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Poly(lactic-co-glycolic acid) nanoparticles as candidate DNA vaccine carrier for oral immunization of Japanese flounder (*Paralichthys olivaceus*) against lymphocystis disease virus

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ABSTRACT

In order to protect DNA vaccine against degradation in alimentary tract of fish, poly(lactic-co-glycolic acid) (PLGA) nanoparticles encapsulating vaccine were prepared using W/O/W emulsification combined with spray drying technique in our laboratory. The characteristics of PLGA nanoparticles were described as follows: (1) shape, spherical; (2) size, <500 nm; (3) yield, ~96.2%; loading percentage, ~0.5%; encapsulation efficiency, ~63.7%; supercoiled conformation percentage, ~65%; (4) release dynamics, gradual release. *In vitro* transfection in SISK cells showed that PLGA nanoparticles could be utilized to transfect eukaryotes. After oral administration, FITC-labeled PLGA nanoparticles were detected in blood of fish, and RNA containing major capsid protein (*MCP*) gene information existed in various tissues of fish 10–90 days. In addition, the analysis of immune parameters in sera of treatment fish showed that: (1) infection rate of LCDV post-challenge, ~16.7%; (2) prophenoloxidase, superoxide dismutase, respiratory burst, lysozyme and antibody levels, increased significantly ($p < 0.05$); (3) activities of serum complement, changed a little ($p > 0.05$). Pearson's correlation displayed that correlation of immune factors mentioned above (not including serum complement) were all positive for fish vaccinated. The data in this study suggested that PLGA nanoparticles were promising carriers for plasmid DNA vaccine and might be used to vaccinate fish by oral approach.

1. Introduction

Japanese flounder is an economically important fish, and it is cultured extensively in Asian countries such as Japan, Korea and China. However, up to date, the aquaculture of Japanese flounder has been hampered by outbreaks of infectious diseases caused by viruses, bacteria, and parasites, which have resulted in serious economic losses. Lymphocystis disease virus (LCDV) is an infectious virus of fish containing a DNA core. Japanese flounder infected by LCDV develops characteristically hypertrophied cells on skin, fins and mouth, which results in fish to lose commercial value. DNA vaccine is a bacterial plasmid DNA which contains promoter and enhancer sequences, the gene of interest, a poly-adenylation sequence, transcriptional termination sequence, antibiotic resistance gene and origin of replication. In general, DNA vaccine is used to establish specific and long-lasting protective immunity against diseases where conventional vaccines fail to induce protection [1]. Based on recombinant viral structural protein, plasmid vaccines have been developed for some of the fish viral diseases such as infectious pancreatic necrosis virus (IPNV), infectious haemorrhagic septicaemia virus (IHSV) and infectious hematopoietic necrosis virus (IHNV) [2]. The study of Husgaro et al. showed that there was a marked increase in specific antibody response following immunization of recombinant partial capsid protein of striped jack nervous necrosis virus (SJNNV) [3]. The results of de las Heras et al. also indicated that alginate microspheres containing a vector with the capsid *VP2* gene could protect salmonid fish against infectious pancreatic necrosis virus (IPNV), and the expression of IFN was more than 10-fold relative to the fish vaccinated with the empty plasmid at 15 days post-vaccination [4]. Our previous studies displayed that the plasmid containing major capsid protein (*MCP*) gene of LCDV induced a protective immune response in Japanese flounder (*Paralichthys olivaceus*), and the levels of polyclonal antisera increased significantly, suggesting that *MCP* effectively neutralized LCDV and recombinant plasmid vaccine confers fish protection against LCDV post-vaccination [5–7].

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As compared with conventional vaccination strategies, DNA vaccines have lots of advantages. Firstly, DNA vaccines possess intrinsic immunostimulatory capacity due to the presence of CpG motifs [8]. Secondly, DNA vaccines resist multiple diseases by constructing a vector encoding several antigens. Thirdly, DNA vaccines could be manufactured in large amounts and administered to targeted diseases. In addition, available clinical tests also indicate that DNA vaccines rarely integrate and have a low probability of creating a tumorigenic event. With regard to the fate of DNA vaccines in fish after oral administration, not much information has been obtained until recently. The rate of degradation of DNA vaccines remains unknown in fish, indicating that it is difficult to assess the immune periods of the transgene protein generated by mRNA. Due to the existence of nucleases in gastrointestinal alimentary canal of fish, DNA vaccines would be degraded during the transport of vaccine, and successful vaccination should evade or lessen the degradation of vaccines effectively before the internalisation of DNA vaccines.

In order to deliver therapeutic drugs, the polymer system has been extensively studied, and many nano-sized drug delivery systems such as liposomes, polymeric micelles, nanoparticles, dendrimers, and nanocrystals have been developed. Biodegradable PLGA, a kind of polyester composed of three different hydroxyl acid monomers (d-lactic, l-lactic, and glycolic acids), has been successfully employed to design drug delivery systems. The system consists of a hydrophobic shell and a drug-containing core, which can be spontaneously generated in a water-in-oil-in-water (W/O/W) emulsion [9]. Major advantages of PLGA nanoparticles are to prolong drug circulation time, increase drug stability, and as well as escape from the reticuloendothelial system due to their nanometer size [10]. When compared to microparticles, nanoparticles generally have relatively high cell uptake, and they could be utilized to accomplish cellular and intracellular targets due to their small size and mobility. Previous studies showed that the uptake rate of 100 nm nanoparticles was 2.5-fold rate than that of 1 μm microparticles, and 6-fold than that of 10 μm microparticles for the Caco-2 cells [11]. Majority of cell types would take up submicron nanoparticles. When compared to submicron nanoparticles, nanoparticles could penetrate through the submucosal layers of a rat intestinal loop model; microparticles were predominantly localized in the epithelial lining [12].

In previous work, three types of pDNA-loaded microspheres (<10 μm) were prepared by emulsification method, and they were administered by oral approach, respectively, and the protective efficacy have been reported [6,7,13]. In order to investigate oral vaccination of nanoparticles, PLGA nanoparticles (<500 nm) containing plasmid vaccine were formulated by the combinative methods of emulsification and spray-drying technique in this study, and they were utilized to vaccinate fish by oral routes. In comparison with previous study, this article described the imbibition of nanoparticles and immune efficacy of nanoparticles, and as well as the changes of immune parameters which were not performed in previous work. Immune results indicated that fish obtained significant immune efficacy after oral administration with PLGA nanoparticles loaded with DNA vaccine.

2. Method and materials

2.1. Polymers and reagents

Poly(DL-lactide-co-glycolide) (L:G = 75:25, M_W 50 kDa, inherent viscosity 0.88 dl g^{-1}) was obtained from Boehringer Ingelheim, Petersburg, VA, USA. Polyethylene glycol (M_W 4 kDa) was purchased from Polysciences Inc., PA. All other chemicals were of reagent grade.

2.2. Preparation of DNA vaccine

A plasmid DNA (pEGFP-N2-LCDV 0.6 kb) containing *MCP* (ORF 0147 L) gene [14] of LCDV was prepared according to the previous method [13]. The concentration of plasmid DNA was set as 1 mg mL^{-1}.

2.3. Synthesis of PLGA nanoparticles loaded with DNA vaccine

Based on the methods of Tian et al. [13] and Zhang et al. [15] with some modifications, PLGA nanoparticles loaded with DNA vaccine were prepared. Briefly, plasmid DNA solution was put into PLGA solution (dissolved in dichloromethane) to produce the primary emulsion (W_1/O), and W_1/O emulsion was poured into polyethylene glycol solution to form $W_1/O/W_2$ emulsion. Subsequently, $W_1/O/W_2$ emulsion was spray-dried using a spray-dryer (Büchi mini spray-dryer, B-191, Switzerland). Blank nanoparticles were made for comparison.

2.4. Nanoparticle characterization

The morphology and size distribution of PLGA nanoparticles was evaluated using scanning electron microscopy (JEOL 6400, Tokyo, Japan). The yield, encapsulation efficiency and loading percentage were investigated according to the method of Tian et al. [13]. The Picogreen® (Molecular Probes, USA) assay was used to quantify plasmid DNA extracted from nanoparticles, and the detecting operation was performed according to the manufacturer's instructions. Samples were analyzed in black 96-well plates (Fisher Scientific International) and read at excitation 485 nm/emission 535 nm using a Wallac Victor2™ 1420 Multilabel Counter (PerkinElmer Wallac, UK). Based on previous formula [7], yield, loading percentage and encapsulation efficiency were calculated.

The supercoiled DNA was assessed by agarose gel electrophoresis, which was described by the method of Tian et al. with minor modifications [13]. The band of supercoiled DNA retrieved was quantified using the Picogreen® (Molecular Probes, USA) according to the manufacturer's instructions. Percentage of supercoiled DNA was calculated as the ratio of DNA in relation to the other conformations in the sample.

2.5. Release dynamics of plasmid DNA from PLGA nanoparticles

The cumulative release of plasmid DNA and swelling ability of PLGA nanoparticles were performed using the method of Tian et al. [13]. The amount of DNA vaccine released was detected using the Picogreen® (Molecular Probes, USA) at intervals of 6 h. Release profiles were calculated in terms of cumulative release (%) versus incubation time.

2.6. In vitro transfection in sea bass kidney cell line (SISK)

The SISK cells, grown on cover slips, were incubated with PLGA nanoparticles (10 particles $cell^{-1}$) in DMEM supplemented with 10% FBS, 50 U Penicillin, and 2 mM L-glutamine. After 48 h of incubation, transfection solution was completely removed; the cells were washed with sterile PBS and cultured in 1 mL of 10% FBS. To measure reporter gene (GFP) expression, the cover slips were observed under a fluorescence microscope (Carl Zeiss, Germany) at the time point of 48 h.

Based on the previous method [13], LCDV isolation and antibody preparation were accomplished. After transfection, SISK cells were cultured for 120 h using Lipofectamine 2000 (Invitrogen). The culturing mixture was centrifuged at 500 *g* for 5 min, and the pellets were harvested and disrupted in 50 mL Laemmli buffer