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Induction of a balanced Th1/Th2 immune responses by co-delivery of PLGA/ovalbumin nanospheres and CpG ODNs/PEI-SWCNT nanoparticles as TLR9 agonist in BALB/c mice

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ABSTRACT

To develop effective and safe vaccines with reduced dose of antigen and adjuvant, intelligent delivery systems are required. Many delivery systems have been developed to enhance the biological activity of cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG ODN) as both immunotherapeutic agents and vaccine adjuvants. In this study we designed a novel CpG ODN delivery system based on single-walled carbon nanotube (SWCNT) functionalized with polyethylenimine (PEI) and alkylcarboxylated PEI (AL-PEI). The physicochemical characteristics, cytotoxicity and cellular uptake studies of these carriers were performed.

All carriers were conjugated with CpG ODN followed by co-delivery with ovalbumin (OVA) encapsulated into poly (lactic-co-glycolic acid) nanospheres (PLGA NSs) to enhance the induction of immune responses. The effect of these formulations on antibody (IgG1, IgG2a) and cytokine (IL-1β, IFN-γ, IL-4) production was evaluated in an *in vivo* experiment. The results showed that all nano-adjuvant formulations had a strong influence in up-regulation of IFN-γ and IL-4 in parallel with high IgG1-IgG2a isotype antibody titers in mice. In particular, SWCNT-AL-PEI nano-adjuvant formulation generated a balanced Th1 and Th2 immune response with more biased toward Th1 response without exhibiting any inflammatory and toxic effects. Therefore this nano-adjuvant formulation could be used as an efficient prophylactic immune responses agent.

1. Introduction

Toll like receptors (TLRs) are the main sensors of innate immunity and their agonists could be formulated as adjuvant platforms both in combination with other components or alone for establishing an effective immune response after vaccination studies. They are located both on plasma membrane (TLR 1, 2, 4, 5, 6) and endosomal compartment (TLR 3, 7, 9) of cells (Haghparast et al., 2016). Cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG ODNs) as TLR9 agonist are short single-stranded synthetic DNA molecule agents which have been used for treatment and prevention of a wide variety of diseases as immunotherapeutic and immunoprophylactic agents. The sequence of this oligonucleotide is similar to those found in bacterial DNA which is recognized by TLR9, inducing innate as well as cell-mediated immune responses (Alignani et al., 2005; Bode et al., 2011). In contrast to anti-sense therapeutics, the effects of CpG ODN can last for an extended period of time even after a brief exposure (Salem and Weiner, 2009). However, natural CpG ODNs having a phosphodiester backbone possess significant disadvantages comprising fast degradation and insufficient delivery to target specific cells or tissues (Hanagata, 2012).

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Recently a great attention has been paid to enhance the immunostimulatory effect of CpG ODNs by increasing the half-life, cellular uptake, delivery to endolysosome and sustained release of CpG ODNs. Chemical modification and various formulations can be used to achieve these purposes. For example substituting the oxygen with sulphur creates phosphorothioate CpG ODNs which could significantly reduce degradation and increase half-life of this oligonucleotide (Brown et al., 1994). On the other hand, free CpG ODNs bear a negative charge which would not allow for their interaction with negatively charged cell surfaces. Thus, many delivery systems have been developed to enhance the efficient delivery and biological activity of CpG ODNs. Many studies suggested that formulation of CpG ODNs in cationic liposomes were more effective than free CpG ODNs in inducing immune responses (Kesarla et al., 2015; Zhou et al., 2014).

Other formulations used for the delivery of either bare CpG ODNs or antigens include PLGA (San Roman et al., 2014), gold nanoparticles (Chen et al., 2014), liposomal nanoparticles (de Jong et al., 2007), silica (Manoharan et al., 2012), protamine nanoparticles (Pali-Schöll et al., 2013), chitosan (Gun et al., 2013), alginate coated chitosan (Borges et al., 2008), boron nitride nanospheres binded to peptide (Zhang et al., 2012) or functionalized by polyethylenimine (PEI) (Zhang et al., 2015) and carbon nanotubes (Bianco et al., 2005a, 2005b; Gunawardana et al., 2015).

Carbon nanotubes (CNTs) are molecular-scaled tubes in which carbon atoms are arranged in condensed aromatic rings. The solubility of CNTs must be improved before they can be used in biological applications (Mali et al., 2011; Sadegh and Shahryari ghoshekandi, 2015).

It was showed that SWCNTs suspensions complexed with CpG ODN (SWCNT/CpG ODN complexes) in 5% dextrose solution were dispersed individually without the formation of considerable aggregates. Intra-tumoral administration of the SWCNT/CpG ODN complexes in mice could enhance the production of inflammatory cytokines in tumor tissues (Zhou et al., 2014). In another study administration of PEG2000-functionalized CNTs was nontoxic and enhanced CpG uptake both *in vitro* and in intracranial gliomas (Zhao et al., 2011; Zhou et al., 2014). Safety and biodistribution of CNTs in various organs and tissues of mice have been examined paving the way for their biomedical applications (Lacerda et al., 2008; Liu et al., 2008; Yang et al., 2007). Hydrophobic nature of CNTs facilitates the interaction of them with antigens or immunostimulatory agents and help to uptake of vaccine component by immunocompetent cells (Bianco et al., 2007).

Poly (lactic-co-glycolic acid) (PLGA) is a biocompatible and biodegradable polymer possessing adjuvant like properties which can enhance the delivery and uptake of antigen by antigen presenting cells (APCs) and the peripheral lymph nodes. Controlled release of antigen encapsulated in PLGA polymer over a long period of time can effectively activate the co-stimulatory signals for optimal immune responses. This effect mainly depends on their solubility, molecular weight, degree of branching and conformation of the polymeric backbone (Shakya and Nandakumar, 2013).

In the present study, we developed a novel CpG ODNs delivery system based on SWCNTs functionalized with alkyl-modified PEI. It was shown that alkylation of PEI with high buffering capacity and DNA condensation ability could increase its transfection efficiency and reduce cytotoxicity by improving hydrophobic–hydrophilic balance of vector (Bishop et al., 2015; Dehshahri et al., 2009a). SWCNTs were carboxylated and PEI was modified with alkylcarboxylate chain. Then SWCNT-COOH was conjugated to alkylcarboxylate PEI *via* amide bond formations between carboxylic acid of SWCNT and amine of PEI.

In the next step CpG ODN/modified SWCNT-alkylcarboxylate PEI polyplexes were co-delivered with ovalbumin (OVA) encapsulated in PLGA to enhance the induction of immune responses. OVA is an important reference protein for immunization and a model antigen for the characterization of antigen uptake, processing and presentation. Encapsulation of OVA in PLGA nanosphere can protect the antigen from degradation and improve the uptake by antigen presenting cells (APCs) resulting in the enhancement of antigen immunogenicity (Elamanchili et al., 2004).

2. Materials and methods

2.1. Materials

Poly (lactic-co-glycolic acid) (PLGA), 50:50, Resomer® RG 502H, Polyvinyl alcohol (PVA; MW 31,000–50,000), albumin from chicken egg white (OVA) grade VI, single-walled carbon nanotubes (SWCNTs) were purchased from Sigma-Aldrich (Munich, Germany). Branched polyethylenimine (PEI; average MW 10 kDa) was purchased from Polyscience, Inc. (Warrington, USA). 6-bromohexanoic acid was obtained from Sigma–Aldrich (Munich, Germany). Spectra/Por dialysis membranes were purchased from Spectrum Laboratories (Houston, USA). Ethidium bromide was obtained from Cinnagen (Tehran, Iran). N-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropy] carbodiimide hydrochloride (EDC) were purchased from Fluka (Steinheim, Germany). Cell Titer 961 aqueous one solution cell proliferation assay (MTT) was obtained from Promega (Madison, USA). CpG ODN was purchased from Bioneer (Daejeon, Korea). All other reagents were of analytical grade and received from commercial sources.

2.2. Cell line and cell culture medium

J774 (murine macrophages) cell line was purchased from the Pasteur Institute of Iran and cultured at 37 °C, 5% CO_2 atmosphere in DMEM high glucose medium supplemented with 1% L-glutamine (2 mM), 100 μg/ml streptomycin, 100 units/ml penicillin and 10% fetal bovine serum (FBS) (Gibco, NY, USA).

2.3. Synthesis of nanoparticles

2.3.1. Preparation of carboxylated SWCNTs

Full-length SWCNTs (2 mg) were oxidized by adding 2.5 M of HNO_3 solution and then the mixture was subjected to reflux at 95 °C for 24 h under constant stirring. The SWCNT–acid mixture was sonicated for 10 min and the reaction was refluxed again for additional 48 h. The resulting mixture was next filtered using a 200 nm polytetrafluoroethylene filter (PTFE, Millipore) and washed thoroughly with water until a pH 7 was obtained. The resulting filtrate on the top surface of the filter collected and was heated in an oven at 65 °C to evaporate the moisture (Wong et al., 2004).

2.3.2. Synthesis of alkylcarboxylate PEI

Synthesis of alkylcarboxylate PEI was performed according to the previous report (Dehshahri et al., 2009a). Briefly, branched PEI 10 kDa (0.3 g) and 6-bromohexanoic acid (0.1 g) were dissolved separately in 5 ml of dimethylformamide (DMF) and the latter solution was added drop-wise at room temperature to a magnetic stirring PEI solution during 2 h to substitute 30% of the surface primary amines of polyethylenimine (MW10 kDa) with 6-bromohexanoic acid. The reaction was allowed to complete for 24 h at room temperature. The unpurified mixture was dialyzed using 3500 Da cut-off Spectra/Por dialysis tubing against three changes of water to remove unreacted reagents, and was subsequently lyophilized (TAITEC Corporation, Japan) (Dehshahri et al., 2009a). Degrees of substitution were determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay by calculating the remaining amines using the standard method (Snyder and Sobocinski, 1975).