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RESEARCH PAPER

Chitosan Film Containing Poly(D,L-Lactic-Co-Glycolic Acid) Nanoparticles: A Platform for Localized Dual-Drug Release

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ABSTRACT

Purpose To characterize and evaluate chitosan film containing PLGA nanoparticles (NPs) as a platform for localized dual-drug release.

Methods Fluorescent Paclitaxel (FPTX), a hydrophobic drug, was incorporated into PLGA NPs. FPTX-loaded PLGA NPs and Carboxyfluorescein (CF), a hydrophilic model drug, were embedded into chitosan films. Release of CF and NPs from chitosan and release of FPTX from PLGA NPs were monitored by fluorescence. The stability of the platform was observed through SEM and dynamic light scattering (DLS).

Results Chitosan films containing CF and FPTX-loaded PLGA NPs showed a biphasic release profile. In the first phase, 78% of CF and 34% of NPs were released within few days. In the second phase, the release was slower, showing an additional release of 22% of CF and 18% of NPs after 3 weeks. SEM images and DLS measurements showed that NP release depends on film degradation rate. FPTX-loaded PLGA NPs showed the release of 19.8% of total drug in 2 days, and no additional release was detected in the next 26 days.

Conclusions The ability of chitosan film containing PLGA NPs to coat gold surface and to incorporate and release two different drugs of different hydrophilicity make it a promising platform for localized dual-drug release.

KEY WORDS chitosan · PLGA · paclitaxel · drug release · implants coating

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INTRODUCTION

Chitosan, a cationic bio-polymer has been widely studied for biomedical application especially due to its biodegradability and biocompatibility. A notable advantage of chitosan is its film-forming capability through solution casting technique. Many studies on drug loading, release and permeation have been performed through chitosan films (1–4). Poly(D,L-lactic-co-glycolic acid) (PLGA) is another biodegradable polymer that has been widely used as nano/microparticles for encapsulation of drugs such as Paclitaxel (5–9). Paclitaxel is one of the most important chemotherapeutic drugs whose fast clearance from the body has hampered its widespread application. The incorporation of paclitaxel in PLGA NPs has been reported as a strategy for increasing its resident time in the tumor and reducing systemic side effects (5–9).

The polymeric coating of biomedical implants like stents, tumor markers, catheters and pacemakers has emerged as a strategy to avoid rejection of these materials that leads to serious inflammation process (10–12). In addition to increased biocompatibility, polymeric coatings also provide the possibility to be loaded with drugs and locally release them, improving treatment efficacy and implant integration (12–14).

To be useful as drug-release coating, the polymer has to present high drug loading and release capacity as well as

high stability when adsorbed on implants. Drug loading and release depend, among other factors, on the interaction between drug and polymer (14). Normally, if they have similar hydrophilicity, they present excellent affinity. Considering that the simultaneous application of two anticancer drugs has shown synergistic effects on cellular death, resulting in higher efficiency of cancer treatment (15–17), platforms that have the ability to deliver multiple therapeutics are quite attractive. However, the simultaneous delivery of drugs with different hydrophilicity is a challenging task due to the non-availability of a single platform that can be not only loaded with hydrophobic and hydrophilic drugs but also have control of their subsequent release.

This work was motivated by the need to create multifunctionality in routinely used implantable devices that improve spatial accuracy in modern Image-Guided Radiation Therapy (IGRT) of prostate, lung, pancreatic and breast cancer. We considered the commonly used gold fiducial that provides unique functionality by enabling tumor tracking to compensate for patient movement (18–20) and potential reduction of the total irradiated volume. Developing gold fiducials that can release radio-sensitizers or cytotoxic compounds over time, similar to drug-eluting stents used in cardiology practice (12–14), would allow fiducials to act as dual-functioning devices, providing both image guidance and localized drug delivery. Such an approach is now feasible using new methods of metallic surface preparation (21–23) developed in the field of nanotechnology and nanomedicine. The potential coating of these fiducials with cytotoxic drugs or radiosensitizers may further enhance the biological effect of radiation therapy (24). Accordingly, the routine introduction of these implantable devices in tumors (e.g. prostate cancer) provides opportunities for combining in-situ delivery of radiosensitization with IGRT.

Here we present chitosan film containing PLGA nanoparticles as a dual drug-release platform for coating gold implants (Fig. 1). We show that chitosan can be loaded with the hydrophilic model drug, 6-Carboxyfluorescein (CF), while PLGA NPs can be loaded with fluorescent paclitaxel (FPTX), a hydrophobic drug. The release profile of CF and PLGA NPs from chitosan film as well as the release of FPTX from PLGA NPs were obtained. The use of the present platform for coating implants was evaluated by analyzing the stability of coated gold slides through studies in buffer solutions.

MATERIALS AND METHODS

Materials

Sodium hydroxide, Hepes, Poly(DL-lactide-co-glycolide) (PLGA; L/G 50/50,MW 40,000–75,000), polyvinyl alcohol (PVA, MW 30,000–70,000), chitosan (MW 40,000 g/mol, deacetylation degree 80%), 6-Carboxyfluorescein (CF), acetic acid, acetic anhydride, methanol and dichloromethane (DCM) were acquired from Sigma-Aldrich. Fluorescent paclitaxel (FPTX) was obtained from Invitrogen. Gold slides 0.5 cm × 1.0 cm were prepared by sputter-coating glass slides with gold.

Methods

Synthesis of Fluorescent Paclitaxel-Loaded Nanoparticles

The PLGA nanoparticles (NPs) containing fluorescent paclitaxel were synthesized by oil-in-water emulsification-solvent evaporation method (8). Initially, 30 μg of FPTX were added to 4 mL of PLGA 2.5% (w/v) solution in DCM. The resulting organic phase was poured into 20 mL of aqueous solution of PVA 1% (w/v) under stirring. The emulsion was stirred at room temperature to evaporate DCM for 6 h. NPs were collected by centrifugation (10,000 rpm, 15 min, 16°C). After washing four times with distilled water, the NPs were suspended in 6 mL of distilled water. Three samples of 100 μL of NP suspension were placed into calibrated vials, dried and weighted. NP concentration in the final suspension was determined as 12 mg NPs/mL.

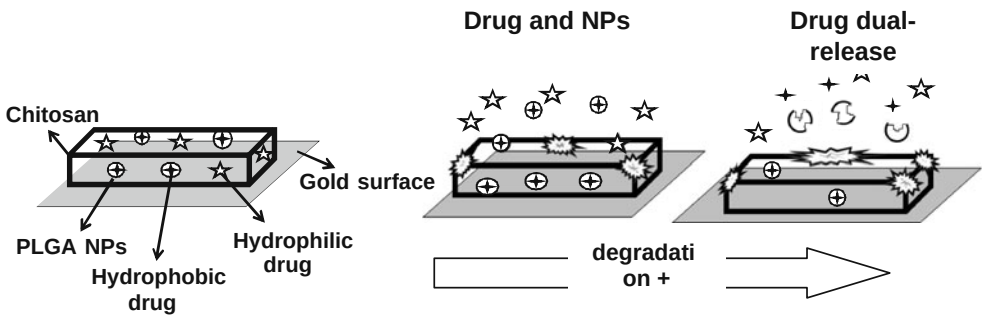

Fig. 1 Representation of drug dual-release platform for hydrophilic and hydrophobic drugs. Release of drugs (✦;★) was measured by fluorescence, and release of drug-loaded NPs (⊕) from film was measured by DLS.

Preparation of Chitosan Films Containing FPTX-Loaded NPs

Four mL of the final NP suspension were centrifuged (10,000 rpm, 15 min, 16°C) and redispersed in 1 mL of acetic acid 2%. The suspension was added to 2 mL of chitosan solution (30 mg/mL in acetic acid 2%). The gel was sonicated for 30 min in order to disperse NPs. One-hundred μL of gel were applied on gold surface and dried under room temperature and atmospheric pressure overnight. The samples were further dried in vacuum for 3 h. In order to control the solubility and biodegradation (2,3), the samples were acetylated through the following steps: a) immersion in sodium hydroxide 1 M, b) wash with methanol, c) immersion in acetic anhydride 1 M, d) wash with methanol. The samples were dried in vacuum and incubated in Hepes buffer solution (pH = 6.0) at 37°C for drug release measurements.

Chitosan Films Containing Carboxyfluorescein (CF)

One mL of a CF solution (1 mg/mL) was added to 10 mL of chitosan solution (30 mg/mL in acetic acid 2%) and mixed under sonication. One-hundred μL of the gel were applied on gold surface and dried under room temperature and atmospheric pressure. For the acetylation, the same procedure described for chitosan films containing NPs was followed.

Characterization of FPTX-PLGA-Loaded NPs

Particle size, size distribution and Zeta potential were measured by dynamic light scattering (DLS) and zeta analysis in Bookhaven 90Plus instrument. Scanning electron microscopy images were obtained for NPs dried on glass cover slips and sputter coated with Pt/Au under vacuum for 30 s (108 Auto, Cressington Sci., USA). The images were obtained in Hitachi S-4800 microscope.

Drug Content in Nanoparticles

Three samples of 100 μL NPs suspension were dissolved in DCM, and FPTX was extracted with acetone/water 50/50. FPTX was quantified by measuring fluorescence and comparing to calibration curve of fluorescence *vs.* FPTX concentration. The drug content was calculated to be 0.03 μg FPTX/mg NPs.

Characterization of Chitosan Film Containing FPTX-PLGA-Loaded NPs

Gold slides coated with chitosan containing FTPX-PLGA NPs were sputter coated with Pt/Au under vacuum for 30 s (108 Auto, Cressington Sci., USA) and analyzed through SEM microscopy (Hitachi S-4800). Samples that were immersed in water for degradation studies were previously dried before submitted to sputter coating with Pt/Au.

Release in Buffer Solution

In order to evaluate the platform for application in cancer therapy, the release assays were performed at 37°C and pH = 6.0, conditions normally found in tumors.

Fluorescent Paclitaxel Release from PLGA NPs

The release of FPTX from NPs was measured in Hepes buffer solution (pH = 6.0) in triplicate. 2.4 mg of NPs were suspended in 2 mL of buffer in microcentrifuge tubes and placed inside an oven at 37°C. At particular time intervals, the tubes were centrifuged at 10,000 rpm (g = 7200) for 15 min. The supernatant was collected and placed in cuvettes for fluorescence measurements. FPTX present in the supernatant was quantified based in a standard curve of fluorescence in function of FPTX concentration.

Carboxyfluorescein Release from Chitosan Films

The release of 6-Carboxyfluorescein from chitosan films was measured in triplicate in Hepes buffer solution (pH = 6.0). The gold slides coated with CF-loaded chitosan film were placed in cuvettes with 2 mL of buffer solution, and the samples were incubated at 37°C. At particular time intervals, the cuvettes were placed in the fluorimeter, and the CF was detected by irradiating the solution at 493 nm and measuring the fluorescence at 520 nm. In order to quantify the CF in solution, the intensity of fluorescence was compared to a standard curve of fluorescence intensity as function of CF concentration. The standard samples of CF solution were also kept incubated at 37°C, and the fluorescence was measured at same time intervals as the analyzed samples.

FPTX-Loaded PLGA NPs Release from Chitosan Films

The release of NPs from chitosan films was measured in triplicate in Hepes buffer solution (pH = 6.0). The gold slides covered with chitosan film containing FPTX-PLGA-loaded NPs were immersed in cuvettes filled with 2 mL of Hepes buffer solution. The samples were incubated at 37°C. At particular time intervals, the cuvettes were placed in the fluorimeter, and FPTX-PLGA NPs were detected by excitation at 493 nm and emission at 530 nm. In order to calculate the percentage of release, the fluorescence of samples was compared to the fluorescence of a FPTX-PLGA NP suspension in the same concentration present into the film. In the last case, the fluorescence was defined as 100% of release.

NP release from films was confirmed through DLS measurements. Gold slides coated with chitosan film containing FPTX-loaded PLGA NPs were incubated in HEPES buffer solution (pH = 6.0) inside cuvettes. At particular time intervals, the cuvettes were placed inside DLS equipment. As the gold slides were localized on the bottom of the cuvettes, the laser beam passed through the solution, allowing the detection of NPs in suspension.

RESULTS

Characterization of FPTX-Loaded PLGA NPs

The size of FPTX-PLGA NPs was analyzed for dry NPs and NPs in suspension through SEM and DLS, respectively. The average diameter of dry NPs was estimated by measuring the diameter of 100 randomly selected particles on enlarged SEM images (Fig. 2A). The values were fitted to a Gaussian distribution (Fig. 2B) giving an average diameter of 511 ± 4 nm. When NPs were in suspension, DLS analysis (Fig. 2C) showed average diameter of 530 ± 17 nm, a value similar to the one obtained through SEM images. Although SEM images showed aggregated NPs, it was clearly demonstrated by comparing DLS and SEM results that the aggregation occurs during the dry processing required to prepare SEM samples, since the average diameter determined through DLS is close to average diameter of NPs individually measured through SEM.

The value of zeta potential was determined to be −16 mV. The charged surface of FPTX-loaded NPs causes NP repulsion and is responsible for maintaining them in suspension through electrostatic stabilization.

Characterization of Chitosan Film Containing FPTX-Loaded PLGA NPs

Through SEM images of chitosan film containing NPs (Fig. 3A) it was possible to observe that NPs are evenly distributed into the film. Furthermore, no clusters of NPs were detected. The average diameter of NPs dispersed into the film was estimated by measuring the diameter of 50 randomly selected particles on enlarged SEM images. The average diameter was calculated to be 460 nm. This value is very close to the average diameter determined through DLS and SEM, indicating that NPs are not aggregated or degraded when incorporated into chitosan film.

Degradation of Chitosan Film Containing FPTX-Loaded PLGA NPs

Gold slides coated with chitosan films containing FPTX-loaded PLGA NPs were immersed in distilled water for 1 h and up to 2 days. Samples were immersed in water instead of buffer solution in order to prevent salt in the buffer from crystallizing on the film's surface, which could prevent detailed analysis of dry films through SEM. In this way, after the incubation in water, the samples were dried in vacuum and sputter coated with Pt/Au for SEM imaging (Fig. 3).

SEM images of samples immersed for 1 h in water showed NPs still incorporated in the film, but the increased roughness compared to the fresh sample (Fig. 3A) indicated that the film had already started to degrade (Fig. 3B). The degradation was more evident for the sample immersed in water for 2 days (Fig. 3C). In this case, a very irregular film surface was clearly visible. Additionally,

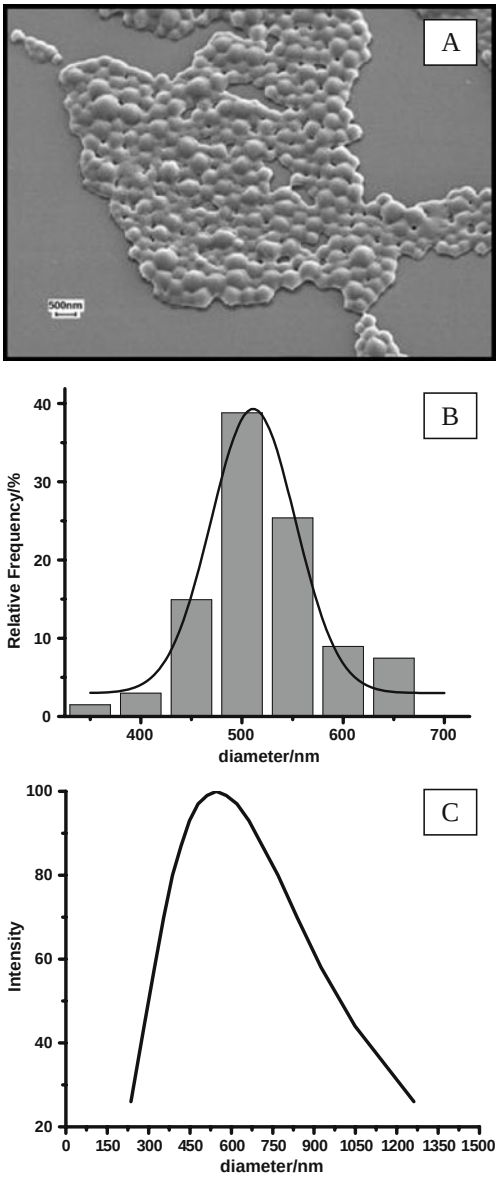

Fig. 2 **A**. SEM image of FPTX-PLGA NPs. Scale bar is 500 nm. Size distribution of FPTX-PLGA NPs determined throughSEM microscopy (**B**) and DLS measurements(**C**).

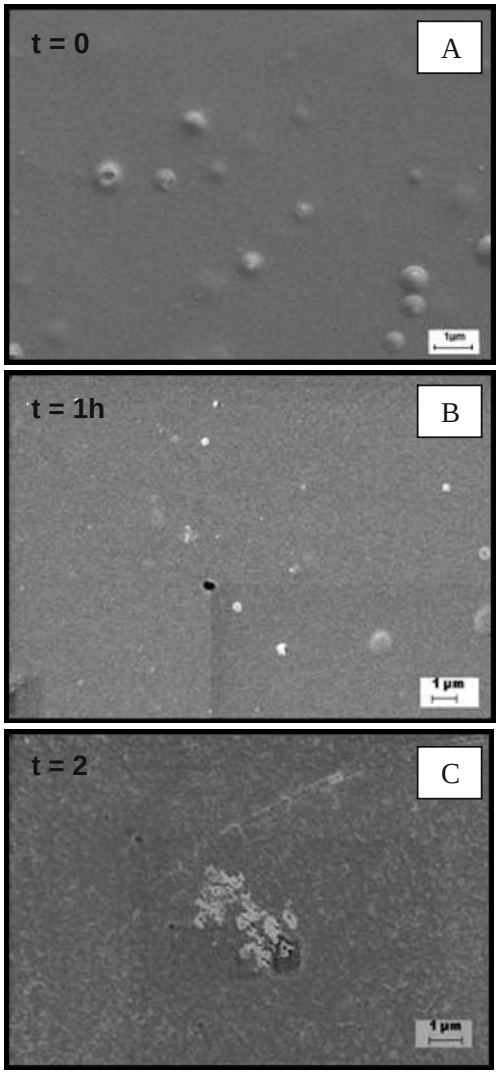

Fig. 3 SEM images of chitosan film containing PLGA NPs showing chitosan degradation. **A**. Fresh film. **B**. Film incubated for 1 h in water. **C**. Film incubated for 2 days in water. Scale bars are 1 μm.

the number of NPs embedded into the film was lower compared to fresh film and film immersed in water for 1 h. These results suggested the erosion of chitosan and NP release from films.

In order to confirm that the film erosion leads to NP release, an experiment was designed to detect *in-situ* NPs released from chitosan films through DLS measurements (Fig. 4). Gold slides coated with chitosan film containing FPTX-loaded PLGA NPs were incubated with Hepes buffer solution (pH = 6.0) inside cuvettes. It was observed that after 1 day of incubation, small particulates of 4 nm on average were released in the solution. After 4 days, it was possible to detect bigger particulates of 1 µm. The average diameter of particulates in suspension after the next 2 days was only 500 nm, suggesting the presence of PLGA NPs in suspension.

Release in Solution

Fluorescent Paclitaxel Release from PLGA NPs

The release profile of PLGA NPs was determined in pH 6.0 and 37°C (Fig. 5). The PLGA NPs released 19.8% ± 0.9% of total drug in 2 days. During the following 26 days, no additional release was detected by fluorescence.

6-Carboxyfluorescein Release from Chitosan Films

In order to investigate the release profile of a hydrophilic molecule from chitosan film, CF was incorporated in chitosan, and after film formation the release profile was determined in pH 6.0 and 37°C (Fig. 6). During an initial phase (phase I), chitosan films demonstrated the release of 78% ± 11% of total drug in 4 days. In the second phase (phase II), CF was slowly released from the film, and an additional 22% ± 3% of drug was released in the next 22 days.

FPTX-Loaded PLGA NPs Release from Chitosan Films

Similar to the release of CF from chitosan films, NP release from films also showed a biphasic pattern. In phase I of release (Fig. 6), 34% ± 16% of total NPs were released in 2 days. In the second phase (phase II; Fig. 6), the release was slower, and an additional 18% of NPs was released in the next 24 days.

DISCUSSION

The conditions used in the preparation of PLGA NPs resulted in NPs of average diameter of 500 nm. The incorporation of drugs in NPs with this size can increase their residence time in tumor tissues. NPs with average diameter in the range of 500 nm are too big to easily pass through normal vessels, but they are still able to diffuse through the tumor (25). Another advantage of PLGA NPs is their capacity to incorporate hydrophobic drugs such as paclitaxel. The hydrophobic interaction between PLGA and paclitaxel guarantees the stability of the drug inside the polymeric matrix even when the material is immersed in aqueous medium. The release profile of paclitaxel from PLGA NPs showed an initial release of 19.8% of total drug in 2 days. During the following days, no additional drug release was detected. One potential explanation is that the drug released in the first few days of incubation with buffer is probably the portion that was adsorbed on NP surfaces. After the initial release, the drug molecules to be released are the ones embedded within NP cores. In this case, drug release depends mainly on NP degradation. The degrada-

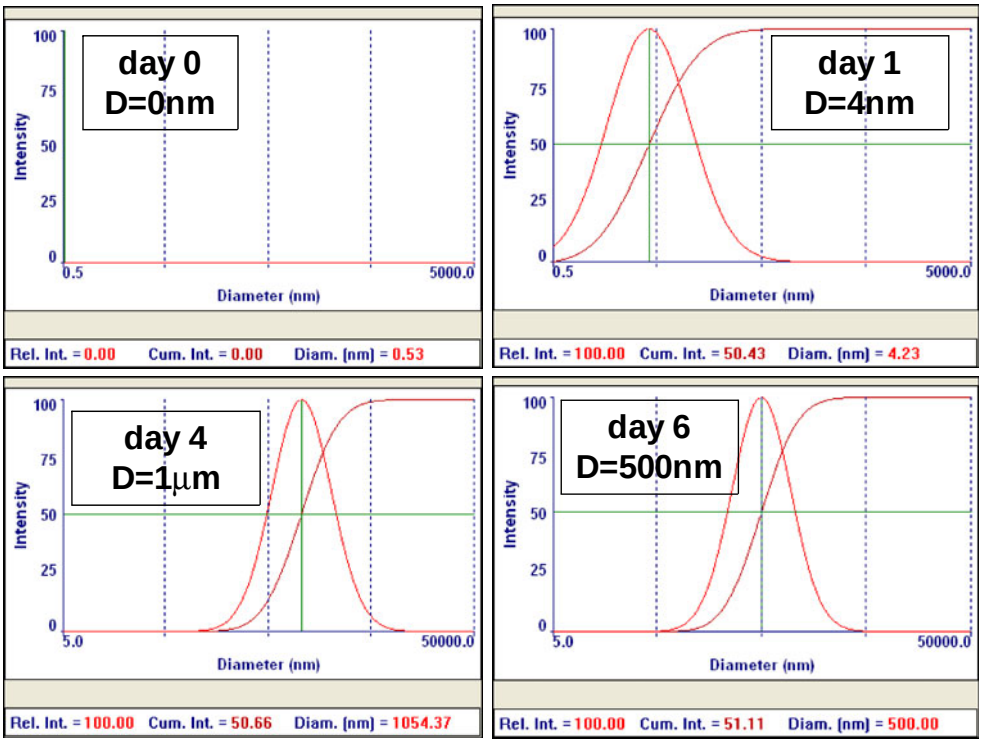

Fig. 4 Size distribution of chitosan fragments and PLGA NPs present in the solution incubated with chitosan film containing PLGA nanoparticles during different time intervals.

tion of PLGA in aqueous medium was reported to be a very slow process during at least 40 days (5,11). The slow drug release from PLGA NPs may allow the distribution of the drug along the tissue, while NPs diffuse through the tumor.

The evaluation of paclitaxel-loaded NP stability showed no aggregation of these NPs when they were in aqueous solution, most probably due to their surface charge of −16 mV that causes repulsion between them. This is an important feature of these NPs, since aggregation could decrease drug release and NP diffusion through tissue.

Considering the observed ability of PLGA to incorporate and release paclitaxel as well as the stability of NPs in solution, the loading of gold implants with these NPs could be a potential technique for the local delivery of hydrophobic drugs. It can be achieved by incorporating paclitaxel-loaded PLGA NPs into chitosan film. The high stability of chitosan films on gold surfaces demonstrated the potential applicability of this polymer on coating gold implants. Furthermore, it was shown that chitosan can be loaded with CF and with PLGA NPs.

The characterization of chitosan films containing PLGA NPs by SEM showed a very uniform NP distribution through the film. This is important to equally distribute the drug to the environment around the gold implant.

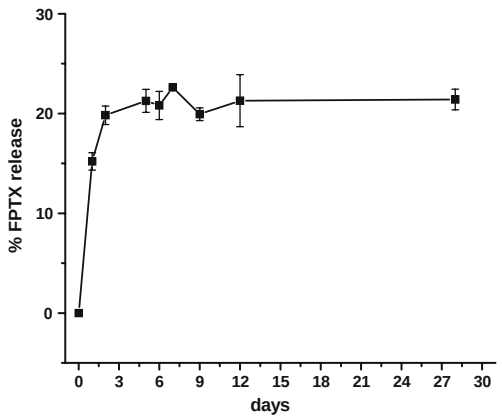

Fig. 5 Release profile of FPTX from PLGA NPs in buffer solution pH 6.0 and 37°C obtained through fluorescence measurements.

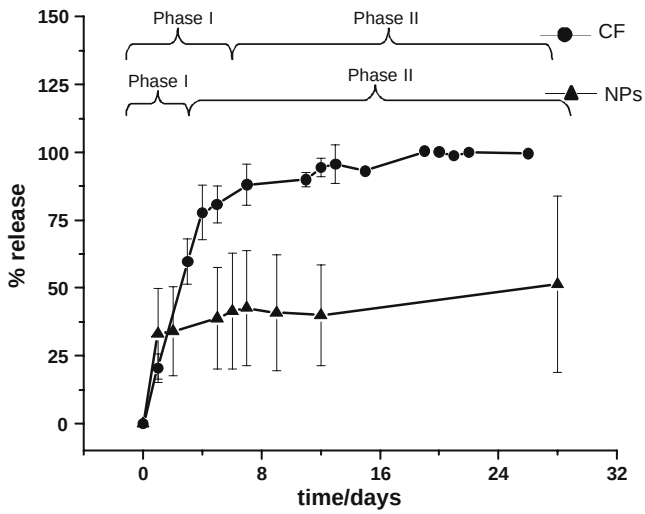

Fig. 6 Release profile of CF and PLGA NPs from chitosan film in buffer solution pH = 6.0 and 37°C, obtained through fluorescence measurements.

The release of CF and PLGA NPs from chitosan film was demonstrated to be a biphasic process. In both cases, a faster release in the initial phase I was followed by a slower release in phase II. In the release profile of CF, the phase I corresponds to the release of CF molecules incorporated on the surface of the film. Following this, the second phase of release (phase II) is attributed to the release of CF molecules diffused within the chitosan film. In this case, the release was not only dependent on the diffusion rate of CF through the film but also on the degradation rate of chitosan film.

The release of NPs from the films was observed to be dependent mainly on the film degradation rate. Comparing fluorescence (Fig. 6) data to DLS measurements (Fig. 4) and SEM images (Fig. 3), it was possible to conclude that the phase I of release was coincident with the period needed to observe effective degradation on film surface and to detect particulates in suspension. SEM images showed the film degradation after 1 h of incubation in water. Degradation was more evident after 2 days. In DLS studies (Fig. 4), particulates of 4 nm were detected after 1 day of incubation in aqueous medium. These particulates are probably small fragments of chitosan film that started to degrade. After 4 days, as the degradation proceeds, bigger fragments of 1 μm were released in suspension. Fluorescence measurements (Fig. 6; phase I) showed that these fragments contained paclitaxel-loaded PLGA NPs. In the sixth day of incubation, the size of particulates in suspension decreased to 500 nm, a value close to the average diameter of PLGA NPs. In this way, it is suggested that chitosan fragments were degraded, and only free PLGA NPs could be detected in suspension. Furthermore, it is noteworthy that no additional fragments of chitosan had been released after the fourth day of incubation. This decrease on film degradation rate is coincident with the slow release phase of NPs from chitosan observed through fluorescence measurements (Fig. 6; phase II).

CONCLUSIONS

The development of new drugs for cancer therapy has been accompanied by efforts to efficiently deliver these drugs to tumor tissues. Coating implants, especially gold fiducial markers, with polymers containing chemotherapeutic drugs can deliver the drug precisely to the tissue under treatment. Local delivery of chemotherapeutic drugs provides a high concentration and decreases the incidence of side effects commonly observed with systemic therapy.

Considering the stability of chitosan film containing PLGA nanoparticles on gold surfaces, this work demonstrates the use of this platform as a coating agent for gold implants. Furthermore, chitosan films could be loaded with hydrophilic model drug (Carboxyfluorescein) and FPTX-loaded PLGA NPs. The release profile of CF and PLGA from chitosan film as well as the release of FPTX from PLGA NPs demonstrated the promising application of this platform for localized dual-drug release.

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