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Ionotropic cross-linked chitosan microspheres for controlled release of ampicillin

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Abstract

The solubility of non cross-linked chitosan in weak acid solutions restricts its utility in microspheres for drug delivery. The primary aim of this study was to produce pentasodium tripolyphosphate cross-linked chitosan microspheres with higher acid resistance for controlled release of ampicillin. The microspheres were prepared by two different microencapsulation procedures (by emulsification and by spray-drying) and characterized by their particle size, surface morphology, stability, drug entrapment efficiency and drug release. The size of the microspheres was <10 μm with a narrow size distribution. The entrapment of ampicillin in the microspheres was more than 80%. Stability of uncross-linked and cross-linked microspheres was affected by the pH of simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.5). The inclusion of the enzymes pepsin and pancreatin did not affect the stability of the microspheres. The inclusion of lysozyme in phosphate buffer saline resulted in increased solubilization. The release of the drug was affected by cross-linking of microspheres with tripolyphosphate (TPP). The cross-linked microspheres were more stable in simulated gastric fluid and showed slower but sustained release of ampicillin. The antimicrobial activity of the released ampicillin was confirmed by *Staphylococcus aureus* bioassay.

Keywords: Ampicillin; Chitosan microspheres; Controlled release; Emulsification-solvent evaporation; Spray-drying; Tripolyphosphate

1. Introduction

Multiple-unit solid dosage forms such as microspheres or beads have gained in popularity as oral drug delivery systems because of more uniform distribution of the drug in the gastrointestinal tract, more uniform drug absorption, reduced local irritation and elimination of unwanted intestinal retention of polymeric material, when compared to non-disintegrating single-unit dosage forms (Lauwo et al., 1990; Bodmeier et al., 1991).

Chitosan, the *N*-deacetylated product of the polysaccharide chitin, is an interesting biopolymer to prepare microspheres owing to its unique polymeric cationic character, good biocompatibility, non-toxicity, biodegradability and its mucoadhesivity and absorption-enhancing effect (Remuñán-López et al., 1998; He et al., 1999). Processing techniques for the preparation of chitosan microspheres have been extensively developed since the 1980s. Four main approaches have been proposed: ionotropic gelation with oppositely charged polyelectrolytes such as tripolyphosphate (TPP) or alginate (Bodmeier et al., 1989; Sezer and Akbûga, 1999; Anal et al., 2003; Anal and Stevens, 2005; Bhopatkar et al., 2005), simple or complex coacervation (Remuñán-López and Bodmeier, 1996), spray drying (Williams et al., 1998; He et al., 1999), and emulsification-solvent evaporation (Thanoo et al., 1992; Shirashi et al., 1993; Lim et al., 1997; Lorenzo-Lamosa et al., 1998; Remuñán-López et al., 1998; Mi et al., 1999). Chitosan microspheres based drug delivery is applicable for systemic (Orienti et al., 2002) as well as for local therapy. In case of oral drug delivery, the use of microspheres loaded with antibiotics would be beneficial for gastric diseases such as peptic ulcer (Lim et al., 1997; Shah et al., 1999; Tozaki et al., 2002), *Helicobacter pylori* (Remuñañ-Lỏpez et al.,

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2000; Portero et al., 2002) and intestinal infections, ulcerative colitis and carcinomas (Tozer et al., 1995; Lorenzo-Lamosa et al., 1998). On the other hand, specific sustained systemic absorption in the intestinal region offers interesting possibilities for the treatment of diseases susceptible to the diurnal rhythm, such as asthma, arthritis or inflammation (Yeh et al., 1995; Désévaux et al., 2002). The antibiotics loaded microspheres can be applied for local wound healing (Conti et al., 2000) and in implants to improve bioavailability (Jameela and Jayakrishnan, 1995).

This research aims at the production of chitosan microbeads for the controlled delivery of the antibiotic ampicillin. A general limitation of the use of chitosan is its high solubility in body fluids. A particular problem related to a low molecular weight compound like ampicillin is the high permeability of the chitosan microbead matrix material. In order to overcome these restrictions, chitosan gel beads and microspheres are generally crosslinked chemically using glutaraldehyde (Berthold et al., 1996; Genta et al., 1997; Jameela et al., 1998) or ethylene glycol diglycidyl ether (Mi et al., 1999). However, residual glutaraldehyde and ethylene glycol diglycidyl ether in the chitosan microspheres give rise to health concerns and can cause undesirable effects including irritation to mucosal membranes. To overcome these disadvantages of chemical crosslinking, researchers have proposed to apply chitosan microspheres reacetylated with acetic anhydride (Remuñañ-Lőpez et al., 2000; Portero et al., 2002). The re-acetylated chitosan microspheres were able to interact closely with the gastric mucosa and to exhibit sustained delivery of entrapped antibiotic (Remuñañ-Lőpez et al., 2000). A third problem related to ampicillin is its short biological half-life of 0.75–1.5 h. Encapsulation in beads might extend its biological availability.

In this research cross-linked chitosan microspheres were developed able to encapsulate and release low molecular hydrophilic antibacterial agents such as ampicillin in a controlled manner. This was achieved by combination of microparticles preparation techniques like emulsification and spray drying with treatment by the cross-linking electrolyte tripolyphosphate TPP. The behavior of ampicillin released from various chitosan and chitosan–TPP microspheres was investigated in simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and phosphate buffer saline (PBS).

2. Materials and methods

2.1. Materials

Chitosan, molecular weight 1×10^6 Da, degree of deacetylation 85% was obtained from shrimp shell (*Penaeus monodon*) after deproteinization and demineralization followed by deacetylation (Stevens, 2002) in the Bioprocess Technology Laboratory, Asian Institute of Technology, Thailand. The following chemicals were bought from commercial suppliers: ampicillin, pentasodium tripolyphosphate (TPP), cottonseed oil (Sigma Chemicals, Spain), and Span 85 (Fluka, Spain). Pepsin and pancreatin powders were supplied by Acros Organics and Carlo Erba Reagent respectively. Simulated gastric fluid (SGF pH 1.2) containing 7 ml HCl, 2 g NaCl with and without 3.2 g pepsin, diluted to 1 l and simulated intestinal fluid (SIF pH 7.5), containing 6.8 g of K_2HPO_4 and 190 ml of 0.2N NaOH with and without 10 g pancreatin, diluted to 1 l were prepared as described in US Pharmacopoeia. Ultrapure water (Milli-Q Plus, Millipore Ibérica, Spain) was used throughout. All other reagents were analytical grade and used without further purification.

2.2. Preparation of chitosan microspheres

The chitosan–TPP microspheres containing ampicillin were prepared by emulsification and spray-drying.

2.2.1. Emulsification technique

Emulsification was carried out as previously described by Remuñañ-Lőpez et al. (2000) except that chitosan was cross-linked with TPP. Cross linking by bifunctional aldehydes were not used in the present study.

Chitosan solution (2%, w/v) was prepared by dissolving chitosan in dilute acetic acid (1%, v/v) at room temperature and filtered through nylon cloth to remove insolubles. The drug ampicillin was dissolved directly into the chitosan solution to a final concentration of 10% (w/w of polymer) and stirred for 1 h to homogeneity. The chitosan-ampicillin solution (10 ml) was mixed with the same amount (10 ml) of acetone. The mixture (9 ml) was then emulsified into cottonseed oil (150 ml) containing 0.1% w/w Span 85 under mechanical stirring (1000 rpm, Jankel & Kankel model, IKA RW 20DZM, Boente, Spain) at 37 °C. The system was maintained under agitation for 14 h to allow complete evaporation of the non-oil solvent. Thereafter, as specified below, the ionic gelification of the chitosan microspheres in the oily suspension medium was achieved by addition of TPP solution in water (1 ml, pH 8.5 ± 0.1) in concentrations corresponding to 4, 8, 12 or 16% of the amount of chitosan (w/w). Finally after a prefixed cross-linking time of 1, 2 or 4 h, the microspheres were isolated by vacuum filtration (0.45 μm PFTE membrane filters, Lida, Spain), washed with equal volume of *n*-hexane and freeze-dried (−30 °C, 24 h; Labconco apparatus, Labconco Corp., USA).

2.2.2. Spray-drying technique

Chitosan microspheres, loaded with various concentrations of ampicillin were prepared by an aqueous spray-drying technique. Chitosan solution (1%, w/v) was prepared in diluted acetic acid (1%, v/v). Various amounts of ampicillin (5, 10, 20, 30, 40 and 50% of chitosan weight w/w) were directly dissolved into the chitosan solution. The mixture was then stirred under magnetic stirring for 1 h to get a homogenous mixture. In some formulations, the 1% chitosan solution (100 ml) was first cross-linked with 50 ml TPP of various concentrations (0, 0.05, 0.075, 0.1 and 0.125% in distilled water) by stirring for 1 h. In these cases, the drug was dissolved in TPP solution. The suspensions of chitosan and drug (150 g) were then spray-dried (Buchi® Mini Spray Dryer, Type 190, Switzerland) through a 0.3-mm nozzle at a feed rate of 2.4 ml/min. The nozzle air pressure was 500 Nl/h. The inlet temperature was 120–125 °C while outlet temperature was about 80 °C. Finally, the product obtained was freeze-dried.

2.3. Morphological characterization of microspheres

The particle size was measured directly by optical microscopy (Olympus BH-2, Tokyo, Japan) on 150 microspheres. The morphology and surface appearance of microspheres were examined by scanning electron microscopy (SEM). The microspheres were freeze-dried, coated with gold palladium to achieve a film of 20 nm thicknesses (Sputter coater, Blazers SCD 004, Liechtenstein) and observed with a scanning electron microscope (Jeol, JSM-6400, Tokyo, Japan).

2.4. Determination of encapsulation efficiency

The drug-loaded microspheres (5 mg) were extracted with 10 ml of 0.1N HCl for 48 h at room temperature, centrifuged at 6000 rpm for 30 min and filtered (0.2 μm nylon filters, Whatman, UK). The filtrate was assayed for drug content by UV-Spectroscopy (Shimadzu RF 5001 PC, Kyoto, Japan) at the wavelength of 203 nm. Encapsulation efficiency was calculated from the actual drug loading of the microspheres and the theoretical drug loading. All samples were analyzed in triplicate.

The encapsulation efficiency was calculated from the following expression:

$$\text{Encapsulation efficiency}\,(\%) = \frac{\text{total amount of drug} - \text{free amount of drug}}{\text{total amount of drug}} \times 100$$

2.5. Evaluation of stability behavior of microspheres in enzymatic solutions

The stability of microspheres was assessed in SGF (pH 1.2), SIF (pH 7.5) and PBS (pH 7.4) containing pepsin (3.2 mg/ml), pancreatin (10 mg/ml) or lysozyme (0.15 mg/ml) respectively as described in Anal and Stevens (2005). Briefly, lyophilized microspheres (5 mg) were dispersed in a capped test tube with 10 ml of freshly prepared SGF, SIF and PBS and incubated at 37 °C with 150 rpm of shaking for 7 days. The time required to solubilize all microspheres was measured using microscopic examination. The results were expressed either by a single value, when nearly equal times were found in the three assays, or by two values corresponding to the minimum and the maximum times. The behavior of the microspheres was observed every hour under the microscope. All the experiments were done in triplicate and the time range was recorded.

2.6. Evaluation of the in vitro drug release

In vitro release studies were performed using the dialysis technique. Dialysis bags (dialysis tubing, MW cut off 12,000 Da, Sigma Chemicals, Spain) containing a suspension of chitosan-ampicillin microspheres (25 mg) in 5 ml of SGF (pH 1.2) or in SIF (pH 7.5) or in PBS (pH 7.4) were placed in 250 ml of the same release medium at 37 °C and stirred at 50 rpm using the USP 23 paddle method. At predetermined times (0.5, 1, 2, 4, 8, 16, and 24 h), 5 ml samples were withdrawn from the release medium and replaced with equal volume of fresh dissolution medium. Samples were filtered (0.2 μm Nylon filters, Whatman, UK) and assayed for drug released by measuring the absorbance at 203 nm. Each experiment was repeated at least three times.

2.7. Microbiological assay for ampicillin

Functional test on the stability of the extracted ampicillin released from the microspheres during dialysis was performed by the test tube serial dilution method (Bailey and Scott, 1970). Test bacterium was *Staphylococcus aureus*. The antibiotic, ampicillin was extracted from microspheres in SGF (pH 1.2). The extracted ampicillin (100 μg/ml) was diluted in phosphate buffer saline solution (5 ml) and serial ten-fold diluted in liquid Mueller–Hinton broth. Duplicate tubes containing 1 ml of each dilution were inoculated with 1×10^5 bacterial cells. The tubes were then incubated at 37 °C for 24 h. Bacterial growth was observed by spectrophotometer at 620 nm. Antibiotic activity was expressed as the minimum inhibitory concentration (MIC), recorded as the highest dilution showing no bacterial growth. Antibiotic not subjected to the microencapsulation procedure was used as control. Blank chitosan microspheres were tested but had no effect on bacterial growth. All experiments were done in triplicate.

2.8. Statistical analysis

Results were analyzed and expressed as mean ± S.D. Statistical analysis was carried out by factorial design (Randomized Complete Block Design, RCBD) for characterization of microspheres. Effects of various cross-linking concentrations on entrapment and release of drug from microspheres were statistically analyzed by one-way ANOVA. The differences were considered significant at the level of $p < 0.05$. SPSS 7.5 for Windows was used for all statistical analysis.

3. Results and discussion

In this paper, results are presented of two microencapsulation methods: emulsification and spray-drying to prepare small chitosan microspheres loaded with ampicillin. Both methods were combined with simultaneous reinforcement with TPP as cross-linking agent. Both methods result in the formation of insoluble chitosan microspheres, without requiring addition of aldehydes. TPP is a non-toxic and multivalent anion that can form cross-links by ionic interaction between positively charged amino groups of chitosan and multivalent negatively charged TPP molecules (Bodmeier et al., 1989; Mi et al., 1999; Shu and Zhu, 2000). The TPP treatment of chitosan microspheres is expected to improve their stability and their applicability in controlled drug delivery.

3.1. Size and morphological characterization of chitosan microspheres

3.1.1. Morphology of chitosan microspheres prepared by emulsification

The shape and surface morphology of chitosan microspheres, prepared by emulsification were observed by scanning electron microscopy (Fig. 1). Most of the chitosan microspheres (approximately >80%) were found to be smooth and spherical (Fig. 1a). A few disintegrated particles were found after cross-linking (Fig. 1b). This may be due to disintegration of some of the microspheres during incubation with aqueous TPP. Particle size analysis done by light microscopic observation reveals that the size of the microspheres prepared by emulsification was in the range of 4–11 μm (Table 1). The size of the microspheres is increased only with the higher concentrations (12 and 16%) of TPP. In the presence of sufficient amount of TPP, fusion of smaller microspheres occurs giving rise to chitosan microspheres of considerable larger size. The multifunctional TPP acts here to facilitate inter-microparticles binding of the chitosan.

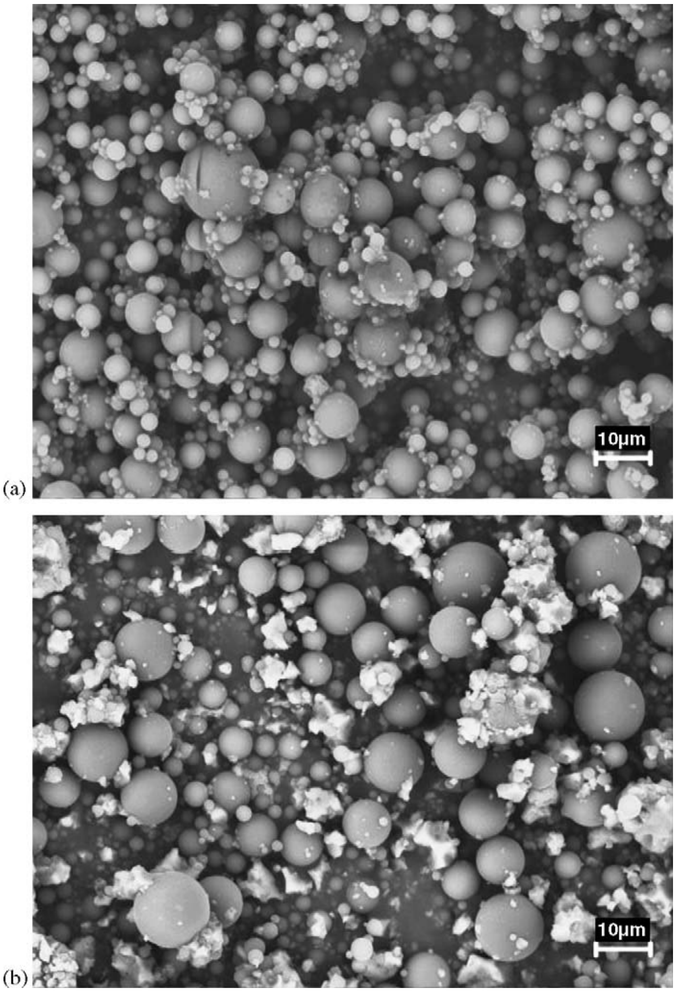

Fig. 1. SEM microphotographs of chitosan microspheres prepared by emulsification method: (a) no TPP, (b) cross-linked with 4% (w/w based on polymer) TPP.

Table 1
Effect of cross-linker TPP concentration (% w/w) on mean size and entrapment efficiency of microspheres prepared by emulsification

TPP concentration (%)	Mean size (μm)	Entrapment efficiency (%)
0	4.4 ± 1.2	85.1 ± 2.8
4	5.5 ± 2.4	76.6 ± 1.9
8	6.4 ± 2.2	73.9 ± 1.8
12	11.2 ± 2.6	65.9 ± 3.6
16	11.4 ± 3	65.2 ± 3.5

Chitosan: ampicillin in the beads: ratio 10:1, cross-linking time: 4 h. Presented data are mean ± S.D., $n = 3$.

Table 2
Effect of drug concentrations on mean size and entrapment efficiency of microspheres prepared by spray drying

Drug concentration (%)	Mean size (μm)	Entrapment efficiency (%)
0	6.2 ± 1.0	0 ± 0
5	7.7 ± 1.1	85.3 ± 1.9
10	8.1 ± 1.6	86.6 ± 2.1
20	8.7 ± 2.2	89.3 ± 1.7
30	8.1 ± 2.6	80.1 ± 1.1
40	8.8 ± 1.9	74.4 ± 2.0
50	9.6 ± 2.1	72.3 ± 1.2

Drug concentrations in % of chitosan concentration (w/w); no cross-linker. Presented data are mean ± S.D., $n = 3$.

3.1.2. Morphology of chitosan microspheres produced by spray-drying method

The microspheres prepared by spray drying were spherical with a regular shape. There was no significant difference ($p > 0.05$) in the size of the microspheres prepared by spray drying in the presence of various drug concentrations (Table 2) and even sprayed after cross-linked with TPP (Table 3). The size range is 7–9 μm in all cases. In this case the TPP has reacted with the chitosan before formation of the beads by spray drying. In spray drying, all droplets in the spray contain more or less the same amount of chitosan. Most of the TPP may present in the interior of the microbeads. Uniform bead size independent of the TPP concentrations can be explained in this way.

3.2. Entrapment efficiency of ampicillin in microspheres

3.2.1. Entrapment efficiency of ampicillin prepared by emulsification

Table 1 shows the loading efficiency of the drug into the microspheres, prepared by emulsification. Statistical analysis

Table 3
Effect of TPP concentration on mean size and entrapment efficiency of microspheres prepared by spray drying

TPP concentration (%)	Mean size (μm)	Entrapment efficiency (%)
0	8.7 ± 2.2	89.3 ± 1.7
0.05	7.6 ± 2.4	84.9 ± 1.4
0.075	7.3 ± 2.6	81.4 ± 1.3
0.1	7.6 ± 2.0	81.8 ± 2.4
0.125	7.4 ± 1.5	79.0 ± 0.9

Chitosan: ampicillin in the beads: ratio 10:2, cross-linking time: 1 h. Presented data are mean ± S.D., $n = 3$.

Table 4
Disintegration times of chitosan–TPP microspheres prepared by emulsification in release media with and without enzymes

TPP concentration (%)	SGF (pH 1.2)		SIF (pH 7.5)		PBS (7.5)	
	Pepsin (+)	Pepsin (−)	Pancreatin (+)	Pancreatin (−)	Lysozyme (+)	Lysozyme (−)
0	10–12	10–12	>170	>170	90–94	>170
4	24–28	24–28	>170	>170	130–140	>170
8	28–30	30–32	>170	>170	145–160	>170
12	30–34	30–36	>170	>170	145–160	>170

Keys: (+) including enzyme and (−) without enzyme in solution. Disintegration times are indicated in hours, mean ± S.D., $n = 4$.

shows that TPP has a significant effect ($p < 0.05$) on the amount of drug entrapped after freeze drying. Lower loading (65–70%) was found at higher concentration of TPP. In absence of TPP, 85% of the drug was entrapped. This could be due to the increased binding of the main groups of the drug to the added TPP that might compete with the incorporation of the drug in the bead matrix.

3.2.2. Entrapment efficiency of ampicillin produced by spray drying

Tables 2 and 3 illustrate the entrapment efficiency of drug into chitosan–TPP microspheres, prepared by spray-drying. The entrapment efficiency of ampicillin was more than 80%. Statistical analysis shows a significant negative effect ($p < 0.05$) of increasing TPP concentration. This could also be due to the increased binding of the main groups of the drug to the added TPP.

3.3. Stability study of chitosan–TPP microspheres

In the next series of experiments, the chitosan–TPP microspheres were incubated in three different disintegration media: SGF with and without pepsin (pH 1.2), SIF with and without pancreatin (pH 7.5) and PBS with and without lysozyme (pH 7.5). The study was conducted at 37 °C. The samples were shaken at 150 rpm during 7 days. Disintegration of microspheres was defined as the disappearance of the bead-like structure as observed by microscopical observation. Thereafter microsphere fragments may have persisted for some time. It cannot be excluded that a small fraction of the drug still was entrapped in these fragments and released only some time after the bead disintegration.

3.3.1. Stability of microspheres prepared by emulsification

The time for various microspheres prepared by emulsification to become completely solubilized, is shown in Table 4. The pH of the media has a noticeable effect on solubilization time. In SGF at pH 1.2, uncross-linked chitosan microspheres disappeared within 10–12 h. TPP delays the degradation of cross-linked microspheres in SGF significantly ($p < 0.05$). The time taken to complete solubilization of microspheres in SGF with and without pepsin was similar. Solubilization times in SIF was >170 h, with and without pancreatin enzyme. The effect of lysozyme, present in PBS, on dissolution of microspheres is significant ($p < 0.05$). Without lysozyme, the dissolution of microspheres did not occur even after 1 week. In the presence of lysozyme, the uncross-linked microspheres dissolved in 94 h but the cross-linked microspheres still remained stable till 140–160 h, depending upon the concentration of TPP. It is concluded that the method of emulsification produces microbeads that are stable in SIF and PBS even in absence of TPP. Stability of beads prepared without TPP in SGF is reasonable (10–12 h) and can be extended for as long as 30 h by incorporation of TPP in the emulsification process.

3.3.2. Stability of microspheres prepared by spray-drying

The time needed for complete disintegration of various microspheres, prepared by spray-drying method is shown in Table 5. The microspheres showed different behavior in the dissolution media. The cross-linked and uncross-linked microspheres agglomerated within 1 h, the uncross-linked microspheres dissolved in SGF, with and without pepsin in 6–10 h. TPP delayed the disintegration of microspheres in SGF. The microspheres in SIF and PBS were much more stable. Addition of lysozyme in PBS resulted in a somewhat faster disintegration. The more stable structure of chitosan–TPP complex may be due to its high degree of interchain linkages. The agglomeration observed and the lower stability in SGF makes the spray drying method less attractive for the preparation of microspheres.

Table 5
Disintegration times of chitosan–TPP microspheres prepared by spray drying in release media with and without enzymes

TPP concentration (%)	SGF (pH 1.2)		SIF (7.5)		PBS (pH 7.5)	
	Pepsin (+)	Pepsin (−)	Pancreatin (+)	Pancreatin (−)	Lysozyme (+)	Lysozyme (−)
0	6–8	8–10	90–96	94–100	80-82	120–130
0.05	12–16	14–18	100–110	110–120	110–120	120–130
0.1	18–24	20–24	120–140	120–130	110–125	140–150
0.125	20–24	20–24	130–140	120–140	125–130	140–150

Keys: (+) including enzyme and (−) without enzyme in solution. Disintegration times are indicated in hours, mean ± S.D., $n = 4$.

3.4. In vitro release

The release of the drug from the chitosan microspheres is strongly affected by the pH of the medium. The release studies with various chitosan microspheres were conducted in SGF, SIF and PBS without enzymes at 37 °C with continuous shaking. The cumulative percentage of ampicillin released from the microsphere formulations was plotted as a function of time.

3.4.1. In vitro release of ampicillin in SGF medium

The release kinetics of ampicillin from chitosan microspheres, prepared by emulsification in SGF (pH 1.2) is illustrated by Fig. 2. In this fluid, these chitosan microspheres swell and gradually dissolve. Almost all entrapped drug was released during 8 h from the uncross-linked microspheres. Addition of TPP during the preparation of the microspheres resulted in prolongation of release. Addition of 4 and 12% (w/w based on chitosan polymer) TPP led to a reduction of 12 and 28% in drug release, respectively. Statistical analysis of ampicillin release in SGF for release values after 8 h of incubation shows that there is a significant effect ($p < 0.05$) of the TPP concentration. The addition of 8% TPP is enough to achieve maximum cross-linkage of chitosan, probably because at that concentration all chitosan amino groups are occupied.

The pattern of release of entrapped ampicillin from chitosan microspheres, in SGF prepared by spray-drying is presented in Fig. 3. Most of the entrapped ampicillin is released after 8 h from uncross-linked chitosan microspheres while 80 and 50% of that release occurred from chitosan–TPP microspheres, cross-linked with 0.05 and 0.125% TPP (w/w of polymer), respectively. These results indicate that the chitosan microspheres are cross-linked by TPP, depending upon its concentration and that the release is reduced after TPP treatment.

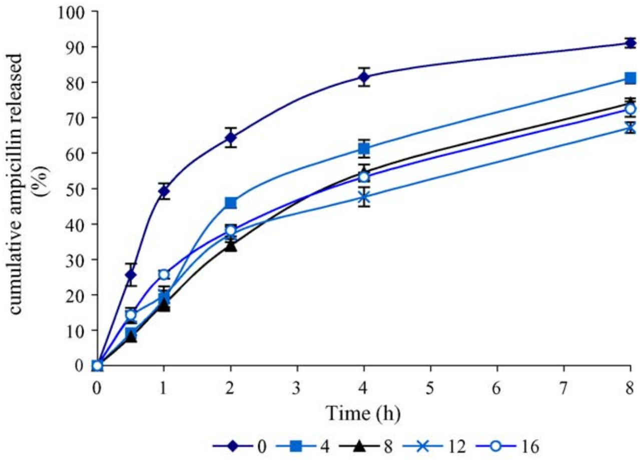

Fig. 2. Ampicillin release from microspheres in SGF pH 1.2; emulsification method. Chitosan ampicillin microspheres (25 mg; 10:1 w/w) were prepared by emulsification including treatment with tripolyphosphate (TPP) solution 0–16%. The microspheres were suspended in and dialyzed against SGF without enzyme. The cumulative release of ampicillin into the dialysis fluid was assayed by its absorption at 203 nm. Mean ± S.D., $n = 3$. For further details see Section 2.

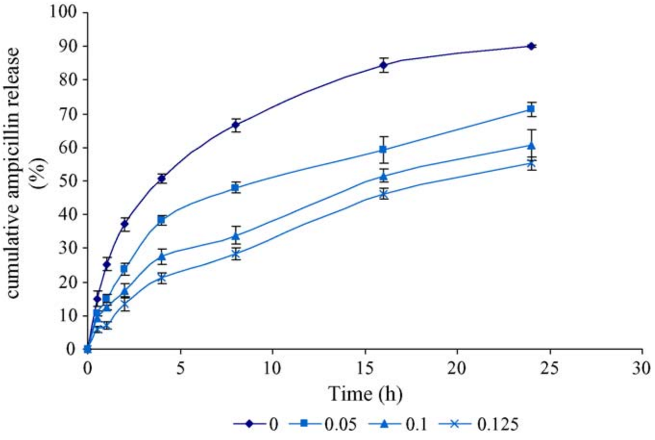

Fig. 3. Ampicillin release from microspheres in SGF pH 1.2; spay-drying method. Chitosan ampicillin microspheres (25 mg 10:2 w/w) were prepared by spray drying including treatment with tripolyphosphate (TPP) solution 0–0.125%. The microspheres were dialyzed against SGF without enzyme. Mean ± S.D., $n = 3$, For further details see Section 2.

3.4.2. In vitro release of ampicillin in SIF medium

The release of ampicillin from various microspheres was also investigated by incubating in SIF (pH 6.5) for 24 h. The microspheres remained intact and degrade only slowly. This is accompanied by gradual release of the drug. In SIF the release of entrapped ampicillin from chitosan microspheres, prepared by emulsification is 75 and 90% from uncross-linked microspheres in 8 and 24 h, respectively (Fig. 4). Only 60% of that release occurred from cross-linked microspheres with 8 and 12% TPP in 8 h, respectively. The release from 8 and 12% TPP cross-linked microspheres was about 88 and 78% respectively in 24 h, compared to the release of non-cross-linked microspheres. The statistical analysis shows that the differences are significant ($p < 0.05$).

Microspheres, prepared by spray-drying release their ampicillin in SIF according to Fig. 5. The microspheres formed

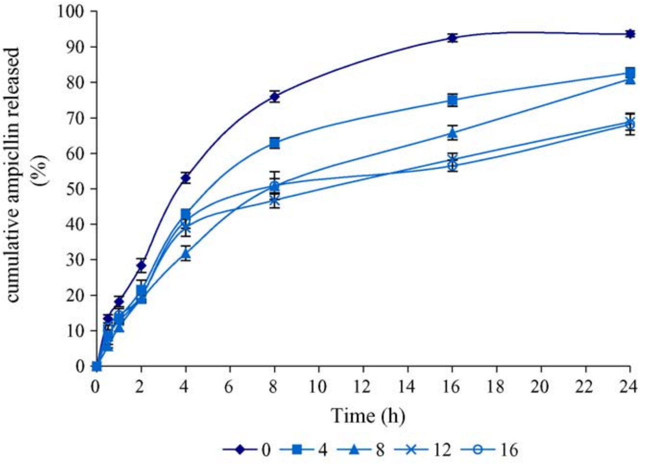

Fig. 4. Ampicillin release from microspheres in SIF pH 7.5; emulsification method. Chitosan ampicillin microspheres (25 mg 10:1 w/w) were prepared by emulsification including treatment with tripolyphosphate (TPP) solution 0–16% The microspheres were dialyzed against SIF pH 7.5 without enzyme. Mean ± S.D., $n = 3$. For further details see legend of Fig. 3 and Section 2.

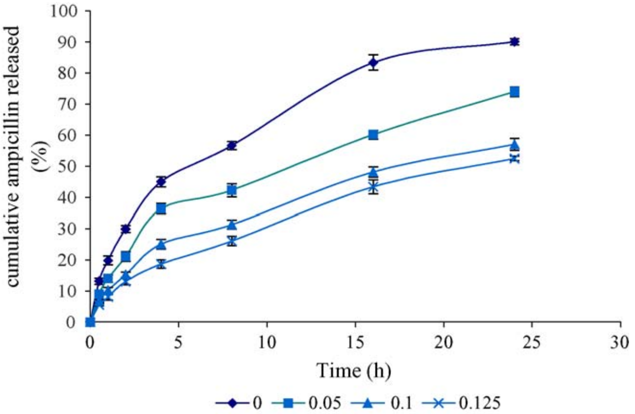

Fig. 5. Ampicillin release from microspheres in SIF pH 7.5; spray-drying method. Chitosan ampicillin microspheres (25 mg 10:2 w/w) were prepared by spray drying including treatment with tripolyphosphate (TPP) solution 0–0.125%. The microspheres were dialyzed against SIF without enzyme. Mean ± S.D., $n = 3$. For further details see legend of Fig. 3 and Section 2.

aggregates in this medium. The release was about 60 and 95% in 8 and 24 h respectively from uncross-linked microspheres. The release was slower from cross-linked chitosan–TPP microspheres about 50% in 24 h.

3.4.3. *In vitro release of ampicillin in PBS medium*

Most release studies are usually conducted in PBS (pH 7–7.5). At this condition, the kinetics might be predictive for release in parenteral applications of ampicillin-chitosan beads for wound healing. Fig. 6 shows the release pattern of chitosan ampicillin microspheres, prepared by emulsification. In 8 h, almost 80% of the entrapped ampicillin was released from uncross-linked microspheres but only 60% was released from microspheres, cross-linked by 8% TPP. The release was slower in case of a higher concentration of TPP was used for cross-linking.

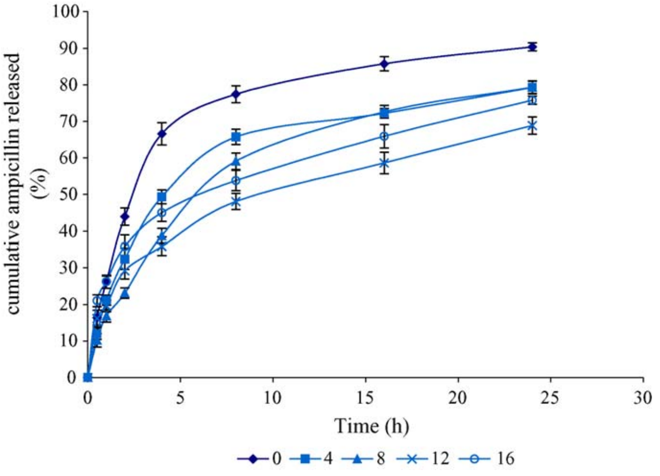

Fig. 6. Ampicillin release from microspheres in PBS; emulsification method. Chitosan ampicillin microspheres (25 mg 10:1 w/w) were prepared by emulsification including treatment with tripolyphosphate (TPP) solution 0–16%. The microspheres were dialyzed against PBS without enzyme. Mean ± S.D., $n = 3$. For further details see legend of Fig. 3 and Section 2.

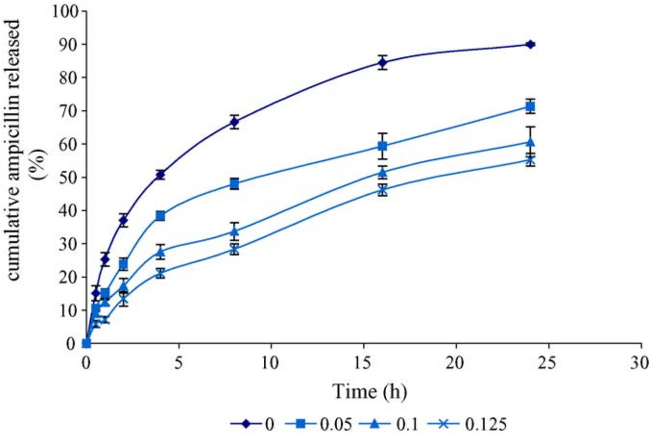

Fig. 7. Ampicillin release from microspheres in PBS; spay drying method. Chitosan ampicillin microspheres (25 mg 10:2 w/w) were prepared by spray drying including treatment with tripolyphosphate (TPP) solution 0–0.125%. Microspheres were dialyzed against PBS without enzyme. Mean ± S.D., $n = 3$. For further details see legend of Fig. 3 and Section 2.

Chitosan–TPP microspheres, prepared by spray-drying release their ampicillin as shown by Fig. 7. The release in 8 h was 67 and 28% from uncross-linked and cross-linked (with 0.125% TPP) microspheres respectively. Most of the entrapped ampicillin was released in 24 h from uncross-linked microspheres while only 50–70% was released from cross-linked microspheres.

3.5. *Microbiological assay for extracted ampicillin*

For the assessment of a possible medical application it is important to confirm the nature of the compound released from the microspheres as ampicillin. This was confirmed by assessing its effectiveness to inhibit the microbial growth of *S. aureus*. The bactericidal action of ampicillin extracted from various chitosan microspheres was tested after appropriate dilution by measuring the minimum inhibitory concentration (MIC) of the drug required to stop the growth of the test bacterium. The minimal inhibitory concentration (MIC) against *S. aureus* for all samples taken from the microsphere release medium was found 12.5 μg/ml as judged from observation of microbial growth by microscopy or by turbidity observation after a suitable period of incubation. The values are in good agreement with the reported MIC value of ampicillin against *S. aureus* of ≥10 μg/ml (Bailey and Scott, 1970). In our earlier report, we produced similar result for extracted ampicillin from various chitosan alginate multilayer beads (Anal and Stevens, 2005).

4. Conclusion

Both emulsification and spray drying of a chitosan solution in combination with TPP result in microbeads that can be used for loading and temporarily containment of a small molecular weight drug like ampicillin. Most of the earlier studies with chitosan microspheres have focused on the production of controlled release systems for proteins. The methods described in this paper allow for the development of delivery systems for small molecules. The emulsification method compared with the spray drying technique is superior regarding the stability of the microspheres. The emulsified microbeads are stable in SGF for 10–30 h, depending on the amount of TPP used. The spray dried microspheres show undesired agglomeration and less stability in SGF. Release properties of both emulsified and spray dried microspheres is equal and can be regulated by TPP dosage during bead preparation. Drug release is completed in 4–8 h in absence of TPP, but can be prolonged for more that 24 h for microspheres prepared in the presence of TPP as cross-linking agent. Both systems have been shown to be amenable for precise regulation of the drug release and opens the way for controlled application of antibiotics either locally or systemic.

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