Therapeutic platforms based on gold nanoparticles and their covalent conjugates with drug molecules

Leonid Vigderman, Eugene R. Zubarev *

Rice University, Department of Chemistry, 6100 Main Street, Houston, TX 77005, USA

A R T I C L E   I N F O

Article history:
Accepted 7 May 2012
Available online 18 May 2012

Keywords:
Gold
Nanoparticles
Nucleic acids
Drug delivery
Covalent attachment
Ligand exchange
Photothermal effect

A B S T R A C T

This review will first look at the various covalent strategies that have been developed to attach drugs to gold nanoparticles as well as the strengths and limitations of such strategies. After examining general strategies for the synthesis of gold nanoparticles and their subsequent covalent functionalization, this review will focus on nanoparticle conjugates for gene therapy, antibacterial, and anticancer applications including the use of gold nanoparticles with intrinsically therapeutic properties. The effects of targeting and cellular uptake of gold nanoparticles will also be discussed.

Contents

1. Introduction .............................................................. 6
2. Gold nanoparticle synthetic strategies .................................................. 6
3. General functionalization strategies ................................................... 6
4. Gold nanoparticles for gene therapy .................................................. 6
   4.1. Spherical nanoparticle conjugates ................................................ 6
   4.2. Delivery effects ........................................................................ 6
   4.3. Cellular response ..................................................................... 6
   4.4. Photothermal release .............................................................. 6
5. Gold nanoparticles for bactericidal applications ............................................. 6
   5.1. Amine-bound antibiotics .................................................... 6
   5.2. Thiol-bound antibiotics ..................................................... 6
6. Gold nanoparticles for anticancer applications .............................................. 6
   6.1. Platinum-based conjugates .................................................. 6
   6.2. Conjugates with small organic molecules ........................................ 6
   6.3. Gold nanoparticles as intrinsically therapeutic agents ............ 6
7. Towards clinical application .......................................................... 6
8. Conclusions .............................................................. 6
Acknowledgement ............................................................. 6
References ................................................................. 6

1. Introduction

Gold nanoparticles have long been considered as possible drug delivery vehicles applicable for therapy of a range of conditions. Among all of these systems, one of the most important elements is developing methodologies to properly functionalize the nanoparticles to have the desired effects. Countless reports exist of gold nanoparticles functionalized...
through the well-known gold–thiol covalent bond [1]. In addition to their excellent functionalization chemistry, gold nanoparticles are attractive for a number of reasons. They can be easily synthesized in a variety of shapes including spherical, rod-like, core–shell, and many others with sizes ranging from 1 nm to more than 100 nm [2–7]. The optical and electronic properties of gold nanoparticles are highly shape- and size-dependent [5,8], which has led to many biomedical applications [9–17]. For instance, nanorods, nanoshells, and other gold nanocrystals are able to absorb light in the near infrared (NIR) region, which makes them particularly useful for the biological purposes given the high transparency of tissue to light of that wavelength [18]. In addition, gold nanoparticles have generally shown themselves to be nontoxic, although there is some indication that non-covalently functionalized particles are not completely benign [19]. There are various strategies for using gold nanoparticles as a drug delivery vehicle, including systems based on covalent binding, drug encapsulation [2,20–23], electrostatic adsorption [24], and other non-covalent assemblies [25]. However, this review will focus only on the direct covalent attachment of several types of drugs to gold nanoparticles for use in gene therapy, bactericidal and anticancer applications (Fig. 1).

2. Gold nanoparticle synthetic strategies

It is first important to look at the general nanoparticle synthesis and functionalization strategies that could be useful for designing a covalent drug delivery system. The first choice that must be made is what shape and size of particle are most desired for the drug delivery application (Fig. 1c). Detailed reviews covering the synthetic strategies for gold nanoparticles have been written, so only a brief review will be given here [4,5,8]. Spherical nanoparticles are often chosen due to their facile synthesis and have been thoroughly studied. Spherical nanoparticles can be made with diameters ranging from 2 to 100 nm. The smallest diameter nanoparticles are typically made by reduction of gold with a strong reducing agent in the presence of thiol molecules [26]. Particles produced by this method are immediately covalently functionalized as they are synthesized. If a functional thiol is used in the initial step, it can be further reacted using standard organic chemistry techniques [27–33]. Gibson et al. have used this technique successfully to synthesize paclitaxel-functionalized gold nanoparticles [34] (Section 6.2). Alternatively, further covalent functionalization can be carried out with a thiol place-exchange reaction with a separate functional thiol [20,35,36]. Though this technique is limited by the necessity for an excess of thiol and does not completely replace the thiol monolayer, it can be useful to install functional thiols as a base for chemical attachment of the desired molecules [37,38].

Small gold nanoparticles make excellent scaffolds for drug attachment, but they generally do not display a strong light absorbance in the visible range. Gold nanoparticles of ca. 5 nm in diameter display large optical absorption due to surface plasmon resonance which makes them very interesting as imaging and detection platforms [4]. Larger nanoparticles in the range of 10–100 nm are usually synthesized by the Turkevich method and are capped with citrate ions [3]. Efficient functionalization of this surface is then carried out by adding a thiol-containing capping agent. This simple procedure has led to the covalent functionalization of nanoparticles with a wide variety of ligands. Larger gold spherical nanoparticles scatter excellently, but absorb light only in the visible range (520–550 nm), which may not be ideal for biological application because of the low transparency of the biological tissue to visible light [18]. On the other hand, gold nanorods and nanoshells, among other morphologies, can be synthesized to have a strong surface plasmon resonance in the near IR region, where the body is much more transparent [18]. Nanoshells, which contain a spherical dielectric core with a thin gold shell, have a citrate coated surface and can be functionalized in a similar way described above [39]. Gold nanorods, on the other hand, are generally synthesized with a cetyltrimethylammonium bromide (CTAB) capping agent, which makes nanorods functionalization much more complicated and it is important to deal with residual CTAB due to its inherent cytotoxicity [40,41]. Several groups have recently developed techniques for covalent functionalization of gold nanorods by carefully controlling their surface chemistry [41–43]. As mentioned earlier, both nanoshells and nanorods can absorb NIR light which can be applicable for imaging or treatment modalities such as photothermal therapy of cancer (Section 6.3).

3. General functionalization strategies

It may be assumed that simply attaching a thiol moiety (Fig. 1a) to the ligand of choice is enough to functionalize any gold surface. However, a single thiol group bound to a gold surface has some limitations, which can actually be useful under some circumstances. As mentioned earlier, thiols bound to a gold surface are subject to exchange with those in solution. This dynamic chemical composition can be utilized as a method of drug release, as was demonstrated by Rotello et al., due to the high levels of intracellular glutathione present in many types of cells [36]. In this case, glutathione partially displaces the thiol monolayer on the nanoparticle surface, thus releasing therapeutic cargo which is covalently bound to the monolayer. This approach demonstrates that more complex

Fig. 1. a) Binding scheme in gold nanoparticle–drug conjugates. b) Types of drugs used. c) Types of nanoparticles used.
systems may be desired if higher stability is required in the system (Fig. 1a). For instance, multiple thiols can be grafted to a single molecule, providing more chemical stability [44,45]. Similarly, multiple binding points can be provided by utilizing a cyclical disulfide which may bind in a multidentate fashion with gold surface to increase the binding strength. Examples of this approach include utilizing a 1,2-dithiane end group, a technique demonstrated to improve DNA-gold nanoparticle conjugate stability [46]. Similarly, thioctic acid is a convenient anchor group due to the presence of the free carboxyl group which can easily be coupled to free amines or alcohols and has been utilized by various groups to create highly stable covalent conjugates of gold nanoparticles [47,48]. Finally, in situ dithiocarbamate formation was shown to be a convenient method of grafting to a gold surface [49,50]. In this technique a molecule with a free amino group is mixed directly with gold nanoparticles and is converted to a dithiocarbamate ligand by addition of carbon disulfide. The dithiocarbamate has a similar bidentate motif with lower propensity for degradation compared to a single thiol. All of these binding motifs represent stronger binding than the traditional gold–thiol bond and are clearly useful in situations where this added strength is desirable. However, there may be times when weaker binding may be required especially if this may function as part of a drug release mechanism. This has been achieved often through the use of non-covalent interactions based on electrostatics, van der Waals forces, hydrophobic effect, hydrogen bonding, etc. which have been used to attach or trap molecules in a gold nanoparticle conjugate, and should be considered as a viable option which is outside the scope of this review. However, if one is looking in the realm of covalent interactions, the bonding between gold and amines can fulfill such a role, although the bond strength is significantly lower, ~6 kcal/mol compared to 47 kcal/mol for thiols, and so it is not a truly covalent bond [52]. Overcoming this weaker binding to effect drug release could be substantially easier than in the case of thiol-bound ligands [53].

4. Gold nanoparticles for gene therapy

Gold nanoparticle-mediated delivery of DNA and RNA has been one of the major research thrusts over the past several years. Because of the centrality of nucleic acids in biological systems, learning to modulate the transcription and translation of DNA and RNA is expected to make gene therapy an important treatment methodology in the near future [54–56]. Thus, being able to deliver a wide variety of oligonucleotides such as plasmids, double stranded DNA (dsDNA), single stranded DNA (ssDNA), and single stranded RNA (ssRNA) could have large therapeutic benefits. However, due to their highly negative charge, these molecules cannot enter the cell without some sort of delivery vehicle, a multitude of which are currently in use or in development [57–61]. Gold nanoparticles of a variety of morphologies have been used for this purpose, including nanospheres, nanorods, and nanoshells. Many of these systems employ non-covalent interactions to attach DNA to the nanoparticles [62–65], but there are several examples where DNA, generally modified with a thiol moiety, is directly attached to the nanoparticle surface.

4.1. Spherical nanoparticle conjugates

One of the first examples of this strategy was demonstrated by Mirkin et al. who were able to efficiently coat citrate-stabilized spherical nanoparticles with a dense layer of ssDNA molecules (Fig. 2) functionalized with either single or multiple thiol groups and use them in gene silencing applications [45]. This conjugate was internalized efficiently by cells, notwithstanding the high negative charge density imparted by the DNA, and the structures were resistant to degradation by proteases and high levels of endogenous glutathione levels which had been shown previously to be able to displace thiols from the surface of gold nanoparticles. Binding of complementary DNA to the grafted ssDNA was strengthened due to the dense packing of DNA around the nanoparticles which led to increased efficacy of the drugs in gene silencing applications. This system is an example where the gold nanoparticle is used not only to facilitate drug delivery, but also function as a critical part of the drug action itself. More recently, further extensions and optimizations of these gold nanoparticle–DNA conjugates were performed. Chemically modified nucleic acids with increased stability and binding properties known as locked nucleic acids (LNA) were used instead of regular DNA (Fig. 2) [66]. This change led to increased melting temperature of the DNA and greatly increased effectiveness as measured by a 50% decrease in the targeted protein levels as compared to a 30% decrease observed with regular DNA at the highest dosage levels.

It is important to understand and predict the structures of nanoparticle conjugates in order to tailor the structure to its particular function, something that is generally very difficult to do. Further studies on oligonucleotide-functionalized gold nanoparticles were conducted that addressed some of these concerns, namely looking at the various factors that affect the structure of the DNA shell around gold nanoparticles as it relates to a variety of factors such as salt concentration and surface curvature of the nanoparticle [67]. The packing density of ssDNA was found to decrease as the size of the particles increases up to about 60 nm, after which it reaches a plateau close to the packing density of the ssDNA on a flat substrate. Moreover, based on the model developed from spherical nanoparticles, it was possible to predict the amount of DNA which could be loaded onto rod-shaped particles with a high degree of accuracy. The covalent attachment of oligonucleotides to gold nanoparticles was found to be extendable to the covalent attachment of RNA to the gold surface as well. Specifically, Mirkin et al. were able to conjugate anti-firefly luciferase siRNA to gold nanoparticles (Fig. 2) and studied their gene knockdown potential compared to standard cationic lipid transfection agents [68]. Because RNA is highly

Fig. 2. Preparation of various types of gold nanoparticle conjugates with DNA, LNA, siRNA, and combined peptide and DNA. Adapted from [45], [66], [68], and [71].
susceptible to nucleases it was necessary to develop a nuclease-free synthesis of gold nanoparticles which was achieved by treating with diethylpyrocarbonate and autoclaving. This citrate gold nanoparticles were able to withstand this treatment, although this procedure might not be transferrable to other nanoparticle systems, especially gold nanorods which have a tendency to reshape into spheres at elevated temperatures. The tight packing of the siRNA due to its covalent coupling to the nanoparticles surface was found to increase its stability in serum as measured by a nearly eightfold increase in half-life as compared to molecular RNA. These particles were also found to be more than twice as effective as traditionally transfected RNA after a period of four days as measured by luciferase fluorescence, again presumably due to the increased resistance to degradation of the siRNA-nanoparticle conjugates. It was similarly possible to regulate mRNA levels by utilizing single stranded DNA—LNA chimera functionalized gold nanoparticles [69]. By binding a small, reporter DNA sequence containing a fluorescent group to the nanoparticle-bound oligonucleotides, the particles gained the ability to detect mRNA when it is bound to the surface. The fluorescence is strongly quenched when the reporter is in proximity to the gold nanoparticle and binding of complementary mRNA leads to desorption of the reporter into solution with concurrent return of the full fluorescent signal. It was the precise nature of the covalent binding to the gold nanoparticle surface along with the well-studied nucleotide binding chemistry that allowed for the development of multifunctional “theranostic” systems. However, it is necessary to mention that this system combines covalent and non-covalent attachment of nucleotides to gold nanoparticles as the first ssDNA strand is covalently attached to the nanoparticle surface while the reporter strand uses well-known DNA hybridization chemistry to build further functionality onto the nanoparticle [69].

4.2. Delivery effects

As mentioned previously, the high negative charge, which differs significantly from most positively charged transfection agents, and the fact that the free DNA does not enter cells by itself, naturally leads to the question of how such particles are able to be internalized by cells. It was found that significant binding of serum proteins upon exposure of the nanoparticles to cell culture medium was occurring [70]. By varying the amount of bound DNA molecules per particle, it was determined that increased levels of bound DNA led to increased recruitment of proteins to the surface of the nanoparticles. Increased
serum protein recruitment levels related directly to increased uptake of the nanoparticles into cells as compared to octaethylene glycol coated nanoparticles. Clearly, for the applications in gene therapy, increased delivery of oligonucleotides into the cell should lead to an increased response. Beyond simply having the capability to deliver the DNA or RNA into cells, increased gene regulation ability would be expected if delivery to the nucleus could be achieved. Mirkin et al. further demonstrated that by co-binding nuclear targeting peptides such as TAT or NLS (Fig. 2), they could increase the perinuclear delivery of the nanoparticle conjugates, although they were still unable to show intranuclear localization of their nanoparticles [71]. This led to an almost 50% increase in gene silencing ability compared to the standard antisense DNA gold nanoparticles given the same degree of nanoparticle uptake.

4.3. Cellular response

When designing drug delivery systems, it is important to understand the cellular response to the delivery vehicle in order to determine unintended effects of the treatment [72]. In particular, modulating the immune response to a delivery vehicle could have important consequences for the effectiveness of the system, especially in the case of nucleotide delivery, in which case foreign DNA is known to elicit a particular type of immune response. Mirkin et al. demonstrated that the innate immune response as measured by quantifying levels of interferon-β (IFN-β) produced in vitro in macrophages is modulated by the structure of the DNA nanoparticle conjugate [73]. Specifically, a greater surface density of bound DNA leads to a lesser immune response in an approximately linear fashion (double DNA density led to half the IFN-β levels). There was an approximately 25-fold difference in IFN-β levels as compared to DNA transfected with standard cationic lipids while maintaining similar gene silencing ability (Fig. 3a). Thus, the tight packing of oligonucleotides around the gold core due to the covalent bonding has clear advantages in modulating the cellular response to the gold nanoparticles. This was shown to be even more generally true in HeLa cells with the examination of genome-wide expression profiling studies of cells treated with several types of gold nanoparticles [74]. The cellular response proved to be minimal in the case of oligonucleotide-functionalized gold nanoparticles (ssDNA, dsRNA, and dsDNA) in terms of either gene expression, cell-cycle progression, or apoptosis induction. However, uptake of unmodified citrate-capped gold nanoparticles was found to have a significantly negative cellular response including the beginnings of apoptosis, which was attributed to the weakly attached citrate capping agent as opposed to the strongly bound, densely functionalized oligonucleotide-nanoparticles. This finding demonstrates the importance of having good control over surface functionality, a fact which naturally lends itself to the use of covalent attachment methods. However, recent studies have shown that cellular response of immortalized lineage-restricted cell lines such as HeLa cells or 293T cells can differ from the response of more biologically relevant systems such as peripheral blood mononuclear cells (PBMC) (Fig. 3b) [75]. Further research needs to be done on the biological effects of nanoparticle-oligonucleotide complexes in *in vitro* as well as *in vivo* experiments before they can be used for real-world gene delivery applications.

4.4. Photothermal release

In some cases, release of the DNA from the delivery vehicle may be desired. A variety of examples exist where non-covalent interactions are used to bind DNA to the nanoparticle allowing for later release of the DNA, such as functionalizing gold nanoparticles to carry a positive charge to which DNA can be electrostatically adsorbed [62–65,76]. Another strategy is to covalently attach a base layer of DNA to the nanoparticle surface and use the self-assembling properties of DNA to further build up the system [77,78]. One popular way of releasing the DNA is by using light-responsive nanoparticles such as nanorods, nanoshells, or nanocages. For these nanoparticles, tuning of their sizes can lead to intense absorbance in the near-IR transparency window. Laser irradiation at the appropriate frequency for these structures can cause photothermal effects as well as the generation of “hot” electrons that can trigger a release of DNA covalently attached to a nanoparticle [79]. Chen et al. employed this strategy by inducing the release of a thiostated Enhanced Green Fluorescent Protein (EGFP) plasmid DNA attached to gold nanorods by femtosecond laser irradiation at the longitudinal plasmon wavelength of the rods (Fig. 4a) [80]. This approach led to melting of the rods with concurrent release of DNA, which was shown to be responsive for the release of up to 80% of the DNA molecules conjugated covalently to the nanorods surface. EGFP fluorescence in cells indicated successful plasmid transfection (Fig. 4b). Wijaya et al. developed a different method for the attachment of thiostated DNA [81] to gold nanorods and were able to demonstrate the nanorods’ use for gene delivery purposes with a similar strategy as Chen et al. [80,82]. They extended this work by synthesizing rods of two distinctly different aspect ratios, and thus different longitudinal

![Fig. 4.](image-url)
peaks, which were functionalized with different oligonucleotide strands. When mixed together, irradiation at the specific longitudinal plasmon of one type of rods led to their selective melting and thus the release of the corresponding DNA sequence (Fig. 4c). In both cases, a femtosecond pulsed laser is required to melt the gold nanorods and release the DNA, presumably by breaking or oxidizing the gold–thiol bond, which represents a significant limitation of this technique. Cytoxicity due to either residual surfactant on the NR surface [41] or due to photothermal heating are also potential issues with this technique [79,80], Still, Chen et al. showed that cytotoxicity could be mitigated by controlling the laser fluence. Clearly the high strength of the gold–thiol covalent bond, while lending itself to applications where that stability is important, may create some limitations when it comes to the release of DNA from nanoparticles. Other systems for DNA release utilize the same covalent technique, but depend on breaking weaker, non-covalent interactions such as those between complementary strands of DNA to effect the release of the DNA [83] or even drugs loaded into DNA [84]. In these systems, gold nanoshells can be excited at lower laser powers to produce the necessary photothermal effect to release DNA hybridized to covalently attached strands. However, Braun et al. demonstrated that while low laser power release of oligonucleotides may be possible, somewhat higher power might be necessary to effect endosomal escape such that the therapeutic effects would be observed [85]. Thus, it is important to understand the desired properties of the gold nanoparticle–drug conjugates before choosing a design strategy.

5. Gold nanoparticles for bactericidal applications

Another important class of gold nanoparticle–drug conjugates has been designed for antibacterial purposes. Many different antibiotic compounds have been developed and are used extensively, therefore it is necessary to understand how gold nanoparticles can improve the efficacy of these molecules (Table 1). It is also important to examine how the covalent attachment chemistry of the antibiotics to gold nanoparticles can affect the properties of the resulting conjugates.

5.1. Amine-bound antibiotics

Several reports exist on gold nanoparticles non-covalently bound with antibiotics and exhibiting enhanced bactericidal activity, including those that involve simple mixing of citrate-capped gold nanoparticles with an antibiotic drug [86–89] and/or reduction of gold chloride in the presence of antibiotics [90]. These specific antibiotics contain free amino groups, which have a strong affinity to gold surface and generally lead to aggregation as evidenced by a color change from red to blue/ purple. In this case the gold–amine binding is not truly covalent as in the case of gold–thiol binding. Although these studies demonstrated an increased activity against some types of bacteria, another report was also published that suggested that such nanoparticle aggregates do not show a measurable enhancement of bactericidal activity [91]. It was suggested that in order for the nanoparticle conjugates to be useful, they should be functionalized in a way that prevents aggregation and leads to a high surface coverage on the surface of nanoparticles. While the gold–amine interaction is weaker than a gold–thiol bond, it is still problematic to mix gold nanoparticles with molecules containing multiple amino groups, i.e. aminoglycoside antibiotics, as it can result in physical cross-linking and uncontrolled agglomeration of particles. It is expected that by moving to a system with only a single amino group, the degree of aggregation could be reduced, although this may not be the only factor affecting the stability of the system. Indeed, Rai et al. used cefaclor, a second-generation cephalosporin, containing a single amino group as the capping agent as well as reducing agent in the synthesis of the gold nanoparticle conjugates [92]. These hybrid structures showed no signs of aggregation, were stable enough to be purified via dialysis, and thus could be properly characterized, indicating the usefulness of an amine–gold binding motif. The conjugates displayed an increased bactericidal activity compared to free cefaclor in both gram-positive and gram-negative bacteria, and they showed lower degradation over time than the free antibiotic drug. Although previous reports indicate that the gold nanoparticles are important only as scaffolds to densely bind antibiotics, Rai et al. suggest that the particle structure acts to amplify the cell membrane damage initiated by the antibiotic, increases the cell penetration into gram-negative bacteria, and disrupts bacterial DNA [92].

5.2. Thiol-bound antibiotics

Using the standard gold–thiol binding approach is also an excellent strategy for binding antibiotics to gold nanoparticles. Early work by Gu et al. reported on coating small 5 nm gold nanoparticles with vancomycin through a phase transfer mechanism [93]. They mixed an aqueous solution of bis(vancomycin) cystamide, with a tolueone solution of quaternary ammonium bromide-coated gold nanoparticles, leading to their phase transfer into aqueous solution (Fig. 5a). These nanoparticles were tested against a variety of vancomycin-resistant enterococci (VRE) as well as some gram-negative bacteria like E. coli and showed excellent activity against all bacterial strains even when vancomycin itself was not effective. The activity of these particles is explained by the effect of multivalency in which multiple vancomycin moieties present enhanced binding to the terminal D-Ala-D-Ala moieties (Fig. 5b) [93–95]. It is also important to note that the exact orientation of the vancomycin is highly important to maximize the binding to bacteria [96], which makes covalent binding an ideal method to attach vancomycin to the nanoparticle systems. Similarly, bis(vancomycin) cystamide was used to functionalize polygonal particles with absorption in the near IR [97]. Such particles were shown to bind to a variety of bacteria types including VRE, MRSA, and PDRAB and once bound, they can be used for photothermal destruction of these bacterial types with high efficiency. This treatment was also found to be nontoxic to non-bacterial cells as determined by MTT assays with human epidermoid carcinoma epithelial cells. As an alternative to antibiotics, photosensitizers can also be covalently conjugated to gold nanoparticles and used to kill bacteria. The light-activated antimicrobial agent (LAAA) toluidine blue O (TBO) was covalently coupled to the carboxyl group on tiopronin-functionalized gold nanorods and found to be highly stable [98]. The exact amount of TBO and tiopronin per each gold nanoparticle was determined by TGA, NMR, and other techniques, facilitated by the discrete structures formed via covalent functionalization. These conjugates demonstrated four-fold decrease in minimum bactericidal concentration under white light or laser illumination as compared to free TBO, which was attributed to an enhanced light absorbance of nanoparticles functionalized by TBO. It is conceivable that a direct covalent link is not required for the enhancement of photodynamic therapy as it is possible that gold nanoparticles may increase the levels of reactive oxygen species [99]. It must be noted that reports have also been made of gold nanorods that have been coated electrostatically with polyelectrolytes and further covalently functionalized for use in photothermal treatment [100] or combined

<table>
<thead>
<tr>
<th>Drug/treatment</th>
<th>Attachment</th>
<th>Tested against</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>Thiol</td>
<td>VRE, gram-negative</td>
<td>[93]</td>
</tr>
<tr>
<td>Vancomycin/</td>
<td>Thiol</td>
<td>VRE, MRSA, PDRAB</td>
<td></td>
</tr>
<tr>
<td>photothermal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin,</td>
<td>Thiol</td>
<td>Gram-positive, gram-negative</td>
<td>[97]</td>
</tr>
<tr>
<td>streptomycin,</td>
<td>Thiol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kanamycin</td>
<td>Thiol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefaclor</td>
<td>Thiol</td>
<td>Gram-positive</td>
<td>[92]</td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>Thiol</td>
<td>Gram-positive</td>
<td>[98]</td>
</tr>
<tr>
<td>O/photosensitization</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
photodynamic and photothermal treatment of bacteria [101]. For gold nanorods, direct covalent functionalization can be more challenging, as mentioned before, so a wider variety of functionalization schemes has been developed.

6. Gold nanoparticles for anticancer applications

Gold nanoparticles carrying anticancer payloads represent another important class of covalent conjugates. A variety of therapeutic approaches have been developed which take advantage of the well-researched covalent chemistry of gold nanoparticles (Fig. 6). Cancer is a major target of new therapeutics because of the drawbacks associated with current treatment strategies which include large systemic side-effects due to the non-specificity of many cancer drugs such as cisplatin [102], paclitaxel [103], tamoxifen [104], and doxorubicin [105]. Gold nanoparticles can address some of these issues in several ways. First, attachment to gold nanoparticles can lead to increased toxicity compared to free drug. This is useful, but must ultimately be combined with increased specificity of the drug conjugate to cancer cells. This can be mediated by passive targeting through enhanced permeability and retention effect (EPR) based on the size of gold nanoparticle conjugates [106] or by active targeting using antibodies or other targeting moieties. In addition, the gold nanostructures themselves can have the ability to kill cells by virtue of intrinsic therapeutic effects such as photothermal effect.

6.1. Platinum-based conjugates

The first type of drug-nanoparticle conjugates that will be discussed is based on platinum compounds. Several examples exist in which a platinum(IV)-nanoparticle complex has been used as a drug delivery vehicle and a prodrug for intracellular release of platinum(II) ions [107–109]. This strategy is beneficial because of the reduced reactivity of Pt(IV) compared to Pt(II) with biological agents and thus higher bioavailability and lower systemic toxicity [110]. Thus far, there exist two reports of platinum complexes covalently attached to gold nanoparticles, which utilize a Pt(IV) prodrug strategy. In the first report, Lippard et al. synthesized oligonucleotide-functionalized gold nanoparticles with a terminal dodecyl amine moiety and attached a carboxyl-containing Pt(IV) complex by using standard carbodiimide coupling chemistry with EDC and NHS (Fig. 7a) [110]. The platinum complex was designed to generate Pt(II) species by means of intracellular reduction of Pt(IV) by glutathione molecules. Subsequent loss of axial ligands would lead to the release of free cisplatin. It was confirmed that the conjugate was taken into cells as was shown for regular DNA-functionalized nanoparticles. Significantly, the anticancer activity of the complexes to several cancer cell types was shown to be equal or greater than the activity of cisplatin, although most of IC50 values were of similar magnitude (Fig. 7b). It is important to note that for nanoparticle drug delivery carriers in general, even having the same activity as the unconjugated drug would be an excellent result if the drug can be delivered more efficiently to cancer site with fewer systemic side effects. A similar strategy was adopted by Min et al. for attaching Pt(IV) prodrugs to gold nanorods [111]. Gold nanorods were first covalently functionalized with a diamino(polyethylene glycol) by using in situ dithiocarbamate formation on one terminus [112] and using the second amino group for coupling with a carboxyl-containing platinum(IV) compound. Cytotoxicity measurements based on the MTT assay for three different cancer lines showed a much higher toxicity compared to free cisplatin, with IC50 values ranging from 9 to 65 times lower. This was correlated to increased intracellular levels of platinum ions in nanorod-treated cells, indicating that the nanoparticle delivery vehicle can be
quite important if it can enhance the cellular uptake of the drug via endocytic pathways (Fig. 7c).

6.2. Conjugates with small organic molecules

Another type of common anticancer therapeutics is based on small organic molecules such as paclitaxel, docetaxel, tamoxifen, etc. Such small molecules either already have reactive functional groups or can be modified in a way which can be used to couple them to gold nanoparticles. As mentioned earlier, several basic strategies can be applied to build the conjugate systems. First, one can synthesize a functionalized nanoparticle to which the drug will be attached through subsequent covalent chemistry. Gibson et al. used such an approach to synthesize well-characterized paclitaxel-functionalized gold nanoparticles and demonstrated the utility of covalent chemistry to accurately characterize the resulting conjugate (Fig. 8) [34]. In this case, the mercaptophenol-functionalized gold nanoparticles were synthesized using the one-phase synthesis first developed by Brust et al. [113]. A carbodiimide-based esterification was used to attach paclitaxel with oligoethylene glycol spacer. It was important to use this flexible spacer, which was attached at the C-7 position of paclitaxel, to allow for proper NMR characterization. Using $^1$H NMR, along with

---

**Fig. 6.** Overview of anticancer drugs covalently conjugated to gold nanoparticles. Dashed lines represent an omitted portion of a linker. Helical segment represents a DNA oligonucleotide. Wavy line indicates an oligo- or polyethylene glycol containing segment.

**Fig. 7.** a) Coupling of Pt(IV) prodrug to oligonucleotide-functionalized gold nanoparticles. b) Cytotoxicity of these nanoparticles to various cell lines. Adapted from [110]. c) Schematic of cell uptake and cisplatin release from gold nanorods conjugated with Pt(IV) prodrug [111].
other characterization methods, it was possible to quantify the number of paclitaxel molecules per nanoparticle, something that could be difficult with non-covalent systems. Hwu et al. synthesized several systems based on paclitaxel functionalized at the C-2 position using a phosphodiester linkage [114]. This compound was attached to a PEG linker containing a thiol group for attachment to citrate-capped gold nanoparticles or maleimide-coated magnetite nanoparticles. The phosphodiester bond was designed to be preferentially cleaved by phosphodiesterases in cancer cells and did not show paclitaxel release in serum alone. Interestingly, while both the gold and magnetite nanoparticles demonstrated high water solubility, only magnetite nanoparticles showed any appreciable release of paclitaxel in the presence of phosphodiesterase, which occurred only after 4 days of treatment. However, this is not surprising given that the gold nanoparticles aggregated at the high salt concentration, unlike their magnetite counterparts. The authors claimed an IC50 value of 0.6 pM for the magnetite nanoparticles, which would be a spectacular break-through considering that the cytotoxicity of regular paclitaxel ranges from 2 to 7 nM [115], although the conditions for the MTT assay were through considering that the cytotoxicity of regular paclitaxel ranges from 2 to 7 nM [115], compared to the free peptide. However, it is important to note that one of the benefits of covalent structures is that they are easily purified and then can be analyzed directly. In this case, it is not clear if the free peptide was still present in the system, which could make it difficult to accurately determine the actual bioactivity of the conjugate.

Recently, Mirkin et al. have synthesized oligonucleotide–gold nanoparticle conjugates with paclitaxel in order to increase its solubility in aqueous media [116]. Paclitaxel was esterified through the C-2 position and coupled to thioldiated, fluorescent DNA, which was immobilized onto citrate-capped gold nanoparticles as described before. Paclitaxel, which is highly insoluble in water, was solubilized by attaching to the gold nanoparticles, including in high-salt buffers. The gold nanoparticle–DNA–paclitaxel conjugates were shown to be internalized by the cells and showed increased cytotoxicity compared to free paclitaxel in MTT assays, and even showed toxicity to paclitaxel-resistant cell lines, suggesting that nanoparticle-mediated cell uptake could fight drug-efflux in drug-resistant cancers. However, comparisons were not made to the standard delivery method (solution in Cremophor EL), which typically displays IC50 values of 2–7 nM [115], compared to the values of 4–100 nM for the nanoparticle conjugates in pure water [116]. Still, the possibility for better targeting, increased effectiveness against the drug-resistant cells, and high solubility without Cremophor EL could make this conjugate a promising paclitaxel-delivery platform.

El-Sayed and coworkers reported the first example of covalent coupling of tamoxifen to gold nanoparticles by attaching it to a thioldiated PEG linker [117]. This procedure was somewhat more involved than the attachment of paclitaxel because of the lack of a simple chemical functionality, such as the free hydroxyl group in paclitaxel. Instead, the tertiary amino group was converted to a secondary amine, and then alkylated with a PEG compound that was subsequently thiolated through several steps. This tamoxifen–PEG–thiol was added to citrate-capped particles, which showed 2.7 times higher potency than that of free tamoxifen. This was shown to be a consequence of the increased cellular uptake rate of the nanoparticle–drug complex compared to free tamoxifen and was not due to any multivalency effects, such as those discussed for antibiotic–gold nanoparticle complexes [93]. Particle uptake was mediated by binding to estrogen receptors, which are overexpressed in 75–80% of breast cancers, suggesting that this strategy could be used to selectively target breast cancers compared to regular cells [117]. Another type of anticancer drug that has been successfully conjugated to gold nanoparticles was a polypeptide drug, Kahalilide F [118]. This thirteen residue peptide drug was synthesized with a cysteine modification that could covalently bind to citrate-capped gold nanoparticles, although it was demonstrated that the amount of peptide loaded onto the nanoparticles was much higher than expected from a purely covalent binding. It was suggested that a multilayer coating of the peptide molecules onto the nanoparticles was responsible for the observed increased loading of the drug. The increased cellular uptake of the nanoparticle system led to a higher anticancer activity when compared to the free peptide. However, it is important to note that one of the benefits of covalent structures is that they are easily purified and then can be analyzed directly. In this case, it is not clear if the free peptide was still present in the system, which could make it difficult to accurately determine the actual bioactivity of the conjugate.

Another interesting example of anticancer drug that has been conjugated to gold nanoparticles is 5-fluorouracil, a drug which inhibits DNA and RNA synthesis [119]. Agasti et al. synthesized fluorouracil–functionalized gold nanoparticles of 2 nm size [35]. The drug was incorporated through a terminal UV-photocleavable ortho-nitrobenzyl (ONB) group. These particles were synthesized by the place exchange reaction of pentanethiol-capped gold nanoparticles with the fluorouracil-containing thiol as well as a zwitterionic thiol for increased solubility. Irradiation of nanoparticles with 365 nm UV light resulted in controllable release of the drug, which exerted its cytotoxic effect only when it was released from the particle surface. The lack of toxicity of the conjugates before the photo-induced release of the ligand could be very beneficial for targeted treatment of cancer in vivo in cooperation with the passive targeting mechanism (EPR effect). Interestingly, unlike most of the other systems, the zwitterionic thiols reduce the cellular uptake [20], indicating that high uptake is not always necessary in the design of gold nanoparticle–drug conjugates. This was also shown to be the case for delivery of a phthalocyanine (Ptc) drug for photodynamic cancer therapy, wherein release of the drug outside of the cell led to more efficient photodynamic therapy compared to internalized covalent nanoparticle–Ptc conjugates [120]. However, this may have been due to poor uptake of the Ptc particles as a result of their PEG coating [41] because the release of drug can be mediated by cell uptake as demonstrated by Rotello and coworkers [36]. In this report, gold particles were functionalized by a place-exchange reaction of octanethiol-capped gold nanoparticles with a cationic thiol and a dye-containing thiol (Bodipy). The presence of the cationic thiols leads to higher cellular uptake of the nanoparticle conjugate as opposed to the zwitterionic system mentioned earlier. It was proven that intracellular glutathione caused the release of
nanoparticle-bound thiols as confirmed by large fluorescence due to free Bodipy dye, which is quenched when attached to the gold nanoparticles surface.

Recently, gold nanoparticles have been covalently grafted with doxorubicin for treatment of drug-resistant breast cancer [48]. Doxorubicin was attached through a thiocystic acid-PEG linker to the surface of 30 nm citrate-capped gold nanorods through a hydrazone group (Fig. 9a). The hydrazone is stable at normal physiological pH, but is hydrolyzed at the reduced pH levels found in endosomes [121]. These nanoparticles inhibited the growth of drug resistant cancer cells by allowing a large uptake of the particles, followed by acid-sensitive release from endosomes which led to a rapid increase in doxorubicin concentration inside the cancer cell (Fig. 9b). This overcomes drug efflux from the cells which is responsible for drug-resistance [122], and increases the cytotoxicity compared to free drug (Fig. 9).

Doxorubicin release was visualized through its fluorescence, though cytotoxicity was not determined. Given that differences between the studies include nanoparticle size, synthesis, and functionalization method it is difficult to draw conclusions and more detailed studies in this area would be useful.

![Fig. 9.](image-url)
6.3. Gold nanoparticles as intrinsically therapeutic agents

In the synthesis of covalently functionalized conjugates designed to destroy cells, gold nanoparticles can act as more than just drug delivery vehicles. The unique properties of these systems mean that attachment of a drug may not be necessary. One area of research that has received a large amount of attention is the use of near-IR active gold nanostructures including nanorods, nanoshells, and nanocages. Several reviews have already been published on this topic and thus will not be discussed in great detail here [2,18,39,40,125]. Briefly, a laser irradiation of anisotropic gold nanocrystals results in a dissipation of energy in the form of heat, which may result in cell death. Importantly, for application of such nanostructures in vivo, it is generally necessary to properly functionalize the nanoparticle surface to mitigate the cytotoxic effects and to target the particles. Vigderman et al. recently reported a cationic thiol exchange for the cytotoxic CTAB on the surface of as-synthesized gold nanorods [41,126]. This surfactant exchange allowed for the loading of more than 2 million gold nanorods per cell, which were visualized by optical (Fig. 10a) and transmission electron microscopy (Fig. 10b). This number is at least 10 times higher than that reported for CTAB-coated rods and therefore such high loading may lead to more efficient photothermal therapy of cancer cells.

El-Sayed et al. demonstrated that covalent attachment of a nuclear targeting peptide to gold nanorods leads to their rapid uptake and subsequent localization in both the cytoplasm and the nucleus [127]. It was later shown that spherical gold nanoparticles conjugated with nuclear targeting peptides cause damage to DNA and lead to cytokinesis arrest resulting in apoptosis of cancer cells [128]. The mechanism of this process was not fully understood, however, and the process was not evident at lower nanoparticle concentrations [129]. More recently, it was demonstrated that masking the cationic charge of nanoparticles by supramolecular assembly of a cucurbit [7] url host over the diaminohexane guest covalently attached to 2 nm gold nanoparticles leads to a loss of their cytotoxic effect [130]. After the uptake of these nanoparticles, cells can be treated with 1-adamantylamine, which more strongly binds the cucurbit [7] url hosts, thereby releasing free cationic gold nanoparticles into the cytosol where they exert their cytotoxic effect.

Evidently, for such systems to be effective in cancer treatment, gold nanostructures must be targeted to cancer cells and avoid accumulation in healthy tissue. As mentioned earlier, the enhanced permeability and retention properties of some tumors lead to the preferential accumulation of nanoparticles in cancer tissue due to the leaky vasculature present in these areas [106,131]. This is a passive targeting mechanism which could be applicable to most nanoparticle drug delivery systems. Active targeting involves covalent attachment of molecules which can bind specifically to cancer cells. Small-molecule based targeting systems can be used, such as tamoxifen-coated particles discussed earlier, which bind specifically to breast cancer cells. In addition, it is possible to use folic acid [132] and polyunsaturated fatty acids [133]. Biopolymers can also be utilized for that purpose as was exemplified by hyaluronic acid [133,134], various peptides [133], and oligonucleotides [133,135]. In addition, gold nanorods covalently functionalized by bombesin peptides, a gastrin-releasing peptide (GRP), were shown to bind with high affinity to breast cancer cells and be taken up by the cells via a GRP receptor-mediated endocytosis process (Fig. 11) [49,136]. Finally, gold nanoparticles can be conjugated to antibodies, which would be expected to increase the specificity of their delivery to cancer in vitro and in vivo experiments. Antibodies are often targeted to overexpressed binding domains on cancer cells as compared to regular cells.

Fig. 10. a) Bright field optical microscopy image of cancer cell treated with gold rods functionalized by cationic thiol. b) TEM image of a section of a cell treated with cationic gold nanorods. Adapted from [41].

Fig. 11. Bombesin-conjugated gold nanorod uptake into GRP-receptor positive cells through receptor-mediated endocytosis and the lack of uptake into GRP-receptor negative cells.
7. Towards clinical application

Much of the work which has been described up to this point is limited to the in vivo testing of therapeutic gold nanoparticle systems. In order to transition these technologies towards clinical applications, it will be necessary to understand their activity in vivo in both animal models and eventually in humans. It is possible that some systems which may be effective in vitro may not only lose their activity in vivo, but may also lead to unexpected side effects. While a detailed examination of these effects is outside the scope of this review, it is important to mention the general factors which must be considered. One of the main issues is the biodistribution of the gold nanoparticles with time, which has been reviewed in detail [143,144]. The EPR effect is often cited as a passive targeting mechanism leading to increased accumulation of nanoparticles inside of solid tumors. However, it is important to address the extent of this effect as well as the possible buildup of particles in other organs. Accumulation is commonly seen in the liver, spleen, and kidneys, which are functioning to clear the gold nanoparticles from the body, but can also occur in other areas including the brain and lungs, which may or may not be desirable [143]. The method of nanoparticle administration could also influence this biodistribution [143]. Furthermore, the nanoparticle size and the surface functionality can have a large effect. Active targeting through antibodies, peptides, or other methods is likely to be crucial, as mentioned earlier, but might not preclude non-specific accumulation in the liver, spleen, etc. The extent of nanoparticle targeting which is acceptable will depend on the application. For instance, laser-induced hyperthermic treatment of tumors will necessitate delivery of nanoparticles to the tumor site, but accumulation of nanoparticles in other areas may not be overly problematic given highly localized laser-treatment area. On the other hand, imperfect delivery of particles functionalized with toxic anticancer drugs could have more side effects. Finally, the study of nanoparticle pharmacokinetics including the clearance of nanoparticles from the body after treatment is still in its infancy [144]. Understanding this clearance and any long-term nanoparticle toxicity, will be necessary for their clinical application. Due to the large effects of size, shape, and surface functionalization on the in vivo activity of therapeutic gold nanoparticles, it will be necessary to carefully evaluate each new system before it can be applied clinically.

8. Conclusions

Covalently modified gold nanoparticles have achieved a great deal of interest as drug delivery vehicles. Their predictable and reliable surface modification chemistry, usually through gold–thiol binding, makes the desired functionalization of nanoparticles quite possible and accurate. A variety of therapeutic molecules have been attached in this manner including various oligonucleotides for gene therapy, bactericidal compounds, and anticancer drugs. Anisotropic gold nanoparticles also exhibit optical and chemical properties that can make them therapeutic in their own right, and yet still require proper surface functionalization to make them useful. The ability to properly purify and accurately analyze the structure of such hybrid inorganic–organic particles should make them an attractive system for continued drug development. Transitioning to testing these gold nanoparticles–drug conjugates from in vitro to in vivo models will be an important step for the future research, and has already been undertaken in some cases. It is expected that enhancing in vivo targeting of nanoparticle–drug conjugates and understanding their biodistribution will be highly important. Just as importantly, understanding and learning how to control the intracellular fate of nanoparticles will be necessary for further development in this field.

Acknowledgement

Financial support by the NSF (DMR-1105878) is gratefully acknowledged by the authors.

References


A.K. Gupta, R.R. Naregalkar, V.D. Vaidya, M. Gupta, Recent advances on surface engineering of magnetic iron oxide nanoparticles and their biomedical applica-


S. Thomas, A. Krüger, Conjugation to gold nanoparticles enhances poly-


H.H. Huang, S. Barua, D.B. Kay, K. Rege, Simultaneous enhancement of photothermal stability and gene transfection efficacy of gold nanorods using poly-


D.A. Gijlhoorn, D.S. Seferos, A.E. Prigodich, P.C. Patel, C.A. Mirkin, Gene regula-


D.A. Gijlhoorn, D.S. Seferos, P.C. Patel, J.E. Millstone, N.L. Rosi, C.A. Mirkin, Oligo-


J.H. Kim, J.H. Yeom, J.J. Ko, M.S. Han, K. Lee, S.Y. Na, J.A. Bae, Effective delivery of anti-miRNA DNA oligonucleotides by functionalized gold nanoparticles, J. Bio-


R. Huschka, J. Zuloaga, M.W. Knight, L.V. Brown, P. Nordlander, N.J. Halas, Light-


E.Y. Kim, R. Schulz, P. Swantek, K. Kunstman, M.H. Malim, S.M. Wolinsky, Gold


S. Jung, J.S. Choi, S.Y. Choi, J. Park, J.H. Seo, J. Park, K. Choy, S.W. Joo, S.L. Lee, Low-toxicity chitosan gold nanoparticles for small hairpin RNA delivery in human lung ade-


J.H. Kim, J.H. Yeom, J.J. Ko, M.S. Han, K. Lee, S.Y. Na, J.A. Bae, Effective delivery of anti-miRNA DNA oligonucleotides by functionalized gold nanoparticles, J. Bio-


R. Huschka, J. Zuloaga, M.W. Knight, L.V. Brown, P. Nordlander, N.J. Halas, Light-


E.Y. Kim, R. Schulz, P. Swantek, K. Kunstman, M.H. Malim, S.M. Wolinsky, Gold


S. Jung, J.S. Choi, S.Y. Choi, J. Park, J.H. Seo, J. Park, K. Choy, S.W. Joo, S.L. Lee, Low-toxicity chitosan gold nanoparticles for small hairpin RNA delivery in human lung ade-


J.H. Kim, J.H. Yeom, J.J. Ko, M.S. Han, K. Lee, S.Y. Na, J.A. Bae, Effective delivery of anti-miRNA DNA oligonucleotides by functionalized gold nanoparticles, J. Bio-


R. Huschka, J. Zuloaga, M.W. Knight, L.V. Brown, P. Nordlander, N.J. Halas, Light-


E.Y. Kim, R. Schulz, P. Swantek, K. Kunstman, M.H. Malim, S.M. Wolinsky, Gold


S. Jung, J.S. Choi, S.Y. Choi, J. Park, J.H. Seo, J. Park, K. Choy, S.W. Joo, S.L. Lee, Low-toxicity chitosan gold nanoparticles for small hairpin RNA delivery in human lung ade-


J.H. Kim, J.H. Yeom, J.J. Ko, M.S. Han, K. Lee, S.Y. Na, J.A. Bae, Effective delivery of anti-miRNA DNA oligonucleotides by functionalized gold nanoparticles, J. Bio-


R. Huschka, J. Zuloaga, M.W. Knight, L.V. Brown, P. Nordlander, N.J. Halas, Light-


E.Y. Kim, R. Schulz, P. Swantek, K. Kunstman, M.H. Malim, S.M. Wolinsky, Gold


S. Jung, J.S. Choi, S.Y. Choi, J. Park, J.H. Seo, J. Park, K. Choy, S.W. Joo, S.L. Lee, Low-toxicity chitosan gold nanoparticles for small hairpin RNA delivery in human lung ade-


