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ScienceDirect

Resource-Efficient Technologies 2 (2016) S25–S38

www.elsevier.com/locate/reffit

Effect of functionalization of polymeric nanoparticles incorporated with whole attenuated rabies virus antigen on sustained release and efficacy

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Received 9 June 2016; received in revised form 4 October 2016; accepted 13 October 2016

Available online 10 November 2016

Abstract

Nanovaccines introduced a new dimension to prevent or cure diseases in an efficient and sustained manner. Various polymers have been used for the drug delivery to increase the therapeutic value with minimal side effects. Thus the present study incorporates both nanotechnology and polymers for the drug delivery. Poly(D,L-lactic-co-glycolic acid)-b-poly(ethylene glycol) was incorporated with the rabies whole attenuated viral antigen using double emulsion (W/O/W) method and characterized by Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). Chitosan-PEG nanoparticles incorporated with the rabies whole attenuated virus antigen (CS-PEG NP-RV Ag.) were prepared using Ionic Gelation method. The CS-PEG NP-RV Ag. was surface modified with biocompatible polymers such as Acacia, Bovine Serum Albumin (BSA), Casein, Ovalbumin and Starch by Ionic Gelation method. The morphology was confirmed by SEM and Transmission Electron Microscopy (TEM). The surface modification was confirmed by Fourier Transform Infrared Spectroscopy (FTIR), Zeta potential. The size distribution of CS-PEG-RV Ag. and surface modified CS-PEG-RV Ag. by respective biocompatible polymers was assessed by Zetasizer. Release profile of both stabilized nanoparticles was carried out by modified centrifugal ultrafiltration method which showed the sustained release pattern of the Rabies Ag. Immune stimulation under in-vitro condition was studied using rosette assay and phagocytosis assay. In-vitro toxicity using human blood and genotoxicity using human blood DNA was also studied to assess the toxicity of the nanoformulations. The results of these studies infer that PLGA-b-PEG nanoparticles, CS-PEG and surface modified CS-PEG nanoparticles may be an efficient nanocarrier for the RV Ag. to elicit immune response sustainably with negligible toxic effect to the human system.

Keywords: Rabies virus; Nanotechnology; Nanovaccines; Biopolymers; Sustained release; Chitosan; PLGA-PEG

1. Introduction

Rabies is the zoonotic disease (a disease transmitted from animals to humans) caused by the rabies virus of genus Lyssavirus, says the World Health Organization (WHO). In several countries, dogs, raccoon dogs, and foxes are considered to be a major vector for rabies [1].

This disease causes acute inflammation on the brain of the infected mammal which leads to death. The disease is ranked endemic on all continents with the highest case incidence in Asia and Africa; it threatens potentially over 3 million people [2].

In 1958, Kissling reported the propagation of the rabies vaccine in hamster cell cultures [3]. This led to the propagation of the virus in cell cultures. Now the virus is generated in chick embryos and attenuated using formaldehyde for vaccination purposes. In the initial stage, the 'vaccination-challenge assay' is widely used for batch release of inactivated rabies vaccine for veterinary use as it attempts to use serological assay [4]. Vaccination against rabies provides immunity to the disease in pre-exposure and postexposure prophylaxis.

Nanovaccines consist of nano-scale based particles attached or formulated with components to which an immune response

Peer-review under responsibility of the scientific committee of TECHNOSCAPE 2016.

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http://dx.doi.org/10.1016/j.reffit.2016.10.007

is desired. Nanovaccines are efficient than the conventional vaccines as they induce the humoral and cell-mediated response [5]. With the help of nanotechnology, non-invasive vaccination can be provided effectively such as oral vaccination for invasive vaccination techniques as it elicits cell-mediated immunity and nasal vaccination technique gives rise to systemic humoral, cellular responses, local and distal secretory immune responses thus making mucosal lining less vulnerable to infection [6,7]. Efficient deliveries in the targeted tissues are achieved through nanotechnology [8]. Nanovaccines have evinced the ability for a number of infectious pathogens, including HIV, malaria, tuberculosis, and hepatitis C.

Nanoparticles are the good candidates to act as an adjuvant when compared to the largely used adjuvant, alum [9]. Physicochemical properties, such as particle size, influence the movement of the nanoparticle to travel through the lymphatic and accumulate in lymph node-resident dendritic cells. Nanoparticles used in vaccine formulations increase the immunogenicity and protect it from its loss and thus act as an adjuvant. Nanovaccines are dreamt to formulate vaccines that require needle-less administration, have long shelf life, are less temperature dependent, have sustained immunogenicity and are one-time dose. This study was done with the view that biopolymers have the potential to increase the therapeutic value of drug delivery with minimal side effects. For the preparation of the nano polymer conjugate double emulsion method and ionic gelation method were used. In vitro toxicity using human blood and genome toxicity were also performed.

2. Materials and methods

2.1. Preparation of poly(D,L-lactic-co-glycolic acid)-poly(ethylene glycol) nanoparticles incorporated rabies whole attenuated virus antigen

2.1.1. Chemicals and reagents

Poly (D,L-lactic-co-glycolic acid) (50:50) 38,000–54,000 mol. Wt. with terminal carboxyl group (PLGA carboxylate); Dichloromethane (DCM) (both were purchased from Sigma Aldrich). Heterobifunctional PEG (amine-PEG-carboxylate) at molecular weight of 3400 g/mol (NOF Corporation, Tokyo, Japan) is stored in dark at −20 °C and *N*,*N*-diisopropylethylamine (DIEA) (Sigma-Aldrich). Conjugation crosslinkers: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) is stored in dark at −20 °C; *N*-hydroxysuccinimide (NHS) is stored at 4 °C (both from Pierce, Rockford, IL). Poly(vinyl alcohol) (PVA) (88% hydrolyzed, ~22 kDa, Fisher), Methanol (Rankem). All the chemicals were of analytical grade and highly purified.

2.1.2. Synthesis of PLGA-b-PEG polymer

There are two ways to produce PLGA-b-PEG diblock copolymers. The conjugation of PLGA homopolymer with a carboxylate end group using EDC and NHS as conjugation crosslinkers method was preferred in this study over melt or solution copolymerization in the presence of monomethoxy-PEG using stannous octoate as a catalyst due to technical easiness and high yield preferences. The modified method is described below [10].

250 mg of PLGA-carboxylate was first dissolved in 1–2 mL of DCM. NHS (3.0 mg) is dissolved in 4.8 mg of EDC in 1 mL of DCM. The PLGA-carboxylate solution was added to the NHS/EDC solution and gentle stirring was carried out in a magnetic stirrer to form PLGA-NHS. Precipitation of PLGA-NHS was done with 20 mL of methanol washing solvent by centrifugation at 3000 rpm for 10 min and residual EDC/NHS was removed. Washing and centrifugation were done twice to ensure purity of the resultant PLGA-NHS. The PLGA-NHS pellet is dried to remove the residual methanol. After drying PLGA-NHS (246 mg) was dissolved in 4 mL of DCM followed by the addition of amine-PEG-carboxylate and DIEA (1:1) mmol. The mixture solution was incubated under stirring at 600 rpm for 24 hours at room temperature. The resultant PLGA-b-PEG block copolymer was precipitated with methanol wash and centrifuged at 3000 rpm for 10 min to remove the unreacted PEG. The PLGA-b-PEG polymer result was lyophilized.

2.1.3. Synthesis of PLGA-b-PEG NP-rabies whole attenuated viral antigen

The modified double emulsion-solvent evaporation method was used for the preparation of PLGA-b-PEG NP-rabies whole attenuated viral antigen. The rabies whole attenuated viral antigen (100 μL) and PLGA-b-PEG polymer (100 mg) were added with DCM (2 mL) and mixed. Further, the solution was sonicated using Probe sonicator at 20 W for one minute. The emulsion occurred was precipitated with PVA (50 mL of 0.1 w/v). Further, it is sonicated at 20 W, 1 min to form W/O/W emulsion. The emulsion is stirred at 200 rpm, 2 h. The resultant PLGA-b-PEG-rabies whole attenuated viral antigen was centrifuged for 3000 rpm, 15 min and washed with deionized water. It was recovered and suspended in PBS buffer for a short time storage.

2.2. Synthesis of chitosan-PEG NP-rabies whole attenuated viral antigen and functionalization of the conjugate with biocompatible polymers

2.2.1. Chemicals and reagents

Chitosan (CS) (48 kDa, Primex Co, Iceland), cross linker Sodium Tripolyphosphate (STPP); Acetic Acid (Sigma Aldrich), rabies whole attenuated viral antigen with potency ≥2.5 I.U. (Rabipur, Novartis vaccine). Acacia; Bovine Serum Albumin (BSA); Casein; Starch; Ovalbumin (were purchased from Ranken). All the chemicals were of analytical grade and highly pure.

2.2.2. Synthesis of CS NP-PEG-rabies whole attenuated viral antigen

The method followed was the modified method of inotropic gelation using STTP as cross linker [11]. CS (25 mg) was taken and acetic acid (250 μL) was added to it. To this mixture deionized water (75 mL) containing PEG (60 μL) rabies whole attenuated viral antigen was added and stirred (3–4 h). The resultant cloudy suspension was centrifuged at 10,000 rpm for 15 min and sonication was done at 20 W at 5 min to recover CS NP-PEG-rabies whole attenuated viral antigen.

2.2.3. Synthesis of CS-PEG NP-rabies whole attenuated viral antigen functionalized with biocompatible polymers

The similar method was followed for functionalization of the CS NP-PEG-rabies whole attenuated viral antigen. For each functionalization each biocompatible polymers (Acacia, BSA, Casein, Starch, and Ovalbumin) was added in 1:1 ratio to the PEG concentration used for the preparation of CS-PEG-RV Ag. After overnight stirring, centrifugation (10,000 rpm for 15 min) and sonication (20 W, 5 min) were done to recover the polymeric nanoparticle functionalized with different biocompatible polymers incorporated Rabies whole attenuated viral antigen.

2.3. Stability study

The method followed for this study was the modified method of Miles et al. [12]. PLGA-b-PEG NP-RV Ag and CS-PEG NP-RV Ag were prepared and stored at 100 °C for one month to assess the structural and storage stability. Antigen stability for CS-PEG NP-RV Ag. and PLGA-b-PEG NP-RV Ag. was revealed by SDS-PAGE method of Laemmli [13]. A clean and rectangular plate was assembled and kept in vertical position by placing it in a gel casting stand. The separating gel mixture was prepared and poured into glass plates uniformly without any leakage. After polymerization of the separating gel, the stacking gel prepared was poured over it and the comb was inserted gently. The set up was left for some time to the gel to polymerize completely. 50 μL of isolated commercially purchased rabies Ag. (Rabipur, Novartis vaccine), 50 μL of PLGA-b-PEG NP-RV Ag. and 50 μL of CS-PEG NP-RV Ag. was mixed with the sample solubilizing buffer (1:1) and the mixture was boiled at 950 °C in a water bath for 5–10 minutes. The samples along with the standard protein marker (29 kDa–205 kDa) were loaded in the respective wells. The gel was electrophoresed at 50–100 V for 3–4 hours. After the electrophoresis, the gel was placed in Coomassie blue stain solution for 1 h and washed thoroughly with distilled water. The stained gel was placed in destaining solution and washed with distilled water and 20 mL of the de-staining solution with 20 mL of distilled water was added and kept overnight incubation. The samples fractioned into bands were observed and their molecular weights were determined using the standard marker used.

2.4. Characterizations

2.4.1. Characterization of PLGA-b-PEG polymer and PLGA-b-PEG NP-whole attenuated RV Ag.

2.4.1.1. Morphology. The surface morphology of the free and the incorporated nanoparticles were characterized by Scanning Electron Microscopy (SEM). The samples were sputtered with Palladium Gold for 30 s under Ploarum Machine (BAC-TEC Model SCDOO5 Switzerland). Afterwards SEM was performed with Carl Zeiss Super 55 (Germany) Field emission SEM with the detector at 15 kV. The magnification was set at 65,000. Samples for Atomic Force Microscopy measurements (AFM) were drop casted on atomically flat mica sheet and imaged. AFM was done with NTMDT (Ireland).

2.4.2. Characterization of CS-PEG NP-rabies whole attenuated viral antigen (RV Ag.) and CS-PEG NP-functionalized with various biocompatible with various incorporated whole attenuated RV Ag.

2.4.2.1. Fourier transform infrared spectroscopy (FTIR). Initial characterization of the nanoparticles was done with Fourier Transform Infrared Spectroscopy (PerkinElmer Spectrum version 10.4.00) for free CS NP-PEG; CS NP-PEG biocompatible polymers and CS NP-PEG-rabies whole attenuated viral antigen; CS NP-PEG-biocompatible polymers-rabies whole attenuated viral antigen to confirm the surface modification. Characterization was performed by recording the absorbance of the samples in the frequency range of 4000 cm^{-1} to 400 cm^{-1} with the resolution of 4 cm^{-1}. The samples were pelletized with KBr before commencing the spectroscopy.

2.4.2.2. Morphology, particle size and ζ potential. The surface morphology of the free and the incorporated CS nanoparticles was characterized by Scanning Electron Microscopy (SEM). The samples were sputtered with Palladium Gold for 30 s under Ploarum Machine (BAC-TEC Model SCDOOS Switzerland). Afterwards SEM was performed with Carl Zeiss Super 55 (Germany) Field emission SEM with detector at 15 kV. The magnification was set at 65,000. Transmission Electron Microscopy was done to get the distinct clear morphology of the nanoparticles. The samples were dispersed in ethanol, sonicated for 30 s; they were placed in the carbon grid and Field Emission TEM was done using Hitachi H-7650 (120 kV). The particle size distribution was analyzed using Dynamic Light Scattering (DLS) technique. The samples prepared using acetic acid were dispersed in saline media and measured by DLS (Horiba partica LA 950) technique. Laser Doppler electrophoresis technique using the same instrument for the determination of Zeta potential was also performed.

2.5. % Yield

The prepared PLGA-b-PEG NP-RV Ag., CS-PEG NP-RV Ag. and surface functionalized CS-PEG-RV Ag. were dried and yield % was calculated by the formula:

$$\text{Percentage yield} = \frac{W1 * 100}{W2} \quad (1)$$

W1 = weight of the dried nanoparticles recovered; W2 = sum of the initial dry weight of starting material.

2.6. Entrapment efficiency

Entrapment efficiency of the antigen (Ag) was determined by filtering a known amount of PEG-PLGA nanoparticles and surface stabilized CS NP-RV Ag. through a filter membrane (0.1 μm) to separate the free Ag. The active Ag. was determined by using the IgG titer. The entrapment efficiency (E) was determined using the values for the total concentration of RV Ag. in the system (free + encapsulated, [RV Ag.]0) and that in the filtrate ([RV Ag.]f) using the equation:

$$E\% = ([\text{RV Ag.}]0 - ([\text{RV Ag.}])f / ([\text{RV Ag.}]0) * 100 \quad (2)$$

2.7. Release profile

Release profile of the antigen was performed by the modified method of centrifugation and ultra-filtration [14]. 50 mg and 100 mg of various surface modified CS NP-PEG incorporated RV Ag. and PLGA-b-PEG NP incorporated RV Ag. were dissolved in 50 mL of 0.2 M/L PBS pH 7.4 containing 0.2% Tween 20, incubated at 37 °C in orbital shaker (REMI) and the respective readings were taken at predetermined time intervals, i.e., 1, 2, 4, 6, 8, 22, 34, 48, 72, 96, 106 hours for CS NP-PEG incorporated RV Ag. and for PLGA-b-PEG NP incorporated RV Ag., the predetermined time interval was every one day for the first 7 days and thereafter every 7th day up to the 70th day. At the predetermined time intervals, 2 mL of the solution was removed and replaced with fresh medium. The sample was centrifuged 40,000 rpm, 15 min and 10,000 rpm, 30 min respectively. The supernatant was analyzed by the rabies IgG titer and OD was taken at 450 nm.

2.8. In-vitro immunogenicity

2.8.1. T-cell rosette formation

In-vitro immunogenicity studies of the CS-PEG NP-RV Ag. were studied by the T-cell rosette formation (TCRF) assay method. The modified method of Baehner et al. [15] was used to perform the assay. In 15 mL centrifugation tube, a buffy coat layer overlaid on 3 mL of Ficoll 400 was taken. The above mixture was centrifuged at 4500 rpm for 40 minutes at 100 °C. Platelets and plasma were discarded carefully such that lymphocytes are left. The obtained lymphocytes were transferred to a sterile centrifuge tube and PBS (1 mL) was added and centrifuged at 4500 rpm for 15 minutes at 100 °C thrice to remove RBCs. The supernatant was discarded and the pellet was suspended in RPMI media and cultured in T25 suspension culture flasks incubated at 37 °C under 5% CO_2 incubator.

Different concentrations of the test concentration were mixed with RPMI media (10, 25, 50, 75 and 100 mg/mL) and added to 12 well plates (pre incubated with T lymphocytes). The plates were incubated at 370 °C under 5% CO_2 incubator for 1 hour. The lymphocytes were mixed with sheep erythrocytes at the rate of 10:1 ratio after the incubation period. Supernatant was removed and the pellet was collected and suspended. The rosette thus obtained was counted in a hemocytometer.

2.8.2. Phagocytic assay

RAW cells suspended in DMEM medium were taken and plated into 96 well late (4×105 cells) per well. The plates containing the RAW cells were incubated at 37 °C, 5% CO_2 incubator. The cells were washed with PBS after the incubation period is over. The cells were washed with HEPES balanced salt solution thrice and incubated at room temperature for 15 minutes. Fluorescent micro particle formulations were incubated for 15 minutes at 37 °C. The nanoparticles which were not phagocytized were removed by washing the cells with cold PBS. The washed cells were fixed with 10% glutaraldehyde. Microscopic examination was carried out by the method of Xu et al. [16] to determine the number of phagocytized cells.

2.9. In-vitro blood compatibility test

2.9.1. PLGA-b-PEG NP, CS-PEG NP and surface modified CS-PEG nanoparticles incorporated with whole attenuated RV Ag

To the 5 mL of human blood taken in five vials at different concentrations, the nanoparticles incorporated with whole attenuated RV Ag. were added and kept in a shaker (3 h) at room temperature. The vials are centrifuged for 10,000 rpm for 10 min and tested for hemolysis. The percentage of hemolysis was determined by the formula of plasma hemoglobin as follows:

$$\begin{aligned}\text{Amount of plasma hemoglobin (mg/dL)} &= 2 * \text{A415} \\ &- (\text{A380} + \text{A450}) * 1000 * \text{dilution factor (E} * 1.655)\end{aligned} \quad (3)$$

where A415, A380, A450 are the absorbance values at 415, 380 and 450 nm, E is the absorptivity value of oxyhemoglobin at 415 nm.

2.10. Genotoxicity

Genotoxicity assay was performed by the modified method of Namasivayam et al. [1]. The human blood DNA was isolated by using a standard protocol. To the purified DNA which was suspended and stored at 20 μg/mL in Tris buffer under pH 7.8 at −20 °C, the aliquots of PLGA-b-PEG-whole attenuated RV Ag. and surface stabilized CS NP incorporated with whole attenuated RV Ag. were added and incubated at 37 °C for 24 hours and electrophoresis was done using Tris acetate buffer at 150 A for 30 minutes.

3. Results and discussion

3.1. Structural and storage stability of encapsulated antigen nano formulation

Antigen incorporated in PLGA-b-PEG NP and CS-PEG NP was stored at 10 °C for one month. SDS-PAGE was done to assess the stability by taking the molecular weight as the parameter. The molecular weight of the rabies vaccine (commercially purchased) and the molecular weight of the formulated vaccine with PLGA-b-PEG NP and CS-PEG NP were exhibited as 66 kDa without any damage or split in the band which reveals that the formulated antigen is stable. Fig. 1 shows the SDS-PAGE of Control (C), PLGA-b-PEG NP-RV Ag. (PPR) and CS-PEG NP-RV Ag. (CPR).

3.2. Characterization

3.2.1. Morphology

3.2.1.1. PLGA-b-PEG polymer and PLGA-b-PEG NP-whole attenuated RV Ag.

3.2.1.1.1. Scanning electron microscopy. Fig. 2 captured in the range of 2 μm shows the clear morphology as a film reveals the structure of polymer (PLGA-b-PEG). Fig. 2 shows the nanoparticle of the PLGA-b-PEG NP-RV Ag. The size of the particles ranges between 90 nm and 1.4 μm. The SEM images confer the micro and nano spheres of PLGA-b-PEG NP-RV Ag.

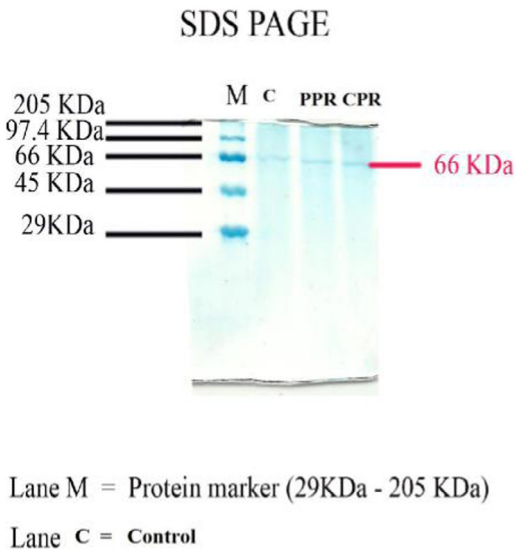

Fig. 1. Structural and storage stability of encapsulated antigen nano formulation.

3.2.1.1.2. Atomic force microscopy (AFM). The Fig. 3A shows the 3D image and Fig. 3B shows the X section at 216 nm and evinces that the PLGA-b-PEG has a film like structure. Fig. 3C and D displays the clear spherical morphology of the PLGA-b-PEG NP RV antigen with the size range of 80 nm–400 nm. Fig. 3E and F display the line profile of PLGA-b-PEG polymer and PLGA-b-PEG NP-RV Ag. respectively. The roughness of 203.607 nm signifies that the nanoformulation has the potential to release the antigen in a controlled and sustained manner.

3.2.2. Fourier transform infrared spectroscopy (FTIR)

3.2.2.1. FTIR studies of free chitosan and functionalized chitosan with biocompatible polymers. Initial characterization was done with Fourier Transform Infrared Spectroscopy (PerkinElmer Spectrum version 10.4.00) for free CS-TTP NP, CS NP-PEG; CS NP PEG-biocompatible polymers to initially confirm the surface modification. The spectra of CS-NP are shown in the Fig. 4A and the spectra of the respective nano polymers were displayed in the Fig. 4B.

The changes in the position of the peaks of the respective polymeric nano formulations with respect to the chitosan nanoparticles were observed to assert the surface modification of chitosan nanoparticles with respective biocompatible polymers. The 1638.2 spectra seen in CS NP attribute to N—H bond shifted to 1650 in CSPEG NP. The peaks in the range of 850–900 cm^{-1} were the result of saccharide structure of CS [17]. There is also a change in the position of the peak from 3447 to 3380 cm^{-1} which shows that there are some changes in the chitosan nanoparticle. An only peak in the range of 1130–1000 cm^{-1} clearly indicates that the chitosan is modified with PEG. Similarly the characteristic range of peaks for the respective biocompatible polymers reveals that the chitosan is functionalized with the respective biocompatible polymers.

3.2.2.2. FTIR for CS-PEG-RV Ag. and CS-PEG-RV Ag. functionalized with biocompatible polymers. The broadening of the peaks in every FTIR shown in Fig. 5 reveals that the presence of the hydroxyl bonds attaches or presents prominently along with the other bonds. This shows the incorporation of the RV Ag. on comparing the peaks absorbed in the free and incorporated CS NP and surface stabilized CS nanoparticles clearly. The additional peaks obtained were due to the presence of respective polymeric coating.

3.2.3. Morphology, particle size and ζ potential

3.2.3.1. Scanning electron microscopy and transmission electron microscopy. SEM and TEM were done for the CS-PEG NP-RV Ag. The SEM shows the three dimensional surface topography of the particles that range around 90–200 nm in size. A characteristic spherical shape for the CS-PEG-RV Ag. was observed. The TEM micrograph shows the distinct shape of the particles, its surface modification and incorporation of the antigen. Refer to Fig. 6A and B.

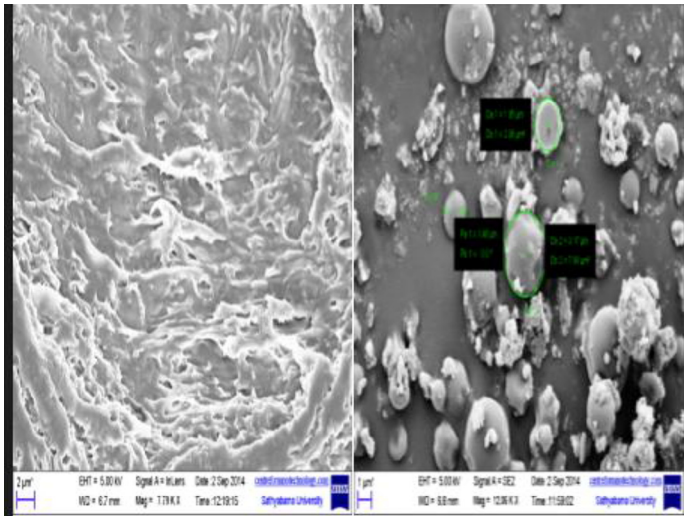

Fig. 2. PLGA-b-PEG polymer and PLGA-b-PEG-RV Ag.

3.2.4. Particle size distribution

The particle size distribution of the CS-PEG-RV Ag. and surface modified CSPEG-RV Ag. with various biocompatible polymers prepared using acetic acid as the solvent through Ionic gelation method was studied. DLS technique was used for measuring the hydrodynamic diameter of the particles and for the determination of particle size distribution. Fig. 7A–F summarizes the results.

3.2.5. Zeta potential

The CS NP are highly positively charged (+40 mV) which indicates the protonation of NH2 functional groups of glucosamine units to $NH3^{+}$ ions in acidic media [18]. When it is dispersed in saline media the charge was reduced to +20 mV. The presence of high positive charge gives rise to low blood compatibility. The present study was done to reduce the high positive charge to low positive charge to make it much better biocompatible. The changes in the charges with respect to

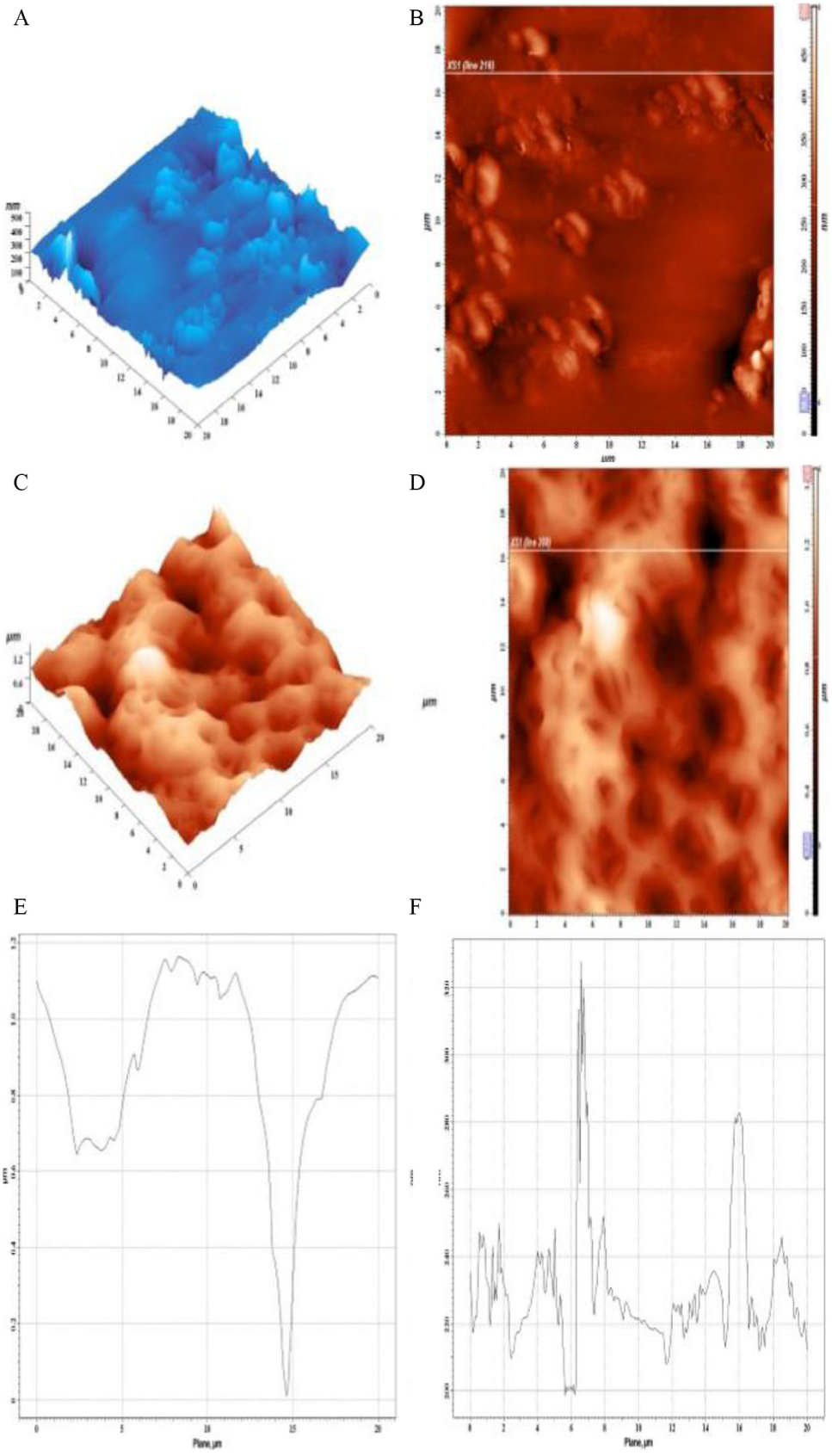

Fig. 3. (A) 3D Image of PLGA-b-PEG polymer. (B) X section at 216 nm. (C) 3D image of PLGA-b-PEG NP-RV Ag. (D) X Section. (E) Line profile of PLGA-b-PEG polymer. (F) Line profile of PLGA-b-PEG-RV Ag.

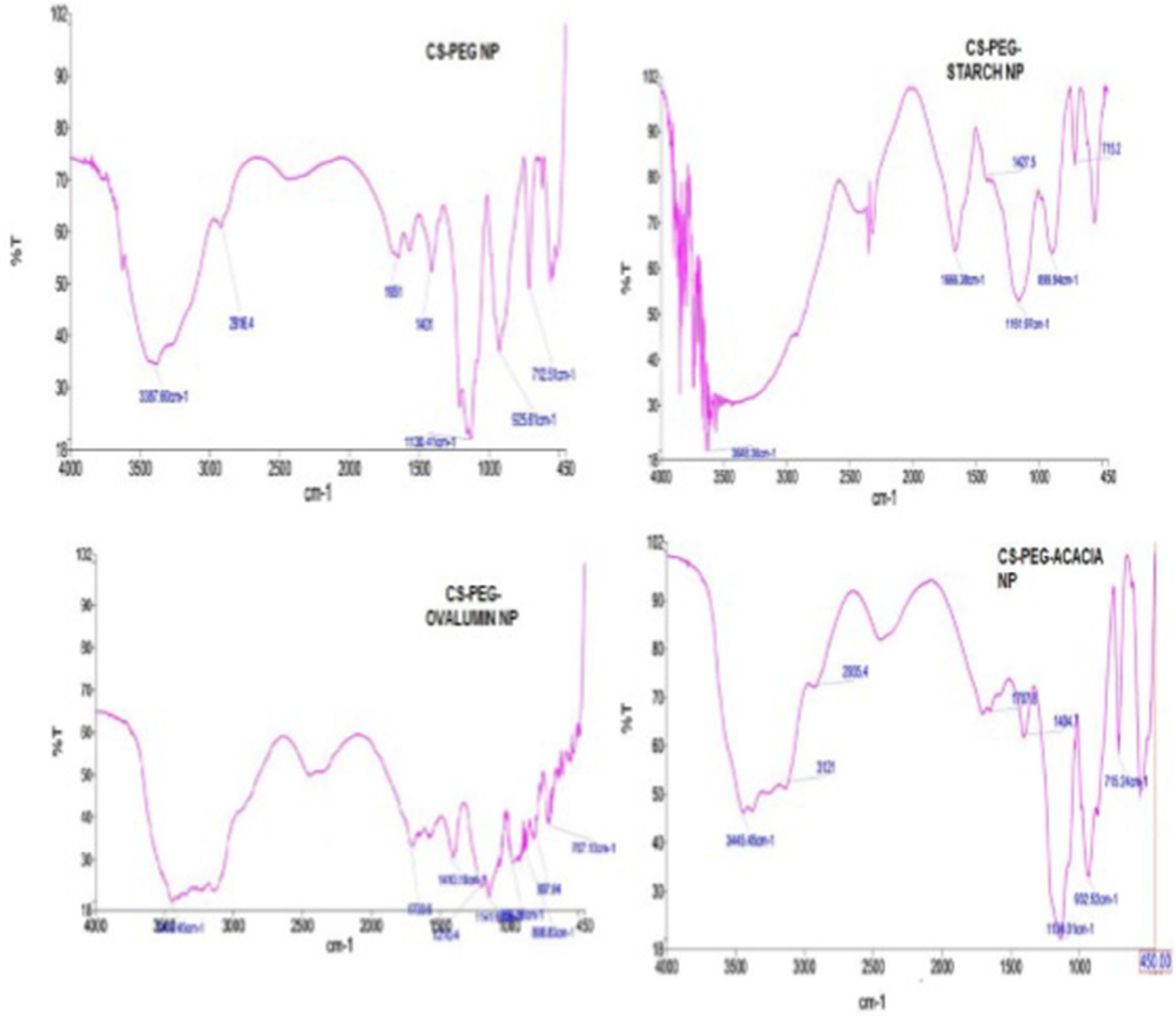

Fig. 4. (A) FTIR of CS NP. (B) FTIR for CS-PEG-RV Ag. and CS-PEG-RV Ag. functionalized with biocompatible polymers.

CS-PEG NP-RV Ag. also confirm the surface medication by biocompatible polymers. From the obtained values it is also suggested that CS and surface modified CS incorporated RV Ag. should be packed as powders (lyophilized) for a long time stability. The dispersion of the particles in .9% saline induces the deprotonating process. In this process it is asserted that Cl^- counter ions in saline migrate to the Gouy layer and interacts with $NH3^+$ groups, resulting in deprotonating which results in the drop of the charges in ζ potential values [18]. Thus it is recommended that dispersing with saline media (.9%) before administration exhibits high biocompatibility (Table 1).

3.3. % Yield

The % of the yield of PLGA-b-PEG NP-RV Ag. CS-PEG NP-RV Ag. and surface functionalized CS-PEG-RV Ag. was calculated and tabulated below (Table 2).

3.4. Entrapment efficiency

Entrapment efficiency of the PLGA-b-PEG NP-RV Ag. and CS-PEG NP and CSPEG NP stabilized with various biocompatible polymers incorporated with RV Ag. was investigated using the formula suggested in the methodology. The results are tabulated below (Table 3).

3.5. In-vitro release profile

3.5.1. In-vitro release profile of PLGA-b-PEG-RV Ag.

In-vitro release profile provides the release of the antigen from the PLGA-b-PEG NP matrix. The cumulative % release of antigen at 37 °C in different time intervals was shown in Fig. 8A–F. The antigen release was found to be 64.9% at the 70th day. From the release profile it is clear that the release of RV Ag. from the nano-sphere was characterized by initial rapid

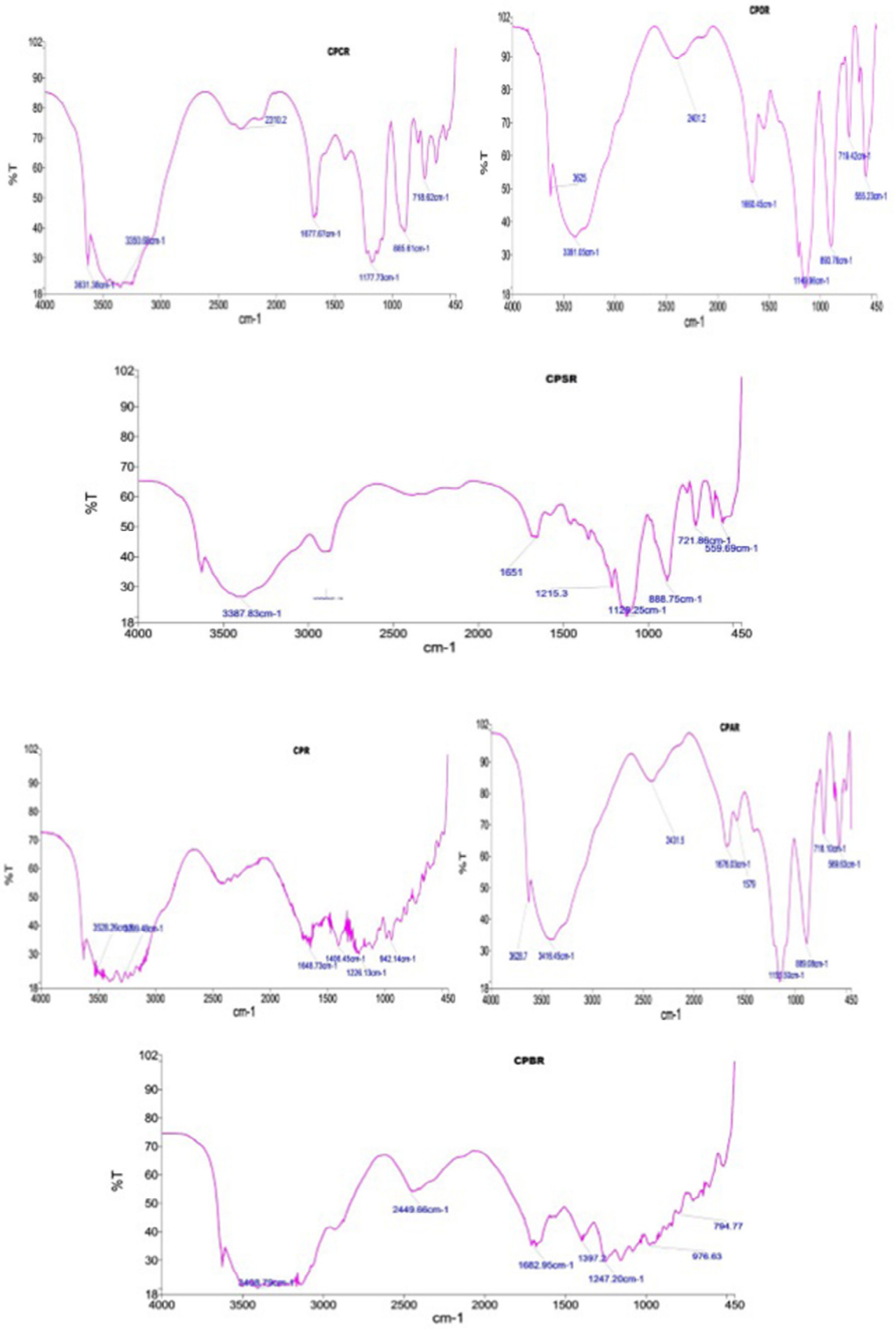

Fig. 5. TIR of CS-PEG-RV Ag and CS-PEG-RV Ag. functionalized with biocompatible polymers.

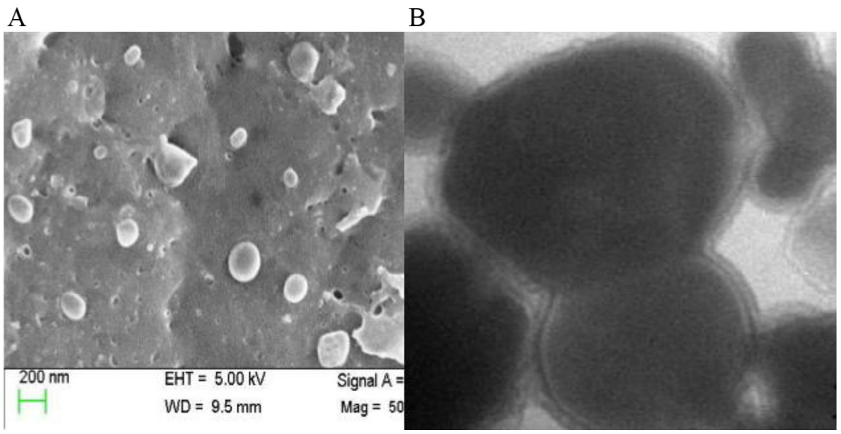

Fig. 6. (A) SEM-CS-PEG NP-RV Ag. (B) TEM-CS-PEG NP-RV Ag.

Table 1
Zeta potential of nano formulation.

Nanoparticle from AA in saline	Potential
1.CPR	+3.5
2.CPAR	−2.4
3.CPBR	−3.6
4.CPCR	−6.9
5.CPSR	−3.8
6.CPOR	−2.1

release (Burst release) phase of about 30%. It is assumed that this burst release is related to drug entrapment near the surface of the nano spheres [19]. This phase is followed by slow release. Around 37% antigen release was recorded for the duration of 50 days. This phase is characterized as sustained release phase.

3.5.2. In-vitro release of CS-PEG NP and surface stabilized CS-PEG NP incorporated with RV Ag.

3.5.2.1. Release kinetics of CS-PEG NP-RV Ag. The in-vitro release profile of RV Ag. entrapped in CS-PEG NP was performed in 0.2 M PBS at 37 °C. The study records the cumulative % of the antigen release shown in Fig. 8A. The initial burst release occurred for 8 hours and the release was about 35%. The release rate slowed down after the first phase of release and attained equilibrium after 10 hours from the commencement of the experiment. The cumulative release until the 80th hour was 43% (approx.) and there was a slight burst release (2nd burst) at the 106th hour. After 106 hours, the amount of antigen released was around 56%. The two phases of burst release of the antigen may be due to the diffusion of the antigen that was entrapped poorly in the matrix of CS-PEG NP while the sustained release may be due to the diffusion of the antigen from the core of the matrix [20].

Table 2
% Yield of polymeric nano spheres.

Polymeric nano spheres	Yield %
1. PLGA-b-PEG-RV Ag.	85.79
2. CS-PEG NP	90.83
3. CS-PEG-Acacia NP	88.72
4. CS-PEG-BSA NP	86.44
5. CS-PEG-Casein NP	88.56
6. CS-PEG-Ovalbumin NP	85.07
7. CS-PEG-Starch NP	87.12

Table 3
Entrapment efficiency (%) of polymeric nano spheres.

Polymeric nano spheres	Entrapment efficiency (%)
1. PLGA-b-PEG NP	73
2.CS-PEG NP	75.33
3.CS-PEG-Acacia NP	71.21
4.CS-PEG-BSA NP	69.15
5.CS-PEG-Casein NP	69.58
6.CS-PEG-Ovalbumin NP	74.12
7.CS-PEG-Starch NP	70

3.5.2.2. Release profile of CS-PEG NP-Acacia-RV Ag. The in-vitro profile of CS-PEG NP-Acacia-RV Ag. was shown in Fig. 8B. It was observed that there was a rapid release (about 36%) within 10 hours. After the 10th hour there was a sustained release pattern until the 80th hour. During this time about 42% of the antigen was released. At the end of the 106th hour about 55% of the antigen was released cumulatively.

3.5.2.3. Release profile of CS-PEG-BSA-RV Ag. The release behavior of CS-PEG-BSANP-RV was shown in Fig. 8C. A slight variation is observed between the 11th hour and 100th hour. Two clear burst release patterns were seen in this kinetics, one at the initial period 10 hours and the other at the final 6 hours. 58% of cumulative release was observed after 106 hours.

3.5.2.4. Release kinetics-chitosan-PEG-casein-rabies antigen conjugate. In the first 9 hours there was a burst release of 33%. After the 10th hour there was a gradual release of the antigen from the matrix. There was a sharp burst release after the 100th hour. Thus a cumulative release of 60% (approx.) of antigen in 106 hours was obtained. The results were plotted and shown in Fig. 8D.

3.5.2.5. Release kinetics-chitosan-PEG-ovalbumin-rabies antigen conjugate. The release kinetics of the CS-PEG-ovalbumin-RV Ag. was profiled in this study and shown in Fig. 8E.

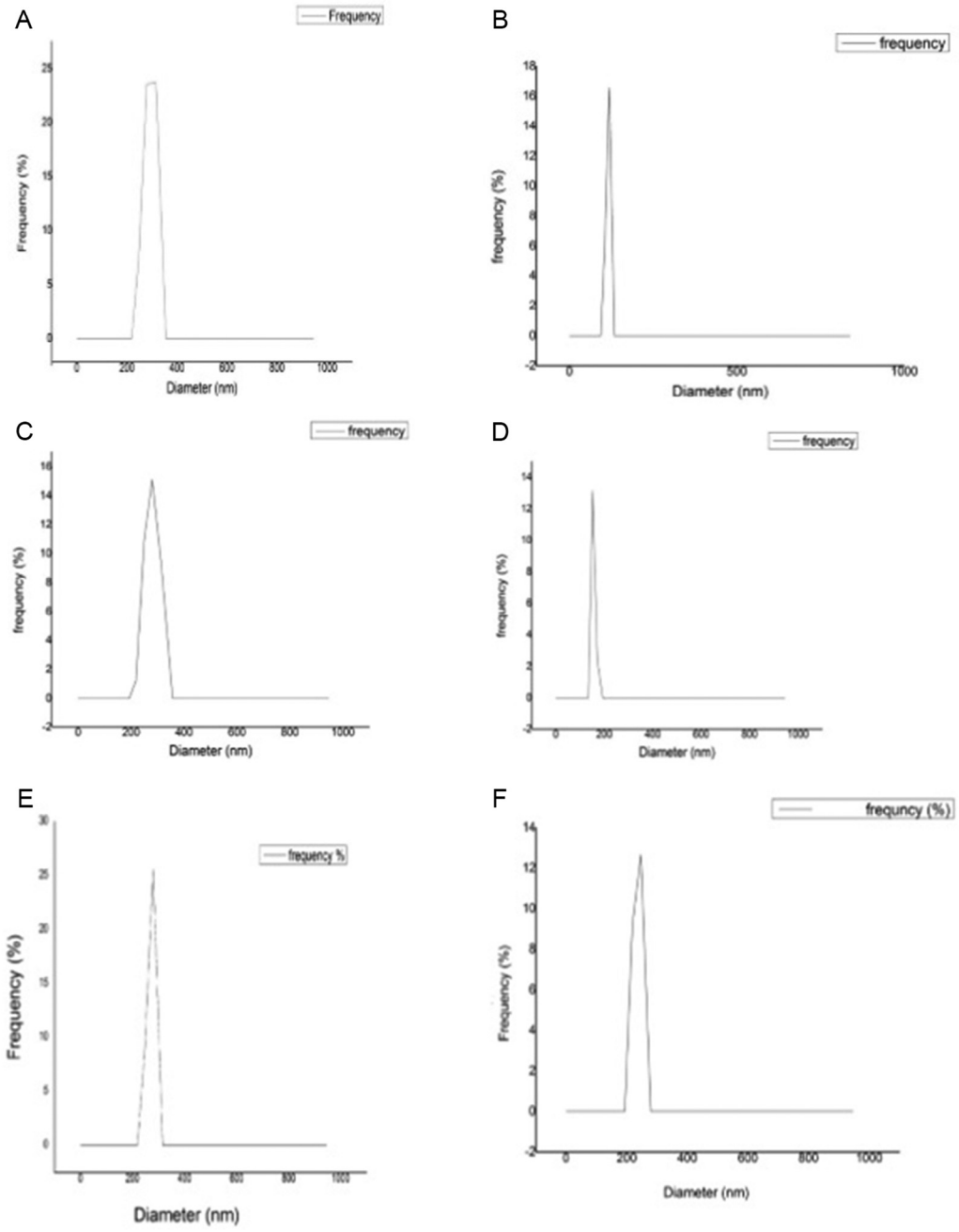

Fig. 7. (A) CSPSR size distribution. (B) CSPAR size distribution. (C) CSPBR size distribution. (D) CSPBR size distribution. (E) CSPOR size distribution. (F) CSPSR size distribution.

The release curve exhibits rapid diffusion of the antigen in less than 9 hours from the beginning of the study. The gradual increase in the release of the antigen when compared to the first 9 hours is seen between the 10th hour and 20th hour (5% approx.). 15% of antigen was released sustainably. From this matrix, a negligible burst of release was observed at the end of 106 hours. Cumulatively there was a discharge of 64% of antigen for 106 hours. Thus CS-PEG-ovalbumin NP shows good sustainable release characteristics.

3.5.2.6. Release profile of CS-PEG-starch-RV Ag. The Fig. 8F reveals that there is a very little fast diffusion of the antigen at the end of the phase of the study. The duration of the sustainable release was lengthier (lasted for 50 hours). There was an initial burst release of about 37% recorded in less than 10 hours for this conjugate. CS-PEG-starch evinces itself as one of the best candidates to serve as a bystander for vaccine delivery.

3.6. In-vitro immunogenicity

3.6.1. T-cell rosette assay

T-cell rosette assay was used to assess the immunological reaction and to identify the T cells where T cells CD2 surface protein is bound to a sugar based LFA-m homolog on the surface of the sheep red blood cell [21]. The number of rosette

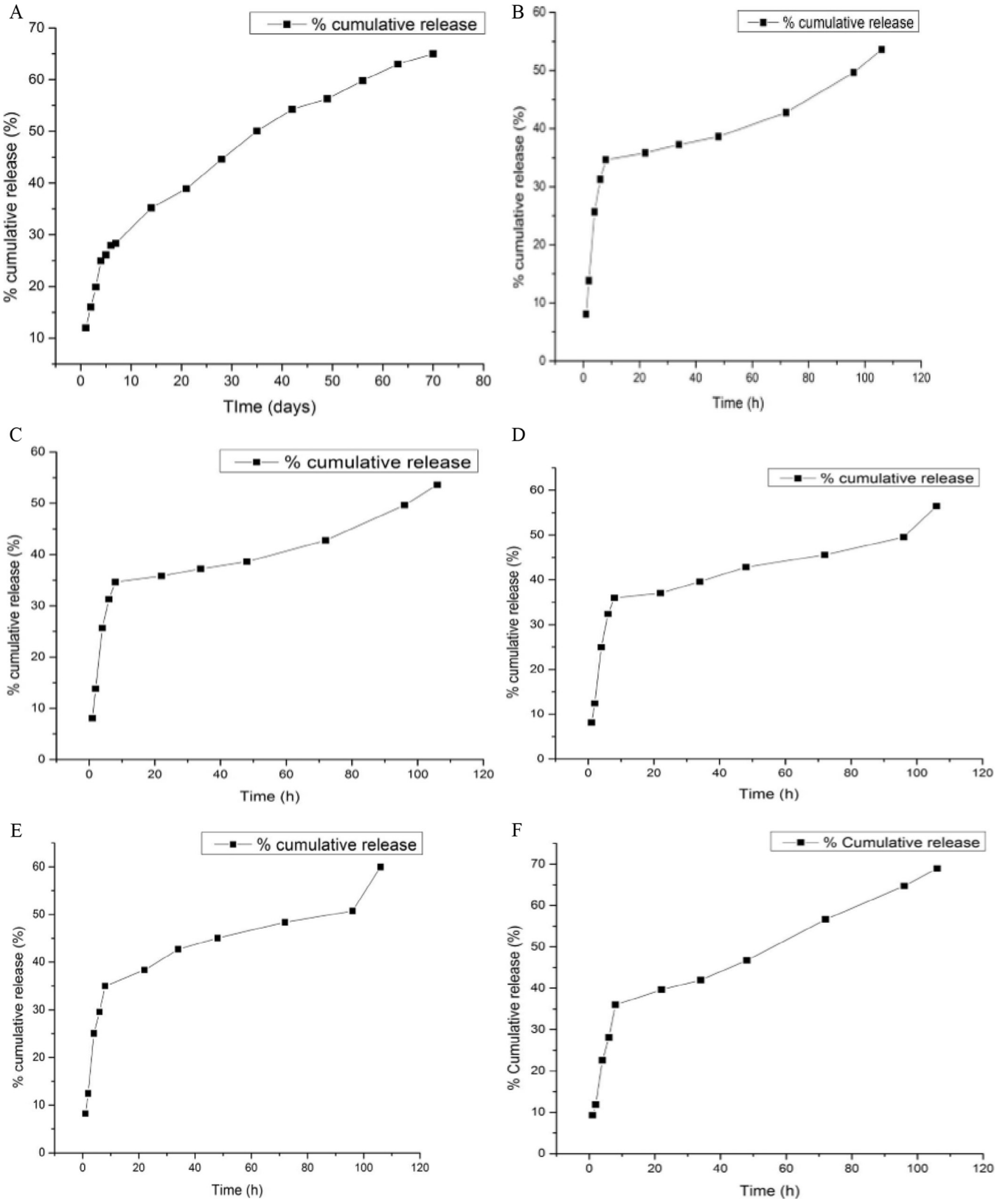

Fig. 8. Release profile. (A) Release profile of PLGA-b-PEG RV Ag. (B) Release profile of CPR. (C) Release profile of CPAR. (D) Release profile of CPBR. (E) Release profile of CPCR. (F) Release profile of CPSR.

Table 4
Number of rosette concentration (mg/mL).

Concentration (mg/mL)	Number of rosettes
10	10
25	28
50	35
75	49
100	60

results for the different concentrations of CS-PEG-RV Ag. was tabulated below (Table 4).

3.6.2. Phagocytosis assay

Phagocytosis assay was done to find out the number of nano conjugates phagocytized. This is one of the best techniques to address the immunogenicity in vitro. The results for the phagocytosis assay were tabulated for CS-PEG NP- RV Ag. below (Table 5).

3.7. In-vitro blood compatibility test

In-vitro blood compatibility test was done to check the biocompatibility of PLGA-b-PEG NP-RV Ag. and CS-PEG NP-RV Ag. in terms of hemolysis. No significant hemolysis (no traces of pale red color in serum) was observed when the human blood is treated with different concentrations of PLGA-b-PEG NP-RV Ag. and CSPEG NP-RV Ag. SEM was taken to ensure that there is no significant hemolysis against the untreated blood with particles (control) and is shown in Fig. 9. Thus, the results can be concluded that PLGAb-PEG NP-RV Ag. and CS-PEG NP-RV Ag. are very biocompatible.

Table 5
Phagocytosis in %.

Concentration (mg/mL)	Phagocytosis (%)
10	38
25	49
50	62
75	76
100	80.7

3.8. Genotoxicity

The genotoxicity assay for PLGA-b-PEG NP-RV Ag., CS NP and surface modified CS NP incorporated RV Ag. was proceeded by the method discussed in the Methodology section. The assay with different nanoparticles tested evinces that there is no clear toxicity at the genomic level in human blood as there are no fragmentations or degradations. A sharp DNA band was observed in the tested DNA as in control. This displays that the nanoparticles are compatible with the human blood DNA. The results were shown in Fig. 10.

4. Conclusion

The use of biodegradable polymers gives the ability to control the time, rate of degradation and antigen release [22]. In this point of view the present study was done. The well-known biocompatible and degradable polymers – poly(D,L-lactic-co-glycolic acid) functionalized with poly(ethylene glycol) (PEG) nanoparticles, chitosan-PEG (CS-PEG) and chitosan-PEG surface modified nanoparticles with biocompatible polymers (Acacia, Bovine Serum Albumin, Casein, Ovalbumin and Starch) were synthesized and loaded with the rabies whole attenuated viral antigen (RV Ag.) using the modified double emulsion-solvent evaporation method and the modified ionic gelation method respectively. The characterization of the nano formulations using Scanning Electron Morphology (SEM) showed the characteristic spherical morphology of the nanoparticles formed. Transmission Electron Microscopy (TEM) of CS-PEG-RV Ag. revealed the distinct image of the nano conjugate formed. Fourier Transform Infrared Spectroscopy (FTIR) studies for the CS-PEG, surface modified CS-PEG, CS-PEG-RV Ag. and surface modified CSPEG-RV Ag. confirmed the incorporation of the antigen and surface modification through the difference in the occurrence of the peaks among the nano formulations. Zeta size for the

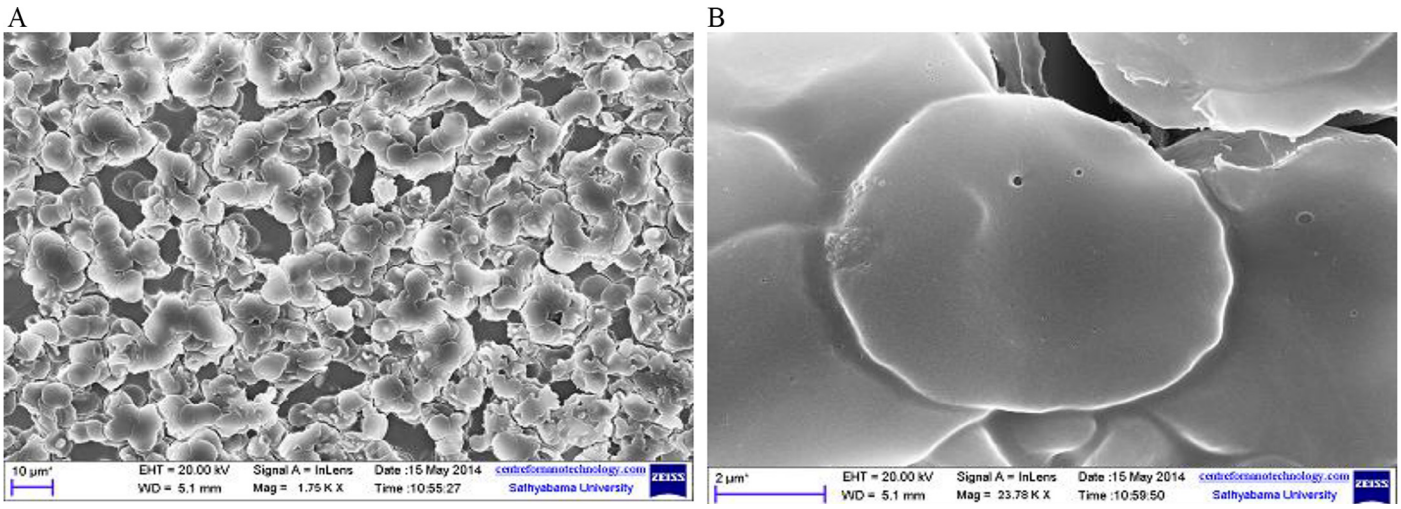

Fig. 9. (A) Blood cell control. (B) Blood cell test.

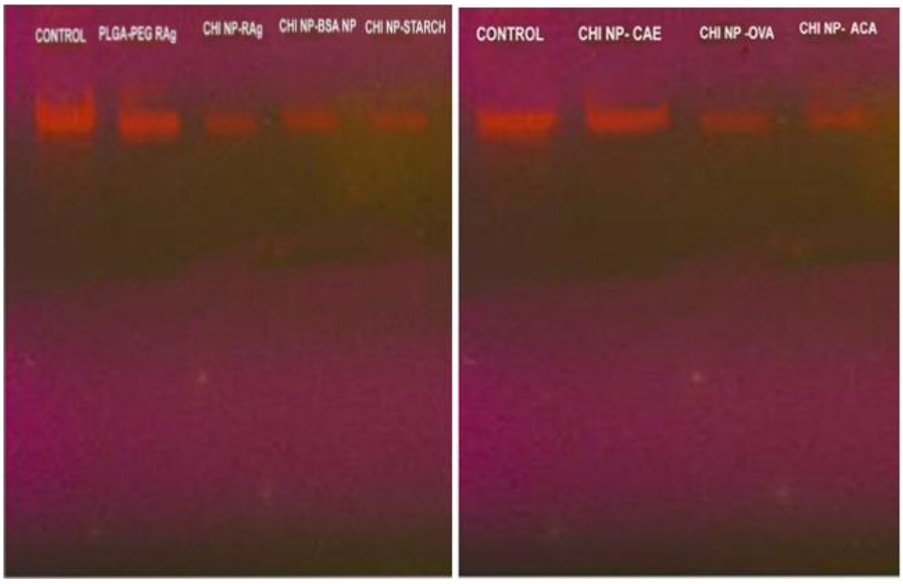

Fig. 10. Genotoxicity effect of formulated nano vaccine.

CS-PEG- RV Ag. and surface modified CS-PEG-RV Ag. revealed the size distribution of the polymeric nano formulation in saline media. A Zeta potential is a term for electro kinetic potential in colloidal systems which has a major effect on various properties of nano drug delivery system [23]. Zeta potential of the CS-PEG-RV Ag. and surface modified CS-PEG-RV Ag displayed lower +ve and −ve charges which show the biocompatibility and confirmation of the surface modification. Antigen stability study was performed for PLGA-b-PEGNP-RV Ag. and CS-PEG NP-RV Ag. by keeping the nano formulations at 100 °C for a month and the structural and storage stability were confirmed by running SDS-PAGE. In vitro immunogenicity by T cell rosette assay and phagocytic assay resulted in 60 T cell rosette for a 100 mg concentration and 80.7% of phagocytosis for a 100 mg concentration of the CS-PEG-RV Ag. respectively. In vitro release profile for the nano formulation showed the pattern of initial burst release in the starting phase followed by sustained release and another burst release was observed for CS-PEG NP and surface modified CS-PEG NP-RV Ag. and only one initial burst release pattern was observed in PLGA-b-PEG NP-RV Ag. In vitro toxicity using blood compatibility test for PLGA-b-PEG NP-RV Ag. and CS-PEG NP-RV Ag. was performed and the experiment resulted in no significant toxicity. Genotoxicity assay using human blood for the respective nano formulations revealed no toxicity. CS-PEG-RV Ag. is thus suggested for pharmaceutical mass production after the successful clinical trials.

Acknowledgment

I acknowledge Centre for Nano Science and Nanotechnology, Sathyabama University, Chennai, Tamil Nadu, India for helping in SEM and AFM analysis. Thanks to IIT Madras and Madras University, Chennai, Tamil Nadu, India for Zeta potential and TEM studies respectively.

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