



Poly(glycerol-co-glyceric acid) Functionalized Nanodiamonds by Nitroxyl Radical-Catalyzed Oxidation of Primary Alcohols in Poly(glycerol) as Scaffolds for Further Conjugation

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Abstract: Poly(glycerol) (PG) functionalization is versatile to make various nanoparticles dispersible in aqueous media. In addition, the abundant hydroxy groups in PG give scaffolds for further modification to introduce suitable functional groups. For example, carboxylic group (–COOH) has been introduced through the reaction of hydroxy (–OH) group in PG with succinic anhydride. However, ester linkage in the

succinate moiety in the PG layer is subjected to hydrolysis. In this work, we develop an alternative way for a novel –COOH containing PG functionalized detonation nanodiamonds (DND-PG-COOH) via oxidation of primary –OH group of PG chain by nitroxyl radical catalysts well-known as TEMPO or its analogues. The content of –COOH is found to be precisely controlled by the amount of oxidant.

Introduction

Poly(glycerol) (PG) functionalization has been recognized as a facile and versatile process to make various nanoparticles (NPs) dispersible in aqueous, or physiological, environments.^[1–10] The simple “grafting-from” process gives NP-PGs through ring-opening polymerization of glycidol on the surface of NPs.^[11,12] Recently, we thoroughly elucidated the PG functionalization of nanodiamonds (NDs), enabling us to control it by tuning the reaction conditions and the ND properties.^[13] The PG coating on NPs has several advantages such as evasion of immune response by preventing the adsorption of serum proteins, or protein corona formation,^[14–17] and further modification to conjugate various functionalities on the abundant hydroxy groups (–OH).^[18–30] In the latter, introduction of another functional group in the PG layer is meaningful as a scaffold to conjugate another functionality. Among the functional groups, carboxylic group (–COOH) is one of the most useful ones to covalently bind various functional moieties such as peptides, proteins including antibodies and small molecules like anticancer drugs.^[27–30] Although carboxylic groups were introduced by the reaction of –OH with succinic anhydride in pyridine,^[14,27–31] the resulting ester linkage is susceptible to hydrolysis in aqueous,

or physiological, environments. In this work, we developed the other means to introduce carboxylic groups in the PG layer through direct oxidation of the primary alcohols (–CH₂OH) in glycerol units by nitroxyl radical catalyst (Scheme 1).^[32–36] The resulting PG-functionalized detonation ND (DND) with carboxylic groups (DND-PG-COOH) should be more chemically robust and less hydrophobic than that prepared from succinic anhydride mentioned above. After the oxidation, the content of carboxylic groups is determined by acid-base titration and is found to be controlled by the amount of the oxidants, sodium hypochlorite (NaClO) and sodium chlorite (NaClO₂), in the presence of nitroxyl radical catalyst such as 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) or its analogues.

Results and Discussion

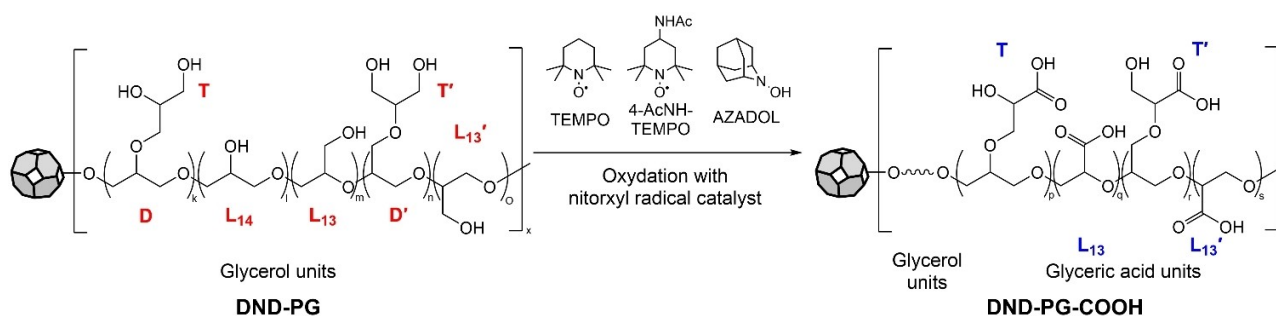
The following two reaction systems were applied to the TEMPO-catalyzed oxidation of DND-PG; NaClO supplemented by sodium bromide (NaBr) under basic conditions (pH ~ 10)^[37] and NaClO₂ with small amount of NaClO under neutral to slightly acidic conditions.^[38,39] Among the various substructures of glycerol unit in DND-PG as indicated in the red letters in Scheme 1,^[13] the primary alcohols are subjected to be oxidized to carboxylic groups in DND-PG-COOH as indicated in the blue letters. In the former reaction system, DND-PG was oxidized in the presence of TEMPO, NaClO and NaBr at pH around 10 and temperature below 5 °C (entry 1 in Table 1 and the details in Experimental). At the beginning, the reaction was so fast that the pH value decreased immediately upon the addition of NaClO solution. Then, the decrease of pH, or consumption of NaClO, slowed down and stopped, when 9.2 mmol of NaClO to 1 g of DND-PG (entry 1 in Table 1) was added. After purification by ultrafiltration, the resulting carboxylic group was confirmed qualitatively by FT-IR at 1734 cm^{–1} due to the stretching vibration of carbonyl group (Figure 1) and quantitatively by acid-base titration to

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Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cnma.202300204>

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Scheme 1. Synthesis of DND-PG-COOH by the oxidation of DND-PG with nitroxyl radical catalyst. Red letters on DND-PG indicate substructure of glycerol units (D: dendritic, L: linear and T: terminal in ref. [13]). Blue letters on DND-PG-COOH indicate substructures with carboxylic groups after CH₂OH in DND-PG is oxidized.

Table 1. Summary of oxidation reactions of DND-PG to DND-PG-COOH with nitroxyl radical catalyst.

Entry	Reagents (mmol) to 1 g of DND-PG (mmol/g)				Solvent (pH) [a]	Reaction time (h)	COOH content in 1 g of DND-PG-COOH (mmol/g)
	Catalyst	NaClO	NaClO ₂				A [b]
1	TEMPO	0.36	9.18	---	Water (10–12)	0.6	1.34
2	TEMPO	0.31	2.91	---	Water (10–12)	0.7	0.06
3	4-AcNH-TEMPO	0.20	0.55	10.14	Acetate (4.7)	48	2.31
4	4-AcNH-TEMPO	0.19	0.22	9.99	Acetate (4.7)	48	2.04
5	4-AcNH-TEMPO	0.15	0.17	7.43	Acetate (4.7)	24	2.60
6	4-AcNH-TEMPO	0.21	0.23	5.18	Acetate (4.7)	48	1.84
7	4-AcNH-TEMPO	0.24	0.27	3.97	Acetate (4.7)	24	1.51
8	4-AcNH-TEMPO	0.25	0.27	1.83	Acetate (4.7)	24	1.17
9	4-AcNH-TEMPO	0.15	0.17	1.26	Acetate (4.7)	23	1.01
10	4-AcNH-TEMPO	0.14	0.17	1.02	Acetate (4.7)	24	0.81
11	4-AcNH-TEMPO	0.11	0.11	0.63	Acetate (4.7)	25	0.47
12	TEMPO	0.16	0.17	5.00	Phosphate (6.8)	21	0.69
13	TEMPO	0.22	0.28	2.95	Phosphate (6.8)	18.5	1.04
14	4-AcNH-TEMPO	0.20	0.22	10.05	Phosphate (6.8)	48	1.36
15	4-AcNH-TEMPO	0.16	0.17	5.01	Phosphate (6.8)	22	0.65
16	4-AcNH-TEMPO	0.15	0.17	1.48	Phosphate (6.8)	22	0.30
17	AZADOL	0.20	0.34	10.07	Acetate (4.7)	45	1.47
18	AZADOL	0.15	0.17	3.00	Acetate (4.7)	23	1.27
19	AZADOL	0.16	0.17	1.03	Acetate (4.7)	21	0.69
20 [c]	4-AcNH-TEMPO	0.16	0.17	0.99	Acetate (4.7)	22	0.78

[a] Acetate: 0.4 M acetate buffer, Phosphate: 0.4 M phosphate buffer.

[b] **A** will be used in Table 2.

[c] ND50-PG was used instead of DND-PG.

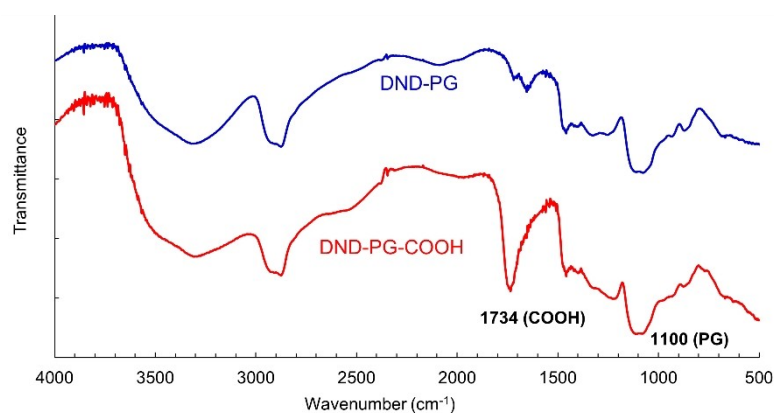
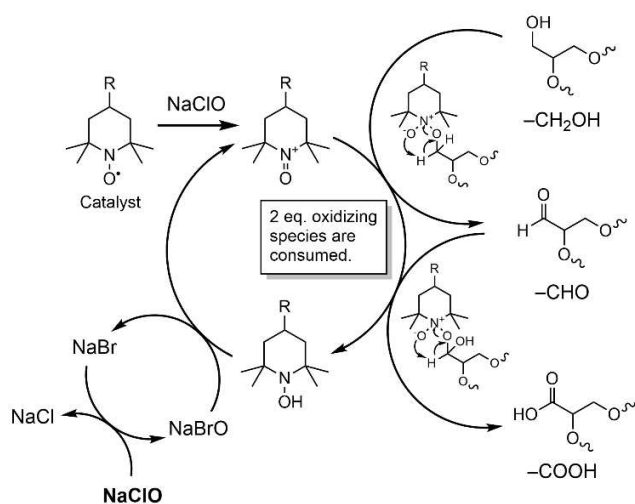


Figure 1. FT-IR spectra of DND-PG and DND-PG-COOH of entry 1 in Table 1 (DRIFT with KBr).

a) With NaClO at basic pH



b) With NaClO₂ at neutral to acidic pH

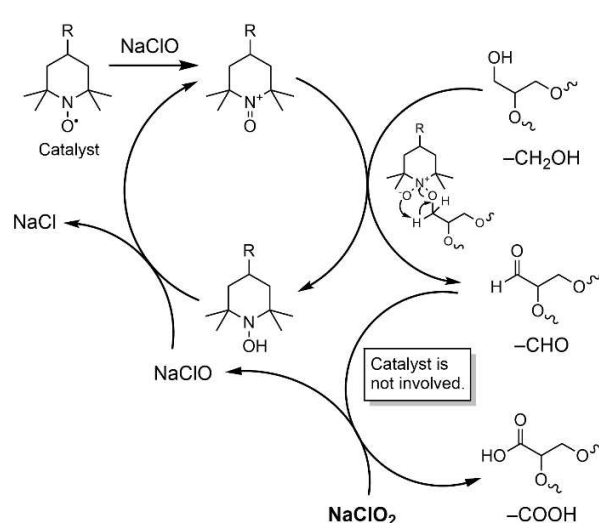


Figure 2. Reaction mechanism of nitroxyl-radical oxidation (a) with NaClO under basic conditions and (b) with NaClO₂ under neutral to acidic conditions.

be 1.34 mmol of COOH in 1 g of the material. When the amount of NaClO was reduced to 2.9 mmol/g (entry 2 in Table 1), almost no COOH was produced (Figure S1a), resulting in poor aqueous dispersibility. The possible reaction mechanism is illustrated in Figure 2a. The oxidation of $-\text{CH}_2\text{OH}$ to $-\text{COOH}$ consists of the following two steps, $-\text{CH}_2\text{OH} \rightarrow -\text{CHO}$ (aldehyde) $\rightarrow -\text{COOH}$. Since *N*-oxoammonium ion is involved in both steps, the reaction of this oxidizing species with $-\text{CH}_2\text{OH}$ should be much faster than $-\text{CHO}$. Hence, $-\text{CHO}$ or its hydrate form remains in the reaction mixture, if a sufficient amount of the oxidizing species is not provided. This limitation in the former reaction system motivated us to apply the latter one using NaClO₂ with TEMPO and small amount of NaClO, realizing the precise control of COOH content. In the reaction, 4-AcNH-TEMPO was applied under acidic conditions (pH 4.7).^[38] The results are summarized in Table 1 (entries 3–11), and FT-IR spectra of the products are shown in Figure S1b–j in Supporting Information to find that the products by two reaction systems are the same. Table 1 and Figure 3 indicate that the COOH content increases as the amount of NaClO₂ increases. In particular, the conversion is nearly stoichiometric, when the amount of NaClO₂ is small (≤ 1 mmol/g); 1.01, 0.81 and 0.47 mmol/g of $-\text{COOH}$ is produced by the addition of 1.26, 1.02 and 0.63 mmol/g of NaClO₂, respectively (entries 9–11 in Table 1). However, the oxidation efficiency decreased gradually, as the amount of NaClO₂ increased. The relationship can be fitted with a natural log approximation (dotted line and equation in Figure 3) in the reactions with 4-AcNH-TEMPO catalyst at pH 4.7 (blue squares). In this system, NaClO₂ oxidizes *in-situ* $-\text{CHO}$ intermediate to $-\text{COOH}$ as shown in Figure 2b. The resulting NaClO (HClO as the reactive species) oxidizes the catalyst to generate *N*-oxoammonium ion and convert $-\text{CH}_2\text{OH}$ to $-\text{CHO}$. NaClO added at the beginning

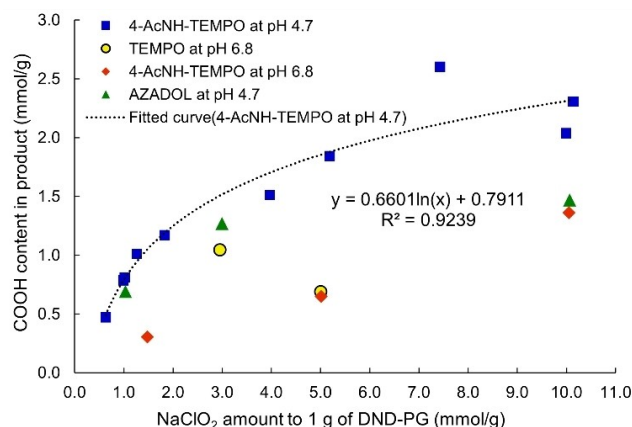


Figure 3. Relationship between the amount of NaClO₂ and COOH content of DND-PG-COOH through the oxidation of DND-PG except for one result (entry 20 in Table 1) from ND50-PG to ND50-PG-COOH by 4-AcNH-TEMPO at pH 4.7 (blue square).

serves as an initiator to oxidize the TEMPO catalyst. The reactions with TEMPO or 4-AcNH-TEMPO at neutral pH (entries 12 and 13, and 14–16, Table 1, respectively) and with 2-hydroxy-2-azaadamantane (AZADOL) at pH 4.7 (entries 17–19, Table 1) exhibited lower reaction efficiency (yellow solid circle, red rhombi and green triangles in Figure 3 and FT-IR spectra in Figure S1k–r, respectively). It may be caused by the differences in the activity of oxidant, or oxidizing species, and the selectivity of catalyst, which will be discussed later.

Colloidal stability of the oxidized products with negative ζ -potential (Table S1) is confirmed by dynamic light scattering (DLS) measurements on number, volume and scattering light intensity bases (Figure 4 for entries 4 and 10, Table 1), indicating that no coarse particles by aggregation have appeared by the reaction. The product contains a trace

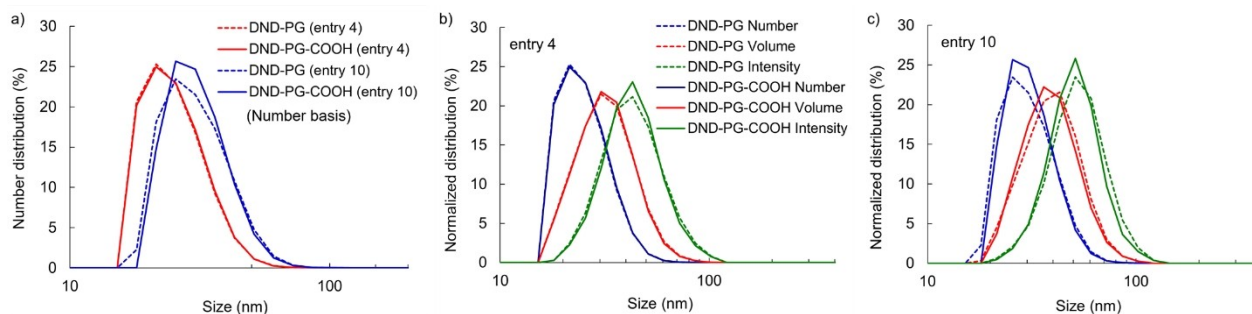


Figure 4. Hydrodynamic diameter of (a) DND-PG (dashed lines) and DND-PG-COOH (solid lines) with red and blue lines corresponding to entries 4 and 10 in Table 1 on number basis, (b,c) DLS of DND-PG (dashed lines) and DND-PG-COOH (solid lines) in entries 4 (b) and 10 (c) in Table 1 on number (blue), volume (red) and scattering light intensity (green).

amount of $-\text{CHO}$ ($\leq 1/1000$ to the amount of glycerol unit) which detected by ^1H NMR (Figure S2), and no free radical derived from nitroxyl radical catalyst is observed by EPR (Figure S3). In addition, the reaction with 4-AcNH-TEMPO at pH 4.7 was applied to ND50-PG (high pressure high temperature (HPHT) ND of 50 nm-size) to give similar result (0.78 mmol/g of COOH content with 0.99 mmol/g of NaClO_2) to DND-PG (entry 20 in Table 1 and FT-IR spectrum in Figure S1s).

In ^{13}C NMR spectra by inverse gated decoupling measurement, the $-\text{COOH}$ and $-\text{CH}_2\text{OH}$ signals in DND-PG and DND-PG-COOH are found around 176 ppm (Figure 5b and d) and around 65 ppm (Figure 5a–d), respectively. Based on the integral of the secondary carbons with ether linkage ($-\text{CH}(\text{OR})-$) around 80 ppm, those of $-\text{COOH}$ are appeared to be 1.05 and 0.25 in Figure 5b and 5d, respectively, after the oxidation of DND-PG (entries 4 and 10 in Table 1, respectively). Simultaneously, the integrals of $-\text{CH}_2\text{OH}$ decreases from 1.50 and 1.44 (Figure 5a and c, respectively) to 0.43 and 0.97 (Figure 5b and d, respectively) after the oxidation (entries 4 and 10 in Table 1, respectively), indicating that the primary alcohols are oxidized to carboxylic acids.

According to our previous report regarding structural analysis of ND-PGs,^[13] $-\text{CH}_2\text{OH}$ contents in DND-PG (DND(+)-PG(m) and -PG(h) with PG/DND weight ratios of 2.43 and 3.58) in entries 4 and 10 (Table 1) are determined to be ca. 5.0 and 5.5 mmol/g, respectively. On the other hand, the COOH contents in the corresponding DND-PG-COOH in entries 4 and 10 (Table 1) are 2.04 and 0.81 mmol/g, respectively. Particularly in entry 4, after most of the $-\text{CH}_2\text{OH}$ in the DND-PG (DND(+)-PG(m)) is oxidized as shown in Figure 5b, only 2.04 mmol/g of $-\text{COOH}$ was produced. This discrepancy implies that some of the PG chains may be lost during the oxidation. Therefore, DND-PG-COOH shown in Figure 5b and 5d (entries 4 and 10 in Table 1, respectively) were compared with the corresponding DND-PG in thermogravimetric analysis (TGA, in air).

As shown in Figure 6, the two-step weight decrease below and above 450°C may result from the degradation of PG chain followed by the combustion of DND core. While oxidation of the DND-PG in entry 10 does not change the

TGA profile (blue lines in Figure 6), the PG content significantly decreases through the oxidation of DND-PG in entry 4 (red lines). This indicates that PG chains should have detached from the PG layer on DND surface at larger degree as the oxidation proceeds from 0.81 mmol/g (entry 10) to 2.04 mmol/g (entry 4). On the other hand, almost no change in hydrodynamic diameters is observed by DLS (Figure 4) before and after oxidation, despite the significant weight loss in entry 4 (Figure 6 and Table S1). This indicates that the PG layer decreases in its density, but the thickness does not change by the oxidation of DND-PG especially in entry 4.

To quantify the PG chains cleaved by the oxidation, molar amounts of glycerol and glyceric acid units on 1 g of DND core in DND-PG and DND-PG-COOH were calculated from the PG/DND weight ratio by TGA and the COOH content by acid-base titration. These results and calculation details are shown in Table 2. In Figure 7, the loss of PG chains in Table 2 correlates quadratically with the COOH content in DND-PG-COOH (A in Table 1). Therefore, little or no PG loss is observed at the COOH content smaller than 1.2 mmol/g. However, more than 20% of PG chain is lost in the DND-PG-COOH with the COOH content at 1.5 mmol/g and more. The loss of PG chain may be attributed to the oxidation of the secondary $-\text{OH}$ in PG chain as shown in Figure 8. When the secondary $-\text{OH}$ is oxidized into ketone, ether linkage to the carbon atoms next to the carbonyl group is subjected to C–O bond cleavage via tautomerization to the enol form. Although TEMPO is known to catalyze the oxidation of primary alcohol due to the steric hindrance in the surrounding of the nitroxyl radical (Figure 2), oxidation at secondary alcohol in the PG layer occurs in some extent. The quadratic relationship shown in Figure 7 may be due to the increase of the proportion of secondary $-\text{OH}$ against primary $-\text{OH}$, which is preferentially oxidized to $-\text{COOH}$, to increase the relative oxidation rate of the secondary $-\text{OH}$. This results in more cleavage of the PG chains. In addition, AZADOL (entries 17–19 in Table 1) with higher catalytic activity and less selectivity to primary alcohol caused larger loss of PG chain than 4-AcNH-TEMPO at pH 4.7 (green triangles in Figure 7),^[36] supporting the above interpretation that the cleavage of PG chain occurs by the oxidation of secondary alcohols shown in

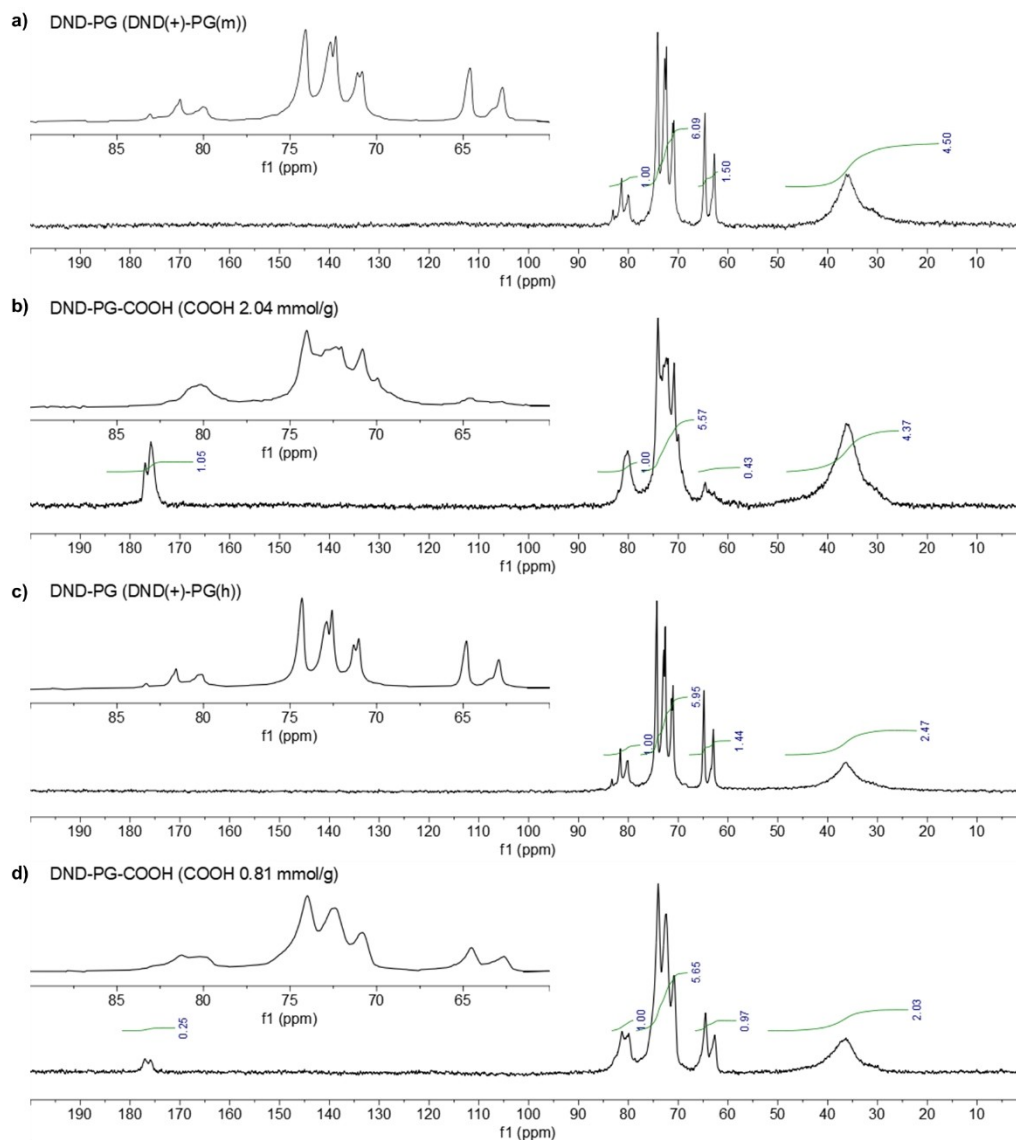


Figure 5. ^{13}C NMR spectra by inverse gated decoupling measurement of (a) DND-PG (raw material in entry 4, Table 1), (b) DND-PG-COOH (COOH content 2.04 mmol/g, entry 4, Table 1), and (c) DND-PG (raw material in entry 10, Table 1) and (d) DND-PG-COOH (COOH content 0.81 mmol/g, entry 10, Table 1). The signal of diamond core at 36.3 ppm is set as a reference.

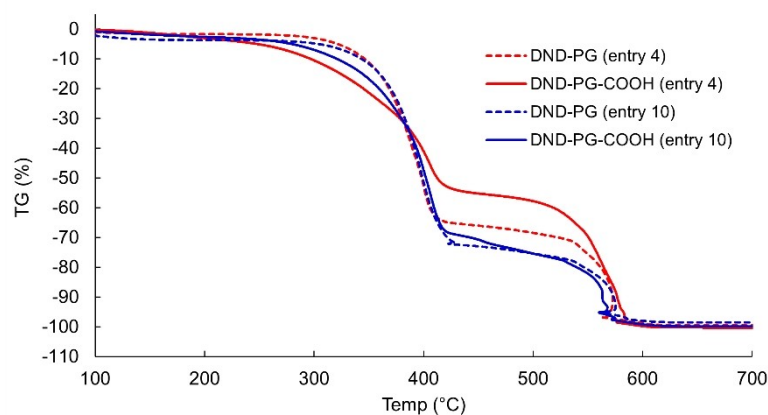


Figure 6. TGA profiles of DND-PG (dashed lines) and DND-PG-COOH (solid lines) with red and blue lines corresponding to entries 4 and 10 in Table 1.

Table 2. Compositions of glycerol and glyceric acid units in DND-PG-COOH listed in Table 1, and the losses of PG chain during oxidation reaction.

Entry ^[a]	Amount on 1 g of DND core in DND-PG		Amount on 1 g of DND core in DND-PG-COOH						Loss of PG chain (%)
	PG chain determined by TGA (g)	Mole amount of glycerol unit (mmol)	PG chain determined by TGA (g)	Glyceric acid unit		Glycerol unit		Total mole amount (mmol)	
				(mmol)	(g)	(g)	(mmol)		
<i>B</i>	<i>C</i> = <i>B</i> / 74.08 × 1000 ^[b]	<i>D</i>	<i>E</i> = <i>A</i> ^[c] × (1 + <i>D</i>)	<i>F</i> = <i>E</i> × 88.06 / 1000 ^[d]	<i>G</i> = <i>D</i> − <i>F</i>	<i>H</i> = <i>G</i> / 74.08 × 1000 ^[b]	<i>I</i> = <i>E</i> + <i>H</i>	(1 − <i>I</i> / <i>C</i>) × 100	
1	2.16	29.1	1.40	3.22	0.28	1.12	15.11	18.33	37.1
2	0.99	13.4	— ^[e]						
3	2.67	36.0	1.74	6.32	0.56	1.18	15.98	22.30	38.0
4	2.43	32.8	1.55	5.18	0.46	1.09	14.71	19.89	39.4
5	3.85	52.0	2.02	7.85	0.69	1.33	17.94	25.80	50.4
6	2.67	36.0	1.90	5.35	0.47	1.43	19.33	24.69	31.4
7	0.99	13.4	0.82	2.75	0.24	0.58	7.80	10.55	21.2
8	2.43	32.8	2.38	3.95	0.35	2.03	27.45	31.40	4.3
9	3.83	51.7	3.85	4.90	0.43	3.42	46.15	51.05	1.2
10	3.58	48.4	3.46	3.61	0.32	3.14	42.35	45.96	5.0
11	3.83	51.7	3.94	2.32	0.20	3.73	50.37	52.69	−2.0
12	3.85	52.0	3.69	3.23	0.28	3.40	45.91	49.14	5.4
13	0.99	13.4	1.02	2.11	0.19	0.83	11.27	13.38	0.1
14	2.43	32.8	2.19	4.34	0.38	1.81	24.37	28.71	12.5
15	3.85	52.0	3.74	3.08	0.27	3.47	46.83	49.91	4.0
16	3.85	52.0	3.76	1.45	0.13	3.63	49.05	50.49	2.8
17	2.43	32.8	1.70	3.97	0.35	1.35	18.27	22.25	32.2
18	3.85	52.0	3.08	5.18	0.46	2.63	35.45	40.63	21.8
19	3.85	52.0	3.46	3.08	0.27	3.19	43.10	46.18	11.2
20	0.85	11.5	0.87	1.47	0.13	0.74	10.05	11.52	−0.4

[a] Experimental conditions are described in the same entry in Table 1.

[b] 74.08: molecular weight of GD.

[c] *A*: COOH content in 1 g of DND-PG-COOH (mmol/g). See the same entry in Table 1.

[d] 88.06: molecular weight of 2,3-epoxypropionic acid.

[e] TGA was not measured. Accordingly, the following calculations were not done.

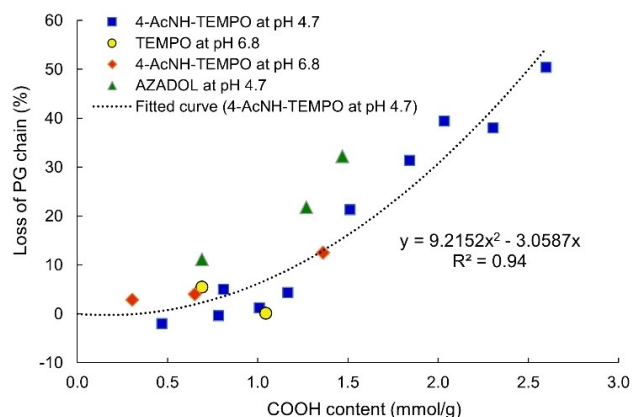


Figure 7. Relationship between the PG chains lost by the oxidation and the COOH content in the resulting DND-PG-COOH except for one result (entry 20 in Table 1) from ND50-PG to ND50-PG-COOH by 4-AcNH-TEMPO at pH 4.7 (blue square).

Figure 8. The reactions using TEMPO or 4-AcNH-TEMPO under neutral conditions gave a different trend; the loss of PG chain

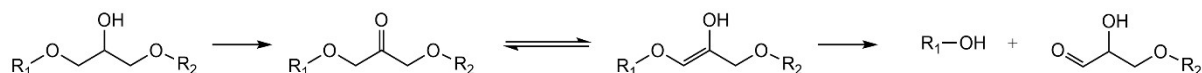


Figure 8. Plausible mechanism for cleavage of ether linkage caused by the oxidation at secondary hydroxy group.

is comparable with the reaction of 4-AcNH-TEMPO at pH 4.7 (yellow solid circles and red rhombi in Figure 7), while the COOH contents to the NaClO₂ amounts are lower and much less relationship (yellow solid circles and red rhombi in Figure 3). This may be because the reactivity of NaClO (HClO) or *N*-oxoammonium ion that react with the primary or secondary −OH may be lower at the neutral pH, while the selectivity is almost the same. Since the performance of the reaction is affected by pH and the amount of NaClO₂, the reaction at stable pH is critical especially for the precise control of COOH content.

Conclusions

We developed the process for poly(glycerol-co-glyceric acid) functionalized DND (DND-PG-COOH), a novel −COOH containing PG functionalized DNDs, via oxidation of primary −OH in PG chain by nitroxyl radical catalysts known as TEMPO. The product should be chemically robust, since the −COOH functionality is incorporated in PG chain. The reaction is so

simple that it can be performed under aqueous conditions with inexpensive fundamental inorganic oxidant like NaClO₂ and NaClO. The amount of –COOH can be precisely controlled by the amount of NaClO₂ in an almost stoichiometric manner for a range of COOH content ≤ 1 mmol/g. On the other hand, the reaction for higher COOH content with large amount of oxidant has a limitation to cleave the PG chains probably due to the oxidation of secondary –OH. Despite the limitation, the product with controlled COOH content can be applied to further functionalization for various applications especially in biomedical field.

Experimental

Materials

Single-digit nanometer-sized water dispersion of DNDs was manufactured by Daicel Corporation (DINNOVARE™). For the modification of DNDs, the following reagents and solvents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka Japan); 2,3-epoxypropan-1-ol (glycidol or GD), sodium hypochlorite (NaClO) solution, hydrochloric acid (HCl), sodium hydroxide (NaOH), ethanolic solution of potassium hydroxide (KOH), acetic acid, sodium acetate, potassium dihydrogen phosphate, ethylene glycol, methanol and ethanol. 2,2,6,6-Tetramethylpiperidine-1-oxyl free radical (TEMPO), 4-acetamido-2,2,6,6-tetramethylpiperidine-1-oxyl free radical (4-AcNH-TEMPO), AZADOL and sodium bromide (NaBr) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo Japan). Sodium chlorite (NaClO₂) was purchased from Sigma-Aldrich Japan G. K. (Tokyo, Japan).

Equipment

NMR spectra were measured with an ECX500 NMR spectrometer (JEOL). FT-IR spectra were recorded on an IR Tracer-100 FT-IR spectrometer (Shimadzu) equipped with DiffusIR DRIFT chamber (PIKE Technologies). Elemental analyses were conducted at Organic Elemental Microanalysis Center of Kyoto University. Thermogravimetric analysis (TGA) was performed with TG/DTA 6200 (SII). DLS measurement was done with a Nanotracs Wave II particle size analyzer (MicrotracMRB). Zeta-potential was measured by ZetaSizer Nano (Malvern, UK). EPR spectra were measured with EMX spectrometer (Bruker).

DND-PG (raw material, entry 1 in Table 1)

An aqueous dispersion of DND with positive ζ-potential, DND(+), was evaporated to dryness. The solid residue was dried at 105 °C *in vacuo* for 2 h. To a suspension of resulting DND powder (1.0 g) in ethylene glycol (15.0 g), GD (45.1 g, 0.61 mol) was added dropwise over 160 min to keep the temperature in the range of 95–100 °C. The resulting black dispersion was stirred at the same temperature for 4 h. After the reaction was cooled below 40 °C, water (40 mL) was added slowly to degrade the unreacted GD. The dispersion was diluted with water to ca. 400 mL and concentrated with ultrafiltration membrane (Ultracel® membrane, 30 kDa) to < 20 mL. The concentrate was diluted and concentrated again, which was repeated five times, and the weight of resulting black water dispersion was adjusted to 100.0 g with water. An aliquot of the dispersion was accurately weighed and dried on heated PTFE sheet. From the weight of the residue, the concentration was determined to be 4.10% (w/w).

To remove free PG, 30.0 g of above water dispersion (net 1.23 g) was ultra-centrifuged at 183400g (50000 rpm) for 2 h. Supernatant (ca. 20 mL) was removed carefully and remained lower layer was diluted and re-dispersed with water (20 mL), and ultra-centrifuged again, which was repeated two times. The resulting lower layer was adjusted to 30.8 g with water, which was used in the following oxidation step. To obtain a sample for analysis (titration), 15.8 g of this aqueous dispersion was once adjusted to pH 1.93 with HCl, then washed with water with the ultrafiltration membrane five times in the same manner as described above to recover 15.8 g of dispersion. An aliquot of the dispersion was accurately weighed (1.5391 g) and dried on heated PTFE sheet. From the weight of the residue (0.0441 g), the sample concentration was determined to be 2.87% (w/w). The net yield of DND-PG was 0.88 g from 1.23 g (net) of crude product.

FT-IR (DRIFT with KBr, cm^{−1}): 3332, 2918, 2875, 1456, 1118, 1075 (C–O). ¹H NMR (500 MHz, D₂O): δ ppm 3.42, 3.50, 3.58, 3.75, 3.88. Elemental analysis: C; 60.75%, H; 5.87%, N; 0.81%, O; 31.79%. TGA (Air atmosphere, 20 °C/min, % weight loss): 50–530 °C; 68.2%, 530–650 °C; 31.6% (PG/DND ratio was estimated to be 2.16).

DND-PG-COOH (oxy-radical oxidation with NaClO under basic condition, entry 1 in Table 1)

Water dispersion of purified DND-PG as described above (15.0 g, 2.87% (w/w), net 0.43 g, PG/DND 2.16) was diluted with water (10.0 mL) and added with NaBr (0.61 g, 5.9 mmol). The mixture was cooled in ice-bath and added with TEMPO (24.5 mg, 0.16 mmol). NaClO solution (effective chlorine concentration: 12.9 wt%) was added slowly with keeping the temperature at around 2 °C (below 5 °C). As pH decreased with the reaction progress, NaClO solution and/or 1 M NaOH was added to keep pH around 10 (10–12). The reaction was stopped when 2.5 g of NaClO solution (net 0.294 g, 4.0 mmol) was added in total for 35 min. After the addition of small amount of methanol to degrade unreacted NaClO, pH was adjusted to 1.97 with 6 M HCl. The dispersion was diluted with water to ca. 400 mL and concentrated with ultrafiltration membrane (Ultracel® membrane, 30 kDa) to < 10 mL. The concentrate was diluted and concentrated again, which was repeated four times, and the weight of resulting black water dispersion was adjusted to 15.0 g with water. An aliquot of the dispersion was accurately weighed and dried on heated PTFE sheet. From the weight of the residue, the concentration was determined to be 2.81% (w/w). The net yield of DND-PG-COOH was 0.42 g. FT-IR (DRIFT with KBr, cm^{−1}): 3273, 2906, 2878, 1734, 1456, 1122, 1080. ¹H NMR (500 MHz, 0.5% NaOD): δ ppm 4.13, 3.97, 3.90, 3.53. Elemental analysis: C; 59.12%, H; 5.29%, N; 0.78%, O; 34.36%. TGA (Air atmosphere, 20 °C/min, % weight loss): 50–487 °C; 57.8%, 487–650 °C; 41.2% (PG/DND ratio was estimated to be 1.40).

The carboxylic acid (COOH) content was determined to be 1.34 mmol by acid-base titration. Aqueous dispersion of DND-PG-COOH (1.6989 and 1.5458 g for two-time operation, net weight 47.7 and 43.4 mg, respectively) was accurately weighed and diluted with 30 mL of ultrapure water. After a few drops of phenolphthalein solution was added as the indicator, titration was done with 0.05 mol/L KOH ethanolic solution (*f* = 1.0). The endpoint was determined when the pink color of indicator was kept up for 30 sec (1.30 and 1.21 mL, respectively). Blank test without sample was also done by the same procedure (0.03 mL). The COOH content was calculated as the following equation.

$$C_{\text{COOH}} = ((T^1 - T^2) \times 0.05 \times f) / (W \times S)$$

where *C*_{COOH}: COOH content (mmol/g), *T*¹: titer of 0.05 mol/L KOH to the sample (mL), *T*²: titer of blank test (mL), *f*: factor value of KOH solution, *W*: weight of sample dispersion (g), and *S*: sample

concentration (wt/wt). By the same procedure, COOH content of raw material (DND-PG) was determined to be 0.04 mmol/g.

DND-PG-COOH (oxy-radical oxidation with 4-AcNH-TEMPO and NaClO₂ under acidic condition, entry 4 in Table 1)

Aqueous dispersion of purified DND-PG (DND(+)-PG(m), 3.23% (w/w), 18.6 g, net 0.60 g, PG/DND 2.43) were added with 0.4 M acetate buffer (pH 4.7, 30 mL), water (10.0 mL), NaClO₂ (content 81%, 671 mg, 6.0 mmol) and 4-AcNH-TEMPO (24.9 mg, 0.12 mmol). NaClO solution (74 μ L, 0.14 mmol) was added into the mixture and the flask was equipped with an air-cooled condenser capped with a universal glass plug, then the reaction was heated at 50 °C for 24 h. After ethanol (1.0 mL) was added to decompose unreacted oxidant, the dispersion was diluted with water to ca. 400 mL and concentrated with ultrafiltration membrane (Ultracel® membrane, 30 kDa) to <10 mL. The concentrate was washed with water once by dilution and concentration with ultrafiltration membrane, and pH was adjusted to ca. 2.0 with 6 M HCl. The mixture was concentrated and then washed with water three times, and the weight of resulting black water dispersion was adjusted to 20.0 g with water. An aliquot of the dispersion was accurately weighed and dried on heated PTFE sheet. From the weight of the residue, the concentration was determined to be 2.49% (w/w). The net yield of DND-PG-COOH was 0.50 g. COOH concentration was determined by acid-base titration to be 2.04 mmol/g. Elemental analysis: C; 60.69%, H; 5.45%, N; 0.79%, O; 32.91%. TGA (Air atmosphere, 20 °C/min, % weight loss): 50–533 °C; 68.7%, 533–650 °C; 31.4% (PG/DND ratio was estimated to be 2.19).

DND-PG-COOH (entry 10 in Table 1)

The oxy-radical oxidation with NaClO₂ was done on DND-PG (DND(+)-PG(h), 3.95% (w/w), 44.3 g, net 1.75 g, PG/DND 3.58) were conducted with 0.4 M acetate buffer (pH 4.7, 35 mL), NaClO₂ (199 mg, 1.7 mmol), 4-AcNH-TEMPO (54.0 mg, 0.25 mmol) and NaClO solution (161 μ L, 0.26 mmol) as described above. The weight of resulting black water dispersion was adjusted to 45.3 g with water. The concentration was determined to be 3.81% (w/w) and net yield was 1.72 g. COOH concentration was determined by acid-base titration to be 0.81 mmol/g. Elemental analysis: C; 57.72%, H; 6.24%, N; 0.63%, O; 35.44%. TGA (Air atmosphere, 20 °C/min, % weight loss): 50–527 °C; 77.4%, 527–650 °C; 22.4% (PG/DND ratio was estimated to be 3.46).

Acknowledgements

We thank Dr. Yajuan Zou (Okayama University, Japan), Mr. Shun Kimiya (Mitsubishi Materials Corporation, Japan), Mr. Yuya Miyake (Hosei University, Japan), Prof. Md. Mahbubor Rahman (University of Rajshahi, Bangladesh), Dr. Giacomo Reina (Empa, Switzerland) and Ms. Jie Yu (Kyoto University, Japan) for useful information and discussion about various knowledge in PG and PG functionalization.

Conflict of Interests

M.N. and N.K. are inventors on Japanese patent application number 2022-028954 submitted by Kyoto University and Daicel Corporation. It covers PG-functionalized nanoparticles in which

at least one –CH₂OH is substituted by –COOH described in this manuscript. Other authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Keywords: Detonation nanodiamonds • Poly(glycerol-co-glyceric acid) • carboxy group • TEMPO oxidation

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Manuscript received: April 25, 2023
 Revised manuscript received: June 21, 2023
 Accepted manuscript online: June 25, 2023
 Version of record online: July 19, 2023
