

DEVELOPMENT AND CHARACTERIZATION OF BIPOLYMER BASED NANOPARTICULATE CARRIER SYSTEM AS VACCINE ADJUVANT FOR EFFECTIVE IMMUNIZATION

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Received: 15 Feb 2013, Revised and Accepted: 04 Apr 2013

ABSTRACT

Now a days poly(lactic-co-glycolic acid) (PLGA) is one of widely used biodegradable polymer in vaccine delivery. However, the use is restricted due to hydrophobic nature and generation of acidic microenvironment upon its degradation, rendering it unfavorable to the encapsulated proteins. In the present study we have synthesized gelatin nanoparticle, Poly(lactic-co-glycolic acid) (PLGA) nanoparticles and PLGA coated gelatin nanoparticles (PGNPs). Where TT (Tetanus Toxoid) is taken as a model antigen and encapsulated to GPNs by double desolvation method, in PGNPs by double emulsification method and PGNPs by phase separation method. Nanoparticles were characterized in vitro for their size, shape, entrapment, and release. FITC-TT conjugate was used to determine entrapment and release from nanoparticles. The average diameter of the PGNPs was found to be around 600 ± 25 nm and protein entrapment efficiency was found to be 76.2%. Protein release experiments indicated sustained release characteristics of developed PGNPs. During stability studies the system demonstrated its capability to prevent denaturation of the encapsulated protein. Finally immunological studies were performed that indicated effective immune response by means of humoral, cellular and mucosal immunity. The results of current study indicate PGNPs as potential candidate for mucosal immunization.

Keywords: PLGA; Gelatin; Nanoparticles; FITC-TT conjugate; PGNPs; vaccine delivery.

INTRODUCTION

Novel strategies are being explored to overcome the limitations associated with the use of conventional vaccines. Polymeric microparticles [1,2,3] and nanoparticles [4,5,6] have been extensively used for vaccine delivery. During last decade biodegradable polymeric systems have emerged as promising candidates since they provide adjuvancity with prolonged release characteristics. When an antigen is associated with these particulate carriers, a stronger immune response compare to soluble antigen is elicited [7,8,9]. Aliphatic polyesters are found to be one of the most attractive biodegradable polymers as their backbones cleave easily by hydrolysis yielding non-toxic products that can be absorbed in and/or eliminated from tissue or cells [10,11]. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), or their copolyesters (PLGA) are widely accepted and explored for the delivery of bioactives as their safety and tissue compatibility are well known with their use.[12,13]

The use of PLA for controlled vaccine delivery has limitations due to its unfavorable physicochemical properties such as hydrophobicity and degradation behavior. PLA degrades into lactic acid that creates acidic microenvironment, causing instability to antigen contained in the delivery vehicle. Additionally, it requires protein stabilizer to protect antigen from organic solvent and high mechanical stresses encountered in encapsulation process. To overcome these limitations various additives, such as antacids and the protein stabilizers have been used.[3,14,16]

The biodegradable polymeric systems may be further categorized into hydrophilic polymeric systems, such as gelatin nano- and microparticles, and hydrophobic polymeric systems, such as PGNPs [17,18] Neither the hydrophilic nor the hydrophobic system is ideal for protein/peptide drug delivery. They each have their own advantages and disadvantages. For example, the hydrophilic polymeric systems are biocompatible with the protein/peptide drugs, but have difficulty achieving sustained drug release. When the systems absorb water and swell, protein/peptide molecules will rapidly diffuse out. In contrast, the hydrophobic polymeric systems have the capability of yielding sustained drug release. However, they are incompatible with the water soluble protein/peptide drugs. The hydrophobicity of the polymers may induce unfolding of protein/peptide molecules; therefore, the protein/peptide drugs may lose their biological activity after being loaded in and then released from the hydrophobic polymeric systems[12,13]. To promote the advantages and overcome the disadvantages of both the hydrophilic and the hydrophobic polymeric systems, we

combined a hydrophilic system, GPNs, with a hydrophobic polymer PLGA is coated over Gelatin nanoparticles by EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride]. This combination creates a new biodegradable system for protein and/or peptide drug delivery.

MATERIALS AND METHODS

Materials

PLGA with a lactide/glycolide ratio of 50:5 (40-75 kDa;) Polyvinyl alcohol (13-23 kDa; 88% hydrolyzed), type A Gelatin (from porcine skin, 300 bloom.) were purchased from Sigma Pvt. Ltd (USA) Tetanus Toxoid (TT) (16.6 mg protein/ml) was kindly gifted by Shanta Biotech Hyderabad, BCA protein estimation kit (KT-31) and protein molecular weight markers were purchased from Bangalore Genei Pvt. Ltd., India. Enzyme linked immunoassay kit (AUSAB) was purchased from Abbott Laboratories, USA. 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.

Preparation of FITC-TT Conjugate

Before the preparation of antigen-loaded formulations, FITC-labeled TT was prepared for the determination of percentage entrapment efficiency and antigen release. A total of 10mg of fluorescein isothiocyanate (FITC) was dissolved in 5ml of carbonate buffer pH 9.5, and 5ml of TT solution was added. The mixture was gently stirred in the dark at 4°C for 20h. The solution was then extensively dialysed against Milli-Q™ water using a 10,000 MW cut-off membrane. The resulting FITC-tetanus toxoid (FITC-TT) solution was freeze-dried and subsequently protected from light and stored at 4°C. Prior to use, the protein was analyzed for the presence of free FITC by ultra-centrifugation (10,000×g for 20min) of an aqueous solution through a 10,000 MW cut-off filter unit (Millipore India Pvt. Ltd., Bangalore, India). Unbound FITC was found to be less than 1.4% of total fluorescence in all cases[15].

Preparation of Gelatin Nanoparticles (GNPs)

GNPs are prepared by two step desolvation method [23]. 0.625 g, of gelatin type A (bloom strength 300) was dissolved in 2.5 ml of water under gentle heating (50° C) with 2ml of TT solution. First desolvation step was initiated by the addition of 12.5 ml acetone. After sedimentation of precipitated gelatin fractions for a certain time, the supernatant consisting of some desolvated gelatin as well as gelatin in solution was discarded, the sediment was redissolved

by addition of 12.5 ml water under heating and the pH was adjusted to 2.5 by HCl (1M). GNPs were formed during second desolvation step by dropwise addition of 25ml acetone under constant stirring (500rpm). After 10 min. 200ul of glutaraldehyde (25%) was added to the reaction vessel to crosslink the nanoparticles, Finally after stirring for 6 hrs the particles were purified by three-fold centrifugation (16000g for 20min.), lyophilized and stored for further studies.

Preparation of PLGA nanoparticles (PNPs)

PNPs were prepared by Double Emulsion-Solvent Evaporation process as reported by Lima et al. with slight modifications.[22]

Aqueous solution phase was vigorously mixed with an organic phase consisting 5% PLGA in DCM (2 ml) by sonication for 30 seconds (Branson Sonicator 250, New Delhi, India). To this water-in-oil emulsion aqueous polyvinyl solution (10mL, 1% w/v) was added and mixed at high speed (RQ-122, Remi Motors, Mumbai) to obtain a w/o/w emulsion. Following solvent evaporation overnight, hardened polymeric nanoparticles were harvested by centrifugation.

Preparation of PLGA coated gelatin nanoparticles (PGNPs)

The system was made by using the phase separation method. By taking the optimized parameters to form GNPs and PNPs the final formulation of PGNP system was prepared.[24]

A 10% (w/w) polymer solution was prepared by dissolving PLGA in DCM. lyophilized GNPs (150 mg) containing 25 mg of TT were suspended in 5 mL of the PLGA solution. The suspension was stirred at 200 rpm. Silicon oil (6 g) was then progressively added to the suspension at a speed of 1 g/min to form an oil-in-oil emulsion. Once the droplets of the PLGA solution containing GNPs reached the desired size (7-10 μ m), the emulsion was transferred with stirring to a beaker containing 2 L heptane.

Finally, the nanoparticles were collected by filtration, washed with heptane, and dried in a vacuum container for 24 h.

Characterization

Morphology

The formulations were observed for their surface morphology by scanning and transmission electron microscopy (SEM and TEM). Scanning electron microscopy was performed using JEOL 6100, Japan. The nanoparticles were placed on the sample holders, sputter coated with gold and then placed in scanning electron microscope. For Transmission electron microscopy specimens were prepared by dropping the dispersion onto carbon-coated EM grids. The grids were held horizontally for 20 s, then tilted at 45 degree to allow excess fluid to drain. The grid was returned to the horizontal position and a drop of phosphotungstic acid (adjusted to pH 4) was added to give a negative stain. The grid was then left to stand for 20 s before removing excess stain as above. Specimens were air-dried and examined using a Philips CM12, Dindhoven Netherlands transmission electron microscope.

Size and Size Distribution Measurement

Gelatin nanoparticle-PLGA nanoparticles (PGNPs) (5 mg) was suspended in 15 mL of distilled water by sonicating the suspension for 10 s using a probe sonicator (Model 2000 U, B. Braun Biotech, Inc., Allentown, PA). The suspension was then evaluated using a particle sizer (CILAS-1064) for average size and size distribution. For each sample the mean value \pm SD of four determinations were established.

Entrapment Efficiency Determination

Percent entrapment of the loaded antigen was determined by directly recovering protein from the PGNPs. The experiment was conducted in triplicate. A dried PGNPs (20mg) was accurately weighed and transferred to a separating funnel. DCM (2ml) was added to the PGNPs sample. After the PGNPs was dissolved, phosphate buffer (pH 7.4, 4ml) was added to the separating funnel. The funnel was capped with stopper and the mixture was agitated in a shaker for one day, then the separating funnel was left hanging until aqueous phase and the organic phase separated completely,

after the phase separation the aqueous layer was collected. Amount of the protein was determined by measuring fluorescence (excitation wavelength: 488nm, emission wavelength: 520nm) in the supernatant using a fluorescence spectrophotometer (RF-5301PC spectrofluorophotometer, Shimadzu). All fluorescence readings were analyzed using a standard curve of FITC-TT in PBS (pH 7.4). To correct for interferences, background fluorescent intensity reading was subtracted (i.e. fluorescent intensity readings for the unloaded nanoparticles treated in the same way as FITC-TT loaded nanoparticles).

Determination of residual PVA

The colorimetric method was used to quantitate the amount of residual PVA content of the PGNPs. In brief, 5 mg of washed and lyophilized PGNPs were hydrolysed in 2 ml of 0.5 N sodium hydroxide solution for 15 min at 60 °C. The solution was neutralized with 900 μ l of 1 N HCl and volume was made up to 5 ml with milli-Q water. Three milliliters of a 0.65 M solution of boric acid, 0.5 ml of a solution of I₂/KI (0.05 M/0.15 M) and 1.5 ml of milli-Q water were added to the neutralized solution and absorbance of the solution was determined at 690 nm after a 15 min incubation (Shimadzu-mini 1240).[25]

In Vitro Protein Release Study

The in vitro release profile of antigen from the PGNPs was preformed in triplicate. The PGNPs (50 mg) was dispersed in 2 ml of PBS (7.4) The dispersion was incubated at 37°C in a shaking water bath with shaking speed of 36 rpm. 1ml supernatant in a vial was withdrawn at various time periods (0,9,18,27,38) and 1ml of PBS was added, the contents of the vial were centrifuged at 4000 rpm for 10 min. The amount of FITC-TT released was determined by measuring the fluorescent intensity of the supernatant. Enzyme solution was replaced each time in the respective vial containing nanoparticles to maintain a constant volume. The fluorescent intensity of the supernatant obtained at different time intervals was measured under the same conditions as described above for the determination of FITC-TT entrapment and the cumulative amount released was calculated from the appropriate calibration curves. For both the studies, background fluorescent intensity reading was subtracted (i.e. fluorescent intensity readings for the unloaded nanoparticles treated in the same way as FITC-TT loaded nanoparticles).

Assessment of structural integrity of antigen.

The SDS-PAGE was performed in order to check the integrity of the attached antigen in the final formulation. The samples were loaded onto SDS electrophoresis assembly (Bio-Rad, USA) using 5% stacking gel and 10% separation gel, run at 60-110 V until the dye band reached the gel bottom. After migration the gel was removed and stained with Coomassie blue to locate respective position of proteins, which was then destained.

The antigen loaded formulations containing a known amount of antigen were incubated with 0.1% w/v SDS (in PBS) at room temperature for 1 hr with gentle shaking and then centrifuged at 4,000 rpm for 25 minutes. The supernatants were used for sample preparation. The whole procedure of electrophoresis was performed as before, except by taking antigen loaded formulations (GNPs, PNPs and PGNPs) simultaneously with pure antigen, instead of antigen alone.

Fluorescence microscopy

The fluorescence microscopy was performed to confirm deposition of selected carrier system in NALT (nasal associated lymphoid tissue) after the nasal administration. Fluorescent Isothiocyanate (FITC) conjugated 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 through nostrils (explain elsewhere in the article), after 30 min the mice were sacrificed. Nasal cavity containing nasal mucosa was cut into pieces 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).

Statistical analysis

All data were expressed as mean±standard deviation. Comparisons among three or more groups were performed by analysis of variance (ANOVA) followed by post hoc Tukey–Kramer test. For comparison between two groups, Student's t-test was applied. A p value less than 0.05 was considered to indicate statistical significance for all comparisons.

RESULTS AND DISCUSSION

Preparation of gelatin nanoparticles.

Gelatin nanoparticles were prepared by two step desolvation method and the various formulations and process variables were checked against the particle size and Poly -dispersivity index. The various formulations shown in Tab. 1 and formulation codes in Tab.2 showed that the formulation code TM-1 is most suitable as it is found to be **63.4±5.8** which is less than 100 nm and PDI ranges roughly **0.135±0.09** which is suitable for the coating of PLGA.

Preparation of PLGA nanoparticles (PNPs)

PNPs were prepared by Double Emulsion-Solvent Evaporation method and the various formulations and process variables were checked against the particle size and Poly -dispersivity index. The

various formulations shown in Tab. 3 and fromulction codes in Tab.4 showed that the formulation code TG-2 is most suitable as it is found to be **694±53** and PDI ranges roughly **0.129±0.013**. which is less than 1000 nm shows their superiority to be administered by nasal route.

Preparation of PLGA coated Gelatin nanoparticles (PGNPs)

A 10% (w/w) polymer solution was prepared by dissolving PLGA in DCM. To this equimolar conc. of EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride] was kept overnight which is considered as the zero length linker to this lyophilized GNPs (150 mg) containing 25 mg of TT were suspended in 5 mL of the PLGA solution. The suspension was stirred at 200 rpm. Silicon oil (6 g) was then progressively added to the suspension at a speed of 1 g/min to form an oil-in-oil emulsion. Once the droplets of the PLGA solution containing GNPs reached the desired size (7-10 μ m), the emulsion was transferred with stirring to a beaker containing 2 L heptane. Finally, the PGNPs were collected by filtration, washed with heptane, and dried in a vacuum container for 24 h.

EDC being the zero length linker conjugate first with the free carboxylic group of PLGA and than with the free amino group of the lyophilized gelatin as shown in the fig.1.

Table 1: Optimization of GNPs

| Formulation Code | Effect of temperature (°C) | Conc. Of Gluteraldehyde (ul) | Rate of desolvant addition (ml/min) | Particle size (nm) | Poly -dispersivity index (PDI) |
|------------------|----------------------------|------------------------------|-------------------------------------|--------------------|--------------------------------|
| TM-1 | 40 | 200 | 3.5 | 63.4±5.8 | 0.135±0.09 |
| TM-2 | 50 | 200 | 3.5 | 114.7±10.1 | 0.199±0.012 |
| TM-3 | 60 | 200 | 3.5 | 303.7±23.87 | 0.212±0.015 |
| GTD-1 | 40 | 100 | 3.5 | 61.4±5.9 | 0.247±0.03 |
| GTD-2 | 40 | 200 | 3.5 | 63.4±5.8 | 0.135±0.09 |
| GTD-3 | 40 | 400 | 3.5 | 79.4±6.9 | 0.167±0.02 |
| RD-1 | 40 | 200 | 3.5 | 63.4±5.8 | 0.135±0.09 |
| RD-2 | 40 | 200 | 5-10 | 129.6±9.3 | 0.231±0.09 |
| RD-3 | 40 | 200 | 10-15 | 212. ±17.3 | 0.181±0.05 |

Table 2: Formulation Codes

| Formulation Codes | Codes |
|-------------------|-------------------------------|
| TM-1,2,3 | Temperature variables |
| GTD-1,2,3 | Gluteraldehyde variables |
| RD-1,2,3 | Rate of Desolvation variables |

Table 3: Optimization of PNPs

| Formulation Code | Polymer Conc. (mg) | % PVA | Stirring Time (hrs) | Particle size (nm) | Poly -dispersivity index (PDI) |
|------------------|--------------------|------------|---------------------|--------------------|--------------------------------|
| PG-1 | 25 | 1.0 | 2 | 497±28 | 0.131±0.09 |
| PG-2 | 50 | 1.0 | 2 | 694±53 | 0.129±0.013 |
| PG-3 | 75 | 1.0 | 2 | 483±45 | 0.143±0.05 |
| PV-1 | 50 | 0.5 | 2 | 521±41 | 0.134±0.04 |
| PV-2 | 50 | 1.0 | 2 | 683±34 | 0.122±0.04 |
| PV-3 | 50 | 2.0 | 2 | 473±22 | 0.151±0.09 |
| ST-1 | 50 | 1.0 | 1 | 519±49 | 0.141±0.05 |
| ST-2 | 50 | 1.0 | 2 | 672±23 | 0.127±0.013 |
| ST-3 | 50 | 1.0 | 3 | 493±34 | 0.153±0.05 |

Table 4: Formulation Codes

| Formulation Codes | Codes |
|-------------------|-------------------------|
| PG-1,2,3 | Polymer variables |
| PV-1,2,3 | PVA variables |
| ST-1,2,3 | Stirring Time variables |

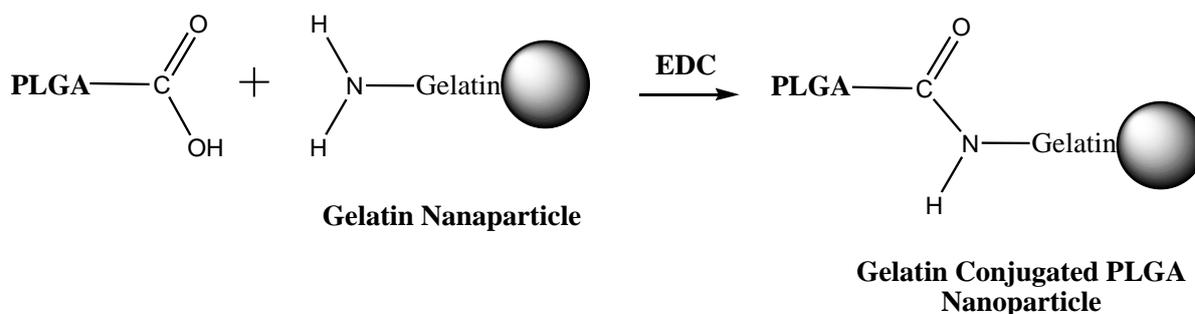


Fig. 1: Conjugation using the carboxyl group of PLGA and the amine-group of Gelatin.

Microscopic Characteristics, Size and Size Distribution

Size and Size distribution was evaluated by Particle sizer (CILAS-1064), 5 mg of PGNP were suspended in 15 mL of distilled water by

sonicating the suspension for 10 s using a probe sonicator (Model 2000 U, B. Braun Biotech, Inc., Allentown, PA). For each sample the mean value \pm SD of four determinations were established, and found to be in the range of 694.49 nm.

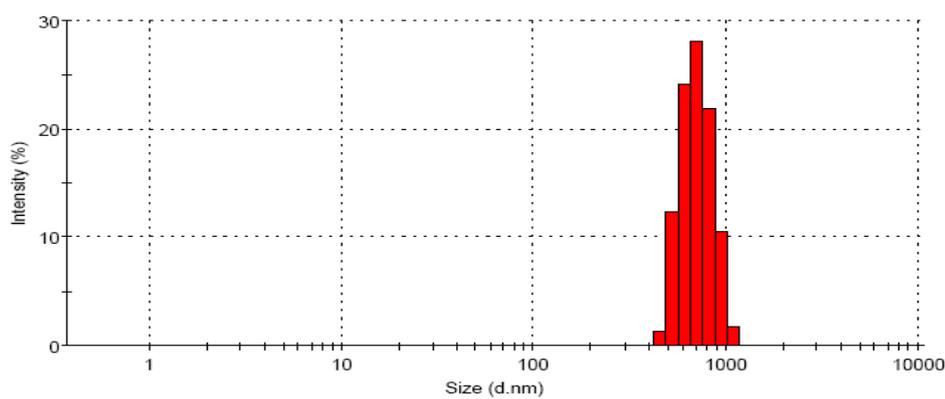


Fig. 1: Size distribution of the optimized formulation of PGNPs (694.49 nm).

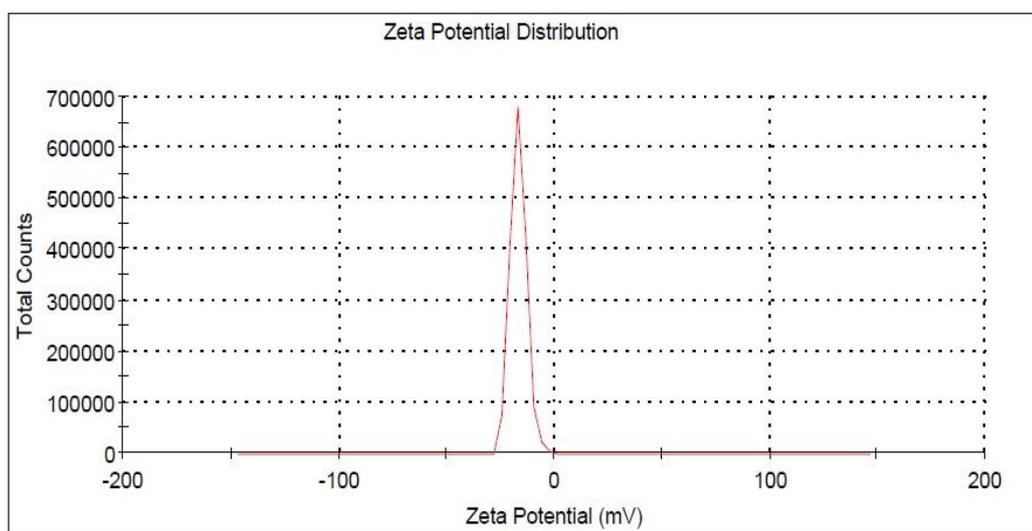


Fig. 2: Zeta potential analysis of final optimized formulation = -11.8mV

Morphology

We additionally analyzed this nanoparticle batch by SEM to visualize the particles and investigate their morphology. SEM analysis demonstrated that PGNPs prepared by the optimized phase separation procedure are colloidal spheres with a smooth surface morphology (Figure 21).

Fig.2.(a) shows a typical population of the unloaded PGNPs prepared by the phase separation method. Fig.1.(b) and (c)

shows TT loaded PGNPs. From these micrographs, one can see that the PGNPs, unloaded and TT-laden are spherical in shape and uniform in size. As seen from the higher magnification SEM image (Fig.2-c) illustrates that the GPNPs were coated by the PLGA matrix and not adsorbed to the surface. If the GPNPs had been on the surface, the surface morphology would have been fairly rough.

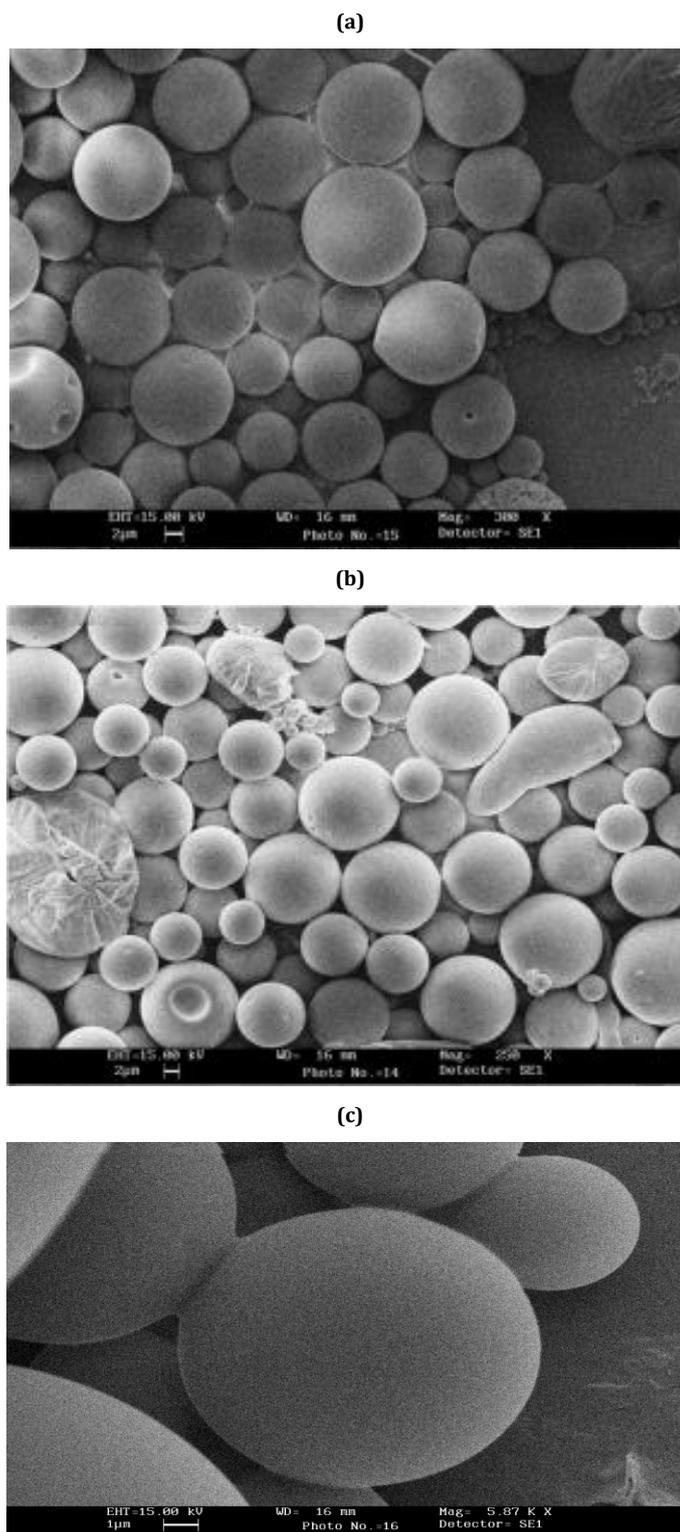


Fig. 2:(a) SEM Photomicrographs of unloaded PGNPs : (b) & (c) TT Loaded PGNPs.

Protein Entrapment Efficiency

Percent entrapment of the loaded antigen was determined by directly recovering protein from the PGNPs. The experiment was conducted in triplicate. A dried PGNPs (20mg) was accurately weighed and transferred to a separating funnel. DCM (2ml) was added to the PGNPs sample. After the PGNPs was dissolved, phosphate buffer (pH 7.4, 4ml) was added to the separatory funnel. The funnel was capped with stopper and the mixture was agitated in

a shaker for one day, then the separating funnel was left hanging until aqueous phase and the organic phase separated completely, after the phase separation the aqueous layer was collected. Amount of the protein was determined by measuring fluorescence (excitation wavelength: 488nm, emission wavelength: 520nm) in the supernatant using a fluorescence spectrophotometer (RF-5301PC spectrofluorophotometer, Shimadzu). All fluorescence readings were analyzed using a standard curve of FITC-TT in PBS (pH 7.4). To correct for interferences, background fluorescent intensity reading

was subtracted (i.e. fluorescent intensity readings for the unloaded nanoparticles treated in the same way as FITC-TT loaded nanoparticles).

$$\text{Entrapment efficiency} = A_{\text{actual}} / A_{\text{theoretical}} \times 100$$

where A_{actual} is the actual amount of TT in each PGNPs determined by the above experiment and $A_{\text{theoretical}}$ is the theoretical amount of TT in each PGNPs calculated from the quantity added in the process which is found to be 72.6% w/w.

Protein Release

The *in vitro* release profile of antigen from the PGNPs was performed in triplicate. 50 mg of lyophilized PGNPs was dispersed in 2 ml of PBS (7.4) The dispersion was incubated at 37°C in a shaking water bath

with shaking speed of 36 rpm. 1ml supernatant in a vial was withdrawn at various time periods in days (0,9,18,27,38) and added 1ml of PBS, the contents of the vial were centrifuged at 4000 rpm for 10 min. The amount of FITC-TT released was determined by measuring the fluorescent intensity of the supernatant. Enzyme solution was replaced each time in the respective vial containing nanoparticles to maintain a constant volume. The fluorescent intensity of the supernatant obtained at different time intervals was measured under the same conditions as described above for the determination of FITC-TT entrapment and the cumulative amount released was calculated from the appropriate calibration curves (Fig.3). For all the studies, background fluorescent intensity reading was subtracted (i.e. fluorescent intensity readings for the unloaded nanoparticles treated in the same way as FITC-TT loaded nanoparticles).

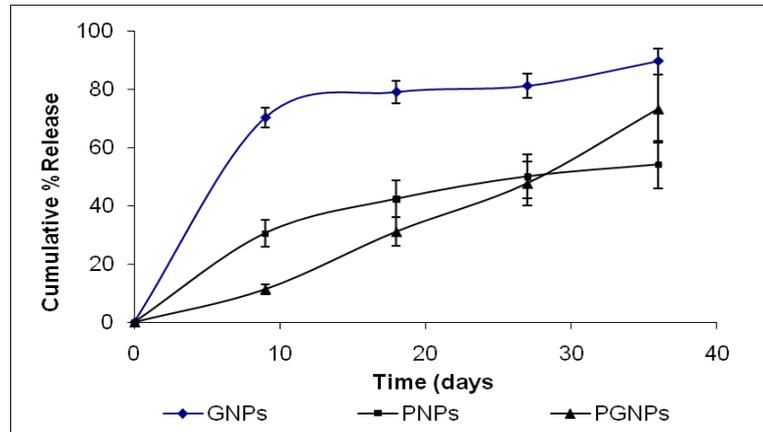


Fig. 3: In-Vitro release profile of GNPs, PNPs, PSNPs.

Assessment of structural integrity of antigen

The SDS-PAGE was performed (as discussed earlier) in order to check the integrity of the attached antigen in the final formulation (Fig.4.). The samples were loaded onto SDS electrophoresis assembly (Bio-Rad,USA) using 5% stacking gel and 10% separation gel, run at 60–110 V until the dye band reached the gel bottom. After migration the gel was removed and stained with Coomassie blue to locate respective position of proteins, which was then destained.

The antigen loaded formulations containing a known amount of antigen were incubated with 0.1% w/v SDS (in PBS) at room temperature for 1 hr with gentle shaking and then centrifuged at 4,000 rpm for 25 minutes. The supernatants were used for sample preparation. The whole procedure of electrophoresis was performed as before, except by taking antigen loaded formulations (GNPs and PGNPs) simultaneously with pure antigen, instead of antigen alone.

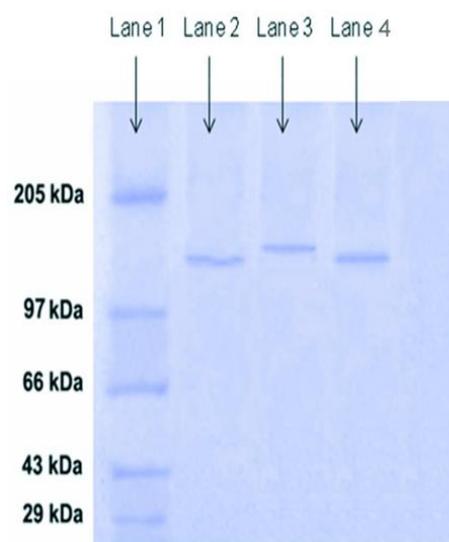


Fig. 4: SDS-PAGE analysis showing structural integrity of release antigen. Lane 1: Marker Proteins(205 kDa myosin, rabbit muscle; 97 kDa phosphorylase B; 67 kDa bovine serum albumin; 43 kDa; ovalbumin; 29 kDa carbonic anhydrase); Lane 2 : Pure TT Lane3 : TT recovered from GNPs ,Lane 4 : TT recovered from PGNPs

Fluorescence microscopy

The fluorescence microscopy was performed to confirm deposition of selected carrier system in NALT (nasal associated lymphoid tissue) after the nasal administration. Fluorescent Isothiocyanate (FITC) conjugated TT was used as a fluorescence marker (Fig. 5). FITC labeled PGNPs were prepared according to the procedure described earlier, using a 0.05% FITC-TT solution in PBS. FITC-TT loaded formulation was administered to mice through nostrils (explain elsewhere in the article), after 30 min the mice were sacrificed. Nasal cavity containing nasal mucosa was cut into pieces 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). Which indicates in Fig. 4. that the formulated PGNPs penetrates the nasal mucosa and were available for the peripheral cells to be expressed.

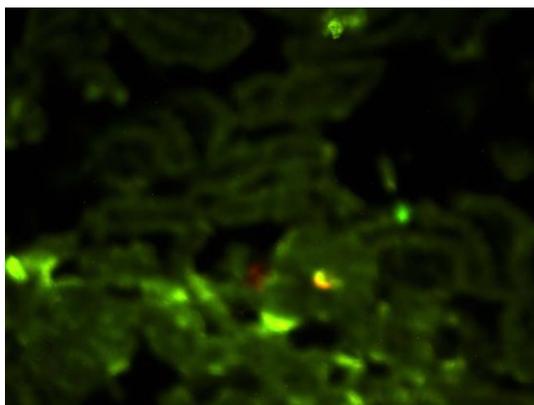


Fig. 5: Fluorescent Microscopy of Nasal Mucosa.

CONCLUSION

A bipolymer based novel biodegradable system for prolonged or controlled release of protein/peptide drugs has been developed. This new system is a combination of a hydrophobic polymer (PLGA) and a hydrophilic polymer (Gelatin). The gelatin is formulated as nanoparticles while the PLGA is coated over the nanoparticles through EDC. FITC-TT conjugates were prepared first before loading it in the system for entrapment efficiency and release profile studies. The experimental results show that the TT-loaded gelatin nanoparticles were successfully coated by PLGA. The coating was conducted by a phase separation method. This coating practice creates a new bipolymer system. The average diameter of the PGNPs is between 650 to 700 nm. TT loading efficiency is 76.2% for the PGNPs prepared by the phase separation method. Release experiments show that only 66% of TT is released from the PGNPs in 18 days, which indicates that this new system possesses sustained release characteristics for protein drugs. This new system also demonstrates the capability of preventing protein drugs from integrity loss or denaturation as shown by the SDS PAGE.

ACKNOWLEDGMENTS

Authors are thankful to Shantha Biotech Ltd. (Hyderabad, India) for providing gift sample of Tetanus Toxoid (TT). Authors are also thankful to AIIMS (New Delhi, India), SICART (CVM, Vallabh Vidyanagar, India), IUC, Indore for SEM, PRC for Fluorescence Spectroscopy.

REFERENCES

1. J.S. Moynihan, D.H. Jones, G.H. Farrar, C.R. Howard, A novel microencapsulated peptide vaccine against hepatitis B, *Vaccine* 19 (2001) 3292-3300.
2. D.T. O'Hagan, M. Singh, Microparticles as vaccine adjuvants and delivery systems, *Expert. Rev. Vaccines* 2 (2003) 269-283.
3. K.S. Jaganathan, P. Singh, D. Prabakaran, V. Mishra, S.P. Vyas, Development of a single dose stabilized poly(D,L-lactic-co-

- glycolic acid) nanoparticles -based vaccine against hepatitis B, *J. Pharm. Pharmacol.* 56 (2004) 1243-1250.
4. Vila, A. Sanchez, M. Tobio, P. Calvo, M.J. Alonso, Design of biodegradable particles for protein delivery, *J. Control. Release* 78 (2002) 15-24.
5. M. Koping-Hoggard, A. Sanchez, M.J. Alonso, Nanoparticles as carriers for nasal vaccine delivery, *Expert. Rev. Vaccines* 4 (2005) 185-196.
6. J. Wendorf, M. Singh, J. Chesko, J. Kazzaz, E. Soewanan, M. Ugozzoli, D. O'Hagan, A practical approach to the use of nanoparticles for vaccine delivery, *J. Pharm. Sci.* 95 (2006) 2738-2750.
7. J.E. Eyles, E.D. Williamson, H.O. Alpar, Immunological responses to nasal delivery of free and encapsulated tetanus toxoid: studies on the effect of vehicle volume, *Int. J. Pharm.* 189 (1999) 75-79.
8. R.S. Raghuvanshi, Y.K. Katare, K. Lalwani, M.M. Ali, O. Singh, A.K. Panda, Improved immune response from biodegradable polymer particles entrapping tetanus toxoid by use of different immunization protocol and adjuvants, *Int. J. Pharm.* 245 (2002) 109-121.
10. A.K. Goyal, A. Rawat, S. Mahor, P.N. Gupta, K. Khatri, S.P. Vyas, Nanodecoy system: a novel approach to design hepatitis B vaccine for immunopotentiality, *Int. J. Pharm.* 309 (2006) 227-233.
11. D.E. Perrin, J.P. English, Handbook of biodegradable polymers, in: A.J. Domb, J. Kost, D.M. Wiseman (Eds.), *Polyglycolide and Polylactide*, vol. 9, Taylor and Francis group, 1997, pp. 1-67.
12. D.M. Abou-Zeid, R.J. Muller, W.D. Deckwer, Degradation of natural and synthetic
13. polyesters under anaerobic conditions, *J. Biotechnol.* 86 (2001) 113-126.
14. J.M. Anderson, M.S. Shive, Biodegradation and biocompatibility of PLA and PLGA
15. nanoparticles, *Adv. Drug Del. Rev.* 21 (1997) 5-24.
16. D.T. O'Hagan, M. Singh, R.K. Gupta, Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines, *Adv. Drug Del. Rev.* 32 (1998) 225-246.
17. B.M. Johnson, W.N. Charman, C.J. Porter, An in vitro examination of the impact of
18. polyethylene glycol 400, Pluronic P85, and vitamin E D-alpha-tocopheryl polyethylene glycol 1000 succinate on P-glycoprotein efflux and enterocyte-based metabolism in excised rat intestine, *AAPS Pharm. Sci.* 4 (2002) E40.
19. Melissa J. Copland, Margaret A. Baird, Liposomal delivery of antigen to human dendritic cells, *Vaccine* Volume 21, Issues 9-10, 14 February 2003, Pages 883-890
20. Sanchez, B. Villamayor, Y. Guo, J. McIver, M.J. Alonso, Formulation strategies for the stabilization of tetanus toxoid in poly(lactide-co-glycolide) microspheres, *Int. J. Pharm.* 185 (1999) 255-266.
21. Tabata, Y.; Ikada, Y. Synthesis of gelatin microspheres containing interferon. *Pharm. Res.* 1989, 6, 422-427.
22. Ruiz, J. M.; Benoit, J. P. In vivo peptide release from poly(D,L-lactic acid-co-glycolic acid) copolymer 50/50 microspheres. *J. Controlled Release* 1991, 16, 177-186.
23. Heya, T.; Okada, H.; Ogawa, Y.; Toguchi, H. Factors influencing the profiles of TRH release from copoly(d,l-lactic/glycolic acid) microspheres. *Int. J. Pharm.* 1991, 72, 199-205.
24. Cohen, S.; Yoshioka, T.; Lucarelli, M.; Hwang, L. H.; Langer, R. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. *Pharm. Res.* 1991, 8, 713-720.
25. S. Sharif, D.T. O'Hagan, A comparison of alternative methods for the determination
26. of the levels of proteins entrapped in poly(lactide-co-glycolide) microparticles, *Int. J. Pharm.* 115 (1995) 259-263.
27. Lima, K.M. and Rodrigues, J.M. Jr., PolyDL-lactide-Co-glycolide nanoparticles as a controlled release antigen delivery system, *Brazilian J. Medical and Biological Research*, 1999, 32:171-180.
28. Coester, C., Nayar, P., Samuel, J., *In-vitro* uptake of gelatin nanoparticles by murine dendritic cells and their intracellular localization. *E.J. of Pharm. and Biopharm.* 2006, 62, 306-314.
29. Li, J. K., Wang, N., Wu, X. S., A biodegradable system based on gelatin nanoparticles and poly(lactic-co-glycolic acid)

- microspheres for protein and peptide drug delivery. *J. Pharm. Sci.* 1997, 86, 891-895.
30. D.P. Joshi, Y.L. Lan-Chun-Fung, J.G. Pritchard, Determination of poly(vinylalcohol) via its complex with boric acid and iodide, *Anal. Chim. Acta* 104 (1979) 153-160.
 31. S. Jain, P. Singh, V. Mishra, S.P. Vyas, Mannosylated niosomes as adjuvant—carrier system for oral genetic immunization against hepatitis B, *Immunol. Lett.* 101 (2005) 41-49.
 32. K.S. Jaganathan, S.P. Vyas, Strong systemic and mucosal immune responses to
 33. surface-modified PLGA microspheres containing recombinant hepatitis B antigen administered intranasally, *Vaccine* 24 (2006) 4201-4211.
 34. C.O. Elson, W. Ealding, J. Lefkowitz, A lavage technique following repeated measurement of IgA antibody in mouse intestinal secretions, *J. Immunol. Methods* 67 (1984) 101-108.
 35. K. Khatri, A.K. Goyal, P.N. Gupta, N. Mishra, A. Mehta, S.P. Vyas, Surface modified liposomes for nasal delivery of DNA vaccine, *Vaccine* 26 (2008) 2225-2233.