Effect of end group modification of DNA-functionalized gold nanoparticles on cellular uptake in HepG2 cells

Surin Hong a, Soomin Park b, Junsu Park b, Jongheop Yi b,∗

a Department of Applied Bioscience, CHA University, Gyeonggi-do 463-863, Republic of Korea
b World Class University Program of Chemical Convergence for Energy & Environment, Institute of Chemical Processes, School of Chemical and Biological Engineering, Institute of Chemical Processes, Seoul National University, Seoul 151-744, Republic of Korea

A R T I C L E   I N F O
Article history:
Received 20 March 2013
Received in revised form 5 August 2013
Accepted 18 August 2013
Available online xxx

Keywords:
Cellular uptake
Dark-field scattering microscopy
DNA-gold nanoparticles
Functionality
Nanoparticle/protein corona
Surface plasmon resonance (SPR)

A B S T R A C T
Understanding the dynamics of the cellular uptake of nanoparticles in human derived (cancer) cells is crucial to the rational design of functional nanoprobes that can be used for the targeting and delivery of drugs. This study reports on the cellular uptake of gold nanoparticles (GNPs) that were functionalized with different oligonucleotide derivatives using HepG2 cancer cells as a model system. DNA oligomers, in which the end group was modified (NH2–, PO3–, OH–, CH3–, and SH– groups) were introduced onto the GNP surface. Then, quantitative and qualitative analyses using each DNA-GNP complex were carried out via dark-field scattering microscopy and ICP-MS measurements. Visualization of microscopic images of single cells indicated that the uptake of DNA-GNPs was highly dependent on the type of functionality of the end group in the DNA-GNP complex; the functionality of CH3– and SH– resulted in less cellular uptake than that for modifications with NH2–, PO3–, OH– for the same incubation time. This result was reinforced by ICP-MS quantitative analysis. These results were also strongly supported by the events of a DNA-GNP/protein corona; the different association and dissociation rates of proteins around the GNPs was dependent on the functionality of the end group in the DNA-GNP complex, providing further evidence for the conclusion that the components on the surface of nanoparticles directly affected cellular uptake. The findings reported herein provide a basis for the understanding of the fate of GNP-based delivery and provide important insights into the rational design of nanoprobes for the effective treatment of various diseases.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Biomedical applications of nanoparticles for use as therapeutic agents in living cells have been growing rapidly over recent years [1–4]. Nanoparticles have also been successfully utilized in novel cancer therapies [5–7]. In these nanoparticle-mediated biotransport systems, gold nanoparticles (GNPs) have received considerable attention due to their biocompatibility and unique optical properties [8–11]. Furthermore, exploiting the optical properties of GNPs for the development of ultrasensitive detection and imaging methods in the field of biomedical science is becoming increasingly important.

Based on the in vivo use of GNPs and novel analytical methods, developing GNPs with functional groups attached for the delivery of exogenous molecules into living cancer cells has become a powerful tool not only for effective delivery and therapeutic purposes, but also for the decoding of fundamental cell mechanisms. Several studies have reported on the successful intracellular delivery of biomolecules such as DNA [12,13], proteins [14], antibodies [15], and polymers [16] by linking GNPs to macropinocytosis or endocytosis pathways. Among these approaches, GNPs loaded with DNA for delivery to live cells have substantial advantages, which include efficient intracellular delivery without the need for transfection or permeabilization reagents and an increased resistance to nuclease degradation. As an example, their efficiency of transporting DNA to the nucleus was reported to be several times higher than that of polymeric compounds such as polyethylenimine [12,13]. In this regard, GNPs functionalized with DNA are attractive vectors for use as a drug delivery system and cellular targeting.

The results of recent investigations indicate that the interactions between living cells and nanoparticles are highly dependent on the behavior of the nanoparticles in biological fluids [17–20]. When nanoparticles are dispersed in a biological fluid, prior to their internalization by a cell, they: (i) become coated by a corona composed of serum proteins and other biomacromolecules, or (ii) aggregate
in a kinetically driven process and form various-sized clusters. Nanoparticles that are used for general therapeutic purposes (10–200 nm size) can be taken up by cells via a micropinocytosis mechanism, which involves endocytosis in which the nanoparticles are internalized via indentations on the cell membrane. However, it is not clear how changes on the surface of GNPs of the delivery vector affect cellular internalization processes, and the surface functionality of GNP complexes and their interactions with living cells are important issues regarding determining mechanisms responsible for this process.

Here, we report on the cellular uptake and a related mechanical study of five GNPs loaded with DNA oligomers (DNA-GNP complexes), in which the end groups of DNA were modified so as to change the hydrophobicity and structure of the molecule. With the objective of investigating the cellular internalization of the system, amine (NH$_3$–), phosphate (PO$_4$–), hydroxyl (OH–), methyl (CH$_3$–), and thiol (SH–) groups, respectively, were used to modify the 3′ end groups of the DNA sequence. The efficiency of the delivery process was evaluated by monitoring the response of human cancer (HepG2) cells using dark-field scattering microscopy and inductively coupled plasma–mass spectroscopy (ICP-MS). Human derived cells have been widely used to better predict human toxicity and the efficiency of a carrier mediated delivery system.

After incubating cells with each DNA-GNP complexes, the different complexes–cell interactions were assessed by the efficiency analysis of cell internalization at different exposure times. Dark-field scattering microscopy was used to obtain images and to track the GNPs in cells as an evanescent wave scattering imaging method. The main advantages of this system are that it permits long-term automated live cell imaging under essentially identical external conditions (CO$_2$, temperature, and humidity) and the uptake of GNPs by a single cell. ICP-MS was also used to quantify and determine the cellular uptake of GNPs, except for non-specific adsorption onto the cell surface.

The results of these analyses show that small changes of the end group in a DNA-GNP complex can lead to drastic alterations in the kinetics for delivery into the cell. We conclude that the different kinetics of cellular uptake can be attributed to different evolutions of DNA-GNP/protein corona in a biological fluid during the internalization process, and this was confirmed by using a GNP-mimetic system with surface plasmon resonance (SPR) measurements. This study provides important information that can be useful in terms of biomedical applications.

### 2. Experimental

#### 2.1. Materials

3-Mercaptopoundecanonic acid (MUA, Sigma Korea Ltd.), N-(3-dimethylamino propyl)-N-ethylcarbodiimide hydrochloride (EDC, Sigma Korea Ltd.), N-hydroxysuccinimide (NHS, Sigma Korea Ltd.), gold nanoparticles (GNPs, 50 nm, BBI International USA Ltd.), and five types of designed oligonucleotides (Korea Ltd.) were used as received. A piranha solution (70% sulfuric acid (H$_2$SO$_4$, Fisher Scientific Korea Ltd.) and 30% hydrogen peroxide (HCl, Sigma Korea Ltd.)) was used to clean the glass substrates on which gold thin films were fabricated.

#### 2.2. Fabrication of functionalized GNPs

The synthesized DNA oligomers were functionalized on the surface of GNPs using a previously reported method [21]. The five types of DNA oligomers were designed as listed in Table 1, in which the 5′-end contained a thiol group and the 3′-end was modified with a variety of different functional groups.

#### 2.3. Cell culture conditions

Human hepatoma HepG2 cells were cultured in RPMI 1640 media with 10% (v/v) fetal bovine serum, 100 unit/ml penicillin, and 100 µg/ml streptomycin. HepG2 cells were grown at 37 °C in a 5% CO$_2$ incubator and sub-cultured at intervals of about 3 days. For the immobilization of HepG2 cells on the substrate, the cover slip was coated with a 17.5 µg/ml collagen (type I from rabbit) solution dispersed in 30% (v/v) ethanol. After sub-culturing, floating cells were stably immobilized on collagen covered cover slips within 1 day.

#### 2.4. Dark field scattering microscopy

Light-scattering images of HepG2 cells targeted with GNPs were obtained with an inverted microscope (Nikon), where the narrow beam of light from a tungsten source was delivered with a dark-field condenser. The scattered light was detected with high contrast using index-matching solutions to reduce background scattering [22]. Only scattered light was collected using the objective (Olympus). A home-made cell well connected to the control box, which contains an inlet and outlet for providing CO$_2$ gas, and is covered with a heat line to control the humidity and temperature, was fabricated for use in capturing images of single live cells.

#### 2.5. Quantification of GNP in cells by ICP-MS experiments

Identical numbers of HepG2 cells were added to each cell culture dish. A solution of DNA-GNP complexes (2 mL) was added to the dishes. After incubation for 24 h, the dishes were rinsed with DPBS (Dulbecco’s phosphate-buffered saline) (2 mL, twice) and 2 mL of Hank’s balanced salt solution to remove non-specific adsorbed GNPs on the surface of cells [23]. The cells were collected and digested in aqua regia for 2 h. The solution was diluted, and the GNP concentration was determined by ICP-MS. Each sample was measured in triplicate.

#### 2.6. SPR measurements

A flow cell (8 µl/min) was mounted on the sensor/prism assembly so that solution of interest could be introduced easily to flow across the Au surface and that switching between different solutions could be accomplished rapidly (SPRlab, K-mac). The SPR system was utilized in the Kretschmann configuration using attenuated total reflection (ATR). Time-resolved SPR angle shifts were measured using the fixed angle method which enabled the reflectance change $ΔR$ to be linearly correlated with the SPR

<table>
<thead>
<tr>
<th>Table 1 Sequence and end-group of DNA oligomer.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA oligomer functionality</td>
</tr>
<tr>
<td>DNA with PO$_4$–</td>
</tr>
<tr>
<td>DNA with NH$_3$–</td>
</tr>
<tr>
<td>DNA with SH–</td>
</tr>
<tr>
<td>DNA with OH–</td>
</tr>
</tbody>
</table>

Initially, Tris-(2-carboxyethyl)phosphine hydrochloride (TECP) (Sigma) was used to activate the thiol-modified DNA oligomers by disulfide bond cleavage. GNP solutions of 3 mL (ca. 4.5 × 10$^{10}$ particles/ml) were mixed with TECP-treated oligonucleotides and functionalization proceeded under dark conditions for 16 h. Finally, Tris-acetate buffer and NaCl solutions were added dropwise for the stability of functionalized GNPs. After 1 day, the oligonucleotide-modified GNPs were added to the culture media.
angle shift, $\Delta \theta_{\text{SPR}}$. Reflectance data at a fixed incident angle were acquired in real time on a computer [24].

3. Results and discussion

3.1. Different end group modified GNP-DNA complexes

The DNA oligomer in the GNP vector was modified at the 3' end group of the molecule, and its cellular uptake in human HepG2 cells was evaluated. To comprehensively characterize the physicochemical properties of the DNA-GNP complexes, size distribution and dispersion characteristics were determined by UV–vis absorption spectroscopy and dynamic light scattering (DLS). UV–vis absorption spectra indicated that, with formation of GNP-DNA complexes, the plasmon absorption maximum shifts to longer wavelength (Figure S1). DLS analyses confirmed that the DNA derivatives were monodispersed. The average GNP size was 50 nm in all cases and the standard deviation was in the monodispersed range (<0.20) (Figure S2). In particular, similar average sizes of GNPs were obtained when the conjugation was carried out in the presence of DNA, as was obtained for the GNP coated with different end groups. This indicates that the GNPs were well suspended, with no detectable aggregation phenomena after their dispersion in water.

3.2. Cellular internalization of different end group modified GNP-DNA complexes

To visualize the uptake of GNPs and DNA-GNP complexes in a single cell, the HepG2 cells were first observed by dark-field microscopy without being processed to avoid the possible production of artifacts produced as the result of cell fixation. To expose cells to GNPs, HepG2 cells were seeded in a home-made sample well of the microscopic system. The cells were then allowed to settle in a humid atmosphere at 37 °C and 5% CO₂ and the GNP solutions were injected into the sample well, where they were allowed to interact.

Fig. 1 shows dark-field scattering images of live HepG2 cells before and after being incubated with GNPs and DNA-GNP complexes. The resonance scattering of a 50 nm-GNP in a uniform dielectric results in a peak in the green region of the optical spectrum. The experimental scattering from DNA-GNP complexes with a true color was observed in this result. The increasing scattering intensity in the images indicates that the GNPs were taken up within the 4 h by the cells and localized in the cytoplasm when delivered by DNA-GNP complexes. Considering the size of the GNPs and the mechanism for the internalization process, which includes cell membrane–nanoparticles interactions and the penetration of cell membrane by nanoparticles, the scattering green regions corresponding to DNA-GNP s in Fig. 1 appear to involve the assembly of GNPs on a region of the plasma membrane for internalization and a region of thoroughly internalized GNPs as well.

For the native GNPs without DNA, no significant cellular uptake of GNPs was observed for 4 h. Since DNA is known to be a good candidate as a delivery vector for cellular uptake, the kinetics of the uptake by cell for native GNPs would be expected to be slower than that of DNA-GNP complexes. In other words, native GNPs would not be expected to be easily delivered into cells as efficiently as DNA-GNP complexes. Interestingly, the initial patterns
for the intracellular distribution of both CH₃— and SH— functionalized DNA-GNPs were similar to that of native GNPs without DNA. Contrary to this, significant increases in scattering intensity were detected for the NH₃—, PO₃—, and OH— functionalized DNA-GNPs in the first 1 h. After 3 h of incubation, the scattering plasmon of NH₃— and OH— functionalized DNA-GNPs in single cells became noticeable. The DNA-GNP complexes were found to cluster in the cytoplasm of cells in large numbers. Most of the internalized particles remained in the cytoplasm and the perinuclear region after a 3 h period of incubation. The results indicate that the uptake of GNPs by HepG2 cells was highly dependent on the type of end-group modification of DNA oligomer and the incubation time used. This is understandable, based on our hypothesis that there is less affinity and binding in the HepG2 cell line with hydrophilic functionality of end group in DNA-GNP complex, leading to a lower overall uptake of particles by the cell for the same incubation time.

3.3. Quantification of cellular uptake of different end group modified GNP-DNA complexes

In order to study the kinetics of cellular uptake of the DNA-GNPs with the exception of non-specific adsorption of the GNPs on the cell surface, ICP-MS experiments were conducted to quantify the amount of GNPs and DNA-GNP complexes with varying end group modifications by cells. We performed time-dependent uptake studies with different DNA-GNP complexes by incubating them with each cell line for different periods of time. In this experiment, we used DPBS/Hank’s balanced salt solution to remove GNPs that were non-specifically adsorbed on the cell surface, and aqua regia as an effective etchant for gold. As shown in Fig. 2, the cellular uptake of DNA-GNPs increased with time. The uptake process of the DNA-GNP complexes can be adequately described by a simple first-order kinetics model with equilibrium rate constants. The amount of DNA-GNPs taken up increased rapidly in the first 2 h, and then increased more slowly from 2 to 24 h, finally reaching a plateau after 24 h. It is likely that the initial rapid increase is due to GNPs that became adsorbed and penetrated to the cell membrane, but were not completely internalized by the cells. Since GNPs that had completely penetrated would be generally internalized, a slow increase in the amount of DNA-GNPs taken up was observed after 2 h. Considering the presence of non-specific adsorbed GNPs in Fig. 1, the tendency of increase in the respective DNA-GNPs is correlated with each other.

For the PO₃— functionalized DNA-GNPs, the amount of GNP uptake was quite significant even after incubation for only 2 h, and the final amount of DNA-GNP taken up per dish reached as high as 1.72 µg for the sample incubated for 24 h. A similar increase and amount of GNP uptake was observed in the NH₃— functionalized DNA-GNPs. For both the OH— and SH— functionalized DNA-GNPs, the initial increases were almost similar for an incubation of 8 h. For the CH₃— functionalized DNA-GNPs, the amount of cellular uptake was minimal after a 1 h period of incubation, and was similar to that of the native GNPs (see Figure S3). Interestingly, after a 12 h incubation time, a significant difference was found between PO₃—, NH₃—, OH— and SH—, CH₃— functionalized DNA-GNPs. The uptake was ca. 2 times higher for the PO₃—, NH₃—, OH— modified DNA-GNPs than for the SH—, CH₃— modified DNA-GNPs. In the native GNPs without a DNA vector, the efficiency of their delivery or uptake was quite low.

Collectively, the results presented here indicate that the functionality of the modified DNA-GNPs is a major factor in determining the extent of cellular uptake. These conclusions are supported by observations of microscopic images of single HepG2 cells, suggesting that the functionality of the colloidal DNA-nanoparticle complexes may be a major factor in determining their uptake by cells. This finding, thus, could be used to control the delivery kinetics of certain drugs into cells as a carrier.

We also examined the cytotoxicity of these nanoparticles. All samples had a cell viability of over 90% after 24 h of incubation with DNA-GNPs, suggesting that none of the DNA-GNP complexes...
3.4. The effect of DNA-GNP/protein corona

It has been reported that nanoparticles are expected to enter cells via endocytic mechanisms and intracellular transport although the rate and mechanism of uptake turns out to be cell-type dependent and vary between nanoparticles and cell surface properties [25]. Before the endocytic pathways, nanoparticles are contacted with biological fluids, and then interact with proteins and other biomolecules, thus forming a dynamic corona. Its compositions are changed with the time due to the continuous association and dissociation of proteins and related molecules [17,26,27]. Importantly, the interactions have a dramatic effect on the extent of internalization, thus, the amount of the proteins on the surface of the particles is one of the major factors occurred in in vivo response.

In order to better understand the effect of the end-group modification of DNA-GNPs on the interaction with proteins and subsequent uptake process of GNP, we developed a simple and effective model of the GNP/protein corona in a body fluid and carried out kinetic studies of protein–nanoparticles interactions using SPR measurements. To prepare the biological mimetic system for interactions between GNP and proteins and measure the time evolution of the interactions, the rates of protein association and dissociation were determined for the different functionalized DNA-GNPs, that are thiol-linked to the gold thin film (Figure S4, similar changes in SPR signal were observed in every GNP sample when the functionalized DNA-GNPs were modified on the gold thin film.). Both the association and dissociation rates are clearly dependent on the end group modification of the DNA-GNPs, as seen in Fig. 3. The data for the PO₃⁻, NH₃⁻, OH⁻ modified DNA-GNPs show that proteins become associated with GNP, in contrast, those for SH⁻, CH₃⁻ modified DNA-GNPs clearly show the opposite, with protein dissociation occurring. Many previous simulation studies for nanoparticle–protein coronas have reported that two distinct temporal regimes are observed when the evolution of the system is monitored for a relatively short time [27–30]. During the very fast initial transient events (<0.05 s), the concentration of the free nanoparticles dramatically decreases due to the rapid formation of

![Fig. 3. SPR studies of DNA-GNP complex–plasma protein interactions. Cartoon of a gold thin film with thiol-tethered particles and associated protein over which buffer is flowing (A), SPR data of plasma proteins injected for 2.4 h (B). The black dot line is criteria line for the discrimination of SPR data between protein association and dissociation.](image)

![Fig. 4. Proposed schemes of the formation of DNA-GNP/protein corona and subsequent cellular uptake of different functionalized DNA-GNP complexes. Due to the different formations of DNA-GNP/protein corona, whether occurring the further association or dissociation after initial association of proteins, the PO₃⁻, NH₃⁻, OH⁻ modified DNA-GNPs (A) can be internalized more easily by the cell membrane than CH₃⁻, SH⁻ modified DNA-GNPs (B).](image)
complexes between the nanoparticles and proteins. It has been also reported that the concentration of these complexes appears to be fairly stable in the first 20 s. Thus, it would be expected that the measured association or dissociation data would be stable for the case of the functionalized DNA-GNPs/protein corona after the initial transient events. During the following 120 min, a redistribution of proteins at the DNA-GNP surface occurs, and the corona composition changes. After 2 h, equilibrium is reached. For the CH$_3$-modified DNA-GNPs, in particular, we also observed a faster dissociation from the SH- modified DNA-GNPs compared with the PO$_3$-OH- modified DNA-GNPs.

In summary, our study demonstrates that the affinity of cellular uptake can be affected by the formation of DNA-GNPs/protein corona and different formations of this protein corona are caused by end group modifications of DNA-GNPs. When the PO$_3$-OH-, OH- modified DNA-GNPs enter into biological fluids, further association of proteins with GNPs occurs after a fast initial association (Fig. 4A). In contrast, the SH- and CH$_3$- modified DNA-GNPs caused the dissociation of proteins (Fig. 4B). Under this condition, the formation of a protein corona with further association would affect the high affinity between the nanoparticles and cell membrane so that the PO$_3$-OH- modified DNA-GNPs would be internalized more easily by cells than other types of DNA-GNPs. This hypothesis is strongly supported by the results obtained; the affinity of cellular uptake remains roughly constant, in agreement with the results shown in Fig. 1 and Fig. 2. Finally, cellular uptake depends on the end-group identity of the DNA-nanoparticle.

4. Conclusions

In conclusion, the findings presented herein demonstrate that GNPs can be effectively utilized as plasmogenic probes for imaging and more importantly for intracellular tracking studies. To investigate the relationship between cellular uptake and the surface functionality of the GNPs, the DNA oligomer on the GNP (DNA-GNPs) surface was modified with different end groups (NH$_3$-, PO$_3$-, OH-, CH$_3$-, and SH-), and quantitative and qualitative analyses using the modified DNA-GNPs were carried out via dark-field scattering microscopy and ICP-MS measurements. Evanescent wave scattering imaging confirmed that the uptake of GNPs by HepG2 cells was highly dependent on the type of surface functionality and there is less affinity and binding to the cells with CH$_3$-, SH- modified DNA-GNPs, leading to a lower uptake by the cell for the same incubation time. The results of quantitative analysis, which are correlated with microscopic images of single HepG2 cells, suggest that changes in the functionality in the DNA-GNPs could be a major factor in the uptake property of the cells. Interestingly, these results were strongly supported by the events of DNA-GNPs/protein corona; different association and dissociation rates of proteins around DNA-GNPs were observed, and this was dependent on the end group modification of the DNA-GNPs. We expect this study will contribute to a better understanding of the intracellular fate of nanoprobes used for therapy and the treatment of diseases.

Authors' contributions

SH carried out the design of the study, fabricated the DNA-GNPs, performed the analysis of dark field scattering images, ICP-MS and SPR, and drafted the manuscript. SP performed the culturing of the cells and participated in the fabrication of the DNA-GNPs. JP participated in the fabrication of the DNA-GNPs and the modification of the gold film surface. JY conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by WCU (World Class University) program through the Korea Science and Engineering Foundation funded by the Ministry of Education, Science and Technology (400-2008-0230), and was also supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Science, ICT & Future Planning (2013-0067).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2013.08.020.

References