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Protein-coated nanocapsules *via* multilevel surface modification. Controlled preparation and microscopic analysis at nanometer resolution†

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We prepared self-assembled vesicles comprising layers of fullerene, alkyl chains, triazole, oligo(ethylene oxide) chains, biotin and avidin molecules. High-resolution scanning electron microscopy using a superhydrophilic indium-tin oxide substrate was found to be useful for nanometer-level structural analysis of the vesicles and individual avidin molecules whose structures are difficult to analyze.

Sub-100 nm capsular objects such as virus capsids displaying proteins on their surface serve as a model of nanometer-sized tools for the detection, reporting and delivery of molecules in a biological environment.[1] Biological nanocapsules are formed by controlled integration of functional parts through multistep covalent and noncovalent chemical processes, which, however, is difficult to achieve in a flask for several reasons: the paucity of methods to prepare sub-100 nm capsular platforms under mild conditions, to modify their structures after their formation in water, and, most importantly, to analyze with molecular precision the structure of such sub-100 nm objects that typically lack structural uniformity and periodicity.[2] We report here that the *ca.* 8-nanometer-radius vesicles of the potassium salt of penta(oct-7-yl)fullerene[3] (**C8(7Y)K**) serve as a platform for construction of protein-covered nanocapsules through multilevel functionalization in a highly controlled manner (Fig. 1), and that their structure can be studied with a state-of-the-art, sub-nanometer spatial resolution, low-accelerating-voltage scanning electron microscope (SEM) equipped with a monochromatic electron source.[4] This SEM combined with the use of a super-hydrophilic and conducting indium-tin oxide (ITO) substrate permitted us to obtain subnanometer resolution images of the vesicles and the small protein molecules. This new SEM technology provided structural information on nonconducting nanoparticles that cannot be obtained using dynamic light scattering (DLS) or conventional microscopy such as atomic force microscopy (AFM).

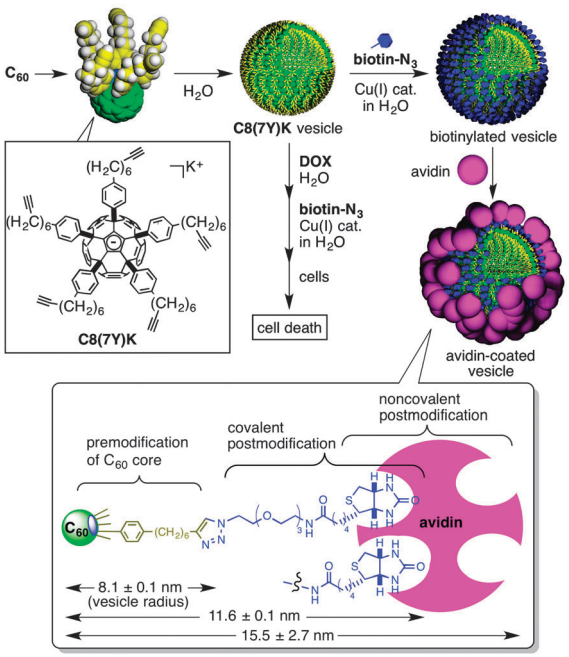

Fig. 1 Preparation of a biotinylated fullerene vesicle from fullerene amphiphile **C8(7Y)K** *via* a click reaction and conjugation with avidin.

To prepare the protein-covered vesicles, we employed a combination of four known sequences: quantitative organocopper addition to form penta(oct-7-yl)fullerene,[5] dissolution of its potassium salt as **C8(7Y)K** in water, installation of biotin groups by a copper(I)-catalyzed click reaction,[6–8] and biotin–avidin molecular recognition (Fig. 1). The fullerene amphiphile is unique in its ability to form stable vesicles on which we can display various functionalities in an aqueous phase.[9] We prepared a 2.0 mM (for fullerene) vesicle solution by slow injection of a THF solution of **C8(7Y)K** into pure water or D_2O followed by removal of THF under vacuum. DLS analysis (cumulant analysis) showed a very narrow size distribution (polydispersity index 0.05), and the

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hydrodynamic radius of the vesicle was determined to be 8.1 ± 0.1 nm. Therefore, one vesicle exposes about 400 fullerene molecules and 2000 alkyne termini on the surface.

We next installed biotin groups on the vesicle surface by the click reaction of the **C8(7Y)K** vesicle with 5 equiv. (for one **C8(7Y)K** molecule) of an azido-tagged biotin–oligo(ethylene oxide) conjugate (biotin-N_3; Fig. 1, top right) in the presence of copper(II) sulfate (50 mol%, 10 mol% per alkynyl group) and sodium ascorbate as a reducing agent.[10] The size distribution was rather narrow (polydispersity index 0.12) and the radius increased by 3.5 nm from 8.1 nm to 11.6 ± 0.1 nm (DLS)—an increase in accordance with the length of the tethered biotin unit (*ca.* 2.8 nm). This biotinylated fullerene vesicle is robust enough to be purified by gel permeation chromatography on Sephadex G50 for removal of unreacted biotin-N_3. No DLS radius change occurred during the purification. A separate set of experiments using a fluorescent probe to estimate the efficiency of the click reaction[11] conducted at a micromolar concentration indicated that the click reaction allowed *ca.* 400 biotin residues per vesicle (ESI†)—a value much higher than the number of 60 required to fully cover the surface with avidin molecules in the next step.

This biotinylation protocol can also be applied to a **C8(7Y)K** vesicle that contains an anticancer drug (Fig. 1, middle).[12] Thus, we added 5.0 μmol of **C8(7Y)K** in THF to an aqueous solution containing 5.3 μmol of doxorubicin (DOX) to form the DOX-containing vesicle, and then attached the biotin groups by the click reaction followed by separation of the vesicles from excess DOX and copper reagents, *etc.*, by chromatography. We incubated this vesicle solution, which contains 2.5 pmol of DOX (25 nM if homogeneously dissolved in the medium), together with human liver carcinoma cells (HepG2) for 48 h, and observed a cell viability of 67 ± 7%. In contrast, the biotin vesicles free of DOX showed a cell viability of 93 ± 3%, and the 25 nM solution of DOX without vesicle encapsulation showed a cell viability of 93 ± 3% (an IC_{50} value of 3.4 μM determined under the same conditions; see ESI†). The experiments showed that the click reaction and the vesicle purification were achieved without the loss of the encapsulated drug.

Finally, we noncovalently bonded avidin molecules to the vesicle surface. Upon mixing the vesicle with avidin in PBS buffer (a fullerene/avidin mole ratio of 1 : 1), the DLS analysis of this solution showed a very broad peak centered at 41 nm. The very large polydispersity index (0.23) suggested vesicle aggregation, and hence gave little information on the structure of the individual vesicle particles. However, the SEM analysis described in the following paragraphs indicates that we have successfully achieved the construction of protein-covered nanocapsules through multilevel functionalization in a highly controlled manner.

Knowing that aggregation is a ubiquitous phenomenon and represents a bottleneck for structural analysis in studies on self-assembled organic materials and protein, we consider it useful to explore the utility of a state-of-the-art SEM that features low acceleration voltage, a monochromatic electron beam (<0.2 eV energy spread), and a subnanometer spatial resolution. Not just allowing for imaging of fine surface details, these features

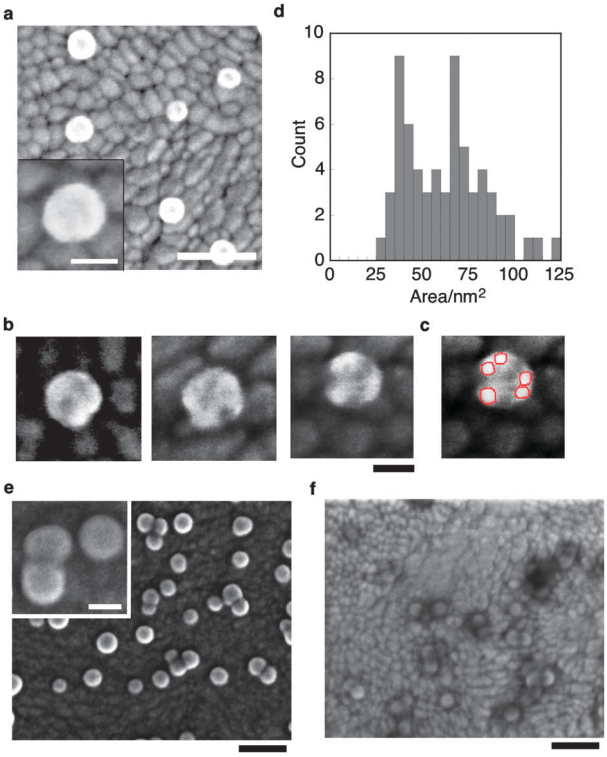

Fig. 2 HR-SEM images of avidin-coated vesicles coated on a microcrystalline ITO/glass substrate. The images were taken on an FEI Magellan 400L XHR SEM equipped with a unicolor monochromatic electron source (<0.2 eV energy spread; except in Fig. 2f). (a) The image taken as the first frame had an electron beam current of 25 pA. Note a nearly homogeneous smooth surface. Scale bar is 100 nm. Inset: a single vesicle with a scale bar of 30 nm. (b) Representative images of vesicles taken after scanning at 25 pA for 3 min. Note the development of fine structures on the surface. The scale bar is 20 nm. (c) The same image as in Fig. 2b, far right, where the bright areas are highlighted in red. (d) Histogram for the 62 bright areas on 36 avidin-covered vesicles. (e) Vesicles and ITO microcrystals after deposition of 1 nm Pd/Pt coating. The scale bar is 100 nm. Inset: single vesicles with a scale bar of 30 nm. (f) Image of the same sample taken with an electron beam with >0.65 eV energy spread. The scale bar is 100 nm.

remove two inherent problems of conventional SEM imaging that have prevented its application in the nanometer-resolution analysis of nonconducting objects: nanometer-thick Pt/Pd coating of the specimen that greatly reduces the quality of the information on the surface structure and electron damage to the specimen.

Fig. 2 shows the images of the avidin-covered vesicle under some representative conditions. Fig. 2a shows the image under the optimum conditions developed in this study, where the vesicle solution was spin-coated (1500 rpm) on a superhydrophilic ITO/glass substrate treated by UV/ozone just before use (a water contact angle of 0°). The coated substrate was dried for 1 h at room temperature before being placed in the SEM chamber kept at 5×10^{-5} Pa at room temperature. Imaging was achieved with an electron beam current of 25 pA and an acceleration voltage of *ca.* 1–2 kV. Note that the high affinity of the ITO surface for the avidin molecules entirely deaggregated the vesicles, allowing us to study individual vesicles and their surface (Fig. 2a and Fig. S7 in ESI†). Note that, on the hydrophobic Si/SiO_2 surface, the vesicles aggregated and fused extensively (contact angle = *ca.* 70°) and could not be coated on gold or graphite. The vesicle radius

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measured for 112 vesicles was 15.5 ± 2.7 nm (Fig. 2b), and we see hints of surface morphology.

The first few scans (*ca.* 3 min at a beam current of 25 pA) caused a change in the surface structure (*e.g.* dehydration of protein), which now shows bright and protruded areas, and dark areas (Fig. 2b and c). The histogram of the bright areas (circled in red in Fig. 2c) showed two peaks at 30–45 nm^2 and 65–75 nm^2 (analyzed for those located nearly in the center of the vesicle image, Fig. 2d). The former is close to the projection area of a single molecule of avidin (25–30 nm^2),[13] and the latter to that of two molecules. Therefore, we consider that the bright areas are due to individual avidin molecules. From these data, we can estimate that at least *ca.* 20 avidin molecules are attached to the vesicle surface.

Fig. 2e illustrates the detrimental effects on the image quality of the conventional Pt/Pd coating (1 nm) of the specimen taken under the same conditions as those used for Fig. 2a. The metal coating covered the avidin molecules and the ITO microcrystals. The coating increased the average vesicle radius by 4 nm to 19.7 ± 2.2 nm (average of 93 vesicles). The average radius of vesicles after 5 nm Pt/Pd coating increased further to 24.6 ± 3.4 nm (ESI†). Therefore, nonconducting substrates such as mica are unsuitable for nanometer-resolution SEM imaging because they inevitably need a metal coating. An SEM using a nonmonochromatic electron source (electron beam with >0.65 eV energy spread) produced the image in Fig. 2f that has an image contrast too low to be useful.

The SEM measurement of the biotinylated vesicle showed a radius of 11.7 ± 1.6 nm, which agrees very well with the hydrodynamic radius determined by DLS (11.6 ± 0.1 nm, see above). Thus, we consider that the SEM measurement provides a reliable alternative for the determination of the size of nanoparticles. In contrast, AFM imaging of the vesicles showed a radius larger by the size of the probe (*i.e.*, 2 nm), and the conventional SEM conditions using Pt/Pd coating also gave a size larger than the real size (see above).

In summary, we have coated the surface of a nanocapsule with avidin molecules through stepwise noncovalent and covalent modifications with good control and precise analysis of each functionalization step. The alkynylated fullerene formed a vesicle, which withstood the conditions of chemical reactions and chromatographic purification, and served as a carrier for drugs. We have found that the combined use of a superhydrophilic ITO/glass as a substrate and the low-accelerating-voltage SEM provides a viable method for imaging nonconducting nanometer-scale objects and proteins with high precision and surface sensitivity. ITO/glass is frequently used in organic electronics research, but it has seldom been used for biological imaging except for one report on micrometer-resolution SEM imaging of cultured cells.[14] We found that the ITO substrate is useful for imaging individual objects that tend to aggregate in water. This sub-nm-resolution SEM technique provides a new single molecule-level approach to the analysis of nm-sized organic molecules and materials,[15] supplementing the AFM technology that gives much lower point resolution and the conventional optics-based microscopy that is useful for molecular ensemble analysis. The subnanometer-resolution electron microscopic imaging of small molecules and proteins is an emerging methodology,[16] and the method reported here will provide a useful platform for future exploration of the potential of SEM.

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Notes and references

1 (*a*) D. Peer, J. M. Karp, S. Hong, O. C. Farokhzad, R. Margalit and R. Langer, *Nat. Nanotechnol.*, 2007, **2**, 751; (*b*) S. Ganta, H. Devalapally, A. Shahiwala and M. Amiji, *J. Controlled Release*, 2008, **126**, 187; (*c*) J. Voskuhl and B. J. Ravoo, *Chem. Soc. Rev.*, 2009, **38**, 495; (*d*) B. Worsdorfer, K. J. Woycechowsky and D. Hilvert, *Science*, 2011, **331**, 589.
2 T. Ogura, *PLoS One*, 2012, **7**, e46904.
3 S. Q. Zhou, C. Burger, B. Chu, M. Sawamura, N. Nagahama, M. Toganoh, U. E. Hackler, H. Isobe and E. Nakamura, *Science*, 2001, **291**, 1944.
4 R. Young, S. Henstra, J. Chmelik, T. Dingle, A. Mangnus, G. van Veen and I. Gestmann, *Proc. SPIE*, 2009, 737803.
5 Y. Matsuo and E. Nakamura, *Chem. Rev.*, 2008, **108**, 3016.
6 (*a*) V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 259; (*b*) C. W. Tornøe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057; (*c*) M. Meldal and C. W. Tornøe, *Chem. Rev.*, 2008, **108**, 2952.
7 (*a*) S. Cavalli, A. R. Tipton, M. Overhand and A. Kros, *Chem. Commun.*, 2006, 3193; (*b*) F. S. Hassane, B. Frisch and F. Schuber, *Bioconjugate Chem.*, 2006, **17**, 849; (*c*) C. Schatz, S. Louguet, J.-F. Le Meins and S. Lecommandoux, *Angew. Chem., Int. Ed.*, 2009, **48**, 2572.
8 H. Isobe, K. Cho, N. Solin, D. B. Werz, P. H. Seeberger and E. Nakamura, *Org. Lett.*, 2007, **9**, 4611.
9 (*a*) T. Homma, K. Harano, H. Isobe and E. Nakamura, *Angew. Chem., Int. Ed.*, 2010, **49**, 1665; (*b*) T. Homma, K. Harano, H. Isobe and E. Nakamura, *J. Am. Chem. Soc.*, 2011, **133**, 6364.
10 C. M. Santos, A. Kumar, W. Zhang and C. Cai, *Chem. Commun.*, 2009, 2854.
11 K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill and Q. Wang, *Org. Lett.*, 2004, **6**, 4603.
12 (*a*) G. Russell-Jones, K. McTavish, J. McEwan, J. Rice and D. Nowotnik, *J. Inorg. Biochem.*, 2004, **98**, 1625; (*b*) J. Chen, S. Chen, X. Zhao, L. V. Kuznetsova, S. S. Wong and I. Ojima, *J. Am. Chem. Soc.*, 2008, **130**, 16778.
13 B. Steiger, C. Padeste, A. Grubelnik and L. Tiefenauer, *Electrochim. Acta*, 2003, **48**, 761.
14 H. Pluk, D. J. Stokes, B. Lich, B. Wieringa and J. Fransen, *J. Microsc.*, 2009, **233**, 353.
15 H. Yabu, M. Kanahara, M. Shimomura, T. Arita, K. Harano, E. Nakamura, T. Higuchi and H. Jinnai, *ACS Appl. Mater. Interfaces*, 2013, DOI: 10.1021/am4003149.
16 (*a*) E. Nakamura, *Angew. Chem., Int. Ed.*, 2013, **52**, 236; (*b*) M. Koshino, T. Tanaka, N. Solin, K. Suenaga, H. Isobe and E. Nakamura, *Science*, 2007, **316**, 853; (*c*) K. Harano, T. Homma, Y. Niimi, M. Koshino, K. Suenaga, L. Leibler and E. Nakamura, *Nat. Mater.*, 2012, **11**, 877.