Development and Characterization of Protein-gold-nanoparticle Conjugates bearing Photocleavable Polymers

Shota Yamamoto¹, Jun Nakanishi² and Kazuo Yamaguchi¹,

¹Department of Chemistry and Research Institute for Photofunctionalized Materials, Kanagawa University, 2946 Tsuchiya Hiratsuka-shi Kanagawa, 259-1293 Japan
²WPI Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science (NIMS), 1-1 Namiki, Tsukuba, Ibaraki, 305-0044 Japan

Keywords: caged compounds, epidermal growth factor, cell signaling, phosphorylation

1. Introduction
Conjugation of diffusible biosignal molecules to solid substrates or soluble nanoparticles (NPs) adds new modalities of biological activities to original ligands due to the changes in diffusivity and intracellular trafficking [1, 2]. For example, Reinhard and coworkers reported that epidermal growth factor (EGF) showed enhanced apoptosis-inducing activity to cancer cells upon conjugating to gold nanoparticles (GNPs) [3]. Therefore, such bioconjugates serve as promising tools not only for fundamental biological studies but also for biomedical applications. However, in most studies, their detailed mechanisms of their actions have not been clarified.

Caged compounds are biomolecules whose activity is temporarily-suppressed by conjugation of a photocleavable protecting group [4]. Upon photocleavage of the protecting group, they retrieve their original activity to switch on biological responses. Because of this feature, caged compounds are useful to spatiotemporally resolve the signal transduction mechanisms of the corresponding biomolecules. As a robust platform for the facile preparation of caged compounds, we have previously reported on GNPs functionalized with photocleavable activated ester together with poly(ethylene glycol) (PEG) [5]. Thus photofunctionalized GNPs can react with primary amines and release them in response to photoradiation. Moreover the biological activity of the primary amines is suppressed while they are conjugated to the GNPs since the bulky nanoparticles as well as PEG chains block the access of immobilized ligand to its cellular reactive sites, whereas the amines gain activity upon releasing from the nanoparticles by photoradiation. In the previous study, we have

Scheme 1. Synthetic route of photoactivatable EGF-GNP conjugates and their activation by photoreleasing PEG.

Received April 1, 2015
Accepted May 11, 2015
demonstrated this strategy for the photocontrolled activation of an intracellular signaling by using diffusible histamine as a model amine compound.

In this study, we applied the similar strategy to develop photoactivatable EGF-GNP conjugates (Scheme 1), aiming to spatiotemporally resolve the molecular mechanisms of their biological actions. The surface of GNPs was functionalized with EGF together with photocleavable PEG (1) to obtain photoactivatable EGF-GNP conjugates. We expected that the biological activity of EGF molecule conjugated to the GNPs was suppressed before irradiation because of the presence of co-immobilized PEG and it became activated by photocleaving the PEG brushes from the surface of nanoparticle. As the proof-of-concept experiments, the photo-induced PEG release from the nanoparticles and the biological activity change of the conjugates were examined by dynamic light scattering and enzyme-like immunosorbent assay (ELISA) experiments, respectively.

2. Method
2.1 Materials
All reagents were purchased from Wako unless otherwise stated. ω-Methoxy-poly(ethylene glycol) amine (mPEG-NH₂, Mw = 5,000) and disuccinimidyl 11,11’–dithiobisundecanoate (DSU) were obtained from Sunbright and Dojindo, respectively. A gold nanoparticle solution (ϕ = 15 nm, BBI solutions) was used as received. A disulfide molecule bearing activated ester via photocleavable 2-nitrobenzyl group, bis(12-(4-(1-(succinimidyloxycarbonyloxy)ethyl)-2-methoxy-5-nitrophenoxy)dodecyl) disulfide (2) was synthesized according to the previous paper [5]. Photocleavable PEG (1) was synthesized by mixing 5 mM of 2, 5 mM mPEG-NH₂, and 10 mM triethylamine at 1:2:1 volume ratio in dimethylsulfoxide (DMSO) at room temperature overnight and the solution was used for the following GNP functionalization without purification.

2.2 Preparation of photoactivatable EGF-GNP conjugates
Six mL of the GNP solution (OD₅₂₀ = 1) was concentrated by spin-filtration with Vivaspin 20 (GE Healthcare, MWCO 30,000) at 9,000 rpm for 30 min, followed by functionalizing with 5 mM of 1 and 5 mM of DSU mixed at 8:2 volume ratio in a 9:1 DMSO/water mixed solution at room temperature. The reacted GNPs were washed with DMSO by centrifugation (16,000 rpm, 60 min, room temperature, 3 times). At the final centrifugation step, GNPs were suspended in 20 μL of DMSO. To this solution, 317 μL of phosphate buffer saline (PBS) containing 20 μL of PBST and 22 μL of EGF solution (0.6 mg/mL) was added, and then it was shaken at 4°C overnight. After reaction, GNPs were washed with PBST by centrifugation (14,000 rpm, 30 min, 4°C, 6 times). The remaining EGF molecule in the system during the washing step was determined by direct ELISA using anti-EGF rabbit antibody (Abcam) and anti-rabbit IgG horse radish peroxidase (Sigma) with 3,3',5,5'-tetramethylbenzidine (TMB) as a substrate. The signal was detected by a plate reader (Biorad).

2.3 Characterization of the photoactivatable EGF-GNP conjugates and their photoirradiation
The average number of EGF molecule immobilized to gold nanoparticle (stoichiometry) was determined from the decrease in the EGF concentration before and after the reaction with the functionalized GNPs. The hydrodynamic radius and polydispersity of the photoactivatable EGF-GNP conjugates were evaluated at room temperature by dynamic light scattering (DLS). The photoactivatable EGF-GNP conjugates were irradiated in the same as those described previously [5]. Briefly, a GNPs solution was placed in a quartz crystal glass cell (OD₅₂₀ = 1) and irradiated with near-UV light for a given energy (3 J and 7 J) by using a Hg arc lamp (USHIO, BA-H250), Optical Modulex (USHIO, H250) and a band-pass filter (λ = 300–400 nm, HOYA).

Fig. 1. Direct ELISA study on the concentration of free EGF after each centrifugation. The data of the first supernatant is not shown because of its extremely high concentration. Error bars represent standard deviations of data from 3 wells.
Fig. 2. DLS study on the change in the particle distribution of photoactivatable EGF-GNP conjugates upon photoirradiation. The conjugates were irradiated with near-UV light for (thin line) 3 J, (bold line) 7 J and (dotted line) without irradiation.

Fig. 3. Photoactivation of biological activity of EGF-GNP conjugates. The conjugates were exposed to HeLa cells after near-UV irradiation for a given energy (0, 3 and 7 J) and the phosphorylation of level of ERK was evaluated by cell ELISA. Error bars represent standard deviations of data from 3 wells. All phosphorylation levels are normalized to the reference condition: 5 min of incubation with 1000 ng/mL free EGF.

2.4 Cell ELISA
HeLa cells were obtained from American type culture collection (ATCC) and cultured in MEM (Sigma) containing 10% FBS (BioWest), 100 units/mL penicillin and 100 mg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂ and subcultured with trypsin every 2–3 days. To start cell ELISA experiments, the cells were seeded in a 96-well plate (20,000 cells/well) in the medium a day before and serum-starved for 4 h in 50 μL of the medium without serum on the day of experiment. Fifty μL of the serum-free medium containing 5 μL of the photoactivatable EGF-GNP conjugate solution irradiated for a given energy (0 J, 3 J or 7 J) was added to each well and incubated for 5 min until an addition of 4% paraformaldehyde in PBS to stop the reaction. After 30 min, the cells were permeabilized by 0.5% triton X in PBS for 20 min, and then blocked with 2% bovine serum albumin (BSA) in PBS. Phospho-ERK1/2 (Thr202/Tyr204) rabbit monoclonal antibodies (CST) and anti-rabbit IgG alkaline phosphatase (Sigma) were used as primary and secondary antibodies, respectively. Each antibody was diluted to 1:1000 and 1:3000 with PBS containing 1% BSA, and the cells were incubated with the solutions for 1 h. Finally, the reacted antibodies were detected by using 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) as a substrate.

3. Results and discussion
3.1 Preparation of photocleavable PEG and photoactivatable EGF-GNP conjugates
Scheme 1 represents the synthetic procedure of the photoactivatable EGF-GNP conjugates and their photoactivation reaction. A mixed solution (8:2, v/v) of 1 and DSU was reacted with concentrated GNPs, thereafter EGF was immobilized via the reaction of N-succinimidyl ester and the amino group of the EGF molecule. After reaction, unreacted EGF molecules were removed from the system by centrifugation, which was monitored as the change in the EGF concentration of each supernatant (Fig. 1). Complete removal of free EGF is critical since we want to focus on the cellular responses induced by the EGF-GNP conjugates rather than free EGF. After centrifugation for 5 and 6 times, there was no change in EGF concentration in the supernatant. The saturated concentration was sufficiently lower than 0.1 ng/mL, which is the minimum EGF concentration that evokes cellular responses in the free form. Therefore, we adapted 6 times centrifugation for the following study. Also, the average number of EGF molecules immobilized to the GNPs surface can be calculated from the EGF concentration of the first supernatant and the GNP concentration determined from the absorbance measurement. By considering the decrease in the EGF concentration being 1.7 × 10⁴ ng/mL and the GNP concentration 4.0 × 10⁻¹¹ mol/mL, the stoichiometry of EGF vs. GNP was determined to be 58 molecule/particle.
3.2 Characterization of the photoactivatable EGF-GNP conjugates and their photoirradiation

The particle size distribution of photoactivatable EGF-GNP conjugates was analyzed by DLS (Fig. 2). The average diameter of commercial GNPs was 16 nm (data not shown), whereas it became 46 nm by immobilizing photocleavable PEG, 1, and EGF to yield photoactivatable EGF-GNP conjugates (Fig. 2, dotted line). When the photoactivatable EGF-GNP conjugates were exposed to near-UV light, we observed gradual decrease in the particle size. After irradiation for 3 J, the average diameter became 37 nm (Fig. 2, thin line). Further decrease in the diameter to 29 nm was observed by irradiation for 7 J (Fig. 2, bold line). These results indicated that release of PEG from the particle surface and successful reduction of the surface PEG density on the particle surface by photoirradiation. We did not further increase the irradiation time, as we knew the photocleavage reaction is almost completed with this dose of photoirradiation from the previous study [5].

3.3 Cell ELISA

Finally, we examined the change in the biological activity of the photoactivatable EGF-GNP conjugates in response to the near-UV irradiation. ERK (extracellular signal-regulated kinase) is an essential serine/threonine protein kinase, which is activated upon stimulation of cells with EGF. Upon activation, it undergoes phosphorylation at the Thr202/Tyr204 residues for the ERK1/2 subtypes. The biological activity changes of photoactivatable EGF-GNP conjugates in response to photoirradiation were evaluated in HeLa cells by cell ELISA based on their ability to phosphorylate these residues. The results are shown in Fig. 3. Before photoirradiation, the activity of ERK was small. However, the activity became progressively larger as increasing photoirradiation time. These results indicate that, before photoirradiation, EGF is buried in PEG brushes, so the activity of EGF is lowered. On the other hand, upon decreasing the surface PEG density by photoirradiation, the activity of the EGF-GNP conjugates increases due to the decrease in the molecular crowding on the surface of nanoparticles. Further enhancement in the difference between the activity before and after photoirradiation will be expected by choosing a suitable molecular weight of PEG chains to completely suppress biological activity at the resting state.

4. Conclusion

We have developed photoactivatable EGF-GNP conjugates by co-immobilizing photocleavable PEG and EGF on the surface of gold nanoparticles. The DLS study demonstrated the release of the grafted PEG as the decrease in the particle size. This reaction is expected to decrease the molecular crowding surrounding the immobilized EGF at the nanoparticle surface. The increase in the biological activity of the conjugates in response to near-UV irradiation was demonstrated in HeLa cells by monitoring the phosphorylation level of ERK by cell ELISA. These results represent usefulness of the present strategy for caging/uncaging EGF-GNP conjugates and their potential applications to spatiotemporally resolved mechanism study of such bioconjugates.

References