Loading of gentamicin onto poly lactic-co-glycolic acid and poly lactic-co-glycolic acid/nano-hydroxyapatite composite microspheres.

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Introduction
Systemic antibiotic therapy has several complications [1] and exerts some adverse effects on several body organs mainly the kidneys, ears and liver. Therefore, finding a method for targeted local delivery of antibiotics can greatly decrease the undesired complications associated with their systemic use. Moreover, systemic use of antibiotics has small efficacy for dental infections unless they are administered in high doses [2]. Thus, local delivery of antibiotics to the desired site can enable greater efficacy at a significantly lower dose for treatment of dental infections. Advances in tissue engineering have paved the way for the use of different antibiotics loaded onto polymer microsphere carriers for dental purposes.

Background and aim: In dental treatments, use of antibiotic carriers can decrease microorganisms more efficiently. In this study, gentamicin (GEN) was loaded onto poly lactic-co-glycolic acid (PLGA) copolymers and PLGA-nano hydroxyapatite (nHA) composite microspheres for a controlled release. The amount of drug release was measured for 20 days.

Materials and methods: In this in-vitro study, microspheres were prepared using the water/oil/water technique and different concentration of GEN loaded onto PLGA copolymers and PLGA-nHA composite microspheres. Loaded microspheres were evaluated morphologically using scanning electron microscopy (SEM). The rate of drug release from the composite microspheres was measured in phosphate buffered saline (PBS) medium for a period of 20 days using UV spectrophotometry (330 nm). Data were analyzed using ANOVA. Also, a microbial culture was carried out of microspheres with the least amount of drug in the first and last day of drug release assessment.

Results: SEM images showed that the microspheres had a smooth surface and the pattern of drug release from the PLGA copolymers and PLGA-nHA composite microspheres loaded with GEN was different in each group at different time points, but this difference only in the PLGA+0.02 GEN group was significant. There was a significant difference between this group and other groups in the amount of released drug at day 6. Also, the results of microbial culture of the group with 0.02 GEN showed the antibacterial effect of these microspheres in the last day of experiment.

Conclusion: The release profiles observed in this study and the well-established biocompatibility of PLGA indicate that the composite microspheres used in this study are suitable for infection control purposes in dentistry.

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Keywords: Gentamicin, Drug delivery, Composite microspheres, Nano-Hydroxyapatite, Poly lactic-co-glycolic acid.
the exposed pulp is covered with a specific material allowing the pulp to form reparative dentin at the exposure site. Formation of a dentin barrier is often followed by dental pulp recess while the tooth remains vital. Calcium hydroxide is the standard pulp capping material for vital pulp treatment [3]. It possesses antibacterial effects and can induce the formation of a microscopic calcified barrier. However, this barrier cannot reinstate a permanent seal and can lead to bacterial leakage. Also, based on the literature, the success rate of vital pulp therapy is not acceptably [4,5] high in the primary teeth [6].

Nano-hydroxyapatite is a bioactive, biocompatible compound highly accepted for use in dental and medical science and has antibacterial effects [7]. Nano-hydroxyapatite is preferred to HA due to its greater surface area and higher solubility [8]. The biological properties of nHA are superior to those of HA. Combining nHA with biocompatible polymers prevents nano powder wash out [9,10].

The efficacy of amoxicillin, vancomycin, erythromycin and doxycycline against Enterococcus faecalis has been well investigated [11,12,13]. Use of systemic and local antibiotic therapy has long been accepted as a standard protocol in medicine and dentistry. In local use of antibiotics, their systemic side effects are prevented and higher therapeutic concentrations can be achieved [14]. Enterococcus faecalis is an anaerobic Gram-positive bacterium and a member of normal flora of the mouth. It is often found in small amounts in unprepared root canals. However, its exact role in the success of root canal treatment has yet to be clearly identified. It is the most commonly isolated bacterium from the root canal system. This microorganism can cause a treatment-resistant infection due to its ability to invade dentinal tubules, resistance against different ecological conditions of the canal and adaptation to unfavorable intracanal conditions [15].

Manufacturing dental cements, which contain biodegradable polymer microspheres for controlled release of antibiotics without affecting the mechanical properties of cements, is increasing [16,17]. In this method, drugs are loaded on the surface and inside synthetic microspheres of variable sizes. These microspheres are biocompatible and biodegradable and can be easily converted into three-dimensional matrices with diverse structures.

Biodegradable synthetic polymers include linear aliphatic polyesters, polyanhydrides and poly (ortho esters); among which, linear aliphatic polyesters namely polyactic acid, polyglycolic acid and their copolymers have extensive applications in local drug delivery systems [18]. Biological products such as glycolic and lactic acid monomeric units are naturally produced and then biodegraded in metabolic pathways of the human body. Their difference is in their structure and rate of degradability. Polyglycolic acid is degraded more rapidly than polyactic acid; however, the PLGA (50:50) has suitable properties for drug delivery. On the other hand, the behavior and the biodegradability of PLGA are controllable and these characteristics are the main advantages of this polymer and the reason for its vast application in medical and dental fields [19-21].

Use of biodegradable polymers as microparticles loaded with medications is a suitable alternative to some complex medical and dental procedures. These microspheres are mostly made of PLGA and are used for in-vitro proliferation of cells and are injected into the injured site for the repair of cartilaginous tissue [22-24].

Several pharmacological and orthopedic studies have investigated the production of PLGA microspheres and assessed the release of loaded antibiotics. In restorative dentistry, pulp-capping agents are used with the aim of eliminating microorganisms from the pulp chamber. Thus, it is particularly important to use antibiotic-loaded materials to minimize the microbial load as much as possible [25].

Our previous studies have been revealed that PLGA 50:50 microspheres are suitable carrier for loading of gentamicin antibiotic [26]. This study sought to assess the rate of release of gentamicin loaded onto PLGA and PLGA-nHA microspheres. In this study, we investigate the role of nano HA in composite microsphere and compared releasing profile in different concentration of gentamicin sulfate solution loaded in PLGA and composite PLGA/nano HA microspheres.

Materials and Methods

Materials

Poly (lactic-co-glycolic acid) and PBS were purchased from Sigma Company (Sigma, Missouri, United States). Gentamicin (GEN) was purchased from Sina Darou Company (Sina Darou, Karaj, Iran). Polyvinyl alcohol (PVA, 87-89% hydrolyzed, mol. wt. 31,000-50,000 g/mol) was purchased (Sigma, United States). Chloroform was purchased from Merck Company (Merck, Darmstadt, Germany). Nano-hydroxyapatite was prepared in the Materials and Energy Research Center.

Methods

This in-vitro experimental study evaluated the release of different concentrations of GEN from PLGA and PLGA-nHA microspheres. Non-probability convenience sampling was carried out.

Microsphere preparation

Water-in-oil-in-water (W/O/W) double emulsion/solvent evaporation technique was used for the preparation of microspheres. Briefly, 1 ml of 15% (w/v) PLGA in chloroform was added to 2 ml of internal water phase [PVA 2% (w/v)] containing different concentrations of GEN [0.1, 0.025 and 0.05% (w/v)] and different concentrations of nHA [0 and 25% (w/w) of polymer]. The mixture was homogenized for one minute at 10,000 rpm to achieve water/oil (W/O) emulsion. After that,
the W/O emulsion was poured into 30 ml of the second aqueous phase containing 0.2% (w/v) PVA (Sigma, PVA, 87-89% hydrolyzed, mol. wt. 31,000-50,000 g/mol) and mixed with stirrer (Heidolph, Schwa Bach, Germany) for two hours to prepare W/O/W emulsion. The microspheres were then collected and washed three times with distilled water by centrifugation (11000 rpm, 10 min) after final washing; the samples were collected and frozen at -15°C. Finally, the samples were lyophilized by freeze-dryer (Christ Alpha 1-2LD Plus, SY4 5NU, UK) for 48 hours at -15°C. Table 1 summarizes the mean concentration of drug released in different groups at different time points.

**Microsphere characterization**

**Scanning Electron Microscopy (SEM):** The size, morphology and microstructure of microspheres were analyzed using SEM (VEGA TESCAN-LMU, Kohoutovice, Czech Republic). Samples were fixed on an aluminum plate and gold coated prior to examination.

**Determination of gentamicin encapsulation efficiency:** To determine the loading efficiency of GEN, 15 mg of the drug-containing microspheres were poured into vials and 5% w/v SDS in 0.1 molar NaOH was added. The solution was mixed with stirrer in order for the microspheres to disintegrate and allow complete release of medication. After 12 hours, the mixture was centrifuged at 8000 rpm for 5 minutes for complete isolation of the remaining particles. The supernatant was collected. The amount of drug extracted from the microspheres was measured and compared with the baseline drug value before loading. The drug encapsulation efficiency was calculated using the following equation:

\[
\text{Encapsulation Efficiency (EE\%) = \left[\frac{\text{drug (encapsulate)}}{\text{drug (total)}}\right] \times 100}
\]

All the tests were performed in triplicate, and the results were reported as mean ± standard deviation (SD). Table 2.

**In-vitro drug release study:** For in-vitro drug release study, 14 mg of different samples were suspended in 3 ml of PBS (pH: 7.4). The plates were then placed in a shaker incubator (Behdad, Tehran, Iran) at 37°C rotating at 100 rpm. At pre-determined times; the PBS was completely extracted and replaced with 3 ml of fresh PBS. The amount of GEN released into the PBS was quantified by UV-V at 330 nm and compared with a standard calibration curve. All drug release studies were carried out three times.

**Bacterial culture:** A 24-hour Staphylococcus aureus culture was prepared in brain heart infusion (BHI) agar at 37°C. Twenty-four hour cultured single colonies were removed and inoculated in saline solution to prepare a 0.5 McFarland standard bacterial suspension. The suspension was streak cultured on BHI agar plate. Using a Pasteur pipette, 100 γ of the control, PLGA+0.02 GEN and PLGA/nHA+0.02 GEN solutions were added to the wells. The plates were then incubated at 37°C. After 24 hours, the plates were evaluated for the formation of growth inhibition zones. Next, another 24-hour culture of Staphylococcus aureus was grown and 0.05 McFarland standard microbial suspensions was prepared. Then, 100 γ of the broth medium was added to each well of the 96-well plate. Then, 100 γ of the highest concentration was added to the first well containing broth medium. Next, 100 γ was removed from the first well and added to the second one and so on; the final 100 γ was removed from the last well and discarded. Serial dilution was carried out as such. Next, 100 γ of the 0.5 McFarland standard microbial suspension was added to each well and then the plate was incubated at 37°C in an incubator (Memmert, Schwa Bach, Germany). After 24 hours, the turbidity was assessed to determine minimum inhibitory concentration. In the two experimental groups of PLGA+0.02GEN and PLGA/nHA+0.02GEN that contained the least amount of GEN, microbial cultures were prepared at the first and last day. The growth inhibition zone of *S. aureus* was

<table>
<thead>
<tr>
<th>Name</th>
<th>Gen concentration (% w/v)</th>
<th>PLGA concentration (% w/v)</th>
<th>nHA concentration (% w/w to polymer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS1</td>
<td>0.1</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>MS2</td>
<td>0.025</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>MS3</td>
<td>0.05</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>MS4</td>
<td>0.1</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>MS5</td>
<td>0.025</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>MS6</td>
<td>0.05</td>
<td>15</td>
<td>25</td>
</tr>
</tbody>
</table>

**Table 1:** Classification of microspheres at different concentration of Drug, Polymer and Ceramic.

<table>
<thead>
<tr>
<th>Gen concentration (% w/v)</th>
<th>PLGA concentration (% w/v)</th>
<th>nHA concentration (% w/w to polymer)</th>
<th>EE% ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>15</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>0.025</td>
<td>15</td>
<td>0</td>
<td>50 ± 2.3</td>
</tr>
<tr>
<td>0.05</td>
<td>15</td>
<td>0</td>
<td>44 ± 1.8</td>
</tr>
<tr>
<td>0.1</td>
<td>15</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>0.025</td>
<td>15</td>
<td>25</td>
<td>30 ± 2.7</td>
</tr>
<tr>
<td>0.05</td>
<td>15</td>
<td>25</td>
<td>20 ± 1.9</td>
</tr>
</tbody>
</table>

**Table 2:** Encapsulation Efficiency percent of different samples.
evaluated.

**Statistical Analysis**

Data were analyzed using SPSS 18. The mean, SD, minimum and maximum concentration of the released drug in different groups at different time points were calculated. The data were analyzed using one-way and two-way ANOVA. Type 1 error was considered as 0.05 and P<0.05 was considered statistically significant.

**Results**

Figures 1 and 2 show SEM images. GEN-loaded PLGA microspheres had a smooth surface without any pores or breakage. The SEM images showed that the size of microspheres containing GEN was approximately 30 microns (values represent mean ± SD).

The mean concentration of the released drug in different samples was plotted as a function of time. Figures 3 and 4 shows the release of GEN from microspheres over 20 days. These figures showed a constant and controlled release profile. Although the pattern of release was different at different time points, overall, the pattern of release was not significantly different among groups and the effects of drug concentration on the amount of released drug were not significant (P=0.26).

One-way ANOVA demonstrated that no significant difference existed in the concentration of released drug at different time points except for day 6, which revealed a significant difference in the concentration of the released drug (P<0.003). On day 6, burst release occurred in the PLGA+0.02 GEN; which was significantly different from the pattern of release in other groups. Drug release in different groups and at different time points is demonstrated in Figure 5.

Since the plasma concentration of drug after systemic use was 24 μg/ml, the mean concentration at day one in the 0.02 GEN group was around 10^{-7} g/ml; which was significantly lower than 24 μg/ml. Thus, no growth inhibition zone was observed in the culture medium. On the last day, this rate reached 10^{-5} g/ml, which was within the effective dose and a growth inhibition zone measuring 16-18 mm was observed. It should be noted that these values are calculated based on the mean release of drug in the same day. If the effective dosage under in-vivo conditions is considered, drug shows a cumulative effect and the drug value from day 6 on will be in the effective therapeutic range. In PLGA-nHA+0.02 GEN group, the mean release on day one was in the range of 10^{-10} and from day 6 on, it reached the effective dose of 10^{-6} g/ml. Thus, the effective dose is released from day 6 on due to the cumulative effect. In PLGA-nHA+ 0.05 GEN, the release was slow until day 8 but from day 10 on, the speed of release accelerated and reached the effective dosage for inhibiting bacterial growth (about 10^{-6}).

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**Figure 1:** SEM micrograph of PLGA microspheres without gentamicin (a), 0.1 g/ml gentamicin (b), and 0.025 g/ml gentamicin (c), 0.05 g/ml gentamicin (d).
Discussion

The current study assessed the GEN release profile when different combinations of drug and PLGA and nHA were used. The results showed that HA had no negative effect on the drug release rate and therefore, it can be used to help pulp regeneration. Moreover, powders containing polymer and antibiotic microspheres may be mixed with mineral trioxide aggregate for efficient use in direct pulp capping and at the site of pulp exposure. Based on the results of the current study, daily drug release profile was variable in different groups but the overall release profile of the groups was similar. However, the release rate was the highest in PLGA+0.02GEN group on day 6, which may be due to the homogenous and uniform distribution of microspheres in this group compared to others. The difference in release rate was not significant on the final day of experiment among groups.

It is believed that nHA has special properties due to its small particle size and huge specific surface area. A significant increase in protein adsorption and osteoblast adhesion to nano-sized ceramic materials was reported by Webster et al [27,28]. SEM images in our study showed that microspheres were 30 μm and had a smooth surface. This result is in agreement with the findings of Sivakumar et al., (2002) who created 16 μm microspheres [29]. However, the difference was in the surface characteristics of microspheres because in our study microspheres had a smooth surface but in their study microspheres had a porous surface. The more porous the surface, the better the results.

Increasing the volume of secondary aqueous phase resulted in higher encapsulation efficiency. As the size of microspheres increases their degradation rate can be more easily controlled; but in our study, the smaller size of microspheres resulted in lower encapsulation efficiency. Also, in our study, different groups had the same drug release profile because the drug was loaded onto homogenous microspheres with almost equal sizes.

The group containing 0.02 GEN, had more microspheres with smaller size and more uniform distribution.

Smaller microspheres have a biphasic release profile with a quick second phase. The three-phasic release profile mostly occurs in larger microspheres; which is the result of heterogeneous destruction. If the particles are of various sizes, the release profile will be single phase. In three-phasic release profiles, the first phase usually includes an initial burst causing the release of non-encapsulated drug molecules or those over the surface of microspheres. The release is followed by small water crack formation and primary degradation of microspheres may also be responsible for the initial burst [30]. However, we did not observe this on SEM images in our study. The second phase includes slow release of the drug. At the same time, polymer destruction continues along with

Figure 2: SEM micrograph of PLGA- nano microspheres without gentamicin (a), 0.05 g/ml gentamicin (b), 0.025 g/ml gentamicin (c).
Loading of Gentamicin in PLGA and PLGA/nano HA microspheres

Water absorption. The third phase is faster and occurs as the result of polymer mass loss due to the destruction of PLGA. The third phase is sometimes called the second burst [31].

Vitro et al. (2007) [32] showed that using ultrasonic method for preparation of microspheres not only increased encapsulation efficiency in size of 20-40 µm but also improved the encapsulation efficiency of GEN to almost 100%; but in our study the ultrasound method was not employed and thus, the microsphere size and the encapsulation efficiency of GEN were lower. In future studies, ultrasonic method can be used to improve encapsulation efficiency.

Sousa et al. loaded PLGA microspheres with amoxicillin [33]. In their study, microparticles measuring 5-38 µm were prepared using a spray-drying technique and different drug-release patterns were observed. Drug composition played a significant role in the controlled release profile. The antibacterial activity of amoxicillin continued even after its encapsulation. As demonstrated by antibiogram results, amoxicillin had antimicrobial effects for 6 hours. Spongy particles in contact with water enhance complete elimination of the formulation from the simulated root canal system. However, this study, similar to the previously discussed ones, was conducted under invitro conditions; which has significant differences with the clinical setting.

**Figure 3:** Drug release curve for gentamicin loaded in PLGA microspheres (a: 0.1 g/ml; b: 0.02 g/ml; c: 0.05 g/ml) at different time points.

**Figure 4:** Drug release curve for gentamicin loaded in nano HA-PLGA microspheres (a: 0.02 g/ml; b: 0.05 g/ml) at different time points.
In addition, the results of the bacterial culture showed that GEN concentration was 24 µg/ml after systemic use. The mean GEN concentration in PLGA+0.02 GEN sample was 7-10 µg/ml on the first day and the inhibition zone was not observed. On the last day, GEN concentration in this sample reached 5-10 µg/ml, which was within the effective range and the inhibition zone was calculated at the range of 16-18 mm. The GEN release from PLGA-nHA+0.02 GEN sample was 10 µg/ml on the first day and it was within the effective range on the eighth day (10⁻⁶ µg/ml). Until the sixth day, the PLGA-nHA+0.05 GEN sample showed prolonged release. Thus, the samples containing nHA have more controlled release profile than pure PLGA microspheres.

PLGA +0.02% GEN showed the highest rate of delivery and burst release on the sixth day, which had significant differences with other groups. This finding can be explained by the fact that less amount of loaded drug causes faster release in comparison to loading 0.05% and 0.1% GEN. According to a study by Imbuluzqueta et al., in 2011 [34], the higher the amount of the drug, the more the interaction with the microspheres and the slower the release profile. In the study of Imbuluzqueta et al, the efficiency of nanoparticles loaded with GEN was approximately 100% and the sustained release was achieved for up to 70 days. This difference is due to the GEN coupling with the anionic AOT salt [(salt-2) BISsulfosuccinate sodium (ethylhexyl)] and the formation of GS-AOT hydrophobic complex.

In our study, double emulsion method was used for drug preparation. However, Prior et al. (2000) [35] prepared GEN particles using the spray drying method. Although they showed continuous burst of drug, it caused agglomeration of particles compromising drug release. Our study showed that by increasing the concentration of GEN, drug loading into the microspheres decreased; this finding is in accord with the results of a previous study by Blanco-Prieto et al., in 2002 [36].

Schneiders et al., in 2006 (Schneiders et al., 2006) evaluated the characteristics of composite microspheres (calcium phosphate PLGA) and observed no reduction in mechanical properties of these cements. Drug loading was also successful. Similarly, nHA and PLGA were mixed; this mixture had no adverse effect on the properties of the two materials, and addition of nHA increased the molecular weight of the composite and better controlled the drug release. It is quite clear that microspheres containing nHA have a slow release profile.

Studies have shown that different formulations, the speed of mixing, chemical composition, surface activator, viscosity of the polymer solution and the volumetric ratio of aqueous phase to organic phase can affect the characteristics and properties of polymer microspheres. Our results showed that double emulsion method was suitable for preparation of PLGA microspheres containing GEN. Also, the release profile showed that GEN molecules were released from the PLGA microspheres via a controlled mechanism by penetration and destruction of polymer in 3 phases: 1. Controlled release via penetration mechanism, 2. Penetration mechanism and simultaneous degradation causing initial burst, and 3. Delayed, controlled and slow drug release. PLGA microspheres have slow, continuous release profile; which is the reason for the popularity of this system. This study appears that PLGA+GEN and PLGA-nHA+GEN may also be mixed with MTA and placed over the exposed site to increase the success rate of pulp capping. Habruken et al, in 2010 confirmed these
findings as well [37].

Future studies are required to assess drug release in the clinical setting. Also, the effect of adding more hydrophilic surfactants on the drug release profile must be evaluated. The release profile of amoxicillin and other antibiotics must be evaluated as well. Assessment of the physical properties of cements containing drug-loaded microspheres would also be an interesting research topic.

Conclusion

In loading GEN onto PLGA and PLGA-nHA microspheres, no difference was noted in the daily release pattern of drug in groups with different concentrations of drug. On day 6, burst release of drug occurred in the PLGA+0.02% GEN group, which was significantly different from other groups. GEN can be loaded onto PLGA-nHA composite microspheres for use in restorative treatments like direct pulp capping.

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