Published in final edited form as:

Biomaterials. 2010 December ; 31(34): 9086–9091. doi:10.1016/j.biomaterials.2010.08.046.

Co-Administration of Protein Drugs with Gold Nanoparticles to Enable Percutaneous Delivery

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Abstract

An interesting nanoscale interfacial phenomenon mediated by gold nanoparticles (Au-NPs) was found, in that co-administration with Au-NPs enables percutaneous delivery of protein drugs. The Au-NPs with a mean size of 5 nm were revealed to be skin permeable, presumably due to the nano-bio interaction with skin lipids and the consequent induction of transient and reversible openings on the stratum corneum. Importantly, when simultaneously applied with Au-NPs, the protein drugs were also granted the ability to penetrate the skin barrier and migrate into the deep layers. This indicated that co-administration with the skin-permeable Au-NPs could mediate proteins across the skin barrier. Such co-delivery effect highlights a simple yet effective method for overcoming the skin barrier for percutaneous protein drug delivery. Employing this method, a noninvasive vaccine delivery strategy was developed, and by topically co-administrating antigens with Au-NPs, robust immune responses were elicited in the tested animals. The results provide the promise for achieving a needleless and self-administrable transcutaneous vaccination.

Keywords

drug delivery; percutaneous protein absorption; gold nanoparticle; co-delivery; transcutaneous immunization

1. Introduction

Skin is regarded as an ideal entry route for non-invasive drug delivery because of its easy accessibility. Yet, the stratum corneum (SC), a composite structure responsible for the skin's barrier function [1,2], normally precludes percutaneous absorption of protein drugs owing to their large size and hydrophilic nature. The lipid-filled extracellular space between corneocytes provides the essential pathway for percutaneous absorption. The skin barrier can be weakened typically by use of chemical permeation enhancers, which interact with the skin lipids and chemically or physically alter the SC structure [3].

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Growing understanding of the biological effects of nanoparticles on the body has led to numerous drug delivery systems meant to improve drug therapy. Rigid metal-based nanoparticles exhibit many distinct biological properties due to their non-deformable shape and much smaller size (e.g. <10 nm), compared to the lipid or polymeric counterparts. For instance, the potential skin penetration of such rigid nanoparticles has recently received much attention [4]. Known for their nanobiological interactions with membrane lipids [5,6], the nanoparticles have shown to modulate membrane lipid phase transitions [7,8] and induce gelled areas on the membrane [9], thereby increasing the lipid fluidity. Since the skin barrier is actually governed by the physical state and structural organization of SC extracellular lipids [10], the lipid-fluidizing functions of nanoparticles play an important role in their ability to alter skin permeability. Metal-based nanoparticles of less than 10 nm in size, including quantum dots [11] and iron oxide nanoparticles [12,13], were revealed to be capable of penetrating through intact skin, due largely to the interaction with the lipid in extracellular space. Hence, the altered condition of skin accompanied by percutaneous penetration of nanoparticles leads to the breach of skin barrier.

Beyond these facts, there is an intriguing yet almost ignored potential that the altered state of skin barrier caused by nanoparticles could benefit percutaneous delivery of drugs. We hypothesized that the skin penetration may occur not only with the nanoparticles themselves but also with the co-administered compounds that will go through with nanoparticles because of the changed skin permeability. If this co-delivery effect does exist, it may provide a strategy for percutaneous drug delivery. Considering their wide applications in drug delivery and the advantages of biocompatibility and fine-tunable sizes, Au-NPs would be an ideal material in this study. In this study, we co-administered various protein drugs with Au-NPs on the skin of mice to investigate the percutaneous co-delivery effect.

2. Materials and methods

2.1 Synthesis and characterization of Au-NPs

Two hundred and fifty microliters of 10 mM HAuCl₄ aqueous solution was added to 10 ml of 20 mM polyvinylpyrrolidone (average MW 40 k, Sigma-Aldrich), followed by addition of 250 µl of 10 mM NaBH₄ solution under vigorous stirring. Reaction mixture was stirred for additional 15 min at room temperature. The resulting Au-NPs were then purified by ultrafiltration (50 k NMWL, Millipore) with water to remove the free agents.

High-resolution transmission electron microscopy (HRTEM) was operated at an accelerated voltage of 300 kV (JEOL 3011, Japan). Samples were prepared by applying diluted nanoparticle suspensions to formvar film-coated copper grids (Ted Pella, USA) dried at room temperature. Particle size and zeta potential were measured using a Malvern Zeta Sizer Nano ZS instrument.

2.2 *In vivo* percutaneous absorption of Au-NPs

The dorsal skin of the Balb/c mice (female, 6–7 weeks old, Simonson Laboratories) was clipped (Andis AGC clipper, USA) 24 hr prior to topical drug administration. The area of naked skin was hydrated for 10 min by covering it with wet gauze. One hundred microliters of the Au-NP suspension (200 µM) in water was applied to the clipped area (about 2 cm²). The applied skin area was maintained moist with water to prevent the Au-NP sample running dry during the course of exposure. After 3 hr, the skin was washed thoroughly with water and harvested for cryosection. The skin sample was embedded using O.T.C. Compound (Tissue-Tek) and rapidly cooled with dry ice. Sections were sliced in a direction toward the outer side of the skin with a cryostat (Microm HM 500 M). Using the silver-enhanced staining method (Silver Enhancer

Kit, Kirkegaard & Perry Laboratories) and Eosin counterstain, the distribution of Au-NPs was examined under a light microscope.

2.3 *In vivo* study of co-delivery of Au-NPs and proteins

Animals were prepared as above. Two model proteins, horseradish peroxidase (HRP, Sigma-Aldrich) and β -galactosidase (β -gal, Sigma-Aldrich), were used. The mixture (100 μ l) of the test protein solution (100 μ g) and the Au-NP suspension (200 μ M) was applied onto the clipped skin area. After 3 hr, the skin samples were prepared as above for a cryosection process. HRP in the skin section was detected by the color development of the peroxidase substrate (Kirkegaard & Perry Laboratories), and β -galactosidase using an x-gal staining method (BetaBlue Staining Kit, Novagen).

2.4 Transcutaneous immunization study

The immunization experiment was performed on Balb/c mice (female, 6–7 weeks) by co-administration of Au-NPs and ovalbumin (OVA). Animals were prepared as above. The direct mixture (100 μ l) containing OVA (500 μ g, Grade V, Sigma) and Au-NPs (200 μ M) in water was applied onto the skin over an area of approximately 2 cm² for 3 hr, followed by extensive washing of the skin. Boosters were administered at both the 2nd and 4th week. In the meantime, intramuscular (i.m.) injection of OVA (50 μ g in 25 μ l of saline) and topical application of OVA (500 μ g in 100 μ l water) without Au-NP co-administration were carried out as positive and negative controls, respectively. Aluminum (25 μ l/mouse, Imject®, PIERCE) was employed as the adjuvant for the i.m. vaccination, and cholera toxin (50 μ g/mouse, List Biological Laboratories) for transcutaneous immunization. The blood samples were collected by retro-orbital bleeding at specified time points. The plasma was isolated by centrifugation (3000 rpm, 5 min) and used for anti-OVA IgG determination by ELISA.

2.5 Anti-OVA IgG assay

Anti-OVA IgG concentrations in plasma samples from the immunized mice were determined by the ELISA method we reported previously [14]. Briefly, 96-well polystyrene plates (Corning Costar) were coated with OVA (50 μ g ml⁻¹ in 0.1 M bicarbonate/carbonate buffer, pH 9.6, 100 μ l per well) and incubated overnight at 4 °C. The plates were washed three times with PBS containing 0.05% Tween-20 (PBST), and were then blocked with 1% bovine serum albumin (BSA)/PBST buffer for 2 hr at 37 °C. After washing the plates with PBST, the plasma samples were added (100 μ l per well at a dilution of 1:100,000) and the plates incubated at 37 °C for 1 hr followed by washing. After addition of peroxidase-conjugated goat anti-mice IgG (H+L) antibody (1:3000, Kirkegaard & Perry Laboratories), the plates were incubated for 30 min at 37 °C and washed thoroughly. One hundred microliters of the substrate solution (TMB Peroxidase Substrate, Kirkegaard & Perry Laboratories) were added to each well and the plates further incubated for 15 min at room temperature. After quenching the reaction by the addition of HCl (1M, 100 μ l per well), the absorbance at 450 nm was measured with reference wavelength of 620 nm using a microplate reader (Bio-tek).

2.6 MTT assay

Human keratinocytes (CCD 1106 KERTr, ATCC) were cultured as described in the Product Instructions from ATCC. Briefly, cells were cultured in KFM medium (Gibco, Invitrogen, USA) in a humidified 5% CO₂ incubator at 37 °C. Cells were planted onto a 96-well plate at a density of 1x10⁴ cells/well, and grown to 80% confluence. Various concentrations of Au-NPs were then added to cells and incubated for 4 hr. After washing cells, MTT reagent (0.5 mg/ml) was added and incubated for 4 hr. Following removal of MTT solution, DMSO was added and absorbance measured at 570 nm with reference wavelength of 630 nm.

3. Results and discussion

The synthesized Au-NPs were morphologically homogeneous and with a mean size of about 5 nm measured by HRTEM (Fig. 1). The hydrodynamic diameter was 11.6 nm, and the zeta potential was -18.3 mV. Over a course of 4 weeks, the nanoparticles remained stable without aggregation.

The skin permeability of these nanoparticles was examined on mice. Au-NPs were found to cross the SC layer of the skin and spread widely over the epidermis layer, and a limited amount of the nanoparticles was also detected in the dermis layer (Fig. 2). Interestingly, no significant accumulation of the nanoparticles was observed within the skin appendages such as hair follicles, implying that the follicle pathway is not the predominant penetration mechanism.

Our results were in accordance with the conclusion of other groups that, for the superfine nanoparticles (i.e. < 10 nm) their skin penetration did not show a significant follicular preference, demonstrating that SC extracellular lipid regions are the important pathway [11–13,15]. But interestingly, follicle pathway was reported the main entry for nanoparticles with sizes range from 40–400 nm [3,16–19]. This difference can be accounted for the new nano-bio interaction caused by reduced size.

The exact skin penetration mechanism of such superfine nanoparticles still remains unknown, with more detail waiting to be explored. On the other hand, knowledge of skin architecture provides a possible explanation for the skin permeability of nanoparticles. For instance, inside skin extracellular space, the lipids arrange themselves in a head-to-head and tail-to-tail configuration, with the nanoscale head-to-head regions likely forming aqueous pores (~ 3 nm in size) [12]. Therefore, albeit acting as the primary physical barrier for the body, the skin is deemed to be nanoporous [20]; it has been estimated that there are approximately 5×10^7 natural intercellular hydrophilic pores per 1 cm^2 of the SC and the aqueous pores through the SC are 0.5–10 nm wide [21]. Importantly, the size of the extracellular space is not static [11]. As evidence, disturbances of the skin lipid state lead to an increase in the skin porosity of aqueous pores [22] and size of the extracellular gaps [11]. It should be noted that the hydrophobic interaction of the Au-NPs and lipids has been demonstrated to be responsible for disruption of the lipid layer structure [8]. As a consequence of the dynamic interaction, skin porosity and permeability increased.

Furthermore, nanoparticles engineered to an exceedingly low size threshold, ranging from 1 to 10 nm, can attain unique physicochemical (e.g., the excess surface energy and active interfacial interaction) and biological properties due to the quantum-size effect [23]. Therefore, the employed Au-NPs, due to its favorable size, may directly induce lipid-modulation and consequent skin penetration ability.

To investigate the skin penetration of the co-administered protein drugs, the model compound, HRP (MW 45 kDa) or β -gal (MW 460 kDa), was co-administered in a simple mixture with Au-NPs on the skin of mice. As displayed in Fig. 3, both compounds penetrated the SC barrier, demonstrating that the Au-NPs are able to create channels through the skin, thereby allowing exogenous compounds of various sizes to penetrate. The HRP that migrated into the skin was indicated by the color development of the peroxidase substrate (3,3',5,5'-tetramethylbenzidine), which is related to the HRP activity, while the β -gal was indicated by the staining of its substrate, x-gal. Both proteins remained primarily in the epidermis layer, though minor amount was found in the dermis layer. Therefore, after overcoming the SC barrier, these molecules appeared to migrate into both the epidermis and dermis layers within 2 hr after topical administration, in a pattern parallel to the distribution of Au-NPs. These results provide solid support for our hypothesis that the increased skin permeability induced by Au-NPs can serve as a potential for drug entry, and that proteins acquired the skin penetrating

ability by a means of co-application with the Au-NPs. The co-delivery effect could be developed into a simple yet effective method for percutaneous drug delivery.

An important finding to note was that the openings in the skin created by topical application of Au-NPs was confirmed to be transient, and after removal of the topical Au-NPs, the skin would return to its original impermeable nature against invasion by pathogen-like or unwanted compounds. In this study, the skin on mice was pretreated by exposure to Au-NPs for 2 hr and followed by thorough washing by tap water for 15 min. Then a fluorescent marker, rhodamine B was applied to the same skin area. Examination of the skin sample indicated that no measurable fluorescence was detected inside the skin, whereas rhodamine B that was simultaneously co-administered with Au-NPs exhibited strong skin penetration (Fig. 4). These findings implied that the skin lipids reverted to their organized structure quickly if the stress of Au-NPs applied on the skin was removed. Furthermore, the small molecule, rhodamine B, was more wide spread within the skin deep layers compared to the proteins, presumably due to the far lower diffusion resistance, which is related to the size effect. Thus, within the same test time line, small molecules were seen quicker migration through the skin layers than macromolecules.

The co-delivered macromolecular compounds accumulated mainly in the epidermis and exhibited a slower migration further into deeper layers within the skin. This distribution pattern suggested a potential application for transcutaneous immunization (Scheme 1), which is defined as induction of immunity by applying vaccine antigens onto the intact skin [24,25]. The epidermis is known for its immunological milieu and is home for mature Langerhans cells, which represent a network of immune cells that constitute 20–25% of the total surface area in human skin [26,27]. Langerhans cells not only process professional antigen-presenting functions to induce antigen-specific T cells for adaptive immune responses, but also initiate a cascade of innate immune responses by sensing these danger signals [28]. Therefore, the epidermis offers an ideal target site of the skin immune system for achieving needleless transcutaneous immunization.

The potential of Au-NPs in facilitating transcutaneous vaccine co-delivery was investigated. Transcutaneous immunization was performed on Balb/c mice by topical co-administration of Au-NPs with a model protein antigen OVA. The results (Fig. 5) demonstrated that the topically applied OVA/Au-NPs physical mixture was highly immunogenic in mice, as anti-OVA IgG levels were gradually yet significantly increased over the course of the immunization schedule. Anti-OVA IgG levels in animals immunized by topical OVA/Au-NPs were robust and consistent, which displayed no significant statistical difference ($p > 0.05$) from those in mice receiving a standard vaccination protocol by intramuscular injection. On the contrary, the control group using topical OVA without co-application of Au-NPs exhibited very low levels of antibody response, apparently due to the poor percutaneous OVA absorption. These results showed that the antigens were able to penetrate into the epidermis layer via the skin-breaching function of Au-NPs, successfully captured by epidermis-residing Langerhans cells and subsequently induced robust humoral immunity responses. Therefore, vaccine antigens can achieve transcutaneous immunization by co-delivery into epidermis with the aid of the skin-permeable Au-NPs.

Needle-involved vaccination remains highly problematic in developing countries due to unsafe injection practices (e.g. needle reuse) and a shortage of healthcare sources. For example, unsafe injections cause 33% of new HBV infections in developing countries for a total of 21.7 million people infected yearly [29]. This co-delivery method could be a solution for this issue, showing promise in achieving simple, noninvasive and low-cost vaccination.

Skin-permeable metal nanoparticles may be used for transdermal drug delivery by modifying the surface with polymers and drug molecules, but few investigations actually reported on protein drugs. This is probably due to the fact that the crosslinking of drugs onto surface of these particles may significantly change their unique physicochemical properties. In addition, the protein loading efficiency onto the rigid nanoparticles via a chemical conjugation is relatively low, and these chemical processes often cause loss in protein drug activity. By contrast, the co-delivery method shows significant advantage over traditional use of nanoparticles in drug delivery by eliminating of complicated and costly processes of integrating drugs into a delivery platform and possibility of compromising in protein stability.

Since the nanomaterials display active interactions with the skin barrier, their potential nanotoxicity also raises a concern [30,31]. Therefore, the *in vitro* cytotoxicity was examined on skin keratinocyte cells (CRL-2309, ATCC). The Au-NPs at a concentration up to 200 μ M was shown to be safe to keratinocytes (Fig. 6), since materials with cell viability more than 80% are often considered as biocompatible [32]. However, as little is known about the systemic toxicity profile of superfine nanomaterials (i.e. < 10 nm) that bear excess surface energy and active interfacial properties, meticulous investigations should be carried out for further safety information.

4. Conclusion

Interaction between the Au-NPs and skin barrier leads to the increase of skin permeability and effectively prompts percutaneous absorption of the co-administered proteins. The advantage of this co-delivery method is that it does not required to “load” drugs into the nanoparticulate system, but simply in a physical mixture instead. Therefore, compromise in activity can be minimized for both protein drugs and nanoparticles because of the exclusion of complicated drug-loading processes. Such co-delivery effect highlights a new strategy for percutaneous protein delivery with obvious advantages in terms of simplicity and cost-effectiveness. It provides a promise in achieving self-administrable transcutaneous immunization, and may especially benefit those areas underserved for medical care.

Acknowledgments

This work was supported in part by NIH R01 Grants CA114612 and NS066945. This work was also partially sponsored by Grant R31-2008-000-10103-01 from the WCU project of South Korea. Victor C. Yang is currently a Participating Faculty in the Department of Molecular Medicine and Biopharmaceutical Sciences, Seoul National University, South Korea. Dr. Yongzhuo Huang is the recipient of the Hundred-talent Program of the Chinese Academy of Sciences.

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Fig. 1.
Transmission electron microscope image of the Au-NPs.

Fig. 2.

In vivo study of skin penetration of Au-NPs. (A) Control. (B) Au-NPs showed high accumulation in the stratum corneum and epidermis. (C) Au-NPs accumulation inside the follicles was found to be minor. Au-NPs are visualized as dark dots by silver-enhanced staining.

Fig. 3.
Percutaneous co-delivery of molecules varying sizes mediated by Au- NPs. (A) HRP alone.
(B) HRP/Au-NPs. (C) β -gal alone. (D) β -gal /Au-NPs.

Fig. 4. Transient skin openings created by Au-NPs. (A) Rhodamine B alone. (B) Rhodamine B, but with Au-NP pretreatment. (C) Rhodamine B and Au-NPs simultaneously applied to the skin. The distribution of rhodamine B was observed using a fluorescence microscope (Olympus BX51).

Fig. 5.

Transcutaneous immunization (TI) with co-administration of OVA and Au-NPs. Mice were primed by co-administration of OVA and Au-NPs, and two boosters were given at the 2- and 4-week marks. Control group (Con) was given topical OVA without Au-NPs, while another group (IM) was given OVA intramuscularly at the 0-, 2- and 4-week marks. (¹ $p = 0.059$, ² $p = 0.620$, ³ $p = 0.197$, $n = 7$)

Fig. 6.
Cytotoxicity study of Au-NPs by MTT assay

Scheme 1.
Transcutaneous antigen delivery mediated by Au-NPs