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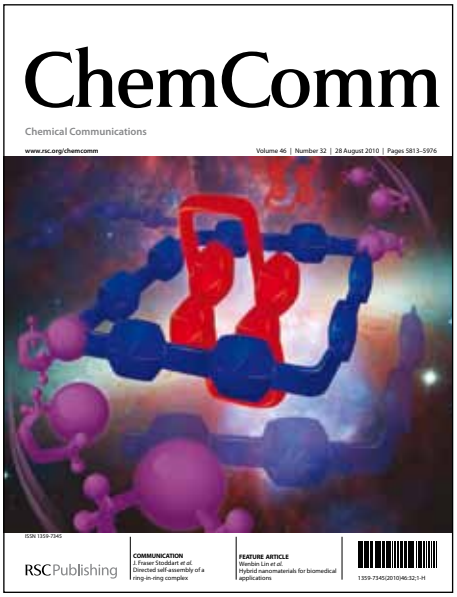

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Enhanced Cellular Uptake of Amphiphilic Gold Nanoparticles with Ester Functionality

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Received (in XXX, XXX) XthXXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Gold nanoparticles (AuNPs) coated with ester-headed or ether-headed PEG ligands were synthesized. Ester-headed AuNPs, but not ether-headed, were transferred from organic phase (CH_2Cl_2) to alkali aqueous phase, indicating that the hydrolysis of the ester moiety triggered the phase transfer of the AuNPs. We found that AuNPs with ester-headed ligands (Ester-AuNPs) were internalized into HeLa cells at a greater level than were ether-headed AuNPs.

Gold nanoparticles (AuNPs) have been the focus of much attention as an attractive material for medical uses,[1] such as a tool for photothermal therapy,[2] biosensing device[3] and drug delivery carrier.[4] To achieve these purposes, the features of the surface ligands displayed on AuNPs play a crucial role because the ligands determine the properties of the nanoparticles,[5] such as their colloidal stability in serum,[6] the level of cellular uptake,[7] cytotoxicitity,[8] and accumulation organs in the body.[8] For effective drug delivery, appropriate functionalization of the carrier surface is particularly important. Hydrophilicity is required for the stable dispersion of nanoparticles in serum media; however, the hydrophobicity of the nanoparticle surface enhances their affinity to the cellular membrane, thereby facilitating its internalization into cells.[5,9] To satisfy these two contradictory requirements and promote cellular uptake, we focused on stimuli-responsive changes to the nanoparticle surface.

There have been several reports on nanoparticles with external stimuli-induced variable surfaces, such as pH,[10] light[11–13] and sugar[14]. For instance, Rotello and coworkers reported a photocleavable ester ligand that can convert a cationic surface to an anionic surface in cells to release drugs upon UV irradiation.[13] However, for the *in vivo* use of such stimuli-responsive nanoparticles, it is preferable that changes to the nanoparticle surface can be induced by biocompatible and internal stimuli without external stimuli.

In this paper, we propose an ester ligand coating as a stimuli-responsive surface to accelerate AuNP internalization into cells via esterase-catalysed hydrolysis of ester moieties within cells. In prodrug technology, ester capping of carboxylic acid has already been used to promote the cellular uptake of drugs.[15] The ester capping of prodrugs can be detached with esterase in cells to produce the original drug. However, the application of the ester capping method has been limited to the internalization of small molecules. Our aim in this study is to clarify whether AuNPs with a terminal ester moiety can effectively enter cells as demonstrated previously using small molecules.

Previously we reported that AuNPs coated with alkyl ether-headed PEG ligands can be transferred from an aqueous phase to chloroform phase due to their flexible PEG ligands.[16] This phase transference property affords a high affinity to the hydrophobic cell membrane from the media; however, it lacks the necessary force to cross the cellular membrane into the cells. In this paper, "ester"-headed PEG ligands were synthesized as a capping reagent for AuNPs and the level of cellular uptake was compared to corresponding ether-headed ligands. Ester-AuNPs are expected to be transferred from the membrane into cells on cleavage of the ester moiety by esterase.

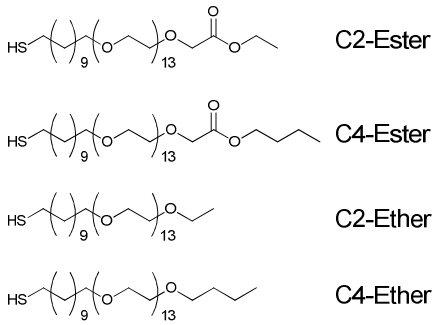

Fig. 1 PEG-derivative ligands for the surface modification of AuNPs.

PEG-derivative ligands bearing ethyl ester (C2-Ester), *n*-butyl ester (C4-Ester), ethyl ether (C2-Ether) or *n*-butyl ether (C4-Ether) at their termini were synthesized as shown in Fig. 1. These ligands were then conjugated to AuNPs of 10 nm in diameter. We chose 10 nm as the diameter for the AuNPs as AuNPs of this size are large enough to simultaneously reach the outer and inner space of cells when they pass through the membranes. The mixed solution of each ligand with BSPP-coated AuNPs (BSPP: Bis(*p*-sulfonatophenyl)phenylphosphinedihydrate dipotassium salt) was let stand for 2 hrs, followed by centrifugal purification to remove free ligands. The hydrodynamic diameters of the four resultant AuNPs were measured by dynamic light scattering (DLS) in a serum-reduced medium (Opti-MEM). BSPP-AuNPs were precipitated soon after dispersion in Opti-MEM; however, C2-Ester-AuNPs and C2-Ether-AuNPs were well-dispersed in Opti-MEM and maintained their original sizes (12.4 nm and 12.7 nm, respectively) for over 20 hrs without aggregation (see DLS time-course in Fig. S1). On the other hand, C4-Ester- and C4-Ether-AuNPs tended to form sub-micron size aggregates within a few hours (ca. 700 nm) but did not precipitate. Therefore, there is a hydrophobic threshold between C2 and C4 that causes the

aggregation of AuNPs.

We examined the phase transfer of C2-AuNPs from phosphate-buffered saline (PBS) to dichloromethane. Dispersions of C2-Ester- and C2-Ether-AuNPs in PBS were slowly mounted on dichloromethane to form a heterogeneous system (Fig. 2a, left). After 2 days, both of AuNPs were transferred from PBS phase to dichloromethane phase (Fig. 2a, middle), which is in agreement with the results of our previous report on alkyl ether-headed AuNPs.[16] Subsequently, a NaOH solution was added to the aqueous layer and let stand for 1 day. The C2-Ester-AuNPs went back to the PBS phase from organic layer, whereas C2-Ether-AuNPs remained in the organic phase even after the addition of NaOH solution (Fig. 2a, right). This phenomenon indicates that the hydrolysis of the ester group at the interface is the driving force of the phase transfer of AuNPs from organic phase to aqueous phase. C4-Ester- and C4-Ether-AuNPs showed a similar tendency in regard to phase transfer (data not shown).

This hydrolysis-triggered phase transfer would support our hypothesis that the esterase promoted AuNP translocation from the hydrophobic membrane to the hydrophilic environment inside cells. In order to confirm that the ester-headed ligands immobilized on AuNPs can be a substrate of esterase, we next examined the hydrolysis of C2-Ester ligands on AuNPs by esterase for 6 hrs. C2-Ester- and C2-Ether-AuNPs were treated with esterase from porcine liver and subjected to electrophoresis on 1.5% agarose gel. Before treatment with esterase, the C2-Ester and C2-Ether-AuNPs showed similar mobility, and only the mobility of the C2-Ester increased after esterase treatment (Fig. 2b), indicating that the C2-Ester ligands on the AuNPs were converted to carboxylic acid. The exchange reaction of the C2-Ester and C2-Ether ligands on BSPP-AuNPs was confirmed by a comparison of their mobility with BSPP-AuNPs. The anionic BSPP-coated AuNPs showed much larger mobility than neutral C2-Ester- and C2-Ether-AuNPs, indicating the replacement of BSPP with PEG-ligands.

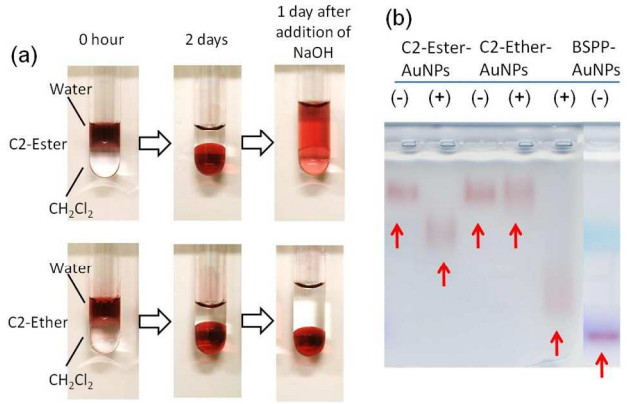

Fig. 2 (a) Phase transfer of AuNPs (Top: C2-Ester-AuNPs, Bottom: C2-Ether-AuNPs), (b) Electrophoresis of AuNPs before (-) and after (+) treatment with esterase.

AuNPs were administrated to HeLa cells and the level of cellular uptake was measured and evaluated by inductively coupled plasma atomic emission spectroscopy (ICP-AES) to compare the influence of the surface ligand. HeLa cells (1.0 x 10^5 cells/well, in Opti-MEM) were incubated with AuNPs (10 nM) at 37°C for the indicated time (3 or 24 hrs). After administration, serum was washed out and AuNPs taken up into the cells were ionized by aqua regia and then subjected to ICP analysis. Fig. 3 shows the relationships among surface ligands, incubation time and the number of AuNPs taken up into HeLa cells per well. After incubation for 3 hrs, few C2-Ether-AuNPs were taken up into the HeLa cells, whereas a large number of C2-Ester-AuNPs were internalized. Extension of the incubation time to 24 hrs resulted in a larger cellular uptake of AuNPs, with the C2-Ester still superior to the corresponding C2-Ether. Although C4-Ester-AuNPs aggregate in Opti-MEM (Fig. S1), the same trend was observed in the comparison with ether derivatives; C4-Ester-AuNPs showed a higher uptake than did C4-Ether-AuNPs. Thus, independent of aggregational status, ester-headed AuNPs were more facile than ether-headed AuNPs with regard to cellular uptake.

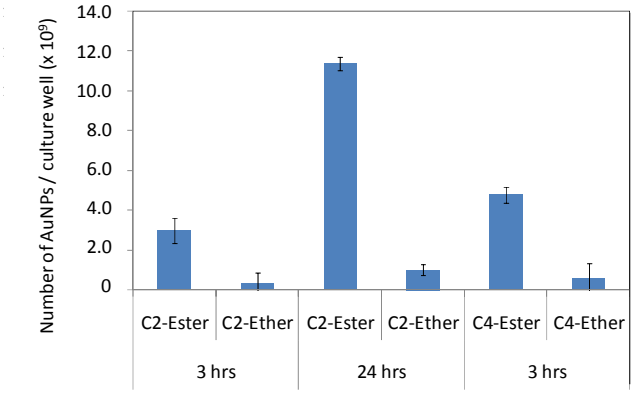

Fig. 3 The number of AuNPs internalized into HeLa cells per well (1.0 x 10^5 cells/well).

Internalization of AuNPs and their location were confirmed by transmission electron microscope (TEM) imaging. Fig. 4 shows a TEM image of HeLa cells after incubation for 3 hrs in the presence of C2-Ester-AuNPs. C2-Ester-AuNPs were mainly found in the multivesicular bodies (MVBs), although a small number of AuNPs were also found within the cytosol (red arrows in Fig. 4, also see Fig. S2 for an enlarged view and additional image). This implies that the main internalization pathway is endocytotic uptake, although some C2-Ester-AuNPs were internalized into cells via membrane permeation. Fig. S3 shows a time course of TEM images of HeLa cells after incubation with C2-Ester-AuNPs. After 5 min, some AuNPs were translocated inside cells near the plasma membrane in a monodispersed state. The internalized AuNPs were observed in small vesicles in the cell at 30 min, and after 1 hr, the AuNPs were gathered in the MVBs. The aggregated C4-Ester-AuNPs were also internalized in cells (Fig. S4). Therefore, the cellular internalization of AuNPs bearing ester functionality was more effective than that of ether derivatives independent of aggregation status.

To verify the mechanism of cellular internalization of AuNPs, C2-Ester-AuNPs were administrated to HeLa cells in the presence of Chlorpromazine hydrochloride, 5-(*N*-ethyl-N-isopropyl)amiloride, or Genistein all of which are well-known as endocytosis inhibitors,[17] at a final concentration of 3, 4, or 40 μM, respectively. In the presence of those inhibitors, the number of C2-Ester-AuNPs taken up into the HeLa cells was clearly reduced after incubation for 3 hrs in all cases (Fig. S5). Consequently, the main mechanism of the internalization was found to be endocytosis. We propose the following hypothesis to rationalize the preferential uptake of ester type ligands. Both hydrophobic ester and ether terminal moieties have high affinity to the cellular membrane. However, the hydrolysis of the ester group by esterase at the cellular membrane creates hydrophilic

characteristic which enables longer retention at the membrane, so promoting their uptake into the cell via endocytosis.

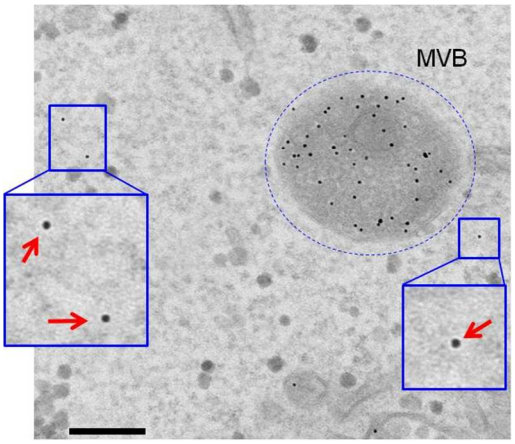

Fig. 4 Ultra-thin section image of HeLa cells observed by TEM. HeLa cells were incubated for 3 hrs in the presence of C2-Ester-AuNPs and then fixed by 2.5% glutaraldehyde. Red arrows indicate nanoparticles within the cytosol. Scale bar: 200 nm.

Conclusions

In conclusion, ester-headed PEG ligands (C2-Ester and C4-Ester) for the modification of the AuNP surface were synthesized and the advantages of these ester ligands over ether-headed PEG ligands (C2-Ether and C4-Ether) with regard to cellular uptake were demonstrated. C2-Ester-AuNPs were monodispersed in an aqueous solution (PBS) and transferred to an organic phase (CH_2Cl_2). C2-Ester-AuNPs in PBS can cross from CH_2Cl_2 to a PBS phase by the addition of NaOH as a stimulus. The detail mechanism underlying their accelerated internalization into cells remains unclear; however, the hydrolysis-triggered aqueous/organic phase transfer suggests that the esterase hydrolysis of ligands could induce the internalization of AuNPs into cells. Our finding regarding the advantages of ester ligands will be applicable to deliver the various functional nanoparticles into cells.

This work was supported by KAKENHI 22655050: Grant-in-Aid for Challenging Exploratory Research. Dr. S. Sekiguchi appreciates the financial support provided by JSPS. We appreciate Ms. T. Kikitsu in Material Characterization Team (RIKEN) for TEM image analysis, Ms. K. Otsuki, Mr. M. Usui, and Ms. A. Abe in Mass Spectrometry Service (RIKEN) for MALDI-TOFMS analysis.

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† Electronic Supplementary Information (ESI) available: [syntheses of ligands, preparation of AuNPs, experimetnals]. See DOI: 10.1039/b000000x/

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1 E. Boisserlier and D. Astruc, *Chem. Soc. Rev.*, 2009, **38**, 1759; D. A. Giljohann, D. S. Seferos, W. L. Daniel, M. D. Massich, P. C. Patel and C. A. Mirkin, *Angew. Chem. Int. Ed.*, 2010, **49**, 3280.
2 Z. Qina and J. C. Bischof, *Chem. Soc. Rev.*, 2012, **41**, 1191.
3 K. Saha, S. S. Agasti, C. Kim, X. Li and V. M. Rotello, *Chem. Rev.*, 2012, **112**, 2739.
4 V. Sokolova and M. Epple, *Angew. Chem. Int. Ed.,* 2008, **47**, 1382; B. Duncanm, C. Kim and V. M. Rotello, *J. Control. Release*, 2010, **148**, 122.
5 S. J. Tan, N. R. Jana, S. Gao, P. K. Patra and J. Y. Ying, *Chem. Mater.,* 2010, **22**, 2239.
6 C. Kinnear, H. Dietsch, M. J. D. Clift, C. Endes, B. Rothen-Rutishauser and A. Petri-Fink, *Angew. Chem. Int. Ed.,* 2013, **52**, 1934.
7 A. Vermai, O. Uzun, Y. Hu, Y. Hu, H.-S. Han, N. Watson, S. Chen, D. J. Irvine and F. Stellacci, *Nature Mater.*, 2008, **7**, 588; C. M. Jewell, J.-M. Jung, P. U. Atukorale, R. P. Carney, F. Stellacci and D. J. Irvine, *Angew. Chem. Int. Ed.,* 2011, **50**, 12312.
8 S. T. Kim, K. Saha, C. Kim and V. M. Rotello, *Acc. Chem. Res.*, 2011, **46**, 681.
9 A. R. Maity and N. R. Jana, *J. Phys. Chem. C,* 2011, **115**, 137; A. Lesniak, A. Salvati, M. J. Santos-Martinez, M. W. Radomski, K. A. Dawson and C. Åberg, *J. Am. Chem. Soc.*, 2013, **135**, 1438.
10 E. W. Edwards, M. Chanana, D. Wang and H. Möhwald, *Angew. Chem. Int. Ed.,* 2008, **47**, 320; Z. Mao, J. Guo, S. Bai, T.-L. Nguyen, H. Xia, Y. Huang, P. Mulaney and D. Wang, *Angew. Chem. Int. Ed.,* 2009, **48**, 4953; Y. Imura, C. Morita, H. Endo, T. Kondo and T. Kawai, *Chem. Commun.*, 2010, **46**, 9206; P. P. Pillai, S. Huda, B. Kowalczyk, and B. A. Grzybowski, *J. Am. Chem. Soc.,* 2013, **135**, 6392.
11 E. A. Osborne, B. R. Jarrett, C. Tu and A. Y. Louie, *J. Am. Chem. Soc.*, 2010, **132**, 5934; J. Buback, M. Kullmann, F. Langhojer, P. Nuerberger, R. Schmidt, F. Würthner and T. Brixner, *J. Am. Chem. Soc.*, 2010, **132**, 16510; Y. Song, C. Xu, W. Wei, J. Ren and X. Qu, *Chem. Commun.*, 2011, **47**, 9083.
12 N. Shao, J. Jin, H. Wang, J. Zheng, R. Yang, W. Chan and Z. Abliz, *J. Am. Chem. Soc.,* 2010, **132**, 725; R. Tong, H. D. Hemmati, R. Langer and D. S. Kohane, *J. Am. Chem. Soc.*, 2012, **134**, 8848; F. Jonsson, T. Beke-Somfai, J. Andréasson and B. Nordén, *Langmuir,* 2013, **29**, 2099.
13 G. Han, C.-C. You, B.-j. Kim, R. S. Turingan, N. S. Forbes, C. T. Martin and V. M. Rotello, *Angew. Chem. Int. Ed.*, 2006, **45**, 3165; C. Subramani, X. Yu, S. S. Agasti, B. Duncan, S. Eymur, M. Tonga and V. M. Rotello, *J. Mater. Chem.*, 2011, **21**, 14156.
14 D. Roy and B. S. Sumerlin, *ACS Macro Lett.*, 2012, **1**, 529.
15 C. A. H. Prata, Y. Zhao, P. Barthelemy,Y. Li, D. Luo, T. J. McIntosh, S. J. Lee and M. W. Grinstaff, *J. Am. Chem. Soc.*, 2004, **126**, 12196; C. C. Woodroofe, A. C. Won and S. J. Lippard, *Inorg. Chem.*, 2005, **44**, 3112; L. D. Lavis, *ACS Chem. Biol.*, 2008, **3**, 203; C. M. LaManna, H. Lusic, M. Camplo, T. J. McIntosh, P. Barthélémy and M. W. Grinstaff, *Acc. Chem. Res.*, 2012, **45**, 1026; L. Tian, Y. Yang, L. M. Wysocki, A. C. Arnold, A. Hu, B. Ravichandran, S. M. Sternson, L. L. L. Looger and L. D. Lavis, *Proc. Natl. Acad. Sci. USA*, 2012, **109**, 4756.
16 S. Sekiguchi, K. Niikura, Y. Matsuo and K. Ijiro, *Langmuir*, 2012, **28**, 5503.
17 M. Tretz, J. Jin, R. Conibere, N. A. Penning, S. Al-Taei, G. Strom, S. Futaki, T. Takeuchi, I. Nakase and A. T. Jones, *J. Control. Release* 2006, **116**, 247; D. Vercauteren, R. E. Vandenbroucke, A. T. Jones, J. Rejman, J. Demeester, S. C. De Smedt, N. N. Sanders and K. Braeckmans, *Mol. Ther.*, 2010, **18**, 561; S. Pan, D. Cao, R. Fang, W. Yi, H. Huang, S. Tian and M Feng, *J. Mater. Chem. B*, 2013, **1**, 5114; E. A. Untener, K. K. Comfort, E. I. Maurer, C. M. Grabinski, D. A. Comfort, and S. M. Hussain, *ACS Appl. Mater. Interfaces* 2013, **5**, 8366.