Nanodiamonds

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Chromatographic Separation of Highly Soluble Diamond Nanoparticles Prepared by Polyglycerol Grafting**

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Diamond nanoparticles, so-called nanodiamonds (NDs), are attracting growing attention, because they have the intrinsic characteristics of diamond together with the unique properties of nanometer-sized particles. In particular, biomedical applications of NDs have been investigated extensively owing to their low toxicity and amenability to various surface functionalizations.[1–7] One of the most promising applications of ND is biomedical imaging on the basis of nonbleaching fluorescence from the diamond core (nitrogen-vacancy, or N-V, center).[4,8,9] In such biomedical applications of ND, it should form a stable hydrosol in a physiological environment, as pointed out by Xing and Dai,[2] and Shenderova et al.[3] in their recent reviews.

Although we successfully prepared a very stable hydrosol of ND functionalized with polyethylene glycol (PEG),[5] its solubility was not sufficient for biomedical applications, for example, as a platform for an imaging probe and drug-delivery system. To increase the solubility, we changed the molecular design and exchanged the linear polyethers PEG for hyperbranched polyols, because the hydroxy group is more hydrophilic than an ether group, and the hyperbranched structure can cover the nanoparticle surface much more densely than a linear chain. Polyglycerol (PG) was adopted as the hyperbranched polyol to be grafted onto the ND surface for the following three reasons: 1) PG shows high biocompatibility as well as high hydrophilicity,[10–13] 2) a PG layer can be readily constructed on the ND surface through ring-opening multibranching polymerization of glycidol initiated at the functional groups on the ND surface,[14,15] and 3) PG can be functionalized further by derivatizing the periphery.[12,13] The recent use of PG for the functionalization of various nanoparticles with potential biological applications[14–18] prompted us to communicate our results. Herein, we report the preparation of PG-functionalized ND (ND-PG). Its extremely high solubility not only in pure water but also in buffer solutions enabled chromatographic separation of the NDs according to size. In view of cancer imaging, size control of nanoparticles is important because of enhanced permeability and retention (EPR) in solid tumors.[19]

The ND used for PG functionalization, designated as ND30 herein, has a 30 nm median diameter and is prepared from bulk diamond synthesized by a static high-pressure–high-temperature (HPHT) method. ND-PG was synthesized through ring-opening multibranching polymerization of glycidol at high temperature (Scheme 1). When the polymerization was initiated at the surface functional groups, such as hydroxy and carboxylic acid groups, the ND surface was covered with PG. However, PG without the ND core, designated as free PG, was also obtained as a side product through self-ring-opening polymerization of glycidol. To retard the side reaction initiated not from the ND surface but from glycidol, we examined the reaction conditions, including the solvent, the temperature, and the presence of an acid or base. When ND in glycidol was stirred at 140 °C under neutral conditions (see the Experimental Section), the reaction mixture became a homogeneous grayish gel, which probably consisted of ND-PG and free PG. A dry black solid was recovered after washing of the gel with methanol five times and subsequent freeze drying; apparently most of the gel, or free PG, included in the reaction mixture had been removed. In our experiments, ND-PG prepared under neutral conditions showed better solubility and formed a more stable hydrosol than ND-PG prepared under acidic or basic conditions. The presence of an acid or base may facilitate initiation of the side reaction, namely, the self-ring-opening reaction of glycidol molecules, because an acid and base increase the electrophilicity of the epoxide and nucleophilicity of the hydroxy group, respectively. Furthermore, the ND-PG product prepared under neutral conditions at lower temperature, for example at 75 °C, exhibited very poor solubility even in pure water.[18] Polymer trees with more and longer branches may be constructed on the ND surface under neutral conditions at 140 °C by suppressing the side reaction. In this way, ND-PG with better solubility is formed.

The solubility of ND-PG in pure water, phosphate-buffered saline (PBS), a phosphate buffer (20 mM, pH 7.0) containing Na_2SO_4 (100 mM; a mobile phase for chromatographic separation), and methanol was not less than 20, 16, 12,

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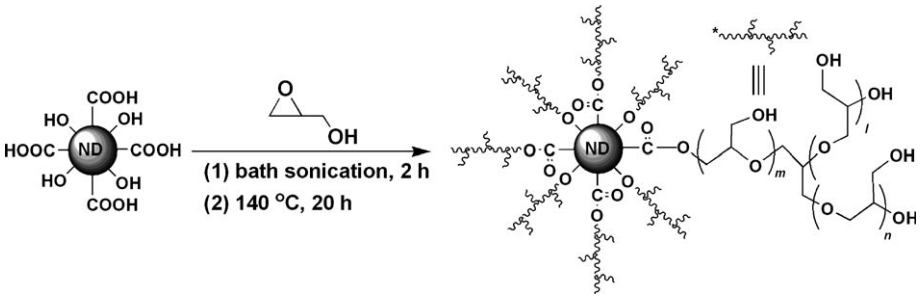

Scheme 1. Synthesis of ND functionalized with hyperbranched polyglycerol through the ring-opening polymerization of glycidol.

and 6 mg mL^{-1}, respectively (see Figure S1 in the Supporting Information). These solutions of ND-PG were very stable; no precipitates and no significant change in the diameter distribution were observed for more than three months. The blackish color of these concentrated ND-PG solutions is considered to originate from a graphitic layer remaining on the ND surface. This layer was detected as a broad peak around 1550 cm^{-1} in the Raman spectrum (Figure 1 d). Since ND-PG showed very high solubility in various solvents, it could be characterized by solution-phase ^{13}C and ^{1}H NMR spectroscopy (Figure 1 a,b).[5]

In the ^{13}C NMR spectrum, diamond carbon atoms are clearly detected at $\delta \approx 35$ ppm in ND-PG, as we reported previously,[5,20] whereas the rest of the resonances are similar to those of free PG prepared in the absence of ND but otherwise under the same conditions. ND-PG and free PG show similar ^{1}H NMR spectra (Figure 1 b). The results of solution-phase NMR spectroscopy indicate that PG and ND coexist in the solution. Taking into account the low solubility of ND without PG grafting, the washing of the nanoparticles five times with methanol to remove most of the free PG, and the peak intensity of diamond carbon atoms relative to PG peaks in Figure 1 a, we conclude that the ND is covered with PG. The FTIR and Raman spectra support this conclusion (Figure 1 c,d): the IR absorptions corresponding to C–H stretching and C–O–C stretching increased greatly after PG functionalization, and a diamond peak was observed at 1332 cm^{-1} in the Raman spectrum. Since the ring-opening polymerization can be initiated at the hydroxy and carboxylic acid groups on the ND surface, we conclude that the PG is covalently immobilized on the ND surface through ether and ester linkages, as shown in Scheme 1. Such covalent immobilization of organic functionalities is possible for carbonaceous materials, such as diamonds and carbon nanotubes.[5,17,18,21–24] This behavior is in marked contrast to that of noncarbonaceous materials, such as iron oxide, iron–gold, and CdTe (quantum dot), in which noncovalent bonds, such as ionic and coordinate bonds, are predominant.[14–16]

To quantify the PG layer on the ND surface, we carried out elemental analysis of ND and ND-PG (Table 1). If all of the ND particles are assumed to have a spherical shape with a 30 nm diameter, each particle was calculated to consist of 2.5×10^6 C atoms.[25] On the basis of the number of C atoms and the results of elemental analysis of the ND, the numbers of H and O atoms were estimated as shown in Table 1. Since the monomer, or glycidol, has the molecular formula $C_3H_6O_2$ (C 48.64 %, H 8.16 %, O 43.20 % (by weight)), the weights of carbon and oxygen (%) in ND-PG should decrease and increase, respectively, as the polymerization proceeds. The number of glycidol units grafted on one ND particle was calculated to be 3.1×10^5 by taking into account the increased number of C and O atoms as a result of polymerization and the results of elemental analysis (see the Supporting Information). On the basis of the molecular weight of glycidol, the molecular weight of the PG layer grafted on the surface of one ND particle was calculated to be 2.3×10^7 (23 MDa), which is comparable to that of the ND core (30 MDa).[12] Thermogravimetric analysis (TGA) gave a similar weight ratio (PG/ND $\approx$ 40:60) in ND-PG (Figure 2) and supported the weight ratio determined by elemental analysis (PG/ND = 23 MDa/30 MDa = 43:57). The PG layer on the ND surface is about 5 nm thick, as estimated from the difference in sizes determined by analysis using dynamic light scattering (DLS) and scanning transmission electron microscopy (STEM), as discussed below. The thick PG layer consisting of a large number of glycerol units on the ND surface affords great solubility to the ND in spite of its relatively large size (30 nm diameter) and enables chromatographic separation.

Table 1: Elemental analysis of ND30 and ND-PG, and estimated number of atoms in each.[a]

Element	ND30		ND-PG	
	Weight [%]	Number of atoms[b]	Weight [%]	Number of atoms[c]
H	1.27	4.2×10^5	3.73	1.9×10^6
C	90.00	2.5×10^6	72.70	3.4×10^6
O	9.42	2.0×10^5	23.08	8.2×10^5

[a] Nitrogen is omitted because of its low content (< 0.1 %). CHN and O were measured independently in CHN and O modes. [b] The number of atoms was calculated on the basis of the number of carbon atoms (2.5×10^6) in a spherical ND with a diameter of 30 nm (see the Supporting Information). [c] The number of atoms was calculated on the basis of the number of glycidol units grafted on one ND particle (see text).

Size-exclusion chromatography (SEC) enabled the size separation of ND-PG under conditions similar to those reported for the length sorting of DNA-wrapped carbon nanotubes.[26] A phosphate buffer (pH 7.0, 20 mM) containing Na_2SO_4 (100 mM) was used as the mobile phase, and elution was monitored with UV light at 254 nm. Eluted material was collected in 0.5 mL fractions (flow rate: 1.0 mL min^{-1}). Typical elution profiles of ND-PG and free PG are shown in Figure 3. A peak at 20–28 min and a tail after 28 min were observed in the chromatogram of ND-PG. On the other hand, the free PG, prepared from glycidol as mentioned above, is eluted at around 35 min under the same conditions. These

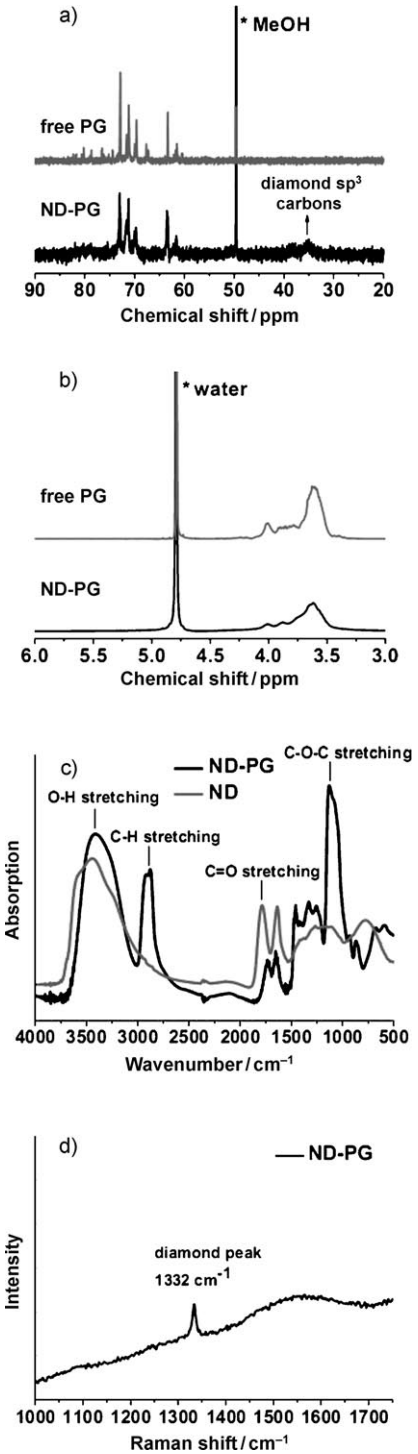

Figure 1. Spectroscopic characterization of ND-PG. a) Solution-phase ^{13}C NMR spectra of ND-PG and free PG in D_2O (methanol was added as an internal standard); b) solution-phase ^{1}H NMR spectra of ND-PG and free PG in D_2O; c) FTIR spectra of ND-PG and ND30 as solids; d) Raman spectrum of ND-PG as a solid.

results imply that the peak at 20–28 min does not correspond to free PG but to ND-PG, and that free PG included in the ND-PG is eluted after ND-PG. Since the diamond cores in the NDs and ND-PG were clearly observed by STEM (Figure 4a,b), fractions 3, 7, 11, 15, and 17 belonging to the peak

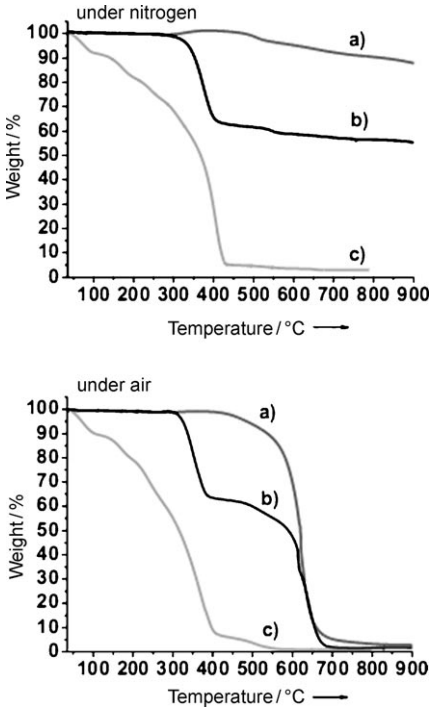

Figure 2. TGA profiles of a) ND30, b) ND-PG, and c) free PG under nitrogen and air.

in the chromatogram in Figure 3 were analyzed by STEM after being desalinized by dialysis against Milli-Q water. In all fractions, diamond cores were confirmed by STEM (Figure 4c–g), and the (111) crystallographic diamond layers were clearly observed in most fractions by high-resolution transmission electron microscopy (HRTEM; Figure 4h; see also Figure S2 in the Supporting Information). Furthermore, the average size of the cores tended to decrease from fraction 3 to fraction 17 (Table 2 and Figure 4). These results indicate that the peak in the chromatogram corresponds to the elution of ND-PG, and that the ND-PG particles were successfully separated according to their size.

The particle sizes of the eluted ND-PG were also determined by DLS (Table 2 and Figure 5). The particle size

Table 2: Particle size of ND-PG before and after chromatographic separation.[a]

Sample	Retention time [min]	Average size (STEM) [nm][a]	Median size (DLS) [nm][b]	Size difference [nm][c]
ND-PG	–	29	47, 52[d]	18, 23
fraction 3	21.0–21.5	67	76	9
fraction 7	23.0–23.5	39	55	16
fraction 11	25.0–25.5	27	38	11
fraction 15	27.0–27.5	18	26	8
fraction 17	28.0–28.5	12	30	18

[a] More than 100 particles in the STEM images were analyzed individually to calculate the mean size. [b] The median size was measured for particles in a buffer, unless otherwise noted. Data are shown in Figure S3 of the Supporting Information. [c] Difference between the sizes determined by DLS and STEM. [d] The median size was measured for particles in Milli-Q water.

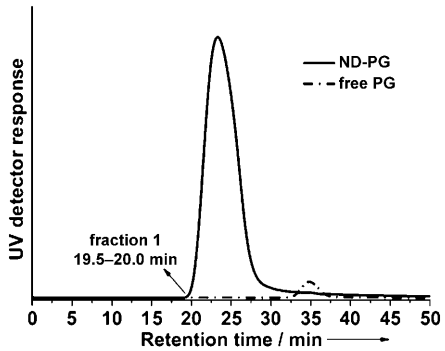

Figure 3. Chromatograms for the elution of ND-PG and free PG.

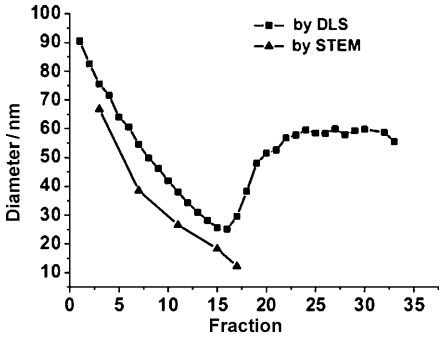

Figure 5. Median and average diameters of the eluted particles, as determined by DLS and STEM, respectively.

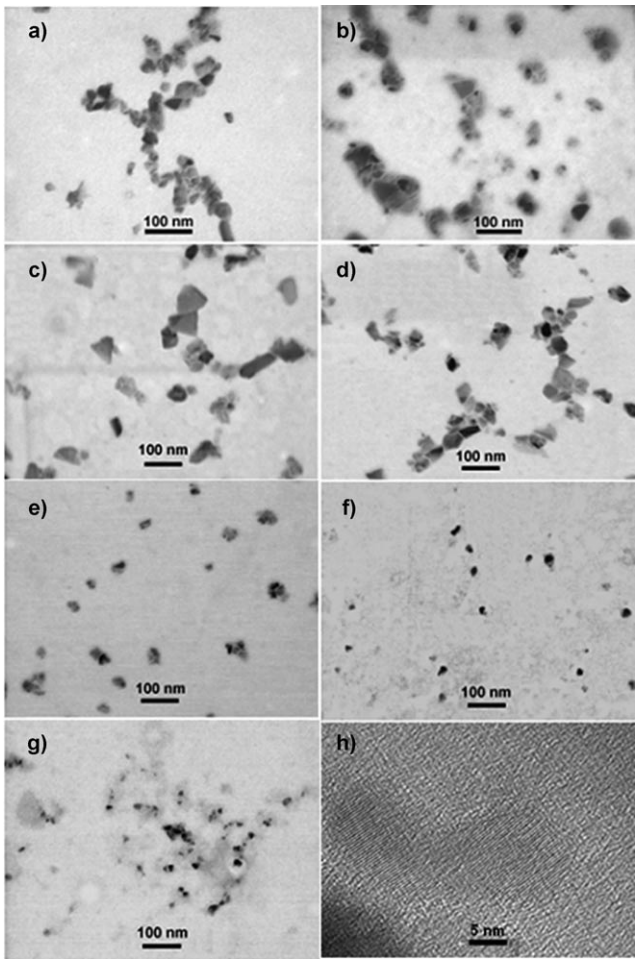

Figure 4. STEM images of a) ND, b) ND-PG before chromatographic separation, c) fraction 3, d) fraction 7, e) fraction 11, f) fraction 15, and g) fraction 17 after chromatographic separation; and h) HRTEM image of fraction 7.

decreased proportionally from 90 to 26 nm in fractions 1–15, or during the retention time of 20–27.5 min, as supported by STEM. However, the size started to increase at fraction 17 (retention time: 28 min) and reached a constant value of approximately 60 nm after fraction 23 (retention time: 31 min). Fractions 17–22 correspond to the tail in Figure 3 and are considered to contain the last elution of the ND-PG and the first portion of free PG. After the constant size had been reached at fraction 23, only free PG was eluted without any contamination by ND-PG. The size increase observed by DLS after fraction 16 can be attributed to the soft structure of free PG without a diamond core. When free PG is eluted through a silica-based stationary phase in SEC, it goes into the pores of the silica beads. Since the smaller particles can enter the pore more deeply than the larger particles, the smaller particles are retained longer than the larger particles. Under a high fluid pressure, free PG is considered to enter the pore more deeply than ND-PG because of its soft structure; it is therefore retained longer than ND-PG. After elution, the free PG is no longer compressed, and it becomes larger than ND-PG (Figure 5). In fractions 16–22, the particle size is considered to be determined by the quantity of ND-PG, with a median size less than 25 nm, and free PG, with a median size of 60 nm. According to the relative increase in free-PG content, the particle size determined by DLS increased from 25 nm for fraction 16 to 60 nm for fraction 23.

In the range of fractions 1–15, DLS and STEM analysis showed the same trend of a proportional size decrease (Table 2 and Figure 5). However, there was a difference of about 10 nm between the average and median sizes determined by STEM and DLS, respectively. Since the size distribution in each fraction, as determined by DLS and STEM analysis, had a near-Gaussian shape, the difference between the median and average sizes of the particles is not so large. Therefore, it may be attributed to the difference between the diameter of the core diamond, as determined by STEM, and the hydrodynamic diameter determined by DLS. Although a gray area, which probably corresponds to the PG layer, was observed at the periphery of the diamond core by STEM (Figure 4), we only measured the size of the core without the gray periphery. On the other hand, it is known that the diameter determined by DLS includes the core and the coverage,[27] if the coverage is dense enough. Therefore, we conclude that the PG layer in ND-PG has a thickness of about 5 nm in a buffer.

In summary, ND particles were covalently functionalized with hyperbranched PG through ring-opening multibranching polymerization of glycidol at high temperature under neutral conditions. The ND-PG thus prepared showed extremely high solubility not only in pure water, but also in buffer solutions. This high solubility in a buffer enabled the chromatographic

purification and size sorting of ND-PG. This process can be scaled up by using larger SEC columns.

Experimental Section

Preparation of ND-PG: The as-received HPHT ND powder with a median diameter of 30 nm was treated with a mixture of concentrated sulfuric acid and 60% nitric acid (3:1 v/v). A suspension of ND30 (50 mg) in glycidol (6 mL) was sonicated in an ultrasonic bath at 25 °C for 2 h, and the resulting well-dispersed suspension was magnetically stirred at 140 °C under an argon atmosphere for 20 h and then left to cool to room temperature. The resulting brownish gel was diluted with methanol (60 mL) in an ultrasonic bath, and the precipitate was recovered after centrifugation at 50400*g* for 2 h. This washing process was repeated four times to remove free PG. The washed precipitate was dialyzed against Milli-Q water to replace residual methanol with water, and then lyophilized to give a gray flocculent solid (73.2 mg).

Size sorting of ND-PG by SEC: The following three columns were connected for SEC separation of ND-PG: Cosmosil CNT-2000, CNT-1000, and CNT-300 (diameter: 7.5 mm, length: 300 mm, Nacalai Tesque Inc.) with pore sizes of 2000, 1000, and 300 Å, respectively. A phosphate buffer (20 mM, pH 7.0) containing Na_2SO_4 (100 mM) was used as the mobile phase with a flow rate of 1.0 mL min^{-1}. After the injection of the solution of ND-PG in the buffer (5 mg mL^{-1}, 1.0 mL), elution was monitored with UV light at 254 nm, and 0.5 mL fractions were collected.

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