Poly(ε-caprolactone) microspheres as a vaccine carrier

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Poly(ε-caprolactone) (PCL), a biocompatible and biodegradable polymer hitherto thought unsuitable for protein delivery because of its poor permeability to macromolecules, is shown to be sufficiently permeable to proteins to function as a vaccine carrier. Using a model antigen such as bovine serum albumin (BSA), we demonstrate that a single injection of BSA-loaded PCL microspheres generates an immune response comparable in magnitude and time kinetics to that of a conventional three-injection schedule of the antigen in a rat model. Unlike polyesters such as poly(lactic acid) (PLA) and poly(glycolic acid) (PGA), PCL degrades slowly and therefore does not generate an acid environment adversely affecting the antigenicity of vaccines and may prove to be promising as a vaccine carrier.

MILLIONS of children die each year from vaccine preventable diseases world-wide, since most vaccines require repeated administration in order to be effective and the compliance of the population in many parts of the world is rather poor[1]. Controlled delivery of vaccines using biodegradable polymeric microspheres as adjuvants has opened up the possibility of immunizing large populations against vaccine preventable diseases with single contact point immunization[2–8]. Polyesters such as PLA, PGA and their copolymers have been extensively studied as microsphere matrices for vaccine delivery[3–8]. One major drawback of these polymers is that their degradation generates extreme acid environment (pH 2–3) in which many vaccines are found to lose their antigenicity[9–10]. PCL is a biocompatible and biodegradable polymer which has been under clinical evaluation for sustained delivery of levonorgestrel world-wide[11]. PCL degrades slowly and, therefore, does not generate an acid environment unlike the PLA/PGA polymers. Although the permeability of macromolecules in PCL is low[11], such low permeability may be sufficient enough for vaccine delivery. This study was therefore undertaken to evaluate PCL as a vaccine carrier.

PCL microspheres containing ca 3% of the model antigen BSA were prepared by a double emulsion technique. One ml of a 5% solution of BSA (Fraction V, Sigma, USA) in water was homogenized with 10 ml of a 10% solution of PCL (average molecular weight 40,000; Polysciences, USA) in dichloromethane (DCM) using a high speed homogenizer (Polytron, Switzerland). This was introduced into 100 ml of a 1% aqueous solution of poly(vinyl alcohol) (PVA, molecular weight 10,000; Sigma, USA) and homogenized again. The dispersion was stirred in a 150 ml round-bottomed flask using a stainless-steel paddle stirrer at 1000 rev. min^{-1} for 3–4 h until all the DCM evaporated. Microspheres were centrifuged, washed with distilled water followed by acetone and air-dried. Particle size distribution was done using a laser-based particle size analyser (Galai, CIS-1, Israel) and SEM was carried out using a Hitachi (Model S-2400, Japan) instrument after sputter-coating the particles with gold in the usual way.

BSA content in the microspheres was estimated by two different methods. The amount of protein migrated into the dispersion medium and the aqueous washings was estimated after making background corrections for PVA and the loading was backcalculated[10]. Estimation was also carried out after alkaline hydrolysis of the polymer using NaOH and extraction by sodium dodecyl sulphate (SDS) which is reported to give accurate estimations[12]. Briefly, 10 mg of the microspheres was shaken with 3 ml of 5% SDS (Sigma, USA) in 0.1 mol dm^{-3} NaOH for 3 days at room temperature. The supernatent after centrifugation was analysed by Lowry's method of protein assay after making background corrections for PVA. Both methods gave a loading of ca 3% BSA within the limits of experimental error.

In vitro release of BSA from microspheres was examined in the following manner. Several 15 mg batches of BSA-loaded microspheres were introduced into 3 ml of phosphate buffer (pH 7.4, 0.1 mol dm^{-3}) in screw-capped vials of 5 ml capacity and incubated at 37°C. In order to overcome the interference due to PVA in protein estimations, blanks containing 15 mg of placebo microspheres were also incubated as controls. Samples were shaken occasionally every day. At definite time intervals, the contents in the vials were filtered and the amount of protein was estimated by Lowry's method. For each time period, a minimum of three vials were estimated and values averaged.

Adult Wistar rats weighing 125–150 g were used for immunogenicity studies. Two groups of 5 rats were immunized intramuscularly either with BSA solution emulsified with an equal volume of Freund's incomplete adjuvant (FIA) (control) or BSA-loaded microspheres suspended in FIA (experimental). The control group received 100 µg BSA emulsified with 50 µl FIA plus 50 µl saline on three occasions separated from each other by an interval of two weeks. The experimental group received microspheres equivalent to 300 µg BSA (ca 10 mg) with 100 µl FIA. When the response to this phase of immunization was observed to have declined,

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100 μg of BSA was administered as a booster dose at the end of 16 weeks in emulsified or encapsulated form (in FIA) to the control and experimental groups respectively. Animals were bled by retro-orbital puncture under ether anesthesia at different time points over a six-month period. Anti-BSA IgG in the serum was estimated using an ELISA standardized in the laboratory (NII) with a Eurogentics MPR A4 (Belgium) ELISA reader. Fresh sera were analysed at the time of collection and stored at –20°C till the termination of the study. The ELISA was then repeated for all samples obtained over the entire period of the study. Comparison between titres was made on the basis of this final assay using the Student's *t*-test. Results were considered statistically significant if $P < 0.05$.

Scanning electron microscopic (SEM) examination of the microspheres prepared showed smooth, spherical microspheres (Figure 1) and particle size analysis showed a volume average size of 3.67 ± 2.25 μm. Figure 2 shows the plot of cumulative amount of BSA released from PCL microspheres against square root of time. There is an initial burst of around 10–12% of the protein which could be mainly from the smaller-sized particles that leach out quickly in a purely diffusion-controlled situation since PCL degrades very slowly[11]. The release of a drug from a matrix type device where the release is diffusion-controlled is given by the Higuchi relation[13],

$$Q = kt^{1/2},$$

where Q is the amount released in time t and k depends on the surface area and diffusion constant. Assuming the surface area and diffusion coefficient to be constant throughout the experiment, a plot of Q versus $t^{1/2}$ should be linear. The plot is fairly linear as shown in Figure 2. Linear regression analysis gave a correlation coefficient of 0.949. Such deviations from linearity are not uncommon in protein release studies carried over extended periods of time, mainly due to the errors involved in estimating the amount of protein released into the dissolution medium accurately. It is noteworthy that in 6 months, only about 60% of the protein is released, which is under sink conditions. On the contrary, the antigen diphtheria toxoid having molecular weight comparable to BSA from PLA microspheres was released to the extent of 90% in 2 months[6]. Similarly, tetanus vaccine incorporated into PLA microspheres was found to be released to the extent of 40–60% in about 3 weeks[7]. In a recent study, using a novel encapsulation technique to prevent the large 'burst effect' that is usually seen with protein-loaded microparticles, McGee *et al.*[8] have found that ovalbumin incorporated into fast degrading polylactide-co-glycolide (PLGA) microspheres is released almost entirely in about 2 weeks. Although the permeability of PLA is 10^4 times less than that of PCL[11], such rapid release of macromolecules from PLA or PLGA matrices is due to polymer erosion. Erosion of these polyesters produces extreme acid environment and the local pH can go down to 2–3 even in a well-buffered medium[10]. The accumulation of acidic degradation end products, lactic acid and glycolic acid has been shown to be responsible for the drop in pH even when initially well-buffered medium is used. Thus, the released protein molecules are subjected to chemical degradation and aggregation in the acidified medium. The protein molecules remaining in the polymer matrix experience an even lower microenvironmental pH due to the higher local concentration of the degradation products[10]. Unfortunately, under such low pH conditions, many vaccines lose their antigenicity drastically. For example, at pH 2.5 and at 37°C, tetanus toxoid loses its antigenicity entirely in less than a week[9]. The antibody titres to tetanus toxoid incorporated into PLA or PLGA microparticles of many different compositions were thus found to be very low compared to alum or FIA prepara-

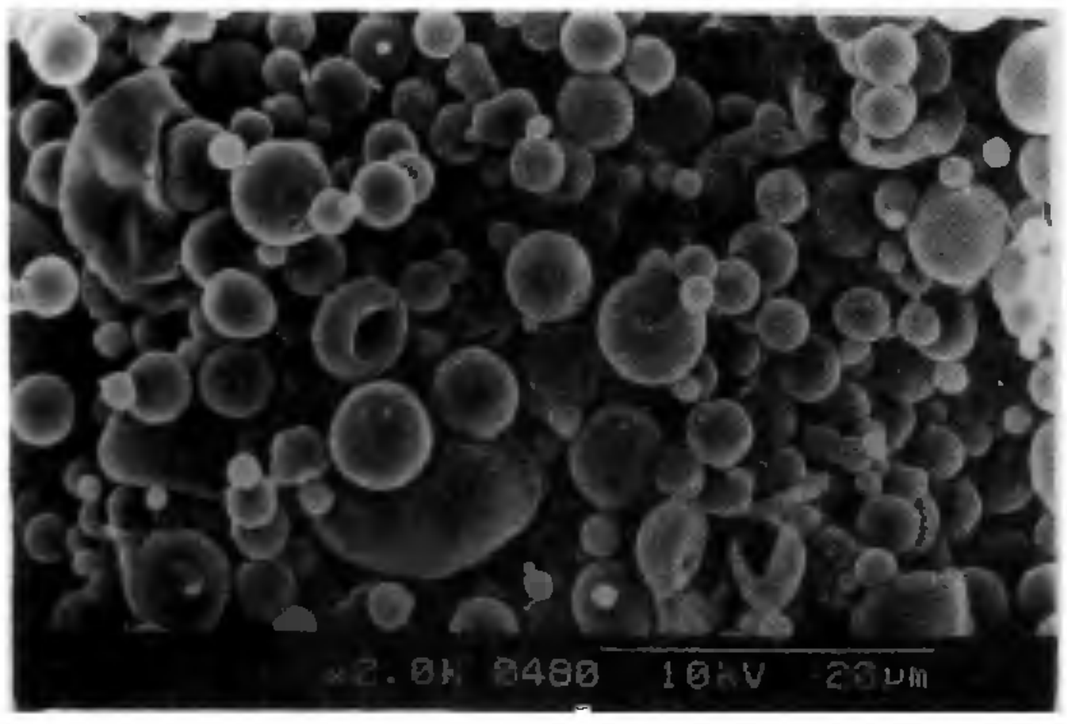

Figure 1. SEM of 3% BSA-loaded PCL microspheres.

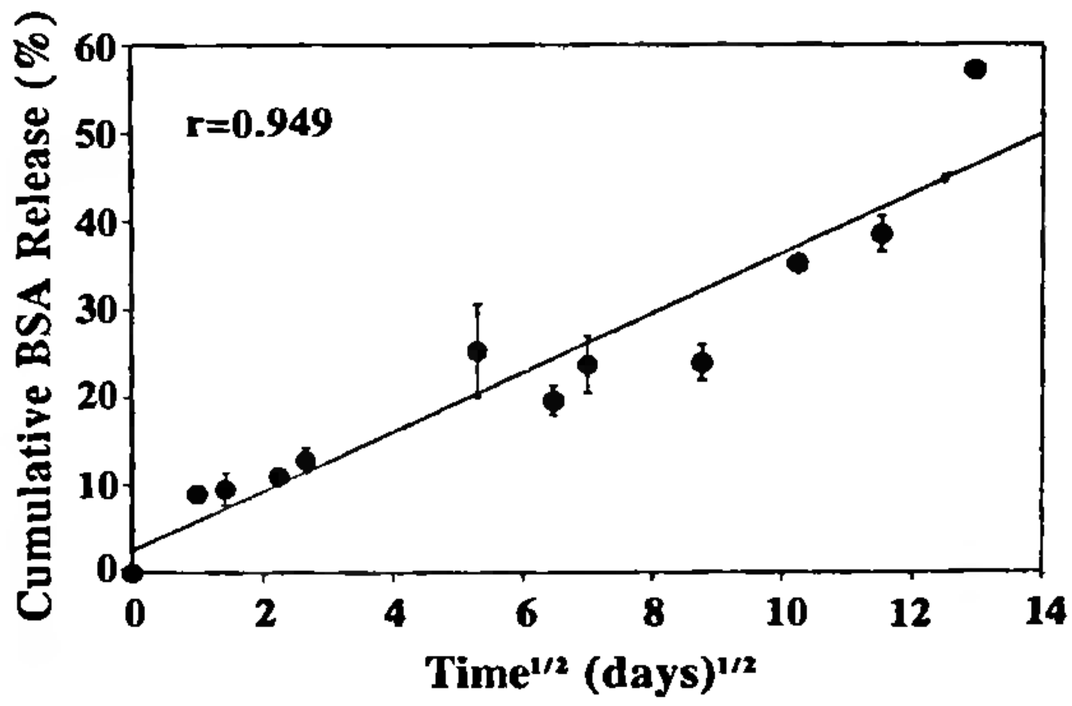

Figure 2. *In vitro* release of BSA into phosphate buffer at 37°C from PCL microspheres plotted against square root of time.

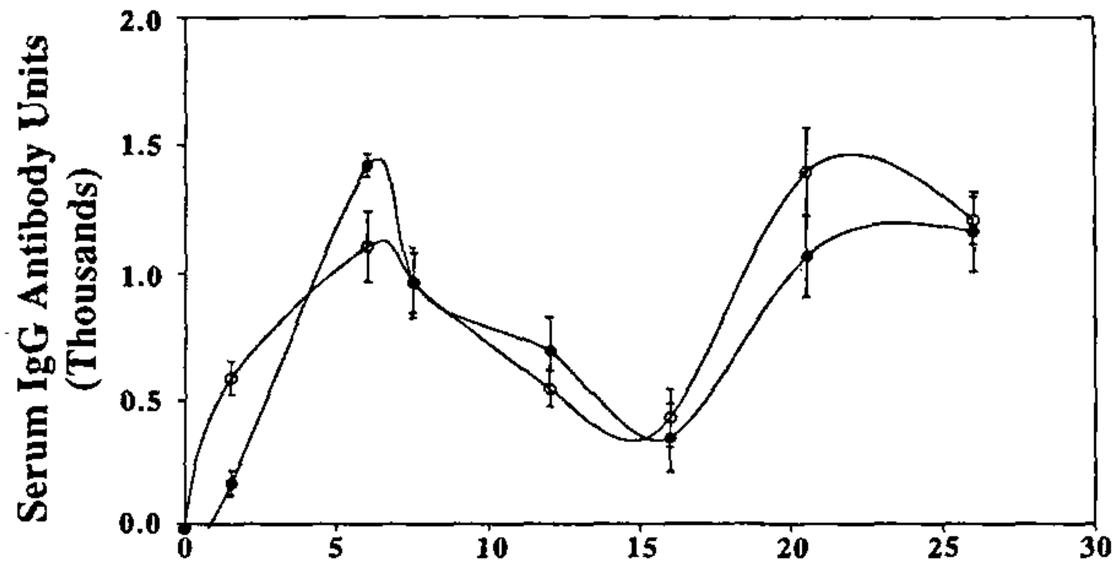

Figure 3. Immune response to BSA encapsulated in PCL microspheres (O) compared with BSA given in FIA (●) in Wistar rats.

tions[9]. We found no change in the pH of the buffer incubated with PCL microspheres over a six-month period, which suggested that protein antigenicity will not be affected by encapsulation in PCL. This observation is consistent with the relatively complete picture of PCL degradation available in the literature[11].

Figure 3 shows the antibody titres of the animals in the control and experimental groups. Both groups showed comparable titres both in terms of time kinetics as well as in magnitude. This implied that a single injection of the microspheres containing the antigen was equivalent to the 3-injection conventional schedule. The kinetics of *in vitro* and *in vivo* release of the antigen from microspheres however, appeared to be different. The prolonged release seen *in vitro* up to six months is not reflected in the *in vivo* response which tapers off after 90 days. This may possibly be due to progressive fibrous capsule encapsulation of the slow degrading microspheres preventing further protein release. When animals were boosted at the end of 16 weeks by administration of 100 μg of BSA in emulsified form (control) or in encapsulated form dispersed in FIA (experimental), titres in both group rose rapidly to similar values indicative of the fact that the delivery system was effective in generating B-cell memory. Comparison of titres over the entire period of study showed that they were not different at 0.05 level.

In this study, we employed FIA as an immunostimulatory vehicle although we are aware that it is not approved by regulatory authorities for human use[14]. This is because, it is well known that injection of empty microspheres in saline does not induce an inflammatory response to attract macrophages and injection of antigen-loaded microspheres in saline generates only a weak primary response[15]. Potent primary response could be seen only when the particles were injected in vehicles such as FIA[8,16]. Although FIA is not yet approved for human use, clinical trials involving several thousands of volunteers have demonstrated its safety and efficacy[17]. Many other immunostimulatory vehicles reported in the literature could possibly play the role of FIA as a vehicle for microsphere-based vaccine carriers.

In conclusion, this pilot study demonstrates that PCL, which was thought to have poor permeability for protein drugs, is sufficiently permeable to proteins from microsphere matrices to generate the desired immune response. Because of its delayed degradation characteristics, the acidic environment that accompanies the rapid biodegradation of PLA or PLGA copolymers is absent. This is extremely important in view of the fact that most vaccines lose their antigenicity at such low pH. The very comparable titres generated by the microsphere modality and the conventional modality presumably is testimony to this fact. Clinical trials of the PCL-based contraceptive delivery system Capronor™ world-wide have demonstrated the safety and efficacy of this polymer[11]. Therefore, PCL and copolymers of PCL with polyesters such as PLA or PGA as a matrix for constructing 'single shot' vaccine delivery system appears to be a promising approach.

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