

Physicochemical Characterization and Carcinoma Cell Interaction of Self-Organized Nanogels Prepared from Polysaccharide/Biotin Conjugates for Development of Anticancer Drug Carrier

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Abstract Self-organized nanogels were prepared from pullulan/biotin conjugates (PU/Bio) for the development of an effective anticancer drug delivery system. The degree of biotin substitution was 11, 19, and 24 biotin groups per 100 anhydroglucose units of pullulan. The physicochemical properties of the nanogels (PU/Bio1, 2 and 3) in aqueous media were characterized by dynamic light scattering, transmission electron microscopy, and fluorescence spectroscopy. The mean diameter of all the samples was less than 300 nm with a unimodal size distribution. The critical aggregation concentrations (CACs) of the nanoparticles in distilled water were 2.8×10^{-2} , 1.6×10^{-2} , and 0.7×10^{-2} mg/ml for the PU/Bio1, 2, and 3, respectively. The aggregation behavior of the nanogels indicated that biotin can perform as a hydrophobic moiety. To observe the specific interaction with a hepatic carcinoma cell line (HepG2), the conjugates were labeled with rhodamine B isothiocyanate (RITC) and their intensities measured using a fluorescence microplate reader. The HepG2 cells treated with the fluorescence-labeled PU/Bio nanoparticles were strongly luminated compared with the control (pullulan). Confocal laser microscopy also confirmed internalization of the PU/Bio nanogels into the cancer cells. Such results demonstrated that the biotin in the conjugate acted as both a hydrophobic moiety for self-assembly and a tumor-targeting moiety for specific interaction with tumor cells. Consequently, PU/Bio nanogels would appear to be a useful drug carrier for the treatment of liver cancer.

Key words: Self-organized nanogels, anticancer drug delivery, pullulan, biotin, HepG2

Anticancer agents in cancer chemotherapy are not as effective as originally anticipated, because of nonspecific toxicity, a lack of tumor selectivity, and the induction of multidrug resistance (MDR) in various tumor cells [7, 11, 24]. Various targeting strategies have already been developed to improve chemotherapy and can be generally categorized as either active or passive targeting [8, 13, 27]. Although not absolutely definitive, active targeting essentially describes systems that use specific interactions (*e.g.*, antigen-antibody interaction and ligand-receptor interactions) or a locally applied external force/energy (*e.g.*, heat, magnetic field, and sonication), whereas passive targeting describes systems that use nonspecific interactions (hydrophobic, electrostatic, and hydrogen interactions) and physical factors (size and mass) for carrier localization [27]. Passive targeting was originally developed based on the discovery of two phenomena: the enhanced permeability and retention (EPR) effect [8, 13] and long-circulating carriers [18], which help improve the accumulation of drug carriers at tumor sites. Thus, various nanocarrier systems have been investigated for anticancer drug delivery in chemotherapy, including self-organized nanogels of polysaccharide derivatives, which are promising candidates because of their good biocompatibility and degradation *in vivo*.

Nanogels are composed of a polycore and outer shell of hydrophilic groups. The dispersed hydrophobic cores can be a depot for a water-insoluble drug, whereas the sea matrix domain works as a surrounding barrier, inhibiting any interaction between the hydrophobic domains and the biocomponents [12, 18, 19, 21]. Na *et al.* [18] previously reported on self-organized nanogels prepared from curdlan/sulfonylurea by intra- and/or intermolecular association in an aqueous solution as an anticancer drug cargo for treating

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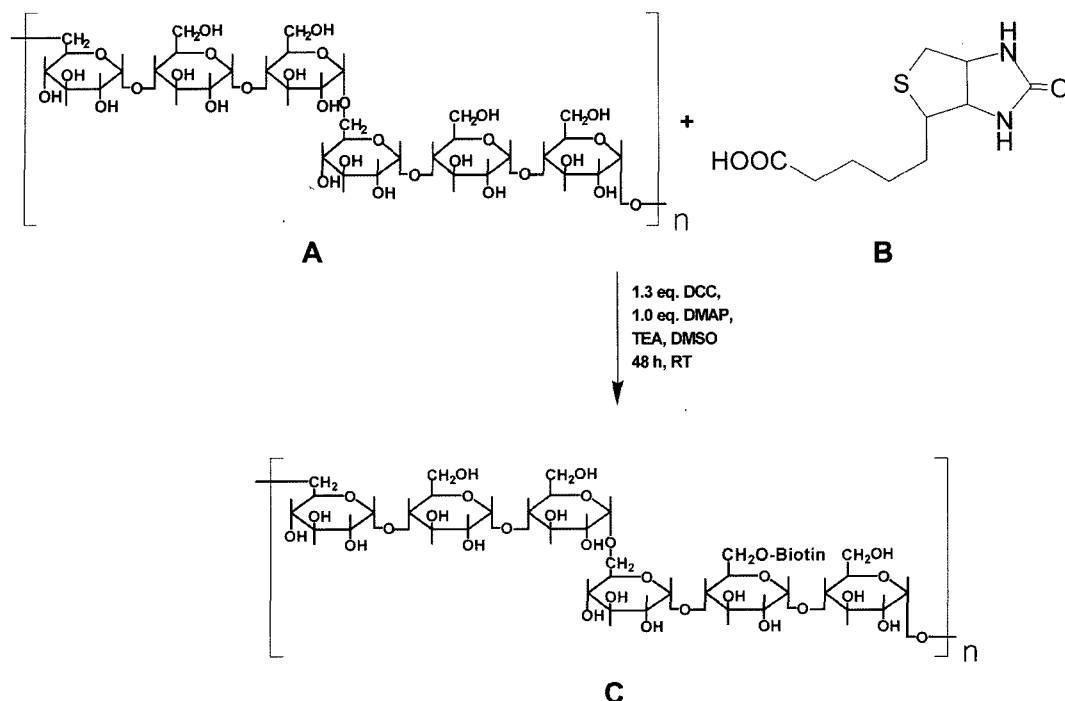


Fig. 1. Chemical structures of pullulan (A), biotin (B), and pullulan/biotin conjugate (PU/Bio) (C).

liver cancer [18]. The sulfonylurea groups create noncovalent cross-linking points through their assembling, resulting in the formation of nanoaggregates with polycore structures. Na *et al.* also reported on self-assembled nanoparticles prepared from pullulan acetate and its derivatives [17, 19, 20] that exhibited effective tumor targeting [19] and pH-sensitivity to tumor acidity [17, 20].

Self-organized nanogels can be delivered to tumor sites by size-dependent passive targeting or active targeting [4, 6]. Various studies have attempted to use active targeting to improve the selectivity for a specific organ and enhance the internalization of drug-loaded nanoparticles into the target cells. Internalization produces more efficient drug therapy, as the drug acts directly in the target cells [18, 19]. Thus, to enhance the internalization of drug carriers into cancer cells, various ligands have been used, such as sugar and vitamin ligands [9, 25]. In particular, vitamin ligands are frequently used because of the high expression levels of their receptors with various tumor cells, their non-immunogenicity, low molecular weight (small size), versatile conjugation chemistry, and economic preparation costs. Furthermore, vitamin receptor-mediated endocytosis often adopts a non-lysosomal route that is advantageous for delivering proteins or genes by avoiding lysosomal enzyme degradation after internalization [10].

Accordingly, to produce a self-organized nanogel with enhanced cancer targeting activity and internalization into cancer cells, the present study investigated the introduction of biotin into pullulan. Pullulan, a linear polysaccharide

with an α-(1-6)-linked maltotriose repeating unit (Fig. 1A), is now commercially available [19], and a variety of pullulan derivatives have already been prepared to study their potential application in the pharmaceutical and biomedical fields. Biotin is a B complex vitamin (Fig. 1B) and growth promoter at the cellular level [1] that has already been used along with its derivatives in cancer studies and tissue engineering [3, 14, 22]. Biotin exists in the liver, kidneys, pancreas, and milk. Furthermore, the biotin content in cancerous tumors is higher than that in normal tissue [2]. Thus, since the rapid proliferation of tumor cells would seemingly require extra biotin, the cell surface receptors for biotin may be overexpressed on tumor cells, although the biotin receptor in a cancer cell has not yet been clearly defined. When biotin is conjugated with other materials via amide or ester linkages, its water-solubility dramatically decreases because of the loss of a carboxylic group. Thus, based on this characteristic of changing from hydrophilic to hydrophobic, it is expected that biotin can act as a hydrophobic moiety to form a self-organized nanogel. Additionally, biotin also spontaneously acts as a targeting moiety for specific interaction with tumor cells.

Therefore, to examine this hypothesis, the physicochemical characteristics of PU/Bio were investigated using a fluorescence microplate reader and confocal laser microscopy to determine the particle size, critical aggregation concentration (CAC), and microviscosity, plus the interaction with a cancer cell line (HepG2) *in vitro*.

MATERIALS AND METHODS

Materials

The pullulan (Mw 100,000) was obtained from Hayashibara, Japan. The biotin, dicyclohexyl carbodiimide (DCC), *N*-hydroxysuccinimide (HOSU), 4-dimethylaminopyridine (DMAP), pyrene, and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were of reagent grade.

Conjugation of Biotin to PU

The biotin was coupled to pullulan by DCC and DMAP-mediated ester formation. The carboxyl groups of biotin (1 g) in dried DMSO were activated by the addition of DCC (600 mg) and DMAP (400 mg) (Fig. 1). Various amounts of activated biotin (100–1,000 mg) were added in 50 ml of dried DMSO containing 1 g of pullulan and reacted for 24 h at room temperature. After 24 h, the reactant mixture was filtered and dialyzed against distilled water for 3 days using a dialysis tube (molecular cutoff 12,000). The distilled water was exchanged at intervals of 3–6 h. The precipitated PU/Bio conjugate was then retrieved by filtration, washed thoroughly with diethyl ether and distilled water, and lyophilized. To completely remove any nonreactants, the lyophilized sample was redissolved in DMSO and dialyzed against distilled water. This process was repeated 3 times. The degree of substitution (DS), defined as the number of biotin groups per 100 anhydroglucose units of PU, was determined by ¹H-NMR. For the NMR measurement, all the samples were dissolved in DMSO-d₆ (Aldrich), and tetramethylsilane (TMS; Aldrich) was used as the reference.

Preparation of Self-Organized Nanogels from PU/Bio Conjugates

The nanoparticles were prepared using a diafiltration method (molecular cutoff 2,000). Each PU/Bio (50 mg) with a different DS was dissolved in 20 ml of DMSO. Each solution was then stirred at room temperature, dialyzed against distilled water for 3 days, and finally filtered using a 0.45-μm filter to remove the precipitated material.

Dynamic Light Scattering (DLS) Measurement

The particle size of the resulting nanoparticles was determined by dynamic light scattering (Zetasizer 3000, Malvern Instruments Ltd., U.K.), using an argon ion laser beam at a wavelength of 488 nm and scattering angle of 90°. Each sample was filtrated through a 0.45-μm filter directly into a precleaned 10-mm-diameter cylindrical cell. The sample concentration was kept at 1.0 mg/ml.

Transmission Electron Microscopy (TEM) Observation

To observe the nanoparticles in a dried state, a drop of the self-organized nanogels in water was placed on a carbon-

coated copper grid, and then dried under a vacuum at 30°C. The samples were observed without any staining using transmission electron microscopy (TEM, Philips CM 30, Philips Electron Optics, Eindhoven, The Netherlands) at 80 kV.

Measurement of Fluorescence Spectroscopy (Pyrene)

A pyrene stock solution (6.0×10⁻² M) was prepared in acetone and stored at 5°C until used. To measure the steady-state fluorescence spectra, the pyrene solution in acetone was added to distilled water to give a pyrene concentration of 12.0×10⁻⁷ M. Next, the solution was distilled under a vacuum at 60°C for 1 h to remove the acetone, and then mixed with solutions of the self-aggregated nanoparticles at concentrations ranging from 1×10⁻⁴ to 1.0 mg/ml. The final concentration of pyrene in each sample solution was 6.0×10⁻⁷ M, which is nearly equal to its solubility in water at 25°C.

Measurement of Anisotropy Value for 1,6-Diphenyl-1,3,5-hexatriene (DPH) in PU/Bio Nanoparticles

The anisotropy value of DPH (2.1×10⁻⁶ M) in the nanoparticles was measured using L-format geometry of detection. The fluorescence anisotropy (*r*) was calculated from the following relationship:

$$r = \frac{(I_{VV} - I_{VV}^s) - G(I_{VH} - I_{VH}^s)}{(I_{VV} - I_{VV}^s) + 2G(I_{VH} - I_{VH}^s)} \quad (1)$$

where *I*^s is the contribution of scattered light from a sample solution in the absence of DPH, *G*(=I_{VH}/I_{HH}) is the instrumental correction factor, and I_{VV}, I_{VH}, I_{HV} and I_{HH} refer to the resultant emission intensity polarized in the vertical or horizontal detection planes (second subindex) when excited with vertically or horizontally polarized light (first subindex) [16, 17]. The excitation wavelength was 360 nm, and the emission was measured at 430 nm.

Cell Culture

The HepG2 was cultured in Dulbecco's modified Eagle's medium (DMEM, 25 mmol/l glucose) equilibrated with 5% CO₂ and 95% air at 37°C. The medium was also supplemented with 10% fetal calf serum (FCS), 50 mg/l streptomycin, and 75 mg/l penicillin sulfate.

RITC Labeling of Conjugates

To a solution of the conjugate (500 mg) in 5 ml of DMSO, 50 mg of rhodamine B isothiocyanate (RITC) and 15 mg of dilaurate dibutyltin were added and allowed to stand for 2 h at 90°C. The mixture was then cooled down to room temperature and added to an excess of ethanol to precipitate the RITC-labeled conjugates. Thereafter, the labeled PU/Bio was dissolved in 30 ml water and dialyzed against 1,000 ml of alkaline water (pH 8.0 with NaOH) for one day, and then against distilled water for 3 days with

three separate changes of water. The final RITC-labeled conjugates were obtained by freeze-drying.

Fluorescence Intensity Analysis (RITC)

The HepG2 was plated in 24-well tissue culture dishes and cultured for 2 days. The RITC-labeled conjugate suspension was then added and allowed to incubate at 37°C for 2 h. The HepG2 was then washed three times with an HEPES-balanced Krebs-Ringer bicarbonate buffer and analyzed using a fluorescence microplate reader (FL600, Bio-Tek, U.S.A.).

Confocal Laser Microscopy

The cells were observed under a confocal laser microscope (Leika, Heidelberg, Germany). A confluent state of HepG2 cells was detached by treating the cells with phosphate-buffered saline (PBS) containing 0.5 mmol/l EDTA for 5 min. The detached cells were then preincubated in an HEPES-balanced Krebs-Ringer bicarbonate buffer (pH 7.4, 3.3 mM of glucose) under 5% CO₂ and 95% air at 37°C for 20 min. This was followed by incubation in the HEPES-balanced Krebs-Ringer bicarbonate buffer containing an RITC-labeled polymer at 37°C for 2 h. Thereafter, the cells (HepG2) were washed with the HEPES-balanced Krebs-Ringer bicarbonate buffer 3 times and placed on a cover glass, which was then gently placed on a glass slide.

RESULTS AND DISCUSSION

The conjugation of pullulan and biotin via the formation of ester linkages was carried out using a conventional carbodiimide reaction, as it is well known that carbonyl compounds preferably bind to hydroxyl groups at C(6) of a polysaccharide. Since pullulan is readily dissolved in an organic solvent, it has attracted attention as a new biomaterial in the biomedical field. The ¹H-NMR spectra showed that biotin was successfully conjugated to pullulan, where the ¹H-NMR (300 MHz, DMSO-*d*₆) presented δ 1.89–2.11 (2H, CH₂ in methylene; -C, -S-C), 2.51 (2H, CH₂ in methylene; -C, -O, -O-C), and 3.37 (H, CH in tetrahydrothiophen) for the biotin, and broad multiple peaks in the range of 3.7 to 5.5 ppm for the pullulan. The OH peak in the carboxylic group of biotin disappeared at δ 11.5. The calculated degree of substitution for each pullulan to biotin (number of biotins per hundred glucose units of pullulan) was 11.34, 18.97, and 23.56.

Physicochemical Characterization of PU/Bio Nanoparticles

Three conjugates, substituting 11 (PU/Bio1), 19 (PU/Bio2), and 24 (PU/Bio3) biotin groups per 100 anhydroglucose units of pullulan, were synthesized. Self-organized nanogels were then prepared from these conjugates using the dialysis method, which has several advantages, including a

small particle size, the prevention of rapid precipitation, and a narrow size distribution [26]. After the dialysis process of the PU/Bio solutions, a considerable change in the light scattering intensity was observed, indicating the formation of nanogels from the conjugates.

The sizes and size distributions of the self-organized nanogels in distilled water were measured by DLS and the morphological structures observed using TEM. Figure 2A shows the particle size and distribution of the PU/Bio2 in aqueous media, where the swollen PU/Bio 2 nanogels at pH 7.4 had a mean diameter of 217 nm with a unimodal size distribution (\pm 182 nm). The morphological structure of the PU/Bio2 nanogels observed by TEM is shown in Fig. 2B, and was spherical with a diameter below 100 nm (dried size).

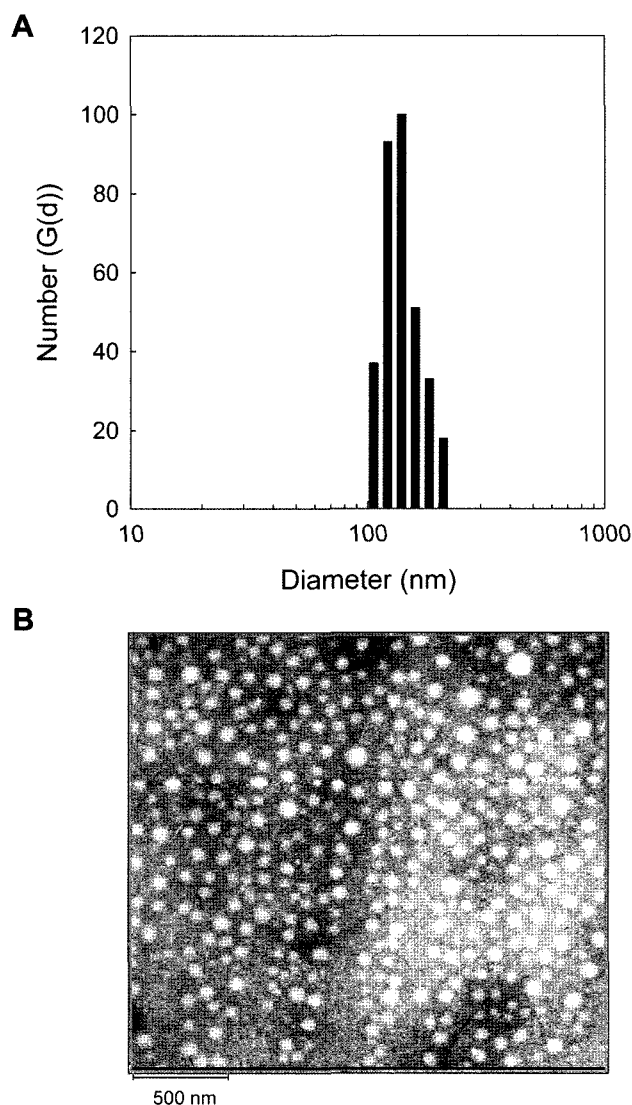


Fig. 2. Particle size distribution measured by dynamic light scattering (A) and transmission electron microscopy (TEM) photograph (scale bar=500 nm) (B) of PU/Bio2 nanogels.

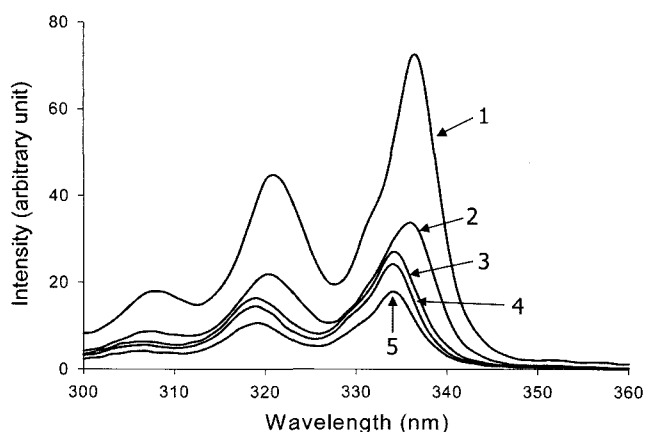


Fig. 3. Excitation spectra of pyrene (6.0×10^{-7} M) in distilled water in the presence of PU/Bio3 as a function of polymer concentration (Emission wavelength was 390 nm). 1, 1.0 g/l; 2, 0.1 g/l; 3, 0.01 g/l; 4, 0.005 g/l; 5, 0.001 g/l.

The self-organized nanogel formation of the PU/Bio conjugates in aqueous media was studied using a fluorescence probe. The fluorescence excitation spectra of the PU/Bio3 nanogels at various concentrations of the polymer in the presence of 6.0×10^{-7} M pyrene are presented in Fig. 3. At low concentrations of the PU/Bio3, the changes in the total fluorescence intensity and shift of the (0,0) band at 334 nm were negligible. However, when the PU/Bio3 concentration increased, an increase in the total fluorescence intensity and red shift of the (0,0) band were clearly observed. This increase in the total fluorescence intensity with the addition of the PU/Bio3 conjugate indicated that the probe was moved

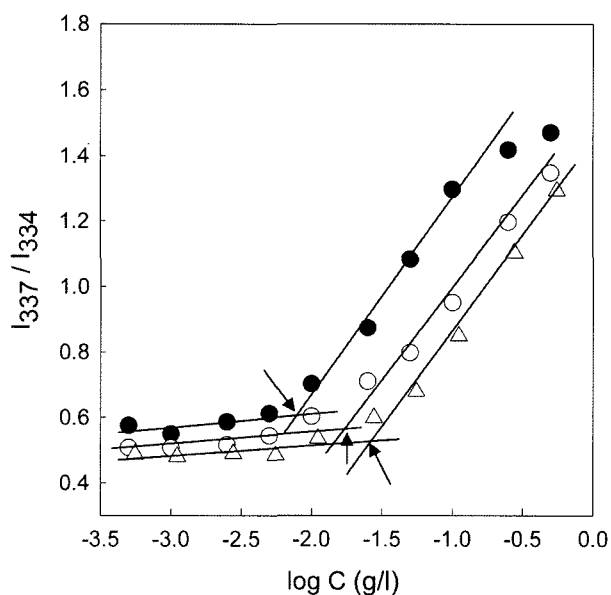


Fig. 4. Plot of intensity ratio I_{337}/I_{334} from excitation spectra vs. $\log C$ of PU/Bio1 (Δ), 2 (\circ), and 3 (\bullet) self-organized nanogels.

Table 1. Properties of self-organized nanogels in aqueous media.

Sample	DS ^a	Particle size ^b \pm SD (nm)	CAC ^c $\times 10^2$ (mg/ml)	r^d (anisotropy values)
PU/Bio1	11	130 ± 114	2.8	0.182
PU/Bio2	19	217 ± 182	1.6	0.196
PU/Bio3	24	275 ± 223	0.7	0.201

^aDegree of substitution of biotin per 100 anhydroglucose units of pullulan (using NMR).

^bMean diameter (intensity average) measured by dynamic light scattering.

^cCritical aggregation concentration determined from I_{337}/I_{334} data.

^dThe value was calculated from Equation 1.

from the aqueous media to the hydrophobic microdomains inside the nanogels. The (0,0) band for pyrene at 334 nm also shifted to 337 nm with the addition of PU/Bio3. The critical aggregation concentration (CAC), which is the threshold concentration of self-assembled nanoparticle formation by intra- and/or intermolecular association, was determined from the change of the intensity ratio (I_{337}/I_{334}) of pyrene in the presence of the PU/Bios. Figure 4 shows the intensity ratio (I_{337}/I_{334}) of the PU/Bio nanogels as a function of the polymer concentration. The CAC values were estimated from the crossover point at lower concentrations.

The physicochemical properties of the self-organized nanogels are shown in Table 1. The mean diameters of the PU/Bio1, 2, and 3 nanogels were 130 ± 114 , 217 ± 182 , and 275 ± 223 nm, respectively. The particle size of the self-organized nanogels increased with an increasing biotin content because of the increase in the aggregation between the biotins. In general, delivery carriers with a diameter larger than approximately 300 nm are known to induce nonspecific scavenging by monocytes and the reticuloendothelial system (RES) [15, 28]. Thus, nanogels can avoid capture by the RES. The CACs showed a dependency on the biotin content in the PU/Bio conjugates (Table 1), which means that the increased hydrophobicity resulting from the introduction of biotin decreased the CAC values. To further investigate the role of biotin as a hydrophobic moiety, the interior structure of the PU/Bio nanogels was examined by measuring the microviscosity using DPH. The microviscosity of the inner core of the particles was determined by measuring the molecular anisotropy as a result of molecular rotational diffusion. The anisotropy value for DPH increased with the core microviscosity owing restricted rotational motion. The r values for the PU/Bio3 nanogels were the highest among the tested samples (Table 1), which meant that the PU/Bio3 nanogels had the most rigid inner core, as a high r value implies an enhanced rigidity of the inner cores of the particles. Therefore, the results confirm that the hydrophobicity of the nanogels was increased when increasing the biotin content in the polymer.

Therefore, it was demonstrated that biotin acted as a hydrophobic moiety to make self-organized nanogels, plus

the degree of biotin substitution in the conjugates controlled the physicochemical properties of the nanogels.

Observation of Interaction Between PU/Bio Nanogels and Carcinoma Cell Line (HepG2) 5

Another role of biotin in the nanogels is the induction of a specific interaction with carcinoma cells. Thus, the PU/Bio conjugate was labeled with RITC as a fluorescence probe, to observe the interaction with HepG2 cells (hepatoma cell line) employed as carcinoma cells. The interactions between the PU/Bio nanogels and the HepG2 cells were quantified using a microplate fluorescence reader. The PU/Bio nanogels exhibited a similar dependency on the biotin content as regards their interaction with the HepG2 cells. The degree of interaction between the PU/Bio nanogels and the cells was higher than that between the RITC-labeled pullulan and the cells (Fig. 5). In particular, PU/Bio2 and 3 interacted more strongly with the HepG2 cells than PU/Bio1. Therefore, this means that the biotin first acted as a hydrophobic moiety to form a self-assembly, and then the biotin exposed on the outer shell of the nanogels acted as a targeting moiety to create a specific interaction with the carcinoma cells.

The internalization of drug carriers loaded with an anticancer drug is an effective way of improving the cytotoxicity of the drug. A higher intracellular content of a drug via endocytosis of the carrier can remarkably increase the cytotoxic effect against the target cells. Therefore, the effect of biotin as a targeting moiety on the internalization of nanogels was also investigated using HepG 2 treated with

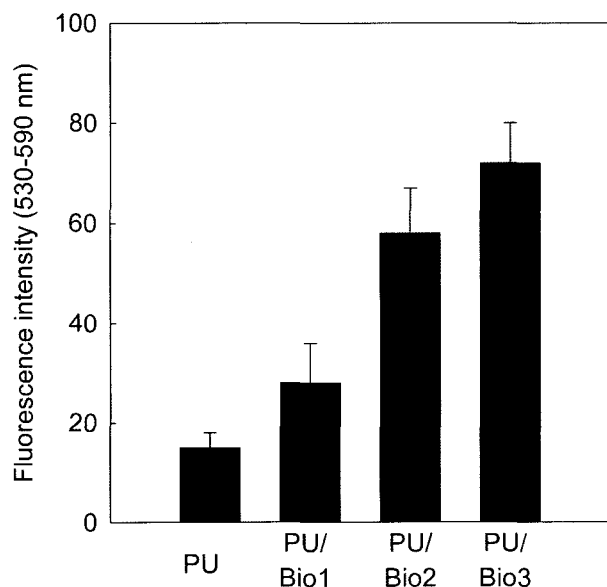


Fig. 5. Changes of fluorescence intensity by specific interaction between cells and nanogels.

A suspension of RITC-labeled conjugate was added and incubated at 37°C for 2 h. The intensity was measured using a microplate reader.

10 µg/ml of nanogels for 2 h. To observe the internalization of the PU/Bio nanogels in the HepG2 cells, confocal laser microscopy was employed. The PU/Bio2 and 3 nanogels exhibited intracellular localization into the HepG 2 cells, whereas the PU/Bio1 nanogels were only slightly localized. The confocal microscopy also indicated that the degree of interaction with the HepG2 cells depended on the biotin

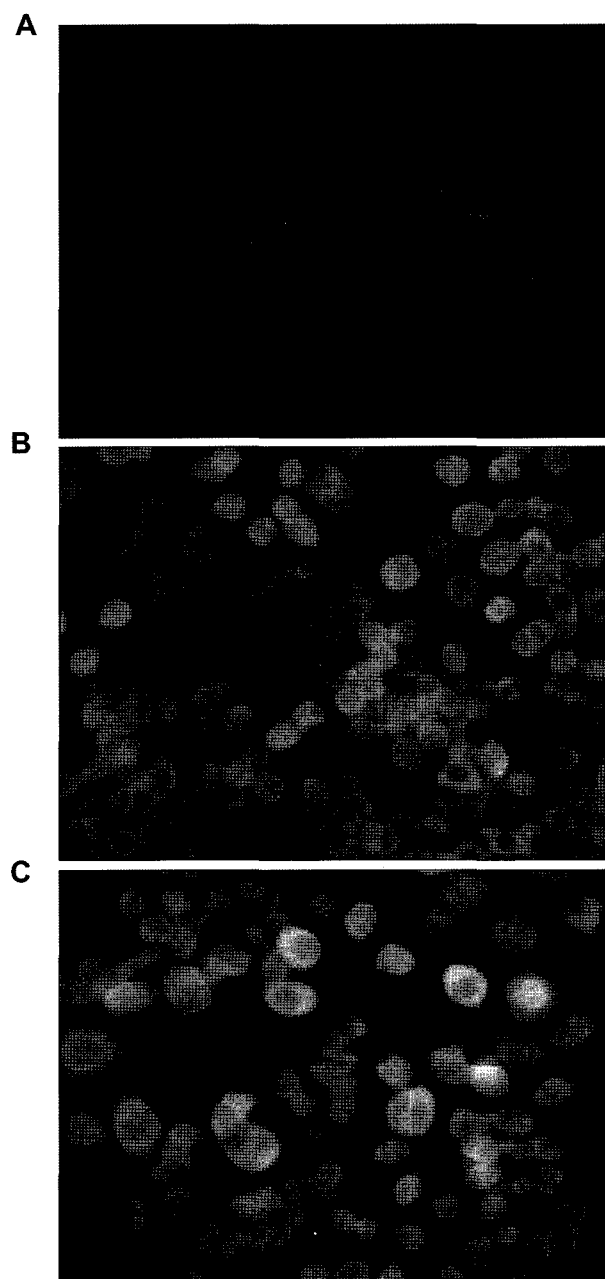


Fig. 6. Different degrees of RITC luminescence according to specific interaction between cells and nanogels.

HepG2 and RITC-labeled polymer concentration was 10 µg/ml. Luminescence image of RITC was observed using a confocal microscope. A. PU/Bio1 and HepG2 cell interaction; B. PU/Bio2 and HepG2 cell interaction; C. PU/Bio3 and HepG2 cell interaction.

content (Fig. 6). In Therefore, the PU/Bio nanogels interacted with the cancer cells via a biotin-cell interaction mechanism, and the internalization of the nanogels with biotin seemed to depend on the biotin content.

In conclusion, self-organized nanogels of pullulan and biotin conjugates (PU/Bio) were prepared to develop an enhanced method of carrying anticancer drugs using the specific interaction between biotin and tumor cells. The average sizes of the PU/Bio nanogels were below 300 nm, with a unimodal size distribution. The measured CAC was lower than the typical critical micelle concentration of low-molecular-weight surfactants in water. The microviscosity increased when increasing the degree of biotin substitution. Accordingly, such results indicate that biotin is a new candidate hydrophobic moiety for the self-organization of water-soluble polymers. The resulting PU/Bio nanogels showed a specific interaction with a hepatoma cell line (HepG2) through a vitamin-cell interaction. Furthermore, confocal images revealed that the nanoparticles entered the cells via a vitamin-cell interaction mechanism. Therefore, PU/Bio nanogels can be recommended as a potential anticancer drug carrier for the treatment of cancer.

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