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PEG–PLA–PEG block copolymeric nanoparticles for oral immunization against hepatitis B

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ABSTRACT

PLA/PLGA nanoparticles are well known as efficient vaccine delivery systems, but they have got limitation in oral vaccine delivery because of their sensitivity to harsh gastric environment. The aim of present study was to improve the stability of PLA nanoparticles in such environment by copolymerizing PLA with PEG. Nanoparticles were formulated using different block copolymers AB, ABA and BAB (where 'A' is PLA and 'B' is PEG) encapsulating hepatitis B surface antigen (HBsAg) to evaluate their efficacy as oral vaccine delivery system. The results of *in vitro* studies engrave the efficiency of copolymeric nanoparticles to retain encapsulated antigen and average particle size even after 2 h incubation in simulated gastric fluid and simulated intestinal fluid. Fluorescence microscopic studies indicated efficient uptake of copolymeric nanoparticles by gut mucosa of immunized mice model as compared to control. Finally copolymeric and PLA nanoparticles, encapsulating HBsAg, were evaluated for their adjuvancity in generating immune response after oral administration. PLA nanoparticles could not generate an effective immune response due to stability issues. On the other hand, oral administration of copolymeric nanoparticles exhibited effective levels of humoral immunity along with the mucosal (sIgA) and cellular immune response (T_H1). The results of *in vitro* and *in vivo* studies demonstrate that BAB nanoparticles depict enhanced mucosal uptake leading to effective immune response as compared to other copolymeric nanoparticles. Present study indicates the efficacy of BAB nanoparticles as a promising carrier for oral immunization.

1. Introduction

Oral vaccination has many intriguing factors that have engrossed researchers for developing effective novel delivery systems for the proteins/antigens (O'Hagan et al., 1987; Delgado et al., 1999; Garinot et al., 2007). Oral vaccination is beneficial as compared to conventional vaccination strategies as it provides local mucosal immunity [secretary IgA (sIgA)] at the site of invasion by microorganisms along with the systemic immunity. sIgA provides dual action, as it averts the invasion of mucosal surfaces by pathogens and simultaneously neutralize the viruses and endotoxins within epithelial cells without causing tissue damage (Kim et al., 2002; Brandtzaeg, 2007; Vyas and Gupta, 2007). Apart from the systemic and local immunity such oral vaccines can provide effective cellular immunization, which is required against viral pathogens like hepatitis B, anthrax, etc. Oral vaccines have some added advantages in terms of patient compliance, avoiding the need of trained personnel for administration and chances of cross contamination using the needle that are associated with conventional vaccines. Being second largest exposed area to microorganisms, generation of immunity at mucosal surfaces is most promising approach for complete eradication of viral infections such as hepatitis B.

Despite of dedicated efforts by researchers the development of efficient oral delivery systems is still a challenge. There are two main hurdles that are needed to be overcome for providing therapeutically effective level of drug after oral administration. First is the instability of delivery system in the harsh GI conditions, whereas the second one is low uptake of delivery systems across thick mucus layer (Fasano, 1998; Page and Cudmore, 2001; Morishita and Peppas, 2006). The novel nanoparticulate carriers have been reported to enhance the bioavailability of drugs (Jung et al., 2000; Debin et al., 2002; Prego et al., 2005; Goyal et al., 2008). Among them PLA/PLGA nanoparticles are most promising and widely accepted delivery systems for the vaccine delivery due to their biodegradable, biocompatible and bioresorbable nature (Anderson and Shive, 1997; Jaganathan and Vyas, 2006a,b; Rajkannan et al., 2006; Gupta et al., 2007). However, they have limited application in oral delivery because they are prone to hydrolysis in harsh conditions of gastrointestinal tract (GIT) that

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include acidic pH and digestive enzymes. Knowing the fact that effectiveness of nanodelivery systems to improve the stability and absorption depends strongly on their polymer composition; physicochemical properties of PLA can be altered by conjugating with polyethylene glycol (PEG), a hydrophilic biocompatible polymer. Such conjugation leads to the amphiphilic copolymer that gives the corona-core structure after the nanoparticles formulation. PLA being the hydrophobic part forms the core where as PEG being the hydrophilic part comes out to from the corona around the PLA core. This PEG corona is capable to alleviate the above mentioned barriers as free PEG chains act as protein repellent to protect the nanoparticles from the enzymatic attack in GIT, on the other hand these free PEG chains penetrate the mucosal layer of GIT which enhance the cellular uptake of the nanocarriers encapsulating the antigenic contents, as indicated by earlier studies (Tobío et al., 2000; Vila et al., 2002; Prego et al., 2006; Hu et al., 2007). The covalent attachment of PEG to PLA can generate three kinds of block copolymers: PLA–PEG, PLA–PEG–PLA and PEG–PLA–PEG; in the present study these are mentioned as AB, ABA and BAB block copolymers, respectively.

We have already shown the efficacy of AB, ABA and BAB block copolymers to stabilize the antigen during the harsh formulation conditions. It was also observed that antigen was stable during the release from nanoparticles owing to the presence of PEG, which prevents the generation of acidic microenvironment resulting from the degradation of PLA to lactic acid (Jain et al., 2009). Among these copolymeric nanoparticles BAB nanoparticles were found to be most promising candidate due to higher PEG density on their surface. The present study aims at demonstrating increased stability of these copolymeric nanoparticles in the gastric environment and evaluating their efficacy in generating complete spectrum of immunity (humoral, cellular and mucosal) after the oral administration.

2. Materials and methods

D,L-Lactide, hexamethylene diisocyanate (HMDI), stannous octoate, polyvinyl alcohol (PVA) (M_w 85–124 kDa) and FITC–BSA were procured from Sigma-Aldrich Pvt. Ltd. (USA). Recombinant hepatitis B surface antigen (HBsAg, M_w 24 kDa) was kindly gifted by Shantha Biotech Ltd. (Hyderabad, India). Analytical grade solvents including dichloromethane (DCM), diethyl ether and toluene were purchased from Merck (India) Ltd. and used as received unless otherwise processed. Toluene was dried over the sodium sulfate and refluxed prior to use. BCA protein estimation kit (KT-31) and protein molecular weight markers were purchased from Bangalore Genei Pvt. Ltd., India. Enzyme linked immunoassay kits (AUSAB and AUSZYME) and cytokines (IL-2, IL-4 and IFN-γ) estimation kit were purchased from Abott Laboratories, USA and eBioscience, respectively. IgG isotyping was performed using sigma isotyping kit (Sigma–Aldrich Pvt. Ltd., USA). All other chemicals and reagents were of analytical grade and purchased from commercial vendors.

2.1. Copolymers synthesis

Different kind of block copolymers of PLA and PEG were synthesized, with varying molar ratio, as described in our previous report (Jain et al., 2009). Fig. 1 exhibits the structures of AB, ABA and BAB block copolymers. PEG–PLA diblock copolymers were prepared by ring opening polymerization (ROP) of D,L-Lactide in the presence of mPEG 6000 (monomethyl ether of polyethylene glycol having molecular weight 6000) using stannous octoate as catalyst (Burkersoda et al., 1997). Briefly, dried mPEG was taken in rotary flask evaporator (Strike 102, Steroglass SRL, Italy) with nitrogen purging (to protect from oxygen and moisture), dissolved with appropriate amount of lactide and 0.05% (w/w) stannous octoate in dried toluene. The mixture was then refluxed at 140 °C for 18 h under nitrogen atmosphere. Afterwards, the solvent was removed under vacuum; resulted product was purified by dissolving in DCM (dichloromethane) and precipitation by cold ethyl ether (−20 °C). The precipitation was repeated twice for the better quality product. Finally, obtained precipitate was dried overnight under vacuum. BAB i.e. PEG–PLA–PEG type triblock copolymer were synthesized by coupling of diblock copolymer using hexamethylene diisocyante (HMDI) (Jeong et al., 1997). ABA i.e. PLA–PEG–PLA type triblock copolymers were synthesized with the similar reaction condition as diblock copolymer, the only change was replacement of mPEG with normal PEG having hydroxyl group at both the ends, so that lactide can get polymerize at both the side (Fig. 1).

2.2. Characterization of copolymers

^{1}H NMR spectroscopy and gel permeation chromatography (GPC) were used to characterize the synthesized copolymers. ^{1}H NMR was performed using Bruker, Avance II 400 MHz, Switzerland instrument. $CDCl_3$ and trimethyl silane (TMS) were used as solvent and internal reference, respectively. The integration values of the peaks corresponding to the PLA methyne protons (CH, δ 5.17 ppm) and PEG methylene protons (CH_2, δ 3.64) were used to determine chain length ratio (LA/EG) and number average molecular weight (M_n) for each copolymer.

Molecular weight (M_w) and polydispersity index (M_w/M_n) of the copolymers were determined by gel permeation chromatography using Perkin Elmer Series-200 instrument, equipped with the refractive index detector. Tetrahydrofuran (THF) was the mobile phase at a flow rate of 1 ml/min and a temperature of 30 °C. Polymers were dissolved in THF, filtered and then injected into column of PL Gel 5 micron, 300 mm × 7.5 mm. Average molecular weights were calculated using a series of polystyrene standards.

2.3. Development of nanoparticles

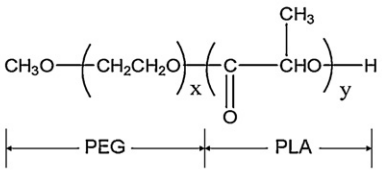
Nanoparticles were prepared by double emulsion solvent evaporation method using optimized parameters as we have reported previously (Jain et al., 2009). Briefly, aqueous phase containing antigen was added drop-wise along vortexing into the organic phase (dichloromethane, DCM) having polymer (4%, w/v). The mixture thus obtained was vortexed for 20 s, followed by probe sonication (Soniweld, India) in ice bath for 60 s at 40 W. To this water-in-oil (w/o) emulsion, 10 ml of 1% (w/v) aqueous polyvinyl alcohol was added, vortexed for 20 s, then probe sonicated in ice bath for 3 min to obtain a w/o/w emulsion. The resultant emulsion was stirred vigorously for 3 h to evaporate organic phase and to obtain the nanoparticles, which were collected by centrifugation at 22,000 × g and washed twice with distilled water to remove PVA and subjected to lyophilization.

2.4. Characterization of nanoparticles

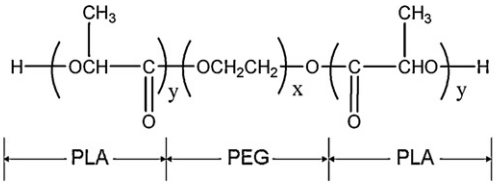
2.4.1. Morphology

The nanoparticles were observed for their surface morphology by transmission electron microscopy (TEM). Specimens were prepared by dropping the dispersion onto carbon-coated EM grids. The grid was held horizontally to allow the molecular aggregates to settle and then tilted to 45° for a while to drain the excess fluid. Then a drop of phosphotungstic acid (pH 4) was added to the grid to give a negative stain. The grid was then kept aside for 20 s before removing excess stain as above. Specimens were air-dried before examination using a Philips Morgagni, Netherlands transmission electron microscope.

I. PLA-PEG Diblock copolymer



II. PLA-PEG-PLA TRiblock Copolymer (ABA)



III. PEG-PLA-PEG Triblock Copolymer (BAB)

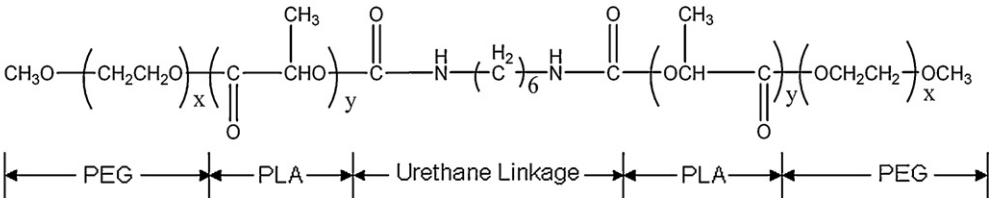


Fig. 1. Structure of (I) AB diblock; (II) ABA and (III) BAB triblock copolymers.

2.4.2. Particle size and zeta potential

The mean diameter and zeta potential of the nanoparticles were determined by dynamic light scattering method using Zetasizer Nano ZS 90 (Malvern Instruments, UK). The zeta potential analysis was performed using samples diluted with 1 mM HEPES buffer (pH 7.4) in order to maintain a constant ionic strength. For each sample the mean value ± S.D. of four determinations were established.

2.4.3. Protein loading efficiency

The loading efficiency of the antigen in nanoparticles was determined by dissolving 20 mg of the nanoparticles in the 2 ml of 5% (w/v) sodium dodecyl sulfate (SDS) in 0.1 M sodium hydroxide solution (Sharif and O'Hagan, 1995). The amount of the antigen was determined by bicinchoninic acid assay using the BCA protein estimation kit (KT-31).

2.5. Stability of nanoparticles in simulated gastric and intestinal fluids (USP 25 NF 20)

The efficiency of developed formulation to remain intact along with loaded HBsAg, was evaluated by incubating them in simulated gastric (USP 25, pH 1.2, pepsin 0.32%, w/v) and simulated intestinal fluid (USP 25, pH 6.8, pancreatin 1%, w/v) at a concentration of 10 mg per ml for 2 h. The concentration of antigenically active HBsAg was determined by AUSZYME monoclonal kit in particles after incubation. The particle size was determined by Zetasizer Nano ZS 90 apparatus (Malvern Instruments, UK).

2.6. In vitro release

To perform the *in vitro* release study the nanoparticles were suspended in phosphate buffered saline (PBS, pH 7.4). Vials containing 40 mg of nanoparticles and 5 ml of PBS were incubated at 37 °C on a constant shaking mixer. To reduce the adsorption of the released protein as well as to prevent the particle aggregation Tween-80 (0.02%, w/v) was added to the release media. At appropriate time intervals 1.0 ml of release medium was collected following centrifugation at 22,000 × *g* for 30 min and 1.0 ml of fresh PBS (pH 7.4) was replaced. The amount of HBsAg released was estimated by AUSZYME monoclonal kit (Abbott Laboratories, Abbott Park, IL, USA).

2.7. Fluorescence microscopy

The fluorescence microscopy was performed to confirm deposition of selected carrier system in gut mucosa after the oral administration. Fluorescent isothiocynate (FITC) conjugated BSA (FITC–BSA) was used as a fluorescence marker. FITC labeled nanoparticles were prepared according to the procedure described earlier, using a 0.05% FITC–BSA solution in PBS. FITC–BSA loaded formulation was administered to mice orally. FITC–BSA solution was also administered orally and was considered as control. After 2 h the mice were sacrificed and approximately 1 cm length of small intestine (illium) containing Peyer's patch were excised, opened longitudinally and pinned flat on corkboard. Tissues were rinsed thoroughly with PBS (pH 7.4) and then cut in small pieces (approximately 1 mm thick) and washed with Ringer's solution. The tissues were fixed in Carnoy's fluid (absolute alcohol:chloroform:acetic acid, 6:3:1 v/v). The tissue blocks were prepared with paraffin wax,

which were subjected to microtomy and mounted on slides and were analyzed after 2 days under fluorescence microscope (Nikon Eclips E 200, Japan).

2.8. Immunological studies

Female BALB/c mice were procured from CDRI, Lucknow, 8–10 weeks old, weighing 20–25 g. BALB/c mice were used to assess the immunogenicity of developed formulations, as they are well established for the immunization studies. Mice were housed in group ($n = 6$) 1 week before the experiments for acclimatization, with free access to food and water with 12 h light/dark cycle. They were withdrawn of any food intake 3 h before immunization. The study protocol was approved by Institutional Animals Ethical Committee of Dr. Hari Singh Gour University, Sagar. The studies were carried out with the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

2.8.1. Immunization

The formulations were administered regarding their groups. The nanoparticles were resuspended in the PBS (pH 7.4) so as to obtain the required concentration of HBsAg. To evoke immune response, preparations equivalent to 20 μg of HBsAg were inoculated three times orally for 3 consecutive days. Conventional alum-HBsAg vaccine was administered subcutaneously. Booster dose was given only to this group (alum based formulation) after 4 weeks of primary immunization.

2.8.2. Sample collection

The blood samples were collected from retro-orbital puncture (under mild anesthesia), at day 14, 28, 42 and 56 after immunization from each animal. The collected blood was allowed to coagulate, so as to separate the serum that stored at −40 °C until tested by ELISA for the estimation of IgG level. The nasal, vaginal and salivary secretions were collected at day 42, after the immunization by the methods described in our previous studies (Jain et al., 2005; Jaganathan and Vyas, 2006a,b). For collection of salivary secretions, mice were injected 0.2 ml sterile solution of pilocarpine (10 mg/ml) intraperitonially. The mice began to salivate after approximately 20 min and the saliva was collected by using capillary tube. To obtain vaginal wash 50 μl of phosphate buffer saline (pH 7.4) containing 1% (w/v) bovine serum albumin (1% BSA–PBS) was introduced into the vaginal tract of non-anesthetized mice using a Gilson pipette. These 50 μl aliquots were withdrawn and reintroduced nine times into the vaginal tract. A second vaginal wash was collected the following day and pooled with the first one. The nasal wash was similarly collected by cannulation by trachea of sacrificed mice. The nasal cavity of sacrificed mice was flushed three times with 0.5 ml of 1% BSA containing phosphate buffer (BSA–PBS, pH 7.4). Intestinal lavage was performed using previously reported method (Elson et al., 1984), briefly four doses of 0.5 ml lavage solution (NaCl 25 mM, Na_2SO_4 40 mM, KCl 10 mM, $NaHCO_3$ 20 mM and polyethylene glycol M_w 3350; 48.5 mM) were administered intragastrically at 15 min intervals using a blunt tipped feeding needle. Thirty minutes after the last dose of lavage solution, the mice were given 0.2 ml pilocarpine (10 mg/ml) intraperitonially. A discharge of intestinal contents occurred regularly over next 20 min, which was collected carefully. These fluids were stored with 100 mM phenylmethyl sulfonyl fluoride (PMSF) as a protease inhibitor at −40 °C until tested by ELISA for secretary antibody (sIgA) levels.

2.8.3. Measurement of specific IgG and IgA response

The anti-HBsAg antibodies in blood samples were determined by enzyme linked immunoassay kit (AUSAB®, Abott Laboratories, USA). Whereas IgG1 and IgG2a isotypes were determined at day 42 using sigma isotyping kit (Sigma–Aldrich Pvt. Ltd., USA). Anti-HBsAg IgG present in sera was estimated using 1/100 dilution the first dilution of serum. To signify actual antibody concentration (antibody titre) in mIU/ml, a standard curve was prepared using the calibrated anti-hepatitis B panel provided by Abott Laboratories. Antibody response was plotted as log anti-HBsAg titre versus time in days. Secretary IgA level in mucosal fluids was determined by ELISA. Briefly, Microtitre plates (Nunc-Immune Plate® Fb96 Maxisorb, Nunc, India) were coated with 100 μl of HBsAg solution at a concentration of 2 μg/ml in carbonate buffer (pH 9.6) by overnight incubation at 4 °C. The wells were washed thrice with PBS-T (0.05%, v/v Tween 20 in PBS) and blocked with 200 μl of blocking buffer (3%, w/v BSA in PBS) by 2 h incubation at 37 °C. The wells were washed thrice with PBS-T. Serial dilutions (100 μl) of mucosal fluids in PBS-BSA (0.1%, w/v) were added and incubated for 2 h at 37 °C, followed by washing three times with PBS-T. 100 μl of HRP conjugated goat anti-mice IgA (Sigma, USA) antibodies were added to each well and again incubation was done for 2 h at 37 °C. After three washings, 100 μl of substrate (OPD, Sigma, USA) (in citrate phosphate-citrate buffer, pH 5.5, containing H_2O_2) was added. Color development was stopped after 30 min by adding 50 μl of 1N H_2SO_4 to each well and measured the absorbance (OD) at 490 nm. The end point titre was expressed as the reciprocal of the last dilution, which gave an optical density at 490 nm above the optical density of negative control (Khatri et al., 2008). Similar protocol was used for the determination of antigen specific anti-HBsAg antibody response.

2.8.4. Estimation of cytokine level

Endogenous levels of cytokines in mice were determined by splenocyte proliferation assay (Sachdeva et al., 2004). For determination of cytokine production, 5×10^6 splenocytes/ml were cultured in a final volume of 200 μl in 96-well flat-bottom plates in the presence or absence of recombinant proteins. Culture supernatants were collected after 48 h for estimation of cytokine concentration (IL-2, Il-4 and IFN-γ). All of these cytokines were measured by using a murine cytokine immunoassay kit (eBiosciences, USA) by following the procedure recommended by the manufacturer.

3. Result and discussion

3.1. Copolymer synthesis and characterization

AB, ABA and BAB copolymers having chain length ratio (LA/EG) 3:1 were synthesized by ring opening polymerization method. Their chain length ratio (composition) and purity (polydispersity index) were determined by ^{1}H NMR and GPC (Table 1), as described in our previous report (Jain et al., 2009). The integral values of peaks at 5.17 ppm (–CH of LA) and 3.64 ppm (–CH_2 of EG), exhibited in corresponding ^{1}H NMR spectra, were used to determine the LA/EG ratio of the product. This LA/EG ratio indicates the ratio of lactide and ethylene repeating units in the respective copolymer. GPC was performed to determine the polydispersity index (ratio of weight average molecular weight and number average molecular weight) of synthesized copolymers and found to be finely low, ensuring the polymers of a particular chain length with low variability. All copolymer exhibited the unimodal mass distribution, which indicates the copolymers were free from unreacted feed materials of polymerization process.

3.2. Characterization of nanoparticles

Particle size, entrapment efficiency and zeta potential of different copolymeric nanoparticles are shown in Table 2. The particles

Table 1
Characterization of AB, BAB and ABA block copolymers.

Polymer	Polymer chain length	LA/EG ratio	^{1}H NMR		GPC[a]	
		In feed	LA/EG ratio Calculated[b]	M_n	M_n	M_w/M_n [c]
AB	PEG136–PLA250	3.0	2.66	21,300	20,751	1.41
ABA	PEG136–PLA500–PEG136	3.0	2.55	42,600	32,146	1.41
BAB	PLA90–PEG91–PLA90	3.25	2.91	15,640	18,148	1.32

M_w stands for weight average molecular weight. M_n stands for number average molecular weight.
[a] Based on polystyrene standards.
[b] Determined from the integration ratio of resonance due to PEG blocks at 3.64 ppm (–O–CH_2CH_2–) and to the PLA blocks at 5.17 ppm (Me–CH*⟨) in the ^{1}H NMR.
[c] Polydispersity of copolymers.

Table 2
Characterization of AB, ABA and BAB copolymer based nanoparticles.

Polymer	Average particle size (nm)	Entrapment efficiency (%)	Zeta potential (mV)	Polydispersity index
PLA	167.2 ± 9.3	39.7 ± 1.8	−26.7 ± 2.1	0.247 ± 0.019
AB	232.8 ± 11.2	30.1 ± 1.6	−5.4 ± 0.21	0.148 ± 0.013
ABA	215.6 ± 12.2	30.2 ± 1.7	−6.2 ± 0.38	0.183 ± 0.016
BAB	109.2 ± 4.6	32.5 ± 0.8	−1.9 ± 0.11	0.113 ± 0.006

Mean ± S.D., $n = 4$.

size was found to be in following order: BAB < PLA < ABA < AB. The lowest size of BAB nanoparticles may be attributed to movement of PLA chain towards the core and the free ends of PEG towards outer side, to form the corona, during the nanoparticles formulation. Such 'U' shape arrangement of chains form more complex core to give lower particle size. The amphiphilic nature of these copolymers imparts stabilizing effect during the primary emulsification step of formulation; leading to narrow size distribution of nanoparticles (indicated by polydispersity index below 0.2 in all cases, Table 2). The spherical shape and particles size was confirmed by transmission electron microscopy (Fig. 2).

Zeta potential was found to be lowest (−26.7 ± 2.1) in case of PLA nanoparticles due to their carboxyl end groups. BAB nanoparticles have shown highest zeta potential (−1.9 ± 0.11) that suggest highest PEG chain density on their surface.

3.3. Stability of nanoparticles in simulated gastric and intestinal fluids (USP 25 NF 20)

Gastrointestinal stability of formulation is the major concern in development of oral vaccine delivery system. Before moving ahead, stability of developed formulations was determined by incubating them in simulated gastric and intestinal fluids, followed by determination of particle size and percent remaining HBsAg. The HBsAg content was determined by AUSZYME monoclonal kit that quantifies the antigenically active HBsAg. Results (Fig. 3) revealed that plain PLA nanoparticles (NPs) get aggregated due to gastrointestinal enzymes and rapidly looses the encapsulated antigen. Moreover, the copolymeric nanoparticles were found to remain segregated with mild increase in their particles size. This stability may be attributed to the presence of free PEG chains on surface, interfering with the enzymes to react with the nanoparticles. Further, the developed nanoparticles exhibited reasonably high content of antigen (nearly 90%) in all the cases, after incubation. No significant difference was observed between different copolymeric formulations.

3.4. In vitro release profile of AB, ABA and BAB type copolymer based nanoparticles

The HBsAg released from different copolymeric nanoparticles is shown in Fig. 4. A triphasic release pattern was observed by these nanoparticles during the *in vitro* release study. The first phase corresponds to the initial fast release in first 24 h, known as burst release, which was due to the amount of antigen present on surface or near to the surface of nanoparticles. This kind of release is advantageous in case of vaccine delivery, as it gives primary immunization effect. The second phase demonstrated constant continuous release of antigen that was controlled by diffusion. The third phase, which started in 4th week, was dominated by the degradation release due to the PLA core and supposed to be responsible for generation of booster kind effect *in vivo*. BAB copolymer showed higher release at approximately each point; this may be due the higher hydrophilicity of nanoparticles provided by BAB copolymer. At the same time plain PLA nanoparticles released ~16% of encapsulated protein by the day 21 and no further significant release was observed. This may be attributed to the degradation of encapsulated antigen due to the harsh conditions posed by PLA.

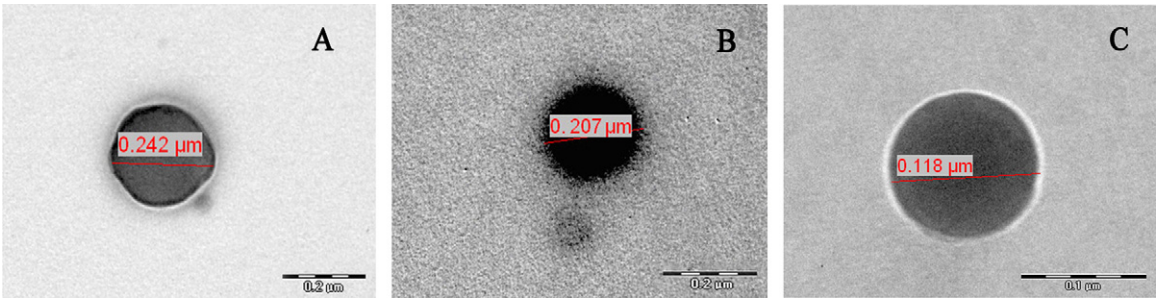


Fig. 2. Transmission electron photomicrograph of copolymeric nanoparticles. (A) AB nanoparticles; (B) ABA nanoparticles; (C) BAB nanoparticles.

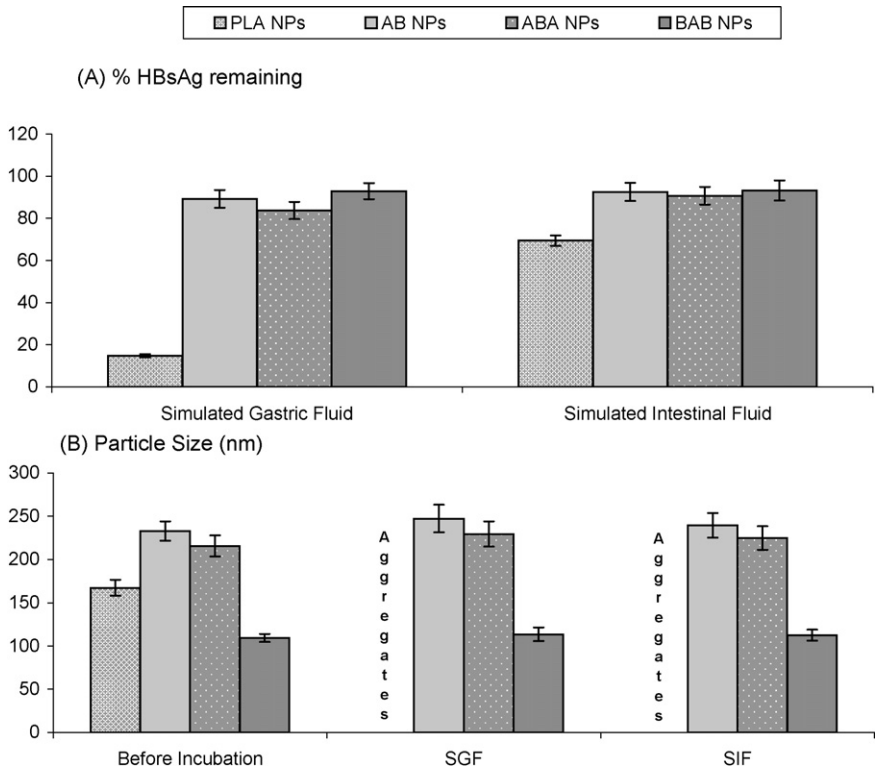


Fig. 3. Stability of nanoparticles in simulated gastric and intestinal fluids. (A) % HBsAg retained within the different nanoparticles after 2 h incubation in the SGF and SIF. (B) Particle size of different formulations before and after incubation in SGF and SIF. All results indicate mean ± S.D. where $n = 4$. AB, ABA and BAB nanoparticles (NPs) did not exhibit significant difference in the particle size after incubation in SGF and SIF as compared to that of before incubation.

3.5. Fluorescent microscopy

Contrary to general concept, which states that hydrophobic surface increases cellular uptake of nanoparticles than hydrophilic one due to its better interaction with lipophilic cell membrane (Brooking et al., 2001; Jani et al., 1990), current study indicates that hydrophilic surface can also exhibit enhanced cellular uptake. The block copolymeric nanoparticles offer free PEG ends on their surface, which penetrate into mucosal surface, enhancing the cellular uptake. These results are in agreement with earlier reports (Tobío et al., 2000; Vila et al., 2002; Jain et al., 2009). In the present study, fluorescent microscopy of Peyer's patches was performed to confirm the uptake of nanoparticles formulated by different copolymers. The images of fluorescent microscopy are shown in Fig. 5. Developed nanoparticles exhibited enhanced uptake by gut mucosa as compared to control. Comparatively, BAB copolymer based nanoparticles showed highest uptake by the gut mucosa supporting highest free PEG chain density on their surface.

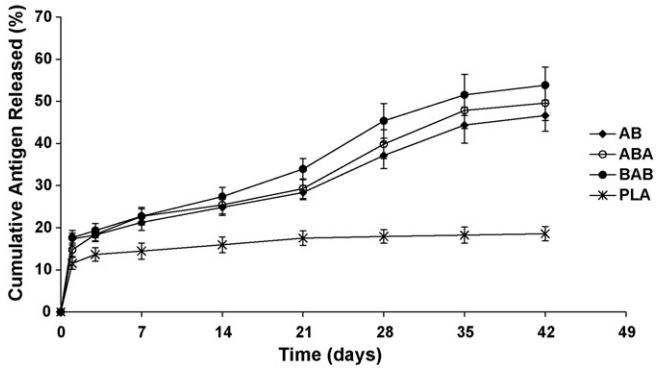


Fig. 4. *In vitro* cumulative release of HBsAg from AB, ABA and BAB nanoparticles ($n = 4$). Each data exhibits statistical significance $p < 0.001$ as compared to PLA nanoparticles.

3.6. Immunological studies

The immunological studies were performed to evaluate the immunization capability of developed formulations after the oral administration. Different groups were designed for the study, each containing 6 mice. Group I kept as control, Group II received conventional alum-HBsAg vaccine, only this group received a booster dose at day 30. Formulations developed from PLA, AB, ABA, BAB based nanoparticles were inoculated three times in consecutive days to the group III–VI via oral route, respectively. Anti-HBsAg antibodies were determined in serum by the use of ELISA kits. IgG titre was calculated from the absorbance obtained as the result of ELISA by the statistical analysis methods, depicted in Fig. 6A. All copolymeric formulations exhibited an abrupt elevated response in 4th week that mimics augmented immune response of subcutaneous administration of alum-HBsAg along with a booster dose at day 28. This elevated response of copolymeric nanoparticle may be attributed to degradation dominated release of antigen from the nanoparticles as shown by *in vitro* release study (Fig. 4). This pulsatile release is prerequisite to generate booster kind effect giving augmented IgG titre in the blood plasma.

The findings of immunological studies indicate the failure of plain PLA nanoparticles in generation of effective immune response after the oral administration. Although hydrophobic nature of PLA nanoparticles favor their effective transport through the epithelial barriers, they might have not been able to keep their intactness by the enzymatic attack at the low gastric pH, which are basic hurdles in the oral delivery of proteins (Fasano, 1998; Morishita and Peppas, 2006). Further, the generation of acidic microclimate, causing instability of encapsulated HBsAg (Pitt et al., 1981; Audran et al., 1998; Johanson et al., 1998), might have added up in fail-

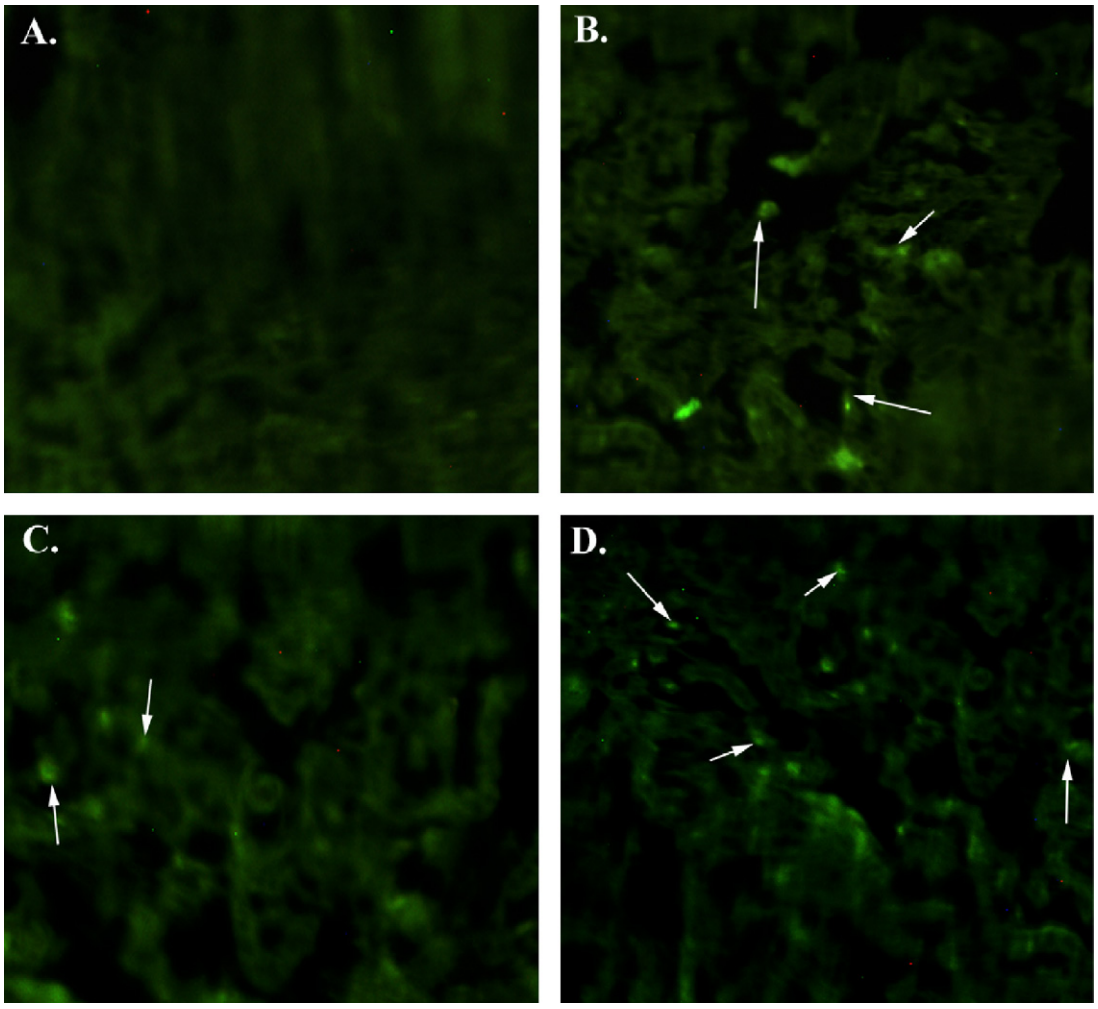


Fig. 5. Fluorescent microscopy images showing the uptake of FITC–BSA loaded different nanoparticles by gut mucosa after oral administration. (A) Soluble FITC–BSA (control); (B) AB nanoparticles; (C) ABA nanoparticles; (D) BAB nanoparticles.

ure of PLA nanoparticles (Jain et al., 2009). Whereas, developed copolymeric nanoparticles exhibited promising result. As shown by *in vitro* stability studies (Fig. 3), the copolymeric nanoparticles were significantly stable in such harsh condition and overcome these preliminary barriers of oral administration. This stability is attributed to the presence of free PEG chains on the nanoparticles surface forming the corona, which shields the antigen containing core (Tobío et al., 2000). As an exception to the hydrophobic thumb rule, these hydrophilic nanoparticles were capable to penetrate the epithelial barrier; as indicated in fluorescent microscopy (Fig. 5). Here free PEG chains again play a major role and get entangled into the mucosal layer to promote the transport, these results can be correlated to the earlier studies (Vila et al., 2004). Alum-HBsAg formulation, that received a booster dose, exhibited highest IgG titre through out the study (Fig. 6A), whereas IgG responses of copolymeric nanoparticles were found only comparative to them (without receiving the booster dose). Although there was no significant difference observed between block copolymer nanoparticles, BAB nanoparticles could be concluded to generate most significant IgG titre as compared to plain PLA nanoparticles.

Developed copolymeric nanoparticles exhibited effective mucosal immunity by the generation of sIgA at local and distal mucosal surfaces (Fig. 6B). The capability of different formulations to provoke sIgA was evaluated after day 42 in different mucosal secretions viz. nasal, salivary, intestinal and vaginal secretion. The results shown that alum-HBsAg failed to produce considerable sIgA titre. Whereas different copolymeric nanoparticles demonstrated significantly higher sIgA titre in all tested mucosal secretions as compared to plain PLA nanoparticles (Fig. 6B).

For the complete eradication of viral infection like hepatitis B, a vaccine should generate the complete spectrum of immunity including humoral, mucosal as well as cellular immune response. Adjuvancity of developed formulation to generate cellular immunity was evaluated by determining the IgG isotypes (IgG1 and IgG2a) in blood serum after day 42 of immunization (Fig. 6C). Alum-HBsAg formulation exhibited effective IgG1 titre but failed to generate IgG2a titre that is the hallmark of T_H1 (cellular) immune response. Whereas developed copolymeric nanoparticles gave mixed T_H1/T_H2 response with good IgG2a/IgG1 ratio indicating both cellular and humoral immune responses. As shown in Fig. 6C, BAB nanoparticles were able to produce most significant IgG2a titre than AB and ABA nanoparticles as compared to PLA nanoparticles.

For further confirmation of cellular immunity, the endogenous cytokine levels (IFN-γ, IL-2 and IL-4) were determined after 42 days of immunization. Interferon gamma (IFN-γ) and interleukin-2 (IL-2) are secreted by T_H1 cells leading to sequential events for the generation of IgG2a, whereas IL-4, IL-5, IL-6 are secreted by T_H2

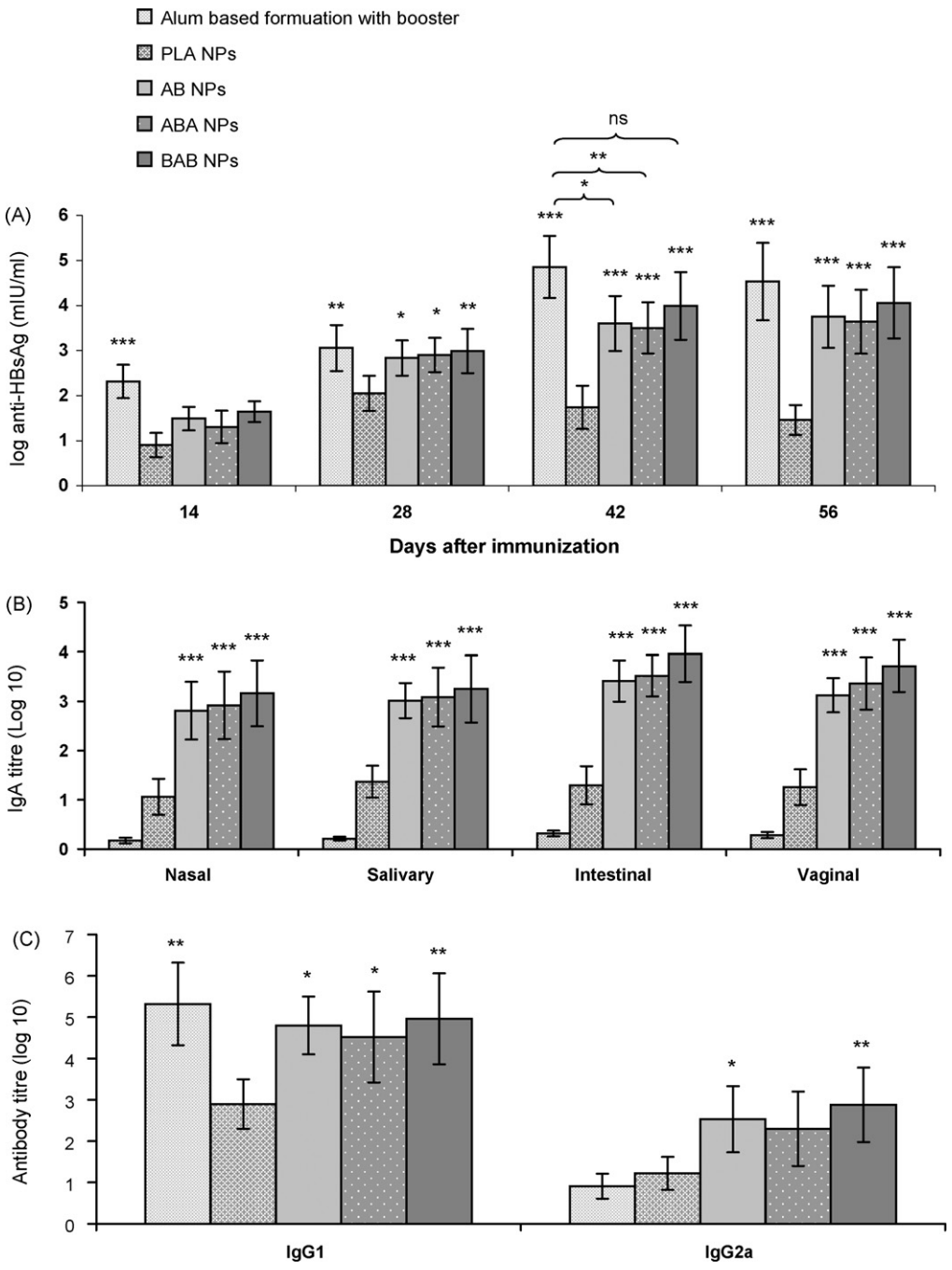


Fig. 6. Immunological studies. (A) Serum anti-HBsAg profile of mice immunized with different formulations containing HBsAg. Only alum based formulation received a booster dose at day 30; (B) sIgA level in the salivary, intestinal, vaginal and nasal secretion after immunization with various formulations at day 42; (C) IgG1 and IgG2a anti-HBsAg antibody isotopes at day 42 in sera of mice. The antibody titre after immunization with PLA, AB, ABA, BAB nanoparticles and alum based formulation were compared. Each data represents mean ± S.D. ($n = 6$). Significance was tested using one-way ANOVA and Tukey–Kremer post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the group treated with PLA nanoparticles (unless otherwise indicated).

cells generating IgG1 isotype. The results of cytokine assay are summarized in Fig. 7. Copolymeric nanoparticles exhibited significantly higher IL-2 and IFN response as compared to the alum-HBsAg and PLA nanoparticles. IL-4 response was dominated by alum-HBsAg (Fig. 7C) that was also depicted by IgG response graph (Fig. 7A), where copolymeric nanoparticles could produce only comparable results.

The results of immunological studies clearly indicate efficient adjuvancity of copolymeric nanoparticles for the generation of complete spectrum of immunity for the effective eradication of viral infection after the oral dosing that too without the need of booster dose. Although the humoral immune response exhibited by the copolymeric nanoparticles was found to be only comparative to the conventional alum-HBsAg vaccine, they were able to produce the effective level of immunity at the mucosal surface, the primary infection site of variety of viruses. Moreover, copolymeric nanoparticles generated effective cellular immune response that was not found with the alum-HBsAg. The results of *in vivo* studies conclude BAB nanoparticles as most promising candidate for oral immunization among developed copolymeric nanoparticles.

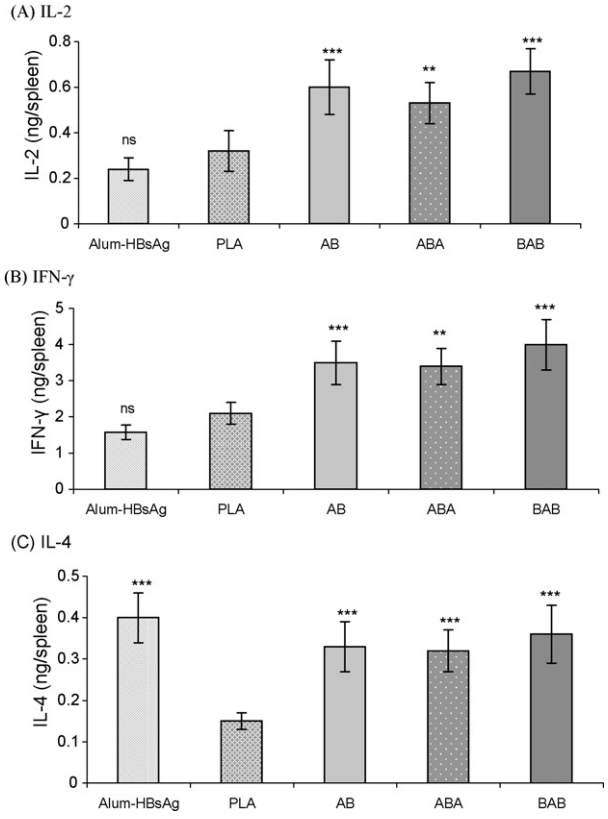


Fig. 7. Cytokine levels of mice immunized with different formulations. (A) Interleukin-2; (B) interferon-γ; (C) interleukin-4. Values are expressed as mean ± S.D. ($n = 6$). Block copolymeric nanoparticles exhibited significantly higher cytokine levels as compared to PLA nanoparticles. As compared to alum-HBsAg vaccine copolymeric nanoparticles exhibited significant higher level of IL-2 and IFN-γ that are the hallmarks of cellular immunity. Each data represents mean ± S.D. ($n = 6$). Significance was tested using one-way ANOVA and Tukey–Kremer post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the group treated with PLA nanoparticles.

4. Conclusion

Immunologists have preferentially used PLA/PLGA nanoparticles as vaccine delivery systems but poor stability of these nanoparticles in harsh GIT conditions has limited their use in oral vaccination. PLA has been copolymerized with PEG to enhance stability and bioavailability of these nanoparticles. Developed nanoparticles of triblock copolymer of PLA and PEG (BAB) demonstrated better stability of nanoparticles into harsh gastrointestinal environments and maintaining the antigenicity of the encapsulated HBsAg. Presence of hydrophilic moiety (PEG) at both end of PLA in BAB copolymer provide efficient cellular uptake and impressively elicit effective immune response. Furthermore, developed copolymeric nanoparticles exhibited all three arms of immunity (cellular, humoral and mucosal) after the oral administration, without need of any booster dose. We foresee that this copolymeric nanoparticulate delivery system can be used in future experiments on a larger scale and as an approach not only to enhance the immune response but also prolong the duration of immunity, however, further studies are required to establish clinical potential.

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