Cleavable conjugation of CpG oligodeoxynucleotides onto microparticles for facile release and cytokine induction in macrophages

Hyo-Eun Jang1 · Heejung Jung1 · Hyejung Mok1

Received: 3 March 2017 / Accepted: 15 May 2017 / Published online: 23 May 2017 © The Korean Society for Applied Biological Chemistry 2017

Abstract The development of biomaterials for efficient intracellular delivery of the nucleic acid-based immune-stimulating molecule, CpG oligodeoxynucleotides (CpG), is crucial for their biological activity. In this study, we successfully fabricated polydopamine-coated porous poly(lactic-co-glycolic acid) microparticles (PPM) for the delivery of CpGs. After conjugation of CpGs to PPMs via cleavable disulfide linkages, CpGs were readily released from CpG-conjugated PPM (PPM-s–s-CpGs) in reductive conditions. Released CpGs exhibited significantly enhanced induction of two cytokines, TNF-α and IL-6, in RAW264.7 cells. CpC-conjugated PPMs showed negligible cytokine induction, whereas CpG-conjugated PPMs exhibited strong induction of the two cytokines in RAW264.7 cells. The PPM-s–s-CpGs can serve as immune-stimulating adjuvants to enhance the immune responses of vaccines.

Keywords Cleavable linkage · CpG oligodeoxynucleotides · Cytokine · Delivery · Porous microparticle

Introduction

Pathogens are detected by host cells via the interactions between biomolecules on the pathogen and pathogen recognition receptors (PRRs), e.g., toll-like receptors (TLRs), on cellular surfaces or intracellular regions of the host cell, which trigger signal transduction pathways and induce local and systemic immune response. Biomolecules of pathogens that are recognized by PRRs are known as pathogen-associated molecular patterns (PAMPs), e.g., host gene product, host-specific protein, and lipopolysaccharides (LPS) [1]. Recently, PAMPs have been popularly investigated as adjuvant materials [2, 3]. The administration of adjuvants with antigens has several advantages, including the induction of immune responses with low doses of antigens, rapid immune response, and enhanced cytotoxic T cell response [4]. Currently, several types of adjuvants, including monophosphoryl lipid A (MPLA), double-stranded RNA, and CpG oligodeoxynucleotides (CpG), have already been under clinical trial [5]. For example, polyriboinosinic:polyribocytidylic acid (poly I:C), a double-stranded RNA, induced increased interleukin-12 production, elevated maturation of dendritic cells, and enhanced memory T cell response by T helper cell 1 and cytotoxic T lymphocytes in an antigen-specific manner [6, 7]. CpG has also been considered as biocompatible adjuvants [8]. While mammalian CpG sequences with methylation have poor binding capacity with TLR-9, bacterial CpG sequences without methylation bind TLR-9 and induce strong immune responses [9–11]. Single-stranded DNA with CpG sequences binds to TLR-9 in endosomes after cellular uptake, which triggers the NF-κB pathway, cytokine induction, and activation of antigen-presenting cells [12]. Currently, several CpGs are under clinical trial [5, 13]. However, CpGs have high molecular weight and negative charge, which hinder endosomal localization for binding to TLR-9. In addition, enzymatic degradation can be an obstacle for the successful in vivo treatment with CpGs. To overcome these challenges,
different types of biomaterials based on lipids/polymers have been designed to protect CpGs from DNase, improve circulation time, and elevate uptake in target cells [8, 14–16].

Poly(lactic-co-glycolic acid) (PLGA), a biodegradable polymer approved by the Food and Drug Administration, has been conventionally used for particle formulation [17, 18]. In our previous study, porous and microsized particles formulated using PLGA via water-in-oil-in-water (W1/O/W2) double emulsion and solvent evaporation processes were coated with polydopamine to conjugate and deliver CpGs via non-cleavable linkages [19]. However, stimuli-responsive behavior and cleavable linkage have not been investigated for the delivery of CpGs using polydopamine-coated porous microparticles (PPMs).

In this study, CpGs were conjugated via cleavable disulfide linkages onto PPMs (PPM-s–s-CpG) for facile interactions with TLR-9. The particle size and morphology of the prepared PPM-s–s-CpG were examined by scanning electron microscopy (SEM). The release profiles of CpGs from PPM-s–s-CpGs were investigated in the presence of a reducing agent, dithiothreitol (DTT). The release of two cytokines, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), by PPM-s–s-CpGs was quantitatively evaluated by enzyme-linked immunosorbent assay (ELISA) after transfection of RAW264.7 cells.

Materials and methods

Materials

Poly(lactic-co-glycolic acid) (PLGA, RG503H) was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany). Branched polyethyleneimine (BPEI, 25 kDa), polyvinyl alcohol (PVA, 13–23 kDa), dopamine-HCl (189.64 Da), cysteamine (77.15 Da), and heparin sodium salt (powder) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Amine-modified CpG (CpG–NH2 1826: 5′-TCCATGACGTTCCTGACGTT-3′) and GpC (GpC–NH2, 5′-TCCATGACGTTCCTGACGTT-3′) at the 3′-end were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amine-modified CpG (CpG–NH2 1826: 5′-TCCATGACGTTCCTGACGTT-3′) and GpC (GpC–NH2, 5′-TCCATGACGTTCCTGACGTT-3′) at the 3′-end were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amine-modified CpG (CpG–NH2 1826: 5′-TCCATGACGTTCCTGACGTT-3′) and GpC (GpC–NH2, 5′-TCCATGACGTTCCTGACGTT-3′) at the 3′-end were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amine-modified CpG (CpG–NH2 1826: 5′-TCCATGACGTTCCTGACGTT-3′) and GpC (GpC–NH2, 5′-TCCATGACGTTCCTGACGTT-3′) at the 3′-end were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amine-modified CpG (CpG–NH2 1826: 5′-TCCATGACGTTCCTGACGTT-3′) and GpC (GpC–NH2, 5′-TCCATGACGTTCCTGACGTT-3′) at the 3′-end were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amine-modified CpG (CpG–NH2 1826: 5′-TCCATGACGTTCCTGACGTT-3′) and GpC (GpC–NH2, 5′-TCCATGACGTTCCTGACGTT-3′) at the 3′-end were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amine-modified CpG (CpG–NH2 1826: 5′-TCCATGACGTTCCTGACGTT-3′) and GpC (GpC–NH2, 5′-TCCATGACGTTCCTGACGTT-3′) at the 3′-end were obtained from Sigma-Aldrich (St. Louis, MO, USA). OligoGreen® ssDNA Quantitation Assay Kit was purchased from Molecular Probes (Eugene, OR, USA). Mouse IL-6 and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from BD Biosciences (Franklin Lake, NJ, USA). Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin (P/S), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). All other chemicals were of analytical grade.

Preparation of PPM-s–s-CpG

Porous spherical PLGA microspheres were formulated according to our previous study [14]. BPEI (2 mg) in 50 μL of deionized water (W1) was first dispersed in 400 μL of methylene chloride containing PLGA (20 mg) via brief sonication for 1 min, using a tip sonicator (Branson Digital Sonifier® 450, Danbury, CT, USA). The primary W1/O emulsion solution was then added drop-wise to 8 mL of aqueous solution (W2) containing 1% PVA before sonication for 150 s. Hardened microspheres via overnight stirring were centrifuged at 10,000 rpm for 10 min, resuspended in deionized water (DW) at a polymer concentration of 1 mg/mL, centrifuged for washing three times with DW, and freeze-dried. For polydopamine coating, dopamine-HCl (300 μg) in DW was added to Tris buffer solution (10 mM Tris–HCl, pH 8.5) containing dried porous PLGA microspheres (300 μg) and mixed by vigorous stirring for 3 h as previously described [20]. The resulting polydopamine-coated and PPMs were purified via centrifugation at 10,000 rpm for 10 min to remove free dopamine.

For the preparation of cleavable CpG oligodeoxynucleotides, 3′-amine-modified CpG (CpG–NH2, 31.2 μg) or 3′-amine-modified GpC (GpC–NH2, 31.2 μg) in phosphate buffered solution (PBS pH 8.0) was mixed with SPDP (156 μg) in dimethyl sulfoxide and stirred overnight at 200 rpm at a molar ratio of oligodeoxynucleotides:SPDP = 1:100. Excessive SPDP was eliminated via dialysis (molecular weight cutoff, MWCO = 3.5 kDa) with DW at room temperature for 1 day. The purified reactant in PBS (pH 8.0) was mixed with cysteamine (38.6 μg) in dimethylformamide and stirred overnight at 200 rpm at a molar ratio of feed oligodeoxynucleotides:-cysteamine = 1:100. The resulting CpG and GpC with disulfide linker (CpG–s–s-NH2 and GpC–s–s-NH2) were purified via dialysis (MWCO = 3.5 kDa) with DW at room temperature for 1 day and freeze-dried.

For the preparation of CpG-conjugated PPMs via cleavable disulfide linkage (PPM-s–s-CpG), PPM (100 μg) in Tris buffer (10 mM, pH 8.5) was mixed with CpG–s–s-NH2 (200 μg) in Tris buffer (10 mM, pH 8.5) and stirred overnight. After incubation, the solution was centrifuged (10,000 rpm, 5 min, 4 °C) to remove unbound CpG–s–s-NH2 and washed with DW and 1 μg/mL heparin solution (100 μL). PPM-s–s-CpGs and non-cleavable PPM–CpG were also prepared using GpC–s–s-NH2 and CpG–NH2 by the same protocol, respectively. PPMs and PPM-s–s-CpG were observed by SEM (Hitachi S-4800, Tokyo, Japan). Dried microparticles on silicon were vacuum-coated with a
thin layer of gold particles. The average size of different microspheres was determined by measuring the diameter of the microspheres.

Time-dependent release profile of PPM-s–s-CpG

PPM–CpG and PPM-s–s-CpG were incubated in the presence of DTT for 10 min. Released CpGs were loaded onto a 15% polyacrylamide gel for polyacrylamide gel electrophoresis (PAGE). To inhibit ionic interactions between the released CpGs and PPMs, heparin (32 μg) in PBS solution was added before gel electrophoresis. To visualize DNA in gels, ethidium bromide was used for staining. The released CpGs were quantitatively measured after the two types of particles, PPM–CpG and PPM-s–s-CpG, and were incubated for predetermined time intervals (0, 0.5, 1, 3, and 5 h) in the presence of DTT (200 mM) in PBS (pH 8.0). After purification with a desalting column (7 kDa), the amount of released CpGs was measured by OliGreen assay according to the manufacturer’s protocol.

Cytokine release

RAW264.7 cells (murine macrophage cell line) were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. RAW264.7 cells were plated in 24-well plates at a density of 8 × 10⁴ cells/well 24 h prior to treatment. PPM-s–s-CpGs and PPM-s–s-GpCs were incubated with cells for 8 h at a final CpG concentration of 3 μg/mL. Then, the medium was replaced with fresh culture medium containing serum proteins, and cells were further incubated for 16 h. The levels of cytokines (IL-6 and TNF-α) released from the cells were quantified by ELISA. Data in this study represent mean values of independent measurements, and error bars represent standard deviations.

Results and discussion

Preparation of PPM-s–s-CpG

In this study, amine-modified cleavable CpG (CpG-s–s-NH₂) was used for covalent conjugation onto PLGA microparticles for the delivery of the immune-stimulating biomolecule, CpG, via phagocytosis into macrophages, as shown in Fig. 1A. In particular, cleavable disulfide bonds were incorporated into CpGs for facile release from microparticles after intracellular uptake. CpG-NH₂ was reacted with SPDP to produce pyridyl-dithiol-activated CpGs. By treatment with excessive cysteamine, pyridine 2-thione was released and CpG-s–s-NH₂ was obtained. To provide sufficient surface area for the conjugation of CpG, porous structures were fabricated using bPEI as porogens, which allowed high loading capacity in our previous study [19]. For facile chemical conjugation of CpG-s–s-NH₂ onto PLGA microparticles, dopamine polymerization was utilized [21]. The amine groups of CpGs formed covalent bonds with PPMs via Michael-type addition and Schiff base reaction [22]. Figure 1B shows the intracellular processes of PPM-s–s-CpGs. After intracellular uptake of PPM-s–s-CpGs via phagocytic pathways, phagosomal fusion with lysosomes resulted in an enzyme-rich environment, e.g., DNase 2. In addition, the intracellular environment exhibited reductive conditions, which are desirable for the release of CpG from the PPMs via the cleavage of disulfide bonds. Released CpG might freely interact with TLR-9, which triggers intracellular pathways related to immune responses. Figure 2 shows the SEM images of PPM and PPM-s–s-CpGs. As previously reported, porous and spherical microparticles with a diameter of around 2 μm were successfully prepared. After the conjugation of CpGs, the porous structure of PPM-s–s-CpG remained similar to that of PPMs.

Time-dependent release profile of PPM-s–s-CpG

To examine whether CpGs could be released from microparticles in reductive conditions, CpG-conjugated microparticles with non-cleavable and cleavable linkages, PPM–CpG and PPM-s–s-CpG, were incubated in PBS with DTT. After 10 min of incubation, each sample was loaded for PAGE. Figure 3A shows the migration of CpGs released from PPMs. While negligible amounts of CpGs were released from both types of microparticles in the absence of a reducing agent, CpGs were successfully released only from PPM-s–s-CpG in the presence of DTT. To investigate the time-dependent release profiles, the released CpGs were quantitatively assessed by OliGreen assay after incubation in the presence of DTT for predetermined times, as shown in Fig. 3B. After 5 h of incubation, 17.0 ± 2.0 and 39.7 ± 7.0 ng of CpGs were released from PPM–CpG and PPM-s–s-CpG, respectively. Considering that the total amounts of CpGs per PPM–CpG and PPM-s–s-CpG were 113.2 and 98.9 ng, respectively, 15.0 and 40.1% of CpGs were released from PPM–CpG and PPM-s–s-CpG after 5 h of incubation. Approximately 2.3-fold higher amount of CpGs was released from PPM-s–s-CpG compared to that released from PPM–CpG, which indicates the facile release of CpGs from PPM-s–s-CpGs by disulfide linkage. According to a previous study, glutathione is involved in the reduction in thiol compounds in
the phagolysosomes of macrophages [23]. It is likely that CpG could be released from PPM-s-s-CpGs via glutathione-mediated reduction in macrophages like RAW264.7 cells.

RAW264.7 cells were treated with PPM-s-s-CpGs for 8 h to examine the released levels of cytokines by ELISA. Figure 4 exhibits the quantitative analysis of two cytokines, TNF-α and IL-6. Figure 4A shows that the amounts of TNF-α triggered by CpG, PPM-s-s-CpGs, and PPM-s-s-GpCs were 548.1 ± 30.1, 3205.5 ± 112.5, and 17.6 ± 10.0 pg/mL, respectively. An 11.7-fold higher induction of TNF-α was observed in cells treated with PPM-s-s-CpGs compared with that in cells treated with free CpG. In addition, noticeable induction of TNF-α was not observed in cells treated with PPM-s-s-GpCs, which suggests that specific cytokine induction is triggered by CpG-mediated pathways. Figure 4B indicates the release of IL-6 induced by PPM-s-s-CpGs. The amount of IL-6 induced by PPM-s-s-CpGs was 13 and 4.8 times higher
than those induced by free CpG and PPM-s-s-GpCs, respectively. Taken together, PPM-s-s-GpCs showed negligible cytokine induction, while PPM-s-s-CpGs exhibited strong induction of TNF-α and IL-6 in RAW264.7 cells. It should be noted that direct interaction of CpG with TLR-9 seems to be crucial for TLR-9 dimerization and following activation according to previous studies [1, 24, 25]. Thus, facile release of CpGs attached onto porous microparticles via cleavable linkage could provide not only high loading efficiency due to sufficient surface area but also favorable interaction with TLR-9 in phagosome. CpGs have elicited several cytokines including TNF-α, IL-12 and IL-6 for macrophages. Expression level of each cytokine has been known to be closely related via intracellular NF-κB pathway [26]. Although only representative two types of cytokines, TNF-α and IL-6, were assessed for macrophages in this study, evaluation study of PPM-s-s-CpGs could be examined using other types of cytokines, e.g., IL-12 and antigen-presenting cells, e.g., dendritic cells in our next study.

In this study, we successfully fabricated CpG-conjugated polydopamine-coated porous PLGA microparticles via cleavable disulfide linkages. CpGs were readily released from PPM-s-s-CpGs in reductive conditions. The released CpGs exhibited significantly enhanced induction of TNF-α and IL-6 in RAW264.7 cells. The PPM-s-s-CpGs can serve as immune-stimulating adjuvants to enhance the immune response of vaccines.

**Acknowledgments** This paper was written as part of Konkuk University’s research support program for its faculty on sabbatical leave in 2017.

**References**

1. Iwasaki A, Medzhitov R (2004) Toll-like receptor control of the adaptive immune responses. Nat Immunol 5(10):987–995
2. Miyaji EN, Carvalho E, Oliveira ML, Raw I, Ho PL (2011) Trends in adjuvant development for vaccines: DAMPs and PAMPs as potential new adjuvants. Braz J Med Biol Res 44(6):500–513
3. Nojiri K, Sugimoto K, Shiraki K, Tameda M, Inagaki Y, Kusagawa S, Ogura S, Tanaka J, Yoneda M, Yamamoto N, Okano H, Takei Y, Ito M, Kasai C, Inoue H, Takase K (2013) The expression and function of toll-like receptors 3 and 9 in human colon carcinoma. Oncol Rep 29(5):1737–1743
4. Petrovsky N, Aguilar JC (2004) Vaccine adjuvants: current state and future trends. Immunol Cell Biol 82(5):488–496
5. Hennessy EJ, Parker AE, O’Neill LA (2010) Targeting toll-like receptors: emerging therapeutics? Nat Rev Drug Discov 9(4):293–307
6. Adams M, Navabi H, Croston D, Coleman S, Tabi Z, Clayton A, Jasani B, Mason MD (2005) The rationale for combined chemo/immunotherapy using a toll-like receptor 3 (TLR3) agonist and tumour-derived exosomes in advanced ovarian cancer. Vaccine 23(17–18):2374–2378
7. Hafner AM, Corthesy B, Merkle HP (2013) Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant. Adv Drug Deliv Rev 65(10):1386–1399
8. Hanagata N (2012) Structure-dependent immunostimulatory effect of CpG oligodeoxynucleotides and their delivery system. Int J Nanomed. doi:10.2147/IJN.S301977
9. Ohno U, Shibata T, Tanji H, Ishida H, Krayukhina E, Uchiyama S, Miyake K, Shimizu T (2015) Structural basis of CpG and inhibitory DNA recognition by toll-like receptor 9. Nature 520(7549):702–705
10. Rutz M, Metzger J, Gellert T, Lupp P, Lipford GB, Wagner H, Bauer S (2004) Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. Eur J Immunol 34(9):2541–2550
11. Vollmer J, Krieg AM (2009) Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. Adv Drug Deliv Rev 61(3):195–204
12. Klinman DM (2004) Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat Rev Immunol 4(4):249–258
13. Karbach J, Neumann A, Wahle C, Brand K, Gnjatic S, Jager E (2012) Therapeutic administration of a synthetic CpG oligodeoxynucleotide triggers formation of anti-CpG antibodies. Cancer Res 72(17):4304–4310
14. Jang HE, Mok H (2016) Polydopamine-coated porous microspheres conjugated with immune stimulators for enhanced cytokine induction in macrophages. Macromol Biosci 16(11):1562–1569
15. Li J, Pei H, Zhu B, Liang L, Wei M, He Y, Chen N, Li D, Huang Q, Fan C (2011) Self-assembled multivalent DNA nanostructures for noninvasive intracellular delivery of immunostimulatory CpG oligonucleotides. ACS Nano 5(11):8783–8789
16. Liu X, Xu Y, Yu T, Clifford C, Liu Y, Yan H, Chang Y (2012) A DNA nanostructure platform for directed assembly of synthetic vaccines. Nano Lett 12(8):4254–4259
17. Houchin ML, Topp EM (2008) Chemical degradation of peptides and proteins in PLGA: a review of reactions and mechanisms. J Pharm Sci 97(7):2395–2404
18. Kloese D, Siepmann F, Willart JF, Descamps M, Siepmann J (2010) Drug release from PLGA-based microparticles: effects of the “microparticle: bulk fluid” ratio. Int J Pharm 383(1–2):123–131
19. Gupta V, Ahsan F (2011) Influence of PEI as a core modifying agent on PLGA microspheres of PGE(1), a pulmonary selective vasodilator. Int J Pharm 413(1–2):51–62
20. Zhou P, Deng Y, Lyu B, Zhang R, Zhang H, Ma H, Lyu Y, Wei S (2014) Rapidly-deposited polydopamine coating via high temperature and vigorous stirring: formation, characterization and biofunctional evaluation. PLoS ONE 9(11):e113087
21. Yang K, Lee JS, Kim J, Lee YB, Shin H, Um SH, Kim JB, Park KI, Lee H, Cho SW (2012) Polydopamine-mediated surface modification of scaffold materials for human neural stem cell engineering. Biomaterials 33(29):6952–6964
22. Chen S, Cao Y, Feng J (2014) Polydopamine as an efficient and robust platform to functionalize carbon fiber for high-performance polymer composites. ACS Appl Mater Interfaces 6:349–356
23. Frezard F, Demicheli C, Ferreira CS, Costa MA (2001) Glutathione-induced conversion of pentavalent antimony to trivalent antimony in meglumine antimoniate. Antimicrob Agents Chemother 45(3):913–916
24. Akira S, Takeda K (2004) Toll-like receptor signalling. Nat Rev Immunol 4(7):499–511
25. Scheiermann J, Klinman DM (2014) Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. Vaccine 32(48):6377–6389
26. Hartmann G, Krieg AM (1999) CpG DNA and LPS induce distinct patterns of activation in human monocytes. Gene Ther 6(5):893–903