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Injectable biodegradable starch/chitosan delivery system for the sustained release of gentamicin to treat bone infections

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ABSTRACT

Starch-conjugated chitosan microparticles were produced aimed to be used as a carrier for the long term sustained/controlled release of antibiotic drugs to control bone infection. The microparticles were prepared by a reductive alkylation crosslinking method. The obtained microparticles showed a spherical shape, with a slightly rough and porous surface, and a size range of 80–150 μm. Gentamicin was entrapped into the starch-conjugated chitosan microparticles and its release profile was studied *in vitro*. Increasing concentrations of gentamicin (from 50 to 150 mg/mL) led to a decrease in the encapsulation efficiency (from 67 to 55%), while drug loading increased from 4 to 27%. A sustained release of gentamicin was observed over a period of 30 days. The release kinetics could be controlled using an ionic crosslinker agent. In addition, a bacterial inhibition test on *Staphylococcus aureus* shows a diameter of the sample inhibition zone ranging from 12 to 17 mm (70–100% of relative activity).

1. Introduction

Chitin and starch are among the most widespread polysaccharides in nature. Chitin (poly β-(1-4))N-acetyl-D-glucosamine), is synthesized by a diverse number of living organisms from arthropods, crustaceans (crab and shrimp shells), to cell walls of fungi (Sinha et al., 2004). Chitosan is a product of partial chitin deacetylation and can be defined as a copolymer of D-glucosamine and N-acetyl-D-glucosamine (Singla & Chawla, 2001). It has been studied and proposed as a biomaterial in a broad range of biomedical applications because of its demonstrated biocompatibility and biodegradability (Illum, 1998; Janes, Calvo, & Alonso, 2001; Rinaudo, 2006). Starch is cheap, abundant and edible polysaccharide, with interesting biodegradable properties (Azevedo & Reis, 2005; Gross & Kalra, 2002). Chitosan and starch have been extensively employed for microencapsulation of bioactive materials (Lim & Sun, 1980) and as a carrier in drug delivery systems (Chandy, Mooradian, & Rao, 1998; Hari, Chandy, & Sharma, 1996; Ramadas, Paul, Dileep, Anitha, & Sharma, 2000).

Bone infection is a significant problem that compromises the healing of bone fractures. In contaminated bone wounds, it is desirable to maintain a therapeutic level of antibiotic for at least 3–6 weeks to control the infection until substantial vascularization occurs in the wound bed. It has been suggested that the treatment of osteomyelitis, an acute or chronic inflammatory process of the bone which can be caused by a variety of microbial agents, most commonly *Staphylococcus aureus*, requires the release of antibiotic at concentrations exceeding the minimum inhibitory concentration for 6–8 weeks (Li, Brown, Wenke, & Guelcher, 2010). Gentamicin, an aminoglycoside antibiotic, possesses a wide antibacterial spectrum, being effective for treating serious infections caused by gram-positive bacteria such as *S. aureus*. Aminoglycoside antibiotics have shown low effectiveness when administered orally (Jana & Deb, 2006). Typically, the human body deals reasonably well with this type of drugs, allowing their rapid clearance from the body. They are absorbed from the small intestine and inactivated very fast in the liver (Touw, 1998). Therefore, the route of their administration can only be done intravenously, intramuscularly or topically (Jana & Deb, 2006) with the inconvenience of fast elimination and the need for repetitive dosages. Attempts to overcome the problems of repeated local drug administration have primarily focused on using slow release formulations of antibiotics. Further critical issues relevant for developing and using slow release antibiotic formulations are the stability of the antibiotic in the

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formulation during the entire release period and the ease of localized administration, (e.g. direct injection in infected bone site). The local application of antibiotics as a slow release system may be an appropriate means to reach high local bioactivity at the contaminated site and low systemic side effects and drug residues. The use of carrier systems with potential capability for the controlled delivery of gentamicin, and other antibiotics generally used to combat osteomyelitis, has been investigated widely, as reviewed by Mouriño and Boccaccini (2010). Thus, the objectives of the present study were to develop a novel injectable system with increased biodegradation for the long term delivery of gentamicin and to test *in vitro* their drug-release properties and the antibacterial activity of the released antibiotic.

2. Experimental

2.1. Materials

Chitosan (medium molecular weight), sodium tripolyphosphate (technical grade, 85%), mineral oil (puriss. p.a.), sodium periodate (ACS reagent, >99.8%), deuterium oxide (D_2O 99.9% D) and deuterium chloride (35 wt%, 99% D) were obtained from Sigma–Aldrich (USA). Soluble potato starch was supplied by ATO (The Netherlands). Gentamicin sulfate from *Micromonospora purpurea* (puriss. p.a.) was purchased from Fluka-Bio Chem. (Switzerland). All other chemicals were of analytical grade and used as received.

The determination of chitosan molecular weight (MW) was conducted by viscosimetric method (Mathew, Brahmakumar, & Abraham, 2006) using 0.3 M acetic acid/0.2 M sodium acetate as a solvent mixture. The viscosity average molecular weight (MW) was calculated based on the Mark–Houwink equation (Terbojevich & Cosani, 1997; Terbojevich, Cosani, & Muzzarelli, 1996). The MW of the chitosan was found to be 385 kDa. The degree of N-deacetylation of the chitosan was determined by ^{1}H NMR spectroscopy method (Lavertu et al., 2003), and was found to be 81.25%.

2.2. Preparation of starch-conjugated chitosan microparticles

2.2.1. Sodium periodate oxidation of soluble starch

The oxidation of soluble starch was performed according to a procedure previously described (Hermanson, 1996). Briefly, soluble starch was dissolved in distilled water (20 mg/mL) in a light protected glass vessel. 0.125 mL of sodium iodate (10 mg/mL) was added per mL of starch solution, allowing the reaction to proceed for 30 min at room temperature under slow magnetic stirring. Finally, glycerin (0.1 mL/mL solution) was added and stirred for additional 10 min. The solution was transferred into a dialysis tube (Dialysis tubing cellulose membrane, MWCO 12 kDa, Sigma-Aldrich, USA) and dialyzed against distilled water for two days with several daily changes. The oxidized starch solution was concentrated by using an ultra-concentrator Amicon® stirred cell 8010 (Millipore, USA) and stored at 4 °C in a light protected glass vessel until further use.

2.2.2. Microparticles preparation

Starch-conjugated chitosan microparticles were prepared by water-in-oil emulsification method, using reductive alkylation crosslinking procedure (Baran, Mano, & Reis, 2004). The preparation procedure is illustrated in Fig. 1. Chitosan solution 1.5% (w/v) was prepared by dissolving the polymer in aqueous acetic acid solution (1%). Chitosan (1.5%, 10 mL) and oxidized starch (2% (w/v), 10 mL) solutions were mixed thoroughly using magnetic stirring, and the pH adjusted to 5.5 by using 0.5 M NaOH. The polymer solution was added dropwise into a glass reactor containing mineral oil with 1% of Tween 80 and emulsified with a top stirrer at 600 rpm for 30 min. After emulsion formation, 30 mL of cold acetone was added slowly and allowed to proceed for 2 h. The microparticles were removed from the reaction medium by filtration and washed with distilled water/acetone mixture (1:1 v/v). After drying at room temperature, the microparticles were transfered to a 0.5 M sodium carbonate buffer solution (pH 8.5) and incubated for 3 h to allow the conjugation between reactive aldehyde groups (oxidized starch) and primary amino groups (chitosan). After repeatedly washing with distilled water, the microparticles were incubated in sodium borohydride solution (0.05% (w/v)) for 1 h to reduce excess of reactive aldehyde groups, and to confer stabilization to the imine conjugation (oxidized starch-chitosan) into covalent bonds.

2.3. Physicochemical characterization

A microparticle suspension (5 mg/mL) was photographed using an optical microscopy Olympus BH2 with polarized light source (Olympus, Japan), using a high-resolution Leica camera for image acquisition (Leica Microsystems GmbH, Germany). For SEM observation, the microparticles were mounted onto metal stubs with a carbon tape and gold sputter-coated (Sputter Coater SC502, Fisons Instruments, UK). The size, shape and surface morphology of the microparticles were analyzed with SEM (Leica Cambridge S-360, UK).

2.3.1. Size distribution

A microparticle dry sample was weighed and then separated through a series of standard sieves (20, 60, 100, 125, 150, 250, 450, 500, 650, 900 and 1000 μm, Linker Industrie-Technik, Germany). The microparticle fraction that has been retained on sieve with certain porous size was collected and weighed, and finally correlated with the total mass of the microparticles.

2.3.2. Nuclear magnetic resonance spectroscopy (^{1}H NMR)

^{1}H NMR spectra were acquired on a Varian Unit Plus 300 MHz spectrometer (USA). For all the samples the following experimental parameters were used: 256 scans were recorded, 5200 Hz filter width, 90° pulse corresponding to a pulse width of 11 μs, 4 s relaxation delay. The experiments were run at 60 °C. For quantitative analysis, the peak area method was used and the start and end points of the integration of each peak were selected manually. For the sample preparation, 10 mg of each sample were exactly weighed into a NMR tube (φ = 0.3 mm) and 1 mL of deuterated solvent was added. Chitosan and starch-conjugated chitosan microparticles were dissolved in 0.98 mL of D_2O and 0.02 mL of deuterium chloride, waiting for complete dissolution, and starch and gentamicin were dissolved in D_2O. The sample was sonicated at room temperature for 30 min and used for ^{1}H NMR measurement.

2.4. Gentamicin loading

For drug encapsulation, gentamicin was directly added to the polymer solution using various initial concentrations of gentamicin (5, 10 and 15 mg/mL of polymer solution) to determine the influence of drug concentration in the encapsulation efficiency (EE). The preparation of the gentamicin loaded microparticles was performed as described in Section 2.2.2 (Fig. 1). Furthermore, the effect of ionic crosslinking on the drug release was investigated. In order to allow microparticles to be further ionic crosslinked, the gentamicin loaded microparticles were immersed into three different concentrations of sodium tripolyphosphate solution (0.5, 1, 2% (w/v)) for 30 min. The microparticles were then removed by filtration and washed several times with distilled water and allowed to dry at room temperature. The reaction medium and washing solutions were stored at 4 °C until further quantification of unloaded gentamicin. The initial concentration of gentamicin used for the

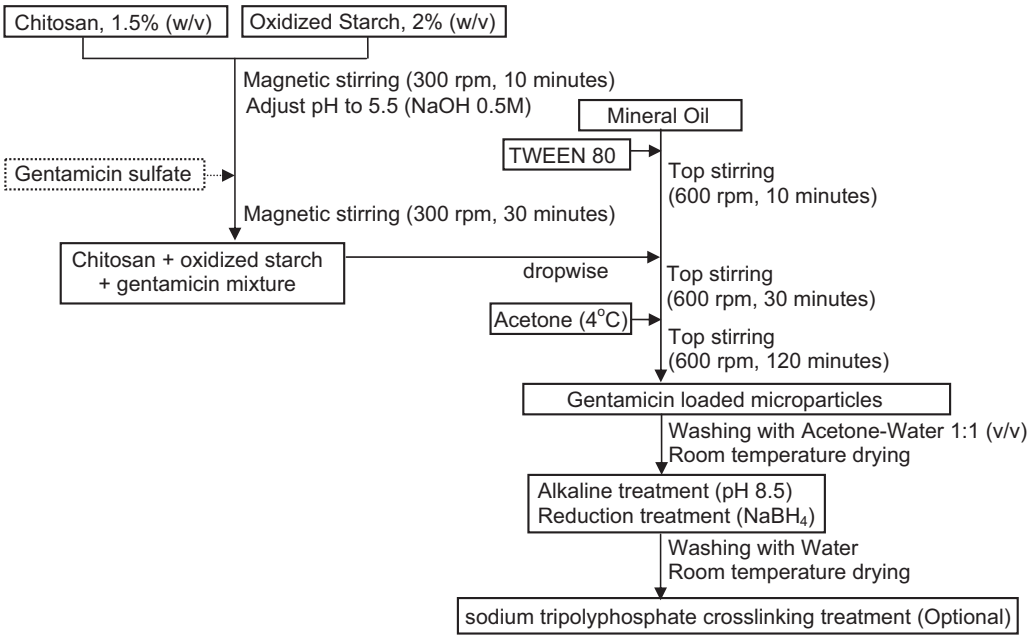

Fig. 1. Schematic diagram showing details of the procedure to prepare starch-conjugated chitosan microparticles.

crosslinking studies was 15 mg/mL. Encapsulation procedures for each initial gentamicin concentration were performed in triplicate.

2.5. In vitro gentamicin release

Pre-weighed gentamicin loaded starch-conjugated chitosan microparticles were suspended in 0.01 M PBS (pH 7.4) using a solid:liquid weight ratio of 2.5:1 (mg/mL). The microparticles were maintained at 37 °C under constant agitation (50 rpm) for 30 days in a shaking water bath. At predetermined time points, 1 mL aliquots were taken and replaced with same volume of fresh PBS solution. All the release experiments were carried out in triplicate, using unloaded starch-conjugated chitosan microparticles as control.

2.5.1. Quantification of released gentamicin

An indirect spectrophotometric method was used for the quantification of gentamicin, using o-phtaldialdehyde as derivatizing agent (Cabanillas, Pena, Barrales-Rienda, & Frutos, 2000). The procedure can be described as follows: o-phtaldialdehyde (0.25 g) was dissolved in a mixture of methanol (6.25 mL) and 2-mercaptoethanol (0.3 mL). The resulting solution was mixed with 0.04 M sodium borate (56-mL, pH 8) buffer solution. For the colorimetric measurement, gentamicin solution, o-phtaldialdehyde reagent and isopropanol were mixed in an equal volume ratio, and incubated for 30 min at room temperature. The absorbance was then measured at 332 nm. The concentration of gentamicin was calculated by using a calibration curve.

For the determination of the encapsulation efficiency and gentamicin loading into the microparticles, the amount of the drug remaining in the reaction medium during the loading process was quantified according to known equations (Wang, Ye, Zheng, Liu, & Tong, 2007).

2.5.2. Antibacterial activity of released gentamicin

The gentamicin released from the starch-conjugated chitosan microparticles was tested for bactericidal activity using *S. aureus* as a pathogenic microorganism. Standard strain of *S. aureus* ATCC 25923 (American Type Culture Collection) was obtained from the Department of Biology, University of Minho, Braga, Portugal. Fresh isolates were cultured for 24 h at 37 °C in LB-Agar (Luria-Bertani Agar, Lennox, Difco Laboratories, USA), immediately before each experiment.

The standard antibiotic disc diffusion method (NCCLS, 2008) in LB-Agar was employed for assessing *in vitro* the antibiotic activity of the released gentamicin against the selected bacteria, by determining the inhibition zones. For this, a bacteria suspension with concentration in the range of 1.5×10^8 UFC/mL was seeded in LB Agar sterile Petri dishes. All samples (released gentamicin solutions from the microparticles up to 30 days) were diluted to 0.25 mg/mL with sterile PBS buffer. Subsequently, 13 mm sterile absorption discs (BBL™ Taxo™ PM Discs, BD, USA) were placed on the agar plates and 20 μL of each sample was directly pipetted onto each disc. A disc impregnated with a solution of sterile PBS was used as control sample, and a standard solution of gentamicin sulfate 0.25 mg/mL was also tested. All agar plates were incubated at 37 °C for 16–18 h. Each sample was tested in triplicate, using different plates. After incubation, the diameters of the inhibition zone were measured using a micrometer.

Relative activity (%) was determined by calculating the ratio between the inhibition zones diameters of released gentamicin and a standard gentamicin solution (considered standard for the maximum inhibition zone) (Liu et al., 2007).

2.6. Short term stability tests

For the stability tests, gentamicin loaded starch-conjugated chitosan microparticles were stored in a well closed container at 4 and 25 °C for different periods of time (2, 4, 8 and 12 weeks). After each time point, samples were resuspended in sterile PBS and the *in vitro* release of gentamicin was analyzed, as well as the microbiological activity of the released antibiotic following the techniques described above.

3. Results and discussion

The general features of the obtained starch-conjugated chitosan microparticles can be observed in Fig. 2. The microparticles present a spherical shape and at higher magnification is possible to observe in more detail a single microparticle with a diameter of about 100 μm (Fig. 2(c)) showing a slightly rough surface

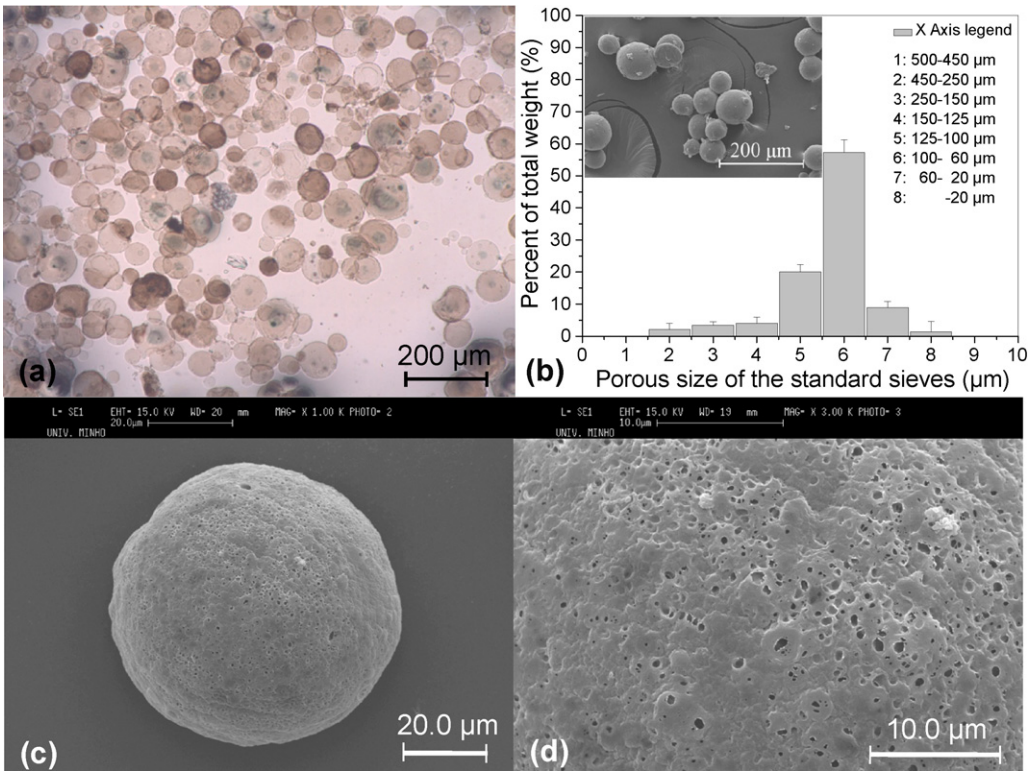

Fig. 2. Polarized LM image (a) and size distribution (ST-CHT, Table 1) quantified by sieve analysis (b) of the obtained starch-conjugated chitosan microparticles. Inset graph in (b) shows the morphologic characteristics of the analyzed sample. SEM micrographs (c, d) of gentamicin loaded microparticles. At higher magnifications (3000×) it can be observed the morphology of the gentamicin loaded microparticles' surface.

and the presence of small pores (around 1 μm size, Fig. 2(d)). No evident differences were observed between unloaded and gentamicin loaded microparticles. Gentamicin loaded microparticles maintained the initial spherical shape and morphological characteristics of unloaded microparticles.

The particles have a narrow size distribution (Fig. 2(b)). Almost 60% of the microparticles are smaller than 100 μm, around 15% are in the range 100–150 μm, and less than 7% are smaller than 60 μm. Thus, a highly homogeneous microparticular system was obtained.

Gentamicin can be identified in the spectrum of loaded microparticles (Fig. 3(a) and (b)). Gentamicin, as a complex mixture of several compounds, gives a very complex ^{1}H NMR spectrum but exhibiting characteristic peaks (Fig. 3(b)) (Deubner, Schollmayer, Wienen, & Holzgrabe, 2003; Winter, Deubner, & Holzgrabe, 2005). From the obtained spectrum, it is possible to identify the main signals as a result of the presence of gentamicin in the developed microparticles. The presence of both polysaccharides (chitosan and oxidized starch) as components of the polymeric matrix was also identified in the microparticles spectrum. For chitosan (Fig. 3(c)) a typical doublet peak, as result of the presence of the deacetylated unit at 5.04 ppm, and a peak (singlet) at 2.18 ppm from the acetylated residue, can be found in the resulting microparticles spectrum (Fig. 3(a)) (Hirai, Odani, & Nakajima, 1991; Varum, Anthonsen, Grasdalen, & Smidsrod, 1991). Finally, as result of the presence of the oxidized starch in the composition of the microparticles, an intense signal at 5.6 ppm can be assigned to the anomeric proton from the α(1 → 4) linkages of the amylose molecules in the starch structure (McIntyre & Vogel, 1991; Zang, Howseman, & Shulman, 1991).

Obviously, the chemical shifts do not match exactly the shift of the gentamicin and starch in aqueous solution, due to the solvent pH used (D_2O with 2% of deuterium chloride). There were no changes on the signals in the loaded microparticles spectra. Moreover, no new peaks have appeared in the spectra when comparing the polymers and drug spectra, indicating that no noticeable interaction between the drug encapsulated with the polymeric matrix had occurred. This may indicate that the drug loading and *in vitro* release is not expected to be affected by any possible chemical interaction between drug and polymer chemical groups.

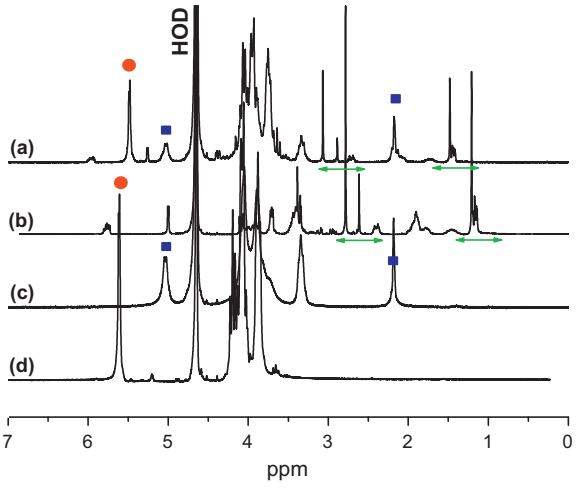

Fig. 3. ^{1}H NMR spectra of gentamicin loaded microspheres (a), gentamicin sulfate (b), chitosan (c) and starch (d). Assigned to gentamicin sulfate the signals at 2.7 and 2.6 ppm, singlets resulting from the protons in the amino methyl groups present in the garosamine and purpurosamine components of gentamicin and a very intense peak at 1.2 ppm assigned to the methyl groups of garosamine molecules (Deubner et al., 2003; Liu et al., 2007; Winter et al., 2005). Characteristic signals as a result of the presence of the different components in the microparticles composition were indicated to facilitate the comparison, i.e., (↔) for gentamicin, (■) for chitosan, and (●) for starch.

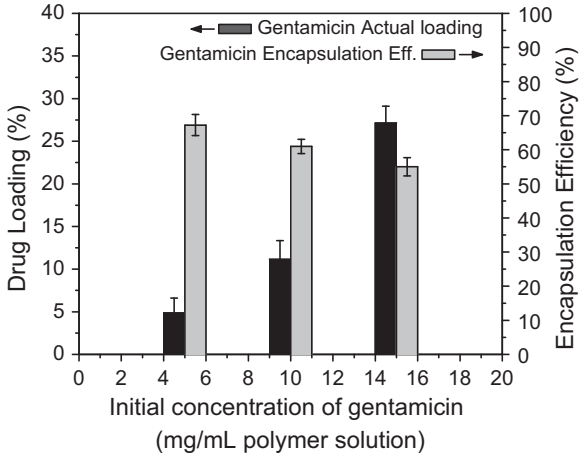

Fig. 4. Effect of initial gentamicin concentration on encapsulation efficiency and drug loading.

3.1. Gentamicin loading into microparticles

As expected, the drug loading increased significantly (from 4.8 to 27.1%) when gentamicin concentration increases from 5 to 15 mg/mL in the polymer solution, leading to a decrease in the EE (from 67.2 to 55%) (Table 1, Fig. 4).

This is a predictable consequence of the increment of the initial amount of drug, which causes a gradual increase of the drug loading until a saturation level is achieved. Moreover, increasing the initial amount of the drug into the polymer solution, the partition of hydrophilic drug molecules solubilized in surrounding aqueous solution increases, which can explain the resulting decrease on the EE. Finally, it must be considered that during encapsulation processes, and when using polymeric microparticles, there is a saturation level for the drug to be entrapped within the polymer droplets during the emulsion formation, and as a result the encapsulation becomes more difficult with the increments on the initial drug concentration.

3.2. Gentamicin release

Gentamicin is a highly soluble compound in aqueous solutions. Thus, the entrapped gentamicin molecules diffused rapidly into the external aqueous solution (Fig. 5(a)). About 54% of the encapsulated drug is released in the first 24 h. The burst release was observed to be followed by two sustained release stages. During the first nine days, 73% of the total entrapped gentamicin was released, being the remaining drug slowly released in the subsequent days. The second stage may be controlled by the swelling of the polymeric matrix (highly hydrophilic in this case) allowing aqueous solution to penetrate through the polymer, resulting in the diffusion of the gentamicin to the medium. Lastly, as result of the extended contact of the polymeric matrix with the surrounding medium, the polymer degradation (by hydrolysis mainly) starts to take place, allowing the remaining drug to be released. After 30 days, 82% of drug was released (Fig. 5(a)).

Although an initial burst release is considered an undesirable behavior when developing drug delivery systems, in the case of delivering antibiotics, a high dose is normally required during the initial period, to combat bacterial infections effectively, and then slowly prolong the release for longer time periods to complete the treatment. Various chemical modifications of the polymeric matrix provide prolonged release of entrapped antibiotics. In this study, we propose the use of a non-toxic ionic crosslinker (sodium tripolyphosphate, TPP). The microparticles were subjected to a crosslinking process that consisted in treating the microparticles with TPP to create ionic crosslinks between the phosphate groups of TPP and amino groups of chitosan. Sodium tripolyphosphate was used at three concentrations to investigate its effect on the release profile of gentamicin from starch-conjugated chitosan microparticles (Fig. 5(a)). The release of gentamicin from the microparticles decreased with increased cross-linking agent concentration. The primary reason for this observation is, increasing the cross-linking density reduces swelling of the microparticles hindering drug release. As the amount of crosslinking increases, the amount of free amino groups on chitosan is less thus decreasing the microparticles water uptake. The release of gentamicin from the matrix involves initial swelling followed by diffusion of the antibiotic. The microparticles produced using higher sodium tripolyphosphate concentrations (2%) were more rigid, had decreased size (Table 1) and showed less swelling in PBS. As a consequence, the initial burst release was slowed down and this behavior was observed up to 30 days. These results demonstrate that ionic crosslinking is a viable strategy for controlling release of gentamicin from starch-conjugated chitosan microparticles. Although the fastest release rate was observed from microparticles without ionic crosslinking, this system was still quite effective to ensure a sustained release of gentamicin after the initial burst stage.

3.2.1. Drug release mechanism

A number of mathematical models have been proposed to describe the mechanisms of drug release from polymeric devices. The Higuchi (Higuchi, 1961), Korsmeyer (Korsmeyer, Gurny, Doelker, Buri, & Peppas, 1983) and Peppas (Ritger & Peppas, 1987; Siepmann & Peppas, 2001) equations can be mentioned as the most widely used to date and are mainly based on the Fickian diffusion equation (Crank, 1975; Fick, 1855). In order to analyze the release mechanism of the gentamicin from starch-conjugated chitosan microparticles, and the effect of the sodium tripolyphosphate crosslinking over the release behavior, the obtained data was processed using the empirical expression proposed by Ritger–Peppas (Ritger & Peppas, 1987).

Table 1 summarizes the values obtained for the diffusion exponent n considering the starch-conjugated chitosan microparticles with and without sodium tripolyphosphate crosslinking treatment. The results show that the microparticles prepared with 2% sodium tripolyphosphate crosslinker exhibited a lower gentamicin diffusion exponent, n. Values of n were calculated and found to be 0.68 for microparticles without sodium tripolyphosphate, and 0.54 for the samples obtained with the highest sodium tripolyphosphate crosslinking density. The drug transport mechanism obtained for all experimental conditions was *Non-Fickian diffusion or Anomalous* (indication of the *superposition of both extreme phenomena: drug diffusion and macromolecular chain relaxation*) (Ritger & Peppas, 1987; Siepmann & Peppas, 2001). This result indicates that neither absolute Fickian diffusion (as result of pure drug diffusion) nor Case II-zero order (because of polymer chain relaxation) was the predominant mechanism in this case, since an Anomalous drug transport was obtained. This can be understood as a close combination of contributions of polymer relaxation and drug diffusion factors that determine the drug release mechanism. In the case of various concentration of sodium tripolyphosphate (0.5, 1 and 2%) the gentamicin release was clearly slowed down by the increase of the sodium tripolyphosphate concentration. Analysing the values obtained for n, the result shows in all cases an anomalous drug transport mechanism (Table 1), from which it can be concluded that the crosslinking density does not show a significant influence in the drug transport mechanism (anomalous in all the cases), affecting solely the diffusion coefficient value.

Table 1
Experimental conditions for the preparation of gentamicin loaded starch-conjugated chitosan microparticles[a]. Values of encapsulation efficiency (EE, %), drug loading (%) and kinetic parameters obtained for the developed microparticular system.

Sample	Gentamicin (mg/mL)	Sodium tripolyphosphate (%)	Size (μm)	Gentamicin loading (%)	EE (%)	Diffusion exponent *n* (r)
ST-CHT[b]	–	–	96.1 ± 12.4	–	–	–
ST-CHT/G[c]-1	15	–	119.4 ± 8.3	n.d.	n.d.	0.68 (0.9998)
ST-CHT/G-2	5	–	103.7 ± 22.9	4.86 ± 1.74	67.24 ± 3.11	n.d.
ST-CHT/G-3	10	–	111.5 ± 18.5	11.14 ± 2.21	60.97 ± 2.08	n.d.
ST-CHT/G-4	15	–	116.6 ± 31.8	27.14 ± 1.98	55.01 ± 2.65	n.d.
ST-CHT-T/G[d]-5	15	0.5	89.3 ± 28.6	n.d.	n.d.	0.60 (0.9980)
ST-CHT-T/G-6	15	1	80.4 ± 22.3	n.d.	n.d.	0.59 (0.9812)
ST-CHT-T/G-7	15	2	81.1 ± 17.9	n.d.	n.d.	0.54 (0.9971)

n.d.: not determined.
[a] Common experimental conditions for all experiments: chitosan–starch ratio: 1.5:2 (w/w). Volume of polymer solution: 10 mL. Mineral oil 70 mL (1% Tween 80, (v/v)). Stirring rate: 600 rpm.
[b] ST-CHT: starch-conjugated chitosan microparticles.
[c] ST-CHT/G: gentamicin loaded starch-conjugated chitosan microparticles.
[d] ST-CHT-T/G: gentamicin loaded starch-conjugated chitosan microparticles ionically crosslinked with sodium tripolyphosphate.

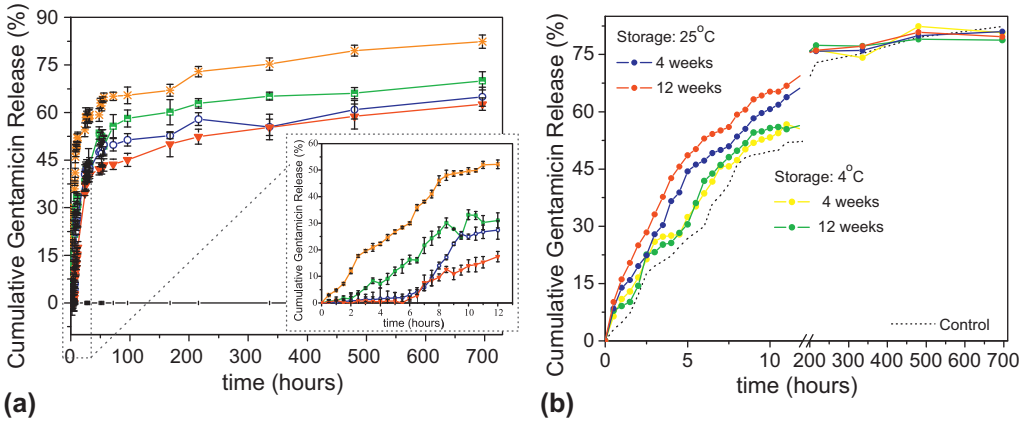

Fig. 5. Release profiles of gentamicin from starch-conjugated chitosan microparticles in PBS at 37 °C. (a) Influence of sodium tripolyphosphate crosslinking concentrations on the gentamicin release profiles: gentamicin unloaded microparticles used as control (ST-CHT, Table 1) (|), Gentamicin loaded microparticles crosslinked with: sodium tripolyphosphate 2% (ST-CHT-T/G-7, Table 1) (▼), sodium tripolyphosphate 1% (ST-CHT-T/G-6, Table 1) (○), and sodium tripolyphosphate 0.5% (ST-CHT-T/G-5, Table 1) (▬), Gentamicin loaded microparticles without sodium tripolyphosphate crosslinking (ST-CHT/G-4, Table 1) (✳). Inset graph in (b) represent the first 24 h of release. **(b)** Release profiles of gentamicin from starch-conjugated chitosan microparticles after being stored for different conditions of time (4 and 12 weeks) and temperature (4 and 25 °C).

3.2.2. *Antibacterial activity of released gentamicin*

One very important factor when designing drug delivery systems is the maintenance of the activity or function of the bioactive molecules entrapped in the polymeric matrix. In the present study, the relative activity of the released gentamicin from the microparticles was screened *in vitro* against *S. aureus* (following standard procedures approved by NCCLS) (NCCLS, 2008).

The obtained results, in values of relative activity (%) and inhibition zones diameters (mm), are shown in Fig. 6.

The diameters of inhibition zones vary from 17 mm for the standard gentamicin solution to 12 mm for the gentamicin released after 30 days of incubation, and the corresponding relative activities change from 100% to 70%. It can be seen that released gentamicin have inhibition zones values similar to the one obtained for the

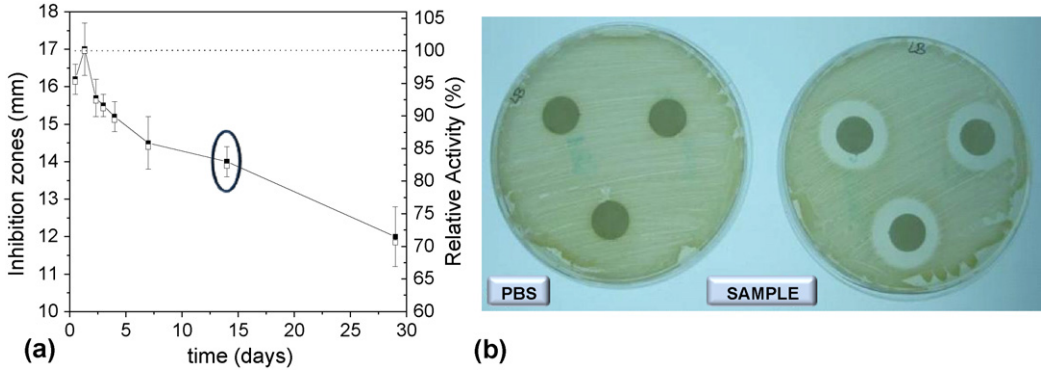

Fig. 6. Inhibition zone and relative activity of released gentamicin from starch-conjugated chitosan microparticles (a). Photographic observation of the antimicrobial activities against *Staphylococus aureus* (b). PBS solution without gentamicin (used as negative control) resulted in no inhibition zone around the impregnated disc. Disc impregnated with gentamicin released after 15 days, as shown in the line graph (360 h). The sample selected for this study was ST-CHT/G-1 (please see Table 1 for details).

standard solution of this antibiotic. Moreover, the obtained results, in terms of diameter inhibition zone (mm) and relative antibactericidal activities (%) are in the same range to the ones reported in the literature for gentamicin sulfate against *S. aureus* (Stigtera, Bezemera, de Grootb, & Layrollec, 2004; Tam, Kabbara, Vo, Schilling, & Coyle, 2006). These results indicated that gentamicin stability was not affected since its antibiotic activity was maintained after the encapsulation and microparticles processing, and also during the release experiments performed for 30 days in PBS at 37 °C.

3.2.3. Effect of storage temperature and time on the release profile and activity of entrapped gentamicin

A slightly faster burst release was observed during the first 12 h, with increasing storage time and temperature (Fig. 5(b)). The plateau of sustained release up of 4 days remained unchanged. This result was more pronounced for the samples stored at 25 °C. The released gentamicin remains active against *S. aureus*, but a decrease in the relative activity was found with increasing storage time.

These results indicate that the sustained release of gentamicin from the starch-conjugated chitosan microparticles does not seem to be significantly impaired. The activity of the gentamicin remains up to 50% for all the samples tested after 30 days of release. It can be concluded that the developed system allows its storage in a controlled environment for at least 30 days. Samples stored for more than 4 weeks maintained a sustained release of gentamicin, and the released antibiotic remains active against *S. aureus*.

4. Conclusions

A successful conjugation of chitosan to oxidized starch was achieved by means of a reductive alkylation procedure and this conjugate system allowed the production of microparticles with spherical shape and uniform size distribution using a water-in-oil emulsion crosslinking method. The potential of the developed starch-conjugated chitosan microparticles for the sustained release applications was evaluated by studying the loading and release of gentamicin sulfate. This system provides a long term sustained release of active gentamicin. Furthermore, the loaded carrier system is highly stable when stored under controlled conditions for periods up to 12 weeks. Biodegradable microparticles loaded with antibiotics, such the ones developed in this study, can provide effective treatments for persistent infections.

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