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# Covalently Linked Au Nanoparticles to a Viral Vector: Potential for Combined Photothermal and Gene Cancer Therapy

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## ABSTRACT

**Hyperthermia can be produced by near-infrared laser irradiation of gold nanoparticles present in tumors and thus induce tumor cell killing via a bystander effect. To be clinically relevant, however, several problems still need to be resolved. In particular, selective delivery and physical targeting of gold nanoparticles to tumor cells are necessary to improve therapeutic selectivity. Considerable progress has been made with respect to retargeting adenoviral vectors for cancer gene therapy. We therefore hypothesized that covalent coupling of gold nanoparticles to retargeted adenoviral vectors would allow selective delivery of the nanoparticles to tumor cells, thus feasibilizing hyperthermia and gene therapy as a combinatorial therapeutic approach. For this, sulfo-*N*-hydroxysuccinimide labeled gold nanoparticles were reacted to adenoviral vectors encoding a luciferase reporter gene driven by the cytomegalovirus promoter (AdCMVLuc). We herein demonstrate that covalent coupling could be achieved, while retaining virus infectivity and ability to retarget tumor-associated antigens. These results indicate the possibility of using adenoviral vectors as carriers for gold nanoparticles.**

Cancer targeted therapies rely on exploiting susceptibility parameters of tumor versus normal cells. The increased susceptibility of tumors to heat makes hyperthermia a feasible treatment option.[1] A variety of heat sources have been explored, including laser light, focused ultrasound, as well as microwaves. More recently, the use of near-infrared-absorbing gold nanoparticles has successfully been applied to reduce tumor burden and increase survival in animal experiments.[2] Selectivity of heat induction is based on enhanced permeability of the tumor vasculature and subsequent retention of the intravenously administered nanoparticles, which can be heated using deep penetrating near-infrared (NIR) laser light.[3] However, enhanced permeability and retention pathophysiology do not occur in all tumors, mandating alternative methods of targeted nanoparticle tumor delivery before successful clinical application can be achieved.

We hypothesized that the recent improvements in targeted adenoviral vectorology might provide the platform for tumor selective delivery of these nanoparticles. We therefore sought

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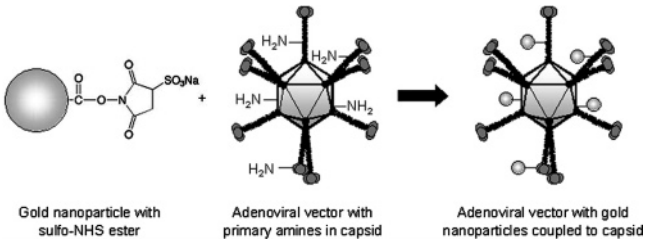


**Figure 1.** Gold nanoparticles can covalently be attached to primary amines present in capsid proteins. The sulfo-NHS ester attached on the 1.3-nm gold particle will react with primary amines of lysine residues present in the adenoviral capsid, resulting in an amide bond between the gold particle and the adenovirus. There are over 10 000 lysines in the proteins that make up the capsid, although not all of these will be accessible for chemical reaction.

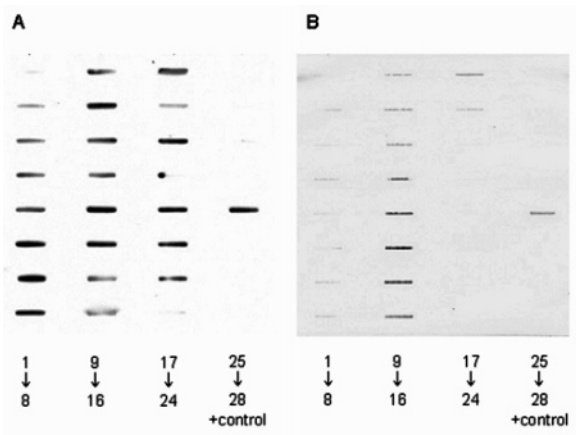


**Figure 2.** Slotblot analysis demonstrating the presence of adenovirus and gold in fractions collected after virus CsCl purification. Gold nanoparticles were first reacted with adenoviral vectors in a 1000:1 ratio. The reaction mixture was then purified using centrifugation over a CsCl gradient, a standard method of viral vector purification. After the bottom of the centrifugation tube was punctured, 28 fractions were collected and analyzed for the presence of adenovirus using staining for the hexon capsid protein (A) and the presence of gold nanoparticles using silver enhancement staining (B). The original reaction mixture prior to purification was used as a positive control (+control).

to establish the feasibility of covalently coupling gold nanoparticles to adenoviral vectors, as a means to achieve the desired targeted localization. We anticipated that this linkage could be achieved without compromising key adenoviral infectivity properties that constitute the functional basis of its tumor-targeting capabilities. This combination of a targeted viral vector with an amplifying nanoparticle payload thus seeks to exploit favorable aspects of each component to realize an optimized anticancer effect. This type of combinatorial system represents a novel paradigm for the design of tumor-targeted nanoparticles.

**Association of Gold Particles with Adenoviral Vectors.** The sulfo-NHS-ester on the gold nanoparticles employed has reactivity toward primary amines that are abundantly present on the adenoviral capsid lysine residues (Figure 1).

For the initial characterization of the feasibility of coupling gold nanoparticles to adenoviral vectors, a ratio of 1000:1 gold:adenovirus (particle:particle) was employed in the synthesis procedure. After the reaction, the mixture was purified using CsCl gradient centrifugation—a standard method for adenoviral vector purification. Fractions collected from the bottom of the centrifugation tube were analyzed for the presence of virus using an anti-hexon antibody (Figure 2A) and for the presence of gold using silver staining (Figure 2B). Although staining for the presence of virus appears to be more sensitive than detection of gold, a comparison of the staining patterns of both slot blots demonstrated co-localization of virus with gold, indicating co-migration of both components through the CsCl gradient. These results suggested a covalent association between the gold nanoparticles and the adenoviral vectors.

During centrifugation of gold-labeled viral particles in a CsCl gradient, a shift in the height of the viral band in the centrifuge tube was observed, which was dependent on the amount of gold nanoparticles employed in the synthesis procedure, indicating an increased density of the viral particles upon gold labeling (Table 1, Figure 3). To exclude aspecific, noncovalent interaction of gold nanoparticles with the viral vectors, a synthesis procedure employing gold nanoparticles labeled with a nickel-nitrilotriacetic acid (Ni−NTA) instead of a sulfo-NHS reactive group was performed. In this reaction, the shift in vector band localization could not be observed (Table 1, Figure 3), confirming the covalent nature of interaction of the sulfo-NHS particles with the virions. It should be noted, however, that the surface charge of sulfo-NHS labeled particles is neutral, whereas the surface charge of Ni−NTA labeled particles is negative. Since adenoviruses have a net negative charge on their capsid surface as well,[4] nonspecific electrostatic absorption of Ni−NTA labeled particles to the virions could be less than electrostatic absorption of sulfo-NHS particles. Nonetheless, the absence of Ni−NTA labeled particles in CsCl purified virions indicates that association of nanoparticles with virions in the gradient is not based on the weight of the particles.

**Electron Microscopy of Gold-Labeled Adenoviral Vectors.** Electron microscopy was used to visualize gold nanoparticles reacted to the surface of adenoviral vectors that were purified by CsCl centrifugation as above. Vectors were deposited onto carbon-coated copper grids; no staining was used in order to avoid occlusion of the 1.4 nm nanoparticles on the surface of the virions. Gold nanoparticles could not

**Table 1.** Positions of Viral Bands in Centrifugation Tubes after CsCl Gradient Centrifugation

| virus−gold combination | distance from bottom to lower band | distance from bottom to upper band (cm) | total distance gradient (cm) |
|---|---|---|---|
| virus alone, no gold nanoparticles | no lower band | 4.3 | 6.6 |
| virus + 10000 Ni−NTA gold nanoparticles | no lower band | 4.2 | 6.6 |
| virus + 10000 Sulfo-NHS gold nanoparticles | 2.8 cm | 4.2 | 6.6 |

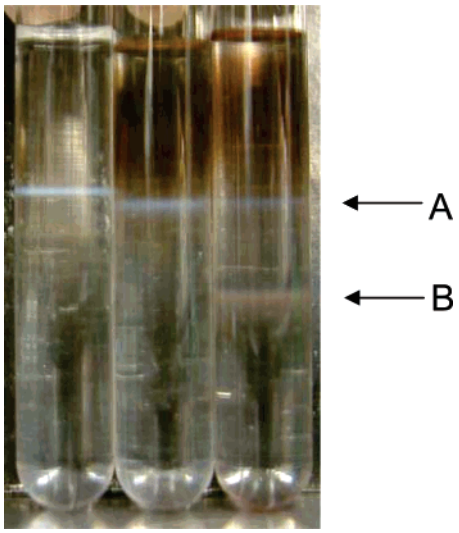


**Figure 3.** Photograph of gold-labeled adenoviral vectors in a CsCl gradient. $1 \times 10^{12}$ viral particles were reacted with either no gold nanoparticles (left), 10 000 Ni−NTA gold particles per viral particle (middle) or 10 000 sulfo-NHS gold particles per viral particle. The gold nanoparticles not reacted to the virus in the middle and right tube remain in the upper portion of the gradient (brown color above arrow A). The white band in the area indicated by arrow A consists of remaining unlabeled virions, the light brown band indicated by arrow B indicates gold-labeled virions and can only be observed in the right centrifuge tube.

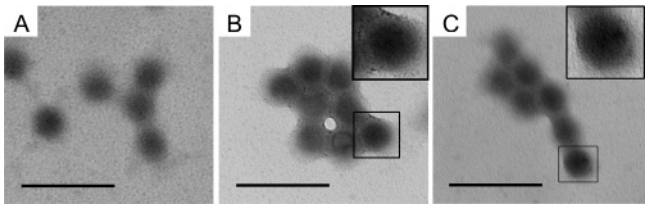


**Figure 4.** Electron microscopy identifies gold nanoparticles associated with adenoviral vectors. Vectors were either unlabeled (A), sulfo-NHS gold nanoparticles labeled (B), or Ni−NTA gold nanoparticles labeled (C). Adenoviral vectors were examined using electron microscopy using a JEOL JEM 1200FX operated at 80 kV. Original magnification 100000×, scale bar 100 nm.

be observed in virus preparations that were either unlabeled (Figure 4A) or labeled with Ni−NTA gold nanoparticles (Figure 4C). One does see some "texture", but this arises from the little bit of underfocus used to better see the virions and is not to be confused with gold particles. In contrast, gold nanoparticles could clearly be observed in the virus preparation labeled with sulfo-NHS gold nanoparticles (Figure 4B, small black dots on the edges of the virions, see magnified insert). The gold particles in Figure 4B have rather sharp boundaries and similar sizes, as opposed to the texture in Figure 4C. This further strengthened the observation of a covalent interaction between the two components.

**Gold-Labeled Adenoviral Vectors Retain Infectivity in HeLa Cells.** Modification of capsid proteins such as the here-described chemical modification with gold nanoparticles may result in a loss of infectivity of the adenoviral vectors. We therefore evaluated gene transfer of a luciferase encoding adenoviral vector AdCMVLuc, labeled with different amounts of gold in HeLa cells. These cells are previously reported to

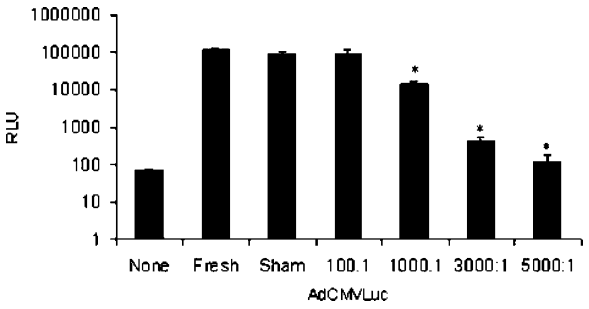


**Figure 5.** Infectivity of gold-labeled adenoviral vectors is retained at lower particle-to-vector ratios. Gold nanoparticles were reacted with AdCMVLuc at ratios of 100:1, 1000:1, 3000:1, and 5000:1. After reaction, HeLa cells were infected with 100 vp/cell and luciferase expression was determined after 24 h. Bars represent mean ± standard deviation. Asterisks indicate $p < 0.05$.

be readily infected with adenoviral vectors.[5] AdCMVLuc infectivity at a multiplicity of infection (MOI) of 25 was not affected by the synthesis procedure itself. This was demonstrated by the comparable levels of luciferase activity of sham-labeled versus fresh, unmodified AdCMVLuc (Figure 5). Further, a gold:adenovirus ratio of 100:1 (particle:particle) also did not affect infectivity. However, higher gold:adenovirus ratios did significantly decrease infectivity compared to unlabeled AdCMVLuc (Figure 5). Results were similar for both lower (5) and higher (125) MOIs (data not shown). This suggested a threshold of the number of gold nanoparticles that can be coupled to adenoviral vector without disrupting the natural infectivity mechanism of adenoviral gene transfer.

**Gold-Labeled Adenoviral Vectors Can Be Retargeted to CEA Expressing Tumor Cells.** A majority of human tumors are deficient in the primary receptor for adenoviral vectors, the Coxsackie adenovirus receptor (CAR), resulting in poor tumor cell transduction. To overcome this hurdle, approaches have been established whereby the adenoviral vector is physically retargeted to alternate receptors on the tumor cell surface. One example is retargeting of adenoviral vectors to the tumor-associated antigen carcino embryonic antigen (CEA), which is overexpressed on several neoplasias such as colon carcinoma. To establish whether gold-labeled adenoviral vectors could still be retargeted to CEA, AdCMVLuc was preincubated with the fusion protein sCAR-MFE, which on one side binds to the adenoviral capsid and on the other side binds to CEA. Gene transfer in MC38−CEA-2 cells, a CEA overexpressing cell line, was markedly increased upon preincubation of fresh, unmodified AdCMVLuc with the sCAR-MFE fusion protein (Figure 6, fresh AdCMVLuc, white versus black bar). Similar to results obtained in HeLa cells, a gold:adenovirus ratio of 100:1 (particle:particle) did not affect retargeting to and infectivity in CEA expressing cells. A gold:adenovirus ratio of 1000:1 significantly but moderately affected retargeting and infectivity, whereas ratios of 3000:1 and higher resulted in infectivity levels lower than untargeted, unmodified AdCMVLuc (Figure 6). This indicated that up to a ratio of 1000 gold nanoparticles per adenoviral vector in the reaction mixture, retargeting to tumor-associated antigens could still be achieved.

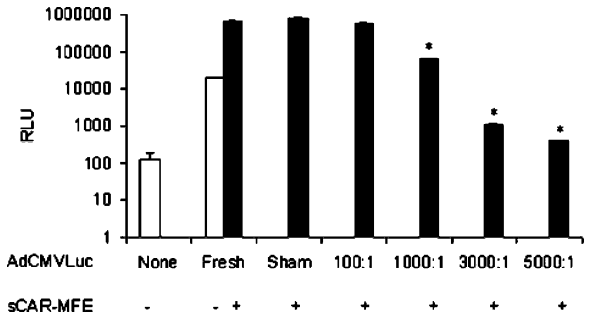


**Figure 6.** Retargeting of gold-labeled adenoviral vectors to CEA is retained at lower particle-to-vector ratios. Gold nanoparticles were reacted with AdCMVLuc at ratios of 100:1, 1000:1, 3000:1, and 5000:1. After reaction, MC38−CEA-2 cells were infected with 100 vp/cell of AdCMVLuc without (white bars) or with (black bars) sCAR-MFE fusion protein preincubation, to retarget the viral vector to the expressed CEA. Luciferase expression was determined after 24 h. Bars represent mean ± standard deviation. Asterisks indicate $p < 0.05$.

It has been recognized that exploitation of multiple treatment modalities will be needed to achieve success in cancer therapy. This has been pioneered using combinations of radiation, surgery, and chemotherapy, which are now standard therapeutic approaches. The addition of gene therapy to the arsenal of available treatment options will lead to a further increase in therapeutic combinatorial opportunities. This has already been demonstrated by synergistic effects between conditionally replicative adenoviruses (CRAds) together with radiation[6] or chemotherapy.[7] To extend this paradigm to a combination of gene therapy and hyperthermia, we herein investigated the possibility to covalently couple gold nanoparticles to adenoviral vectors, without affecting virus infectivity or retargetability. The herein established gold-labeled vectors can thus next be analyzed for hyperthermia-mediated tumor cell death induction.

It has been known that rapidly proliferating cells, such as tumor cells, are more sensitive to heat shock than slowly proliferating cells through a variety of mechanisms, including mitotic delay, cell cycle arrest, and plasma membrane damage.[8,9] The selectivity of hyperthermia for tumors is further conveyed by a tumor's usually limited blood supply, thus creating an environment with hypoxia and low pH, which is not found in healthy tissue.[1] One of the mechanisms by which hyperthermia has been induced is near-infrared absorption and subsequent heating of gold nanoparticles[10−12] or nanoshells.[3] So far, tumor selectivity of these particles has been achieved based on enhanced permeability and retention of particles due to "leaky" tumor architecture.[3] Although the tumor selectivity obtained by these mechanisms is promising, the specificity that would be obtained by covalent conjugation of the gold nanoparticles to tumor-targeted adenoviral vectors, as demonstrated in this paper, would be beneficial for an increased tumor to healthy tissue ratio and thus a better therapeutic index. This is particularly valid for smaller solid gold nanoparticles that are expected to interfere to a lesser extent with virus infectivity and retargeting compared to gold nanoshells, which have a size between 100 and 140 nm.

In the current work we have employed relatively small gold nanoparticles, for which effective heating will require extremely short (femto- or at least picosecond) laser pulses.[13] Alternatively, "nanoclusters" can be formed, where gold nanoparticles are clustered together, resulting in increased absorption efficiency.[13] Indeed, the proximity of the three-dimensional location of gold nanoparticles within one virus ("bio-nanoclusters") may provide the condition for plasmon−plasmon resonances that accompany increased absorption and a shift to the near-IR range. Nevertheless, we plan to explore the possibility of larger solid gold nanoparticles to be incorporated in the adenoviral platform.

Considerable advancements in tumor-specific targeting of adenoviral vectors for gene therapy have been achieved using both bispecific adapter molecules[14] as well as genetic capsid modification.[15] Furthermore, adenoviral vectors are compatible with chemical modification, as shown with fluorophores[16] or poly(ethylene glycol) derivatives.[17] This led us to explore the possibility of covalently coupling gold nanoparticles to targeted adenoviral vectors and thus retarget the nanoparticles to tumor-associated antigen expressing tumor cells, as demonstrated in this paper. The achieved targeting to tumor cells now creates the opportunity to test the therapeutic effects of near-infrared (NIR) laser-induced hyperthermia-mediated tumor cell death. These data further provide a rational basis to investigate the synergistic therapeutic gains accrued by a combination of hyperthermia with gene therapy. For example, hyperthermia-sensitive promoters such as hsp70 can drive the expression of cytokines such as interleukin-12 or tumor necrosis factor-alpha.[18] Furthermore, hyperthermia induced viral replication of CRAds might be a feasible strategy, thus combining multiple ways of cell death induction using a single vector.

Another important issue that still needs to be addressed is the optimal spatial location of the particles for most effective cancer cell killing. One option might be a location near the cell membrane (during initial vector binding and internalization) or even inside the membrane itself, since only a low laser energy is required for plasma membrane damage through thermal denaturation or bubble formation phenomena. This will lead to immediate cell death, mainly through necrosis.[19] Another option might be the nucleus (after complete penetration of the vector into the cells), where laser-induced nuclear damage may lead to cell damage through other mechanisms such as apoptosis, cell cycle arrest, etc.[8,9]

Along with adenoviral vectors, other methods of tumor-selective targeting have been exploited in the literature, such as direct conjugation of therapeutic modalities to antibodies[19] or inclusion of such modalities in liposomal formulations.[20] However, some potential limitations of directly conjugating gold nanoparticles to tumor-targeted antibodies include the limited number of modalities that can be attached without disrupting antibody specificity, whereas liposomes can be modified with particles to a higher extent, forming metallosomes.[21] These metallosomes may have a favorable body distribution and hence tumor specificity, as well as decreased immunogenicity compared to viral vectors. However, efficacy of adenoviral gene transfer is still unparalleled in in

vivo systems, favoring the use of adenoviral vectors for possible combinatorial approaches of hyperthermia with gene therapy, as described above.

In addition to gold, we are currently exploring the feasibility of coupling other metal nanoparticles to adenoviral vectors, such as iron−platinum (FePt) nanoparticles. These nanoparticles have magnetic properties that make them ideal for either imaging of particle localization (e.g., using MRI techniques), or magnetic-induced hyperthermia for cell killing, analogous to the gold NIR approach.[22,23]

Finally, to further improve on the herein established gold conjugated adenoviral vector paradigm, other methods of coupling nanoparticles to the adenoviral surface need to be investigated. The relatively nonspecific coupling via the sulfo-*N*-hydroxysuccinimide reactive group on the gold particle to primary amines of lysine-residues present in the capsid interferes with virus infectivity and retargetability at higher gold−virus ratios. Genetic manipulation of the nanoparticle binding locales will allow us to specifically conjugate particles to capsid proteins not involved in virus infectivity or interaction with targeting methods. Such modifications will allow for an increase in payload capacity of the virus, i.e., the number of nanoparticles that can be coupled without negatively affecting the virus, and thus create a wider therapeutic window. For example, nickel−nitrilotriacetic acid (Ni−NTA) modified gold particles,[24] used in the here-described experiments as a negative control, would be capable of binding six histidine residues that have carefully been inserted in certain capsid proteins, such as hexon[25] or pIX.[26] Ultimately, combination of multiple modalities within one viral particle, i.e., a targeting site in genetically modified virus capsid proteins allowing physical localization of the vector with the tumor cell, a nanoparticle binding site allowing induction of hyperthermia and imaging opportunities, combined with tumor-specific induction of therapeutic gene expression and viral replication may prove to be an optimal platform for cancer therapy.

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**Supporting Information Available:** Detailed description of the materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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