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Improved sustained release of antigen from immunostimulatory DNA hydrogel by electrostatic interaction with chitosan

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ABSTRACT

Immunostimulatory DNA hydrogel (sDNA hydrogel) containing unmethylated cytosine-phosphate-guanine (CpG) sequences has been demonstrated to be a useful antigen delivery system, which can effectively induce an antigen-specific immune response through stimulation of the innate immune system. However, relatively rapid release of antigens from the sDNA hydrogel limits its potential. To enhance the potency of the sDNA hydrogel *via* improvement of its sustained release property, we selected chitosan, a biocompatible cationic polymer which electrostatically interacts with DNA, and mixed it with the sDNA hydrogel. Compared to unmixed sDNA hydrogel, sDNA hydrogel mixed with chitosan (Chitosan-sDNA hydrogel) was more stable, tougher, had more bound water, released a model antigen ovalbumin (OVA) more slowly *in vitro*, and provided longer retention of OVA at the injection site after intradermal injection into mice. Intradermal immunization of mice with the OVA-loaded Chitosan-sDNA hydrogel resulted in the induction of a higher level of OVA-specific IgG in serum compared with OVA-loaded sDNA hydrogel with no chitosan. These results indicate that the Chitosan-sDNA hydrogel is an improved sustained release formulation for efficient induction of antigen-specific immune responses.

1. Introduction

Toll-like receptor 9 (TLR9) is the receptor for DNA containing the unmethylated cytosine-phosphate-guanine (CpG) sequence, or "CpG motif." Ligation of TLR9 results in the release of T helper type 1 and proinflammatory cytokines, maturation/activation of professional antigen-presenting cells, and stimulation of humoral and cellular immune responses (Akira and Takeda, 2004; Kumagai et al., 2008). Based on this immunostimulatory mechanism of action, substantial potential of CpG motif-containing DNA (CpG DNA) as adjuvants for the treatment of cancer and infectious and allergic diseases has been demonstrated in many studies (Vollmer and Krieg, 2009; Klinman et al., 2009).

Through the use of structural DNA nanotechnology (Mohri and Nishikawa, 2014; Zhang et al., 2014; Chao et al., 2014; Linko et al., 2015), our group previously developed a DNA hydrogel, which was an enzymatically catalyzed assembly of CpG motif-containing X-shaped DNA (Nishikawa et al., 2011). We extended this technology and developed a novel method to prepare a ligase-free, self-gelling, injectable DNA hydrogel. Immunostimulatory DNA hydrogel (sDNA hydrogel) was prepared by the self-assembling of CpG motif-containing hexapod-like DNA (Nishikawa et al., 2014). Intradermal injection or intranasal administration of this sDNA hydrogel loaded with antigens showed high potency and low toxicity as a vaccine adjuvant (Nishikawa et al., 2014; Mizuno et al., 2015).

The efficient induction of antigen-specific immune responses was at least partly due to the sustained release of antigens from the sDNA hydrogel. However, the half-life of the antigens released from the sDNA hydrogel was less than 4 h under *in vitro* conditions. Considering the well-known fact that sustained release of antigens is effective for increasing antigen-specific immune responses

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(Petrovsky and Aguilar, 2004; Nochi et al., 2010; Chen et al., 2013; Adams et al., 2015), there may be potential to improve the potency of sDNA hydrogels by increasing the duration of antigen release. To investigate this potential, we cationized the negatively charged antigen OVA so that it could electrostatically interact with DNA. The cationized OVA was more slowly released from the sDNA hydrogel, and intratumoral injections of this formulation were found to be more potent than a non-cationized OVA/sDNA hydrogel in suppressing the growth of EG7-OVA tumors in mice (Umeki et al., 2015).

Sustained release of not only antigens but also CpG DNA would be useful to maximize the potency of sDNA hydrogels. Prolonged TLR signaling could be used as a continuous warning signal to the immune system through sustained release of proinflammatory cytokines (Celis, 2007; Yang et al., 2004). In fact, slow release of TLR ligands including CpG DNA results in enhanced antigen-specific immune responses (Yang et al., 2004; Jewell et al., 2011). To achieve improved sustained release of both antigens and CpG DNA, it would be useful to optimize the function of the sDNA hydrogel itself. In addition, this approach can be easily applied to various antigens regardless of their electric charge and other physicochemical properties.

The application of electrostatic interactions between oppositely charged polymers is a frequently used approach to obtain reversible polyelectrolyte complexes (Bhattarai et al., 2010; Agnihotri et al., 2004). For example, chitosan, a cationic polymer, interacts with a polyanionic tripolyphosphate *via* electrostatic force and leads to ionic gelation (Agnihotri et al., 2004). Therefore, cationic polymers are expected to electrostatically interact with an anionic sDNA hydrogel to modify its function.

Based on these considerations, we aimed to stabilize an sDNA hydrogel *via* electrostatic interactions between cationic polymers and DNA to improve sustained release of antigens and CpG DNA. Chitosan, a major natural biodegradable polysaccharide, was selected as a biocompatible cationic polymer, and the effect of chitosan on physicochemical properties of the DNA hydrogel was examined. Polyethylene glycol and sodium alginate were used as neutral and anionic polymers, respectively, for comparison. A chitosan-mixed sDNA hydrogel was loaded with OVA, a model antigen, and properties of the chitosan-mixed sDNA hydrogel with regard to sustained release and induction of antigen-specific immune responses were investigated.

2. Materials and methods

2.1. Chemicals

Ultrapure medical grade water-soluble chitosan glutamate (PROTASAN UP G213), which was nontoxic *in vitro* and *in vivo* (Chenite et al., 2000; Molinaro et al., 2002), was purchased from FMC BioPolymer (Philadelphia, PA, USA). Polyethylene glycol (POLYOX WSR 303) was obtained from Dow Chemical Company (Midland, MI, USA). Sodium alginate (sodium alginate 300–400) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of the highest grade available and used without further purification.

2.2. Animals

Male ICR mice (6 weeks old) and C57BL/6 mice (5 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

2.3. Preparation of DNA hydrogel and polymer-mixed DNA hydrogel

All phosphodiester oligodeoxynucleotides (ODNs) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The hexapodna and DNA hydrogel were designed and prepared using the ODNs as reported in our previous study (Nishikawa et al., 2014). Briefly, three types of immunostimulatory hexapodna (sHexa) with potent CpG motifs, namely, sHexa-1, sHexa-2 and sHexa-3, and two types of non-immunostimulatory hexapodna (nsHexa) with no CpG motifs, nsHexa-1 and nsHexa-2, were prepared. Each hexapodna was prepared by annealing of a mixture of six ODNs with 40 base lengths. The sequences of ODNs are summarized in Table S1 in our previous report (Nishikawa et al., 2014). Immunostimulatory and non-immunostimulatory DNA hydrogels (sDNA hydrogel and nsDNA hydrogel, respectively) were obtained by mixing equimolar amounts of sHexa-1 and sHexa-2 or nsHexa-1 and nsHexa-2, respectively, at a concentration of 7 or 22 μg DNA/μl as used in previous studies (Nishikawa et al., 2014; Mizuno et al., 2015). The size of the DNA hydrogel used in all the experiments was in millimeter range. sHexa solution was prepared by mixing sHexa-1 and sHexa-3.

To prepare polymer-mixed DNA hydrogels, three different polymer solutions, i.e., chitosan glutamate solution, polyethylene glycol solution, and sodium alginate solution, were prepared in advance at a concentration of 20 μg/μl in Tris-EDTA buffer containing 150 mM NaCl. The viscosities and Zeta potentials of each polymer solution at the final concentration in the polymer-mixed DNA hydrogel were measured using a viscometer (RheoSense, Micro VISC, San Ramon, CA, USA) and a Zetasizer (ZEN3600, Malvern Instruments, Worcestershire, UK), respectively. The polymer solutions were mixed with sHexa-1 or nsHexa-1 in a BD ™ self-contained U-100 29G syringe (Becton Dickinson, San Jose, CA, USA), followed by mixing with sHexa-2 or nsHexa-2, respectively. Next DNA hydrogels mixed with chitosan glutamate (Chitosan-DNA hydrogel), polyethylene glycol (PEG-DNA hydrogel) or sodium alginate (Alginate-DNA hydrogel) were obtained. The concentration of polymers in the DNA hydrogel was set at 7 or 15 μg/μl, which was the maximum concentration of the polymers under the current preparation conditions where the DNA concentration was 22 or 7 μg/μl, respectively. Hydrogel formation was confirmed by 6% polyacrylamide gel electrophoresis as previously reported (Nishikawa et al., 2014).

2.4. Preparation of DNA hydrogel and chitosan-DNA hydrogel loaded with OVA

OVA was mixed with sHexa-1 and sHexa-2 to obtain the OVA-loaded sDNA hydrogel (OVA/sDNA hydrogel). To prepare the OVA-loaded Chitosan-sDNA hydrogel (OVA/Chitosan-sDNA hydrogel), OVA was added to sHexa-1, followed by mixing with chitosan glutamate solution and sHexa-2 in a 29-gauge syringe. The concentration of OVA was set at 2.2 μg OVA/μl through all the experiments. Hydrogel formation was confirmed by 6% polyacrylamide gel electrophoresis as previously reported (Nishikawa et al., 2014).

2.5. DNA release from DNA hydrogel in vitro

sDNA hydrogel, Chitosan-sDNA hydrogel, PEG-sDNA hydrogel, and Alginate-sDNA hydrogel were prepared at concentrations of 22 μg DNA/μl and 7 μg polymer/μl (polymer to DNA ratio of 0.3), or 7 μg DNA/μl and 15 μg polymer/μl (polymer to DNA ratio of 2). Then, 10 μl of the preparations were placed into the upper chamber of a Transwell (Product#3460, 0.4-μm pore size; Corning Inc., Corning, NY, USA), and 1000 μl of phosphate buffered saline (PBS) was added into the bottom chamber, followed by incubation at 37 °C.