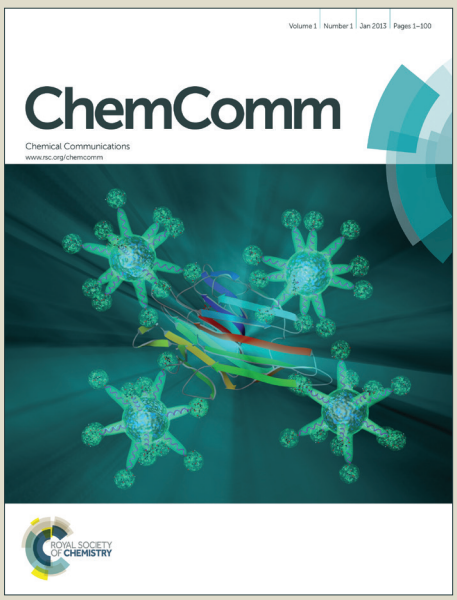
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Chemical Communications

RSCPublishing

COMMUNICATION

Cite this: DOI: 10.1039/xoxxoooooox

Efficient Delivery of siRNAs by Photothermal Approach using Plant Flavonoid-inspired Gold Nanoshells**

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

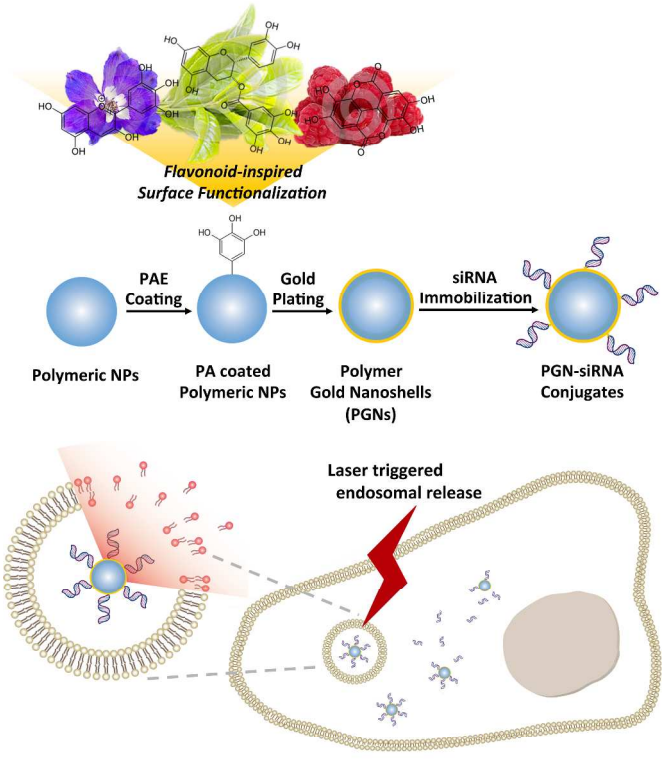
www.rsc.org/

Eun Hye Jeong[a], Ji Hyun Ryu[b], Hansaem Jeong[a], Bora Jang[a], Ho Yeon Lee[a], Seonki Hong[b], Haeshin Lee[b], and Hyukjin Lee[a]*

Polymer gold nanoshells (PGNs) are prepared by a novel plant-inspired flavonoid surface modification method. The PGNs show a dramatic photothermal property, which can facilitate the endosomal escape and delivery of siRNA into the cytoplasm of cells. Efficient gene silencing has been achieved with siRNA immobilized PGNs, suggesting the potential applications of *in vitro* gene regulation by an external NIR stimulus.

Photothermal effects of gold based nanostructures such as nanoshells and nanorods have drawn much attention in the field of drug delivery systems due to their unique physicochemical property that can trigger environmentally responsive drug release upon an exposure to NIR laser.[1] Up to date, an external control over the drug release from carriers to regulate the cellular behaviors remains highly challenging. Especially the intracellular delivery of nucleic acid drugs such as antisense DNA (ODN) or siRNA can be aided from the site-specific release of these molecules into the cellular cytoplasm for therapeutic gene regulations.[2] Since the lack of endosomal release followed by acidic/enzymatic degradation of siRNA is one of major drawbacks in current delivery systems, a photothermal approach to disrupt the early endosome and release the cargo materials into the proper site can be an ideal solution.

Previously various gold nanoshells have reported to deliver anti-cancer drugs (doxorubicin), antibodies (Cetuximab), ODNs, and siRNA.[3] All these studies have shown and emphasized on the possibility of enhancing the drug efficacy under photothermal condition. In order to synthesis heat-inducing gold nanoshells, various synthesis approaches have been developed including: 1) Au galvanic exchange at the surface of Ag precursor nanoparticles, 2) reduction of Au seeds on template polymeric/inorganic nanoparticles.[4] However aforementioned approaches often require specific template materials and multi-step surface functionalization process to prepare the gold nanoshells. Therefore it is necessary to develop a versatile surface modification method to generate a thin gold layer on a wide range of materials.



Scheme 1. Graphical illustration of Polymer Gold Nanoshell (PGN) Formation and NIR laser triggered siRNA delivery using PGN-siRNA conjugates

Herein, we report the facile preparation of polymer gold nanoshells (PGNs) by the surface modification of polymer nanoparticles with pyrogallol 2-aminoethane (PAE). Previously our group has reported a plant flavonoid-inspired molecule for material independent-surface modification.[5] In addition to the adhesive property of the pyrogallol moiety in PAE, it exhibits strong metal binding affinity and the reducing capability of noble metals enabling facile metal shell formation for potential photothermal therapy.[6] Also, the amine group in PAE further strengthens the unique material-independent surface modification properties as previously demonstrated in poly(dopamine) and poly(norepinephrine).[7] Thus, we hypothesized that the PAE-mediated functionalization can generate a thin gold shell layer on various polymeric nanoparticles and investigated the use of PGN systems for the delivery of siRNAs by facilitating the endosomal release of siRNAs by a photothermal approach (**Scheme 1**). Surface modification of commercially available polymer nanoparticles (polymethylmethacrylate (PMMA), polystyrene (PS), Poly(lactic-co-glycolic acid) (PLGA)) was conducted by a simple immersion to the PAE solution (1 mg/ml in 10 mM Tris buffer) for 2 hrs. Elemental analysis of PAE functionalized nanoparticles was shown in **Figure S1**. X-ray photoelectron spectroscopy (XPS) revealed a successful surface modification in all three different polymer nanoparticles. The tested three nanoparticles commonly exhibited the appearance of C1s, N1s, and O1s signals that is specific for PAE molecules (theoretical N1s/C1s ratio should be 0.125 and the ratio of N1s/O1s should also be 0.333).

Particle stability after PAE surface functionalization was investigated by DLS and electron microscopy measurement (Figure 1a, Figure S2). All tested polymeric nanoparticles exhibited small increase in diameter (~ 5 nm) with neutral zeta potential values after the surface modification. Their morphology remained unchanged and no particle aggregation was observed during the preparation. PAE functionalized polymer nanoparticles were then utilized to grow the gold seeds on their surfaces without addition of any reducing agent. The growth of gold seeds on the polymer nanoparticles was verified by TEM analysis as shown in **Figure 1b**. After further reduction of tetrachloroaurate (III) ions on polymeric nanoparticles, we were able to generate a thin gold layer that can fully cover the surface of polymeric nanoparticles. UV/VIS spectrum analysis exhibited the successful generation of gold seeds and shells on polymer nanoparticles (**Figure 1c**). The surface plasmon of gold seed formation on polymer nanoparticles was observed at 518 nm as well as the gold shell formation at 810 nm. Especially the strong adsorption spectrum of PGNs at NIR region suggests the possible photothermal release of nanoparticles from the endosomal compartments.[8] The photothermal potential of prepared PGNs was investigated by NIR laser irradiation (808 nm). Various laser intensity was applied to the PGN solution and temperature increase was measured for 20 min (**Figure 1d**). Compared to polymer nanoparticle in deionized water (DW), PGNs showed markedly higher temperature increases at any given time (5, 10, 15, 20 min) along with varied laser intensities (0.5, 1.5, 2.33 W/cm^2).

In order to utilize the PGNs as siRNA delivery carriers, we immobilized siRNAs (anti-GFP) by thiol-Au interaction, followed by a modified salt-aging method for 12 hrs to fully immobilized anionic RNAs onto the Au surface.[9] We observed small increases in diameter (from 115 nm to 150 nm) after immobilizing the siRNAs without any aggregation problem. It was expected that the siRNA immobilized PGNs would exhibit enhanced serum and salt stability due to the strong polyanion layer formation on PGNs.[10] Total number of siRNAs immobilized on a single PGN was estimated to be 130 molecules after the treatment of 0.5M DTT for 30 min. It is hypothesized that cytosolic GSH would able to release the immobilized siRNAs from the PGNs.[11] Therefore the dissociation of siRNAs from the gold surface by the thiol exchange reaction was investigated under the cytosolic GSH concentration (**Figure 2a**). From PAGE analysis, the release of siRNAs was verified under GSH concentration of 10 mM for 2 hrs. Unlike siRNA immobilized PGNs, the GSH treated sample showed a similar migration band pattern as compared to negative control, thus indicating successful dissociation of siRNAs from the PGNs under cytosolic GSH condition.

Before the gene silencing experiment, we evaluated the cellular toxicity of PGN-siRNA conjugates under NIR exposure. HeLa cells were incubated with PGN-siRNA conjugates (200 nM) for 2 hrs and two different NIR laser intensities (1.5 and 2.4 W/cm^2) were applied in the course of 20 min. Cellular toxicity was measured

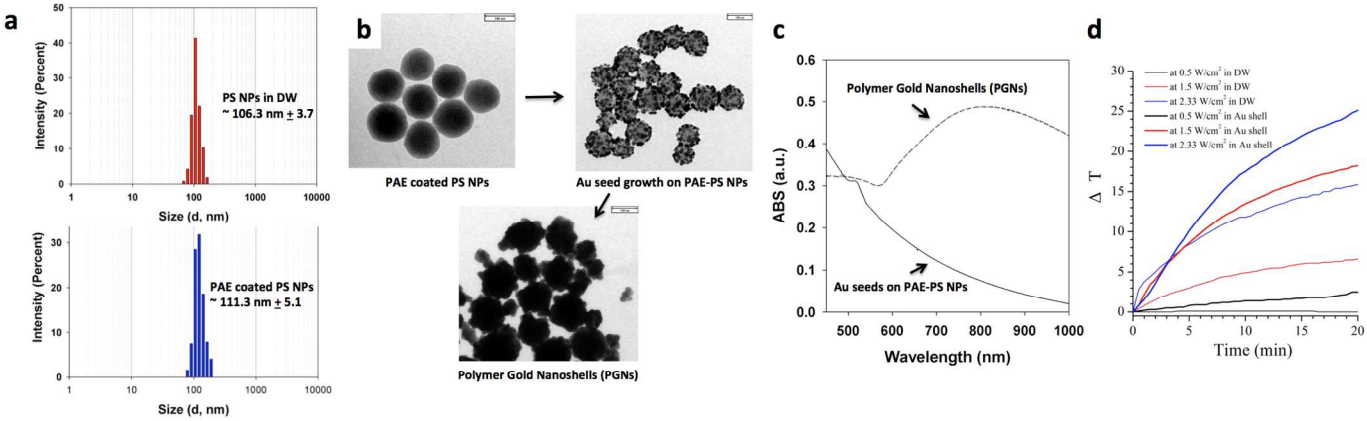


Figure. 1. Characteristics of Polymer Gold Nanoparticles (PGNs): (a) DLS measurement of PS NPs and PAE coated PS NPs; (b) TEM images of PAE coated PS NPs, Au seeds on PAE-PS NPs, and PGNs; (c) UV/VIS spectrum of PGNs showing strong absorption at NIR spectrum; (d) Photothermal property of PGNs by the NIR laser treatment at three different laser intensities (0.5, 1.5, 2.33 W/cm^2)

after 12 h incubation followed by a laser irradiation (**Figure S3**). The PGN-siRNA conjugates have shown negligible viability changes in HeLa cells under the tested NIR exposure condition. For up to 10 min exposure, no viability change was observed in both light intensity of 1.5 and 2.4 W/cm^2. After 20 min exposure, only slight decrease of cell viability (79.4 %) was observed at the higher intensity of 2.4 W/cm^2

To test the intracellular delivery of siRNAs and gene silencing, various concentrations of siRNA immobilized PGNs (siRNA concentration: 25, 50, 100 nM) were treated to the GFP expressing HeLa cells. After 2 hr incubation, NIR-laser induced photothermal treatment was applied for the laser-treatment group with a laser intensity of 1.5 W/cm^2 for 5 min. Both cells of NIR-laser treated and non-treated groups were further incubated for 1 days and their relative fluorescence (FL) intensity was evaluated by FACS analysis (n=3). As shown in **Figure 2b**, GFP-HeLa cells treated with the NIR-laser markedly showed enhanced GFP silencing, resulting more than 27.5 % reduction of FL intensity at 25 nM siRNA concentration as compared to the 10.8 % of non-laser treated GFP-HeLa cells. NIR-laser treated GFP-HeLa cells also exhibited dose-responsive GFP gene silencing achieving up to 51.7 % reduction of FL intensity at 100 nM of siRNA concentration. Intracellular trafficking and co-localization of PGN-siRNA conjugates with endosomal markers also supported the facilitated release of siRNAs into the cytoplasm under NIR exposure (**Figure S4**). This result is well matched with a previous study that more strong cy5 labeled siRNA signals were observed throughout the cytoplasm after a laser treatment.[2]

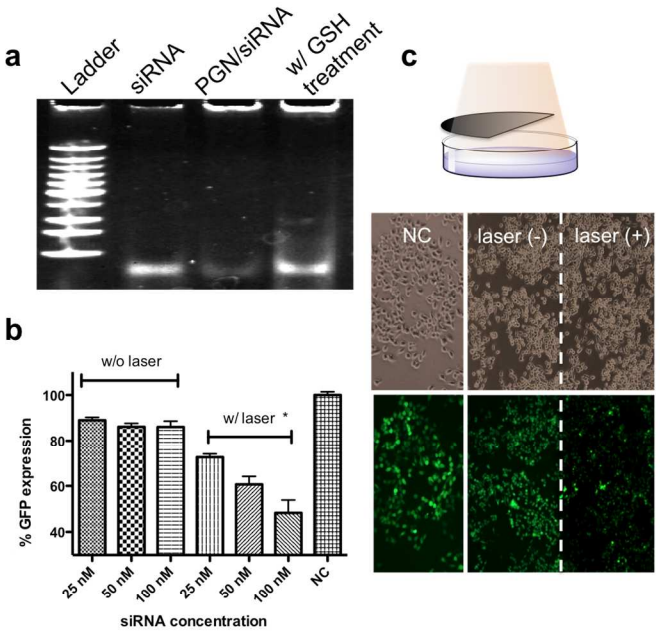


Figure 2. (a) GSH sensitive release of siRNAs from PGN-siRNA conjugates (GSH ~ 10 mM); (b) GFP gene silencing in HeLa cells treated with three different concentrations of PGN-siRNA conjugates without and with NIR-laser exposure (n=3, *statistical difference of $p < 0.05$ as compared to the non-laser treated group); (c) Spatial GFP gene regulation by a photo-masking of NIR-laser exposure on the monolayer cell culture. (NC: negative control without treatment).

In addition, the spatial control of gene expression on the monolayer cultured GFP-HeLa cells has been exploited using a photo-masked cover lid, which can expose the NIR-laser on the selective region of cell monolayer **(Figure 2c)**. After treatment with the NIR-laser (1.5 W/cm^2) for 5 min, the cells were incubated for 24 hrs and fluorescence images were obtained. The images have revealed that the only cells resided on the clear region of culture plate exhibited the selective GFP silencing as compared to that of photo-masked region. From the result, it is confirmed that the spatial regulation of GFP expression can be achieved using PGN-siRNA conjugates by an external NIR stimulus.

In conclusion, we have demonstrated a facile preparation method of gold nanoshell formation on various polymeric nanoparticles, which can be applied for the delivery of siRNAs. Photothermal behaviors of prepared PGNs have been exploited that they can induce enough heat generation to facilitate the endosomal release of cargo materials, thus, our model drug, siRNA can be directly delivered to the cytoplasm to trigger the RNA interference (RNAi). This study exemplifies the usefulness of versatile gold shell synthesis on polymer nanoparticles by providing multiple functionalities such as facile surface immobilization, dramatic changes of optical property, and finally the photothermal behavior. Our approach can be applicable on various studies requiring the external control of drug release from carriers.

**This work is supported by the National Research Foundation of South Korea: Grant number (2012K1A1A2A01056092; 2012M3A9C6049834; 2012R1A1A1039030), and the Ministry of Health & Welfare of South Korea (HI12C0174).

Notes and references

[a]Graduate School of Pharmaceutical Sciences, College of Pharmacy, Ewha Womans University, Seoul 120-750, Republic of Korea.
[b]Department of Chemistry, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, Republic of Korea.
*Corresponding author's contact email: hyukjin@ewha.ac.kr

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

1 A. Wijaya, S.B. Schaffer, I.G. Pallares, and K. Hamad-Schifferli, *ACS Nano*, 2009, **3**, 80.; R. Huschka, J. Zuloaga, M.W. Broun, P. Nordlander, and N.J. Halas, *J. Am. Chem. Soc.*, 2011, **133**, 12247.
2 G.B. Braun, A. Pallaoro, G. Wu, D. Missirlis, J.A. Zasadzinski, and M. Tirrell, *ACS Nano*, 2009, **3**, 2007; W. Lu, G. Zhang, R. Zhang, L.G. Flores, Q. Huang, J.G. Gelovani, and C. Li, *Cancer Res.*, 2010, **70**, 3177.
3 J. Yang, J. Lee, J. Kang, S.J. Oh, H. Ko, J. Son, K. Lee, J. Suh, Y. Huh, and S. Haam, *Adv. Mater*, 2009, **21**, 4339; D.D. Young, H. Lusic, M.O. Lively, J.A. Yoder, and A. Deiters, *Chembiochem*, 2008, **9**, 2937.

4 J. Yang, J.Y. Lee, and H.P. Too, *J. Phys. Chem. B.*, 2005, **109**, 19208; W. Shi, Y. Sahoo, M.T. Swihart, and P.N. Prasad, *Langmuir*, 2005, **21**, 1610

5 S. Hong, J. Yeom, I.T. Song, S.M. Kang, H. Lee, and H. Lee, *Adv. Mater. Interfaces*, 2014, 1400113

6 H. Ejima et al. *Science* 2013, **341**, 154

7 H. Lee et al. *Science* 2007, **318**, 426; S. Hong et al. *Angew Chemie Int. Ed.* 2013, **52**, 9187

8 X.H. Huang, P.K. Jain, I.H. El Sayed, and M.A. El Sayed, *Photochem. Photobiol.* 2006, **82**, 412; O. Ekici, R.K. Harrison, N.J. Durr, D.S. Eversole, M. Lee, and A. Ben Yakar, *J. Phys. D: Appl. Phys.* 2008, **41**, 185501.

9 D.S. Seferos, D.A. Giljohann, C.S. Thaxton, A.K.R. Lytton-Jean, M.S. Han, and C.A. Mirkin, *Science*, 2006, **312**, 1027; S.J. Hurst, A.K.R. Lytton-Jean, C.A. Mirkin, *Anal. Chem.*, 2006, **78**, 8313.

10 D.A. Giljohann, et al. *Angew Chem Int Ed* 2010, **49**, 3280; C.H. Choi, L. Hao, S.P. Narayan, E. Auyeung, and C.A. Mirkin, *PNAS* 2013, **110**, 7625;

11 H. Lee, K. Lee, I.K. Kim, and T.G. Park, *Biomaterials*, 2008, **29**, 4709.

