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ORIGINAL ARTICLE

Evaluation of antibacterial and cytotoxic effects of nano-sized bioactive glass/collagen composites releasing tetracycline hydrochloride

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Abstract

Aims: To evaluate the antibacterial efficacy of silicate bioactive glass nanoparticles/collagen composites functionalized with tetracycline hydrochloride (TCH).

Methods and Results: Different concentrations of tetracycline hydrochloride (TCH) were incorporated on silicate bioactive glass nanoparticles/collagen composites by dipping these biomaterials for 48 h at 37°C in a solution of simulated body fluid (SBF) plus 0·05, 0·20 or 0·35 mg ml^{-1} of the antibiotic. TCH release was assessed in double-distilled water at 37°C up to 72 h. The antibacterial activity of the samples has been evaluated in two ways: inhibition zone test and plate count method. The experiments were performed *in vitro* up to 48 h on four staphylococci strains (*Staphylococcus aureus* ATCC29213, ATCC25923, ATCC6538P and *Staphylococcus epidermidis* ATCC12228). The new composites were also tested for cytotoxicity on MG-63 human osteosarcoma cells. The results showed that the incorporation and release of TCH was dependent on the initial concentration of TCH in SBF. The biomaterials also inhibited the *Staph. aureus* cell growth even though the efficacy was similar for all concentration. On the other hand, no cytotoxic effects were found on osteoblast-like cells, even at the highest concentration.

Conclusions: Considering all results, it can be concluded that the new composite acts as a suitable bioactive carrier of TCH and could have potential in the prevention of biomaterial related infections.

Significance and Impact of the Study: The results suggest a potential application as wound dressing.

Introduction

Infections after tissue trauma (i.e. burns, ulcers) and surgery represent an enormous economic burden and a high vulnerability for patients health (Reilly *et al.* 2001; Paocharoen *et al.*2009). *Staphylococcus aureus* (*Staph. aureus*) is one of the most important pathogen involved in this kind of infections (Onche and Adedeji 2004). When wound healing is delayed because of an infection, the treatment can become a challenge. The development of new therapeutic methods is therefore necessary for the treatment of this problem.

It has been proposed that a local and prolonged delivery of an antibiotic can reduce the side effects and risk of overdose, increasing also the concentration that can be effectively delivered to a targeted site (Mouriño and Boccaccini 2010). In that way, a systemic administration could be reduced significantly, and in addition, biofilm

formation can be prevented because of the relatively high concentration of antibiotics that can be employed locally. Other advantages include continuous action, reduced toxicity and a convenient situation for patients since frequent systemic administration can be reduced (Zhao *et al.* 2008).

Tetracycline hydrochloride (TCH) belongs to a group of broad-spectrum antibiotics that act interfering with protein synthesis (Sapadin and Fleischmajer 2006). Tetracycline is also well known to have binding affinity to bone. Mechanistically, the antibiotic binds with hydroxyapatite by forming complexes with calcium ions (Misra 1991; Dashti *et al.* 2010).

Bioactive glasses have been investigated widely as carriers for many drugs including antibiotics (Mouriño and Boccaccini 2010; Hum and Boccaccini 2012; Arcos and Vallet-Regí 2013). In particular, TCH was incorporated onto bioactive glasses mainly by sol gel method (Andrade *et al.* 2006, 2009; Zhao *et al.* 2008; Cavalu *et al.* 2013) and also tested for their antibacterial activity *in vitro* and *in vivo* (Domingues *et al.* 2004). Data showed that the drug release behaviour was strongly dependent on the bioactive glass system and the CaO content in some cases (Zhao *et al.* 2008). In general, glass–tetracycline samples showed rapid drug release kinetics (Andrade *et al.* 2006, 2009) and presented a favourable effect in terms of antibacterial and bioactive behaviours of the glasses (Domingues *et al.* 2004).

Recently, Miola *et al.* (2012) have reported the incorporation of carbenicillin onto a bioactive glass and its corresponding glass-ceramic by a co-precipitation method during the bioactivity process in simulated body fluid (SBF).

Type I collagen is the most abundant protein of the human body and the main organic component of bone. Due to its biocompatibility, biodegradability, biological properties and natural role in tissue formation, collagen results a favourable matrix for on-site drug delivery (Ruszczak and Friess 2003).

In the present work, different concentrations of tetracycline hydrochloride were incorporated onto nano-sized bioactive glass/collagen composites using the SBF immersion method introduced by Miola *et al.* (2012). The new biomaterials were investigated on their antibacterial efficacy against four staphylococci strains. The cytotoxicity of the materials on MG-63 human osteosarcoma cell line was also studied.

Materials and methods

Materials

Bioactive glass nanoparticles (n-BG, 20–30 nm) of nominal composition close to 45S5 Bioglass® (46 wt% SiO_2, 23 wt% Na_2O, 27 wt% CaO, 4 wt% P_2O_5) fabricated by flame spray method (Brunner *et al.* 2006) were kindly supplied by W. Stark, ETH Zurich, Switzerland. Type I collagen bovine membranes (100 μm of thickness) were supplied from Laboratorio Celina (Buenos Aires, Argentina). Tetracycline hydrochloride in powder form (Sigma-Aldrich, ST. Louis, MO) was used as received. Hank′s balanced saline solution (HBSS) without Ca^{2+} and Mg^{+} was obtained from Life Technologies (Carlsbad, CA).

Tissue culture materials were purchased from Corning (Princeton, NJ), Dulbecco′s Modified Eagles Medium (DMEM), TrypLE™ from Gibco (Gaithersburg, MD), and foetal bovine serum (FBS) from Internegocios SA (Argentina). MTT salt was purchased from Sigma Chemical Co (ST. Louis, MO).

Preparation of composites

A total of 100 mg of 45S5 n-BG was employed to coat type I collagen bovine membranes (9×9 cm^2). Each time, 10 mg of 45S5 n-BG was sonicated for 5 min in 20 ml of isopropanol, and then, aliquots of 3 ml were used to coat the membranes and left to dry in air at room temperature. At the end, samples of 3×3 cm^2 were obtained.

Tetracycline incorporation

Initially, a calibration curve was performed by measuring UV absorbance of tetracycline hydrochloride (THC) solutions ranging from 0·01 to 0·1 mg ml^{-1} at 350 nm (UV-Vis, Varian 500SCAN) (Dashti *et al.* 2010).

The incorporation of TCH was performed at 37°C in simulated body fluid (SBF), which was prepared following the Kokubo protocol (Kokubo and Takadama 2006). The initial antibiotic concentrations were 0·05, 0·20 and 0·35 mg ml^{-1}. Coated n-BG membrane samples were incubated for 48 h in 30 ml of SBF supplemented with the above concentrations of TCH. In that way, the antibiotic could co-precipitate and bond to 45S5 n-BG particles. At different times, the absorbance of aliquots of TCH-SBF solution was measured. The initial volume of the solution (30 ml) was maintained by replacing the extracted amount from a solution made at the start of the experiments and maintained in similar conditions. At the end of the uptake, the samples, now on TCH composites, were washed in double-distilled water and allowed to dry at room temperature. The concentrations of TCH incorporated were calculated by subtracting the remaining TCH concentration at the different time points from the initial concentration of the immersion solutions.

The composites obtained using the above coating processes were named as: TCH-005, TCH-020 and TCH-035, according to the initial concentration of the antibacterial agent in SBF.

Release of tetracycline

The release evaluation was performed in bi-distilled water to avoid any influence on the release kinetic both from the environment and from the from further going of the bioactivity process that might occur on the material surface of the bioactive glass (Miola *et al.* 2012).

Tetracycline hydrochloride composites were soaked in 30 ml of bi-distilled water at 37°C in static conditions for 72 h. TCH release was calculated by measuring the UV absorbance of 4 ml extracted at regular time intervals, which were then replaced back into solution. Tests were performed in triplicate, calculating the averages and standard deviations.

Surface analysis of the samples

The materials obtained were morphological characterized by scanning electron microscopy (SEM). For this, biomaterials were fixed with a 2·5% glutaraldehyde 0·1 mol l^{-1} PBS solution overnight at 4°C. The samples were then washed with distilled water and sequentially dehydrated through a graded series of ethanol solutions. After mounting on stubs and gold sputtering, the samples were examined by scanning electron microscopy (JSM 6480 LV, JEOL Ltd, Tokyo, Japan).

Bacterial cultivation and preparation of inoculum

The following strains were used in this study: *Staph. aureus* ATCC29213, *Staph. aureus* ATCC25923 and *Staph. aureus* ATCC6538P. *S. epidemidis* ATCC12228 was used as negative control as this strain has shown resistance to TCH (Zhang *et al.* 2003). All strains were grown for 24 h in Mueller-Hinton broth (Britania S.A., Buenos Aires, Argentina) at 37°C. For the experiments, bacterial cells suspensions were adjusted to 6–7 log CFU $ml^{-1.}$

Antibacterial efficacy

For antibacterial activity tests, composites samples in disc form of 5 mm in diameter were obtained with a paper punch circle (area = 0·20 cm^2). The performance of samples was evaluated in two ways: inhibition zone evaluation and plate count method.

For inhibition zone evaluation, 100 μl of the described suspension was seeded on Mueller-Hinton agar plates on which samples have then been placed with the coated and treated surface in contact with the plate. As control, TCH unloaded discs were used.

After 24 h incubation, inhibition zones were observed as a halo around samples where bacteria have not grown. Inhibition zones' area was measured in millimeters (mm). All tests were performed in duplicate, and the average and standard deviation were calculated.

For plate count method, the experiments were carried out in Hank's balanced saline solution (HBSS) without Ca^{2+} and Mg^{+}. The TCH composites samples were incubated for 48 h at 37°C in 1 ml of cellular suspensions. Each staphylococci suspension in absence of biomaterial served as controls. Samples were collected after 24 and 48 h of incubation, and the viability of cells at 37°C was assessed by counting in Mueller-Hinton agar plates. At the end of each period, the pH value of the culture was determined with pH indicator paper given the relevance of pH value, as indicated by previous results obtained on 45S5/agar-gelatin films (Rivadeneira *et al.* 2013). The results were expressed as $\log_{10}$ CFU $ml^{-1} \pm$ SD. All tests were performed in triplicate, and the average and standard deviation were calculated.

MG-63 osteoblast-like cell culture and incubations

MG-63 human osteosarcoma cells were grown in DMEM containing 10% FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37°C in 5% CO_2 atmosphere. Cells were seeded in a 75 cm^2 flask, and when 70–80% of confluence was reached, cells were subcultured using 1 ml of TrypLE™ per 25 cm^2 flask. Cells were counted in an improved Neubauer haemocytometer, and viability was determined by the exclusion Trypan Blue (Sigma, St. Louis, MO) method; in all cases, viability was higher than 90%. For experiments, cells were grown in multiwell plates. When cells reached the desired confluence, the monolayers were washed with DMEM and were incubated under different conditions according to the experiments.

Antibiotic extracts

Antibiotic extracts were obtained by immersing previously sterilized TCH composites disc of 0·20 cm^2 into 1 ml of complete culture medium at 37°C for different periods: 3, 6 and 24 h. The sterilization process was carried out by placing the disc under UV light for 20 min each side. It has been previously demonstrated that degradation of tetracycline hydrochloride in aqueous solution is independent of light, but is accelerated by the temperature increase. However, the degradation after 24 h incubation at 37°C is <80% (Wu and Fassihi 2005). An aliquot of the complete culture medium without any

disc was incubated under the same conditions and used as control.

Cytotoxicity of TCH composites on MG-63 osteoblast-like cells

Cell viability was evaluated by the conversion of the tetrazolium salt MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) to a coloured formazan by mitochondrial dehydrogenases. The MTT assay was carried out following a report previously published (Mosmann 1983). Briefly, $2{\cdot}5 \times 10^4$ cells per well were seeded in a 96-multiwell dish, and they were allowed to attach for 24 h. Afterwards, the culture medium was removed, and the cells were treated for 24 h with the different extracts obtained as described above. Following exposure, medium was changed and cells were incubated with 0·5 mg ml^{-1} MTT under normal culture conditions for 3 h. Cells were lysed in DMSO (100 μl per well), and colour development was measured spectrophotometrically in a Microplate Reader (7530, Cambridge Technology, Inc., Karlstad, Sweden) at 570 nm. Cell viability is shown graphically as the percentage of the control value (assuming data obtained from the untreated cells as 100%).

Results

Surface analysis of samples

Figure 1 shows SEM micrographs of the surfaces: uncoated Type I collagen membrane (a); glass-coated collagen membrane (b); a glass-coated collagen membrane exposed to SBF for 48 h (c); and a glass-coated collagen membrane exposed to SBF containing tetracyline for 48 h (d).

As shown in the SEM micrographs, a continuous grained coating was created on samples. Pores were created as a consequence of the irregular surface coating. In the case of composite plus 0·05, 0·25, and 0·35 mg ml^{-1} concentrations of TCH (b–d), after soaking in SBF for 2 days, the morphology of the surfaces reflects some modification probably because of calcium and phosphate deposition (Marelli *et al.* 2010).

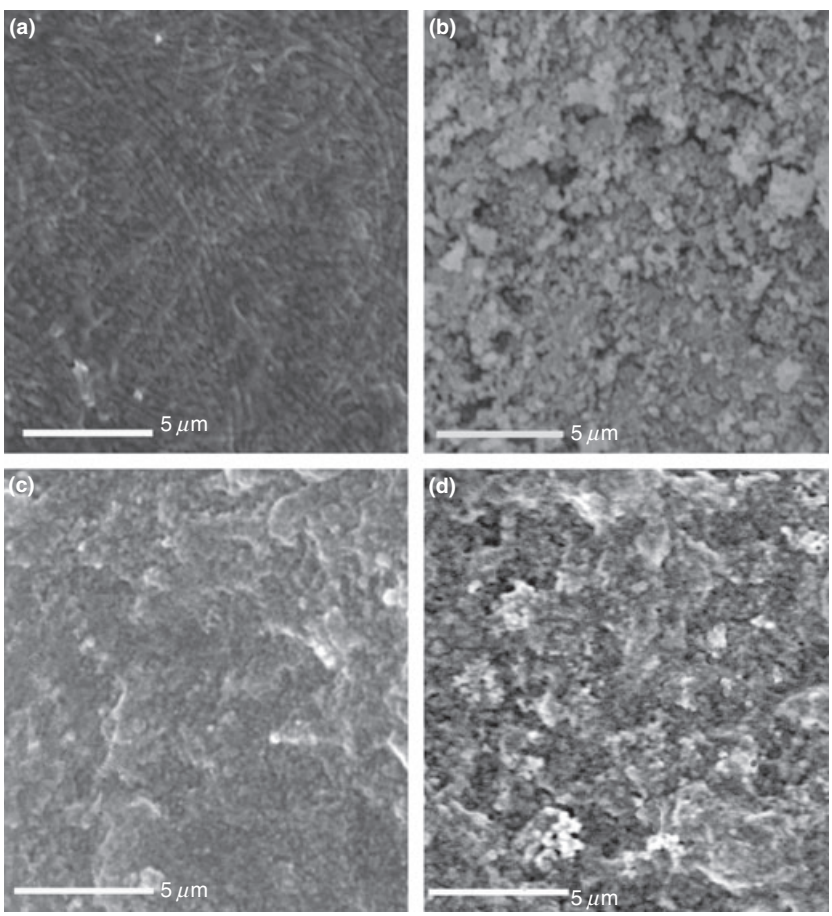

Figure 1 Scanning electron microscopy (SEM) photographs of samples: uncoated Type I collagen membrane (a), glass-coated collagen membrane (b), glass-coated collagen membrane exposed to simulated body fluid (SBF) for 48 h (c), glass-coated collagen membrane exposed to SBF containing tetracyline for 48 h (d).

TCH incorporation and release

Tetracycline hydrochloride incorporation values for composites are shown in Fig. 2. The experiment was performed as a function of time up to 48 h. This time was chosen as the cut-off because the majority of uptake took place within this period. As can been seen in Fig. 2, the antibiotic incorporation on the TCH composites was strongly dependent on the initial concentration of TCH and time on SBF solution. At the end of the experiments, the total amount of TCH incorporated on composites cm^{-2} was $0{\cdot}110 \pm 0{\cdot}007$, $0{\cdot}314 \pm 0{\cdot}012$ and $0{\cdot}628 \pm 0{\cdot}009$ mg for TCH-005, TCH-020 and TCH-035, respectively.

Figure 3 shows the release of TCH from composites as function of time. The TCH release behaviour was similar for all concentrations. The extent of release was proportional to the concentrations. The majority of release took place during the first 24 h. Within the first 6 h, for example, more than half of the concentration of TCH was released. After 1 day, the amounts of TCH become constant or gradually decreased. Taking the time of maximum release (24 h), the final amounts of released TCH were 0·060, 0·083 and 0·100 mg cm^{-2} for TCH-005, TCH-020 and TCH-0035, respectively.

Antimicrobial efficacy of TCH composites

Table 1 summarizes the results obtained by inhibition zone evaluation. As shown, TCH-005, TCH-020 and TCH-035 did inhibit the growth of *Staph. aureus* strains. As expected, no inhibition zone was detected for the tetracycline-resistant strain *Staph. epidermidis* ATCC12 228. The control composites showed no antibacterial effect and therefore no inhibition zone. The area around the coated discs increased in size with increasing the concentration of TCH.

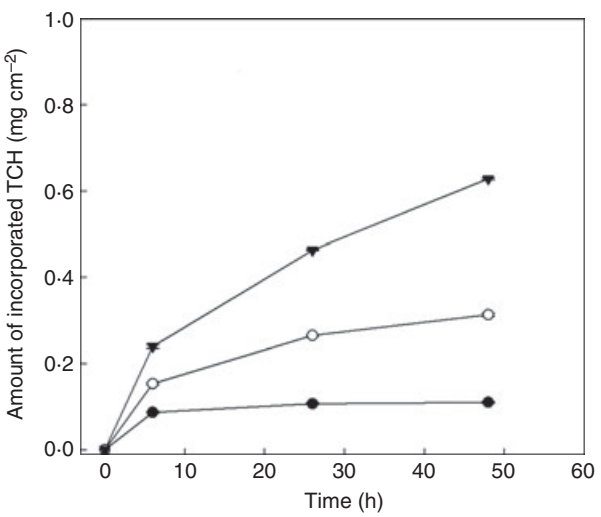

Figure 2 Tetracycline hydrochloride (TCH) incorporation on silicate bioactive glass nanoparticles/collagen composites as a function of time in simulated body fluid (SBF) aqueous solutions: (●) 0·05; (○) 0·20 and (▼) 0·35 mg ml^{-1} concentrations.

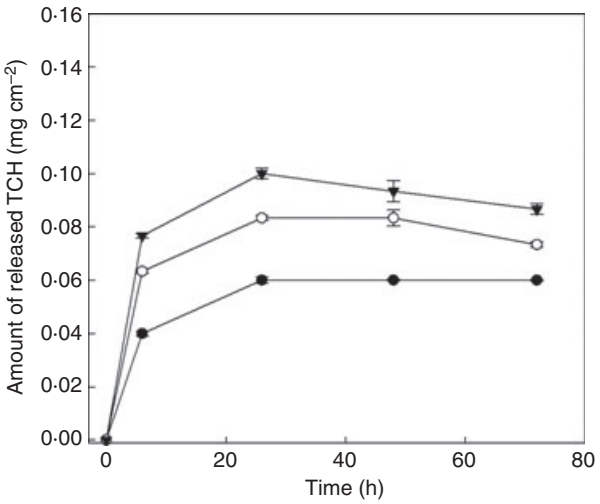

Figure 3 Tetracycline hydrochloride (TCH) release profile from TCH composites as a function of time: (●) TCH-005; (○) TCH-020 and (▼) TCH-035.

Figure 4 (a–d) shows the viability of staphylococci cells in the presence of the TCH composites as a function of time. The behaviour of the strains in presence of TCH composites was similar. The cell viability was significantly inhibited ($P < 0{\cdot}05$) on the three *Staph. aureus* strains in comparison with the control after 24 h of incubation. A prolonged incubation period, up to 48 h, did not increase the reduction in cell growth. Interestingly, the efficacy of composites was not found to be dependent on the TCH concentration, as no statistically relevant differences were found between TCH-005, TCH-020 and TCH-035. The aqueous pH values did not show variations. In addition, no large differences in inhibition strength between the *Staph. aureus* strains were observed after 24 or 48 h of incubation period.

Cytotoxicity effect of TCH composites on osteoblast-like cell

The results obtained from MTT assay showed that there was no significant effect on viability of MG-63 cells in the presence of TCH solutions obtained from TCH composites samples over the studied incubation periods.

Discussion

The strategy of producing biomaterials with an antibiotic delivery function has been studied from the early 50 s

Table 1 Inhibition zones (in mm) around tetracycline hydrochloride (TCH) composites (n = 2)

	Zone of inhibition (mm)			
THC composites	*Staphylococcus aureus* ATCC29213	*Staphylococcus aureus* ATCC25923	*Staphylococcus aureus* ATCC6538P	*Staphylococcus epidermidis* ATCC12228
Control	–	–	–	–
TCH-005	7·50 ± 0·70	8 ± 1·41	6	–
TCH-020	11	10·5 ± 0·70	8 ± 1·41	–
TCH-035	12·50 ± 0·70	14·5 ± 2·12	12 ± 1·41	–

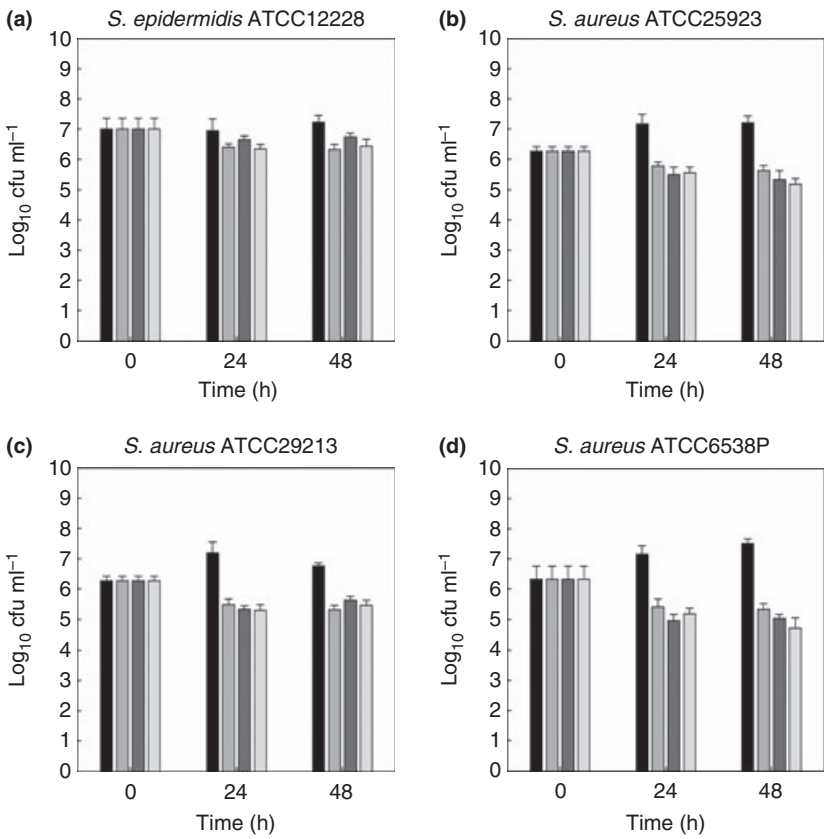

Figure 4 Viable counts of *Staphylococcus epidermidis* ATCC12228 (a), *Staphylococcus aureus* ATCC25923 (b), ATCC29213 (c) and ATCC6538P (d) in presence of composites: (■) control, (▢) TCH-005; (▩) TCH-020 and (▢) TCH-035.

(Colton and Ehrlich 1953). Since then, different classes of materials, bioinert, bioactive or biodegradable, have been investigated as carriers for local antibiotic delivery (Nandi *et al.* 2009; Cover *et al.* 2012; Giavaresi *et al.* 2012; Hum and Boccaccini 2012).

In this work, different concentrations of tetracycline hydrochloride were successfully incorporated onto silicate bioactive glass nanoparticles/collagen composites following a co-precipitation method. The TCH uptake over the time of incubation leads to consistent antibiotic incorporation. This was expected as the drug uptake is greatly influenced by the kinetics of the surface reactions of bioactive glass and the amount of THC incorporated on the composites surfaces is in line with the soaking time in SBF (Miola *et al.* 2012). The results showed that high concentrations of TCH in solution led to high quantities of the antibiotic in the coating of the TCH composites. The majority of the incorporation took place within the initial 48 h. This behaviour is in accordance with results of previous research (Stigter *et al.* 2002, 2004; Oyane *et al.* 2006; Dashti *et al.* 2010).

The release rate of the antibacterial agent from TCH composites also correlated well with the initial concentration of TCH. The TCH releasing effect was maintained at least for 72 h. Similar trend of release behaviour of TCH was observed for the different samples. In general, the release curves showed an initial burst effect followed by a slow release profile. The fast release is mainly caused by

the dissolution of the antibiotic located at the surface of the glass nanoparticles, and the slow release may be attributed to the chemically adsorbed drugs. This phenomenon is in agreement with other related system releasing TCH (Andrade *et al.* 2006, 2009; Zhao *et al.* 2008; Dashti *et al.* 2010).

In terms of efficacy, the percentage of TCH incorporated was higher when the initial TCH concentration in solution was the lowest. This is in contrast with the results of Dashti *et al.* (2010) who reported that the percentage of release of THC from an inorganic bovine-derived hydroxyapatite (Bio-Oss®; Geistlich Pharma AG, Wolhusen, Switzerland) correlates with the initial concentration. When comparing the release of different drugs from biomaterials, factors such as porosity of the carrier materials, amount of water, solubility of the drug among others determine the release rate (Stigter *et al.* 2002, 2004; Oyane *et al.* 2006).

The MIC for on *Staph. aureus* ATCC29213 and ATCC25923 is $\leq 0{\cdot}5$ μg ml^{-1} (http://www.eucast.org/antimicrobial_susceptibility_testing/qc_tables/; Novy *et al.* 2011). The levels of drug released achieved by TCH composites exceeded this concentration several fold. The inhibition halos observed around the *Staph. aureus* strains confirm that the quantity of TCH released from the composites exceeded the MIC for the drug. The plate count method also demonstrated a significant reduction in the viable count of *Staph. aureus* strains when exposed to TCH composites up to 48 h. In addition, the reduction in cell growth was not time or dose dependent. This behaviour is in line with Dashti *et al.* (2010) who reported no statistical differences in *Staph. aureus* NCTC6571 cell growth inhibition when TCH concentrations were in the range of 0·015–0·065 mg ml^{-1}. In a previous research (Rivadeneira *et al.* 2013), we could determine that 45S5 Bioglass®/agar-gelatin composite films were able to inhibit the cell growth of the four staphylococci strains studied in this work. This effect was correlated with an alkalinization of the media as it was reported by previous research (Allan *et al.* 2001; Waltimo *et al.* 2007; Hu *et al.* 2009). In this work, the pH value was not alkalinized in presence of TCH composites; therefore, the antimicrobial effects are related only with TCH. On the other hand, the results showed that there was not a significant reduction in the cell viability of MG-63 human osteosarcoma cell line in the presence of different TCH extracts obtained from TCH composites.

Previous research work carried out *in vitro* has shown that tetracycline derivatives induce apoptosis selectively in cultured monocytes and macrophages but not in mesenchymal cells including MG-63 in range of concentrations between zero and 0·05 mg ml^{-1} in both serum-containing and serum-free culture conditions (Bettany and Wolowacz 1998). Duewelhenke *et al.* (2007) showed that 0·4 mg ml^{-1} of TCH was necessary for 50% inhibition of proliferation of MG-63 cells. In the study carried out by Dashti *et al.* (2010), 0·01 mg ml^{-1} of TCH was well tolerated by HOS human osteosarcoma cell line. Higher concentrations (0·1 and 1 mg ml^{-1}) resulted in the inhibition of cell proliferation. In this work, the maximum concentration of TCH released was 0·03 mg ml^{-1} and that could explain the null cytotoxicity observed.

Taken an overview of the results, the composites may have a potential application as wound dressing. In an infection situation, a wound dressing must respond immediately to the presence of large number of bacteria. The results found in this work shows that the composites are able to inhibit cell concentration up 10^6 CFU ml^{-1} (Fig. 4). Moreover, the burst released observed within the first hours makes the composite compatible to manage large number of bacteria cells in a very short time. Silver ions, which are the most commonly used topical antimicrobial agent in burn wound care products, can cause lethal damage to both keratinocytes and fibroblasts (Poon and Burd 2004; Burd *et al.* 2007). Moreover, the concentrations of TCH released are out of the range of cytotoxicity effects reported in previous works. The amount of TCH released by TCH composites was several folds higher than the MICs required for TCH for the *Staph. aureus* strains evaluated. This is important since according to the literature (Campoccia *et al.* 2010), the antibiotic needs to be locally released at concentrations exceeding several times (usually 10 times) the minimum inhibitory concentration (MIC) for the concerned pathogen. Future investigations could support the potential of these composite as wound dressings.

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Conflict of interest

No conflict of interest declared.

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