Chitosan Beads as Templates for Layer-by-Layer Assembly and their Application in the Sustained Release of Bioactive Agents

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ABSTRACT: Uncoated chitosan beads and chitosan beads coated with hyaluronic acid/chitosan (HA/Ch) multilayers, were used to investigate the controlled release of gentamicin sulphate (GS). Greater encapsulation efficiency was observed for the layer–by-layer multilayer coated beads. The *in vitro* drug release was in a slower sustained manner compared with noncoated chitosan beads. The differences in *in vitro* drug release results may be explained by the barrier effect of the coating that impedes diffusion of GS and supporting complementary water uptake. These findings indicated that a slower sustained release of gentamicin can be obtained using multilayer coatings of HA/Ch on chitosan beads and that this process could be used as a drug delivery system. In addition, agglomerates of these bead could provide a porous support in tissue engineering applications.

KEY WORDS: layer-by-layer, chitosan beads, bead coatings, drug release, gentamicin delivery, controlled delivery, tissue engineering, scaffold.

INTRODUCTION

One of the major challenges in tissue engineering and drug delivery is to develop materials capable of delivering specific drugs by

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sustained release. Particulate systems provide a suitable form for such purposes due to size, surface area, and physicochemical properties [1].

Recently, interest has arisen in the use of layer-by-layer (LBL) self-assembly methods to develop new drug delivery systems [2–8]. The LBL method is based on the alternately deposition of oppositely charged species onto a charged substrate, leading to materials with unique functional properties. The LBL application has been applied in various substrates ranging from planar [9] to nonplanar [6,10]. A wide variety of polymeric carriers has been investigated for the delivery of different types of molecules, such as, latex particles [10], inorganic particles [11], drug crystals [12], proteins [13], and cells [14]. Similarly, films [15], microcapsules [16], and nanospheres [17] have been coated using this technique.

In the case of capsules, the substrate decomposes after layer deposition. However, particles must not dissolve during the LBL coating [16,18]. Using the LBL method [19,20], after the drug particles/crystals are coated, the drug release was found to be dependent on the number of layers, the solubility, and size of the crystals as well as the type of polymers used for the coating layers [20,21].

Biodegradable microspheres or beads offer attractive advantages as drug delivery systems [22]. Apart from their size, surface area, and physicochemical properties [1], they do not require surgical removal from the body [23] and are able to deliver bioactive agents, to act as cell carriers and to provide structural support [1]. Chitosan particles have been used for the construction of scaffolds prepared by simple agglomeration of the particles [24] and for the delivery of a wide range of drugs [25,26]. However, in many cases, a slow sustained release is not accomplished. In fact, hydrogels systems, such as chitosan-based materials, swell considerably within a short period of time and a burst release of >50% of the encapsulated molecule is often observed. Various methods have been investigated to overcome this problem; one is LBL assembly. Whether the bioactive agent is located in the bulk or encapsulated in polyelectrolyte multilayered films [27], the release behavior is dependent on the permeability or breakdown of the multilayer structure [28]. Gentamicin loaded chitosan microspheres have been single coated by hyaluronic acid (HA) [29] and the *in vitro* drug release of the drug occurred over 5 h, thus, providing a potential stable sustained release. Alginate beads containing a thermoresponsive polymer exhibited a slower release profile of indomethacin when previously coated with a chitosan layer [30].

Gentamicin sulphate (GS), used to treat bacterial infections and osteomyolite, was used as a model antibiotic system by Lecaros et al. [31].

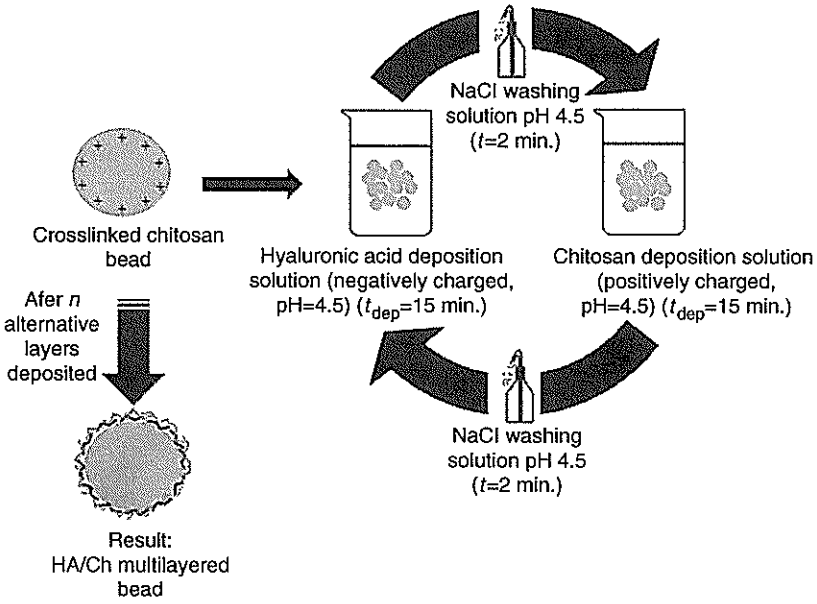

Figure 1. HA/Ch LBL assembly on chitosan crosslinked beads.

GS has been encapsulated in micro- and nanoparticles of poly(lactide-co-glycolide) [32], in microporous poly(ε-caprolactone) matrices [33] and even in scaffolds [34]. Previous *in vitro* release studies of gentamicin incorporated into chitosan microspheres reported fast release [29].

In the present work, a HA/Ch multilayered coating system was applied as a drug release system to produce a slow and sustain drug release system using multilayer assembly. Schematically represented in Figure 1 is the LBL coating procedure used for chitosan crosslinked beads. The morphological characterization was performed by scanning electron microscopy (SEM). Additionally, cytotoxicity tests were performed to evaluate whether the amount of glutaraldehyde used to crosslink the beads induced some degree of cytotoxicity.

MATERIALS AND METHODS

Materials

Medium molecular weight chitosan (Ch) (with a degree of deacetylation of 83.8%, determined by H-NMR), glutaraldehyde (Glu) and GS salt were purchased from Sigma-Aldrich. HA, from *Streptococcus equi.* sp.

with an average molecular weight of 1.63×10^6 Da, was obtained from BioChemika Fluka. All other chemicals were reagent grade and used as received. Acetic acid glacial (CH_3COOH), sodium hydroxide pellets (NaOH) and sodium chloride were purchased from Panreac Quimica SA. Mouse lung fibroblast-like M cells, used for the cytotoxicity tests, were obtained from European Collection of Cell Cultures (ECACC, UK).

Preparation of Chitosan-based Beads and LBL HA/Ch Deposition

Chitosan beads were prepared as previously described [24]. Briefly, a 1.5% (w/w) chitosan solution was dissolved overnight in a 2% (v/v) acetic acid solution. The solution was extruded through a needle using a syringe pump at a constant rate of 4 mL/h into an alkaline bath (1 M NaOH) under continuous stirring (350 rpm). Chitosan beads (*A*) were formed on contact with the alkaline solution. After stirring for 30 min, the beads were washed with distilled water: the beads were sieved and put in a 0.025 M glutaraldehyde solution for 30 min to crosslink the beads. Crosslinked chitosan beads (A30) were dispersed in distilled water for further use.

LBL deposition was performed by the dipping method. 0.5% (w/w) HA solution was dissolved in 0.15 M NaCl and stirred overnight at room temperature. Similarly, 1.5% (w/w) Ch solution was prepared under the same conditions. Both solutions were then adjusted to pH 4.5 with either 0.1 M CH_3COOH or 0.1 M NaOH. Each layer on the A30 beads was deposited by the alternatively suspending the beads in HA or Ch solution for 15 min and then stirred for 2 min in 0.15 M NaCl (pH adjusted to 4.5). The last layer was washed with distilled water. Several different layers were deposited on A30 [5 and 10 bilayers (M5x and M10x), respectively].

Evaluation of Cytotoxicity of Glutaraldehyde Crosslinked Chitosan Beads

Crosslinked Beads

Three different concentrations of the glutaraldehyde in distilled water were prepared; 0.025 M, 0.1 M, and 0.175 M. The chitosan beads (100 mg) were filtered using a thin sieve and immersed into each concentration of glutaraldehyde for 30 min after which, the beads were filtered again and thoroughly rinsed with distilled water. All three batches of beads were then autoclaved at 110°C for 30 min to sterilize the before conducting the MTS test.

Preparation of Extracts

Extracts were obtained by immersing the beads from each formulation (beads ca. 2 mm) in 50 mL plastic tubes with 10 mL of Dulbecco Modified Eagle Medium (DMEM, Sigma, USA), with 1% of an antimicotic/antibiotic solution (A/B, Gibco, UK) containing 10,000 U/mL penicillin G sodium, 10,000 mg/mL streptomycin sulphate and 25 μg/mL amphotericin B as Fungizones® in 0.85% saline without fetal bovine serum (FBS) and without phenol red previously prepared and put in a water bath at 37°C, 60 rpm for 24 h. The extracts were filtered using a 0.45 μm syringe filter (Schkeicher & Schuell Microscience, Germany). In the case of latex rubber, discs was immersed in DMEM culture medium following the same procedure as described above for the test materials. Extracts were prepared in accordance with Gomes et al. (2001) [35].

Cell Culture

For this cytotoxicity assay, mouse lung fibroblastic-like cells were used (obtained from ECACC, UK) and cultured in basic medium: DMEM (Sigma-Aldrich, USA) without phenol red supplemented with 10% of FBS (Gibco, UK) and 1% A/B (Gibco, UK) solution. The L929 cell were incubated at 37°C in an atmosphere containing 5% of CO_2. A cell suspension was prepared with ~6.6×10^4 cells/mL. After reaching 80% of confluence (~48 h), the L929 cells were seeded (8th passage) onto 96-well plates. The culture medium in each well was replaced by 150 μL of extraction fluid (material extracts and the response of the cells was evaluated at 24, 48, and 72 h, with the three different crosslinker concentrations. Six replicates were examined per sample. The L929 cells relative viability (%) was determined for each extract concentration and compared with tissue culture polystyrene (TCPS). Latex rubber discs (Dermagip, WRP) were used as a positive control of cellular death while TCPS were used as a negative control of cellular death.

MTS Solution

The MTS solution was prepared with DMEM (Sigma, USA) without red phenol and without FBS in a 1:5 ratio (MTS:DMEM). After removing the extracts, 200 μL of MTS solution was added to the cells and incubated for 3 h at 37°C in an atmosphere containing 5% of CO_2. The absorbance was read with a microplate reader (BIO-TEK – Synergy HT) at 490 nm. The results were expressed in terms of relative viability (%) when samples were compared to TCPS for each time period.

Encapsulation Efficiency and Actual Bead Drug Loading

Dried beads (100 mg of A30, M5X, and M10X, respectively) were immersed in 3 mL of PBS containing 300 mg of GS (100 mg/mL solution). After 48 h, the GS solution was removed using a 1 mL insulin syringe (BD-Microfine U-100 insulin); the beads were then dried for 3 days. The GS loaded bead samples (A30GS, M5xGS, and M10xGS) were weighed. The amount of gentamicin left in the loading medium was assayed using o–phtaldialdehyde as a derivatizing agent [36]. Briefly, the o–phtaldialdehyde (2.5 g) was dissolved in methanol (62.5 mL) and 2-mercaptoethanol (3 mL) in a light protected flask. This solution was added to 0.04 M of sodium borate (560 mL) in distilled water in a dark glass bottle and protected from light with aluminium foil. Similar proportions of gentamicin solution, isopropanol and o–phtaldialdehyde reagent were mixed and stored for 30 min at room temperature before quantifying under a UV-multiwell microplate reader (Bio-Tek Instruments – Synergy HT), at the maximum absorbance of gentamicin-o-phtaldialdehyde complex at 332 nm [36], with previous determination of a calibration curve.

The small amounts of GS residues that adhered to the surface of the equipments/utensils used to remove the beads were also taken into account for determination of encapsulation efficiency and actual drug loading.

The percent encapsulation efficiency of each sample of 100 mg of beads was calculated using Equations (1) and (2), as follows:

$$\text{Encapsulation efficiency (\%)} = \frac{[\text{encapsulated drug}]}{[\text{total initial drug}]} \times 100 \quad (1)$$

$$\text{Encapsulation drug} = [\text{total initial drug}] - [\text{free drug after loading}] \quad (2)$$

The initial amount of drug used in this procedure was determined taking into account that 100 mg of beads was used to absorb GS (100 mg/mL in a 3 mL PBS solution) and the mass of free drug corresponds to the mass of drug that did not absorb and was determined by UV.

The percentage of actual drug loading of each sample of 100 mg of beads was calculated by Equation (3), as follows:

$$\text{Drug loaded (\%)} = \frac{[\text{encapsulated drug Wt}]}{[\text{encapsulated drug Wt}] + \text{dried beads Wt}} \quad (3)$$

Determination of Bead Size and Morphology

The beads size and morphology was determined using a SEM (Leica, Cambridge S360). Samples were dehydrated in different increased concentrations of ethanol (25, 30, 50, 70, 80, 90, and 100%, for 30 min), separately dried at room temperature, mounted on metal studs using double sided conducting adhesive tape and vacuum coated with gold in a sputter coating unit.

In vitro Drug Release Studies

In vitro release of gentamicin from the beads formulations were carried out at 37°C in 10 mL PBS under continuous agitation. At fixed intervals, an aliquot (1 mL) of the release medium was withdrawn and fresh buffer of phosphate buffer saline (PBS) was replaced each time. The procedure used to quantify the amount of gentamicin on the beads was by the same quantitative colorimetric assay explained before and using the UV-multiwell microplate reader at 332 nm of absorbance.

The results were expressed as a percentage of the drug released as shown below in Equation (4):

$$\%\ \text{drug released} = \frac{[\text{amount of drug released at time } t, \text{mg}]}{[\text{total amount of encapsulated drug, mg}]} \quad (4)$$

RESULTS AND DISCUSSION

Crosslinked chitosan beads were used as the substrate for the deposition of HA/Ch using LBL self-assembly. The layered assembly showed no cytotoxic effects and the LBL material absorbed the GS. Subsequent, *in vitro* drug release and encapsulation efficiency studies indicated that this material provided suitable sustained release of the drug.

Cytotoxicity of the Crosslinked Chitosan Beads

Cellular viability and proliferation were determined using fibroblastic cell cultures with the chitosan beads exposed to MTS solution for 72 h. The aim of this assay was to determine whether the concentration of gluteraldehyde used had a impact, such as, causing cytotoxic responses by the materials developed.

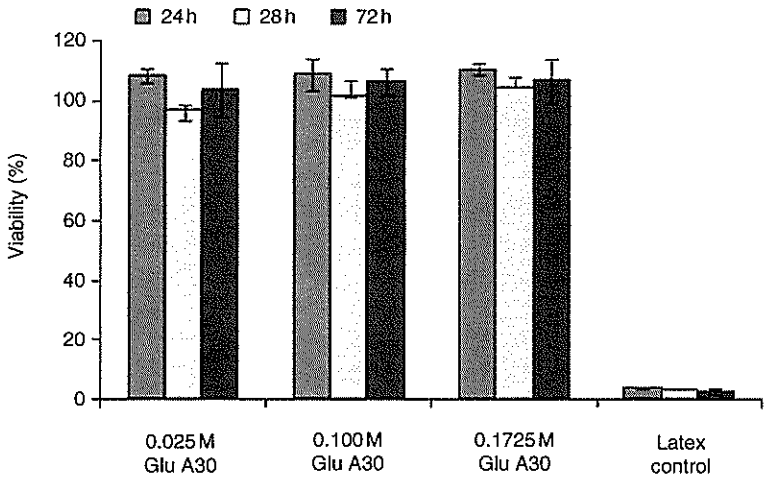

Figure 2. Percentage of cell viability of fibroblast cells on chitosan beads crosslinked with different concentrations of glutaraldehyde (Glu) determined by MTS.

The MTS results are shown in Figure 2 indicate that the leachables obtained from crosslinked beads exposed to 0.025 M, 0.10 M, or 0.1725 M gluteraldehyde do not exhibit cytotoxic effect compared to latex cell death positive control. Although, none of the samples produced toxic leachables, the lowest concentration of gulteraldehyde was used to crosslink the chitosan beads for further study.

Encapsulation Efficiency and Actual Drug Loading

Encapsulation efficiency and drug loading are important parameters to consider when evaluating the properties of the drug release of beads. The efficiency and the drug loading in the developed beads are listed in Table 1. The drug efficiency and the drug loading of each sample are shown in Figure 3. It is clear that M5xGS and M10xGS had the highest drug efficiency with 55.96% and 52.24% compared to the uncoated system, A30GS, which was 39.9%. The latter may be explained by the fact that GS is positively charged similar to chitosan, which induced repulsion between them [29]. Multilayer coated beads, M5xGS and M10GS, were efficient than A30GS because of the presence of the negatively charged hyaluronic acid. Complementary swelling tests, that will be reported in more detail elsewhere, showed that the coated beads take up a lot more water than the uncoated ones. This explains the higher loading capacity of the beads as the drug is introduced by swelling the dry beads in the drug solution.

Table 1. Data for the determination of the encapsulation efficiency and actual drug loading of the beads.

Sample	Total amount of drug to be encapsulated (mg)	Amount of free drug (unloaded) (mg)	Amount of dried beads (mg)	Drug efficiency (%)	Actual loading (%)
A30GS	723	434.37	241.02	39.92	54.49
M5xGS	909	400.29	303.15	55.96	62.66
M10xGS	909	434.09	303.06	52.24	61.04

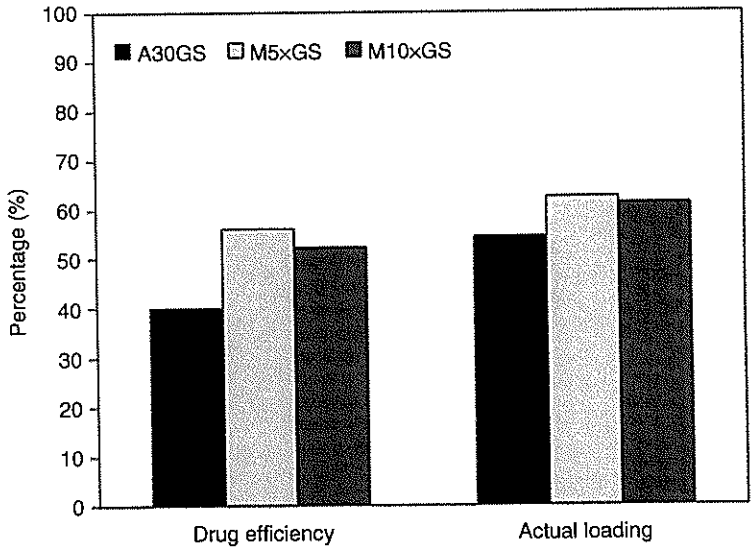

Figure 3. Drug efficiency and actual loading of GS loaded beads.

Determination of Size and Morphology

The size and morphology of the gentamicin loaded beads were evaluated by SEM. The general morphology of the chitosan beads is shown in Figure 4. The beads were spherical in shape and the surface appears to be smooth (Figure 4(a)); the average diameter of the beads was ~500 μm. After the layering procedure and the inclusion of the drug, the beads exhibited a rougher surface, while keeping their spherical shape (Figure 4(b)). In this case, the beads have an average diameter of ~800 μm. SEM micrographs of the surface of unloaded and loaded GS multilayer coated beads are displayed in Figure 5, with insets for each image.

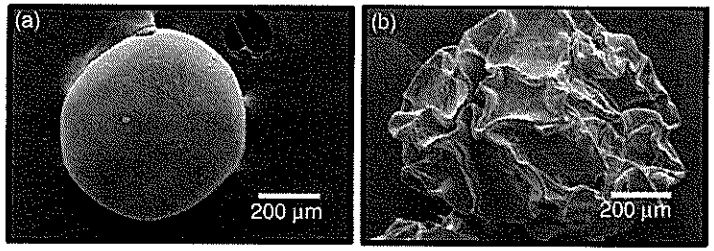

Figure 4. Representative SEM micrographs showing the general morphology of obtained beads: (a) A30 and (b) M5xGS.

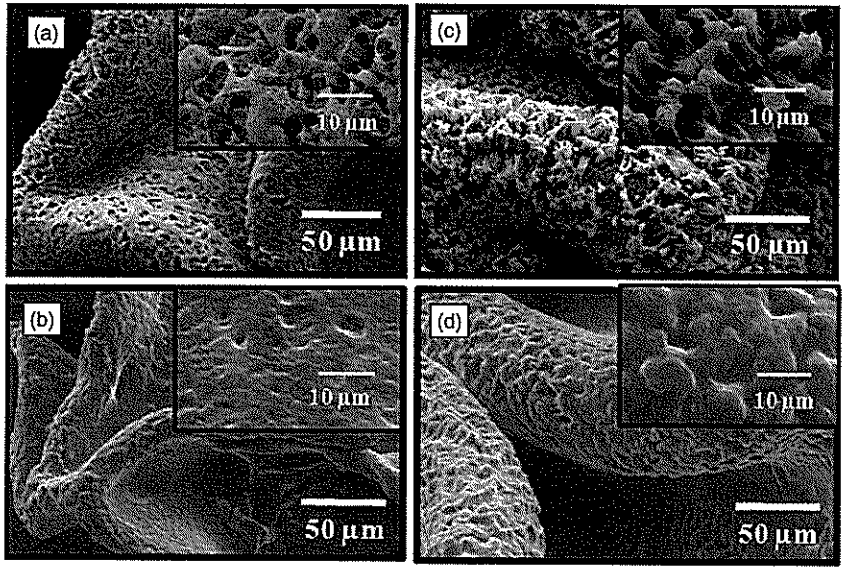

Figure 5. SEM micrographs of the surface of unloaded and loaded GS multilayer coated beads: (a) M5x; (b) M5xGS; (c) M10x; (d) M10xGS.

In vitro Drug Release Studies

The *in vitro* release profiles of GS from the three different beads' formulations are shown in Figure 6. Empty beads were also tested to determine if any materials released could interfere with the drug detection; no effect was detected. For all formulations, the release of the drug from the beads was almost accomplished within 48 h. A30GS released most of its initial drug content within 6 h. However, both M5xGS and M10xGS demonstrated a distinct release profile, when compared with the uncoated system. Despite the initial burst observed,

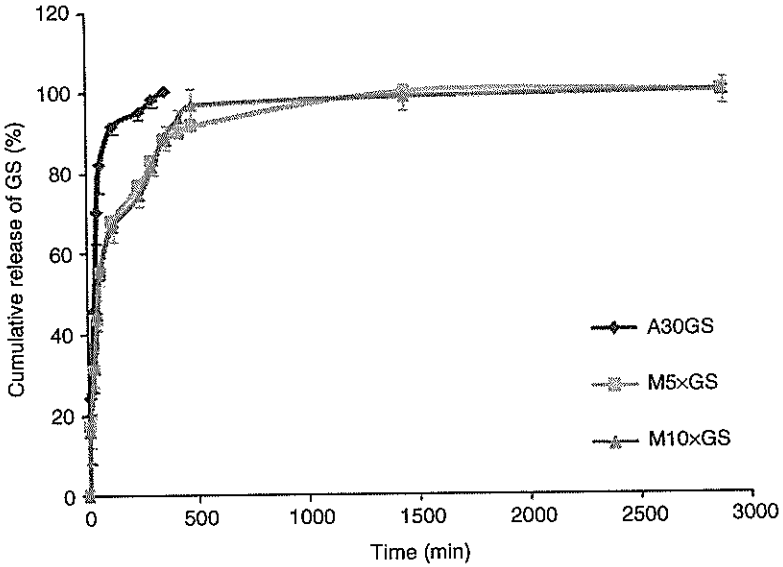

Figure 6. Release profiles of GS from crosslinked chitosan and multilayered beads.

a slow release phase was also observed. The release profiles for M5xGS and M10xG, in Figure 6, are similar with 92 and 97% release, respectively, within 8 h. After 24 h, the release from both types of beads was 100%.The initial burst was probably due to the dissolution and release of the drug absorbed on or near the surface of the beads [37] or due to the diffusion of gentamicin particles that were trapped in the voids within the multilayered matrix [29,36], that were observed in the SEM images (Figure 5(b) and (d)). Lim et al. inferred that the release of gentamicin is dependent on the presence of cracks on the surface of the beads, such that, slow trapped the drug slowly release. It was also reported that HA beads swell faster than chitosan when in contact with the dissolution medium and form a gel diffusion layer that obstructs the transport of the drug from the matrix, hence producing a more sustained release effect [29]. Desai et al. (2006) reported that the gel layer texture and thickness also play a role in the drug release process [38]. Considering that M10x and M5x are capable of absorbing more water implies that more drug was absorbed and, therefore, provided more drug to be released. This is in accordance with the *in vitro* drug release behavior. However, since M5xGS and M10xGS have similar drug release profiles (Figure 6), with each layer acting as a gel diffusion layer impeding or to slow the diffusion of GS through the layers, due to HA's viscoelastic properties [39], there is no reason to build up 10 bilayers of

HA/Ch on chitosan beads. The electrostatic architecture of the coating may also act as a barrier to the diffusion of the drug, also providing a slower sustained release.

CONCLUSIONS

A unique drug delivery, based on the slow sustained release of gentamicin from a multilayer coated system consisting of hyaluronic acid/chitosan layers on chitosan beads, was developed. The crosslinked chitosan beads did not show any cytotoxicity; consequently, these beads were applied as substrates for the alternately deposition of HA/Ch. GS was absorbed by the biomaterial developed and *in vitro* drug release studies showed a slow sustain release for up to 48 h, in contrast, the uncoated crosslinked chitosan beads released all of the drug within 6 h. Thus, the multilayer coated beads demonstrated slower release kinetics as compared to A30GS. The results also indicate that the structure of M5xGS is as effective as M10xGS, with similar loading capabilities and drug release profiles. The HA/Ch multilayers on crosslinked chitosan beads are potentially useful for encapsulating bioactive agents to provide slow sustained release for tissue engineering and drug delivery applications.

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