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Effective polymer adjuvants for sustained delivery of protein subunit vaccines

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ABSTRACT

We have synthesized thermogelling cationic amphiphilic pentablock copolymers that have the potential to act as injectable vaccine carriers and adjuvants that can simultaneously provide sustained delivery and enhance the immunogenicity of released antigen. While these pentablock copolymers have shown efficacy in DNA delivery in past studies, the ability to deliver both DNA and protein for subunit vaccines using the same polymeric carrier can provide greater flexibility and efficacy. We demonstrate the ability of these pentablock copolymers, and the parent triblock Pluronic copolymers to slowly release structurally intact and antigenically stable protein antigens in vitro, create an antigen depot through long-term injection-site persistence and enhance the in vivo immune response to these antigens. We show release of the model protein antigen ovalbumin in vitro from the thermogelling block copolymers with the primary, secondary and tertiary structures of the released protein unchanged compared to the native protein, and its antigenicity preserved upon release. The block copolymers form a gel at physiological temperatures that serves as an antigenic depot and persists in vivo at the site of injection for over 50 days. The pentablock copolymers show a significant fivefold enhancement in the immune response compared to soluble protein alone, even 6 weeks after the administration, based on measurement of antibody titers. These results demonstrate the potential of these block copolymers hydrogels to persist for several weeks and sustain the release of antigen with minimal effects on protein stability and antigenicity; and their ability to be used simultaneously as a sustained delivery device as well as a subunit vaccine adjuvant platform.

1. Introduction

Much recent work in vaccine design has focused on the delivery of pathogenic subunits such as proteins or DNA in place of a live, attenuated or killed organism. Such subunit vaccines are typically safer than live or attenuated viruses, but need an adjuvant to boost the immune response [1]. Recently, synthetic polymeric adjuvants have been explored as they have the advantage of serving simultaneously as adjuvants as well as sustained delivery devices for release of the antigen, without some of the potential toxicity concerns associated with microorganism-derived adjuvants [1]. The need for the development of a vaccine delivery system where release kinetics can be controlled in vivo is implied by empirical evidence that suggests that sustained delivery may be a factor in adjuvanticity [2–4].

Amphiphilic block copolymers that spontaneously gel at physiological temperatures have great potential as injectable sustained delivery systems [5]. The phase behavior of Pluronics that contain a central hydrophobic block of polyoxypropylene (POP), flanked by two hydrophilic blocks of polyoxyethylene (POE), and their responsiveness to changes in solution conditions have been widely studied [6–8]. At high concentrations, Pluronics exhibit a thermoreversible gel phase at physiological temperatures caused by the association of micelles [7]. We have synthesized pH-dependent pentablock copolymers based on Pluronics and poly(diethylaminoethylmethacrylate) (PDEAEM) blocks, with tertiary amine groups in their outer cationic blocks, that retain the thermogelling characteristics of the poloxamers while also providing pH as an additional tuning mechanism for controlling release [7]. The release rates from these gels can be controlled by changing the concentration of polymer or the surrounding pH. An increase in block copolymer concentration leads to a decrease in release rates [9]. The pentablock copolymers also exhibit slower release rates than the Pluronics and the release rate can be potentially controlled by varying the length of the cationic blocks [10]. This may have implications for

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vaccine design by providing a controlled release system for the antigen.

In addition to serving as a delivery vector, these block copolymer hydrogels can serve as vaccine adjuvants by enhancing the immunogenicity of the antigen. A suspected mechanism of adjuvanticity of conventional adjuvants, such as incomplete Freund adjuvant, is the depot effect, where antigen release is sustained over a period of several days or weeks from the injection site [11]. A known property of these block copolymer hydrogels is their ability to slowly release their payloads through the disassociation of aggregated micelles. Furthermore, hydrophobic portions of polymers, such as the central hydrophobic POP blocks in block copolymers, are thought to interact with pattern recognition receptors that comprise innate immunity [12]. We have also successfully functionalized the bromo-terminated polyacrylate end blocks with mannose, which has the potential to increase activation of antigen presenting cells [13]. In this study we use the model antigen ovalbumin (OVA), which is one of the most widely used protein for vaccine studies [14]. The benefits of using the ovalbumin model include well-established immunological assays, cross-platform comparison with other antigen delivery systems and a relatively low cost compared to the use of more medically relevant proteins [15–17]. There are a number of in vitro and in vivo vaccine models focused on this protein, including fluorescently labeled protein, OVA-specific tetramers and a mouse model with OVA-specific T cells [18–20]. Future work will focus on transitioning to medically relevant proteins, such as the hemagglutinin protein of the highly pathogenic avian influenza H5N1 virus. To investigate this adjuvant effect of block copolymers, we have investigated the ability of the block copolymers to enhance antigenicity by measuring the humoral immune response of mice immunized against OVA.

The development of polymers that offer sustained released has added additional challenges, such as the risk of degradation of the payload days to months after administration from the prolonged exposure to the polymeric delivery vector [21]. The cationic blocks of the pentablock copolymers form complexes with DNA and enable efficient DNA delivery [22–24]. The same polymers can also be used for simultaneous delivery of proteins to form multifunctional delivery platforms for subunit vaccines. However, these proteins are highly intricate structures that must maintain integrity in order to properly function [21]. Proteins are exposed to multiple stresses during the entrapment or encapsulation process, including elevated temperatures, vigorous agitation and exposure to organic solvents or acidic environments [21,25]. In addition to manufacturing methods, proteins are also susceptible to degradation after administration caused by changes in the microenvironment from polymer degradation products [21]. Taken together, these data suggest that antigen stability is an important consideration when constructing a subunit vaccine.

Our research group has previously demonstrated a novel method for synthesis of the pentablock copolymer family, using cuprous oxide nanoparticles as an easily removable catalyst [26]. This method reduces the cytotoxicity of the polymer, providing an advantage for potential in vivo biomedical applications. Additionally, we have shown the ability to easily functionalize the bromo-terminated polyacrylate end blocks with mannose, which has the potential to increase the activation of immune cells, such as dendritic cells and macrophages, through increased pattern recognition receptor recognition [13]. Based on these promising results, we now focus on evaluating the potential of the pentablock copolymer platform as a delivery system and adjuvant for subunit vaccine formulations based on proteins. It has previously been shown that the cationic end blocks of the pentablock copolymer can complex with DNA for nuclear delivery [23,24,27]. Here, we investigate the potential for the delivery of protein in subunit vaccine formulations, possibly leading to gene and protein co-delivery with the polymer.

The work here investigates in detail the preservation of structure and function of protein released in vitro from the pentablock copolymer vaccine platform. This allows us to build on our earlier work in using these pentablock copolymers for DNA delivery [24,28] and developing a unique injectable polymer carrier that potentially can be used for the simultaneous delivery of both DNA and protein for subunit vaccines, for maximum efficacy. Protein was released from the thermogelling block copolymers through polyethylene glycol diacrylate barrier gels to mimic an in vivo tissue environment, and protein primary, secondary and tertiary structures were examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), circular dichroism and ultraviolet–visible (UV–vis) spectroscopy, respectively. The antigenicity of the released protein was determined using an enzyme-linked immunosorbent assay (ELISA) to measure the ability of released OVA to be recognized by a monoclonal antibody. To investigate the persistence of these formulations in vivo, mice were administered fluorescently labeled block copolymer hydrogels and its persistence was analyzed using a live animal imaging system. Taken together, the following studies investigate the ability of the pentablock copolymer system to serve as a new ideal vaccine adjuvant by providing sustained delivery of the protein antigen release with preservation of its structure and function, and its abilities to persist in vivo and to enhance the immune response to the antigen.

2. Materials and methods

2.1. Materials

DEAEM, polyvinylpyrrolidone (M_n = 40,000), Pluronic F127 (M_n = 12,600, 70% poly(ethylene oxide)), OVA protein (44 kDa) and antibodies were purchased from Sigma–Aldrich (St. Louis, MO). Slide-A-Lyzer™ dialysis cassettes and Micro BCA protein assay kits were purchased from Pierces Biotechnology (Rockford, IL). Poly(ethylene glycol) diacrylate (M_w = 4000) was purchased from Polysciences Inc. (Warrington, PA). Pluronic F127 and Irgacure 2959 photointiator were purchased from BASF (Florham Park, NJ). Alexa Fluor® 647 alkyne was obtained from Life Technologies (Grand Island, NY). All other materials, including Spin-X UF™ concentrators and 6-well plate Transwell® microplate membrane inserts, were purchased from Fisher Scientific (Pittsburgh, PA). The syntheses of N-propyl-pyridynyl methanimine (NPPM) from 1-propylamine and 2-pyridinecarbaldehyde and of cuprous oxide nanoparticles from copper acetate, sodium borohydride and polyvinylpyrrolidone have been described previously [26,29,30]

2.2. Pentablock copolymer synthesis

The synthesis of PDEAEM pentablock copolymers with cuprous oxide nanoparticles has been reported previously [26]. Briefly, a solution of cuprous oxide nanoparticles (0.24 g, 1.68 mmol) in 50 ml of toluene was briefly sonicated in a round-bottom flask. Pluronic macroinitiator (10 g, 0.78 mmol) was added before a rubber stopper was securely fastened with a cable tie. The reaction flask was degassed by vacuum/argon three times to remove air. The NPPM ligand (0.5 ml, 3.40 mmol) and DEAEM monomer (4 ml, 2.53 mmol) were injected into the reaction flask and several freeze–pump–thaw cycles with liquid nitrogen were used to further remove oxygen. The reaction was carried out inside of a water bath at 70 °C and 300 rpm (PMC 720 Series DataPlate digital hot plate). After 20 h the reacted product was passed through a column