Preparation of Photo-Reactive Azidophenyl Chitosan Derivative for Immobilization of Growth Factors

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ABSTRACT: Photoreactable chitosan derivative have been prepared by introducing azido phenyl groups to amino groups of low molecular weight chitosan to facilitate the immobilization of growth factors. The prepared azidophenyl chitosan was characterized by Fourier transform infrared (FTIR), proton nuclear magnetic resonance (^{1}H NMR), scanning electron microscope (SEM). Cytotoxicity of prepared azidophenyl chitosan derivative was confirmed by MTT assay. Cell culture on the surfaces coated azidophenyl chitosan derivative was also carried out and cell adhesion was observed between azidophenyl chitosan derivative and cells. And the released rate of protein from azidophenyl chitosan derivative was measured using albumin instead of growth factors for check on effect of the prepared azidophenyl chitosan derivative. As a result of that, relations among the concentration of azidophenyl chitosan derivative, releasing period of albumin and the quantity of released albumin was confirmed. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 117: 3029–3037, 2010

Key words: biological applications of polymers; biomaterials; drug delivery systems; stabilization; supports

INTRODUCTION

Some of the proteins can be used as a medicine with high efficiency and less side effect. However, the proteins are unstable and lose their activities depending on physical, chemical conditions,[1] and proteolysis *in vitro*. To solve these problems for using proteins to various medical fields, immobilization of proteins is needed. Some effects of the immobilized proteins were already reported. For example, the immobilized growth factors such as immobilized epidermal growth factor (EGF)[2–4] and insulin[5,6] showed a transduction of signal for longer than free EGF and insulin did. Mitogen activated protein kinase (MAPK) activation by free EGF decreased with the passage of time, while MAPK was activated continuously by immobilized EGF.[5,6] Moreover, proteins can be used less than free proteins by immobilization when those are applied to patients as a kind of medicine. This mean that it is possible to minimize potential side effects generated by using excess proteins.[7]

Entrapment of proteins in polyacrylamide gel and covalent binding technique have been used mainly for immobilization of proteins.[8] Entrapment of proteins in polyacrylamide gel can maintain the enzymatic function by offering a wet micro-environment and accommodate proteins of size up to 400 kDa. However, that method has the drawbacks of lower reaction rate.[9] Although covalent binding technique has several advantages, for example, proteins can be immobilized tightly on supports via chemical bond formation by this technique, it still has the drawbacks. When proteins are attached to supports for immobilization, site specific binding occurs between proteins and supports. There are possible chances that one or more active sites of proteins are covered and activators are interrupted to bind those active sites. Therefore, specific activities related to covered active sites of proteins could not or less activated.

In these days, the photo-immobilization techniques have been comprehensively researched for applying in many fields.[10–13] The photo-immobilization can make higher activity, higher cross-linking efficiency, and better folded conformation of proteins can be conserved.[14] The specific activities of proteins are not lost or decreased because the site specific binding is not formed proteins and supports.

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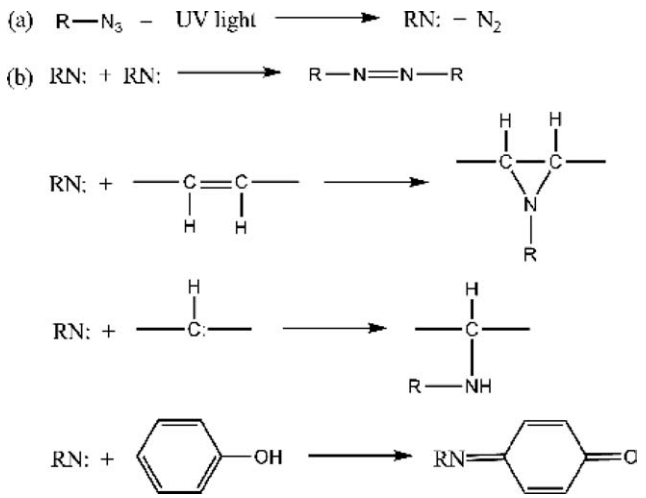


Figure 1 Scheme of nitrene group formed by UV light (a) and various reactions of nitrene group with other compounds (b).

There are several preferred photoactivatable agents for protein immobilization. In this study, the applicability of photo-immobilization by chitosan derivative containing aryl azides as a photoactivatable agent was investigated. Aryl azides were used because those can be manipulated in ambient light and can be activated at 350–360 nm of light. The use of 350–360 nm of light avoids the damage of protein or DNA.[8] Chitosan was selected along with unique biological properties such as biocompatibility, biodegradability to harmless products, nontoxicity, physiological inertness, antibacterial, haemostatic, fungistatic, antitumoral, and anticholesteremic properties.[15–21] Photoreactable chitosan derivative have been prepared by introducing azido phenyl groups to amino groups of low molecular weight chitosan to facilitate the immobilization of growth factors. The prepared azidophenyl chitosan was characterized by Fourier transform infrared (FTIR), ^{1}H NMR (proton nuclear magnetic resonance), SEM (scanning electron microscope). To confirm biocompatibility, MTT assay was carried out. Cell culture on the surfaces coated azidophenyl chitosan derivative was also carried out to observe adhesion between azidophenyl chitosan derivative and cells. The released rate of protein from azidophenyl chitosan derivative was measured using albumin instead of growth factors to check the effect of the prepared azidophenyl chitosan (Fig. 1).

EXPERIMENTAL

Materials

Chitosan from crab shell was purchased from Jakwang Co. (Suwon, Korea) and the degree of deactylation (DAc) was about 88%. Chitosan was used without any purification. Acetic acid, sodium nitrite, ammonium hydroxide, sodium tetrahydridoborate, methanol, acetone, and ether for preparation of low molecular weight chitosan were obtained from Duksan pure chemical Co. (Ansan, Korea). For preparation of *N*-(4-azidobenzoyloxy) succinimide, *N*-hydroxysuccimide, *N*,*N*-dicyclohexylcarbodiimide, diethylether were obtained from Wako Pure Chem. Ind. (Tokyo, Japan), 4-azidobenzoic acid was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan), and dioxane was obtained from Samchun pure chemical Co. (Seoul, Korea). 2, 2-azobis (isobutyronitrile) was obtained from Wako Pure Chem. Ind. (Tokyo, Japan). Poly (ethylene glycol) methacrylate and 4-azidophenyl methacrylamide was obtained from Sigma-Aldrich to prepare azidopoly (ethylene glycol) (Az-PEG) following the reported method by Yoshihiro Ito, et al., at acta biomaterialia.[22] For cell culture, protein releasing test, and MTT assay, DMEM (Dulbecco's Modified Eagle's Medium) was obtained from WelGene , and fetal bovine serum (FBS) and penicillin-streptomycin were obtained from GIBCO. Trypsin-EDTA (ethylenediaminetetraacetic acid), (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) MTT formazan, and bovine serum albumin (BSA) were obtained from SIGMA-Aldrich. Cell culture, cover slip, sterile thermanox plastic (15 mm diameter) was purchased from NUNC. Six well and 96 well were obtained from each Becton Dickinson Labware and TPP Immunomaxi (Berne, Swiss). COS7 (African Green Monkey SV40-transf'd kidney fibroblast cell line) cells which are cells of African green monkey kidney derived from CV-1, a simian cell line (*Cercopithecus aethops*) and grow as monolayers were obtained from the Riken cell bank. 3T3 (mouse embryonic fibroblast cell line) cells were obtained as a gift from the college of medicine in Kyungpook National University, Korea. Ultra violet (UV) light source was purchased from Ushio, Japan (Model: Spot cure-9). On every UV irradiation experiments of this study, the distance between UV light lamp and sample was 5 cm and intensity was 48 mW/cm^3 with that distance.

METHODS

Preparation of low molecular weight chitosan

Chitosan was dissolved in 4% v/v acetic acid solution with strong agitation. $NaNO_2$ (4.35 g) dissolved in 43.47 mL distilled water was added dropwise to the chitosan solution in an ice bath. After stirring for 2 h, the chitosan solution was neutralized (pH 7) by NH_4OH. $NaBH_4$ (4.77 g) was added to reduce the sample. Neutalization (pH 7) was carried out by 1N-HCl. Depolymerized chitosan filtered and concentrated by evaporation. Mixed solution of water and methanol was added to induce precipitation with stirring and stored at 4°C to increase the precipitation

yield. The precipitate was obtained by centrifugation. Obtained precipitate was washed 3 times with methanol and washed 1 time with acetone and ether. The precipitates were finally dried under vacuum. These chitosan powders were dissolved in deionized water and ultra-filtered using YM10 (molecular weight cut-off 10,000 Da) and YM3 (molecular weight cut-off 3,000 Da) membranes (Amicon). The chitosan fraction solution which were concentrated through ultra-filtration system, were thoroughly evaporated and powdered after washing by ether and acetone. Of some chitosan fractions obtained from the above reaction, a single chitosan fraction with about M_w 10,000 was used to prepare azidophenyl chitosan derivative.

Determination of molecular weight and DAc (degree of deacetylation)

The molecular weight of low molecular weight chitosan prepared was determined with a high performance liquid chromatography (Gilson, France) with a pullulan standard (column, Shodex Asahipak GS-320, 30 cm $\times$ 7.6 cm). The mobile phase consisted of 0.2 M CH_3COOH and 0.036 M CH_3COONa. The sample, with a concentration of 0.1% (w/v), was loaded and eluted with a flow rate of 0.5 mL/min at 25°C. The eluted peaks were detected by an RI (refractive index) detector (132 RI detector, Gilson, France). Main macromolecular structure and DAc of low molecular weight chitosan were identified using FTIR and ^{1}H NMR analysis, respectively. The FTIR spectra were taken on KBr pellets in a FTIR spectrometer (FTIR 8400s, Shimadzu, Japan). ^{1}H NMR spectra were recorded on NMR spectrometer (Gemini 2000, 300 MHz, Varian) using D_2O as solvents. 10 mg sample was placed into a 5 mm NMR test tube with 0.5 mL D_2O solution.

Preparation of *N*-(4-azidobenzoyloxy) succinimide

N-(4-azidobenzoyloxy) succinimide was prepared according to the previously reported method.[23] *N*-hydroxysuccinimide (210 mg) was dissolved in dioxane (20 mL). 4-azidobenzoic acid (300 mg) and *N*,*N*-dicyclohexylcarbodiimide (DCC, 380 mg), dissolved in dioxane (10 mL) were added gently to the solution of *N*-hydroxysuccinimide in an ice bath. The reaction mixture was stirred for 12 h at room temperature. The white precipitant, formed in the reaction mixture, was filtered off, and the filtrate was evaporated under reduced pressure. Finally, yellow *N*-(4-azidobenzoyloxy) succinimide powder was obtained. It was dried under vacuum and recrystallization was carried out twice using dioxane and diethyl ether.

Synthesis of azidophenyl chitosan derivative

The prepared chitosan oligomer (0.20 g, M_w = 10,000) was dissolved in 5 mL of distilled water. The synthesized 0.11 g of *N*-(4-azidobenzoyloxy) succinimide, dissolved in as small amount of dioxane as possible, was added to the chitosan oligomer solution. The reaction was performed for 24 h at room temperature with stirring, and the reaction mixture was evaporated under reduced pressure. The reaction products were washed with acetone to remove the remained *N*-(4-azidobenzoyloxy) succinimide. Main macromolecular structure of azidophenyl chitosan derivative was identified using FTIR and ^{1}H NMR analysis, respectively. The FTIR spectra were taken on KBr pellets in a FTIR spectrometer (FTIR 8400s, Shimadzu, Japan). ^{1}H NMR spectra were recorded on NMR spectrometer (Gemini 2000, 300 MHz, Varian) using D_2O and CD_3COOD as solvents. Sample (10 mg) was placed into a 5 mm NMR test tube with 0.5 mL D_2O/CD_3COOD solution.

Cytotoxicity test of azidophenyl chitosan derivative irradiated to UV

For the cytotoxicity test of azidophenyl chitosan derivative irradiated to UV light usual MTT assay method was not available. Because azidophenyl chitosan derivative irradiated to UV light was not soluble in water. Various concentrations of azidophenyl chitosan derivative solution (5%, 10%, 15%, and 20%) samples were prepared, and the samples were irradiated to UV light for various times intervals (1 min, 3 min, 5 min, and 7 min) on 6-well culture plates to be cast over the surfaces of wells. 3T3 (mouse embryonic fibroblast cell line) cells were cultured in Dulbecco's modified eagle's medium (DMEM) contained 10% FBS, 1% penicillin-streptomycin at 37°C in a 5% CO_2 / 95% atmospheric air. The cells were seeded into 6-well culture plates coated with azidophenyl chitosan derivative at 1.0 $\times$ 10^4 cells per well. The medium was then replaced with 200 μL of the appropriate test medium (serum-free DMEM medium), and the plates were incubated at 37°C in a 5% CO_2 / 95% atmospheric air for the 48 h. In one plate, uncoated with azidophenyl chitosan derivative, the cells were also cultured as a control group in the same conditions mentioned above. MTT formazan was added to each plate and plates were incubated for 4 h. DMSO (dimethyl sulfoxide) was added to each plate, and the mixtures in plates were moved into 96 well. After then, usual MTT assay method was carried out.

Observation of the surface of azidophenyl chitosan derivative—BSA by scanning electron microscope

The mixture of azidophenyl chitosan derivative and BSA solutions were irradiated to UV light. The surface of azidophenyl chitosan derivative and BSA conjugate was observed by SEM (S-3400N, Hitachi,

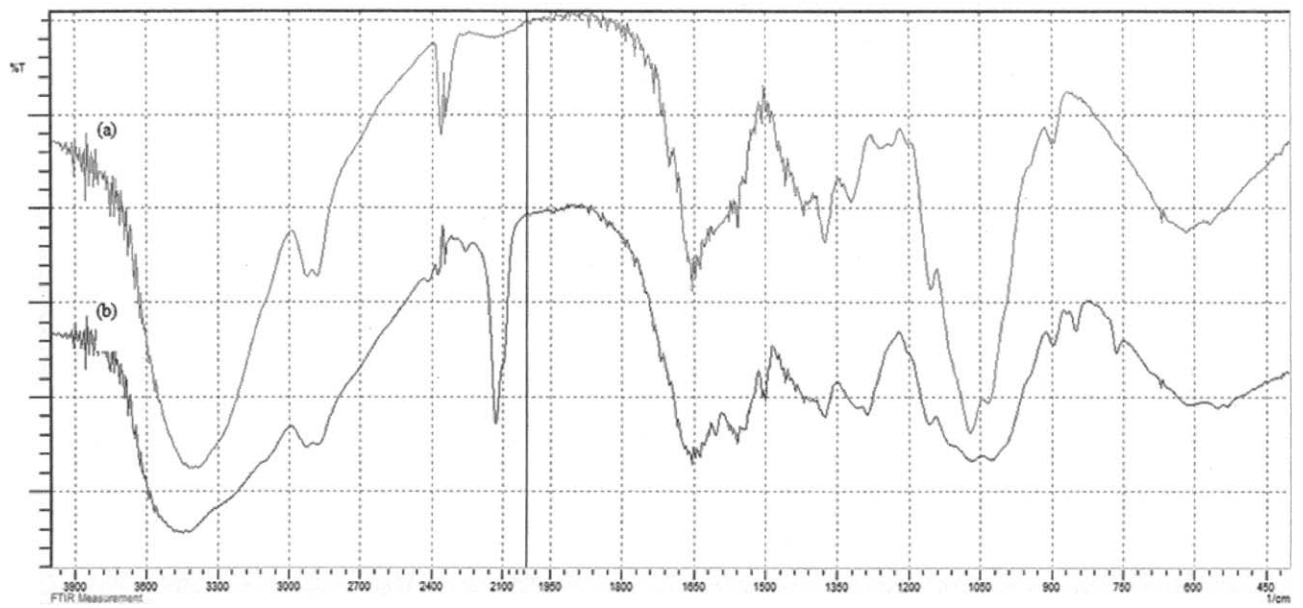


Figure 2 FTIR spectrum of low molecular weight chitosan (a), FTIR spectrum of azidophenyl chitosan derivative (b).

Japan) with an accelerating voltage 15.0 [Fig. 2(a,b)] and 20.0 kV [Fig. 1(b)]. Azidophenyl chitosan derivative irradiated to UV light were also observed by SEM.

Cell culture

Cell culture was carried out for additional characterization of azidophenyl chitosan derivative. Adhesion between azidophenyl chitosan derivative and cells was examined by observing the quantity of cell on the surfaces of Thermanox™ plastic cover slips coated with the azidophenyl chitosan derivative. Azido-poly (ethylene glycol) (Az-PEG) was prepared by following the reported method by Yoshihiro Ito et al., at acta biomaterialia.[22] Az-PEG inhibits the attachment of cell on surface. Az-PEG was used for coating the cover slips to get clear result. Without Az-PEG, the character of cell attachment cannot be observed clearly because there are some materials, which help cell attachment are contained on the cover slip. The cover slips without any treatment and the cover slips coated with Az-PEG were placed on 12-well culture plates and used as a blank and a negative control, respectively. The cover slips, coated with the 0.1, 0.5, and 5% concentrations of azidophenyl chitosan derivative on the top of Az-PEG-coated cover slips by irradiation through UV light, were washed with distilled water for 60 min and placed on 12-well culture plates for cell culture. COS7 (Green Monkey SV40-transf'd kidney fibroblast cell line) cells were cultured in DMEM (Wako Pure Chem. Ind., Japan) containing 10% FBS. The cultured cells were treated with PBS (phosphate buffer saline) containing 0.25 wt % trypsin and 0.9 m*M* EDTA. The cells were washed with the culture medium and suspended in this medium. The cell suspension was added to sample plates, which had been sterilized with 70% ethanol. The cells were incubated at 37°C under 5% v/v of CO_2 for 19 h. Same number of COS7 cells were seeded into each well and cultured in high glucose DMEM containing 5% FBS for 48 h at 37°C. After 48 h cultivation, the surfaces of the cover slips were observed to evaluate the adhesion between azidophenyl chitosan derivative and cells by a microscope and compared to the negative control.

Protein releasing test

Various concentration (5%, 10%, 15%, 20% w/v) of azidophenyl chitosan derivative solution sample groups were prepared (four samples per same concentration for one group, totally 16 samples, four groups). BSA 200 μg was dissolved in each sample solution. Each sample groups were irradiated to UV light for 1, 3 , 5, and 7 min. Every sample was washed with PBS three times to remove BSA not immobilized by azidophenyl chitosan derivative and 1 mL of PBS was added to every sample. The quantity of released BSA, dissolved in PBS, was measured using Bradford assay (Bio-rad protein assay, Bio Rad laboratories) by UV/VIS spectrophotometer (Optizen 3220UV, Mecasys Co. Korea) in every 12 h. Samples prepared with same conditions mentioned above without BSA were used as controls for each sample. After every measurement, 1 mL of PBS was added to every sample.

RESULTS AND DISCUSSION

FTIR analysis of low molecular weight chitosan

FTIR spectrophotometer analysis was carried out for the confirmation of the main molecular structure of low molecular weight chitosan. The IR spectra of low molecular weight chitosan were investigated in comparison with the IR spectra of chitosan polymer. The spectral profiles of low molecular weight chitosan are similar to the one of chitosan polymer. It is considered that the main macromolecular structure of chitosan polymer was conserved on the structure of low molecular weight chitosan even after the depolymerization by sodium nitrite.

^{1}H NMR and GPC analysis of low molecular weight chitosan

Deacetylation degree (DAc) of chitosan polymer and low molecular weight chitosan were determined by ^{1}H NMR spectroscopy. DAc of chitosan polymer and low molecular weight chitosan were evaluated by using the integral intensity of the CH_3 residue on the acetyl group ($I_{acetyl\text{-}H}$) and the sum of the integral intensities of H_2, H_3, H_4, H_5, H_6, and $H_{6'}$ protons ($I_{H2\text{–}H6}$).[24]

$$\mathrm{DAc}(\%) = [1 - ((1/3)\mathrm{I}_{\mathrm{acetyl\,H}}/(1/6)\mathrm{I}_{\mathrm{H2-H6}})] \times 100$$

The resonance at 2.04 ppm was due to the CH_3 residue on the acetamide group and the resonances of H_2–H_6 occurred in the range 3.1–4.0 ppm.[25] DAc values of chitosan polymer and low molecular chitosan were 87.62 and 86.24%, respectively. The weight average molecular weights (M_w) of low molecular weight chitosan were determined by HPLC (high performance liquid chromatography) analysis with GPC (gel permeation chromatography) as the main separation mode. M_w of low molecular weight chitosan was about 10,973.

Synthesis of azidophenyl chitosan derivative

FTIR spectrophotometer analysis was carried out for the confirmation of the molecular structure of prepared azidophenyl chitosan derivative. The IR spectrum of prepared azidophenyl chitosan derivative was investigated in comparison to the IR spectrum of low molecular weight chitosan (Fig. 2). The IR spectrum of low molecular weight chitosan and chitosan polymer depict the characteristic absorption bands. According to the FTIR spectra, the spectral profiles of prepared azidophenyl chitosan derivative were similar to spectra of low molecular weight chitosan and chitosan polymer. It is considered that azidophenyl chitosan derivative still has pyranose structure of low molecular weight chitosan and chitosan polymer. IR spectrum of azidophenyl chitosan derivative demonstrates the characteristic absorption band at near 2125 cm^{-1}, which represent the presence of azido group. To confirm the structure of prepared azidophenyl chitosan derivative, ^{1}H NMR spectroscopy analysis was also carried out (Fig. 3). The ^{1}H NMR spectrum of azidophenyl chitosan derivative was investigated in comparison to the ^{1}H NMR spectra of low molecular weight chitosan. Aromatic ring was depicted at 7.0–8.0 ppm in ^{1}H NMR spectrum of azidophenyl chitosan derivative. In contemplation of preparation process of azidophenyl chitosan derivative, it was sure that the aromatic ring depicted at 7.0–8.0 ppm in ^{1}H NMR spectrum was benzene ring as a part of azidophenyl group. Therefore, it is considered that azidophenyl chitosan derivative were prepared successfully.

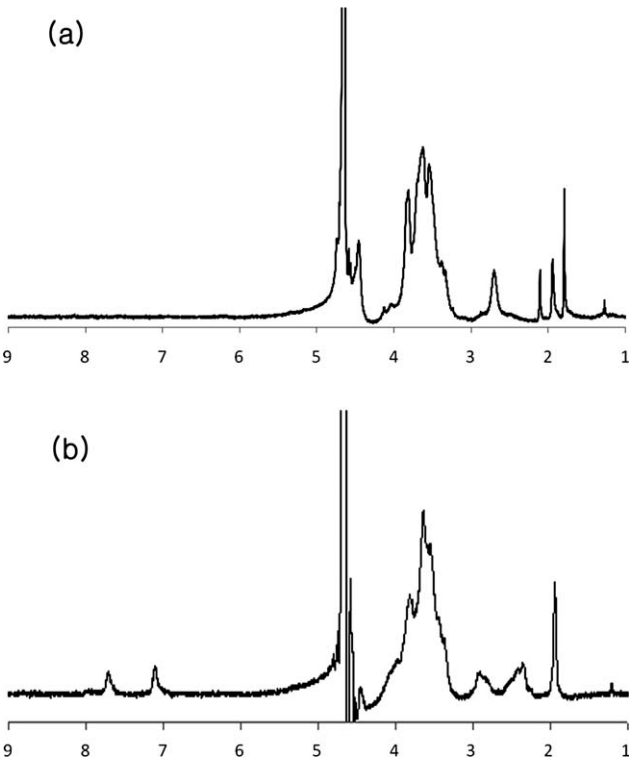
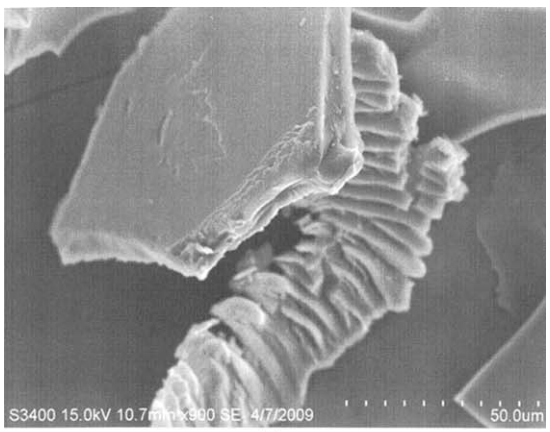
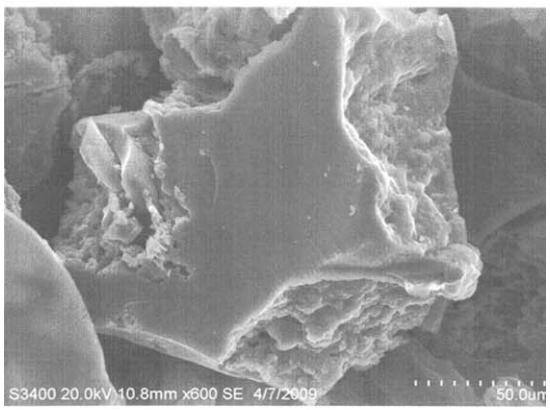


Figure 3 300 MHz ^{1}H NMR spectra of low molecular weight chitosan in D_2O (a), 300 MHz ^{1}H NMR spectra of azidophenyl chitosan derivative in D_2O in the presence (b).

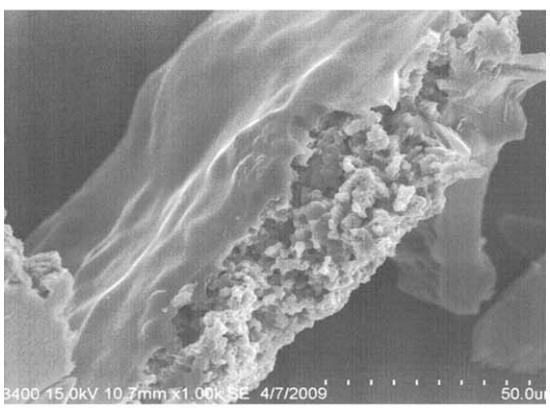
Azidophenyl group was introduced to amino group of low molecular weight chitosan during preparation of azidophenyl chitosan derivative. The introducing degree of azidophenyl group to amino group was also determined by the ^{1}H NMR spectrum of azidophenyl chitosan derivative. The equation for DAc of low molecular weight chitosan, mentioned above, was used and applied to design an equation for determination of the introducing degree. To determine the introducing degree, following equation was used.



(a)



(b-1)



(b-2)

Figure 4 Scanning electron microscope (SEM) of azidophenyl chitosan derivative irradiated to UV light (a) and azidophenyl chitosan derivative—BSA (b-1, b-2).

$$D(\%) = (1/4 \times I_{\text{benzene H}})/(1/6) \times I_{\text{H2-H6}}) \times 100$$

If the introducing rate is 100%, one azidophenyl group per one unit of chitosan should be contained in the structure of azidophenyl chitosan derivative. The introducing rate of azidophenyl chitosan derivative to chitosan can be calculated by using the number of chitosan unit to the number of azidophenyl group ratio. The above equation was designed for calculating the ratio. In the structure of azidophenyl chitosan derivative, one benzene ring contains four hydrogens. Therefore, integral intensity which expresses the hydrogens on the benzene ring is divided by four and the result can be estimated as the relative number of benzene rings against the number of chitosan units. One chitosan unit contains six hydrogens. By same method as the case of benzene ring mentioned above, the relative number of chitosan units against the number of benzene ring can be calculated. As a result, the introducing rate of azidophenyl group to chitosan can be estimated or calculated by division of two values. The calculated values was around 20%.

Observation of the surface of azidophenyl chitosan derivative—BSA by scanning electron microscope

The results of SEM of azidophenyl chitosan derivative and azidophenyl chitosan derivative –BSA are shown in Figure 4. As the external appearance, it seems that BSA was coated or surrounded by UV light exposed azidophenyl chitosan derivative. It is expected that azidophenyl chitosan derivative can possibly provide physical protection to growth factors based on protein from some of physical conditions and proteinases, which would denature growth factors or negatively affect to activities of growth factors.

Cytotoxicity test of azidophenyl chitosan derivative irradiated to UV light

For application of azidophenyl chitosan derivative to some growth factors which have been used as a medicine, it must be confirmed that azidophenyl chitosan derivative is non-cytotoxic. As a result of MTT assay, it is considered that all azidophenyl chitosan derivative samples were non-cytotoxic on the proliferation of 3T3 (mouse embryonic fibroblast cell line) cells cultured in serum-free DMEM medium at 48 h after the initial addition of sample solutions.

When prepared azidophenyl chitosan derivative are used for immobilization of growth factors actually, it will be irradiated to UV light before use. Because of this reason, only cytotoxicity of azidophenyl chitosan derivative irradiated to UV light was carried out. Cytotoxicity test used for azidophenyl chitosan derivative was slightly different to usual MTT assay. When azidophenyl chitosan derivative was irradiated to UV light, it was not dissolved in water, acidic solvents, and organic solvents which are able to be used for MTT assay as a solvent for interesting compounds or materials. Therefore, azidophenyl chitosan derivative was casted over the surfaces of 6 wells as mentioned methods part above

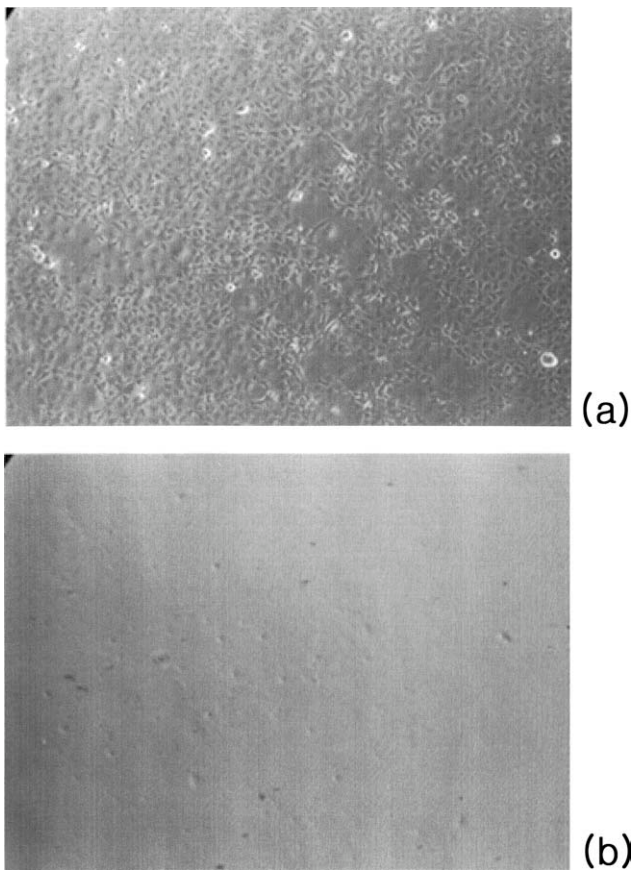


Figure 5 The surfaces of thermanox plastic cover slips (a) and themanox plastic cover slips coated with Az-PEG only (b) (×100). Same number of COS7 cells were seeded and cultured on the double coated surfaces for 48 h.

rather than dissolved in solvents and added to medium for cytotoxicity test. Because, in actuality, azidophenyl chitosan derivative will be served with same insoluble character as one of azidophenyl chitosan derivative irradiated to UV light and used for cytotoxicity test, it is considered that results of cytotoxicity test carried out in this study was quite reliable.

Cell culture

On the surfaces of thermanox plastic cover slips, attachment of COS7 (Green Monkey SV40-transf'd kidney fibroblast cell line) cells was observed while not on the surfaces of thermanox plastic cover slips coated with Azido-poly (ethylene glycol) (Az-PEG) (Fig. 5). It means that Az-PEG was prepared successfully because Az-PEG was known that it inhibits cell adhesion and proliferation. On the cover slips, coated with the 0.1, 0.5, and 5% concentrations of azidophenyl chitosan derivative on the top of Az-PEG-coated cover slips, attachment of COS7 cells was observed. As a result, it is considered that cells adhered quite strongly to azidophenyl chitosan derivative (Fig. 6)

Concentrations of growth factors are important factors for increasing of reaction rate between receptors of cell and growth factors and of growth factors activities. Easy way to increase the concentration of growth

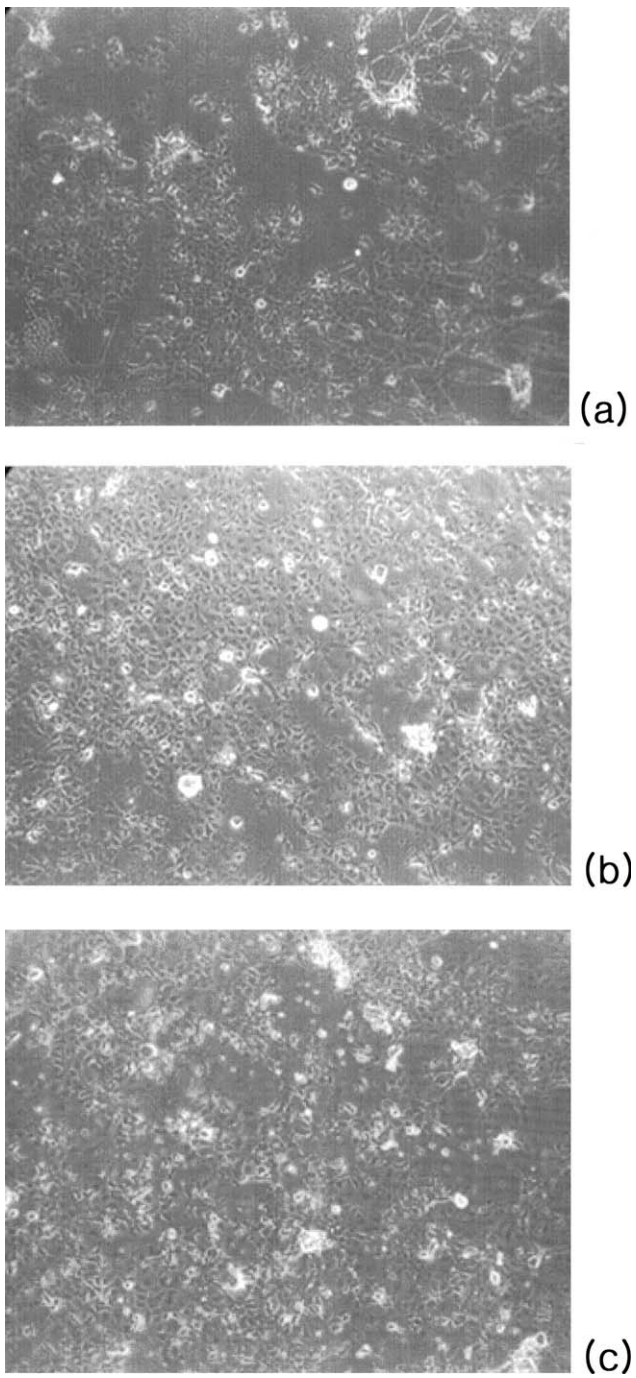


Figure 6 The surfaces of thermanox plastic cover slips coated with Az-PEG and 0.1% (a), 0.5% (b), and 5% (c) (wt/v) of azidophenyl chitosan derivative (×100). After coating with azidophenyl chitosan derivative the plastic cover slips were washed with distilled water for 60 min. Same number of COS7 cells were seeded and cultured on the double coated surfaces for 48 h.

TABLE I
Samples Prepared for Protein Releasing Test

Concentration in aqueous / Irradiation time to UV light	5%	10%	15%	20%
1 min	A1	B1	C1	D1
3 min	A2	B2	C2	D2
5 min	A3	B3	C3	D3
7 min	A4	B4	C4	D4

The percentage means the concentration of azidophenyl chitosan in aqueous. The time on vertical row means the time that azidophenyl chitosan was irradiated to UV light. The amount of BSA for immobilization was 200 μg in all samples.

factors is to use more growth factors. However, to use more growth factors could make some possible problems in commercial part and side effects. As decreasing the amount used for growth factors, it is possible to minimize of potential side effects generated from using of an excess proteins.[7] If immobilized growth factors are released on the surfaces of cells which are close to receptors, local concentration of growth factors around receptors will be increased. Therefore, in this case, fewer amounts of growth factors will be needed than other cases. According to the results, it is possible that azidophenyl chitosan derivative immobilizing growth factors adhere to surfaces of cells. It is expected that azidophenyl chitosan derivative will be able to make to reduce the amount of growth factors needed when same level of activities are required as one of free growth factors or higher activities than free growth factors when same amount of growth factors are used. Moreover, it is expected that the higher activity will be caused by a higher local concentration of growth factors on the surface.[26]

Protein releasing test

To find out optimum UV irradiation time and concentration of azidophenyl chitosan derivative for the highest growth factor immobilization efficiency of azidophenyl chitosan derivative, protein releasing test was carried out. The samples were divided into four groups according to concentrations. The samples with same concentration were irradiated to UV light for different time. Higher concentration of samples than 20% (wt/v) was not able to be prepared due to solubility of azidophenyl chitosan derivative. It seems that the saturation point of azidophenyl chitosan derivative is 20% (wt/v). The amount of released BSA was generally decreased as time goes. In early time of the test, quite a little amount of BSA was released and there was large change in the amount of BSA released. However, after around 36 h, the amount of BSA released was remarkably decreased compared to the amount in early time of the test and there was little change of the amount of BSA released although amount of BSA released was not fixed. According to results, the correlation between the amount of immobilized BSA and concentration of azidophenyl chitosan derivative used for immobilization was found out clearly. Average amounts of BSA released by each group were about 36.6 μg in A group, 94.0 μg in B group, 134.5 μg in C group, and 163.0 μg in D group. Because the amount of BSA released can be thought as the amount of immobilized BSA by azidopheyl chitosan derivative, it is considered that, in average of each group, about 18.3% in A group, 47.0% in B group, 67.3% in C group, and 81.5% in D group of BSA compared with the amount of BSA used for immobilization initially was immobilized by azidophenyl chitosan derivative. This suggests that the amount of immobilized BSA is increased as concentration of azidophenyl chitosan derivative used for immobilization is high (the results are summarized in Tables I and II). However, there was no obvious correlation between the irradiation time to UV light of azidophenyl chitosan derivative and the amount of immobilized BSA. BSA of each group was released for around 276 h in A group, 336 h in B group, 420 h in C group, and 432 h in D group. It is indicated that BSA may be released for longer duration with higher concentration of azidophenyl chitosan derivative used for immobilization. As a result, the highest efficiency in the amount and the longest releasing duration of growth factors are expected when the concentration of azidophenyl chitosan derivative

TABLE II
The Results of Protein Releasing Test

Groups of samples / Categories of results	A group	B group	C group	D group
The average amount of released BSA in mass	36.6 μg	94.0 μg	134.5 μg	163.0 μg
The amount of released BSA in percentage	18.3%	47.0%	67.3%	81.5%
A period of time BSA releasing	276 h	336 h	420 h	432 h

The results suggest that the amount of immobilized BSA and a period of time BSA releasing are increased as concentration of azidophenyl chitosan derivative used for immobilization is high.

used for immobilization is the highest within the limits possible.

CONCLUSIONS

The azidophenyl chitosan derivative which has photoreactivity was successfully prepared for immobilization of growth factors. Azidophenyl chitosan derivative irradiated to UV light was sufficiently noncytotoxic on 3T3 cells. It was found that azidophenyl chitosan derivative irradiated to UV light surrounded BSA. It is expected that physical protection against physical and chemical conditions can be provided for the growth factors, which would denature growth factors or negatively affect the activities of growth factors. Cell adhesion on the surfaces of azidophenyl chitosan derivative irradiated to UV was also found. Therefore, the higher activities are expected by a higher local concentration of growth factors on the surfaces. The higher immobilization efficiency in the amount and the longer releasing duration of BSA were found as azidophenyl chitosan derivative with higher concentration was irradiated to UV light and used for immobilization. It is probable that azidophenyl chitosan derivative is thought as a potent candidate for immobilization reagent and will have an application to immobilization of growth factors.

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