 nm lasers, respectively. Using this photothermal release system the researchers were able to control a gene circuit to produce a desired protein by laser irradiation, switching the circuit on with one laser and off with the other.

Optoacoustic imaging has also helped to demonstrate the utility of siRNA-AuNPs. When this imaging technique is used with AuNPs, light pulses provoked the thermoelastic expansion of the tissues where AuNPs are located producing ultrasound signals that are collected and used to form an image. Although this imaging technique is not as precise as others nanomaterial-based methods,¹⁷⁵ one of the advantages of optoacoustic imaging is that it can be combined with photothermal therapy using the same AuNP.¹⁷⁰ For example, Taruttis *et al.*, employed a liposome-gold nanorod nanocarrier for siRNA for multispectral optoacoustic tomography (MSOT) in 4T1 and HT29 tumor models and delivered siRNA against PLK1 to cause apoptosis of tumor cells.¹⁷⁶ Irradiation of hollow gold nanoshells

with NIR light have also been employed to produce RNAi in human embryonic stem cells.¹⁷⁷

Although several interesting studies on the effect of siRNA delivery and photothermal treatment against cancer have been performed *in vitro*,^{67,122,178} hereafter we will focus on *in vivo* studies because they represent a more reliable model for such a complex disease as cancer. Some studies employed spherical AuNPs with siRNA for laser irradiation^{90,98} but as they possess an LSPR band in the visible region, in this case adjacent (non-targeted) tissues can be damaged by the irradiation. In order to avoid this unwanted effect, the use of anisotropic AuNPs that absorb in the NIR region is preferable because the absorption profile of biological structures and living organisms is significantly lower between *ca.* 750 and 1200 nm.¹⁷⁹ It should be taken into consideration that the irradiation conditions can strongly affect the results. For example, Braeckmans and colleagues compared the siRNA transfection efficacy of AuNPs depending on laser energy and how pores in the membrane were created either through direct heating or by 'vapour nanobubbles' produced around the AuNPs at higher laser potency.¹⁸⁰ In the same direction, Wayteck *et al.* compared siRNA delivery by vapour-mediated photoporation using AuNPs and nucleofection; showing a three-fold higher number of efficiently transfected cytotoxic T cells in the case of photoporation.¹⁷³

Different siRNA knockdown strategies have been employed in combination with photothermal anticancer treatment, including reduction of angiogenesis and suppression of metastasis. Kwon and colleagues employed AuNPs with siRNA against VEGF administered intratumorally in PC-3 tumor bearing mice in order to deplete angiogenesis and obtain tumor ablation by laser irradiation, obtaining a complete regression of the tumor.⁹⁸ Other authors made use of siRNA to suppress metastasis while cancer cells were compromised by photoablation. In this instance gold nanorods with docetaxel and siRNA against p65 to block the nuclear factor kappa B pathway (important in immune response and altered in several types of cancer) were administered by intratumoral injection in orthotopically implanted 4T1 tumors. In this case, an 84% decrease of tumor growth rate was achieved and the amount of metastatic lesions found in the lungs of mice was decreased by more than 14 times.¹⁶³ In the same line, Yang *et al.* employed siRNA against the glycolytic pyruvate kinase muscle isoenzyme PKM2 that is overexpressed in tumor cells. Here, the siRNA was carried in a layer-by-layer chitosan-gold nanorods assembly that reduced cell migration *in vitro* and accumulated considerably in the tumor after tail vein injection in mice.¹⁰²

Another clever strategy consists of using siRNA to increase cancer cell thermal susceptibility during optical hyperthermia treatment. Although there are *in vitro* studies that employ siRNA and AuNPs separately to increase heat susceptibility of cells before undergoing laser irradiation,¹⁸¹ *in vivo* models typically use AuNPs functionalized with siRNA in a combined

manner and one such study, by Lu and colleagues, reported the use of gold nanostars functionalized with siRNA against heat shock protein 72, a malignancy-specific-overexpressed thermotolerance-related chaperone. The nanostars were covered by hyaluronic acid and were injected intravenously resulting in the complete reduction of luminescence and tumor volume after laser irradiation in MDA-MB-231/Luc bearing mice models.⁶³ In this study, nanostar biocompatibility and hemocompatibility was also studied, without showing haemolysis. A similar study performed by Li and co-workers focused on gold nanorod-siRNA targeting BAG3, a gene that blocks heat-shock response. Particles were administered intratumorally and, after laser treatment, tumor growth was clearly reduced in a xenograft model.⁶² Apart from nanorods and nanostars, hollow gold nanoshells functionalized with siRNA against heat shock protein 70, intravenously administered, have been employed in a similar strategy, showing a decrease in tumor volume and weight in nude mice bearing U87MG.⁶⁴

Oncogenes are genes that codify for proteins that control cell cycle and are overexpressed in cancer cells. siRNA has also been used to prevent the division of cancer cells and to induce apoptosis. Gold nanorods carrying doxorubicin and siRNA against mutant K-Ras were administered intratumorally in pancreatic adenocarcinoma xenograft mice models. Following irradiation, the tumor volume was decreased significantly.¹⁶⁰ Recently, Zhu and colleagues have developed gold nanorods covered with thermosensitive polymers that are internalized by the tumor cells when they are irradiated and functionalized with DNA Y-motifs, a special structure that binds the siRNA and doxorubicin until it is exposed to miRNA and ATP (such as in the cellular cytoplasm) provoking the release of doxorubicin and the siRNA against the over-expressed proto-oncogene PLK1 to produce a synergistic inhibition of tumor growth in mice.¹²⁰

However, probably the most relevant photothermal treatment study was performed by Conde *et al* (Figure 8)¹²⁹. In this study a triple-combination therapy was designed involving interference, drug and phototherapy. Gold nanorods functionalized with avastin, a vascular endothelial growth factor (VEGF) inhibitor, and gold nanospheres functionalized with siRNA against K-Ras, both of them also functionalized with a targeting peptide, were entrapped in a hydrogel patch that enhanced the stability and provided local delivery of embedded nanoparticle. When laser irradiated, mediated the conversion of near-infrared radiation into heat, causing the release of the chemotherapeutic as well as thermally induced tumor cell damage. VEGF is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis while K-Ras is a gene that performs an essential function in normal tissue signaling, but its mutation is considered to be an essential step in the development of many cancers. This hydrogel was implanted adjacent to the colorectal tumor in a mouse model and irradiated, causing a total reduction in the luciferase signal and achieving the survival of all the mice treated with the triple therapy compared to 100% mortality of the untreated mice. The authors also studied the efficacy of the triple therapy both

without tumor resection and after tumor resection, leading to a clear luciferase expression decrease compared to controls. To complete this study, a complete expression kinetics study of 20087 genes in local triple therapy was performed, showing 3589 genes altered 72 hours after the therapy. These studies achieve a new depth in the level of the study of AuNPs for siRNA by attempting to understand how the treatment affects metabolism taking one step further towards facilitating their implementation in a clinical setting.

3.4 Entrapment of AuNPs in organic composites

The aim of encapsulating drugs is usually to enhance drug stability, provide a sustained release, improve their effectiveness, decrease drug toxicity and/or enhance drug bioavailability.¹⁸² A variety of nanomaterials can be employed for this purpose, for example lipidic and polymeric derivatives, such as liposomes, solid lipid nanoparticles, dendrimeric nanocarriers, block copolymer micelles, cyclodextrins or hydrogel nanoparticles. However drugs encapsulated in proteins, silica or carbon nanomaterials are also common. It should be noted how siRNA encapsulation has provided the best results until now compared with other siRNA carriers, providing different clinical trials with positive conclusions (Table 1). For this reason, **entrapment** of AuNPs for siRNA delivery provide advantages stemming from both nanoparticle and encapsulating material, by increasing the protection of AuNPs and siRNA and providing a sustained release.

Different **entrapment** materials have been employed to capture siRNA-conjugated AuNP composites. Kong and colleagues employed a mixture of different cationic lipids to encapsulate hydrophobic AuNPs, forming a composite with a highly cationic surface charge that complexed efficiently to siRNA and were able to achieve gene silencing *in vitro* with a lower toxicity than cationic carrier polyethyleneimine.¹²⁷ Oh and co-workers co-**entrapped** siRNA and Au nanorods in Bovine Serum Albumin (BSA) nanocapsules and after grafting with PEG and anti-ErbB-2 antibodies they were able to silence the anti-apoptotic protein Bcl-2 in the target cells during approximately 72 hours and observed a synergistic effect when nanorods were laser irradiated.¹²² Polymer-encapsulated nanoparticles have also been employed successfully to provide RNA interference. For example, Son *et al.* developed a strategy to precisely control the assembly of AuNP in groups of two, three or more particles by the interaction of complementary sense and antisense strands of siRNA just controlling the number of single stranded siRNAs per AuNP. These RNAi-AuNP nanoconstructs were **entrapped** in branched polyethyleneimine that were administered by intratumoral injection providing anticancer efficacy in mice.⁹⁸ In another interesting work, Park and colleagues siRNA and AuNPs synthesized *in situ* on a siRNA/PEI complex were encapsulated in PEGylated hyaluronic acid, showing that AuNPs were crucial to provide siRNA stability against **RNases** in rat serum solution and demonstrating an accumulation of the nanocomplex in tumors by fluorescence imaging after tail administration in mice.¹⁸³ Thereafter the researchers showed that this nanomaterial was able to reduce fluorescence protein expression in tumor bearing mice. In another study, gold nanoclusters were coated with polyarginine and were encapsulated in the siRNA against nerve growth factor (NGF) showing efficacy as anticancer treatment against pancreatic cancer in mice.¹⁸⁴

One further approach is the use of niosomes for drug **entrapment**. Niosomes are formed by a bilayer structure similar to biological vesicles and are supposed to be biodegradable, biocompatible and nonimmunogenic.¹⁸⁵ For siRNA delivery, Mandal and colleagues employed acidic milieu-sensitive multi-lamellar gold niosomes to downregulate Akt in human breast

therapy-resistant cancer xenograft tumor models.¹²⁴ Other approaches to increase the stability and provide local delivery can be applied, such as the employment of hydrogel patch with the embedded nanoparticles that was implanted subcutaneously on top of the mammary tumor.¹²⁹ **Entrapment** of AuNP/siRNA constructs appears to provide all of the aforementioned advantages, but could also improve the choice of routes of administration depending on the properties of the **entrapping** material, for example by taking advantage of oral administration thanks to the use of the **entrapping** chitosan polymer.^{93,186} Overall, encapsulation of AuNPs could provide an additional level of control to AuNP delivery, stability and permit the use of new routes of administration avoiding the feared unknown side effects of nanoparticle accumulation.

Conclusions and future perspectives

Although AuNP-based siRNA delivery remains at a fundamental stage of development, far from being employed in a clinical setting, the innovative approaches described herewithin are testament to the many promising benefits of using nanocarriers for siRNA interference applications. It is clear that the highly modular nature of AuNP-based siRNA delivery systems offers several key advantages, since one or more design strategies can be exploited for any given application. For example, in this review we have shown how it is possible to harness active targeting methodologies based on multi-functional nanoparticles, co-delivery of other pharmaceuticals, entrapment in organic composites (for protection or targeted delivery) of the AuNPs as well as photothermal-triggered release. AuNPs also offer an improved protection to siRNA (especially against RNases) along with the possibility to achieve site-specific targeting. In addition, the observed efficacy of AuNP-based siRNA delivery in many reported *in vivo* studies (especially for anticancer treatments) is particularly relevant as they provide promise for future clinical trials. But perhaps their most important disadvantage is that AuNPs do tend to accumulate in tissues and organs such as the liver and spleen and, moreover, despite the 'inert' nature of gold, there is a clear lack of knowledge concerning the long-term toxicological consequences of such deposits in organs and tissues. One of the grand challenges for this area should therefore be to shed light on the longer-term toxicological effects of AuNP accumulation *in vivo*. Furthermore, the historic approval of (Onpattro) Patisiran from Alnylam on 10th of August 2018 as the first-ever FDA-approved siRNA therapy is sure to serve as a driving force to once again intensify research in nanomaterial-based siRNA delivery. Importantly, the long wait for Onpattro approval demonstrates the need to continue research into alternative precision therapies in this promising field. In this respect, engineering AuNP-based siRNA nanocarriers for routes of administration that will pose lower risks of accumulation *in vivo*, for instance topical and oral administration, could provide a significant opportunity for AuNP-mediated siRNA delivery in a clinical setting.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

AA reviewed the literature and co-wrote the manuscript; SGM provided overall guidance to the development of the review and co-wrote the manuscript; ISS, LDM, SGM, JMF critically reviewed and contributed ideas to various sections. All authors read and approved the final manuscript.

- 1 A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello, *Nature*, 1998, **391**, 806–811.
- 2 S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl, *Nature*, 2001, **411**, 494–498.
- 3 A. P. McCaffrey, L. Meuse, T.-T. T. Pham, D. S. Conklin, G. J. Hannon and M. a Kay, *Nature*, 2002, **418**, 38–9.
- 4 D. V. Morrissey, J. A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, K. Hartsough, L. Machemer, S. Radka, V. Jadhav, N. Vaish, S. Zinnen, C. Vargeese, K. Bowman, C. S. Shaffer, L. B. Jeffs, A. Judge, I. MacLachlan and B. Polisky, *Nat. Biotechnol.*, 2005, **23**, 1002–1007.
- 5 S. A. Leachman, R. P. Hickerson, P. R. Hull, F. J. D. Smith, L. M. Milstone, E. B. Lane, S. J. Bale, D. R. Roop, W. H. I. McLean and R. L. Kaspar, *J. Dermatol. Sci.*, 2008, **51**, 151–157.
- 6 G. R. Devi, *Cancer Gene Ther.*, 2006, **13**, 819–829.
- 7 Z. Wang, D. D. Rao, N. Senzer and J. Nemunaitis, *Pharm. Res.*, 2011, **28**, 2983–2995.
- 8 E. Bernstein, A. A. Caudy, S. M. Hammond and G. J. Hannon, *Nature*, 2001, **409**, 363–366.
- 9 T. A. Rand, K. Ginalski, N. V. Grishin and X. Wang, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 14385–9.
- 10 S. L. Ameres, J. Martinez and R. Schroeder, *Cell*, 2007, **130**, 101–112.
- 11 D. W. Bartlett and M. E. Davis, *Nucleic Acids Res.*, 2006, **34**, 322–333.
- 12 F. T. M. D. C. Vicentini, L. N. Borgheti-Cardoso, L. V. Depieri, D. De MacEdo Mano, T. F. Abelha, R. Petrilli and M. V. L. B. Bentley, *Pharm. Res.*, 2013, **30**, 915–931.
- 13 R. L. Kanasty, K. A. Whitehead, A. J. Vegas and D. G. Anderson, *Mol. Ther.*, 2012, **20**, 513–524.
- 14 J. J. Turner, S. W. Jones, S. A. Moschos, M. A. Lindsay and M. J. Gait, *Mol. Biosyst.*, 2007, **3**, 43–50.
- 15 A. D. Judge, V. Sood, J. R. Shaw, D. Fang, K. McClintock and I. MacLachlan, *Nat. Biotechnol.*, 2005, **23**, 457–462.
- 16 Y. Huang, J. Hong, S. Zheng, Y. Ding, S. Guo, H. Zhang, X. Zhang, Q. Du and Z. Liang, *Mol. Ther.*, 2011, **19**, 381–385.
- 17 S. Lau, B. Graham, N. Cao, B. J. Boyd, C. W. Pouton and P. J. White, *Mol. Pharm.*, 2012, **9**, 71–80.
- 18 R. Kanasty, J. R. Dorkin, A. Vegas and D. Anderson, *Nat. Mater.*, 2013, **12**, 967–977.
- 19 C. Schmidt, *Nat. Biotechnol.*, 2007, **25**, 273–275.
- 20 R. W. Esmond and A. K.-H. Chung, *Nanotechnol. Law Bus.*, 2014, **11**, 15–28.
- 21 P. K. Kaiser, R. C. A. Symons, S. M. Shah, E. J. Quinlan, H. Tabandeh, D. V. Do, G. Reisen, J. A. Lockridge, B. Short, R. Guercioli and Q. D. Nguyen, *Am. J. Ophthalmol.*, 2010, **150**, 33–39.
- 22 M. E. Davis, J. E. Zuckerman, C. H. J. Choi, D. Seligson, A. Tolcher, C. A. Alabi, Y. Yen, J. D. Heidel and A. Ribas, *Nature*, 2010, **464**, 1067–1070.
- 23 S. A. Leachman, R. P. Hickerson, M. E. Schwartz, E. E. Bullough, S. L. Hutcherson, K. M. Boucher, C. D. Hansen, M. J. Eliason, G. S. Srivatsa, D. J. Kornbrust, F. J. D. Smith, W. I. McLean, L. M. Milstone and R. L. Kaspar, *Mol. Ther.*, 2010, **18**, 442–446.
- 24 M. R. Zamora, M. Budev, M. Rolfe, J. Gottlieb, A. Humar, J. DeVincenzo, A. Vaishnav, J. Cehelsky, G. Albert, S. Nochur, J. A. Gollob and A. R. Glanville, *Am. J. Respir. Crit. Care Med.*, 2011, **183**, 531–538.
- 25 J. Dannull, N. R. Haley, G. Archer, S. Nair, D. Boczkowski, M. Harper, N. De Rosa, N. Pickett, P. J. Mosca, J. Burchette, M. A. Selim, D. A. Mitchell, J. Sampson, D. S. Tyler and S. K. Pruitt, *J. Clin. Invest.*, 2013, **123**, 3135–3145.
- 26 B. Schultheis, D. Strumberg, A. Santel, C. Vank, F. Gebhardt, O. Keil, C. Lange, K. Giese, J. Kaufmann, M. Khan and J. Dreves, *J. Clin. Oncol.*, 2014, **32**, 4141–4148.
- 27 K. Fitzgerald, M. Frank-Kamenetsky, S. Shulga-Morskaya, A. Liebow, B. R. Bettencourt, J. E. Sutherland, R. M. Hutabarat, V. A. Clausen, V. Karsten, J. Cehelsky, S. V. Nochur, V. Kotelianski, J. Horton, T. Mant, J. Chiesa, J. Ritter, M. Munisamy, A. K. Vaishnav, J. A. Gollob and A. Simon, *Lancet.*, 2014, **383**, 60–68.
- 28 T. Golan, E. Z. Khvalevsky, A. Hubert, R. M. Gabai, N. Hen, A. Segal, A. Domb, G. Harari, E. Ben David, S. Raskin, Y. Goldes, E. Goldin, R. Eliakim, M. Lahav, Y. Kopleman, A. Dancour, A. Shemi and E. Galun, *Oncotarget*, 2015, **6**, 24560–24570.
- 29 J. M. Benitez-Del-Castillo, J. Moreno-Montañés, I. Jiménez-Alfaro, F. J. Muñoz-Negrete, K. Turman, K. Palumaa, B. Sádaba, M. V. González, V. Ruz, B. Vargas, C. Pañeda, T. Martínez, A.-M. Bleau and A. I. Jimenez, *Investig. Ophthalmology Vis. Sci.*, 2016, **57**, 6447–54.
- 30 J. Conde, A. Ambrosone, Y. Hernandez, F. Tian, M. McCully, C. C. Berry, P. V. Baptista, C. Tortiglione and J. M. De La Fuente, *Nano Today*, 2015, **10**, 421–450.
- 31 S. H. Ku, S. D. Jo, Y. K. Lee, K. Kim and S. H. Kim, *Adv. Drug Deliv. Rev.*, 2016, **104**, 16–28.
- 32 F. Eberle, K. Giessler, C. Deck, K. Heeg, M. Peter, C. Richert

- and A. H. Dalpke, *J. Immunol.*, 2008, **180**, 3229–37.
- 33 S. A. Dar, A. Thakur, A. Qureshi and M. Kumar, *Sci. Rep.*, 2016, **6**, 1–8.
- 34 L. B. Couto and K. A. High, *Curr. Opin. Pharmacol.*, 2010, **10**, 534–542.
- 35 Y. Deng, C. C. Wang, K. W. Choy, Q. Du, J. Chen, Q. Wang, L. Li, T. K. H. Chung and T. Tang, *Gene*, 2014, **538**, 217–227.
- 36 S. Wilhelm, A. J. Tavares, Q. Dai, S. Ohta, J. Audet, H. F. Dvorak and W. C. W. Chan, *Nat. Rev. Mater.*, 2016, **1**, 1–12.
- 37 E. P. Thi, C. E. Mire, A. C. H. Lee, J. B. Geisbert, J. Z. Zhou, K. N. Agans, N. M. Snead, D. J. Deer, T. R. Barnard, K. A. Fenton, I. MacLachlan and T. W. Geisbert, *Nature*, 2015, **521**, 362–365.
- 38 H. Meng, M. Liong, T. Xia, Z. Li, Z. Ji, J. I. Zink and A. E. Nel, *ACS Nano*, 2010, **4**, 4539.
- 39 J. H. Lee, K. Lee, S. H. Moon, Y. Lee, T. G. Park and J. Cheon, *Angew. Chemie - Int. Ed.*, 2009, **48**, 4174–4179.
- 40 N. W. S. Kam, Z. Liu and H. Dai, *J. Am. Chem. Soc.*, 2005, **127**, 12492–12493.
- 41 L. Zhang, Z. Lu, Q. Zhao, J. Huang, H. Shen and Z. Zhang, *Small*, 2011, **7**, 460–464.
- 42 M. V. Yezhelyev, L. Qi, R. M. O'Regan, S. Nie and X. Gao, *J. Am. Chem. Soc.*, 2008, **130**, 9006–9012.
- 43 J. Li, Y. C. Chen, Y. C. Tseng, S. Mozumdar and L. Huang, *J. Control. Release*, 2010, **142**, 416–421.
- 44 A. Elbakry, A. Zaky, R. Liebl, R. Rachel, A. Goepferich and M. Breunig, *Nano Lett.*, 2009, **9**, 2059–64.
- 45 H. Lee, A. K. R. Lytton-Jean, Y. Chen, K. T. Love, A. I. Park, E. D. Karagiannis, A. Sehgal, W. Querbies, C. S. Zurenko, M. Jayaraman, C. G. Peng, K. Charisse, A. Borodovsky, M. Manoharan, J. S. Donahoe, J. Truelove, M. Nahrendorf, R. Langer and D. G. Anderson, *Nat. Nanotechnol.*, 2012, **7**, 389–393.
- 46 Y. Ding, Z. Jiang, K. Saha, C. S. Kim, S. T. Kim, R. F. Landis and V. M. Rotello, *Mol. Ther.*, 2014, **22**, 1075–1083.
- 47 M. Faraday, *Philos. Trans. R. Soc. London*, 1857, **147**, 145–181.
- 48 L. Vigderman and E. R. Zubarev, *Chem. Mater.*, 2013, **25**, 1450–1457.
- 49 B. Pelaz, V. Grazu, A. Ibarra, C. Magen, P. del Pino and J. M. de la Fuente, *Langmuir*, 2012, **28**, 8965–70.
- 50 J. Piella, N. G. Bastús and V. Puentes, *Chem. Mater.*, 2016, **28**, 1066–1075.
- 51 X. Cheng, X. Tian, A. Wu, J. Li, J. Tian, Y. Chong, Z. Chai, Y. Zhao, C. Chen and C. Ge, *ACS Appl. Mater. Interfaces*, 2015, **7**, 20568–20575.
- 52 L. Shang, K. Nienhaus and G. Nienhaus, *J. Nanobiotechnology*, DOI:10.1186/1477-3155-12-5.
- 53 S. Hirn, M. Semmler-Behnke, C. Schleh, A. Wenk, J. Lipka, M. Schäffler, S. Takenaka, W. Möller, G. Schmid, U. Simon and W. G. Kreyling, *Eur. J. Pharm. Biopharm.*, 2011, **77**, 407–416.
- 54 W. H. De Jong, W. I. Hagens, P. Krystek, M. C. Burger, A. J. A. M. Sips and R. E. Geertsma, *Biomaterials*, 2008, **29**, 1912–1919.
- 55 G. Alfranca, Á. Artiga, G. Stepien, M. Moros, S. G. Mitchell and J. M. de la Fuente, *Nanomedicine (Lond.)*, 2016, **11**, 2903–2916.
- 56 Y. Wang, K. C. L. Black, H. Luehmann, W. Li, Y. Zhang, X. Cai, D. Wan, S.-Y. Liu, M. Li, P. Kim, Z.-Y. Li, L. V. Wang, Y. Liu and Y. Xia, *ACS Nano*, 2013, **7**, 2068–2077.
- 57 J. Yue, T. J. Feliciano, W. Li, A. Lee and T. W. Odom, *Bioconjug. Chem.*, 2017, **28**, 1791–1800.
- 58 L. M. Liz-Marzán, *Langmuir*, 2006, **22**, 32–41.
- 59 M. E. Stewart, C. R. Anderton, L. B. Thompson, J. Maria, S. K. Gray, J. A. Rogers and R. G. Nuzzo, *Chem. Rev.*, 2008, **108**, 494–521.
- 60 N. Li, P. Zhao and D. Astruc, *Angew. Chemie - Int. Ed.*, 2014, **53**, 1756–1789.
- 61 J. Conde, A. Ambrosone, V. Sanz, Y. Hernandez, V. Marchesano, F. Tian, H. Child, C. C. Berry, M. R. Ibarra, P. V. Baptista, C. Tortiglione and J. M. de la Fuente, *ACS Nano*, 2012, **6**, 8316–8324.
- 62 B. K. Wang, X. F. Yu, J. H. Wang, Z. Bin Li, P. H. Li, H. Wang, L. Song, P. K. Chu and C. Li, *Biomaterials*, 2016, **78**, 27–39.
- 63 S. Wang, Y. Tian, W. Tian, J. Sun, S. Zhao, Y. Liu, C. Wang, Y. Tang, X. Ma, Z. Teng and G. Lu, *ACS Nano*, 2016, **10**, 8578–8590.
- 64 Z. Wang, S. Li, M. Zhang, Y. Ma, Y. Liu, W. Gao, J. Zhang and Y. Gu, *Adv. Sci.*, 2016, **4**, 1600327.
- 65 K. Rahme, M. T. Nolan, T. Doody, G. P. McGlacken, M. A. Morris, C. O'Driscoll and J. D. Holmes, *RSC Adv.*, 2013, **3**, 21016.
- 66 Y. T. Chang, P. Y. Liao, H. S. Sheu, Y. J. Tseng, F. Y. Cheng and C. S. Yeh, *Adv. Mater.*, 2012, **24**, 3309–3314.
- 67 X. Huang, A. Pallaoro, G. Braun, D. Morales, J. Zasadzinski and N. Reich, *Nano Lett.*, 2014, **14**, 2046–2051.
- 68 C.-T. Chien, C.-Y. Liu, Z.-W. Wu, P.-J. Chen, C.-L. Chu and S.-Y. Lin, *J. Mater. Chem. B*, 2014, **2**, 6730–6737.
- 69 M. Lee, N. Kim, J. Lee, D. Lim, W. Suh, H. Kim, J. Park and J. Jeong, *Macromol. Res.*, 2015, **23**, 670–677.
- 70 Z. Wang, H. Wu, H. Shi, M. Wang, C. Huang and N. Jia, *J. Mater. Chem. B*, 2016, **4**, 2519–2526.
- 71 Y. Chen, M. Xu, Y. Guo, K. Tu, W. Wu, J. Wang, X. Tong, W. Wu, L. Qi and D. Shi, *Nanotechnology*, 2017, **28**, 025101.
- 72 Q. Ni, Z. Teng, M. Dang, Y. Tian, Y. Zhang, P. Huang, X. Su, N. Lu, Z. Yang, W. Tian, S. Wang, W. Liu, Y. Tang, G. Lu and L. Zhang, *Nanoscale*, 2017, **9**, 1466–1474.
- 73 M. Mohammadniaei, T. Lee, B. G. Bharate, J. Yoon, H. K. Choi, S. Jeong Park, J. Kim, J. Kim and J. W. Choi, *Small*, 2018, **14**, 1–13.
- 74 J. H. Choi and B. K. Oh, *J. Microbiol. Biotechnol.*, 2014, **24**, 1291–1299.
- 75 X. Liu, N. Huang, H. Li, Q. Jin and J. Ji, *Langmuir*, 2013, **29**, 9138–9148.
- 76 P. del Pino, B. Pelaz, Q. Zhang, P. Maffre, G. U. Nienhaus and W. J. Parak, *Mater. Horiz.*, 2014, **1**, 301–313.
- 77 B. D. Chithrani, A. A. Ghazani and W. C. W. Chan, *Nano Lett.*, 2006, **6**, 662–668.
- 78 L. Treuel, D. Docter, M. Maskos and R. H. Stauber, *Beilstein J. Nanotechnol.*, 2015, **6**, 857–873.
- 79 M. C. Lo Giudice, L. M. Herda, E. Polo and K. A. Dawson, *Nat. Commun.*, 2016, **7**, 13475.
- 80 P. Zhao, N. Li and D. Astruc, *Coord. Chem. Rev.*, 2013, **257**, 2903–2916.

- 638–665.
- 81 S. D. Perrault, C. Walkey, T. Jennings, H. C. Fischer and W. C. W. Chan, *Nano Lett.*, 2009, **9**, 1909–1915.
 - 82 N. T. Chen, K. C. Tang, M. F. Chung, S. H. Cheng, C. M. Huang, C. H. Chu, P. T. Chou, J. S. Souris, C. T. Chen, C. Y. Mou and L. W. Lo, *Theranostics*, 2014, **4**, 798–807.
 - 83 Z. Zhang, L. Wang, J. Wang, X. Jiang, X. Li, Z. Hu, Y. Ji, X. Wu and C. Chen, *Adv. Mater.*, 2012, **24**, 1418–1423.
 - 84 A. N. Kharlamov, A. E. Tyurnina, V. S. Veselova, O. P. Kovtun, V. Y. Shur and J. L. Gabinsky, *Nanoscale*, 2015, **7**, 8003–8015.
 - 85 J. Conde, F. Tian, Y. Hernández, C. Bao, D. Cui, K. P. Janssen, M. R. Ibarra, P. V. Baptista, T. Stoeger and J. M. de la Fuente, *Biomaterials*, 2013, **34**, 7744–7753.
 - 86 S. Labala, A. Jose and V. V. K. Venuganti, *Colloids Surfaces B Biointerfaces*, 2016, **146**, 188–197.
 - 87 L. Han, J. Zhao, X. Zhang, W. Cao, X. Hu, G. Zou, X. Duan and X. J. Liang, *ACS Nano*, 2012, **6**, 7340–7351.
 - 88 E. Zhao, Z. Zhao, J. Wang, C. Yang, C. Chen, L. Gao, Q. Feng, W. Hou, M. Gao and Q. Zhang, *Nanoscale*, 2012, **4**, 5102.
 - 89 K. Niikura, K. Kobayashi, C. Takeuchi, N. Fujitani, S. Takahara, T. Ninomiya, K. Hagiwara, H. Mitomo, Y. Ito, Y. Osada and K. Ijiri, *ACS Appl. Mater. Interfaces*, 2014, **6**, 22146–22154.
 - 90 S. Son, J. Nam, J. Kim, S. Kim and W. J. Kim, *ACS Nano*, 2014, **8**, 5574–5584.
 - 91 A. Chaudhary and S. Garg, *Mater. Sci. Eng. C*, 2017, **80**, 18–28.
 - 92 X. Huang, Y. Lai, G. B. Braun and N. O. Reich, *Small*, 2017, **13**, 1602473.
 - 93 S. H. Kang, V. Revuri, S. J. Lee, S. Cho, I. K. Park, K. J. Cho, W. K. Bae and Y. K. Lee, *ACS Nano*, 2017, **11**, 10417–10429.
 - 94 M. Oishi, J. Nakaogami, T. Ishii and Y. Nagasaki, *Chem. Lett.*, 2006, **35**, 1046–1047.
 - 95 C. A. Mirkin, R. L. Letsinger, R. C. Mucic and J. J. Storhoff, *Nature*, 1996, **382**, 607–609.
 - 96 M. Jiwaji, M. E. Sandison, J. Reboud, R. Stevenson, R. Daly, G. Barkess, K. Faulds, W. Kolch, D. Graham, M. A. Girolami, J. M. Cooper and A. R. Pitt, *PLoS One*, 2014, **9**, e99458.
 - 97 J. Conde, F. Tian, Y. Hernandez, C. Bao, P. V. Baptista, D. Cui, T. Stoeger and J. M. de la Fuente, *Nanoscale*, 2015, **7**, 9083–9091.
 - 98 S. Son, N. Kim, D. G. You, H. Y. Yoon, J. Y. Yhee, K. Kim, I. C. Kwon and S. H. Kim, *Theranostics*, 2017, **7**, 9–22.
 - 99 S. N. Barnaby, A. Lee and C. A. Mirkin, *Proc. Natl. Acad. Sci.*, 2014, **111**, 9739–9744.
 - 100 Z. J. Lu and D. H. Mathews, *Nucleic Acids Res.*, 2008, **36**, 104–108.
 - 101 Y. Yi, H. J. Kim, P. Mi, M. Zheng, H. Takemoto, K. Toh, B. S. Kim, K. Hayashi, M. Naito, Y. Matsumoto, K. Miyata and K. Kataoka, *J. Control. Release*, 2016, **244**, 247–256.
 - 102 Z. Yang, T. Liu, Y. Xie, Z. Sun, H. Liu, J. Lin, C. Liu, Z. W. Mao and S. Nie, *Acta Biomater.*, 2015, **25**, 194–204.
 - 103 R. Masood, I. Roy, S. Zu, C. Hochstim, K.-T. Yong, W.-C. Law, H. Ding, U. K. Sinha and P. N. Prasad, *Integr. Biol.*, 2012, **4**, 132–141.
 - 104 H. S. Kim, Y. J. Son, W. Mao, K. W. Leong and H. S. Yoo, *Nano Lett.*, 2018, **18**, 314–325.
 - 105 L. Kong, J. Qiu, W. Sun, J. Yang, M. Shen, L. Wang and X. Shi, *Biomater. Sci.*, 2017, **5**, 258–266.
 - 106 B. Shah, P. T. Yin, S. Ghoshal and K. B. Lee, *Angew. Chemie - Int. Ed.*, 2013, **125**, 6310–6315.
 - 107 S. E. Lee, D. Y. Sasaki, Y. Park, R. Xu, J. S. Brennan, M. J. Bissell and L. P. Lee, *ACS Nano*, 2012, **6**, 7770–7780.
 - 108 N. Panwar, C. Yang, F. Yin, H. S. Yoon, T. S. Chuan and K. T. Yong, *Nanotechnology*, 2015, **26**, 365101.
 - 109 K. H. Min, Y. H. Kim, Z. Wang, J. Kim, J. S. Kim, S. H. Kim, K. Kim, I. C. Kwon, D. O. Kiesewetter and X. Chen, *Theranostics*, 2017, **7**, 4240–4254.
 - 110 R. Huschka, A. Barhoumi, Q. Liu, J. A. Roth, L. Ji and N. J. Halas, *ACS Nano*, 2012, **6**, 7681–7691.
 - 111 R. Bahadur K.C., B. Thapa and N. Bhattarai, *Nanotechnol. Rev.*, 2014, **3**, 269–280.
 - 112 H. J. Kim, H. Takemoto, Y. Yi, M. Zheng, Y. Maeda, H. Chaya, K. Hayashi, P. Mi, F. Pittella, R. J. Christie, K. Toh, Y. Matsumoto, N. Nishiyama, K. Miyata and K. Kataoka, *ACS Nano*, 2014, **8**, 8979–8991.
 - 113 Y. Xiao, R. Jaskula-Sztul, A. Javadi, W. Xu, J. Eide, A. Dammalapati, M. Kunnimalaiyaan, H. Chen and S. Gong, *Nanoscale*, 2012, **4**, 7185.
 - 114 Z. Tang, Y. Wang, P. Podsiadlo and N. A. Kotov, *Adv. Mater.*, 2006, **18**, 3203–3224.
 - 115 S. K. Lee, B. Law and C. H. Tung, *Macromol. Biosci.*, 2017, **17**, 1600294.
 - 116 A. M. Paul, Y. Shi, D. Acharya, J. R. Douglas, A. Cooley, J. F. Anderson, F. Huang and F. Bai, *J. Gen. Virol.*, 2014, **95**, 1712–1722.
 - 117 S. K. Lee and C. H. Tung, *Adv. Funct. Mater.*, 2013, **23**, 3488–3493.
 - 118 M. Uz, S. K. Mallapragada and S. A. Altinkaya, *Rsc Adv.*, 2015, **5**, 43515–43527.
 - 119 B. Esteban-Fernández De Ávila, C. Angell, F. Soto, M. A. Lopez-Ramirez, D. F. Báez, S. Xie, J. Wang and Y. Chen, *ACS Nano*, 2016, **10**, 4997–5005.
 - 120 P. Zhang, C. Wang, J. Zhao, A. Xiao, Q. Shen, L. Li, J. Li, J. Zhang, Q. Min, J. Chen, H. Y. Chen and J. J. Zhu, *ACS Nano*, 2016, **10**, 3637–3647.
 - 121 R. Kotcherlakotaa, D. J. Srinivasanb, S. Mukherjeea, M. M. Haroon, G. H. Dar, U. Venkatraman, C. R. Patra and V. Gopal, *J. Mater. Chem. B*, 2017, **5**, 7082–7098.
 - 122 J.-H. Choi, H.-J. Hwang, S. W. Shin, J.-W. Choi, S. H. Um and B.-K. Oh, *Nanoscale*, 2015, **7**, 9229–9237.
 - 123 J. Gilleron, W. Querbies, A. Zeigerer, A. Borodovsky, G. Marsico, U. Schubert, K. Manygoats, S. Seifert, C. Andree, M. Stöter, H. Epstein-Barash, L. Zhang, V. Koteliansky, K. Fitzgerald, E. Fava, M. Bickle, Y. Kalaidzidis, A. Akinc, M. Maier and M. Zerial, *Nat. Biotechnol.*, 2013, **31**, 638–646.
 - 124 S. Rajput, N. Puvvada, B. N. P. Kumar, S. Sarkar, S. Konar, R. Bharti, G. Dey, A. Mazumdar, A. Pathak, P. B. Fisher and M. Mandal, *Mol. Pharm.*, 2015, **12**, 4214–4225.
 - 125 S. K. Mudedla, E. R. Azhagiya Singam, K. Balamurugan and V. Subramanian, *Phys. Chem. Chem. Phys.*, 2015, **17**, 30307–30317.
 - 126 X. Xie, S. Xu, P. Pi, J. Cheng and X. Wen, *Am. Inst. Chem.*

- Eng. J.*, 2018, **64**, 810–821.
- 127 W. H. Kong, K. H. Bae, S. D. Jo, J. S. Kim and T. G. Park, *Pharm. Res.*, 2012, **29**, 362–374.
- 128 L. Fan, Y. Zhang, F. Wang, Q. Yang, J. Tan, R. Grifantini, H. Wu, C. Song and B. Jin, *Biomaterials*, 2016, **76**, 399–407.
- 129 J. Conde, N. Oliva, Y. Zhang and N. Artzi, *Nat. Mater.*, 2016, **15**, 1128–1138.
- 130 T. A. Ignatowski, R. Aalinkeel, J. L. Reynolds, B. B. Nair, D. E. Sykes, C. P. K. Gleason, W. C. Law, M. J. Mammen, P. N. Prasad, S. A. Schwartz and S. D. Mahajan, *J. Neuroimmune Pharmacol.*, 2015, **10**, 136–152.
- 131 D. Zheng, D. A. Giljohann, D. L. Chen, M. D. Massich, X.-Q. Wang, H. Iordanov, C. A. Mirkin and A. S. Paller, *Proc. Natl. Acad. Sci.*, 2012, **109**, 11975–11980.
- 132 H. W. Child, Y. Hernandez, J. Conde, M. Mullin, P. Baptista, J. M. de la Fuente and C. C. Berry, *Nanomedicine (Lond.)*, 2015, **10**, 2513–2525.
- 133 J. L. Reynolds, W. C. Law, S. D. Mahajan, R. Aalinkeel, B. Nair, D. E. Sykes, K. T. Yong, R. Hui, P. N. Prasad and S. A. Schwartz, *J. Neuroimmune Pharmacol.*, 2012, **7**, 673–685.
- 134 H. Nemati, M. H. Ghahramani, R. Faridi-Majidi, B. Izadi, G. Bahrami, S. H. Madani and G. Tavoosidana, *J. Control. Release*, 2017, **268**, 259–268.
- 135 X. Zhao, Q. Huang and Y. Jin, *Mater. Sci. Eng. C*, 2015, **54**, 142–149.
- 136 W. Feng, H. Guo, T. Xue, X. Wang, C. Tang, B. Ying, H. Gong and G. Cui, *RSC Adv.*, 2016, **6**, 60171–60179.
- 137 P. S. Randeria, M. A. Seeger, X.-Q. Wang, H. Wilson, D. Shipp, C. A. Mirkin and A. S. Paller, *Proc. Natl. Acad. Sci.*, 2015, **112**, 5573–5578.
- 138 S. Sajeesh, J. Y. Choe and D. ki Lee, *Nanomedicine (Lond.)*, 2017, **12**, 2271–2286.
- 139 T. Lobovkina, G. B. Jacobson, E. Gonzalez-Gonzalez, R. P. Hickerson, D. Leake, R. L. Kaspar, C. H. Contag and R. N. Zare, *ACS Nano*, 2011, **5**, 9977–9983.
- 140 P. Majumder, S. Bhunia and A. Chaudhuri, *Chem. Commun.*, 2018, **54**, 1489–1492.
- 141 S. Rietwyk and D. Peer, *ACS Nano*, 2017, **11**, 7572–7586.
- 142 P. Majumder, S. Bhunia, J. Bhattacharyya and A. Chaudhuri, *J. Control. Release*, 2014, **180**, 100–108.
- 143 Y. Jiang, S. Huo, J. Hardie, X.-J. Liang and V. M. Rotello, *Expert Opin. Drug Deliv.*, 2016, **13**, 547–559.
- 144 W. G. Kreyling, A. M. Abdelmonem, Z. Ali, F. Alves, M. Geiser, N. Haberl, R. Hartmann, S. Hirn, D. J. De Aberasturi, K. Kantner, G. Khadem-Saba, J. M. Montenegro, J. Rejman, T. Rojo, I. R. De Larramendi, R. Ufartes, A. Wenk and W. J. Parak, *Nat. Nanotechnol.*, 2015, **10**, 619–623.
- 145 M. Pérez-Hernández, M. Moros, G. Stepien, P. del Pino, S. Menao, M. de las Heras, M. Arias, S. G. Mitchell, B. Pelaz, E. M. Gálvez, J. M. de la Fuente and J. Pardo, *Part. Fibre Toxicol.*, 2017, **14**, 1–20.
- 146 S. M. Dizaj, S. Safari and A. Y. Khosroushahi, *Nanoscale Res. Lett.*, 2014, **9**, 1–9.
- 147 S. K. Surapaneni, S. Bashir and K. Tikoo, *Sci. Rep.*, 2018, **8**, 1–12.
- 148 Y. H. Bae and K. Parkb, *J Control Release*, 2011, **153**, 198–205.
- 149 M. Mitra, M. Kandalam, J. Rangasamy, B. Shankar, U. K. Maheswari, S. Swaminathan and S. Krishnakumar, *Mol. Vis.*, 2013, **19**, 1029–38.
- 150 S. Acharya and R. A. Hill, *Nanomedicine Nanotechnology, Biol. Med.*, 2014, **10**, 329–337.
- 151 L. Gamrad, C. Rehbock, A. M. Westendorf, J. Buer, S. Barcikowski and W. Hansen, *Sci. Rep.*, 2016, **6**, 28709.
- 152 K. A. Fitzgerald, K. Rahme, J. Guo, J. D. Holmes and C. M. O'Driscoll, *J. Mater. Chem. B*, 2016, **4**, 2242–2252.
- 153 N. Li, H. Yang, Z. Yu, Y. Li, W. Pan, H. Wang and B. Tang, *Chem. Sci.*, 2017, **8**, 2816–2822.
- 154 H. Zhu, W. Liu, Z. Cheng, K. Yao, Y. Yang, B. Xu and G. Su, *Int. J. Mol. Sci.*, 2017, **18**, 2029.
- 155 C. Bechara and S. Sagan, *FEBS Lett.*, 2013, **587**, 1693–1702.
- 156 N. Chanda, V. Kattumuri, R. Shukla, A. Zambre, K. Katti, A. Upendran, R. R. Kulkarni, P. Kan, G. M. Fent, S. W. Casteel, C. J. Smith, E. Boote, J. D. Robertson, C. Cutler, J. R. Lever, K. V. Katti and R. Kannan, *Proc. Natl. Acad. Sci.*, 2010, **107**, 8760–8765.
- 157 E. C. Dreaden, L. A. Austin, M. A. Mackey and M. A. El-Sayed, *Ther Deliv*, 2012, **3**, 457–478.
- 158 S. Labala, A. Jose, S. R. Chawla, M. S. Khan, S. Bhatnagar, O. P. Kulkarni and V. V. K. Venuganti, *Int. J. Pharm.*, 2017, **525**, 407–417.
- 159 R. A. Sperling and W. J. Parak, *Philos. Trans. R. Soc. A Math. Phys. Eng. Sci.*, 2010, **368**, 1333–1383.
- 160 F. Yin, C. Yang, Q. Wang, S. Zeng, R. Hu, G. Lin, J. Tian, S. Hu, R. F. Lan, H. S. Yoon, F. Lu, K. Wang and K. T. Yong, *Theranostics*, 2015, **5**, 818–833.
- 161 L. Ding, R. Sun and X. Zhang, *Oncotarget*, 2017, **8**, 21200–21211.
- 162 M. Li, Y. Li, X. Huang and X. Lu, *J. Biomater. Sci. Polym. Ed.*, 2015, **26**, 813–827.
- 163 D. Wang, T. Wang, Z. Xu, H. Yu, B. Feng, J. Zhang, C. Guo, Q. Yin, Z. Zhang and Y. Li, *Small*, 2016, **12**, 488–498.
- 164 F. Perche, Y. Yi, L. Hespel, P. Mi, A. Dirisala, H. Cabral, K. Miyata and K. Kataoka, *Biomaterials*, 2016, **90**, 62–71.
- 165 C. J. Bishop, S. Y. Tzeng and J. J. Green, *Acta Biomater.*, 2015, **11**, 393–403.
- 166 M. E. Muroski, J. M. Kogot and G. F. Strouse, *J. Am. Chem. Soc.*, 2012, **134**, 19722–19730.
- 167 J. Liu, C. Detrembleur, B. Grignard, M. C. De Pauw-Gillet, S. Mornet, M. Treguer-Delapierre, Y. Petit, C. Jérôme and E. Duguet, *Chem. - An Asian J.*, 2014, **9**, 275–288.
- 168 E. Polo, P. del Pino, B. Pelaz, V. Grazu and J. M. de la Fuente, *Chem. Commun.*, 2013, **49**, 3676.
- 169 A. Squassero, Á. Artiga, C. Morasso, R. R. Jimenez, R. M. Rapún, R. Mancuso, S. Agostini, A. Hernis, A. Abols, A. Lin, A. Gualerzi, S. Picciolini, M. Bedoni, M. Rovaris, F. Gramatica, J. M. De Fuente and R. Vanna, *Anal. Bioanal. Chem.*, 2018, 1–13.
- 170 J. Han, J. Zhang, M. Yang, D. Cui and J. M. de la Fuente, *Nanoscale*, 2016, **8**, 492–499.
- 171 M. Pérez-Hernández, P. Del Pino, S. G. Mitchell, M. Moros, G. Stepien, B. Pelaz, W. J. Parak, E. M. Gálvez, J. Pardo and J. M. De La Fuente, *ACS Nano*, 2015, **9**, 52–61.
- 172 D. Heinemann, M. Schomaker, S. Kalies, M. Schieck, R.

- Carlson, H. M. Escobar, T. Ripken, H. Meyer and A. Heisterkamp, *PLoS One*, 2013, **8**, e58604.
- 173 L. Wayteck, R. Xiong, K. Braeckmans, S. C. De Smedt and K. Raemdonck, *J. Control. Release*, 2017, **267**, 154–162.
- 174 R. S. Riley, M. N. Dang, M. Marie, B. Abraham, L. Gundlach and E. S. Day, *Nano Lett.*, , DOI:10.1021/acs.nanolett.8b00681.
- 175 F. Li, C. Li, J. Liu, X. Liu, L. Zhao, T. Bai, Q. Yuan, X. Kong, Y. Han, Z. Shi and S. Feng, *Nanoscale*, 2013, **5**, 6950–6959.
- 176 A. Taruttis, N. Lozano, A. Nunes, D. A. Jasim, N. Beziere, E. Herzog, K. Kostarelos and V. Ntziachristos, *Nanoscale*, 2014, **6**, 13451–13456.
- 177 X. Huang, Q. Hu, G. B. Braun, A. Pallaoro, D. P. Morales, J. Zasadzinski, D. O. Clegg and N. O. Reich, *Biomaterials*, 2015, **63**, 70–79.
- 178 J. Shen, H. C. Kim, C. Mu, E. Gentile, J. Mai, J. Wolfram, L. N. Ji, M. Ferrari, Z. wan Mao and H. Shen, *Adv. Healthc. Mater.*, 2014, **3**, 1629–1637.
- 179 Y. Huang, A. C. Chen and M. Hamblin, *SPIE Newsroom*, 2009, **9**, 1–3.
- 180 R. Xiong, K. Raemdonck, K. Peynshaert, I. Lentacker, I. De Cock, J. Demeester, S. C. De Smedt, A. G. Skirtach and K. Braeckmans, *ACS Nano*, 2014, **8**, 6288–6296.
- 181 M. R. K. Ali, H. R. Ali, C. R. Rankin and M. A. El-Sayed, *Biomaterials*, 2016, **102**, 1–8.
- 182 N. A. Liechty, W. B., Kryscio, D.R., Slaughter, B. V. and Peppas, *Annu. Rev. Chem. Biomol. Eng.*, 2010, **1**, 149–173.
- 183 R. Heo, H. Y. Yoon, H. Ko, J. M. Shin, J. Jeon, Y. S. Chae, Y. M. Kang, D. Kim, D. S. Lee and J. H. Park, *Chem. Commun.*, 2015, **51**, 16656–16659.
- 184 Y. Lei, L. Tang, Y. Xie, Y. Xianyu, L. Zhang, P. Wang, Y. Hamada, K. Jiang, W. Zheng and X. Jiang, *Nat. Commun.*, 2017, **8**, 15130.
- 185 D. A. Seleci, M. Seleci, J. G. Walter, F. Stahl and T. Scheper, *J. Nanomater.*, , DOI:10.1155/2016/7372306.
- 186 Á. Artiga, S. García-Embid, L. De Matteis, S. G. Mitchell and J. M. de la Fuente, *Front. Chem.*, 2018, **6**, 1–10.
- 187 E. C. Dreaden, A. M. Alkilany, X. Huang, C. J. Murphy and M. A. El-Sayed, *Chem. Soc. Rev.*, 2012, **41**, 2740–2779.