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Magnetic stimulus responsive vancomycin drug delivery system based on chitosan microbeads embedded with magnetic nanoparticles

Ankita Mohapatra,[1] Michael A. Harris,[2] David LeVine,[2] Madhav Ghimire,[3] Jessica A. Jennings,[2] Bashir I. Morshed,[1] Warren O. Haggard,[2] Joel D. Bumgardner,[2] Sanjay R. Mishra,[3] Tomoko Fujiwara[4]

[1]Electrical and Computer Engineering, University of Memphis, Memphis, Tennessee 38152
[2]Biomedical Engineering, University of Memphis, Memphis, Tennessee 38152
[3]Physics, University of Memphis, Memphis, Tennessee 38152
[4]Chemistry, University of Memphis, Memphis, Tennessee 38152

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Abstract: Local antibiotic delivery can overcome some of the shortcomings of systemic therapy, such as low local concentrations and delivery to avascular sites. A localized drug delivery system (DDS), ideally, could also use external stimuli to modulate the normal drug release profile from the DDS to provide efficacious drug administration and flexibility to healthcare providers. To achieve this objective, chitosan microbeads embedded with magnetic nanoparticles were loaded with the antibiotic vancomycin and stimulated by a high frequency alternating magnetic field. Three such stimulation sessions separated by 1.5 h were applied to each test sample. The chromatographic analysis of the supernatant from these stimulated samples showed more than approximately 200% higher release of vancomycin from the DDS after the stimulation periods compared to nonstimulated samples. A 16-day long term elution study was also conducted where the DDS was allowed to elute drug through normal diffusion over a period of 11 days and stimulated on day 12 and day 15, when vancomycin level had dropped below therapeutic levels. Magnetic stimulation boosted elution of test groups above minimum inhibitory concentration (MIC), as compared to control groups (with no stimulation) which remained below MIC. The drug release from test groups in the intervals where no stimulation was given showed similar elution behavior to control groups. These results indicate promising possibilities of controlled drug release using magnetic excitation from a biopolymer-based DDS. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 106B:2169–2176, 2018.

Key Words: chitosan, magnetic hyperthermia, magnetic nanoparticle, vancomycin, smart

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INTRODUCTION

In the past few decades, pharmaceutical research has progressed significantly in various smart drug delivery systems (DDS) that are aimed at controlling dosage and drug localization.[1] Traditional systemic delivery of antibiotics is not always effective in achieving minimum inhibitory concentration (MIC) required for sustained treatment at the target site of injured tissue, thereby requiring stronger or repetitive dosages for efficacy in preventing infection.[2] The reason for insufficient concentration is usually due to avascular nature of the injured tissue, dissipation of drug into nontargeted tissues, dilution due to vascular circulation, or opsonization by Mononuclear Phagocytic System. There have been numerous approaches to enhance pharmacokinetic efficiency and longevity of pharmaceutical products *in vivo*.[1] A popular method is to use a biocompatible, biodegradable DDS that allows longer bioavailability and localization of drug at potent concentrations at the site of interest. Chitosan has been extensively studied as a drug carrier because of positive characteristics such as biodegradability, noncytotoxicity, intracellular permeability, biocompatibility, and its ability to entrap drugs.[3–5]

Although these DDS prolong the lifetime and efficacy of drug compared to naked drug, these DDS characteristically have a continuous first-order elution profile until the drug reserve is exhausted.[6] Slow degrading or nondegrading carriers such as poly-methylmethacrylate may continue releasing antibiotics at subtherapeutic levels after the drug payload is drained, increasing the risk of antibiotic resistance.[7] Several modifications have been proposed to make such DDS responsive to a variety of stimuli, which would enable on-demand dosage optimization to actual therapeutic requirement.[8–10] Also, a drug boost at later time points may increase drug elution to therapeutic levels, thereby

Correspondence to: A. Mohapatra; e-mail: mhapatra@memphis.edu
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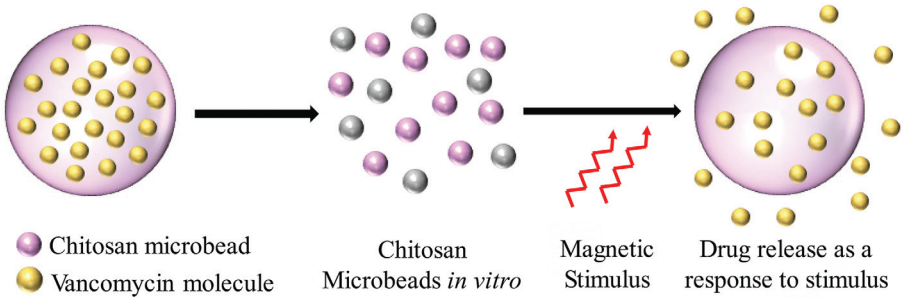

FIGURE 1. Conceptualized framework for the DDS responsive to external stimuli.

increasing efficacy and reducing the risk of drug-resistant bacterial strains.[11]

A DDS that is responsive to an exogenous stimulus of high-frequency alternating magnetic field, for on-demand drug releases has been developed (Figure 1).[12] The DDS is in the form of microbeads fabricated from chitosan, cross-linked with poly-ethylene glycol dimethacrylate (PEGDMA) and embedded with magnetic nanoparticles (MNP). As chitosan microbead itself does not respond to an external magnetic stimuli, MNP were embedded at the time of chitosan microbead preparation to enable the DDS to demonstrate hyperthermia phenomenon. The antibiotic vancomycin was loaded into microspheres to study drug release profiles with and without magnetic stimulation. A long term study was also performed through magnetic stimulation applied to microbeads after 12–15 days *in vitro*, in which a controlled increased drug delivery occurred even after initial first order elution profiles had been reached. This external noninvasive stimulation approach may have the benefit of using a noninvasive stimulus to maintain the antibiotic release profile for extended durations of infection prevention.

MATERIALS AND METHODS

MNP-loaded chitosan microbeads were prepared by an emulsion cross-linking method as described below, followed by the setup used for providing magnetic stimulus.

Preparation of MNP

The Fe_3O_4 MNP were made by following the nonsurfactants method described by Kang et al.[13] MNP were imaged by a transmission electron microscope (TEM, Jeol JEM 1200) and the sizes of 500 individual particles in random fields of view were measured with ImageJ. D8 Advance (Bruker Inc.) diffractometer was used to measure X-ray diffraction (XRD) with Cu-Kα ($\lambda = 0.15418$ nm) radiation. A magnetization curve for the same samples was obtained by VSM-130 (Dexing Magnet Tech. Co.) at room temperature.

Preparation of chitosan microbead

A modified water-oil emulsification technique reported by Jain et al.[14] was followed to make chitosan microbeads.[14] The preparation of the chitosan DDS can be divided into three phases: (1) Preparation of chitosan solution, (2) emulsification/cross-linking step of chitosan microbeads, and (3) washing stage.

In phase 1, a solution of 4 wt % chitosan, 2 wt % MNP (Fe_3O_4), 1% volume glacial acetic acid and 0.8% vancomycin was made. Then, 1 g MNP was added to 46 mL deionized water in a 50 mL centrifuge tube and sonicated. The mixture was then added to 2 g chitosan (Chitopharm S) and 0.4 g vancomycin (MP Biomedicals). After that, 0.5 mL glacial acidic acid was added and the solution was stirred well. The solution was set up on an impeller and left to stir overnight.

In phase 2, 2 g Span 80 surfactant (Sigma Aldrich), 75 mL light mineral oil, 75 mL heavy mineral oil (Fisher Scientific) and 15 mL PEGDMA Mn = 550 (Sigma Aldrich) were combined in a beaker. This solution was placed on a hot plate and stirred by an impeller for a few hours. Then, 15 mL chitosan solution from phase 1 was injected into the mixture. The hot plate was set to 60°C and left for 24 h.

In the last phase, the excess oil was drained and the beads were centrifuged, followed by thorough washing with hexane, methanol, and acetone. After the final wash, the beads were resuspended in approximately 10 mL of acetone and poured into a glass petri dish to dry.

The same process was followed without adding the Fe_3O_4 nanoparticles for making chitosan microbeads without MNP. Both kind of microbeads were imaged with a scanning electron microscope (SEM, XL30 ESEM) after fixing them on a carbon tape and coating with 5 nm Au/Pd on a sputter-coater (EMS 5502X).

Procedure for stimulus

A MagneTherm instrument (nanoTherics, UK) was used to provide magnetic stimulation (Figure 2). It consists of

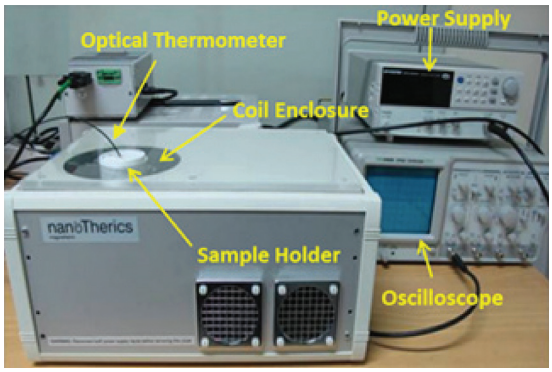

FIGURE 2. Photograph of the Magnetherm equipment for magnetic stimulation.

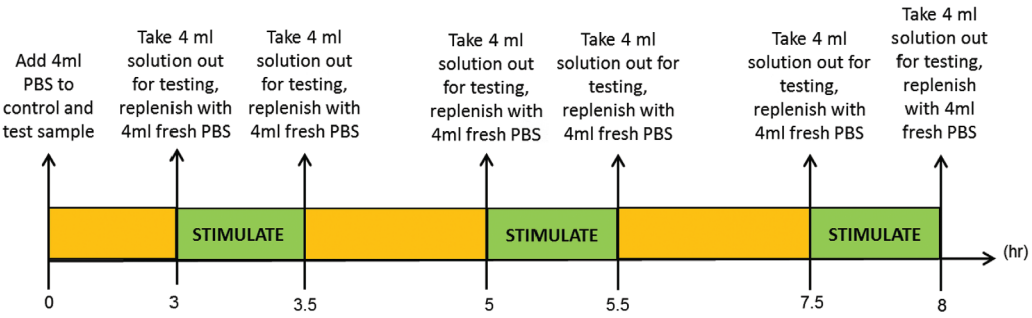

FIGURE 3. Short term elution study timeline of hyperthermia experiments on samples with MNP.

interchangeable 9 and 17 turn coils with ten different capacitor banks, each of which is characterized by a specific resonant frequency and maximum magnetic flux density. The coil is water-cooled and positioned around a sample holder. A fiber optic thermometer (Optocon, Germany) was used for temperature measurements of media surrounding the samples.

A preliminary temperature rise test with five frequency/intensity combinations was performed and compared to determine the setting that caused the highest temperature rise in a sample of MNP. Based on results of this test, stimulus parameters for all DDS experiments were set at 109.9 kHz/25 mT, as this setting has produced the maximum temperature rise in this test.

Experiments on chitosan microbeads with MNPs

Short term elution study. The total duration of this experiment was 8 h, with test groups stimulated at third, fifth and seventh h for 30 min as shown in Figure 3. The DDS was divided into ten samples of 100 mg each, of which five were assigned as control (nonstimulated) and five as test groups. Four milliliter was added to each sample. The phosphate-buffered saline (PBS) is completely refreshed with the same volume of fresh PBS every 1 h.

Long term elution study. To evaluate clinically relevant application of this DDS elution and stimulation over a period of several days at a point when drug release is depleted or below active concentrations, the DDS samples were placed in 4 mL PBS and media was refreshed daily for 11 days and were then stimulated for 60 min on day 12 and day 15, as depicted in Figure 4. The experiments consisted of six control (nonstimulated) and six test samples, each with 100 mg MNP-chitosan microbeads. Hundred microliters from the PBS was collected before and after stimulation on day 12 and 15. Additional 100 μL samples were also collected on day 13 and day 16.

General hyperthermia study. To investigate if the samples would exhibit a similar drug release when treated to the same temperature increment by other means, five DDS samples (test groups) were also subjected to a rapid temperature rise of 16°C in an incubator, which was the same temperature rise observed through magnetic hyperthermia. The experimental timeline was same as showed in Figure 3. A separate batch of five samples was not given any stimulation and were labeled as control groups for comparing drug elution. All samples in both groups had 100 mg microbeads in 4 mL PBS.

Experiments on chitosan microbeads without MNP

In order to demonstrate the role of MNP in aiding drug release, experiments above were conducted on beads without MNP. In these set of experiments, there were five control and five test samples containing 100 mg of microbeads. Due to the very fine and light nature of these beads, complete media refreshment was not possible without pipetting out several microbeads each time. To avoid introducing errors due to nonconsistent sample weight, a slightly different timeline was followed, as shown in Figure 5. Stimulations were given at 3rd, 5th and 24th h, each session of 30 min. At $t = 0$, 10 mL of PBS was added to all samples. Then, 120 μL of the supernatant was collected before and after the stimulation sessions.

Data collection, calibration and analysis

High performance liquid chromatography (HPLC; Dionex UltiMate 3000 HPLC, Thermo Scientific, Waltham, MA) was used for analyzing vancomycin amount released from the collected supernatant. The release data between stimulated

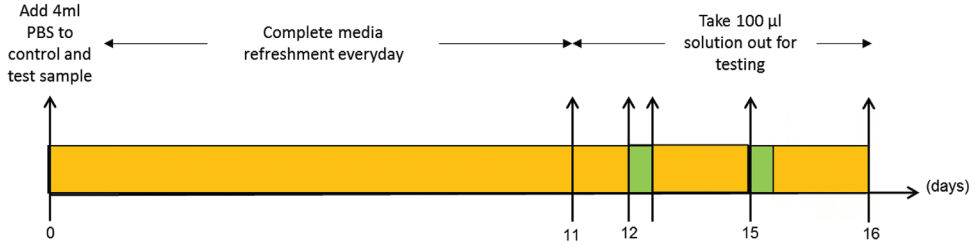

FIGURE 4. Long term elution study timeline of hyperthermia experiments on samples with MNP.

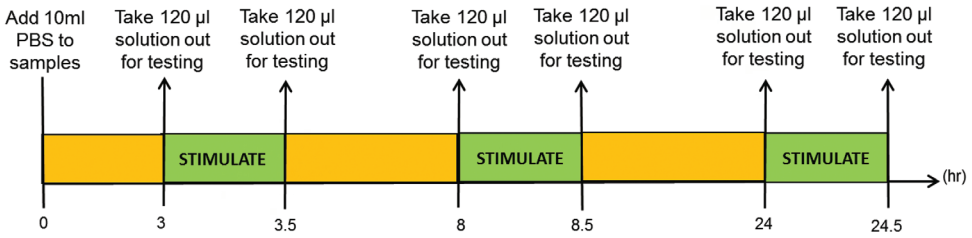

FIGURE 5. Timeline of hyperthermia experiments on samples without MNP. Vertical arrows represent sampling instances for HPLC tests.

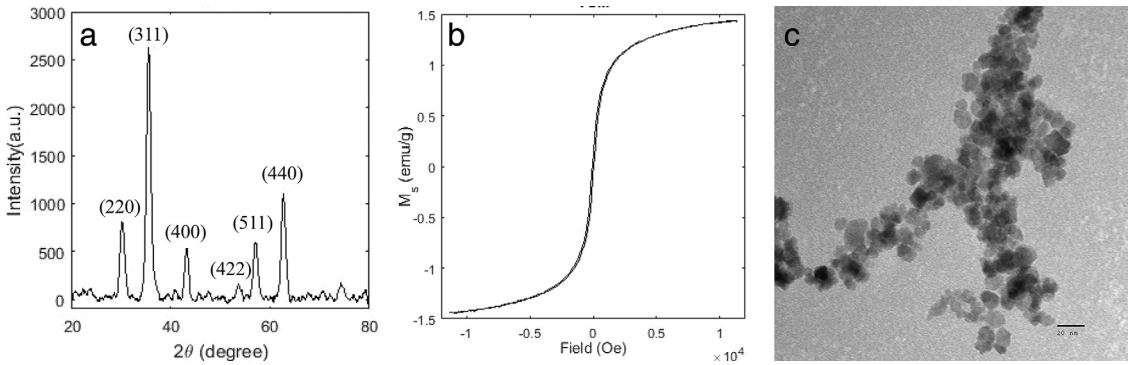

FIGURE 6. (a) XRD pattern, (b) magnetization versus field curve, and (c) TEM image of Fe_3O_4 MNP.

and nonstimulated groups were analyzed by the nonparametric Mann Whitney test. The significance level for assessing significant differences in drug elution was fixed at 5%.

RESULTS

MNP characterization

The XRD [Figure 6(a)] confirms the presence of Fe_3O_4 phase and magnetization curve [Figure 6(b)] show magnetic nature of the MNP up to 1.2 T field. Size analysis of TEM imaging [Figure 6(c)] revealed the size of MNP distribution to be 10.89 ± 2.67 nm.

Chitosan microbead characterization

The microbeads were imaged using an SEM (Figure 7) and the size distribution of these particles was 288.4 ± 62.2 µm.

A Fourier transform infrared spectroscopy (FTIR) for the chitosan/MNP microbeads is shown (Figure 8). The observed peaks were C–O–C stretching (1070 cm^{-1}), C–H rocking (1400 cm^{-1}), amide II (1530 cm^{-1}), N–H Scissor (1550 cm^{-1}), C=O (1640, 1720 cm^{-1}), C–H (2850, 2920 cm^{-1}) and N–H Stretch (3350–3280 cm^{-1}), OH (2700–3600 cm^{-1}).

The XRD of chitosan, vancomycin and chitosan microbeads with MNP are shown in Figure 9.

Experiments on chitosan microbeads with MNP

A temperature elevation of 16°C was recorded after each magnetic stimulation from MNP loaded chitosan.

Short term elution study. The eluted concentration as a function of time is graphed in Figure 10, which shows a statistically significant drug increase in stimulated samples. After stimulation at each of the three instances, the test groups released significantly higher amount of vancomycin compared to control ($p \leq 0.008$). In the periods when no

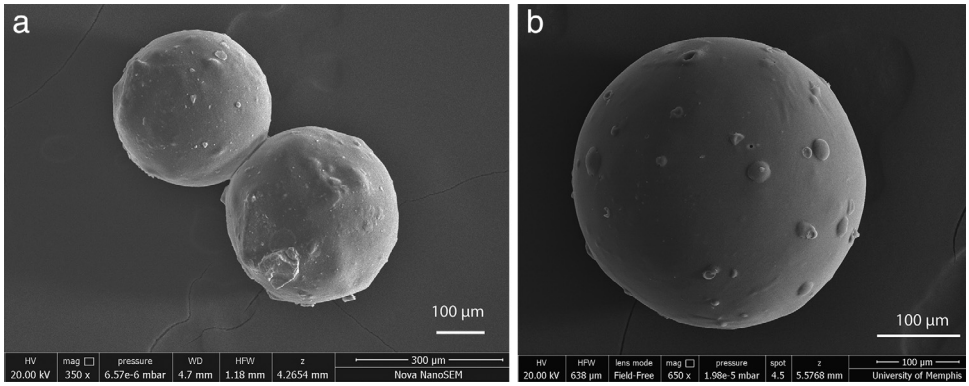

FIGURE 7. SEM image of chitosan microbeads (a) with MNP and (b) without MNP.

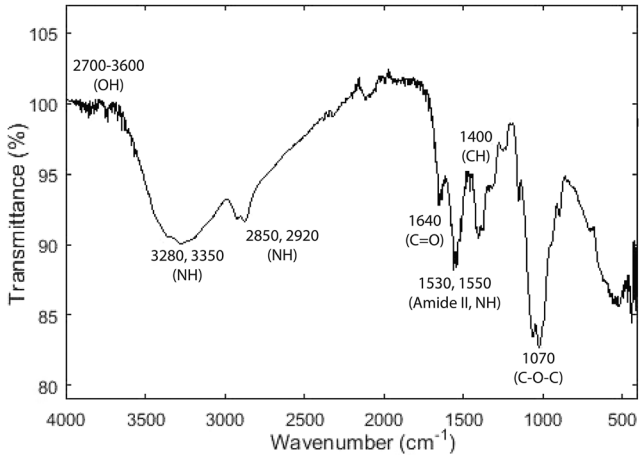

FIGURE 8. FTIR of chitosan microbead containing MNP, vancomycin and PEGDMA.

stimulation was given, the test groups released similar amounts of drug to control levels ($p > 0.05$).

Long term elution study. After 12 days of passive dissolution of antibiotics, vancomycin release from microparticles dropped below theoretically effective minimum inhibitory values against *Staphylococcus aureus S. aureus*. Magnetic stimulation of these microbeads for 30 min at days 12 and 15 caused an increase in vancomycin release to above the theoretically effective MIC of 1 μg/mL against *S. aureus* (Figure 11). For both stimulation events on day 12 and day 15, the test groups released statistically significant higher amounts of vancomycin ($p = 0.002$). In the nonstimulation periods, there was no statistical difference in drug elution between both groups ($p > 0.05$).

General hyperthermia study. No significance differences were detected between both groups (Figure 12).

Experiments on chitosan microbeads without MNP

The vancomycin concentration detected at each sampling points is shown in Figure 13. There was no statistically significant difference in drug release between magnetically stimulated and nonstimulated MNP-free chitosan beads ($p > 0.05$). The temperature rise did not exceed 3°C, which was the normal temperature rise observed in the MagneTherm chamber when the coil is generating a 25 mT magnetic field at 109.9 kHz, without any samples.

DISCUSSION

The DDS developed was responsive to a high frequency magnetic field, releasing increased amounts of antibiotics when stimulated. We were also able to boost antibiotic levels above MIC after several days by stimulation. Magnetic excitation was chosen over other stimulation modalities because this stimulation approach does not require any direct physical contact with the patient and is already being used for other needed clinical external stimulations, such as regenerating bone by stimulating external fixation devices.[15] The cross-linking process produced microbeads of the size that could be delivered through larger gauge syringes. While general diffusion of drug occurred between these stimulations, the ability to increase release with external, noninvasive means shows promise clinically.

XRD of the Fe_3O_4 MNP [Figure 6(a)] show the peaks are consistent with those reported in literature.[16,17] Size of MNP produced were similar to other reported nanoparticles.[16,17] The hysteris loop of the MNP [Figure 6(b)] confirms its superparamagnetic behavior. The major peaks of chitosan [Figure 9(a)], vancomycin [Figure 9(b)] and MNP [Figure 6(a)] are prominent in XRD of the chitosan-MNP microbeads [Figure 9(c)], supporting the presence of these constituents in the DDS.[18,19] It was further affirmed by FTIR (Figure 8) which showed amine groups and hydroxyl groups corresponding to chitosan and vancomycin, respectively. The C–O–C stretching confirms the inclusion of polyethylene glycol in the DDS.

In this DDS, vancomycin as well as chitosan can bind to PEGDMA through Michael addition reactions. Figure 14(a,b) show chemical structures of chitosan and vancomycin, respectively. Similar to chitosan, vancomycin has two amino groups. Those amino groups react with the crosslinker PEGDMA as shown in Figure 14(c), in the phase 2 step of microbeads preparation. After the reaction, some vancomycin should be immobilized through degradable ester bond. Ester hydrolysis is known to accelerate at elevated temperature in the

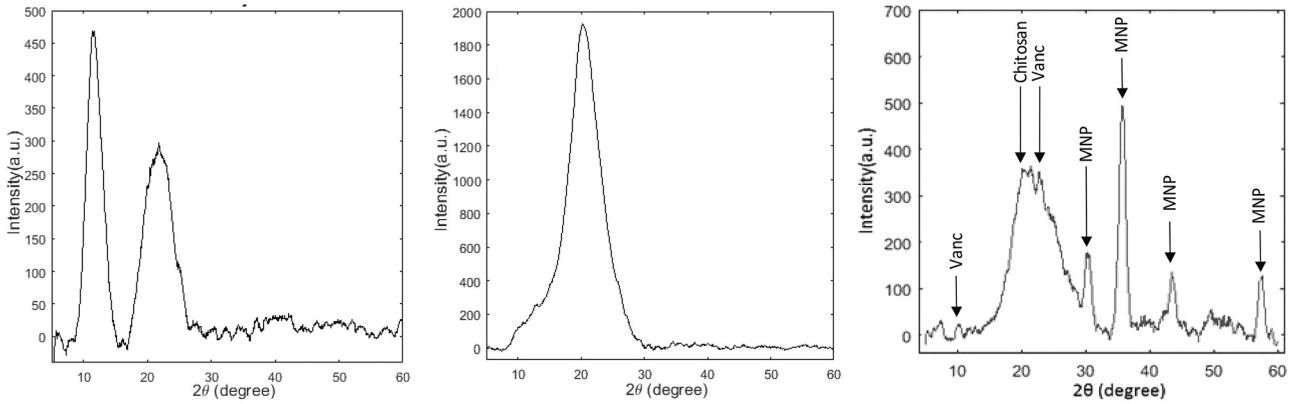

FIGURE 9. XRD plots of (a) vancomycin, (b) chitosan with PEGDMA and vancomycin, and (c) chitosan with PEGDMA, vancomycin, and MNP.

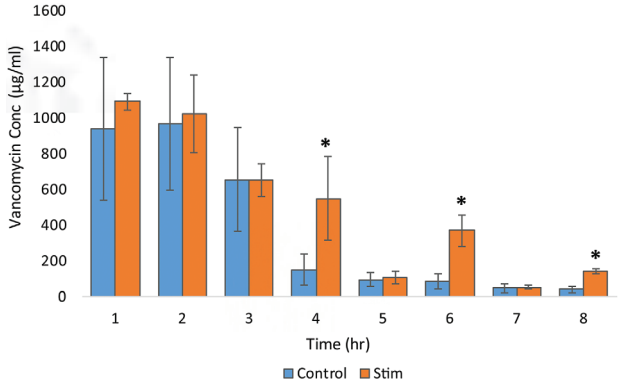

FIGURE 10. Concentration of vancomycin over time with (Stim) and without (Control) stimulation for short term elution study with stimulation given at third, fifth and seventh hour. Data represented is average ± standard deviation. Asterisks (*) represent statistically significant differences between stimulated and control groups, $p < 0.05$.

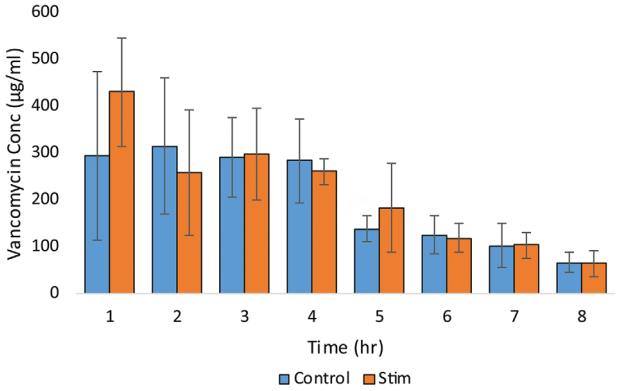

FIGURE 12. Concentration of vancomycin over time with and without stimulation in an incubator. Assuming 5% confidence level, the difference in vancomycin elution were not significant between test and control groups.

presence of acidic water. The chitosan microbeads may have confined slightly acidic water from the emulsion procedure. Therefore, it is very likely that this conjugated ester bond was cleaved as a result of hyperthermia, causing vancomycin release. In addition, as the crosslinked chitosan– polyethylene glycol bond is also cleaved by heat, diffusion of vancomycin could also be promoted by this stimulus.

In general hyperthermia tests on chitosan/MNP beads (Figure 12), although the samples underwent the same elevation in temperature, the absence of a significant difference in vancomycin elution from test groups compared to test groups suggests that magnetic field is necessary for causing response. The dependence of drug release on magnetic field was further confirmed when chitosan microbeads without MNP were subjected to the same stimulation parameters (Figure 13) but failed to show a significant variation in elution profile of vancomycin in comparison to control groups. It is known that on excitation with an alternating magnetic field, MNPs generate and dissipate heat due to core relaxation losses.[20–23] In the chitosan/MNP DDS, the localized temperature rise around the MNP on a nanoscale is likely to be much higher than 16°C, which might be sufficient for cleaving the bonds. Further tests are needed to explore the exact mechanism causing vancomycin release.

Magnetic hyperthermia has been extensively explored for smart therapeutic applications like cancer therapy and drug delivery, leading to successful clinical trials on human subjects with brain/prostate cancer.[24,25] Also, since MNP, like magnetite, have been proven to be efficient magnetic resonance imaging contrast agents,[26–28] they facilitate drug targeting, localization and confinement.[29] Some of our preliminary tests further indicated that the DDS exhibits favorable cyto-compatibility.[12]

The repeated increases in release upon stimulation in the elution studies is relevant clinically because it gives the care provider freedom to administer dosage as per each patient's unique needs, without intravenous procedures each time.) Ethylene-vinyl acetate copolymer polymeric matrices loaded with insulin and MNP showed similar passive elution of active insulin as release of antibiotic in this DDS, with higher activity in glucose lowering after stimulus of magnetic field.[30] Finotelli et al.[31] loaded insulin in alginate/chitosan microbeads with magnetite nanoparticles and showed insulin release tripled in stimulated groups with respect to nonstimulated groups in response to a magnetic field.

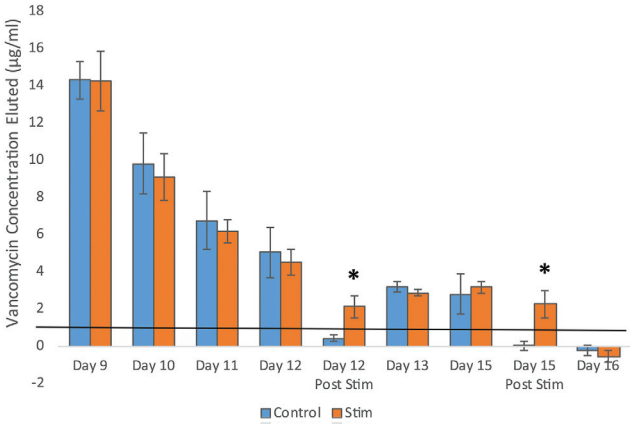

FIGURE 11. Concentration of vancomycin over time with (Stim) and without (Control) stimulation for a long term elution study with stimulus given on day 12 and day 15. Data represented is average ± standard deviation. Asterisks (*) represent statistically significant differences between stimulated and control groups, $p < 0.05$.

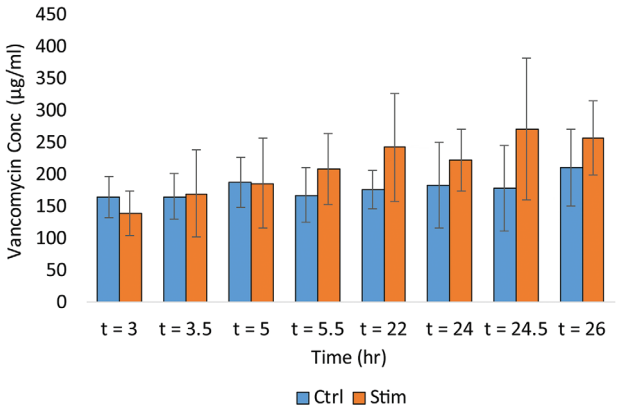

FIGURE 13. Amount of vancomycin eluted by chitosan microbeads without MNPs. Assuming 5% confidence level, the difference in vancomycin elution were not significant between test and control groups.

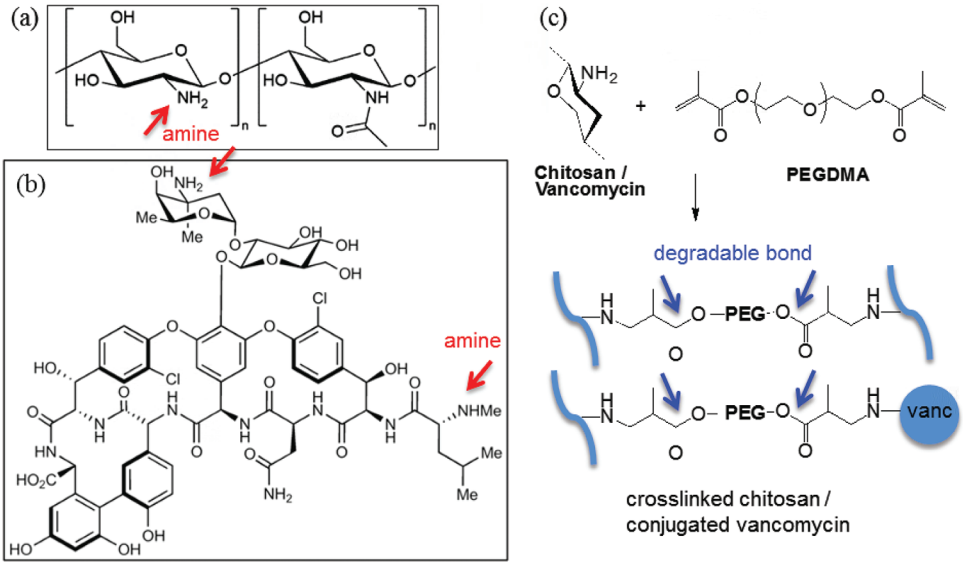

FIGURE 14. Chemical structures of (a) chitosan, (b) vancomycin, and (c) reaction mechanism of PEGDMA crosslinker with chitosan and vancomycin.

Katagiri et al.[32] designed polyelectrolyte hollow multilayered shells containing dye, coated with Fe_3O_4 MNP and an amphiphilic bilayer that released dye upon magnetic stimulation which was attributed to heat-induced change in phase of amphiphile membrane, rather than any structural fissure. Preliminary SEM and macroscopic observations of the DDS used in this study suggests that stimulation does not cause damage to the structure of microbeads. This is in contrast to some magnetically responsive DDS, such as Fe_3O_4/poly(-allylamine) polyelectrolyte microcapsules, that formed microcavities on the DDS surface on application of a high frequency magnetic field and exacerbated into major ruptures with time, eluting drug in significant amounts.[33] Koppolu et al.[34] designed MNP cores with outer multilayered shells of the temperature-responsive polymer poly(*N*-isopropylacrylamaide) (PNIPAAm) and poly(D,L-lactideco-glycolide) as carriers of both curcumin and bovine serum albumin (BSA); while curcumin showed a sustained release profile over 13 day, BSA could be burst-released from PNIPAAm layer by elevating temperature.[34]

CONCLUSION

The results demonstrated that the DDS responded to stimulus by discharging significant amount of drug compared to control nonsimulated samples. It was also observed that the DDS did not respond to general hyperthermia, indicating that any *in vivo* thermal fluctuations will not affect drug elution. The experiments further suggest that this DDS has the potential to burst-release higher amount of drugs on multiple instances of stimulus, several hours or days apart as needed, and thus might enable us to maintain or control drug concentrations in the targeted treatment location. It can aid in targeting drug directly to problem areas, preventing systemic toxicity. The DDS also has the capability to be guided, localized and confined at the target site by a static magnetic field.[35] Once at the site, drug can be released, when required, by an external alternating magnetic field as demonstrated in this work. These features would greatly assist clinicians in controlling drug delivery, dosage timings and local concentration to match the clinical needs of the patient.

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