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RESEARCH PAPER

# Evaluation of Poly (1, 6-bis-(p-carboxyphenoxy) Hexane-co-sebacic Acid Microspheres for Controlled Basal Insulin Delivery

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## ABSTRACT

***Purpose*** To develop poly 1,3-bis-(p-carboxyphenoxy) hexane-co-sebacic acid (p(CPH/SA)) microspheres for controlled basal insulin delivery and evaluate their *in vivo* efficacy and toxicity.

***Methods*** A series of CPH/SA copolymers with molar ratios 20/80, 40/60, and 50/50 were synthesized and characterized. The stability of encapsulated insulin and the fraction of insulin released from microspheres were assessed by different analytical techniques. The skin from the injection site of rats was examined microscopically for histomorphological changes.

***Results*** Increasing the molar ratio of CPH/SA significantly ($p < 0.05$) improved insulin loading and controlled insulin release. However, dimer aggregates of insulin were observed as CPH/SA molar ratio increased. Co-encapsulation of zinc oxide with insulin inhibited dimer aggregate formation and further controlled insulin release. Insulin was stable after entrapment into microspheres and during *in vitro* release studies. Administration of microsphere formulations CPH/SA 40/60 and 50/50 with zinc oxide controlled insulin release and maintained basal insulin levels for 42 days in rats. Skin sections showed minimal inflammation with no evidence for histomorphological changes and toxicity.

***Conclusions*** Insulin-loaded CPH/SA microspheres demonstrated considerable potential as controlled delivery system for insulin. Copolymer microspheres maintained basal insulin levels for 42 days and were biodegradable and biocompatible.

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## INTRODUCTION

Diabetes Mellitus, a chronic disease characterized by elevated plasma glucose continues to be a growing epidemic in both developed and developing countries. Recent findings indicate that by the year 2030, about 439 million worldwide will have diabetes (1). Basal insulin accounts for almost half of daily insulin output and plays an important role in maintaining plasma glucose levels within a narrow concentration range. At a rate of 0.5-1 U/h, basal insulin is secreted constantly between meals and throughout night contributing to serum insulin levels of about 5–15 U/mL (2). Basal insulin secretion regulates endogenous glucose production from the liver and the uptake of glucose in the peripheral insulin-sensitive target tissues. A controlled delivery system for basal insulin would benefit all type I and II diabetic patients since it is secreted in a continuous and constant fashion.

For the last two decades polymeric microspheres made from poly (lactic-co-glycolic acid) (PLGA) have been widely evaluated for controlled insulin delivery (3–6). In general, limitations including high initial burst, protein instability, and incomplete release profiles curtail the use of microspheres made from PLGA for controlled protein delivery. While various individual approaches have been reported to overcome these limitations, developing an approach that could overcome all the above-mentioned limitations still remains a major challenge. In recent years, polyanhydride polymers are being extensively studied for controlled protein delivery (7–10). Polyanhydrides have hydrophobic backbones and anhydride linkages which are hydrolytically labile (11). The degradation mechanism is one of the prominent

features which makes polyanhydrides desirable for drug delivery applications. In polyanhydrides, chain scission occurs mainly at the polymer matrix surface and the mass is translocated from periphery to the interior as the matrix degrades (12). The stability of the encapsulated drug is improved due to minimal water interaction prior to release and an ideal surface erosion can contribute to near zero-order release profiles (13). In addition, desired release rates can be achieved by making simple changes in the polymer backbone. A broad variety of polyanhydrides, *i.e.*, aliphatic, aromatic, unsaturated, aliphatic-aromatic, and anhydrides based on poly(ester), poly(ether), fatty acid, and amino acid have been presented over the years for drug delivery applications (14). Two clinically used drug delivery devices based on polyanhydrides are Gliadel® and Septacin™ (15). Gliadel® contains carmustine to treat brain tumor and has poly 1,3-bis-(p-carboxyphenoxy) propane-co-sebacic acid (p(CPP/SA)(20/80)) as polymeric carrier. Septacin™ implant employs poly(erucic acid dimer-sebacic acid) (p(EAD/SA)(50/50)) as polymeric carrier and delivers gentamicin for treating osteomyelitis.

The overall goal of the present work was to develop microsphere delivery system based on poly 1,6-bis-(p-carboxyphenoxy) hexane-co-sebacic acid p(CPH/SA) copolymer that could provide controlled basal insulin delivery for a prolonged duration. We previously explored the potential of CPP/SA copolymers for controlled basal insulin delivery (16). Insulin release was controlled over 30 days from CPP/SA 50/50 microspheres upon a single subcutaneous injection in diabetic rats. In an effort to further control insulin release and reduce injection frequency, the applicability of CPH/SA copolymer, a copolymer that is closely related to CPP/SA is evaluated in this work. The difference between CPP/SA and CPH/SA copolymers is the number of methylene groups in the aromatic monomer (Fig. 1). The aromatic CPP monomer has three methylene groups and the aromatic CPH monomer has six methylene groups. The longer the backbone chain length, the greater the hydrophobicity of the polymer and slower the erosion rate. We hypothesized that the more hydrophobic CPH/SA copolymer should further control insulin release over a long time period. In addition, insulin released from the microspheres should be sufficient enough to mimic endogenous basal insulin levels. Recently Determan *et al.* (7) demonstrated the efficacy of CPH/SA copolymers for the controlled delivery of bovine serum albumin labeled with fluorescein isothiocyanate.

Very little information is available in literature regarding the protective effect of various metal salts and other excipients on protein stability when encapsulated in highly hydrophobic polymers. As mentioned above, the longer backbone chain length in the CPH monomer imparts more hydrophobicity to the CPH/SA copolymer. Proteins when exposed to hydrophobic surfaces tend to unfold leading to the formation of irreversible covalent aggregates. Therefore, retaining insulin stability in CPH/SA microspheres is a major challenge. Here, we also evaluate whether a zinc salt could circumvent the formation of insulin aggregates by preventing insulin adsorption to the highly hydrophobic CPH surfaces. The hypothesis behind employing a zinc salt is that the zinc salt should stabilize insulin by forming hexamers, the most stable species of insulin. CPH/SA copolymers were synthesized with different molar ratios of CPH and SA (20/80, 40/60, and 50/50) to observe their effect on stability and release profile of insulin. A double emulsion solvent evaporation method was used for insulin encapsulation into CPH/SA microspheres. The stability of insulin encapsulated and released *in vitro* was determined by employing various comprehensive and sensitive investigative techniques. *In vivo* release profiles of insulin were evaluated in diabetic rats.

## MATERIALS AND METHODS

### Materials

Human recombinant insulin (Incelligent SG®) was obtained from Celliance Corporation (Norcross, GA). Sebacic acid, p-hydroxybenzoic acid, and 1,6-dibromohexane were procured from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Trifluoroacetic acid, polyvinyl alcohol (PVA; 30–80 kDa), zinc oxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), streptozotocin, mannitol, carboxymethylcellulose, methylene chloride, citric acid, α-cyano-4-hydroxycinnamic acid, sodium citrate, fluorescein

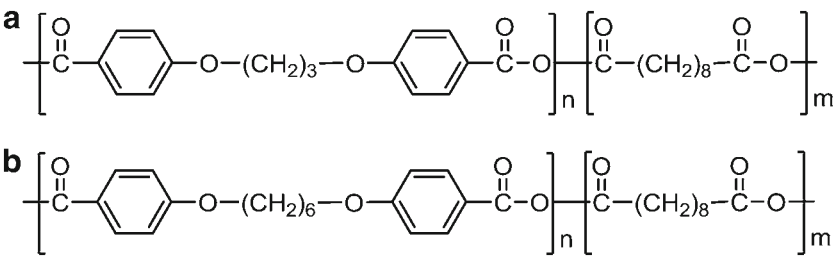


**Fig. 1** Chemical structure of (**a**) poly 1,3-bis-(p-carboxyphenoxy) propane-co-sebacic acid (p(CPP:SA)) copolymer and (**b**) poly 1,6-bis-(p-carboxyphenoxy) hexane-co-sebacic acid (p(CPH:SA)) copolymer.

isothiocyanate (FITC), acetic anhydride, and acetonitrile were purchased from Sigma (St. Louis, MO). Radioimmunoassay kit was purchased from Millipore (St. Charles, MO). All other reagents used in this work were of analytical grade and used as received.

## Methods

### *Synthesis and Characterization of CPH/SA Copolymers*

CPH synthesis was carried out with 1,6-dibromohexane as reported previously (16). Prepolymers of CPH and SA were synthesized as described in literature (17,18). The synthesized prepolymers were then copolymerized at different molar ratios by melt polycondensation and characterized as described earlier (16).

### *Preparation and Characterization of Microspheres*

The water/oil/water (w/o/w) double emulsion solvent evaporation technique was used to encapsulate insulin into CPH/SA microspheres. Briefly, 10 mg of insulin in 100 µl of phosphate buffered saline (PBS, 10 mM, pH 7.4) was mixed with dichloromethane (2 mL) containing 200 mg of the copolymer. A Silverson L4RT Homogenizer (Silverson Machines, East Longmeadow, MA) was used to homogenize the mixture at 4000 rpm for 1 min to form an uniform w/o emulsion. Under constant stirring the formed w/o emulsion was transferred into 100 mL of PVA aqueous solution (1% *w/v*) and further emulsified at 2000 rpm for 4 min. Following the formation of w/o/w double emulsion, 300 mL of PVA aqueous solution was added and stirred at 25°C for 3 h to evaporate dichloromethane. Microspheres were separated by centrifugation (3000 rpm, 10 min) and the residual PVA and insulin were removed by washing the microspheres with 20 ml of double distilled water for at least three times. The microspheres were stored at −20°C after lyophilization for 24 h. For the preparation of microsphere formulations with zinc oxide, 100 µl of 0.3 mM zinc oxide (prepared in 0.1 M HCl) was added to PBS with insulin prior to primary emulsification. Homogenization and microsphere recovery procedures were followed as described above. The prepared microspheres were characterized for their size, morphology, insulin loading, and insulin distribution within CPH/SA microspheres as described in our earlier publication (16).

## *In Vitro* Release Studies

CPH/SA microspheres (10 mg) were suspended in 1 ml of 10 mM PBS (pH 7.4, 0.01% $NaN_3$) and placed in a water bath with temperature set at 37°C. The samples were under mild agitation over the entire study period. The release medium was removed after centrifugation at specific time points and analyzed using the micro bicinchoninic acid (BCA) protein assay kit (Pierce Inc., Rockford, IL) (19). The microsphere samples were replenished with equal amounts of fresh buffer. The amount of insulin released from the microspheres are reported as mean ± SD from quadruplicate measurements.

### *Insulin Stability Studies*

The stability of the encapsulated insulin and the fraction of insulin released at various time points was evaluated using Fourier Transform Infrared Spectroscopy (FTIR), Circular Dichroism spectroscopy (CD), Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), High Performance Liquid Chromatography (HPLC), Size-Exclusion HPLC (SE-HPLC), and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) techniques as described in our earlier publications (16,20).

## *In Vivo* Studies

The pharmacokinetics and pharmacological activity of insulin were assessed in adult male Sprague–Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing about 125–150 g. The rats were handled and treated in compliance with NIH guide for the Care and Use of Laboratory Animals. The rats were made diabetic by administering a single subcutaneous injection of streptozotocin at a dose of 55 mg/kg and were confirmed to be diabetic if the blood glucose levels were≥400 mg/dl. Twenty four rats were randomized into two treatment groups ($n$=6 per group) and two control groups ($n$=6 per group). One treatment and control group received CPH/SA 40/60 microspheres encapsulated with insulin and zinc oxide and blank CPH/SA 40/60 microspheres, respectively. The other treatment and control group received CPH/SA 50/50 microspheres encapsulated with insulin and zinc oxide and blank CPH/SA 50/50 microspheres, respectively. The dose for both treatment groups was 60 U/kg. The control group received equivalent weight of the respective blank microspheres. Microsphere suspensions were prepared by dispersing microspheres in saline (2% carboxymethylcellulose, 2% mannitol) and were vortex-mixed before administration. Microspheres were injected subcutaneously at neck region of the rats using standard 23-guage needles. After overnight fasting, the following morning blood samples (approximately 400 µl) were drawn *via* the tail vein. The blood samples were

centrifuged within 1 h to separate serum. Cetrifugation was carried out for 10 min at 3000×g and the serum samples were stored at −20°C until insulin measurements were performed. Serum insulin levels were determined by radioimmunoassay and blood glucose levels were measured with Elite glucometer (Bayer Corporation, Elkhart, IN).

### *In Vitro* Cytotoxicity Studies

The *in vitro* cytotoxicity of the various CPH/SA microsphere formulations and their degradation products was evaluated using the MTT assay. Human embryonic kidney (HEK293) cells (American Type Culture Collection, Rockville, MD) in media were added to a 96-well plate ($8\times10^3$ cells/well) and incubated for 24 h at 37°C in a 5% $CO_2$ incubator. Following incubation, cells were treated with CPH/SA microsphere formulations at different concentrations (10 and 50 μg per well), and incubated for 24 and 72 h. After incubation, 10 μl of freshly prepared MTT reagent was added to each well. The plate was shaken gently and incubated again for another 4 h. Following incubation, the supernatant was discarded and 100 μl of dimethyl sulfoxide was added and swirled gently. The absorbance was measured using a microplate reader at 570 nm. Percentage cell viability was calculated using the following formula: cell viability (%)=(experimental absorbance)/(control absorbance)×100.

### *In Vivo* Biocompatibility Analysis

*In vivo* biocompatibility was assessed by studying rat skin tissue for inflammatory changes after the administration of microsphere formulations. Microspheres were injected subcutaneously on the back near the neck region. One rat was injected with saline to serve as control. At the end of day 1 and 90 post injection, rats were euthanized by intraperitoneal injection of Nembutal (pentobarbital sodium) at a dose of 100 mg/kg. Using a scalpel skin samples were removed from the injection site and fixed in 10% buffered formalin solution. Skin sections of about 5 microns thick were stained with hematoxylin and eosin and observed under a light microscope. Skin sections were evaluated for chronic inflammation, cell damage, and fibrous capsule formation.

### Statistical and Pharmacokinetic Analysis

The experimental results were analyzed with one-way analysis of variance and Student's *t*-test at a significance level of 0.05. The results from *in vivo* experiments are presented as mean ± S.D ($n$=6).

## RESULTS

### Characterization of CPH/SA Copolymers and Microspheres

Table I shows the characteristics of the prepolymers and CPH/SA copolymers synthesized by melt polymerization condensation. The composition of the copolymers were determined from $^1$H nuclear magnetic resonance spectra by integrating the CPH aromatic proton peaks from approximately 6 to 9 ppm and SA methylene proton peaks at 1.32 ppm. The molar CPH and SA segments in the copolymers were in close agreement with the feed molar ratios. Higher molar ratios of CPH (>50%) in the CPH/SA copolymer shows an elevation in crystallinity and melting point and hence restrict the solubility in organic solvents (21). Therefore, CPH/SA copolymers with higher CPH ratios were not synthesized. Gel permeation chromatograms of obtained copolymers exhibited an unimodal mass distribution indicating that the obtained copolymers were pure with no low molecular weight fractions of cyclic oligomers or any other unreacted feed materials.

Various batches of microspheres were prepared with CPH/SA 20/80, 40/60, and 50/50 copolymers. In order to assess the effects of zinc oxide in CPH/SA microspheres, three additional batches of CPH/SA microspheres (20/80, 40/60, and 50/50) were prepared by adding zinc oxide to insulin prior to encapsulation. The molar ratio of zinc added to insulin hexamer was 4:1 (4 $Zn^{2+}$/hexamer). Table II illustrates the properties of the microspheres made using various CPH/SA copolymers. The particle size of the microspheres were observed to be uniform and were between 40.1 and 47.4 μm. Microspheres prepared from CPH/SA copolymers irrespective of their molar ratios exhibited smooth surface morphologies (Fig. 2). In addition, there were no visible pores observed. Coencapsulation of zinc oxide was found not to affect

**Table I** Weight Average Molecular Weight ($M_W$), Melting Point Temperature ($T_m$), and Polydispersity Values of the CPH/SA Copolymers Synthesized by Melt Polycondensation Technique

| Polymer | Feed molar ratio | Found molar ratio[a] | $M_W$ | Polydispersity | Tm (°C) |
|---|---|---|---|---|---|
| SA | - | - | 22000 | 2.9 | - |
| CPH | - | - | 27000 | 2.4 | - |
| CPH/SA (20/80) | 20/80 | 18/82 | 20000 | 2.5 | 78.2 |
| CPH/SA (40/60) | 40/60 | 38/62 | 24000 | 3.1 | 64.1 |
| CPH/SA (50/50) | 50/50 | 47/53 | 16000 | 2.3 | 51.6 |

$M_W$ and polydispersity, determined by gel permeation chromatography

$T_m$, determined by differential scanning calorimetry

[a] determined by $^1$H nuclear magnetic resonance spectroscopy

**Table II** Composition and Overall Characteristics of Microspheres Prepared from CPH/SA Copolymers

| Polymer | Zinc compound | Encapsulation efficiency (%)[a] | Mean particle size ($\mu$m)[b] |
|---|---|---|---|
| CPH/SA (20/80) | - | 69.7 ± 0.3 | 40.1 ± 12.4 |
| CPH/SA (40/60) | - | 74.3 ± 0.3 | 43.9 ± 17.1 |
| CPH/SA (50/50) | - | 77.9 ± 1.2 | 47.4 ± 15.2 |
| CPH/SA (20/80) | Zinc oxide | 81.8 ± 0.7 | 41.7 ± 14.6 |
| CPH/SA (40/60) | Zinc oxide | 85.1 ± 0.1 | 40.3 ± 16.8 |
| CPH/SA (50/50) | Zinc oxide | 91.2 ± 0.4 | 42.4 ± 11.3 |

[a] means ± S.D., $n$=4

[b] means ± S.D., $n$=100

microsphere size and surface morphology. Confocal microscopy images revealed homogeneous distribution of insulin in the matrix of microspheres, for all three formulations (Fig. 3). This also demonstrates that there were no interactions (*i.e.*, adsorption) between insulin and the hydrophobic copolymers during the microsphere preparation process. Also, the molar ratios of the copolymers were not found to affect insulin distribution across the microsphere matrix.

### *In Vitro* Release Profiles

Figure 4a displays insulin release from various CPH/SA microspheres formulations prepared without the addition of zinc oxide. The initial burst of insulin from CPH/SA microspheres was about 50% and within 3 weeks the entire amount of encapsulated insulin was released. The initial burst was about 30% for CPH/SA 40/60 microspheres and 27% for CPH/SA 50/50 microspheres. Insulin release decreased rapidly after the initial burst for both formulations and a slow release was seen thereafter. Lower initial burst and a more controlled release profile of insulin were seen from CPH/SA microspheres with zinc oxide (Fig. 4b) when compared to similar formulations without zinc oxide. For CPH/SA 20:80 microspheres with zinc oxide, an initial burst of 33% was seen and the release of insulin was controlled over a month. Initial bursts decreased significantly ($p$ <0.05) when the molar ratio hydrophobic monomer CPH increased to 40 and 50%. Both CPH/SA 40/60 and 50/50 microspheres showed released profiles that were superimposable and released approximately 94 and 89% of encapsulated insulin by day 60.

### Insulin Stability Analysis

The stability of insulin encapsulated and released from CPH/SA microspheres were evaluated using various analytical techniques at different time points. Conformational changes in the secondary structure of insulin encapsulated within the CPH/SA microsphere matrices were monitored by FTIR spectroscopy. Table III displays the percentage of the secondary structure contents including α-helix, β-sheet, and unordered structures of insulin quantified from the amide I region. The individual assigned bands for α-helix (1658–1659 $cm^{-1}$), β-sheet (1630, 1690, 1642–1643 $cm^{-1}$), and unordered structures (1649, 1669 $cm^{-1}$) were used for the quantification. Conservation of α-helix is commonly

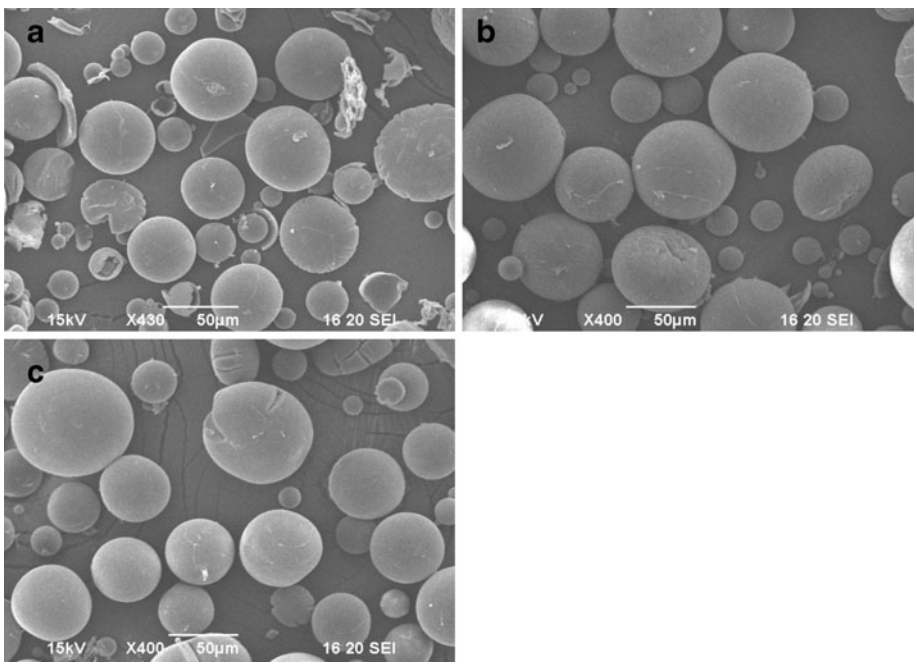


**Fig. 2** SEM photographs of polyanhydride microspheres loaded with insulin: (**a**) CPH:SA 20:80; (**b**) CPH:SA 40:60; (**c**) CPH:SA 50:50.

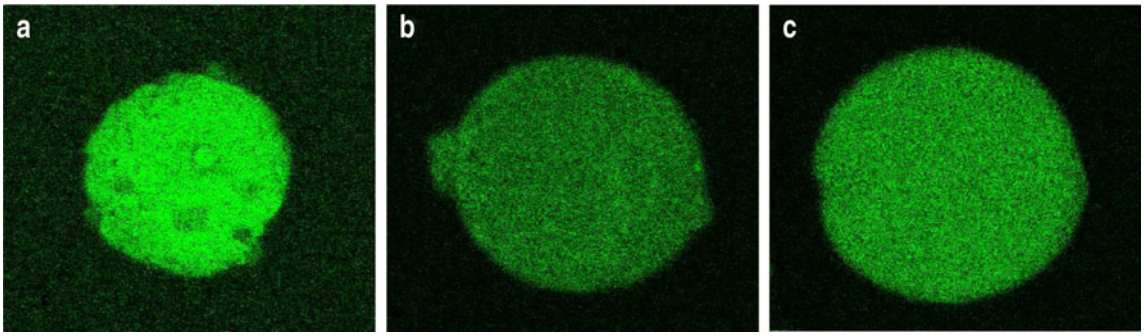


**Fig. 3** Confocal microscopy images of polyanhydride microspheres loaded with FITC-insulin: (**a**) CPH:SA 20:80; (**b**) CPH:SA 40:60; (**c**) CPH:SA 50:50.

used to judge the secondary structure of proteins (22). Secondary structure content determination of native insulin resulted in 42% α-helix and 13% β-sheet. A slight decrease in α-helix content was observed for insulin encapsulated in CPH/SA microspheres without zinc oxide. Slightly higher β-sheet content for insulin was noticed corresponding to the slightly lower α-helix content. CPH/SA microsphere formulations with zinc oxide exhibited α-helix content similar to native insulin with no discernible amplification of β-sheet content.

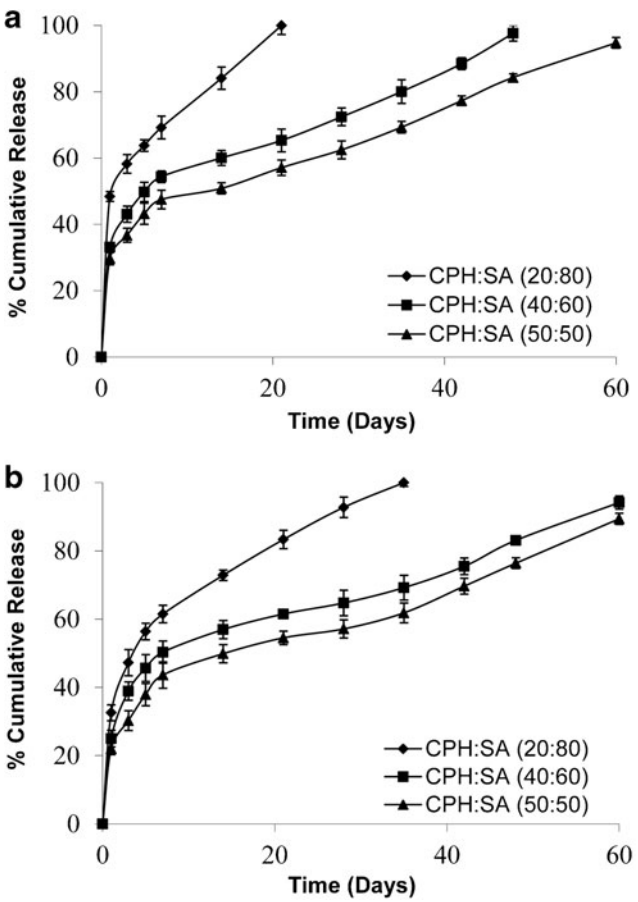


**Fig. 4** *In vitro* release profiles of insulin from (**a**) CPH:SA microspheres and (**b**) CPH:SA microspheres with zinc oxide.

*In vitro* release study samples obtained on day 7 were analyzed for insulin stability by analytical techniques. SDS-PAGE experiments under non-reducing conditions were carried out to analyze the primary structure of insulin. SDS-PAGE patterns of insulin released from various CPH/SA microspheres with and without zinc oxide are shown in Fig. 5. As expected, a single protein band at 6 kDa was observed for native insulin which was a freshly prepared insulin solution. Similar protein bands at 6 kDa were observed for insulin released from various CPH/SA microsphere formulations. No protein bands for covalent dimers or high molecular weight aggregates were observed. The mass spectrum of insulin released from CPH/SA microspheres and mass spectrum of native insulin are shown in Fig. 6. For native insulin, the molecular ion $[M + H]^+$ is represented by the peak at 5808 m/z and the molecular ion $[M + H]^{2+}$ is represented by the peak at 2918 m/z. The mass spectra of insulin released from the CPH/SA microspheres matched with the spectrum of native insulin. No

**Table III** Secondary Structure of Insulin Encapsulated in CPH/SA Microsphere Formulations with and without Zinc Oxide as Determined by FTIR

| Formulation | Secondary structure content (%) | | |
|---|---|---|---|
| | α-helix[b] | β-sheet[b] | Unordered[ab] |
| Native insulin | 42 ± 1 | 13 ± 2 | 45 ± 1 |
| CPH/SA (20/80) | 38 ± 2 | 19 ± 1 | 43 ± 6 |
| CPH/SA (40/60) | 38 ± 1 | 14 ± 1 | 48 ± 1 |
| CPH/SA (50/50) | 37 ± 3 | 15 ± 1 | 48 ± 4 |
| CPH/SA (20/80) + Zn | 40 ± 3 | 15 ± 4 | 45 ± 1 |
| CPH/SA (40/60) + Zn | 41 ± 3 | 14 ± 4 | 45 ± 2 |
| CPH/SA (50/50) + Zn | 41 ± 2 | 16 ± 1 | 43 ± 8 |

[a] Unordered structures include random coils and turns

[b] The ± values are the standard deviations calculated by analyzing three individual spectra

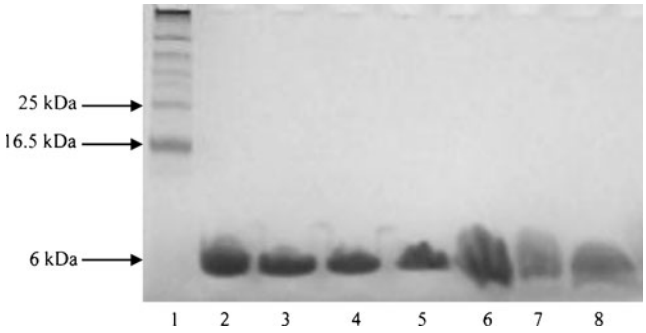


**Fig. 5** SDS-PAGE analysis of insulin released from CPH:SA microspheres at day 7. Lane 1: standard molecular marker, lane 2: fresh insulin solution, lanes 3–5: insulin released from CPH:SA 20:80, 40:60, 50:50 microspheres, and lanes 6–8: insulin released from CPH:SA 20:80, 40:60, 50:50 microspheres with zinc oxide.

additional peaks that represent insulin degradation products were observed. Similar peak characteristics in the mass spectra for insulin encapsulated with zinc oxide in CPH/SA microspheres were obtained (data not shown).

Conformational changes in the secondary structure of insulin during the release from CPH/SA microspheres were monitored by Far-UV CD spectroscopy. The CD spectra of insulin released from CPH/SA microspheres were compared with the CD spectrum of native insulin. The CD spectrum of native insulin exhibited two minima at 208 and 223 nm, indicating a dominant α-helical structure (Fig. 7a, b). All the CD spectra of insulin released from CPH/SA microspheres (Fig. 7a) showed predominant α-helical structure identical to that of native insulin. Similar results were observed for insulin released from CPH/SA microspheres encapsulated with zinc oxide (Fig. 7b). The results obtained from CD measurements were in agreement with FTIR results.

*In vitro* release study samples obtained on day 14 were analyzed for the presence of any degradation and aggregation products by HPLC and SE-HPLC. Desamido insulin and covalent dimers are the two predominant chemical degradation products of insulin found when insulin was encapsulated in polymeric microspheres. HPLC chromatogram of native insulin (Fig. 8) showed a single absorbance peak at 11 min representing insulin monomer with a molecular weight of 6 kDa. HPLC chromatograms of insulin released from CPH/SA microspheres (Fig. 8) also exhibited a single absorbance peak and were indistinguishable from that of native insulin. Similar chromatograms were obtained for insulin released from CPH/SA microspheres encapsulated with zinc oxide (data not shown). The amount of insulin aggregates detected by SE-HPLC are reported as percentage in Table IV. The percentages were calculated by

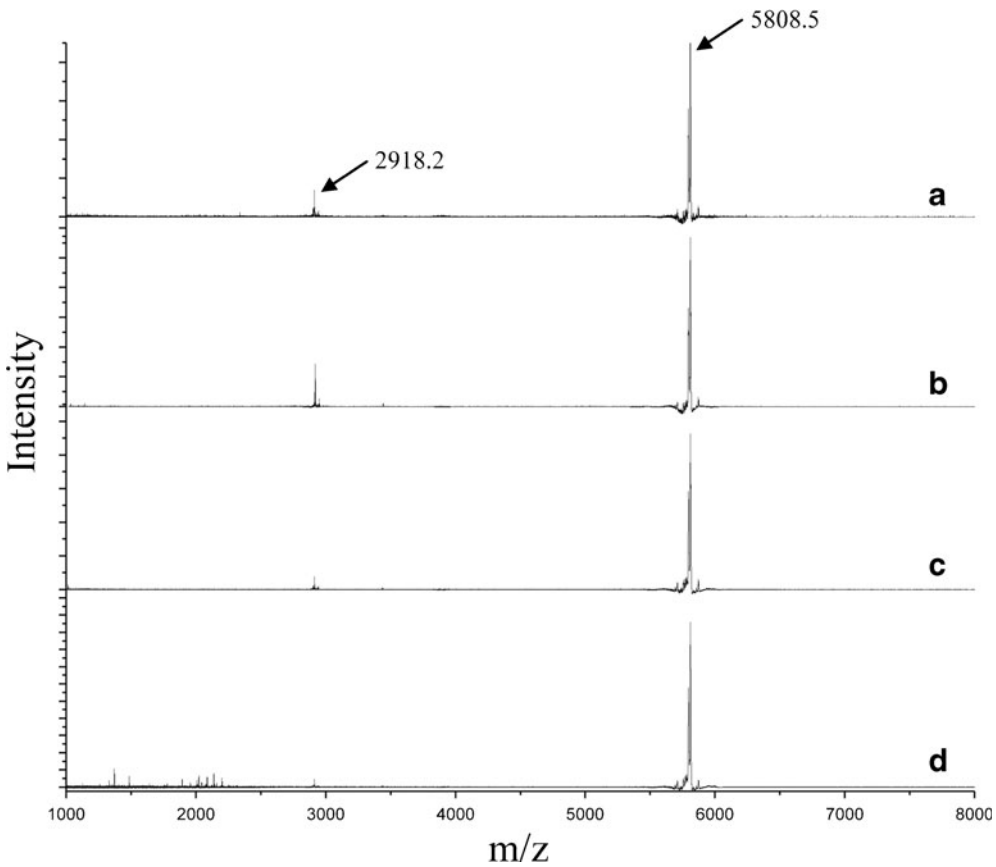


**Fig. 6** MALDI-TOF mass spectrum of native insulin (**a**) and insulin released at day 7 from CPH:SA microspheres: (**b**) 20:80; (**c**) 40:60; (**d**) 50:50.

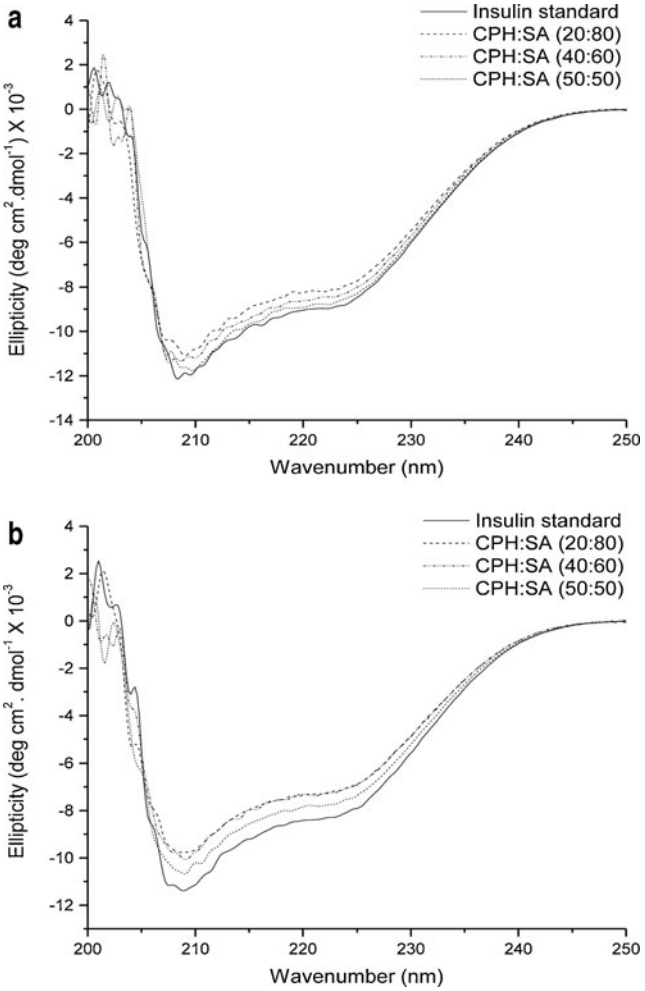


**Fig. 7** Far-UV CD spectra of insulin released from (**a**) CPH:SA microspheres and (**b**) CPH:SA microspheres with zinc oxide at day 7. Insulin standard refers to a freshly prepared insulin solution.

dividing the respective peak area of the monomers and dimers by total area. A small percentage of dimers were seen for insulin released from CPH/SA microspheres without zinc oxide.

### *In Vivo* Studies

For *in vivo* studies, microsphere formulations of CPH/SA 40/60 and 50/50 with zinc oxide were chosen since they showed very low initial bursts and prolonged insulin release over 2 months *in vitro*, and preserved the stability of the encapsulated insulin. Following a single subcutaneous injection, each of the formulations were evaluated by measuring the blood glucose levels and serum insulin for several weeks. Rats in two groups injected with blank CPH/SA 40/60 and 50/50 microspheres served as positive controls. The baseline serum insulin levels in streptozotocin induced diabetic rats ranged between 4 and 5 μU/mL. The serum insulin levels following the administration of microsphere formulations CPH/SA 40/60 and 50/50 microspheres are illustrated in Fig. 9a. The serum insulin levels peaked at 1 day after dosing for both formulations indicating the initial burst of insulin as observed *in vitro*. By day 14, serum insulin levels gradually dropped and reached levels of about 15 and 12 μU/mL for microsphere formulations CPH/SA 40/60 and 50/50, respectively. For both the formulations, the serum insulin levels were about 7 μU/mL by day 60. The *in vivo* release profiles for the formulations were very close, except for some difference during the 14–28 day period.

Figure 9b shows the changes in blood glucose levels that occurred over 60 days following the administration of microsphere formulations CPH/SA 40/60 and 50/50. The levels of blood glucose in diabetic rats prior to treatment were observed to be above 450 mg/dL. Changes in blood glucose levels were consistent with serum insulin levels. At 1 h, the blood glucose levels for rats injected with microsphere formulations CPH/SA 40/60 and 50/50 were 277 and 293 mg/dL, respectively. A considerable glucose lowering activity until day 42 was noticed for both formulations and the blood glucose levels returned close to pre-treatment levels by day 60. For comparison, insulin solution (2U/kg) was administered subcutaneously, and serum insulin and blood glucose levels were measured up to 12 h after injection. Maximum concentrations of insulin were observed at 2 h and then rapidly declined to baseline levels within 12 h. The $C_{max}$ and $t_{max}$ were 122 μU/ml and 2 h, respectively. Blood glucose levels dropped to 139 mg/dl within 2 h of injection and returned to pretreatment levels within 12 h.

### *In Vitro* Cytotoxicity

All of the six CPH/SA microsphere formulations prepared were evaluated *in vitro* cytotoxicity using MTT assay. The MTT assay was performed after 24 and 72 h of incubation with CPH/SA microspheres (10 and 50 μg), with medium cultures serving as control. Incubation of HEK293 cells with CPH/SA microspheres did not significantly ($p>0.05$) affect cell viability at any concentration (Fig. 10a, b). When compared to 10 μg microsphere concentrations, decreased cell viability was seen for 50 μg concentrations at both time points, but the inhibitory effect was insignificant. Formulations with zinc oxide showed higher cell viability in comparison to the formulations without zinc oxide. Cell viabilities were seen to decrease as the CPH monomer ratio was increased in the copolymers. However, the decrease in cell viabilities was found to be not significant ($p>0.05$).

### *In Vivo* Biocompatibility

Figure 11 shows the micrographs of histology sections taken at day 1 and day 90 post injection, respectively. Analysis of the tissue site 1 day after microsphere injection revealed

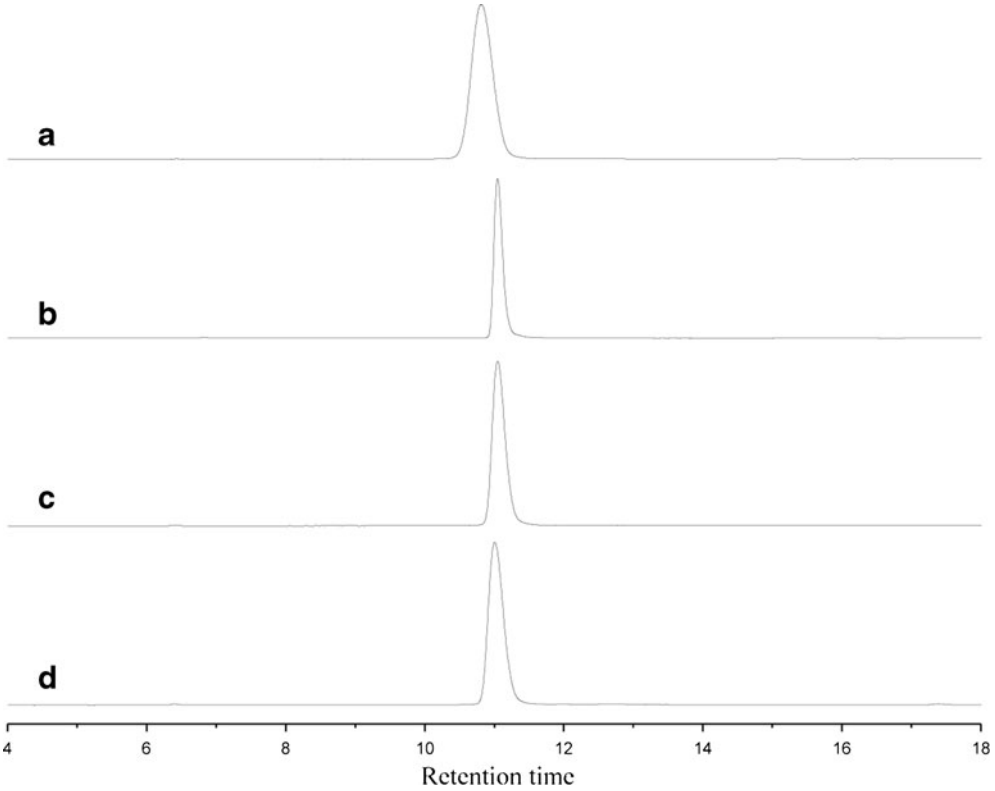


**Fig. 8** HPLC chromatograms of native insulin (**a**), insulin released from CPH:SA 20:80, 40:60, and 50:50 microspheres (**b**, **c**, and **d**) on day 14.

inflammatory responses and infiltration of neutrophils due to tissue trauma resulting from needle injection. The acute and chronic inflammatory responses for biodegradable microspheres are of relatively short duration, *i.e.*, from 1 to 2 weeks (23). Tissue samples did not exhibit rough endoplasmic reticulum or swollen mitochondria. At day 90, skin tissue sections taken from rats injected with CPH/SA 40/60 and CPH/SA 50/50 microspheres showed the absence of macrophages and giant cells (Fig. 11c, d). This means that the microspheres degraded completely over time. The tissue sections also appeared to be very similar to the control tissue (Fig. 11e).

**Table IV** Aggregation of Insulin Released from CPH/SA Microspheres at day 14, as Determined by SE-HPLC

| Formulation | Monomer (%)[a] | Dimer (%)[a] | HMWP |
|---|---|---|---|
| Native insulin | 100 | 0 | ND |
| CPH/SA (20/80) | 99.52 | 0.48 | ND |
| CPH/SA (40/60) | 98.18 | 1.82 | ND |
| CPH/SA (50/50) | 96.53 | 3.47 | ND |
| CPH/SA (20/80) + Zn | 99.99 | 0.01 | ND |
| CPH/SA (40/60) + Zn | 99.99 | 0.01 | ND |
| CPH/SA (50/50) + Zn | 99.99 | 0.01 | ND |

*HMWP* high molecular weight products

*ND* none detected

[a] Average values obtained from replicate analysis ($n=3$)

## DISCUSSION

An encapsulation efficiency of about 69% was seen for CPH/SA 20/80 microspheres without zinc oxide (Table II). The low encapsulation efficiency can be attributed to higher molar ratio of SA leading to increased hydrophilicity of the microspheres. The SA-SA bonds are highly water labile and as they come in contact with the secondary aqueous phase during microsphere fabrication, fractions of SA can be expected to undergo hydrolytic degradation. Diffusion of insulin into the secondary aqueous phase occurs as SA degrades resulting in reduced encapsulation. Further, the low molecular weight and hydrophilic nature of insulin aids quick diffusion into the secondary aqueous phase. An encapsulation efficiency of about 81% was achieved with the addition of zinc oxide. The molecular weight of insulin hexamers (~36 kDa) formed with the addition of zinc oxide and their poor aqueous solubility at neutral pH (24) prevented insulin diffusion into the secondary aqueous phase leading to increased insulin encapsulation. It is obvious that fabrication of microspheres by employing CPH/SA copolymers with high SA content can result in poor insulin encapsulation efficiency and hence an optimal ratio must be considered in order to obtain high encapsulation efficiencies. As the molar ratio of hydrophobic component CPH was increased in the copolymers, encapsulation efficiency increased which is due to the increased hydrophobicity of

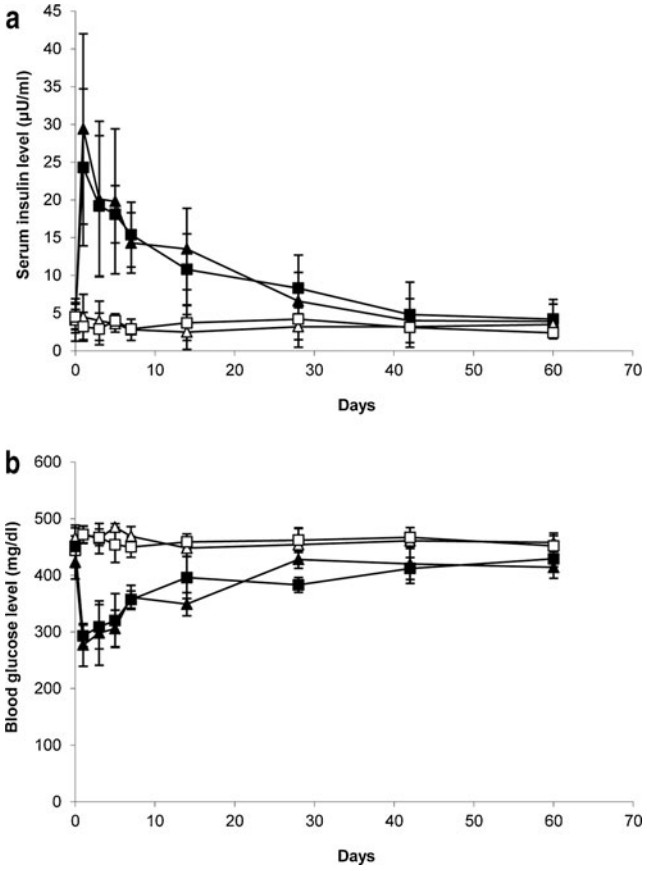


**Fig. 9** (**a**) Serum insulin and (**b**) blood glucose levels in diabetic rats after a single subcutaneous administration of insulin loaded CPH:SA microspheres. CPH:SA 40:60 microspheres with zinc oxide (*Black up-pointing triangle*), CPH:SA 50:50 microspheres with zinc oxide (*Black Square*), blank CPH:SA 40:60 microspheres (*Increment*), and blank CPH:SA 50:50 microspheres (*White square*). Data are expressed as mean ± SD, and $n=6$. Serum insulin levels were determined by radioimmunoassay and blood glucose levels were determined using the Glucometer Elite kit.

the CPH monomer preventing insulin diffusion into the secondary aqueous phase. Higher encapsulation efficiency and microsphere yields were observed for microspheres prepared from CPH/SA 50/50 copolymer. Significant increase in encapsulation efficiencies were observed for CPH/SA microsphere formulations prepared with zinc oxide compared to the microsphere formulations prepared in the absence of zinc oxide. Microsphere formulation CPH/SA 50/50 with zinc oxide showed the maximum encapsulation efficiency (91%) when compared to other formulations (Table II).

Initial burst release of insulin was seen from all the microsphere formulations (Fig. 4a, b). Since, the polyanhydrides degrade by erosion of the surface, initial burst is usually unexpected. Initial insulin burst observed with all the CPH/SA microsphere formulations could be explained by the size of the microspheres prepared and the lower molecular weights of the copolymers. A high initial burst of about 50% was observed from the microsphere formulation CPH/SA 20/80 and this increased burst is likely due to higher SA content (80%) in the copolymer. SA degradation occurs as soon as the microspheres are exposed to the release medium thereby releasing insulin molecules present at the surfaces. Further, the degradation of SA-SA bonds creates pores in the erosion front releasing insulin molecules entrapped near the microsphere surfaces. In addition, microspheres smaller in size have increased surface area which results in rapid uptake of the release medium, degradation, and insulin release. Insulin release rate was controlled following the initial burst and the release rate was dependent on the copolymer degradation rate. Since the copolymer is hydrophilic in nature, the SA-SA bonds degrade and erode more quickly than the CPH-CPH bonds. Quicker erosion and solubilization of the SA monomers creates pores in the matrix through which the release medium penetrates to the matrix core allowing the encapsulated insulin to diffuse out from the matrix. As soon as the erosion of the SA monomers is complete, a partially eroded matrix containing only CPH-CPH bonds is left and the remaining insulin was released as the matrix eroded over time.

As the amount of CPH in the copolymers was increased, initial burst decreased significantly ($p<0.05$) and insulin release was observed to be controlled. Increase in the number of CPH-CPH bonds in the copolymer increases the hydrophobicity of the copolymer and decreases the degradation rate by inhibiting release medium penetration to the matrix core. It can be clearly seen from Fig. 4 that insulin was initially released from the matrix surface as a result of SA degradation and depletion followed by a slow continuous release due to the erosion of CPH-CPH bonds. After 1 week, the release kinetics proceeded close to zero order until 50 days indicating the surface erosion property of polyanhydrides. The CPH/SA microspheres with zinc oxide revealed significantly ($p<0.05$) lower initial bursts and greater controlled release of insulin in comparison to the CPH/SA microspheres without zinc oxide (Fig. 4b). The lower initial bursts observed are due to the poor solubility of the insulin hexamers and their high molecular weight which prevented diffusion through the pores as the matrix surfaces start to erode. Protein solubility is a critical factor in determining the extent of initial burst from the microspheres. Results from a previously published study (25) have shown that high molecular weight proteins are released slower as compared to the release of low molecular weight proteins, which is consistent with our results. The release of insulin from CPH/SA microspheres with zinc oxide was more controlled. The presence of zinc oxide in the microsphere matrix affected the degradation rate of the copolymers. Zinc oxide has low aqueous solubility and must have led to lower uptake of the release medium resulting in gradual hydration and degradation. In addition, a bivalent metal ion such as zinc can neutralize the carboxylic acids that are created during CPH/SA hydrolysis and hence reduce the

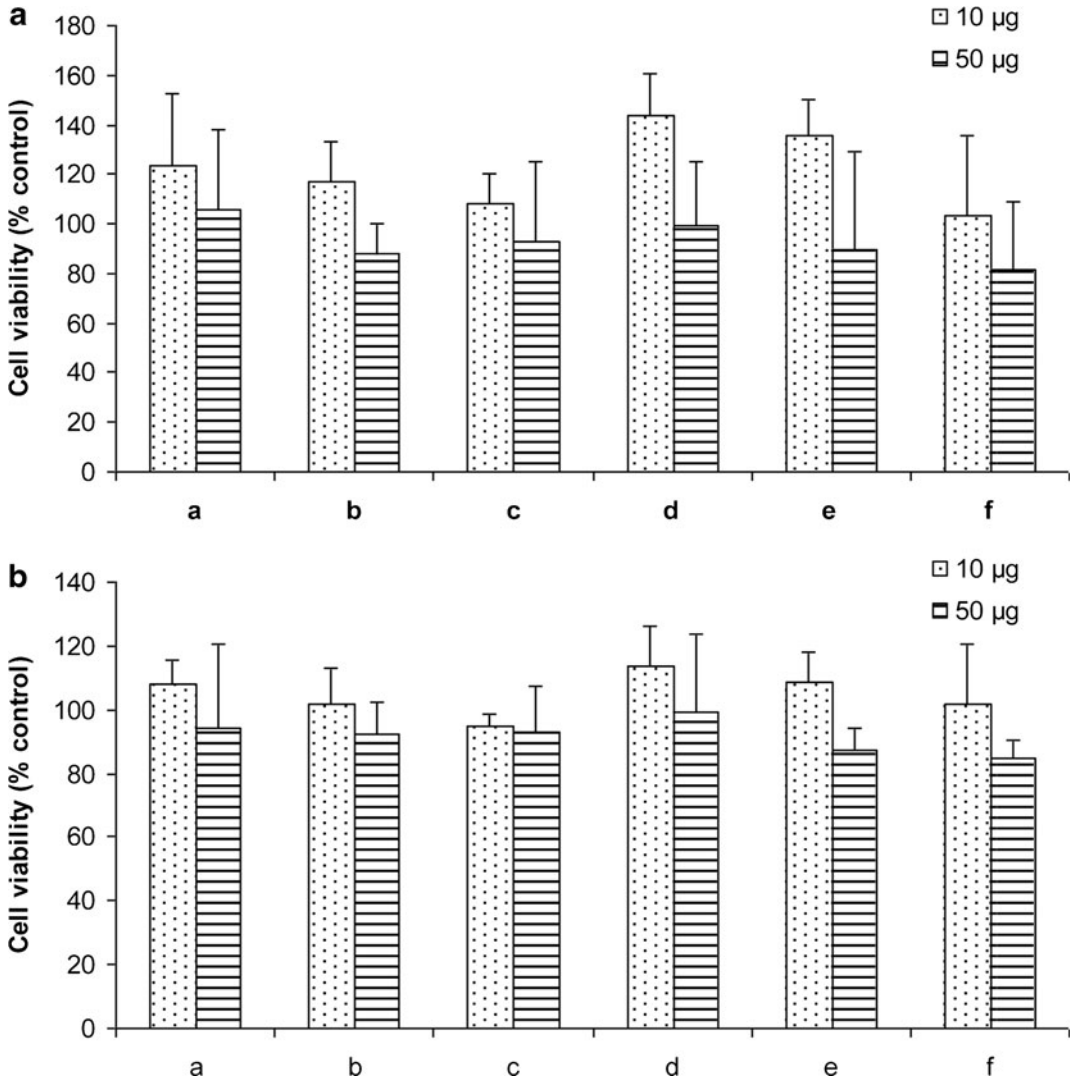


**Fig. 10** Cell viability assay of microspheres fabricated from CPH:SA copolymers with varying molar ratios. Cell viability was measured at (**a**) 24 h and (**b**) 72 h of incubation. Untreated cells were used as control and the results shown are the means ± SD for each group ($n$=4). Key: a-c: CPH:SA 20:80, 40:60, 50:50 microspheres containing insulin, d-f: CPH:SA 20:80, 40:60, 50:50 microspheres containing insulin and zinc oxide.

degradation rate of the CPH/SA copolymer. Insulin release is further controlled by lower solubility and slow dissociation of insulin hexamers. The *in vitro* results clearly suggest that the addition of a bivalent metal ion influenced CPH/SA degradation and insulin release rates.

It is extremely important that the stability of protein therapeutics in microspheres is maintained beginning from the formulation step until its release, since even a small conformational change may affect the protein's biological activity. Stability is a major challenge especially when proteins are encapsulated in polymeric microspheres using the w/o/w double emulsion technique. The primary emulsification step (where the w/o emulsion is formed) is identified as the major step for protein inactivation and aggregation (26,27). The hydrophobic interface created between the water-organic solvent leads to adsorption of proteins at the interface which results in protein unfolding and aggregation. In addition, the shear used for the emulsification can contribute to protein denaturation and aggregation. Hence, maintaining the native conformation of a protein during the encapsulation process is highly important, which otherwise may lead to protein aggregation and immunogenicity. Apart from the microsphere fabrication step, the stability of a protein encapsulated in polymeric microspheres could be affected to a greater extent during the long-term controlled release phase. Exposure to moisture, polymer degradation products, and adsorption to hydrophobic polymeric surfaces can trigger protein degradation and aggregation. Since polyanhydrides degrade by surface erosion, the encapsulated protein molecule is not exposed to moisture until the erosion front of the polymer degrades. Hence, denaturation and aggregation of the protein due to hydration is not a major issue with polyanhydride polymers. Polyanhydrides degrade into acidic monomeric units which can lower the

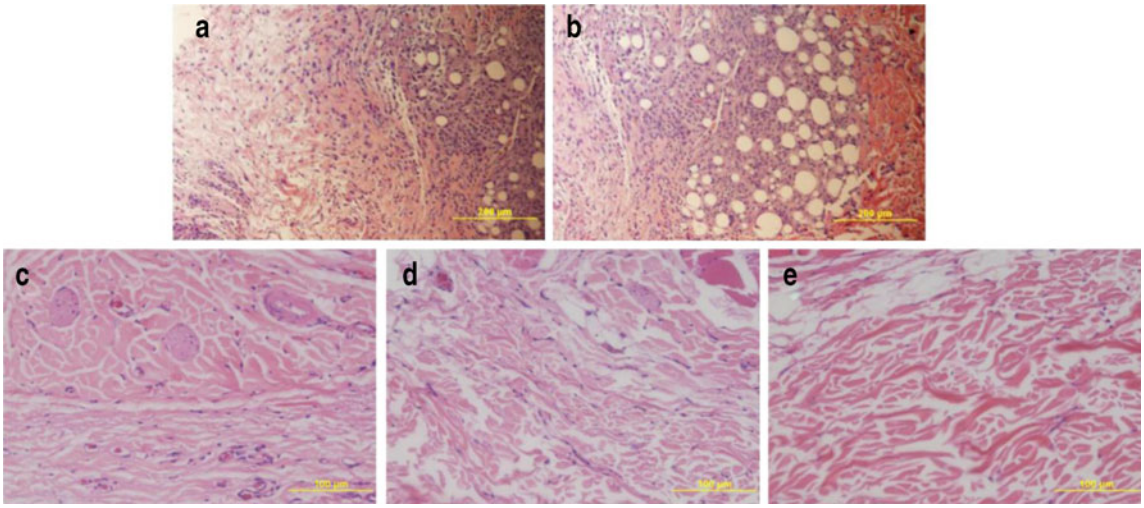


**Fig. 11** Microphotographs of skin tissue sections taken from rat injected with (**a**) CPH:SA 40:60, and (**b**) CPH:SA 50:50 microspheres at day 1; Microphotographs of skin tissue sections taken from rat injected with (**c**) CPH:SA 40:60, and (**d**) CPH:SA 50:50 microspheres at day 90; (**e**) Microphotograph of skin tissue section of control rat (untreated).

surrounding pH and induce denaturation or aggregation. Unlike other bulk eroding polymers such as polyesters, an acidic microenvironment within the degrading matrix is not created and the protein stability is not affected due to low pH microenvironments. However, the stability of proteins incorporated into polyanhydrides has to be carefully considered due to the acylation of nucleophiles such as primary amines and hydroxyl groups (28). In addition, covalent or noncovalent protein aggregates may be formed due to strong hydrophobic interactions between the protein and the polymer. In particular, since CPH is a highly hydrophobic monomer, the possibility of protein interaction or exposure to these monomer surfaces is relatively high. Therefore, for developing a successful controlled release system, it is mandatory that a protein retains its stability during device fabrication and release.

FTIR analysis of insulin encapsulated in CPH/SA microspheres (Table III) implies that minor structural transitions occurred during microsphere fabrication and clearly explains the exposure of hydrophobic regions of insulin to the w/o interfaces. However, a significant decrease in the α-helix or β-sheet content was not observed suggesting that no conformational changes in secondary structure occurred during the microsphere preparation process. This can be explained by the homogenization time used for creating the w/o primary emulsion. Homogenization of the primary emulsion for 1 min decreased the residing time for insulin molecules at the w/o interfaces and therefore did not allow any major structural transitions to occur. On the other hand, microsphere formulations with zinc oxide showed α-helix content similar to native insulin. The results clearly explain the increased stability of hexamers. In the presence of zinc ions, three insulin dimers associate to form a hexamer. As a hexamer is formed, the hydrophobic amino acid residues are buried in the dimer which otherwise are exposed on the surface of the dimer. Therefore, during the primary emulsification step, the hexamers prevented the exposure of non-polar amino residues to the w/o interfaces thereby preserving the secondary structure of insulin. In addition, the hexamers as the most hydrophilic species of insulin (29) are less prone for adsorption at the w/o interfaces, and therefore prevent aggregation and denaturation of insulin at the interfaces.

SDS-PAGE analysis showed that the primary structure of the released insulin was unchanged. In addition, MALDI-TOF MS results confirmed the absence of covalent insulin dimers and any other degradation products at day 7 indicating the chemical integrity of the released insulin. According to far-UV CD none of the formulations exhibited secondary structural changes. No degradation products were observed by HPLC for insulin released from various microsphere formulations. SE-HPLC analysis of the samples from CPH/SA microsphere formulations without zinc oxide revealed a small percentage of covalent insulin dimers (Table IV). As the hydrophobicity of the copolymer increased, the percentage of dimer aggregates increased suggesting that the dimer aggregates were formed as a result of insulin adsorption to CPH surfaces that are highly hydrophobic. At neutral pH, zinc free insulin in an aqueous solution, depending on concentration may exist as monomers, dimers, or hexamers with the first two forms being predominant. With monomer and dimer being the most hydrophobic species of insulin, adsorption to the CPH surfaces is highly likely. The formation of covalent insulin dimer aggregates provide evidence for insulin adsorption to CPH surfaces. In contrast, CPH/SA microspheres prepared with the incorporation of zinc oxide showed no evidence for covalent dimer aggregates. This supports our hypothesis that occurrence of insulin in the

hexameric form prevents the exposure of non-polar amino acids to the hydrophobic surface of CPH. Therefore, adsorption of insulin and formation of aggregates was prevented. In summary, results from HPLC and SE-HPLC experiments confirm that insulin stability was retained with the addition of zinc oxide.

A gradual decrease in serum insulin levels demonstrated the slow surface erosion phenomenon of the polyanhydride polymers. Normal basal serum insulin levels range from 5 μU/ml to 15 μU/ml and the formulations achieved desired insulin levels up to 42 days. The results indicate that both the microsphere formulations delivered almost similar amounts of insulin into the systemic circulation which is obviously due to a small difference in the CPH molar ratio between the two copolymers. A sharp decrease in blood glucose levels post administration of the microspheres shows the rapid absorption of insulin released from the formulations injected at the subcutaneous site. Similar to serum insulin release profiles, a very marked difference in blood glucose levels was not seen in rats receiving microsphere formulations CPH/SA 40/60 and 50/50. The hypoglycemic activity of insulin was observed until the 42 day time period. Based on the results it can be concluded that the pharmacological activity of insulin encapsulated in CPH/SA microspheres was preserved until release. As explained before, insulin molecules encapsulated in the CPH/SA 40/60 and 50/50 microspheres are not exposed to water during the release period. In addition, insulin molecules present as hexamers prevent aggregation and adsorption to the hydrophobic polymer. Addition of zinc oxide and the formation of hexamers is considered to have a major impact on insulin stability in CPH/SA 40/60 and 50/50 microspheres.

*In vitro* cytotoxicity tests offer the opportunity to assess the biocompatibility of materials that are being used for biomedical applications. The results demonstrated that the cells did not undergo any viability change under given conditions and CPH/SA copolymers regardless of their molar ratios were not toxic. Histological examination of skin tissues obtained from rats receiving CPH/SA 40/60 and 50/50 microspheres revealed no histomorphological evidence of toxicity. Minimal to mild inflammatory response was present with no signs of tissue necrosis.

## CONCLUSIONS

The hydrophobic characteristics of CPH/SA copolymers have limited their use in controlled drug delivery applications. In this work, we showed that CPH/SA copolymers can be successfully used to develop microsphere delivery systems and can control insulin release for up to 42 days in diabetic rats. Based on the results, one could easily alter the CPH/SA copolymer composition and modify the release of insulin to last anywhere from days to weeks. Thus, CPH/SA copolymers are promising candidates for developing systems to deliver various protein and peptide molecules in a controlled fashion. However, potential interactions between CPH and other protein and peptide therapeutics must be taken into consideration. Investigation of the influence of CPH on other protein and peptide therapeutics and excipients could bring about new insights to controlled drug delivery from these copolymers. Biocompatibility assessments *in vitro* and *in vivo* implied that the degradation products of CPH/SA copolymers are not toxic and the copolymers are completely biocompatible. In conclusion, this work demonstrates a new possibility of delivering basal insulin for 42 days using microspheres prepared from hydrophobic CPH/SA copolymers without any influence on insulin stability.

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