

A highly water-soluble C₆₀-NVP copolymer: a potential material for photodynamic therapy†

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Water-soluble C₆₀ polymers, which have the highest solubility of fullerene reported to date (7.8 mM of C₆₀ in water), were prepared *via* copolymerization, and showed significant visible light-induced O₂^{•-} generation and DNA cleavage, indicating the potential of these water-soluble fullerene derivatives as agents for photodynamic therapy.

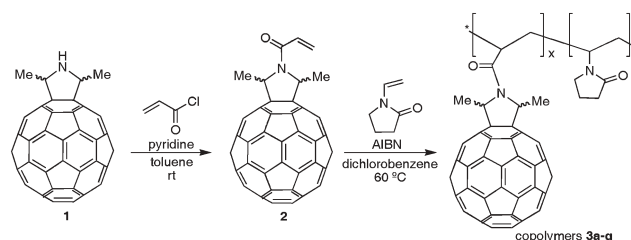
Fullerene-based materials are currently of great interest for applications that take advantage of their unique electronic properties or biological activities. In the context of biologically active fullerenes, reported activities include HIV-1 protease inhibition,^{1–3} radical scavenging activity⁴ and neuroprotective effects.⁵ In addition, the powerful photoinduced biological activities^{6–9} of C₆₀ have been of interest due to the remarkably high quantum yield of photoexcitation reaction¹⁰ and the relatively long excitation wavelength (S–S absorption: 530, 920 nm, T–T absorption: 400, 740 nm), and C₆₀ has been regarded as a potential scaffold for photodynamic therapy. The hydrophobicity¹¹ of C₆₀, however, has impeded further biological application, despite the large number of water-soluble C₆₀ derivatives that have been reported.¹² In our own research, we have developed methods to solubilize C₆₀ and C₇₀ in aqueous solution by using a non-toxic detergent, poly(vinylpyrrolidone) (PVP).¹³ The resulting solutions showed *in vitro* biological activities including DNA-cleaving,¹⁴ cytotoxicity¹⁵ and antibacterial activity under visible light irradiation.¹⁶ In addition, we observed photoinduced generation of reactive oxygen species (ROS) such as O₂^{•-} and [•]OH in these aqueous fullerene solutions under physiological conditions as key active species responsible for photoinduced DNA cleavage by fullerenes.^{17,18}

These studies indicated that C₆₀ is an effective photosensitizer with high quantum yield of ROS generation and could be a lead structure for photodynamic therapy. For the further development of fullerene-based photodynamic therapy, the selected targeting of the organ or tissue is an important consideration. A first level of selectivity is possible by choosing a specific wavelength of the light for irradiation at a defined area of the body. For a second level of targeting, we considered taking advantage of the enhanced permeation and retention (EPR)¹⁹ effect of polymers, which is useful for targeting polymeric drugs to tumor tissue by selective accumulation into tumor tissue due to leaky vasculature. In this

study, we report the synthesis, characterization, and preliminary biological activities of highly water-soluble C₆₀-derived polymer. To achieve this, we prepared the first C₆₀-*N*-vinylpyrrolidone (NVP) copolymer, which consists of C₆₀ incorporated covalently into a PVP chain *via* radical polymerization. As a result, we achieved the highest level of fullerene solubility in water (7.8 mM) yet reported, and which enables preparation of aqueous solutions even higher in concentration than saturated C₆₀ solution in toluene (4 mM). The use of a non-ionic PVP polymer, which has long been demonstrated to be non-toxic, offers a promising approach to bringing the demonstrated biological properties of fullerenes into a medically-relevant environment.

We chose dimethylfulleropyrrolidine **1** as a convenient starting material for further elaboration to a C₆₀-derived monomer.^{20,21} Intact C₆₀ is reported to react with styrene and other olefins by radical polymerization to form copolymers, but is also known to quench radicals and inhibit polymerization. Using the secondary amine of **1** as a synthetic handle, we incorporated an acrylamide, which is highly reactive in radical polymerization reactions and was expected to increase the efficiency of C₆₀ incorporation in the copolymer. Using monomer **2**,²² the copolymerization with *N*-vinylpyrrolidone (NVP) (Scheme 1) was carried out using a common radical initiator, 2,2'-azobisisobutyronitrile (AIBN), and with various feed ratios of C₆₀ and NVP (1 : 50, 75, 100, 150, 200, 250 and 300 in mol ratio), to compare the properties of the resulting copolymers.

Due to the radical quenching activity of C₆₀ reported,^{23,24} somewhat larger amounts of AIBN (0.2 equiv. of feed NVP) than commonly employed were used for the radical polymerizations. During the reaction, the mixtures solidified and were reconstituted in CHCl₃. The synthesized polymer was subsequently precipitated by the addition of Et₂O. At the end of each reaction, no C₆₀ monomer **2** was detected in the reaction mixture by TLC, indicating that all of monomer **2** was incorporated into the copolymer. Each copolymer obtained was characterized by GPC, DLS and solubility tests.



Scheme 1 Synthesis of copolymers of *N*-vinylcarbonyldimethylfulleropyrrolidine **2** and *N*-vinylpyrrolidone.

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Table 1 Synthesis and characterization of water-soluble C₆₀-NVP copolymers

Entry	Feed ratio of C ₆₀ -NVP (mol)	Yield (%)	Mn (kDa) ^a	Solubility (mg mL ⁻¹)	[C ₆₀] (mM) ^b	[PVP] (%w/v)
1 (3a)	1 : 50	43	41	0.04	0.0063	0.0035
2 (3b)	1 : 75	53	41	0.05	0.0055	0.0045
3 (3c)	1 : 100	66	30	80	6.7	7.4
4 (3d)	1 : 150	84	43	120	6.9	11
5 (3e)	1 : 200	96	39	180	7.8	17
6 (3f)	1 : 250	96	46	200	7.0	19
7 (3g)	1 : 300	95	51	240	7.0	23

^a Determined by GPC analysis. ^b Estimated as monomer.

In the reactions with relatively low feed ratios of NVP (Table 1, entries 1 and 2), the yields were low and the solubilities of the resulting copolymers (**3a** and **3b**) in water were not high enough for biological assays. When 100 or greater equivalents of NVP to C₆₀ were employed, the solubility of copolymers in water increased significantly (Table 1, entry 3). Especially in reactions with higher feed ratio of NVP (C₆₀ : NVP = 1 : 200–300, entries 5–7), almost quantitative yields of copolymers with high solubility in water were obtained, giving dark brown aqueous solutions. The solubility of each polymer was determined by measuring the OD₃₀₀ of the solution as shown in Fig. 1. The amount of C₆₀ and PVP in the saturated solution was calculated (Table 1). The highest solubility of C₆₀ was observed in copolymer **3e** (C₆₀-NVP (1 : 200)), which was equivalent to 7.8 mM of C₆₀ (estimated as monomer) in water with 17% of PVP. This solubility in water is the highest level reported for fullerenes. By GPC analysis, the molecular weight of each copolymer was estimated to be in the range of 30–50 kDa (Table 1). Since the EPR effects of polymers in tumors are generally observed in molecules with a MW greater than 20 kDa, these C₆₀ copolymers are expected to be suitable for assays against cancer. The size of the particle of each polymer was analyzed by DLS analysis in DMF, DMSO and water.²⁵ The diameters of particles of copolymers **3d–g** in water and **3b–g** in DMF or DMSO

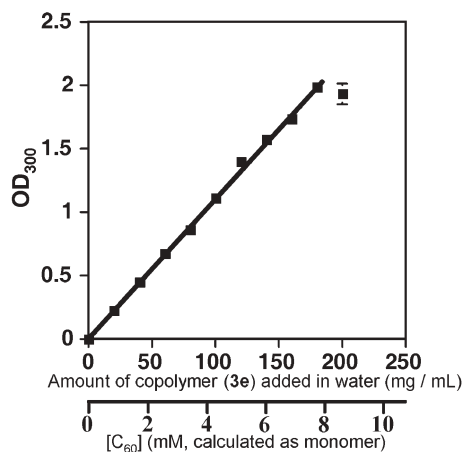


Fig. 1 Solubility curve of C₆₀-NVP copolymer (1 : 200, **3e**). Copolymer was weighed and water was added to prepare 1 mL of solution. Each solution was filtered through a cotton-plug filter followed by a membrane filter. The filtrate was diluted with water 100 times to measure the OD₃₀₀. The solubility of the copolymer was estimated as 180 mg mL⁻¹ and the calculated concentration of C₆₀ as monomer was 7.8 mM.

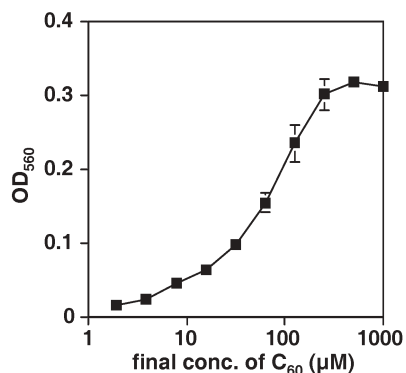


Fig. 2 Dose-dependent superoxide (O₂^{•-}) generation by C₆₀-NVP copolymer **3e** detected by NBT assay. Irradiation: 200-W photoreflexor lamp (35,000–37,000 lux), in 50 mM phosphate buffer (pH 6.5) in the presence of 10 mM NADH at 0–5 °C.

were around 10–20 nm which is reasonable for single molecules whereas those of **3c** in water and **3a** in DMF or DMSO were 80–90 nm, indicating aggregation of the molecules.

The copolymer **3e**, which showed the highest solubility of fullerene, was chosen for further studies including O₂^{•-} generation and DNA cleavage tests. The O₂^{•-} generating property of copolymer **3e** was tested by the nitro blue tetrazolium (NBT) assay. Under visible light irradiation and in the presence of physiological concentrations of NADH as a biological electron donor, blue formazan formation was observed in a dose-dependent manner (Fig. 2). Also no increase in OD₅₆₀ was observed without light, indicating that photoexcitation of the C₆₀ in the copolymer is responsible for the O₂^{•-} generation.

A photoinduced DNA cleaving test was selected as a good preliminary indicator for the potential of these water-soluble copolymers as photodynamic therapeutic agents against cancer. Fig. 3 shows the results using pBR322 supercoiled DNA. As shown in lane 1, copolymer **3e** showed significant DNA cleaving activity to give nicked form II in the presence of physiological concentration of NADH and under visible light irradiation. As expected, this cleavage was observed in a dose-dependent manner of polymer concentration and light irradiation.

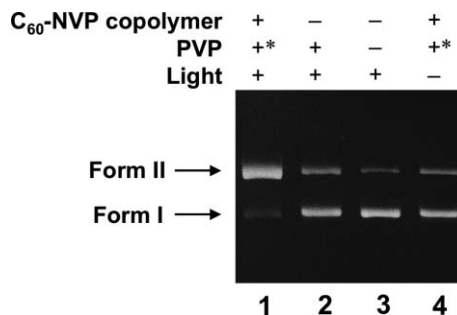


Fig. 3 Photoinduced DNA cleavage by water-soluble C₆₀-NVP copolymer **3e**. The pBR322 supercoiled plasmid was incubated with copolymer in TDC buffer in the presence of 10 mM of NADH for 4 h at 37 °C under irradiation by a 200-W photoreflexor lamp (60,000–63,000 lux). Lanes 1 and 4: C₆₀-NVP copolymer: 23 mg mL⁻¹ (estimated C₆₀ concentration as monomer: 1 mM); lane 2: control experiment with 2.2% PVP; lane 3: blank experiment without chemicals. *As a part of C₆₀-NVP copolymer.

In summary, a new class of water-soluble C₆₀ copolymers with high water-solubility was synthesized and the bioactivities were studied. The polymers were obtained in good yield and showed the highest level of solubility reported, even higher than saturated C₆₀ in toluene. The molecular weights of the copolymers were high enough (30–50 kDa) to likely exhibit the EPR effects in tumor tissues. The confirmed biological activities include photoinduced O₂^{•−} generation and DNA cleavage, establishing that this water-soluble C₆₀ copolymer possesses the photosensitive reactive oxygen generation causing the DNA damage. Taken together, these results suggest that the novel, highly water-soluble C₆₀-containing polymers will have potential as new materials for photodynamic therapy. Further studies including *in vivo* biological tests are ongoing.

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- Preparation and selected spectroscopic data of **2**. To a solution of *cis*- and *trans*-**1** (77 : 23 mixture, 34 mg, 0.043 mmol) in toluene (20 mL), acryloyl chloride (TCI, 40 μ L, 45 mg, 0.46 mmol) and pyridine (Alfa Aesar, distilled, 2 mL) were added and stirred for 2 min under N₂ atmosphere. The reaction progress was monitored by TLC (SiO₂, toluene–EtOAc (10 : 1), R_f: *cis*, *trans*-**1**: 0.19, *cis*-**2**: 0.44, *trans*-**2**: 0.37). The reaction mixture was separated by silica gel column chromatography (SiO₂: 50 g, developed with hexane, toluene–EtOAc, eluted with toluene–EtOAc (30 : 1)) to give a brown solid (*cis*- and *trans*-**2**, 30.3 mg), which was further purified by preparative TLC (toluene–EtOAc (10 : 1)) to give *cis*-**2** (20.4 mg, 0.024 mmol) and *trans*-**2** (8.2 mg, 0.0097 mmol) (total 0.034 mmol, 79%); *cis*-**2**: mp > 260 °C; IR (neat) 2969 cm^{−1}, 2925, 2848, 1649, 1609, 1415, 1376, 1303, 1277, 1213, 1185, 1099, 972; ¹H-NMR (CDCl₃, 400 MHz) δ 7.01 (dd, *J* = 10.29, 16.74 Hz, 1H, vinyl), 6.63 (dd, *J* = 16.74, 1.84 Hz, 1H, vinyl), 5.93 (dd, *J* = 10.29, 1.84 Hz, 1H, vinyl), 5.80 (br.q, *J* = 5.99, 2H, CH), 2.32 (d, *J* = 5.99, 6H, CH₃); ¹³C-NMR (CDCl₃, 100 MHz) δ 168.1 (C=O), 154.8, 152.0, 147.7, 146.6, 146.6, 146.4 (3 peaks overlap), 145.8 (2 peaks overlap), 145.8, 145.7, 145.6, 145.5, 144.8, 144.7, 143.4, 143.0, 142.9, 142.4, 142.4, 142.3 (2 peaks overlap), 142.1, 142.0, 140.4, 140.0, 137.0, 135.9, 130.6 (vinyl), 129.2 (vinyl), 74.0 (sp³ quaternary), 64.6 (CH), 20.2 (CH₃); MS (FAB, NBA) *m/z* 846 ([M + H]⁺); HR-MS (ESI) 868.0772 obs., 868.0732 calcd. for C₆₇H₁₁NONa; *trans*-**2**: mp > 260 °C; IR (neat) 3007 cm^{−1}, 2967, 2925, 2851, 1666, 1614, 1407, 1378, 1338, 1264, 1227, 1186, 1123, 972; ¹H-NMR (CDCl₃, 400 MHz) δ 6.97 (dd, *J* = 10.3, 16.7, 1H, vinyl), 6.74 (dd, *J* = 2.00, 16.7, 1H, vinyl), 6.20 (q, *J* = 7.06, 2H, CH), 6.00 (dd, *J* = 2.00, 10.3, 1H, vinyl), 2.28 (d, *J* = 7.06, 6H, CH₃); ¹³C-NMR (CDCl₃, 100 MHz) δ 164.1 (C=O), 155.8, 152.1, 147.7, 146.6, 146.5, 146.4, 146.3, 146.2, 145.8, 145.6, 145.6 (2 peaks overlap), 145.5, 144.9, 144.8, 144.6, 143.5, 142.9, 142.9, 142.5, 142.4, 142.3 (2 peaks overlap), 142.2, 141.8, 140.4, 140.2, 137.6, 134.5, 130.2 (vinyl), 127.8 (vinyl), 73.6 (sp³ quaternary), 64.1 (CH), 24.7 (CH₃); MS (FAB, NBA) *m/z* 846 ([M + H]⁺); HR-MS (ESI) 868.0730 obs., 868.0732 calcd. for C₆₇H₁₁NONa.
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