

## 論文の内容の要旨

論文題目      Nanoparticles covered with photoreactive phospholipid  
polymer for intracellular delivery of      bioactive  
proteins

(細胞内へ生理活性タンパク質を送達する光反応性リン脂質ポリマーナノ粒子)

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The cell engineering for tissue-regenerated medicine strongly requires the maintenance of cell viability and different functions, encouragement of cell proliferation, modulation of cell migration, and regulation of cellular adhesion. Since proteins are regarded as the important molecules in living cells, they provide valuable tools for controlling cellular behaviors associated with tissue formation. The problems with proteins, which limit their practical use in controlling cell behaviors, are the mode of delivery. The high molecular weight, enzymatic susceptibility and hydrophilicity are main obstacles to the use of proteins. These properties make proteins difficult for internalizing into cells, and easy degradation before the proteins can take effects on the cell functions. To solve these problems, a novel carrier for protein is necessary, which can take proteins into cells, protect protein activity, control release rate, and reduce biological side effects.

The use of stimuli-responsive nanoparticles offers an interesting opportunity for protein delivery, where the delivery system becomes an active participant. Among these stimuli, photoinduced responses have attracted much attention, since it is one of the most desirable methods for the easy, rapid, and efficient control of material properties by tuning the wavelength and energy of photoirradiation. Photoreactive nanoparticles could release the entrapped proteins at the desired time and location. Some systems focusing on photoinduced dissociable protein carriers have been reported, which demonstrated that photoreactive nanoparticles offered new perspectives in controlled release that would be difficult to realize using other stimuli. These interesting examples employed instability and less biocompatible photoreactive moieties, which would be harmful to the cells. In some cases, it takes several days for the carriers to release most of the loaded proteins. This low release rate is similar to that of conventional protein release systems, in which proteins are released from carriers by diffusion.

The objective of this study is to develop an effective carrier for controlled protein delivery to cells,

which can induce efficient intracellular protein release upon photoirradiation. To achieve this purpose, a cytocompatible protein delivery system is demonstrated based on a copolymer composed of 2-methacryloyloxyethyl phosphorylcholine (MPC) units and photocleavable (PL) units (PMB-PL). The MPC unit is hydrophilic and biocompatible, which can maintain the bioactivity of protein and inhibit non-specific protein adsorption. The PL unit has a carboxylic group in the side chain, which provides a site for conjugating proteins and cell-penetrating peptides (CPP). The nitrobenzyl group of PL unit is rapid photocleavable by photoirradiation at a wavelength of 365 nm, which allows the fast release of protein. Using the PMB-PL, a smart carrier for protein delivery can be prepared.

To prepare the photoreactive nanoparticles, the first step is to design the suitable polymers. The photocleavable monomer, methacrylate with 4-[4-(1-methacryloyloxyethyl)-2-methoxy-5-nitrophenoxy]butyric acid (PL), was synthesized by the condensation reaction of methacryloyl chloride and hydroxyethyl photolinker under dark conditions. The water-soluble polymer poly (MPC-*co*- *n*-butyl methacrylate (BMA)-*co*-PL) (PMB-PL) was synthesized by a conventional radical polymerization method using  $\alpha$ ,  $\alpha'$ -azobisisobutyronitrile (AIBN) as an initiator. Poly (MPC-*co*-BMA) (PMB) was also synthesized as the control sample. The photoreactivity of PMB-PL and PMB were evaluated by the UV/Vis spectra. Before photoirradiation, the spectrum of PMB-PL showed a broad  $n-\pi^*$  transition centered at 350 nm. Excitation of this solution led to a dose-dependent loss of the 350 nm band, near ultraviolet light (340-420 nm). After the PMB-PL solution was treated with photoirradiation for 180 sec, two new adsorption transitions appeared at 268 and 378 nm. These adsorption bands most probably belonged to the 3-methoxy-6-nitrophenyl compound. This result suggests that the PMB-PL in bulk solution undergoes a photoreaction that is characteristic of the nitrobenzyl ester. Comparing PMB-PL with PMB solution showed less absorbance intensity. The absorbance intensities of PMB-PL and PMB in ethanol solution at 268 nm in different irradiation time were measured. After photoirradiation, the absorbance intensity of PMB-PL at 268 nm increased significantly on the first 60 sec and almost stayed the same on the next 240 sec, while the absorbance intensity of PMB kept near 0 all the time. This result indicated that PMB-PL could response rapidly to the photoirradiation. The photochemical reaction due to the nitrobenzyl ester could almost be finished within 60 sec. Measurement of the surface tension of polymer aqueous solution is useful to evaluate the conformation of polymer in water. The surface tension of polymer was measured in a water solution using the Wilhermy method. The surface tension of the MPC polymer solution began to decrease gradually with the increasing polymer concentration. The surface tension of PMB-PL and PMB solution was about 72 dyn/cm (the surface tension of water) when the concentration was lower than 0.01 mg/mL, and approached to 50 dyn/cm when the concentration was 10 mg/mL. It suggested that the amphiphilic MPC polymer PMB-PL could form polymer aggregation in water. It is possible to prepare the polymer nanoparticles using the PMB-PL as a suspension stabilizer in aqueous solution. These results demonstrated that the PMB-PL was qualified to be prepared as photoreactive nanocarriers.

The polymer nanoparticles covered with amphiphilic PMB-PL polymers were prepared by the solvent evaporation technique in an aqueous medium with poly(L-lactic acid) (PLA). During the procedure of nanoparticle preparation, PMB-PL was a suspension stabilizer due to the forming ability of aggregation. The PLA/dichloromethane droplets were suspended in the aqueous solution with the PMB-PL aggregation. When dichloromethane was removed from the suspension by evaporation, PLA was precipitated at the interface of PMB-PL aqueous medium. At this interface, the hydrophobic units of PMB-PL form entanglements with PLA. A stable PMB-PL coating layer could be formed on the surface of PLA core. The diameter of the PMB-PL/PLA nanoparticles was about 130 nm, with a narrow size distribution. The TEM image of the nanoparticles indicated the spherical shape of these nanoparticles. The  $\zeta$  potential of the nanoparticles was -9.3 mV, while that of the PLA nanoparticles was about -60 mV. This is due to the electrical neutrality of the phosphorylcholine groups, which formed an inner salt between phosphate anions and trimethylammonium cations. The XPS spectrum showed a phosphorus peak at 133 eV, a nitrogen peak at 403 eV, a carbon peak at 285 eV, and an oxygen peak at 532 eV. These peaks were attributed to methyl or methylene groups. XPS analysis and  $\zeta$  potential measurements revealed that the surface of PMB-PL/PLA nanoparticles was covered with phosphorylcholine groups. The PMB-PL/PLA nanoparticles had good dispersion in an aqueous medium because of the hydrophilicity of the phosphorylcholine groups.

Loading and release behavior of proteins is very important for practical application of a protein delivery system; therefore, the loading and release process using fluorescein isothiocyanate (FITC) labeled bovine serum albumin (BSA) (FITC-BSA) were monitored by fluorescence spectroscopy. After the carboxylic group was activated, the nanoparticles were mixed with the FITC-BSA solution. After washing the nanoparticles with PBS, the fluorescence intensity of nanoparticles was evaluated. The strong fluorescence could be observed from nanoparticles before photoirradiation. This suggested that the activated PL group on the surface of nanoparticles formed strong covalent bonding with the amino groups of FITC-BSA, which made the immobilization of proteins successfully. The loading amount of proteins was 8.6  $\mu\text{g/mL}$ . After photoirradiation for 180 sec, the fluorescent intensity of nanoparticles decreased significantly. The photoirradiation cleaved the covalent bonding between nanoparticles and proteins, and made the proteins released to the surrounding PBS solution. The released amount of FITC-BSA was 7.5  $\mu\text{g/mL}$ , that is, the release degree of BSA from the nanoparticle is more than 90 %. The relationship between the immobilized protein amount and the concentration of protein solution was investigated. The concentration of nanoparticles used in this experiment was 10 mg/mL, and the concentration of FITC-BSA was 0.01 mg/mL, 0.05 mg/mL and 0.10 mg/mL, respectively. The result showed that the immobilized proteins were similar, about 3  $\mu\text{g/mL}$ , while the protein concentrations of proteins were different. This result indicated that dilute protein solution was effective to immobilize proteins onto the nanoparticles.

To investigate the release degree of protein from nanoparticles, the protein-immobilized nanoparticles

were treated with photoirradiation for different time, ranged from 10 sec to 180 sec. The release degree of protein was over 60% while the photoirradiation started only 10 sec. When the irradiation increased to 30 sec, the release degree reached about 90%. Using light as a trigger, the release of protein was much more efficient. The short photoirradiation time could also reduce the harm to cells when the immobilized proteins need to be intracellular delivered.

As the protein carriers to perform intracellular delivery, the nanoparticles should have low cytotoxicity, and could be internalized into cells. Before introducing nanoparticles into cells, the influence of photoirradiation on the cells was evaluated. Hela cells were applied with photoirradiation (28 mW/cm<sup>2</sup>) for 3 min and continued to be cultured for 24 h. The cell viability was over 99%, which was almost the same as that of the cells without the treatment of photoirradiation. The result suggested that photoirradiation in the weak density and short time would not influence the cells. But this photoirradiation was effective to release proteins. After culturing PMB-PL/PLA nanoparticles with cells for 6 h, the cell viability was as high as 97%, which indicated that the cytotoxicity of PMB-PL/PLA was limited. By immobilizing the cell penetrating peptide, octaarginine (R8) on the nanoparticles, the nanoparticles could be internalized into cells. By increasing the concentrations of nanoparticles when culturing with cells, the cell uptake of nanoparticles was improved. More proteins were brought into cells along with nanoparticles when the concentration of proteins was high during the immobilization process. After applying the cells with photoirradiation, proteins got better distributions inside cells, which suggested the intracellular protein release.

In conclusion, the photoreactive copolymer PMB-PL was synthesized. The PMB-PL quickly responded by photoirradiation. The PMB-PL/PLA nanoparticles were designed and successfully prepared by solvent evaporation method using PMB-PL as suspension stabilizer. Proteins could be immobilized onto PMB-PL nanoparticles and released upon photoirradiation in high efficiency. The nanoparticles could be internalized into cells by immobilized with cell penetrating peptides, and released inside cells by photoirradiation. Also, the good cytocompatibility of the nanoparticles was obtained. These results indicated that nanoparticles covered with the photoreactive PMB-PL can be applied for preparing the smart carriers for loading and delivery of proteins into cytoplasm.

For the future perspectives, to evaluate the bioactivity of proteins after intracellular release, the proteins will be replaced with alkaline phosphatase (ALP). ALP can react with the substrate 4-methylumbelliferyl phosphate. By measuring the amount of the product, the activity of the released proteins can be evaluated, and the availability of this intracellular protein delivery system can be confirmed. The final goal of this research is to control cell functions by intracellular protein delivery. To achieve this, two kinds of proteins have been chosen as candidates to be released inside cells, ribonuclease A (RNase A) and protein kinase A (PKA). RNase A leads to cell death and PKA induces cell growth. The cell viability is expected to be different compared with the control sample after intracellular delivered and released these two proteins, respectively.