Hyaluronan conjugation of antigenic protein to modify immunogenic information

S. Mochizuki\textsuperscript{a}, A. Kano\textsuperscript{a}, A. Yamayoshi\textsuperscript{a}, A. Maruyama\textsuperscript{a,\textit{b},*}

\textsuperscript{a}Institute of Materials Chemistry and Engineering, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
\textsuperscript{b}CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi-shi, Saitama 332-0012, Japan

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Abstract

Hyaluronan is an anionic polysaccharide and a major component of extracellular matrices. The major physiological role of hyaluronan (hyaluronic acid, HA) is thought to be a space filling material and HA is an attractive biomaterial due to its biocompatibility and biodegradability. We chemically modified ovalbumin (OVA), a well defined protein antigen, with HA to reduce its immunogenicity. HA was enzymatically fragmented by a hyaluronidase and different sizes of fragments ($M_n = 3000$ and 9000) were obtained. The enzyme-digested HA fragments have a reducing end, which was used for coupling with OVA through reductive amination reaction. Colorimetric assay for free amino groups in OVA indicated that the conjugated number of HA fragments was maximally 10 and 6 per OVA, for the smaller ($M_n = 3000$) and larger ($M_n = 9000$) HA fragments, respectively. The HA contents of the conjugates were calculated as 40 and 55 wt\%, respectively. We also obtained HA conjugates that have different length but almost the same number of HA fragments on OVA. The mice injected with the HA-conjugated OVA showed considerably lower OVA-specific IgE production than those with native OVA, implying reduced immunogenicity of OVA by the HA-modification. The data suggest that HA is a useful biomaterial for modification of immunological information in antigenic proteins.

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1. Introduction

It has been demonstrated that the antigen-specific immune tolerance can be induced by a protein antigen that was modified with serum proteins [1] or synthetic macromolecules [2–5]. Especially, polyethylene glycol (PEG) derivatives have been well studied for protein modification. In the early study, the aims of protein modification with PEG were to give it stability and reduce its immunogenicity in medical processes [6]. Indeed this idea has been successful in using foreign protein for medical application, for instance, adenosine deaminase [7,8], interleukin-2 [9,10], asparaginase [11,12], interferon [13,14], and so on. Based on this idea, Lee et al. tested the capability of PEG-antigen conjugates to induce antigen-specific immune tolerance by measuring antigen-specific IgE levels [2]. They showed that the mice administered PEG-antigen conjugates intravenously were insensitive against the native antigens, whereas they were normal against another antigen. Although a number of studies have been made on the tolerogenicity of the PEG-antigen conjugates, its mechanisms were poorly understood. Lee and Sehon described that the lack of allergenicity and antigenicity of the PEG-antigen conjugates was paralleled by their non-immunogenicity [2].

Hyaluronan or hyaluronic acid (HA), one of the glycosaminoglycans, is a linear high molecular weight polysaccharide consisting of alternating N-acetyl-\(\beta\)-D-glucosamine and \(\beta\)-D-glucuronate residues linked at the 1–3 and 1–4 positions, respectively [15]. HA is one of the major components of the extracellular matrices found in highly differentiated tissues. HA is a major constituent of some tissues, such as vitreous of mammalian eye, synovial fluid,
and skin. At least, one of the roles of HA is believed to be a space filling material. Unique hydrodynamic and physico-chemical properties of high viscoelasticity and high capacity for holding water probably played important roles in living body. Thus HA is an attractive material for biocompatible and biodegradable polymers. Increasing studies of HA for drug delivery and tissue engineering application [16,17] have been studied.

We have interested in HA as a new type of material for protein modification to modify immunological information of antigenic proteins. It is expected that HA gives reduced antigenicity to the protein leading to specific immune tolerance. In this report, we described preparation of HA-ovalbumin (OVA) conjugates having different lengths and coupling degrees of HA molecules. The effect of HA-modification on immunogenic responses against OVA in mice was studied.

2. Experimental details

2.1. Materials

OVA, Bovine testicular hyaluronidase (Type I-S) and alcian blue were purchased from Sigma Chemical Co (St. Louis, MO). 2, 4, 6-trinitrobenzene-sulfonic acid (TNBS) and sodium cyanoborohydride (NaBH3CN) were purchased from Sigma Chemical Co (St. Louis, MO). 2, 4, 6-trinitrobenzene-sulfonic acid (TNBS) and sodium cyanoborohydride (NaBH3CN) were pur- chased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). High-molecular weight HA (5.9 × 10⁵), obtained as its sodium salt (sodium hyaluronate), was kindly provided by Denki Kagaku Kogyo Co., Ltd (Tokyo, Japan). BALB/c mice (male, 7 weeks old) were purchased from Sankyo Laboratory Service Co. Inc (Tokyo, Japan).

2.2. Enzymatic hydrolysis of HA

High-molecular weight HA was partially degraded by hyaluronidase to low-molecular weight fragments [18]. Hyaluronidase (60 mg) and HA (3 g) were mixed together in 300 mL of water. The solution was stirred at 50 °C for a desired time (1–20 h). Then, the solution was boiled for 5 min to terminate the reaction and allowed to cool to room temperature. After the filtration of the cooled mixture thorough a 0.45 μm filter, the resulting HA fragments were obtained by freeze-drying.

2.3. Synthesis of HA-OVA conjugates

The obtained HA fragments were conjugated to OVA by reductive amination using NaBH3CN as a reducing agent (Scheme 1). The OVA (14–27 mg) and HA (10–58 mg) were mixed in 1.2 mL of sodium borate buffer (0.1 M, pH 8.5) containing 0.4 M NaCl. The reaction mixture with NaBH3CN (200 mM) was incubated at 40 °C for 5 days. The conjugates were obtained by ultrafiltration using a Centriplus® YM-30 or YM-50 (molecular weight cutoff of 30,000 or 50,000; Millipore Co., Billerica, MA). PEG-OVA was also prepared as described above, using PEG (PEG- aldehyde; Mn = 2000) kindly provided by Nippon Oil and Fats Co., Ltd. (Tokyo, Japan).

2.4. Gel permeation chromatography (GPC)

GPC was carried out using a JASCO 880-PU pumping system (Tokyo, Japan) at the flow rate of 0.8 mL/min with OH pak SB-806 M and OH pak SB-804 columns (Showa Denko Co., Ltd, Tokyo, Japan). About 50 mM phosphate buffer (pH 7.2) containing 0.2 M NaCl and 1 mM EDTA was used as a mobile phase. The eluate was detected by a reflective index (RI) detector (RI-930, JASCO) and a multiangle light scattering (LS) detector (Dawn–DSP, Wyatt Technology Co., Santa Barbara, CA). RI and LS signals were subjected to calculate the number-average (Mn) molecular weight according to the instruction manual (Wyatt Technology Co.) for Dawn-DSP.

2.5. Polyacrylamine gel electrophoresis (PAGE)

The protein concentration was determined by bicinchoninic acid (BCA) assay (PIERCE Biotechnology, Inc., Rockford, IL). About 2 μg of the protein samples were incubated with 2% SDS and 4% 2-mercaptoethanol at 95 °C for 5 min, and separated by the 8% SDS-PAGE (at 100 V, for 2 h). Then, Coomassie Brilliant Blue (CBB) staining was performed as described in [19]. Non-reducing native PAGE analysis was performed as described above without SDS. About 15 μg of proteins and HA (15 μg) were separated by a 6% acrylamide gel for 40 min at 100 V. After the electrophoresis, the gel was incubated with 0.5% aqueous solution of alcian blue for 30 min, then washed with deionized water [20,21].

2.6. Determination of the free amino groups of OVA in a conjugate

The conjugates (0.1–1 mg/ml) were diluted to 100 μl with PBS and an equal volume of 0.1 M borate buffer (pH 8.5) and 0.1% TNBS was added. After incubation at 40 °C for 2 h, the reaction was stopped by the addition of 100 μl of 10% SDS and 50 μl of 1 N HCl and measured OD at 340 nm [22]. The concentration of amino groups in the conjugate was determined from a standard curve constructed with known concentration of OVA. It has been reported that number of free amino groups is 21 [23]. The protein concentration was determined by the BCA assay, which is not affected by amino groups. Number of free amino groups on an OVA was calculated by comparing concentration of free amino groups and proteins.

2.7. Evaluation of immune reaction of mice preinjected with HA–OVA conjugate

Six groups of four mice were subjected for measuring of the specific IgE production. The mice were pretreated intravenously with 100 μl of PBS, 50 μg of OVA, 50 μg of
HA–OVA (sample 3 in Table 1) and 50 μg of PEG–OVA (sample 6 in Table 1) in 100 μl of PBS each. The mice, pretreated intraperitoneally with 50 μg of PEG–OVA (sample 6 in Table 1) in 100 μl of PBS were also prepared. After 15 days, a mixture of 10 μl of OVA (10 μg) and 100 μl of aluminum hydroxide gel adjuvant (alum adjuvant; 5 mg) [24] was injected to mice intraperitoneally. A group of mice was kept without any treatments. Five days later, the mice were bled from a tail artery and OVA-specific IgE in serum was measured by the modified enzyme linked immunosorbent assay [25]. In brief, 96-well plates were incubated with rat anti-mouse IgE antibody (Sanbio b.v. Co., Uden, Netherlands), the capture antibody, for 2 h at r.t. and blocked with 5% BSA in PBS overnight at 4 °C. The 100 fold-diluted sera were incubated with the capture antibody in the 96-well plate for 1 h, followed by native OVA. The bound OVA was detected by rabbit anti-OVA antibody (Rockland Immunochemicals, Inc., Gilbertsville, PA), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (PIERCE), and developed by ImmunoPure TMB Substrate Kit (PIERCE) according to the manufacturer’s instruction.

3. Results and discussion

3.1. Synthesis of HA–OVA conjugates

Most polysaccharides are useful for site specific chemical modifications of biopolymers because they have one reducing end per molecule; namely, they are classified into semitelechelic polymers [26,27]. The polysaccharides whose glycosyl bonds are hydrolyzed enzymatically or chemically are also semitelechelic, enabling us to control molecular weight of polysaccharides for bioconjugation. We previously prepared graft polymers having polylysine backbones and HA graft chains (PLL–g-HA). PLL–g-HA copolymers having different lengths and substitution degrees of HA (Mn = 1600, 2300 and 3800) were successfully prepared [28]. According to the previous study, we adjusted incubation time with hyaluronidase to obtain HA fragments with controlled Mn. HA fragments (Mn = 3000–9000) which have 8–24 repeats of constituting disaccharide unit were obtained and used for OVA conjugation.

The hydrolyzed HA fragments were conjugated with OVA by reductive amination between the reducing end of
HA and amino groups of OVA using NaBH₃CN as a reducing agent (Scheme 1). The reaction conditions for conjugation between OVA and HA were summarized in Table 1.

### 3.2. Chemical characterization of HA–OVA conjugates by GPC and gel electrophoresis

Fig. 1a shows the representative GPC profiles of OVA, reaction mixtures of HA–OVA \( (M_n = 3000) \) before and after ultrafiltration. After the reaction, the OVA peak detected by RI was shifted to small elution volume side, suggesting an increase in molecular weight due to coupling of OVA with HA (solid line and dashed line). The chromatogram clearly indicated presence of unreacted HA which eluted at 23–24 ml. By performing the ultrafiltration (dotted line), the HA–OVA conjugate was largely separated from unreacted HA. The incubation of HA or OVA alone with NaBH₃CN under the same conditions caused no change in their GPC profiles, which suggested that neither degradation nor side reaction occurred during the coupling reaction (data not shown). The GPC profile of the resulting conjugate also suggests that the conjugate was synthesized and purified without undesired aggregations. When OVA was reacted with the larger fragments \( (M_n = 9000) \) of HA, the increased molecular weight of OVA was confirmed, but the purification was not confirmed distinctly (Fig. 1b) owing to large molecular weight distribution of HA.

Next, we characterized the HA–OVA conjugates by polyacrylamide gel electrophoresis (PAGE). Reducing SDS–PAGE analysis (Fig. 2a) showed slower migration of the OVA reacted with HA than that of native OVA. The OVA reacted with HA at higher feed ratio migrated much slower. On the other hand, faster migration of HA–OVA conjugates than that of native OVA was observed in native PAGE (Fig. 2b). Since HA is a negatively charged polymer, the HA fragments conjugated on the OVA likely contributed to the faster migration. It is known that alcian blue specifically bind to acidic saccharides [22]. Increasing alcian blue staining of the HA–OVA conjugates in Fig. 2b indicates increasing amount of HA reacted with the OVA. Also the result of the PAGE analysis showed that unreacted HA was removed from the conjugate. Taken together, it is conceivable that the enzymatically digested HA fragments were covalently coupled with OVA by reductive amination reaction. The coupling ratio can be controlled by the feed ratio of HA.

In contrast, the OVA reacted with the larger HA fragments \( (M_n = 9000) \) migrated slower (Fig. 2c). These results may be due to increased bulkiness of HA–OVA conjugates. These behaviors of HA–OVA conjugates depending on HA length may be interesting point for the protein modification strategy. Further characterization is required to understand the physicochemical and hydrodynamic properties of oligo HA and its protein conjugates.

The percentage of amino groups reacted with HA was estimated by the TNBS assay as described in the Experimental part. The results were summarized in Table 1. When the OVA was reacted with the small or large HA fragments at the same molar feed, the number of HA fragments coupled with OVA was 10 and 6, respectively (sample 3 and 5). The results indicated the lower reactivity of the larger HA fragments than the smaller one. However, the weight fraction of the HA in the resulting conjugates was 40 and 55 wt%, respectively (sample 3 and 5), indicating higher HA content of the later than the former. We also obtained conjugates that have almost the same number of HA fragments, but different length of HA fragments (sample 1 and 4). Consequently, various types of HA–OVA conjugates with the differences in HA size and weight fraction were available with our
conjugation method. With similar method, we have prepared the PEG–OVA conjugates with desired PEG content.

3.3. Evaluation of immune response of mice preinjected with HA–OVA conjugate

The effect of the HA or PEG modification on immune response of mice was observed as follows. First, PBS, native OVA, the HA–OVA conjugates (sample 3) or the PEG–OVA conjugates (sample 6) were intravenously administered in mice. The mice administered with the PEG–OVA conjugates intraperitoneally were also prepared. Next, the mice received native OVA with the alum adjuvant intraperitoneally. The antigenicity of the conjugates was evaluated by measuring IgE level in the sera (Fig. 3). As expected, the mice pretreated with native OVA considerably produced OVA specific IgE after the 2nd OVA challenge. When the mice were pretreated with the HA–OVA conjugates, the serum IgE level was significantly reduced. Of interest, the level was even lower than that in the mice pretreated with the PEG–OVA intraperitoneally, and almost the same level to the mice pretreated with PBS. These results indicate that the antigenicity of OVA was reduced by the HA modification. On the other hand, we did not observe remarkable effects of intravenously injected PEG–OVA on IgE production (Fig. 3). Lee et al. demonstrated that the PEG–OVA injected intravenously reduced immunogenicity of hapten-OVA conjugates [2]. The chemical composition of the conjugates, the dose and the schedule of antigen administration in our study were, however, different from that in Lee’s report.

Fig. 1. Gel permeation chromatograms of (a) OVA (solid line), reaction mixture of OVA and HA ($M_n = 3000$) (dashed line) and the mixture after the ultrafiltration (dotted line), (b) OVA (solid line), HA ($M_n = 9000$; dashed line) and the reaction mixture after the ultrafiltration (dotted line). Chromatograms detected by RI are shown.
As mentioned above, the conjugation of OVA with HA did not produce any aggregates nor precipitates. Furthermore, the reactivity of the anti-OVA antibody to the HA–OVA was not changed significantly (in our preliminary experiments, data not shown). These observations suggest that the conjugation with HA hardly changed the conformation of OVA. However, even slight changes of the surface character of OVA, such as electric charges,
hydrophilicity or steric hindrance, would influence recognition and processing by immune cells and may affect its immunological responses. In this study, we obtained different OVA conjugates in the length and coupling degree of HA. The evaluation of these conjugates may elucidate the roles and effects of HA modification on immune responses.

4. Conclusions

In this study, we prepared the HA–OVA conjugates by reductive amination between the reducing end of HA and amino groups of OVA. We could obtain several types of HA–OVA conjugates with various chain lengths and the densities of HA. The resulting conjugates were characterized by the GPC and the PAGE analysis. No degradation and aggregation of OVA were observed. The in vivo study using mice suggested that the pretreatment with HA–OVA conjugate considerably reduced immunogenic activity of native OVA. HA is an attractive biomaterial to modify not only immunological but also various information of proteins.

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