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Poly D, L-lactide-co-glycolic acid (PLGA) entrapped chitin on innate and adaptive immune responses in *Epinephelus bruneus* against *Vibrio alginolyticus*

Ramasamy Harikrishnan[1], Chellam Balasundaram[2], Moon-Soo Heo[1,*]

[1]Marine Applied Microbes and Aquatic Organism Disease Control Lab, Department of Aquatic Biomedical Sciences, School of Marine Biomedical Sciences & Marine and Environmental Research Institute, Jeju National University, Jeju 690-756, (SOUTH KOREA)

[2]Department of Animal Science, Bharathidasan University, Tiruchirapalli - 620 024, Tamil Nadu, (INDIA)

E-mail: msheo@jejunu.ac.kr; rhari123@yahoo.com

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ABSTRACT

The efficacy of poly D, L-lactide-co-glycolic acid (PLGA)-entrapped chitin (CI) on innate and adaptive immune response in kelp grouper (*Epinephelus bruneus*) against *Vibrio alginolyticus* was investigated at weeks 1, 2, and 4. The myeloperoxidase (MPO) activity significantly increased in chitin (CI) and PLGA-entrapped chitin (PLGA+CI) on week 4. The serum haemoagglutinating activity and haemolysin activity were significantly enhanced in CT and PLGA+CI on weeks 2 and 4. The respiratory burst (RB) activity and bacterial agglutination activity were increased significantly in chitin (CI) and PLGA-entrapped chitin (PLGA+CI) from weeks 1 to 4. The cumulative mortality was found low in PLGA-entrapped chitin with 15% whereas 30% and 25% in PLGA and chitin. The present results suggest that PLGA-entrapped chitin microsphere act as immunostimulant that enhance the innate and adaptive immune system in kelp grouper against *V. alginolyticus* infection.

KEYWORDS

Chitin;
Epinephelus bruneus;
Immune response;
Poly D, L-lactide-co-glycolic acid (PLGA);
Vibrio alginolyticus.

INTRODUCTION

Groupers are popular and high quality marine sea food in Asian aquaculture and they are also good candidates for intensive aquaculture because of their desirable taste, hardiness, and rapid growth[1,2]. The large size of grouper brooders (30-60 kg per fish) was high market value (=USD 30,000 per fish)[3]. Grouper farming has suffered many diseases including nervous necrosis and sleepy disease[4,5], as well as vibriosis caused by *Vibrio alginolyticus* and *V. carchariae*[6-8]. Traditional disease control strategies employ antibiotics and chemical disinfectants, but these are no longer recommended practices due to the emergence of bacterial resistance, which concerns over environmental impacts and wildlife protection. In this regards vaccinations have been indicated as an effective prophylactic method for disease control in aquaculture[9]. They are some methodological problems insofar as they may be very expensive, stressful for fish as well as pathogen specific[10].

Full Paper

Recently a remarkable success has been achieved with nanoparticles such as PLGA [poly(d,l-lactide-co-glycolide)], chitin, and chitosan in aquaculture; they are good biocompatibility, biodegradability, cellular binding capability, antimicrobial activity, anti-cancer, and wound healing effect[11-14]. In this regards, biodegradable nanoparticles are efficient drug delivery systems for proteins, vaccine or peptides that extensively investigated in the last decades[15-17] which are more stable and elicit both humoral as well as cellular immunity in mammal[18]. PLGA, chitin, and chitosan polymers are already approved by the US Food and Drug Administration (FDA), such as biodegradable structures, implantable screws, pins, drug delivery devices, and tissue engineering scaffolds[19,20]. The natural and synthetic of these polymers are currently being used biomedical application[21,22]. PLGA-based systems are promising candidate for vaccines in that PLGA microspheres may simplify vaccination programs, enhance efficiency[23,24], and increase the immunogenicity of the encapsulated antigen[25]. A novel type of composite microspheres of alginate-chitosan-PLGA, developed recently for improving the stability of encapsulated proteins and increasing the subsequent release drug amount[27].

Chitin is a widespread polysaccharide, poly-β(1?4)-*N*-acetyl-$_D$-glucosamine in nature after cellulose which is an insoluble, non-toxic, biodegradable, and biocompatible polymer found in the exoskeletons of crustaceans and in the cell walls of fungi, insects, and yeast. Although only few study indicate that PLGA, chitin and its derivatives especially chitosan have been shown to possess immunostimulating properties such as the enhances the innate and adaptive immune response in fish and shellfish against diseases[28-40]. Further they can stimulate the nonspecific resistance to *Escherichia coli* infection, suppress tumour growth, show antiviral and anti-*Candida albicans* activities, possess adjuvant properties (enhancing the specific immunity) and stimulate cytokine production[41,42] and, to our knowledge, there was no report have determined the effect of PLGA entrapped with chitin on immune system in fish through intraperitoneal administration. Therefore we investigate for the first time to evaluate PLGA-entrapped chitin on innate and adaptive immune responses in *E. bruneus* against *V. alginolyticus*.

MATERIALS AND METHODS

Chemicals

PLGA 50:50 [poly(d,l-lactide-co-glycolide)] copolymer (inherent viscosity 0.17 dl/g in hexafluroisopropanol) was purchased from Birmingham Polymer Inc. (Birmingham, AL, USA). Chitin (poly[1?4]-*β*-N-acetyl-$_D$-glucosamine) powder purified from crab shells was purchased from Sigma. Polyvinyl alcohol (PVA) (87-89% hydrolyzed, 31000-50000 g/mol) was purchased from Merck (Darmschtadt, Germany). The organic solvents used were of HPLC grade.

Preparation of PLGA-entrapped chitin microsphere

PLGA-entrapped chitin microsphere was formulated using a double emulsion-solvent evaporation technique[43] with small modification. Briefly, aliquots of 5 ml of aqueous solutions containing 3% chitin (w/v) were was emulsified with 150 mg of PLGA in chloroform solution (5% w/v) followed by vortexing for 3 min to get a primary emulsion. The primary emulsion was further emulsified in an aqueous PVA solution (15 ml, 5% w/v) to form an oil-in-water emulsion. For preparation of microparticles, the emulsion was homogenized for 3 min and stirred for overnight at room temperature to allow the evaporation of organic solvent. Microparticles were recovered by normal centrifugation at 5000 xg for 20 min (SIGMA 3K30, Germany). The process of centrifugation was repeated three times to remove excess PVA and un-encapsulated chitin. The recovered microparticle suspensions were lyophilized for two days (-80 °C and <10 mm mercury pressure (LYPHLOCK, Labconco, Kansas City, MO) to get lyophilized powder for further use.

Vibrio alginolyticus

V. alginolyticus was isolated from moribund olive flounder and cultured in 50 ml tryptic soy broth (TSB) supplemented with 2.5% NaCl for 24 h at 30 °C, and then centrifuged at 7000 g for 15 min at 4 °C[7]. The supernatant fluid was removed and the bacterial pellet was re-suspended in 0.85% NaCl at 3.4×10^7 colony-forming units (cfu) ml^{-1} using plate courting as the stock bacterial suspension stored 14 °C for challenge. The presence of pathogen was confirmed by the relevant biochemical and molecular (RT-PCR) tests[44].

Immunization protocol

Healthy kelp grouper, *Epinephelus bruneus* (34.2 ± 2.7 g) were purchased from Jeju Island and acclimatized in the wet laboratory of Environmental Research Institute, Jeju National University kept with 500 L aerated recirculation sea water. The fish were acclimatized two weeks prior to the start of the experiment and fed with formulate diet. One-third of water was renewed daily and the water temperature, pH, and salinity were 26 ± 1 °C, 7.7 ± 0.8, and 31.2 ± 1.3‰, respectively were measured during the experimental period. The fish were divided into three groups of 25 each in triplicate and were immunized/injected separately each with 0.1 ml of PLGA, chitin (CI), and combined with both PLGA-encapsulated chitin (PLGA+CI). The vaccination was repeated 3 times at 2-week intervals. Another two groups of 25 fish each were maintained separately for controls and injected with PBS (C) or bacteria (I). After the last vaccination, all groups were injected intraperitoneally (i.p.) with 100 µl PBS containing *V. alginolyticus* at 3.4 x 10^7 cfu ml^{-1} except control (C). Six fish of all the treated groups including the control group were bled at weeks 1, 2, and 4 post-injection and anaesthetised with MS-222 ($NaHCO_3$ and tricaine methanesulphonate; Sigma Chemicals) 1:4000 in dechlorinated water for 2 min to collect blood for immunological assay. A group 20 fish were used separately in each treatment to record the cumulative mortality and relative percent survival (RPS) over 30 days[45].

Preparation of anti-grouper-globulin rabbit serum

The rabbit anti-grouper globulin was prepared by the following method of Swain et al.[46] using sera obtained from healthy adult grouper. The serum was collected from healthy grouper and pooled and added equal volume of saturated ammonium sulphate solution. They were mixed with the pooled sera drop by drop and then placed on a magnetic stirrer overnight at 4 °C. The sample mixture was centrifuged at 10,000 xg for 10 min at 4 °C and the precipitate and dissolved with 5 ml carbonate-bicarbonate buffer (pH 9.6). Then the sera were centrifuged at 10,000 xg for 10 min at 4 °C. Then collect the pellet and the volume was made to 2 ml with carbonate-bicarbonate buffer (pH 9.6). The globulin solution was dialyzed using dialysis membrane purchased from Snakeskin, Pierce Chemical Company, USA with 7000 molecular weight cut off against PBS (pH 7.2) for 72 h at 4 °C. After the globulin was collected and raised in a New Zealand white rabbit by the following method of Lund et al.[47].

Immunological assays

The myeloperoxidase activity was determination with 15 µl of serum and diluted in 135 µl of Hank's balanced salt solution (HBSS; Ca^{2+}, Mg^{2+} free). After 50 µl of 20 mM, TMB (3, 30,5,50-tetra methyl benzidine) and 5 mM H_2O_2 were added. The reaction was stopped after 2 min by adding 50 µl of 4 M sulphuric acid and read the optical density (O.D) at 450 nm[48] using UV-VIS spectrophotometer (Thermo Spectronic, UK). The respiratory burst activity was measured by the reduction of nitro blue tetrazolium (NBT) by intracellular superoxide radicals[49]. For bacterial agglutination test, to-fold serial dilutions of 25 µl fish serum was made and add equal volume of PBS in each well then add 25 µl of formalin killed *S. iniae* (3.7×10^8 cfu ml^{-1} suspension) conducted in 'U'-shaped microtitre plates according to Behera et al.[27]. The haemagglutination activity of serum samples was carried out using a standard method of Blazer and Wolke[50] and Behera et al.[27] using 'U'-shaped microtitre plates by serial two-fold dilution. The haemolytic titre of serum was determined as described previously[5] and Behera et al.[27] in HA titre by using fresh sera from all the groups.

Statistical analysis

The statistical analysis system (SAS) software (version 6.12) was used to analyse in each data (SAS, 1991). One-way analysis of variance followed by Duncan's multiple range tests were done to compare the variations in various immune parameters at significance level of difference ($p < 0.05$) in different injected groups. The mean standard error (±S.E) of assayed parameters was calculated for each group of fish.

RESULTS

Myeloperoxidase (MPO) activity

The myeloperoxidase (MPO) activity did not significantly increased in any immunization groups on weeks 1 and 2 when compared to the control against patho-

gen. In fish immunized with chitin (CI) and PLGA-entrapped chitin (PLGA+CI) was significantly increased the MPO activity on week 4. However, it did not significantly increase in PLGA on week 4 (Figure 1).

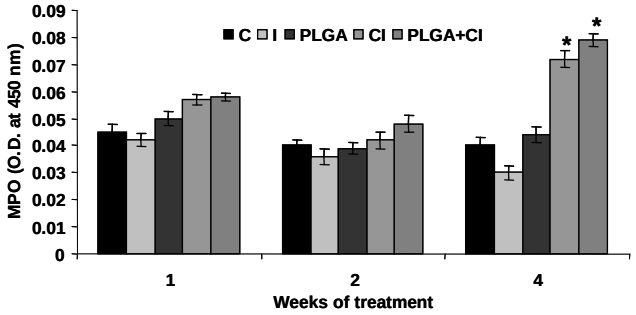

Figure 1 : Myeloperoxidase activity of *E. bruneus* (n = 6) control (C), infected (I), injected with PLGA, chitin (CI), and PLGA-encapsulated chitin (PLGA+CI) against *S. iniae*. Data (mean ± SE; *P < 0.05) difference from the control is indicated with asterisks.

Respiratory burst (RB) activity

The respiratory burst (RB) activity did not significantly enhanced in PLGA and chitin immunization group on first week against pathogen. The RB activity was significantly enhanced in fish immunization with chitin (CT) and PLGA-entrapped chitin on weeks 2 and 4 and but not in PLGA against pathogen when compared to the control (Figure 2).

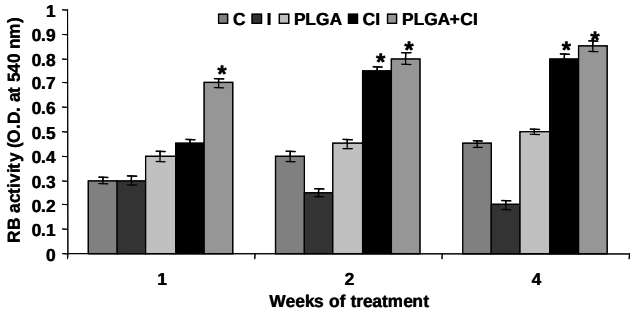

Figure 2 : Respiratory burst (RB) activity of *E. bruneus* (n = 6) control (C), infected (I), injected with PLGA, chitin (CI), and PLGA-encapsulated chitin (PLGA+CI) against *S. iniae*. Data (mean ± SE; *P < 0.05) difference from the control is indicated with asterisks.

Serum haemoagglutinating titre

The serum haemoagglutinating titre did not significantly increased in any groups on first week when compared to the control. The haemoagglutinating titre was significantly enhanced in fish immunized with chitin (CT) and PLGA-entrapped chitin on weeks 2 and 4 whereas not in PLGA (Figure 3).

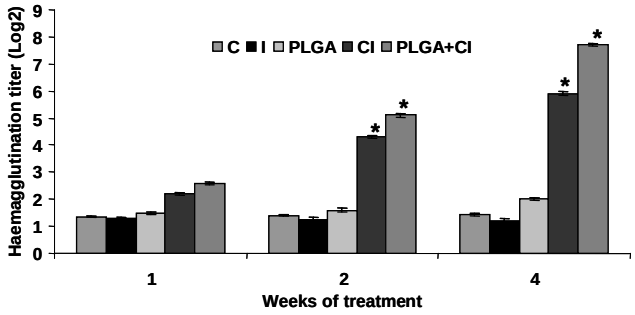

Figure 3 : Serum haemoagglutinating activity (titre) of *E. bruneus* (n = 6) control (C), infected (I), injected with PLGA, chitin (CI), and PLGA-encapsulated chitin (PLGA+CI) against *S. iniae*. Data (mean ± SE; *P < 0.05) difference from the control is indicated with asterisks.

Serum haemolysin titre

The serum haemolysin titre did not significantly increase any groups on fist week as compared to the control against pathogen. It was significantly enhanced on weeks 2 and 4 in chitin (CT) and PLGA-entrapped chitin but not in PLGA (Figure 4).

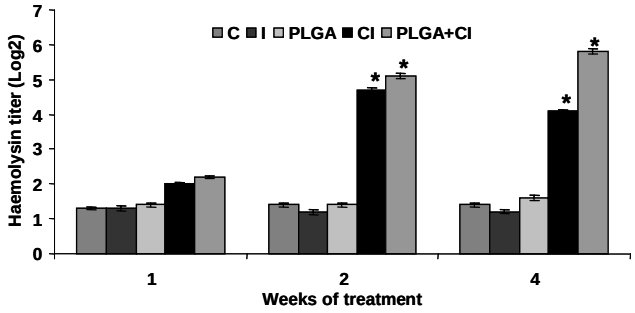

Figure 4 : Serum haemolysin activity (titre) of *E. bruneus* (n = 6) control (C), infected (I), injected with PLGA, chitin (CI), and PLGA-encapsulated chitin (PLGA+CI) against *S. iniae*. Data (mean ± SE; *P < 0.05) difference from the control is indicated with asterisks.

Bacterial agglutination titre

The bacterial agglutination titre did not significantly increased in PLGA at any time. On the other hand, the bacterial agglutination titre significantly increased in fish immunization with chitin (CI) and PLGA-entrapped chitin from weeks 1 to 4 when compared to the control (Figure 5).

Mortality

The cumulative mortality was found low in fish immunization with PLGA-entrapped chitin with 15% against pathogen for 30 days. However, the mortality was 30% and 25% in fish immunization with PLGA

and chitin. In fish infected and non-immunization group was 80% mortality while no mortality in control group (Figure 6).

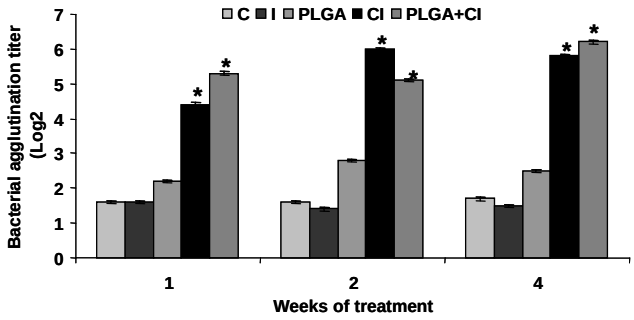

Figure 5 : Bacterial agglutination activity (titre) of *E. bruneus* (n = 6) control (C), infected (I), injected with PLGA, chitin (CI), and PLGA-encapsulated chitin (PLGA+CI) against *S. iniae*. Data (mean ± SE; *P < 0.05) difference from the control is indicated with asterisks.

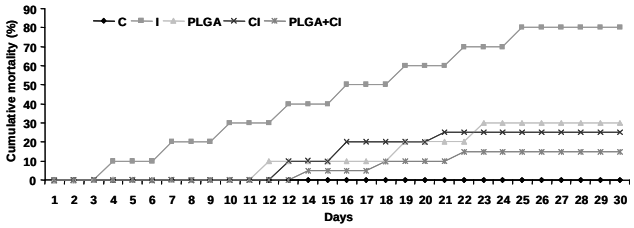

Figure 6 : Cumulative mortality (%) of *E. bruneus* (n = 20) control (C), infected (I), injected with PLGA, chitin (CI), and PLGA-encapsulated chitin (PLGA+CI) against *S. iniae* for 30 days.

DISCUSSION

The myeloperoxidase (MPO) activity significantly increased in fish immunized with chitin (CT) and PLGA-entrapped chitin on week 4. Similarly the MPO activity was increased in rohu after immunization with PLGA encapsulated outer membrane proteins (OMP) of *Aeromonas hydrophila*[27]. However, MPO activity did not significantly increased in any groups on weeks 1 and 2. The respiratory burst (RB) activity was significantly enhanced in the present study in chitin (CT) and PLGA-entrapped chitin from weeks 1 to 4 and but not in PLGA. The present results are agreement in Japanese flounder oral immunization with PLGA encapsulated DNA vaccine significantly enhanced the RB activity against lymphocystis disease virus[29]. Since O_2^- is the first product released during the respiratory burst, O_2^- concentration has been accepted as an accurate parameter to quantify the intensity of a respiratory burst[51]. Jeney and Anderson[52] reported that the total number of NBT-positive cells in blood kept rising after treatment with immunostimulant. These cells might be the neutrophils that still retain the capacity for production of oxidative radicals.

In fish, like in mammals, the immune system can be modulated both *in vitro* and *in vivo*. Among the enhancers, the chitin has been shown enhanced immune system in fish when administered *in vitro* but to act as a very good immunomodulator *in vivo*[49,53,54]. Chitin and its derivatives administration through intravenous, intraperitoneal or oral in mammalian and fish *in vivo* can stimulate the respiratory burst[32,55,56]. PLGA are prepared from lactide and glycolide, which are cyclic esters of lactic acid and glycolic acids[57]. PLGA microspheres and other nanospheres are promising delivery systems for proteins, peptides, and DNA vaccines[58]. PLGA particles have been shown to be taken up *in vivo* by the main antigen presenting cells (APCs) in mammals, dendrittic cells (DCs)[59,60] and using PLGA nanoparticles as antigen delivery vehicles have shown to enhance antigen-presentation efficiency by 10-100 fold[61], and also increasing cytotoxic T-cell activity. Mammalian DCs, the main APCs, showed an adjuvant-like maturation with expression of major histocompatibility complex (MHC) class II and expression of costimulatory molecules on DCs stimulated with PLGA particles[60]. Studies in mammalian DC also show increased maturation after stimulation of PLGA microparticles alone[60].

PLGA particles as antigen carrier have been evaluated and high potencies in activating the adaptive immune response in mammals[61,62]. PLGA particles are used for intracellular delivery of antigens, their actual uptake by fish phagocytic cells (macrophages), the main APC in fish[63,64]. PLGA have shown to be phagocytosed by a number of mammalian cells[59] by various endocytic routes, like phagocytosis, pinocytosis or by receptor-mediated endocytosis, and the process seems to be saturable[65,66]. PLGA has been proven to be a very useful antigen delivery system in mammals since it provides long lasting immunity[62,67,68]. PLGA microparticles have been found to enhance phagocytosis by macrophages[69] and neutrophils[60] in mouse and human, respectively. Comparable results were also found by the use of PLGA alone in mammal[59] as well

as in fish, rainbow trout[70]. Chitin administration through intraperitoneal injection in trout and salmon significantly enhanced the lysozyme activity[71-73]. Phagocytic activity was increased in trout injection with chitin against *V. anguillarum*[56]. In rainbow trout, chitin stimulated macrophage activities[74] and in seabream it stimulated the main innate immune responses, including respiratory burst, phagocytic and also cytotoxic activities[56].

The serum haemoagglutinating activity and haemolysin activity were significantly enhanced in this study with chitin (CT) and PLGA-entrapped chitin on weeks 2 and 4 but not in PLGA and all groups on first week. The present results are agreement in rohu immunization with PLGA encapsulated OMP of *A. hydrophila* that enhance the haemoagglutinating titre and serum haemolysin titre[27]. Another study indicates that in Japanese flounder oral immunization with PLGA encapsulated DNA vaccine and plasmid vaccine enhanced the antibody titre against lymphocystis disease virus[29-31]. The antibody level remained high even one year after injection through subcutaneous route in mice with PLGA microparticles which indicate control the release of antigen over a period of several weeks[75]. Similar results were also found when PLGA was used as carrier for peptide vaccine in mammals[76]. Moreover, the superiority of PLGA microspheres over alum adjuvant in eliciting high antibody responses was seen in mice through subcutaneous administration[77].

The bacterial agglutination titre significantly increased in the present study in chitin and PLGA-entrapped chitin from weeks 1 to 4. The present results are agreement in rohu immunization with PLGA encapsulated OMP of *A. hydrophila*[27]. Enhanced NK or NCC activity by chitin has been described in mammals[42,55,78] and fish[32,56] in both *in vitro* and *in vivo*, respectively. On the other hand, the bacterial agglutination titre did not significantly increased in PLGA at any time. The cumulative mortality was 15% in PLGA-entrapped chitin whereas 30% and 25% in PLGA and chitin. Based on the results of the present study, it can be concluded that the administration of PLGA-entrapped chitin enhances kelp grouper immune activity through innate and adaptive immune response such as respiratory burst, serum haemoagglutinating, haemoagglutinating, and bacterial agglutination titre against *V. alginolyticus*. The mechanisms by which PLGA-entrapped chitin modulates the kelp grouper immune system remain unclear and further detailed molecular and immunological studies should be carried out.

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