Complexation of C_{60} Fullerene with Cholesteryl Group-Bearing Pullulan in Aqueous Medium

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Water-soluble complex between C_{60} fullerene and cholesteryl group-bearing pullulan (CHP) was prepared. C_{60} fullerene was dissolved in pyridine (10% v/v) in advance and then mixed with an aqueous CHP suspension (0.1 mg ml^{-1}). The particle size of the formed complexes varied from 60 nm to 150 nm by the concentration of aqueous pyridine in final solution. The complex could retain its integrity for a long period of time without destruction upon heating or freezing.

The use of fullerenes for biological purpose has been gained more attentions in the past decade due to its ability of converting oxygen from triplet state to singlet state when exposed to light.[1] The mechanism has been proposed to the direct deactivation of enzymes in the target cell, relating to antiviral activity or DNA cleavage.[2] However, the low water solubility of fullerene makes it less accessible to the living system. Thus, an increase in water solubility of fullerenes leads to an increase in the cellular uptake of fullerene, and this makes phototherapy more effective. Many attempts for this purpose have been tried by complexation and solubilization with cyclodextrins,[3] calixerenes,[4] Tween-20,[5] Triton X-100,[6] phospholipids[7] and polyvinylpyrrolidone[8] or by encapsulation in liposomes[6] and vesicles.[9] However, those previous methodologies could not much improve the water solubility of fullerene. To overcome this problem, we include the new technique to solubilize C_{60} fullerene in greater content.

Self-aggregates of hydrophobilized polysccharides[10] as developed by ourselves have shown a promising candidate to form supramolecular assembly with fullerenes in water. Hydrogel nanoparticles[11] formed by self-aggregates of hydrophobized polysaccharide are capable to encapsulate not only bio-macromolecules like proteins[12] or enzymes[13] but also the small hydrophobic molecules, such as neocartinostatin chromopher[14] or doxorubicin.[15] This versatile complexation ability of hydrophobized polysaccharide is very promising as a potent drug carrier even for fullerenes.

Stock solution of CHP (CHP-108-0.9, 1.0 mg ml^{-1})[11] (Nippon Oil and Fats Co., Tsukuba, Japan) was prepared in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4) containing 3 mM NaN_3. The solution was stirred at 50-55 °C for 48 h following by sonication at 40 W for 5 min on an ice/water-bath using a probe type sonifier (TOMY, UR-200P, Tokyo).

Simple mixing of fullerene powder in an aqueous CHP suspension did not lead to complexation between the two. Therefore, complexation between C_{60} fullerene and CHP was made by quickly injecting a pyridine solution of fullerene (400 µl, 0.5 mg ml^{-1}) into an aqueous CHP suspension (9.6 ml, 0.05 mg ml^{-1} in PBS). The resulting mixture was stirred at room temperature for 48 h and then dialyzed four times against 3000 ml of PBS buffer using a Spectra/Por® molecular dialysis membrane (molecular weight cut-off = 3000, Amicon, Inc., Beverly, MA USA). UV-visible spectrum of the complex is given in Figure 1. We will discuss in more detail about the electronic spectra[16] of the complex in the coming full paper. Of course, that the CHP self-aggregate does not take place any significant change upon the mixing with pyridine under the conditions employed was certified in advance.

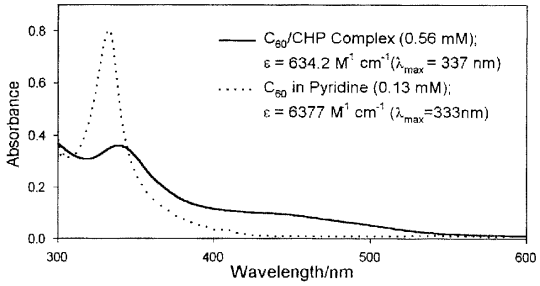

Figure 1. UV-visible spectra of CHP/C_{60} complex and C_{60} fullerene in pyridine.

The dialyzate was then submitted to a high performance size exclusion chromatography (HPSEC, Tosoh Ltd., Tokyo, Japan) with Superose® 6HR 10/30 column (Pharmacia biotech, Uppsala, Sweden). Detection was made by UV-visible absorption and RI. The chromatogram of the sample (Figure 2) showed two peaks; the first one, which was detected by both UV (at 337 nm) and RI, was the CHP/C_{60} complex. This peak was well separated from that of free CHP self-aggregate (Figure 2a). The mixture was then chromatographically separated by Sephacryl S-500 column (Φ17 mm × 40 mm, Pharmacia) at flow rate of 1.0 ml min^{-1}. Collected and purified fractions at R_t 17 min was found to contain C_{60} fullerene and completely overlapped with the peak of free CHP self-aggregate (Figure 2b). HPSEC confirmed that more than 90% of fullerene initially adopted was complexed with CHP.

The concentration of C_{60} fullerene complexed with CHP spectroscopically determined. Extraction of fullerene from the complex was carried out as follows. First, 0.5 ml of saturated NaCl solution was mixed with 0.1 ml of the CHP/C_{60} sample solution, and then 2 ml of toluene was added in a glass vial. The resulting mixture was sonicated at 40 W for 20 seconds. The content of fullerene extracted in toluene was photometrically determined at 335 nm.

The complexation between C_{60} fullerene and parent pullulan was also tried by the same method. However, no complexation was observed at all, and most of fullerene molecules were recov-

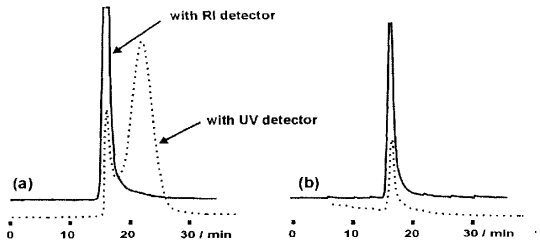

Figure 2. HPSEC chromatograms for the CHP/C_{60} complex before (a) and after (b) purification using Sephacryl S-500 chromatography. Solid line is given by UV detection at 337 nm and dotted line is by RI detection.

Table 1. The hydrodynamic diameter and the weight ratio of CHP to C_{60} content in the CHP/C_{60} complex.

In complex formation			Particle size	Coefficient[a]	Recover / %		Weight ratio of polysaccharide to fullerene / %w/w
CHP wt%	C_{60} wt%	Aqueous pyridine concentration / vol%		k_1/k_2^2	CHP	C_{60}	
1.0	0.2	**2**	152.0±5.5	1.004	24	91	1.30±0.07
1.0	0.2	**4**	101.3±4.1	0.6823	26	94	1.39±0.06
1.0	0.2	**10**	59.6±2.5	0.7676	33	96	1.73±0.03
1.0	0.2	**20**	60.6±2.6	0.7345	30	93	1.61±0.09
1.0	0.2	**30**	60.7±3.1	0.9307	29	93	1.54±0.07

[a]Polydispersity obtained by cumulants analysis.

ered as precipitates upon membrane filtration employing 0.2 μm pore size filter.

The particle size of the CHP/C_{60} complex was determined by dynamic light scattering (DLS-700, Osaka Electronics, equipped with a vertically polarized 5-mM He-Ne laser, 633 nm). Relation between the diameters of the complex and the concentration of pyridine during the complexation is shown in Table 1. The particle size of the complex decreased from approximately 150 nm to 60 nm as the pyridine content in the resulting mixture increased from 2% to 10%. No significant change of the particle size was observed under the pyridine content over 10%. This would suggest importance of the solubility of fullerene in aqueous pyridine. Unlike free C_{60} fullerene aggregates which are polydisperse particles and precipitate out under low ionic strength,[14] the CHP/C_{60} complex was rather stable. Neither aggregation nor precipitation of C_{60} molecules dissociated from the complex was observed even for more than three months at room temperature. The size of the complex particles was fairly monodisperse in PBS buffer, and the weight ratio of polysaccharide to fullerene of the complex was found to be 1.30 – 1.73.

To examine the stability of the complex, the CHP/C_{60} complex suspension was freeze-dried and then re-dispersed in Milli-Q water. After very short period of sonication (at 40 W for 10 s), sample suspension was submitted to HPSEC and DLS. HPSEC data showed neither free CHP monomer nor CHP self-aggregate itself, and the particle size retained unchanged during the lyophilization.

Cryo-tem imaging of the comlex was carried out using a JEM4000SFX (JEOL, Japan). The CHP/C60 suspension was deposited onto a carbon microgrid that was hydrophilized in advance with plazma beam irradiation for 30 s. After blotting excess amount of the sample, the specimen was immersed in liquid propane for thermal fixation. The cryo-tem images were then taken at the acceleration voltage 400 kV and the defocus by 3 μm in depth from the right position. The specimen temperature to obtain images was kept at 14-20 K. The practical resolution limit of the images was allowed to 1 nm. This was confirmed by the contrast transfer function at the acceleration voltage and the defocus value. The cryo-tem image also showed the monodispersity of the particles in a narrow size distribution of 10 to 40 nm (Figure 3). The image showed further aggregation of small particles to give larger aggregates.

As the results, we can suggest a potential technique for solubilizing water insoluble fullerene in water by simple complexation with hydrophobized polysaccharides without any chemical modification[17] of fullerene. This will provide a convenient method to study the effect of fullerene in biological environment. Hydrophobized polysaccharide is very promising as a vehicle for biologically active fullerene.

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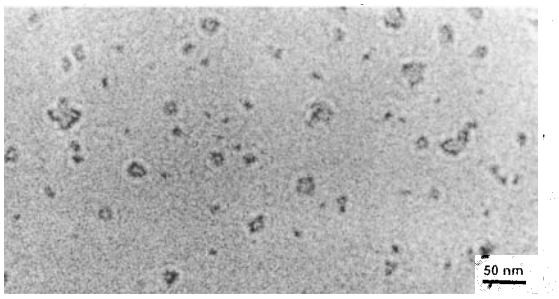

Figure 3. TEM-microgram of CHP/C_{60} complex (magnification, 180 x 10^3).

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