

Radiation induced formation of poly (N-isopropyl acrylamide)-bovine serum albumin covalent conjugates and their immunogenicity

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Poly (N-isopropyl acrylamide) was bioconjugated to bovine serum albumin by ⁶⁰Co γ -rays at pH 7. The covalent binding mechanism of the radiation induced poly (N-isopropyl acrylamide)-bovine serum albumin conjugate was analyzed by high performance liquid chromatography while the immunization property was analyzed by ELISA tests. High performance liquid chromatography results showed the conjugate formation of polymer with protein and also the increase in the radio stability of the protein as a result of this formation. The immunization results of irradiated conjugates in Balb/c mice were significantly higher than the free BSA immunization results. This system is attractive for application of a novel immunogenic model system in vaccine technology.

Keywords: Immunization, Irradiation, Polyelectrolytes, Proteins, Vaccine.

INTRODUCTION

The bioconjugation of synthetic polymers with biomolecules finds various implementations in many different areas such as biotechnology, medicine, pharmacy and engineering. Furthermore, while the solid state (water insoluble) polymeric systems are used in functional implementations like tissue engineering and cell generation, the water soluble polymer-biomolecule bioconjugates are lately being used in protein purification, enzyme stability, drug delivery systems, biosensor production, intracellular transfer of DNA, and protein modification [1-15].

A new approach is brought about for the resolution of the synthetic vaccine problem. The strong effect of the synthetic polyelectrolytes (PEs) (negatively or positively charged polymers) on the organisms' immune systems qualifies these PEs to be used as carrier matrix (and adjuvant) for microbes and virus antigens (peptides), and a tool for the synthesis of high antigen-specific immunogenic bioconjugates (and biocomplexes). Such synthetic macromolecular bioconjugates (and biocomplexes) display protective immunity against numerous diseases such as tuberculosis, flu, salmonella, working as a synthetic polymeric vaccine system.

Recent *in vivo* studies have shown that the bioconjugates which are synthesized by conjugation of proteins and neutral uncharged synthetic polymers (like polyvinyl alcohol, poly-N-vinylpyrrolidone, polyethylene glycol, dextran, etc.

dissolved in water) have relatively poor immunogenic properties in contrast to natural protein antigens. Additionally, it has been proven that the proteins are more stable within longer periods. The conjugation of polyethylene glycol with albumin and liver catalase enzyme decreases the immune reactivity of these proteins. Polymers with such structure do not have adjuvant characteristics. Nevertheless, the high molecular weight synthetic PEs, negatively or positively charged polymers or polymers that are charged at the physiologic conditions of the medium, polybases, polycations, polyacids and their copolymers show similar adjuvant effects independently of the chemical structure of their monomers (auxiliary chemicals which may enter into the organism with the antigens to increase the immunity of the organism). As the complexes or conjugates of different antigens with PEs are immunized to the living organism, the number of formed antigen-specific antibodies increases excessively, and they show protective characteristics on living organisms against viruses acting like a polymeric vaccine [1, 6, 12, 16].

In bioconjugate formation of the antigens with neutral polymers, the surface of the biomolecule is covered with a neutral and flexible hydrophilic lid. Closing the antigen determinants in such a manner makes the antigen molecule inert in the immune procedure [17-19].

Poly (N-isopropyl acrylamide) which carries characteristics of PEs was bioconjugated with bovine serum albumin by ⁶⁰Co γ -rays at pH 7 in order to develop synthetic polymeric vaccine model

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systems. The conjugation of poly (N-isopropyl acrylamide) and bovine serum was analyzed by high performance liquid chromatography, the immunization property was analyzed by ELISA tests.

EXPERIMENTAL

Materials

Poly (NIPAAm) (Mw: 60 kDa) was supplied by Prof. Dr. Yoshihito Osada (Hokkaido University, Sapporo, Japan). Bovine serum albumin (BSA) (Mw: 66 kDa, pI: 4.9) was purchased from Sigma Chemical Company (St. Louis, USA).

Synthesis of Poly (NIPAAm) - BSA Bioconjugates

The mixture of 0.1% poly (NIPAAm) and 0.15% BSA prepared in phosphate buffer solution was exposed to radiation at 100, 300, 500, 700 Gy from a ^{60}Co γ -ray source to form poly (NIPAAm)-BSA conjugate. The same procedure was previously used to prepare 0.1% poly (NIPAAm) and 0.15% BSA solutions each.

Preparation of Phosphate Buffer Solution

In order to prepare 1 l of phosphate buffer solution, each 1.1998 g of NaH_2PO_4 and 2.6807 g of Na_2HPO_4 was dissolved in 500 ml ultra pure water. After mixing these two together, 8.766 g of NaCl was added to the solution. The pH of the prepared solution was adjusted to 7 by adding 0.1 M NaOH solution.

Immunization

Poly (NIPAAm) - BSA bioconjugates were used as immunogens. Free BSA was utilized for controlling purposes. Eight week-old Balb/c mice were immunized with each of the bioconjugates by intravenous injections. The blood was collected into a microfuge tube with sodium citrate and centrifuged at 6000 g to remove red blood cells. A set of dilutions of the serum (1/50 and 1/100) was made in phosphate-buffered saline (PBS). The serum samples were tested by ELISA [20].

ELISA

To assay BSA specific antibodies, ninety-six well polystyrene plates (NUNC-immunoplates) were coated with 200 ng poly (NIPAAm)-BSA bioconjugates in parallel with BSA in 100 μl PBS. Coated plates were incubated at 4°C overnight. The plates were washed two times with wash buffer (0.005% Tween-20 in PBS). Then, 0.2% casein in PBS was added to the wells, and the plates were incubated at 37°C followed by washing as above. The mouse serum in dilution buffer was added to

each well and the plates were incubated at 37°C for 1 h. The plates were washed two times with wash buffer. Alkaline-phosphatase conjugation of polyvalent goat-antimouse Ig (Sigma) in 1/750 dilution buffer was then added to each well and incubated for 1 h at 37°C. After repeating the wash step five times with wash buffer as above, the substrate buffer (1mM ZnCl_2 , 1mM MgCl_2 , 0.1M glycine, pH 10.4) and 1 mg/ml *p*-nitrophenyl phosphate were added. After 45 min, the absorbances at 405 nm were determined.

Gamma-Radiolysis

Gamma-radiolysis of the aqueous solutions of poly (NIPAAm) - BSA bioconjugates was performed by using a ^{60}Co γ -source (Picker 9 V). 5 ml sample solutions were irradiated at a position of 10 cm away from the source. The dose rate was measured to be 28.61 Gy/h as determined by Fricke dosimetry [21].

Gel Filtration HPLC

Poly (NIPAAm), BSA and the prepared poly (NIPAAm) - BSA were analyzed by HPLC (gel filtration chromatography) before and after irradiation.

The system consisted of a Bio-Sil Sec 250 column (7.8 mm \times 300 mm), pump (LC-10Ai) and automatic sample injector (SIL-10Ai HPLC). The eluent was monitored at 214 and 280 nm by using UV detector (SPD-10Ai). A phosphate buffer containing 0.1 mol/l NaCl was used as a mobile phase at a flow rate of 1.0 ml/min at room temperature. The calibration of the column was performed by using a protein kit from Sigma Chemical Co., St. Louis, MO, USA, namely, thyroglobulin (670 kDa), immunoglobulin (155 kDa), ovalbumin (44 kDa), myoglobulin (16.9 kDa), and vitamin B12 (1.35 kDa).

Dynamic Light Scattering Method

Photon correlation spectroscopy with a Zetasizer Nano ZS instrument equipped with 4.0 mV He-Ne laser operating at a wavelength of 633 nm and a temperature of 25°C (Malvern Instruments, UK) was used to examine the average size and the size distribution of polymer and bioconjugate of protein-polymer. Before DLS measurement, each solution was filtered through a 0.2 μm RC-membrane Sartorius filters to remove the impurities in the solutions.

RESULTS AND DISCUSSION

Gel filtration HPLC was used for the poly (NIPAAm), BSA and poly (NIPAAm) - BSA

bioconjugates HPLC chromatograms of poly (NIPAAm) and BSA are given in Figure 1 and Figure 2, respectively.

Figure 1 summarizes the gel filtration HPLC results of the free polymers before and after 700 Gy irradiation. As seen from the figure, while the unirradiated free polymer shows a peak in 7.38 minutes, the polymer solution irradiated with 700 Gy shows no peak at all. It could be said that polymers are forming water insoluble aggregates by forming crosslinks in themselves at this irradiation dose.

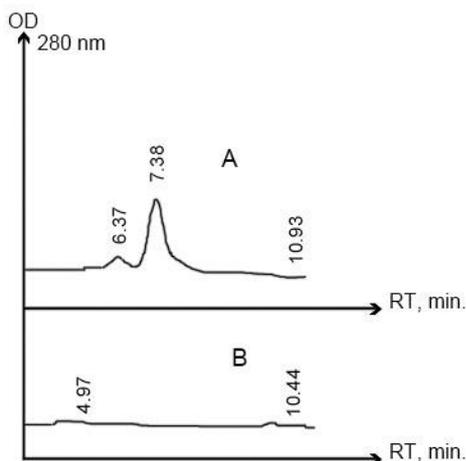


Fig. 1. HPLC results of the unirradiated (A) and irradiated (B) solutions of poly (NIPAAm) at irradiation dose (Gy): 700.

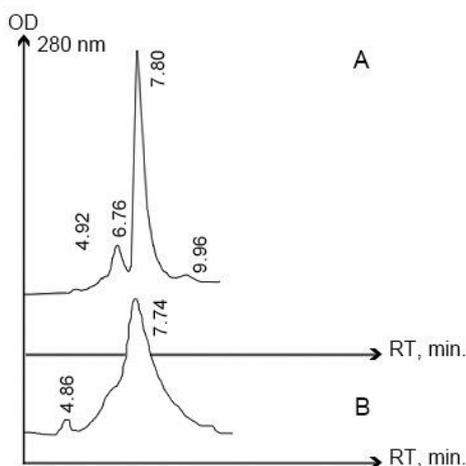


Fig. 2. HPLC results of the unirradiated (A) and irradiated (B) solutions of BSA at irradiation dose (Gy): 700.

The gel filtration results of free BSA before and after irradiation at 700 Gy are shown in Figure 2. As the figure indicates, the retention time (RT) value of the protein irradiated at 700 Gy decreased and the peak generated was significantly deformed. From this, we can suggest that BSA has denatured and its molecular

weight has increased due to the 700 Gy irradiation strength.

The gel filtration HPLC results of the poly (NIPAAm) - BSA mixture with and without irradiation at 700 Gy are shown in Figure 3. As it can be understood from the figure, the polymer and the protein formed a bioconjugate by binding after 700 Gy irradiation so there was a shift in peak retention time (B), and the protein molecule in the conjugate structure was protected against irradiation (it is radiostable), which means that it has not denatured.

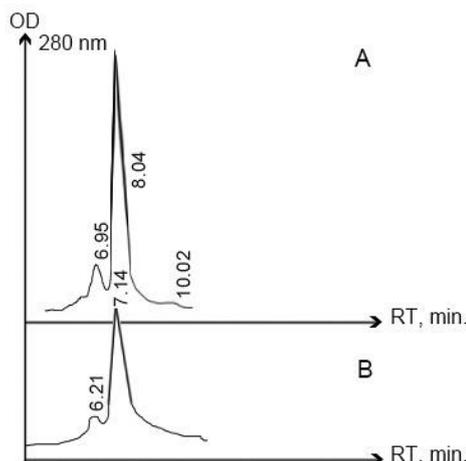


Fig. 3. HPLC results of the unirradiated (A) and irradiated (B) solutions of a poly (NIPAAm) - BSA mixture at irradiation dose (Gy): 700.

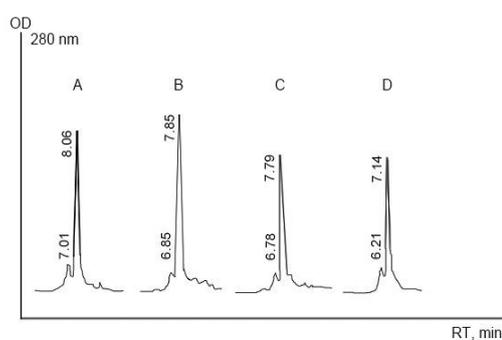


Fig. 4. HPLC results of the irradiated solutions of poly (NIPAAm) - BSA mixture at different irradiation doses (Gy): 100 (A), 300 (B), 500 (C), 700 (D).

HPLC results of the irradiated solutions of the poly (NIPAAm) - BSA mixture at different irradiation doses (Gy): 100 (A), 300 (B), 500 (C), 700 (D) are shown in Figure 4. Homogenous conjugates are formed once radioactive ray between 100-700 Gy was applied to the poly (NIPAAm) - BSA mixture. The figure shows that, as the radiation strength increases, the RT values of the peaks of the generated conjugates decrease, indicating that the generated bioconjugate had a higher molecular weight than the components in the mixture. In addition, it should be mentioned that the best

bioconjugates are obtained at 700 Gy irradiation.

If the irradiation dose increases, the hydrodynamic diameter also increases due to increased interaction between the PE of BSA. (Figure 5).

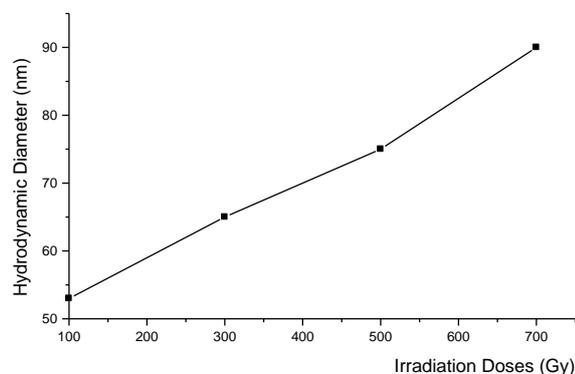


Fig. 5. Hydrodynamic diameter of poly (NIPAAm)-BSA bioconjugate at different irradiation doses (100-700 Gy).

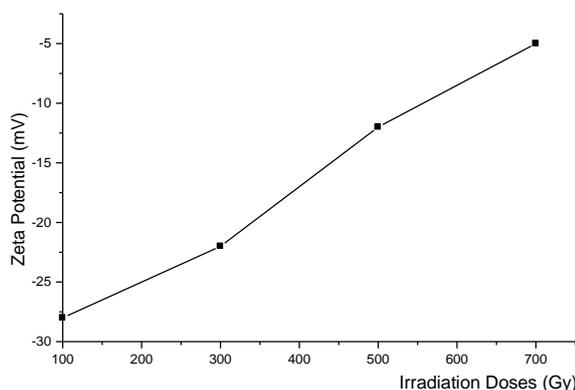


Fig. 6. Zeta potentials of poly (NIPAAm) - BSA bioconjugate at different irradiation doses (100-700 Gy).

The dynamics of formation of BSA specific antibodies (OD_{405 nm}) in the blood serum of mice immunized with poly (NIPAAm) - BSA bioconjugate at different irradiation doses (100-700 Gy), and pure BSA are shown in Figure 7. As seen from the figure, the immunization results of the bioconjugates generated by irradiating at 100, 300, 500 Gy radiation strengths are close to each other, the antibody level obtained against the bioconjugate irradiated with 700 Gy is the highest, and a significantly higher value of the conjugate is observed as compared to the free BSA.

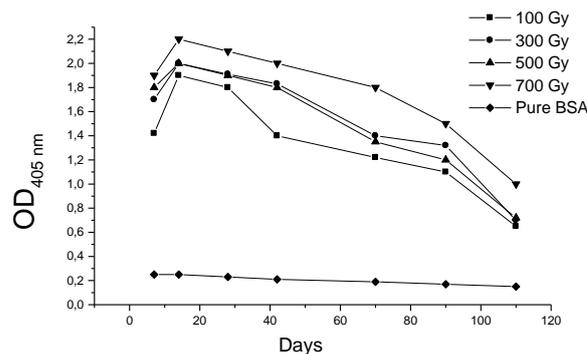
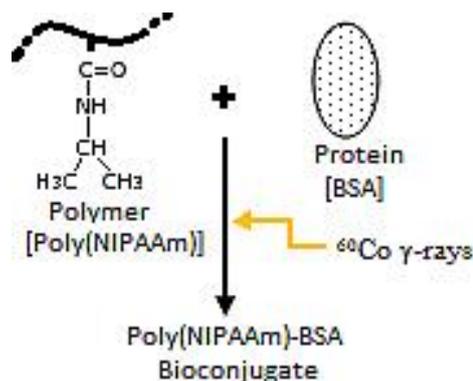


Fig. 7. The dynamics of formation of BSA specific antibodies (OD_{405 nm}) in the blood serum of mice immunized with poly (NIPAAm) - BSA bioconjugate at different irradiation doses (100-700 Gy) and pure BSA.

CONCLUSION

As the results of the HPLC and ELISA tests showed, BSA was successfully conjugated to poly (NIPAAm) at pH 7 under radiation doses of 100-700 Gy without any degradation in its structure. It is seen that the best bioconjugate was generated at a radiation strength of 700 Gy. No denaturation was observed in the structure of BSA protein in this bioconjugate, which indicates an increase in its radio stability. The most important point in the increase of the polymer-protein radio stability is that the polymer macromolecules form a cover on the protein and thus, the protein molecule is protected. This bioconjugate and synthetic vaccine model system was developed by ⁶⁰Co γ -rays.



Schema. The hypothetical structure of water soluble bioconjugate irradiated with 700 Gy.

According to this, by binding the suitable synthesized antigenic peptide of a disease with polymer instead of BSA protein in the bioconjugate process, a synthetic vaccine can be developed by using the carrier matrix and adjuvant PEs for the microbes or virus antigens against that disease.

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РАДИАЦИОННО ИНДУЦИРАНО ОБРАЗУВАНЕ НА КОВАЛЕНТНО СПРЕГНАТИ ПОЛИ – (N-ИЗОПРОПИЛ АКРИЛАМИД)-АЛБУМИНИ ОТ ГОВЕЖДИ СЕРУМ И ТЯХНАТА ИМУНОГЕННОСТ

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Поли (N-изопротил акриламид) е био-спрегнат към албумин от говежди серум при облъчване с γ -лъчи от източник ⁶⁰Co при рН 7. Анализирани са механизмите на радиационно индуцираното спрягане с помощта на ВЕТХ, а имунизационните свойства са анализирани с ELISA-тест. Резултатите от ВЕТХ показват образуването на спрегнат полимер с протеина и в резултат – повишена радио-стабилност на протеина. Резултатите за имуногенността на облъчваните конюгати в мишки Balb/c mice са значително по-добри от тези при BSA-имунизационните резултати. Тази система е привлекателна за приложението на нов имуногенен модел в технологията на ваксините