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Novel Antibacterial Nanofibrous PLLA Scaffolds

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Abstract

In order to achieve high local bioactivity and low systemic side effects of antibiotics in the treatment of dental, periodontal and bone infections, a localized and temporally controlled delivery system is crucial. In this study, a three-dimensional (3D) porous tissue engineering scaffold was developed with the ability to release antibiotics in a controlled fashion for long-term inhibition of bacterial growth. The highly soluble antibiotic drug, Doxycycline (DOXY), was successfully incorporated into PLGA nanospheres using a modified water-in-oil-in-oil (w/o/o) emulsion method. The PLGA nanospheres (NS) were then incorporated into prefabricated nanofibrous PLLA scaffolds with a well interconnected macroporous structure. The release kinetics of DOXY from four different PLGA NS formulations on a PLLA scaffold was investigated. DOXY could be released from the NS-scaffolds in a locally and temporally controlled manner. The DOXY release is controlled by DOXY diffusion out of the NS and is strongly dependent upon the physical and chemical properties of the PLGA. While PLGA50-6.5K, PLGA50-64K, and PLGA75-113K NS-scaffolds discharge DOXY rapidly with a high initial burst release, PLGA85-142K NS-scaffold can extend the release of DOXY to longer than 6 weeks with a low initial burst release. Compared to NS alone, the NS incorporated on a 3-D scaffold had significantly reduced the initial burst release. *In vitro* antibacterial tests of PLGA85 NS-scaffold demonstrated its ability to inhibit common bacterial growth (*S.aureus* and *E.coli*) for a prolonged duration. The successful incorporation of DOXY onto 3-D scaffolds and its controlled release from scaffolds extends the usage of nano-fibrous scaffolds from the delivery of large molecules such as growth factors to the delivery of small hydrophilic drugs, allowing for a broader application and a more complex tissue engineering strategy.

Keywords

doxycycline; controlled delivery; nanofiber; scaffold; tissue engineering

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Introduction

Chronic inflammation diseases caused by bacterial infection such as periodontitis and septic arthritis remain common in the United States and throughout the world. For example, approximately half of the adult population in the US suffers chronic periodontitis. This disease is initiated by pathogenic infection on the tooth's surface as a microbial biofilm which gradually leads to the destruction of the periodontal tissue, supporting alveolar bone, and eventually leading to tooth loss. [1] In the treatment of periodontitis, antibiotics such as tetracycline (TCL) and metronidazole have been frequently used.

Doxycycline (DOXY) is a well known broad-spectrum antibiotic, which is effective against both gram-positive and gram-negative bacteria, protozoa, and various anaerobes. [2] As a tetracycline analogue, it can work as a bacteriostatic which is capable of inhibiting the bacterial protein synthesis at the ribosomal sites. [3] Compared to TCL, DOXY has certain advantages such as a longer half-life, higher lipid solubility, and greater oral absorption. [4] It also shows strong inhibition of matrix metalloproteinases (MMPs) both *in vitro* and in clinical studies. [5-9] After being introduced to the clinical area in 1967 [10], DOXY has been frequently used in treating destructive periodontal diseases such as juvenile periodontitis and acute periodontal abscesses. It has been used against periodontal infection and enhancing bone regeneration after periodontal disease. [11] It has also been used to prevent bacterial infection related to septic arthritis. [10,12] However, there are some concerns over possible side effects such as gastro-intestinal disturbance, esophageal erosion, and photosensitivity when administered orally. [13-18] Therefore, in order to reach the infection deep inside tissue with an effective drug concentration and to circumvent the systemic side effects, controlled local delivery of DOXY is desired.

Many attempts have been made in the area of controlled delivery of the DOXY/TCL family and certain progress has been achieved. Drug encapsulation methods such as mixing, emulsification, and electrospinning have been used. Delivery systems in the forms of particles, stripes, hollow fibers, gels, and cements have been fabricated. [19-25] However, most systems release all the encapsulated DOXY from hours up to 2 weeks. While a high initial burst release is acceptable for a highly hydrophilic antibiotic such as DOXY/TCL in order to immediately achieve effective concentration, it is also necessary to attain a long-term sustained release to fight against chronic infection and avoid repeated local drug administration. How to achieve both reduced initial burst and extended release duration remains a challenge.

Controlled release of proteins has been widely investigated. [26-31] Our laboratory has previously developed a novel growth factor delivery system. The growth factors were encapsulated into PLGA nanospheres and the nanospheres were then incorporated into three-dimensional (3D) macro-porous and nano-fibrous PLLA scaffolds. [32-34] The function of this delivery system is two fold: First, it serves as an osteoconductive scaffold. With its suitable pore structure and nano-fibrous architecture similar to collagen, it improves cell attachment, proliferation and differentiation. [35,36] Second, it stimulates vascularization or bone formation by localized controlled delivery of a bioactive angiogenic or osteogenic growth factor. [32,33] In this paper, we incorporated DOXY-containing PLGA nanospheres into the nano-fibrous scaffolds to evaluate their anti-bacterial capacity. The combination of the osteoconductive properties of the macro-porous and the nano-fibrous scaffold with its ability to release antibiotics could be a good candidate for local treatment of oral and periodontal diseases.

1. Materials and Methods

2.1 Materials

Doxycycline hyclate, D-fructose, mineral oil, and sorbitanmonooleate (span 80) were obtained from the Sigma-Aldrich Chemical Company (St. Louis, MO). PLGA copolymers with LA/GA ratio of 50:50 (Lakeshore Biomaterials™, PLGA 50-6.5K, Mw=6.5kDa; PLGA 50-64K, Mw=64KDa); PLGA copolymers with a LA/GA ratio of 75:25 (Lakeshore Biomaterials™, PLGA 75-113K, Mw=113kDa) and the PLGA copolymers with a LA/GA ratio of 85:15 (Lakeshore Biomaterials™, PLGA 85-142K, Mw=142kDa) were purchased from SurModics Pharmaceuticals Inc. (Birmingham, AL). Poly(L-lactic acid) (PLLA) with inherent viscosity of 1.6 dl/g was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(acrylic acid) (M_w = 5000) and poly(vinyl alcohol) (PVA) (88 mol% hydrolyzed, M_w = 25,000) were obtained from Polysciences Inc. Hexaglyceryl polyricinolate (Hexaglyn PR-15) was provided by the Barnet Products Corp (Englewood Cliffs, NJ). Acetonitrile and petroleum ether (BP 60-95°C) were obtained from Acros Organic (Morris Plains, NJ). Dichloromethane (DCM), cyclohexane, hexane, tetrahydrofuran (THF) and Fisherbrand regenerated cellulose dialysis bags (nominal MWCO 3500) were purchased from Fisher Scientific (Pittsburgh, PA).

2.2 DOXY containing PLGA nanospheres (NS) and NS-incorporated nano-fibrous scaffold (NS-scaffold) preparation

Doxycycline-containing PLGA NS were fabricated using a modified water-in-oil-in-oil (w/o/o) emulsion method. [37,38] Briefly, 80 mg PLGA was dissolved in 1 ml solvent mixture of acetonitrile/DMC (3:2 in volume ratio). 100 μ l aqueous solution of certain amount of DOXY was added into the above solution and emulsified with a probe sonicator at 50 W (Virsonic 100, Gardiner, NY). This primary w/o emulsion was then emulsified into 30 ml of mineral oil containing 4% hexaglyceryl polyricinolate under sonication at 90 W to form the w/o/o emulsion. The resulting secondary emulsion was magnetically stirred at room temperature for 12 h to evaporate the solvents. The spheres were collected by centrifugation and washed three times with petroleum ether and freeze dried. Four PLGA formulations: PLGA50-6.5K, PLGA50-64K, PLGA75-113K and PLGA 85-142K were used for the *in vitro* release study. The macroporous PLLA nano-fibrous scaffolds with dimensions of 7.2 mm in diameter and 2 mm in thickness were prefabricated by a combination of phase separation and sugar leaching techniques previously described. [34] The PLGA NS were incorporated onto PLLA scaffolds using a post seeding method. [32,33] Briefly, PLGA NS were dispersed in hexane with 0.1% span 80 and were seeded onto NS-scaffolds drop wise. Then, the scaffolds were subject to vapor of a mixed solvent of hexane/THF (volume ratio 9:1) for 30 minutes. The scaffolds were vacuum-dried for 3 days to remove the solvent.

2.3 Characterization of PLGA NS and NS-scaffolds

The morphology and size of the nanospheres and the scaffolds (before and after NS incorporation) were examined using scanning electron microscopy (Philips XL30 FEG SEM). The nanospheres were dispersed in hexane and deposited onto a metal stub. SEM figures of these spheres were taken after gold coating. Average diameters of all type of PLGA spheres studied were calculated based on the sizes measured from the SEM images using ImageJ software (National Institutes of Health). The sizes of PLGA50-6.5K and PLGA 85-142K spheres were also measured by dynamic light scattering (DLS) in an aqueous solution for comparison (Delsa Nano C Particle Size Analyzer, Beckman Coulter Inc). The drug content and encapsulation efficiency were determined by UV spectrophotometric analysis. Briefly, 10 mg DOXY-encapsulated nanospheres (or one NS-scaffold) were dissolved in dichloromethane. Then, 10 ml PBS was added to extract DOXY. The extraction process was repeated several times. The solution was filtered through a 0.45

μm filter to remove polymer debris and then the DOXY concentration was measured using a UV spectrophotometer (Hitachi U2910) at the λ_{max} value of 274 nm. PBS was used as the control.

2.4 In vitro drug release studies

The release profiles of DOXY-loaded NS or NS-scaffolds with four PLGA formulations were investigated in PBS (0.1 M). 10mg NS were suspended in 1 ml PBS and placed within a dialysis bag. The dialysis bag was kept in a glass flask containing 20 ml PBS. One NS scaffold was suspended in 1 ml PBS in a glass vial. Both the glass vial and the glass flask were covered in aluminum foil and shaken at 50 rpm at 37°C. At designated time points (1, 3, 5, 7, 10, 14, 21, 28, 35, 42 days), the release medium was withdrawn and replaced with pre-warmed fresh PBS. The released amounts of DOXY were analyzed using a UV spectrophotometer at the λ_{max} value of 274 nm.[23] For DOXY release through the dialysis bag from NS alone, PBS was used as the control. For DOXY release from NS-incorporated scaffolds, blank PLGA NS (without DOXY) were immobilized onto the scaffolds and were used as the controls. The absorbance values of the controls were subtracted from those of the DOXY containing samples.

2.5 In vitro Anti-bacterial activity of NS-scaffold

The antibacterial assay was performed as previously described. [39] The overnight incubated bacteria solution (*S.aureus* and *E.coli*) was diluted with sterile Mueller-Hinton Broth and reached an absorbance value between 0.1 and 0.2 at 625 nm (equivalent to 0.5 McFarland standard). First, the bacterial clone formation assay was performed using the Mueller-Hinton agar (1.5%) plate. The scaffolds with or without DOXY nanospheres were placed in the center of the melting agar just before solidification. The DOXY was allowed to diffuse from the scaffold into the agar for 4 hours before 100 μL inoculum was spread over the agar plate. The plates were incubated at 37 °C for 5 days. The bacterial growth on plates was visualized directly on the plate. Second, the released drug solutions, collected from different scaffold groups at different time points, were mixed with 2X Mueller-Hinton Broth and overnight inoculum (the bacterial final absorbance value was around 0.1 at 625nm). All tested solutions were incubated at 37 °C on a shaker (50 rpm). The absorbance at 625 nm was monitored after 12 h of incubation. The percentage of bacterial inhibition was calculated using the following equation.

Bacterial inhibition (%) = $\text{abs}(I_c - I_s) / (I_c) \times 100$, where I_c and I_s are the absorbances of the control bacterial solution without drug and bacterial solutions with released drug respectively at 625 nm after 12 h [39].

3 Results

3.1 PLGA nanospheres loaded PLLA nano-fibrous scaffolds

PLGA NS prepared from a modified w/o/o double emulsion process were of smooth surface (Fig.1A). The combination of the phase separation and sugar sphere templating techniques allowed the formation of PLLA scaffolds with a high porosity (98%), multi-level pore structures and nano-fibrous architecture of the pore walls. The scaffolds prepared for this study have regular spherical macro-pores 250-425 μm in diameter with well interconnected inter-pore openings of $\sim 100 \mu\text{m}$ (Fig. 1B). The scaffold walls are entirely made of the PLLA nanofibers with the diameter range of 50-500 nm (Fig. 1C), which is similar in size to native collagen fibers. Fig. 1D and Fig. 1E show a cross section of the scaffolds after the DOXY loaded NS incorporation, and the pore wall surface at a higher magnification. From SEM observation, it is clear that the macro-pores and inter-pore opening structure of the scaffolds

were well preserved and the nano-spheres were uniformly distributed throughout the nano-fibrous pore walls.

Four different PLGA NS formulations were examined for encapsulation efficiency and release behavior. Two of the copolymers had the same LA/GA ratio of 50/50 but different molecular weights, PLGA50-6.5K (Mw: 6.5K) and PLGA50-64K (Mw: 64K). The other two had different LA/GA ratios: PLGA75-113K with a LA/GA ratio of 75/25 (Mw: 113K) and PLGA85-142K with a LA/GA ratio of 85/15 (Mw: 142K). The effects of processing parameters such as polymer concentration, volume ratio of drug solution to inner oil phase (w/o_1), volume ratio of inner oil to outer oil phase (o_1/o_2), the addition of PAA on the DOXY encapsulation efficiency (EE) of PLGA85-142K NS are presented in Table 1. It was observed that the EE of DOXY increased with an increase in the polymer concentration from 3%-8%, no significant difference between 8% and 10%. With the increase of aqueous drug volume, the encapsulation efficiency decreased. The higher polymer concentration may entrap the aqueous DOXY droplets more efficiently (higher EE), while the greater aqueous DOXY volume destabilizes the emulsion (lower EE). The increase in the volume ratio of outer oil phase to inner oil phase did not change EE significantly. It was found that the addition of poly(acrylic acid) (PAA) as a co-encapsulation factor did not have significant effect on the EE of the DOXY.

The diameter and the DOXY encapsulation efficiency of NS of four PLGA formulations with the same other processing parameters are listed in Table 2. Based on SEM measurement, the average diameter of the spheres increased with the increase of LA/GA ratio and/or PLGA molecular weight, with PLGA50-6.5K NS had the smallest diameter of 300 nm and PLGA85-142K NS had the largest diameter of 730 nm. Based on DLS measurement, the diameter of PLGA50-6.5K NS and PLGA 85-142K NS was 400 nm and 890 nm respectively in aqueous solution, possibly due to some degree of swelling or/and certain discrepancy between the two different methods. The changes in size and shape of similar PLGA spheres in aqueous solution over long-term culture were reported previously by our lab [40] and others [37]. Such PLGA spheres swelled first and then deformed from their original spherical shape into irregular shape with a rough surface texture over time. Eventually, they collapsed and disintegrated as the degradation continued. [40] EE of DOXY was not changed significantly among different PLGA NS formulations. The larger diameter of PLGA85-142K NS was possibly resulted from the higher viscosity of the DOXY/PLGA85-142K polymer emulsion compared to those of other polymers (Table 2).

3.2 In vitro DOXY release kinetics

In vitro release profiles of DOXY from PLGA NS with four PLGA formulations were displayed in Fig. 2A and release profiles of DOXY from NS-scaffolds were shown in Fig. 2B. Compared to release profiles of DOXY from PLGA NS alone, the release from the scaffolds showed a similar release pattern with decreased initial burst release and prolonged release period. In general, the DOXY release profiles of NS-scaffolds showed two phases: an initial burst release within the first day followed by a slow and sustained release over time. There were also significant differences in both the burst release rate and sustained release rate of different PLGA formulations. Both PLGA50-6.5K NS-scaffold and PLGA50-64K NS-scaffold released approximately 70% of DOXY within the first day and 95% of DOXY within two weeks, while the PLGA75-113K NS-scaffold (with a higher LA/GA ratio and higher molecular weight) had a lower burst release (50%) in the first day and a slower sustained release of DOXY (85% within two weeks, 95% within 6 weeks). The PLGA85-142K NS-scaffold (with the highest LA/GA ratio and the highest molecular weight) had the lowest burst release (25%) in the first day and kept a continuous slow release for longer than 6 weeks (80% within 6 weeks). This two-phase release profile of PLGA nanospheres, characterized by an initial burst release followed by a sustained slow

release, indicated that DOXY was dispersed in a polymeric matrix inside the PLGA nanospheres. [41,42] The initial burst release was reduced with the increase of the PLGA LA/GA ratio and the PLGA molecular weight. PLGA85-142K NS-scaffolds with the lowest burst release and the longest release period was selected for *in vitro* anti-bacterial test.

3.3 In vitro anti-bacterial effect of DOXY NS-scaffolds

The antibacterial activity of the released DOXY was investigated using bacteria inhibition experiments. *S.aureus* and *E.coli* were used in the current study since they represent the most common Gram-positive and Gram-negative bacteria in the human body. The growth of *S.aureus* and *E.coli* bacteria can be visualized directly from the plate to assess the anti-bacterial function of all scaffold groups (shown in Fig. 3). It was observed that the agar plates with the BSA scaffolds were totally covered with *S.aureus* or *E.coli* after 5 days of incubation. In contrast, the growth of *S.aureus* and *E.coli* were both inhibited at regions around the DOXY adsorbed scaffolds and DOXY NS scaffolds. The bacteria inhibited areas were much larger on the plates with the DOXY NS-scaffolds than DOXY adsorbed scaffolds.

The data from the long-term liquid bacterial culture (*S.aureus* data shown in Fig. 4 and *E.coli* data shown in Fig. 5) demonstrated that the antibacterial activity of DOXY lasted for more than 6 weeks in the NS controlled release group (showed 70% inhibition for *S.aureus* and 40% inhibition for *E. coli* at Day 42), while that of the adsorbed DOXY decreased rapidly after 14 days (showed 18% inhibition for *S.aureus* and 9% inhibition for *E.coli* at Day 42) and the BSA scaffold had no antibacterial effect even from the very beginning (showed less than 20% inhibition for *S.aureus* and less than 10% inhibition for *E. coli*). The antibacterial activity of the scaffold measured over time was the measurement of the overall antibacterial capacity of the scaffold, including the reduced DOXY amount released at a later time and possibly also some biological activity loss over time. These results indicated that the encapsulation of DOXY in the nanospheres not only can control the DOXY release rate and prolong its release duration but also retain its antibacterial activity.

4 Discussion

The main objectives of this work were to fabricate the DOXY loaded NS with good encapsulation efficiency, to incorporate the NS onto a nano-fibrous scaffold, to release DOXY in a controlled manner, and to retain the antibacterial activity. In previous publications from our lab, novel nano-fibrous scaffolds capable of delivering growth factors were successfully developed. [32,33] While water-in-oil-in-water (w/o/w) double emulsion method can be readily used to entrap proteins into nanospheres, it is difficult to entrap very hydrophilic small molecules such as doxycycline into NS with an acceptable encapsulation efficiency (less than 5% showed in Table 3) since the high solubility of the drug in the external phase and its small size makes it easy for the majority of the drug to diffuse to the external phase during the emulsion and solvent evaporation process and results in very low encapsulation efficiency. [42] Some researchers made modifications to w/o/w method [23] or used other encapsulation method [21] to achieve a higher DOXY encapsulation efficiency. However, the EE increase was limited and the size of the spheres obtained became much larger, usually in the range 100 μm . [23] The DOXY loaded spheres with this size may be used alone for antibacterial treatment, but they are too large to be incorporated into the nano-fibrous scaffolds. [34] overcome these limitations, a modified w/o/o emulsion and solvent evaporation method successfully adopted to encapsulate DOXY into spheres. By changing the external continuous phase from water to an organic solvent mixture, the diffusion of the drug was reduced due to the decreased solubility of the drug in the external phase, and the encapsulation efficiency was greatly improved. It was also discovered that the release profile of DOXY from the PLGA NS-scaffolds was mainly determined by the

release from NS which can be adjusted by tailoring the chemical composition and molecular weight of the PLGA copolymers. The PLGA85 scaffolds with the highest molecular weight and highest LA/GA had the lowest initial burst release (25%) and longest release duration of DOXY (longer than 6 weeks). This trend consistent with our lab's previous publications on the release profiles of growth factors NS-scaffolds. [32,33] The difference in release profiles of PLGA NS-scaffold was possibly resulted from both the difference in the NS diameter and PLGA degradation PLGA85-142K NS with the largest NS diameter had the smallest specific surface area and the longest average travel distance for the encapsulated DOXY to diffuse out of the spheres, resulting in the lowest burst release during the earlier release stage. It is also possible that the highest viscosity of PLGA85 polymer solution also reduced the diffusion of DOXY to outer oil phase during the emulsion and solvent evaporation process so that the least amount of the drug was simply absorbed on or near the NS surface, resulting in a reduced initial burst release. PLGA 85-142K NS also had the slowest degradation rate due to the highest molecular weight and LA/GA ratio, which contributed to the slower release rate of DOXY. It was discovered that incorporating DOXY loaded PLGA NS into PLLA nano-fibrous scaffold effectively reduced burst release and slowed down the DOXY release rate. The reduced burst release of DOXY from nano-fibrous scaffold may have resulted from the adsorption desorption of DOXY on the nanofibers before diffusing out of the scaffolds due to the high surface area ($\sim 100 \text{ m}^2/\text{g}$) of the nano-fibrous scaffolds.

The antimicrobial experiment demonstrated the biological function of the DOXY released from PLGA NS-scaffolds for a long duration (six weeks), which is a great improvement for the long-term treatment of dental, periodontal and bone infection or MMP-inhibition since the commercially available DOXY releasing gels typically release all DOXY within 2 weeks. [43] These novel DOXY NS incorporated nano-fibrous scaffolds were intended for tissue engineering applications where microbial infection may be involved. While the macro-porous and nano-fibrous scaffolds may promote the tissue regeneration, the DOXY NS in the scaffolds may also protect the tissue from microbial invasion. Since DOXY is also an inhibitor of multiple MMPs such as MMP-8,-9 and -13, DOXY NS-scaffold may also be able to protect engineered bone from MMP destruction in situations with chronic inflammation in diseases such as periodontitis and rheumatoid arthritis. [44]

The 3-D NS-scaffolds developed in this work have shown the capability of controlled delivery of DOXY and inhibition of bacterial growth for a prolonged period. This success has demonstrated the scaffolds to be capable of controlled delivering small hydrophilic drug molecules as well as large proteins. With the versatile delivery capability and macro-porous and nano-fibrous structure, the scaffolds may allow for the implementation of more comprehensive strategies in tissue engineering.

Conclusion

Results of *in vitro* release and anti-bacterial experiments suggest that the developed drug-containing nano-fibrous scaffolds are capable of effectively delivering doxycycline in a controlled fashion with prolonged duration. These biodegradable PLLA scaffolds have well interconnected macroporous and nano-fibrous structure and can inhibit common bacterial growth for more than 6 weeks with the incorporation of DOXY containing PLGA NS. Release kinetics from the scaffolds was found to be determined by PLGA formulation. The incorporation of the NS into scaffolds reduced the burst release. PLGA85-142K NS-scaffold has the lowest initial burst release and the longest release period compared to other formulations investigated. Taken together with earlier studies, the NS-scaffold system can be used as a delivery system for both large proteins and small drug molecules for various complex tissue engineering applications.

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Fig.1.

Characterization of PLGA85-142K Nanospheres (NS) and PLLA nano-fibrous scaffold (NS scaffold) before and after nanospheres incorporation. (A) SEM view of DOXY containing PLGA 85-142K NS; (B, C) SEM views of the PLLA scaffold before NS incorporation at 100x and 10,000x magnifications respectively; (D, E) SEM views of PLLA scaffold after NS incorporation at 100x and 10,000x magnifications respectively.

Fig.2.

In vitro release kinetics of DOXY from NS (A) and from NS incorporated nano-fibrous PLLA scaffolds (B): in 10 mM PBS with DOXY loading of 100 μg /scaffold. Each data point represents a mean $\pm$ standard deviation (n=3).

Fig.3.

Agar petri dish cultivated with *S. aureus* (upper) and *E. coli* (lower) with scaffold samples in the center after 5 days of incubation. Scaffolds on the left side were seeded with BSA containing NS. Scaffolds in the center absorbed 100 μ g DOXY. Scaffolds on the right side were seeded with NS containing 100 μ g DOXY.

Fig.4.

Long term *S. aureus* growth inhibition test for release solution from three groups of scaffolds. Each data point represents a mean $\pm$ standard deviation (n=3).

Fig.5. Long term *E. coli* growth inhibition test for release solution from three groups of scaffolds. Each data point represents a mean $\pm$ standard deviation (n=3).

Table 1

Effects of various processing parameter on encapsulation efficiency of DOXY in PLGA 85-142K nanospheres. Each EE data point represents a mean $\pm$ standard deviation (n=3). Note: w: water phase; o₁: inner oil phase (PLGA solution); o₂: outer oil phase (mineral oil solution)

PLGA85-142K Concentration	w/o ₁	o ₁ /o ₂	PAA concentration	EE (%)
3%	1:10	1:30	0	13.3 $\pm$ 5.2
5%	1:10	1:30	0	15.3 $\pm$ 4.1
8%	1:10	1:30	0	25.3 $\pm$ 3.0
10%	1:10	1:30	0	25.9 $\pm$ 3.9
8%	1:4	1:30	0	8.6 $\pm$ 2.1
8%	1:5	1:30	0	10.8 $\pm$ 3.2
8%	1:10	1:30	0	25.3 $\pm$ 3.0
8%	1:20	1:30	0	25.4 $\pm$ 3.5
8%	1:10	1:10	0	22.6 $\pm$ 4.8
8%	1:10	1:15	0	24.4 $\pm$ 3.8
8%	1:10	1:30	0	25.3 $\pm$ 3.0
8%	1:10	1:30	2%	24.4 $\pm$ 3.3
8%	1:10	1:30	4%	23.5 $\pm$ 4.5
8%	1:10	1:30	8%	23.1 $\pm$ 5.6
8%	1:10	1:30	16%	25.7 $\pm$ 3.1

Table 2

Effects of PLGA composition on sphere diameter with the same other processing parameters (8% polymer concentration, 4% PR-15, 1:10 W/O₁, 1:30 O₁/O₂). Each data point represents a mean $\pm$ standard deviation. Sphere diameters were measured using SEM.

Polymer	LA/GA ratio	Mw (kDa)	EE (%)	Sphere diameter(nm)
PLGA 50-6.5K	50/50	6.5	22.1 $\pm$ 4.8	300 $\pm$ 110
PLGA 50-64K	50/50	64	24.4 $\pm$ 3.5	520 $\pm$ 150
PLGA 75-113K	75/25	113	23.1 $\pm$ 4.4	570 $\pm$ 130
PLGA 85-142K	85/15	142	25.3 $\pm$ 3.0	730 $\pm$ 160

Table 3

Encapsulation efficiencies (10% polymer concentration, 1% PVA, $w_1/o = 1:10$, $o/w_2 = 1:30$). Each data point represents a mean $\pm$ standard deviation. Note: w_1 : aqueous drug solution, o : PLGA solution, w_2 : aqueous PVA solution.

Polymer	LA/GA ratio	Mw (kDa)	EE (%)	Sphere diameter(nm)
PLGA 50-6.5K	50/50	6.5	3.2 $\pm$ 1.8	280 $\pm$ 70
PLGA 50-64K	50/50	64	2.7 $\pm$ 0.5	320 $\pm$ 100
PLGA 75-113K	75/25	113	3.1 $\pm$ 1.4	370 $\pm$ 90
PLGA 85-142K	85/15	142	2.3 $\pm$ 1.0	410 $\pm$ 120