Title: Sustained antibacterial activity of doxycycline-loaded poly(D,L-lactide-co-glycolide) and poly(ε-caprolactone) nanoparticles
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Doxycycline (DXY) is a well-known broad-spectrum semisynthetic antibiotic, derived from oxytetracycline (Figure 1) [1] . It is bacteriostatic, inhibiting bacterial protein synthesis by disrupting tRNA and mRNA at the ribosomal sites [2] . DXY is used against a wide range of Gram-positive and Gram-negative bacteria such as *Escherichia coli* , *Hemophilus influenzae* , *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, *Chlamydia psittaci* and *Chlamydia trachomatis* . It is prophylactic against *Bacillus anthracis* (anthrax) and also malaria and filaria. DXY is frequently used to treat chronic prostatitis, sinusitis, syphilis, Chlamydia and pelvic inflammatory diseases. It is efficiently used in the treatment of bubonic plague (caused by *Yersinia pestis*), Lyme disease, ehrlichiosis and Rocky Mountain spotted fever [3] .

The most important drawback of current antibiotic therapy is their limited ability to reach their site of action. They also have other limitations such as instability in the biological environment and premature loss through rapid clearance and metabolism [4] . Moreover, high concentrations of these agents may be toxic to healthy tissues. Thus, to increase the therapeutic efficacy of these antibiotics, modern drug-delivery systems play an important role in controlled delivery of these agents to the target site of the body at a therapeutically optimal rate and concentration. These controlled release systems are able to maintain optimum therapeutic drug concentration in the blood with minimum fluctuation giving predictable and reproducible release rates for a longer period of time, enhancing the duration of activity of drugs with a short half-life, eliminating the side effects of frequent dosing and limiting wastage of drugs, and providing an optimized therapy and improved patient compliance [5] . All of the features are strictly related to the nature of materials that constitute the continuous matrix of the delivery system. Various types of functional nanosystems, such as carbon nanotubes, quantum dots, polymeric micelles, dendrimers, metallic nanoparticles, solid lipid nanoparticles and liposomes, are being extensively explored for their potential ability to provide a controlled release of encapsulated drugs [6] . Other nanocarriers such as solid lipid nanoparticles or liposomes have drawbacks including a limited drug loading capacity and drug expulsion during storage [7,8] . Polymeric nanoparticles offer an attractive alternative approach for drug delivery owing to their biocompatibility, nonimmunogenicity, nontoxicity, biodegradability, simple preparation methods, physicochemical stability and drug-targeting properties [9,10] . Polymeric nanoparticles enable the drug to be released efficiently, releasing the drug from the polymeric matrix by diffusion, swelling or polymer erosin, or a combination of these processes [5] . These polymeric nanoparticles are being used for the efficient delivery of 5-flurouracil, periodontal delivery of tetracycline and insulin delivery [5] . Recently, nanoparticle-encapsulated antibiotics were reported to improve efficacy, reduce toxicity, prolong half-lives and alter the tissue distribution of these antibiotics [11] . The effectiveness of nanoparticles in eradicating *Helicobacter pylori* was studied by Umamaheshwari *et al.* using

mucoadhesive gliadin nanoparticles containing amoxicillin [12] . Patil *et al.* have encapsulated DXY in nanosized hydrophilic polymer-lipid combination particles (LIPOMER) with a high entrapment efficiency (91%) and postulated that the combination of a hydrophilic polymer with lipid would permit a high drug entrapment efficiency due to the hydrophilic polymer. They have evaluated the splenotropic potential of these DXY-containing LIPOMER in the treatment of intracellular splenic infection [13] . Zeidner and coworkers reported the advantage of sustained release formulation of DXY over the oral administration for the prophylaxis of tick bite infection of *Lyme Borelliosis* [3] . Goodson *et al.* have developed a tetracycline-loaded ethylene vinyl acetate fiber formulation which showed sustained release of tetracycline for up to 9 days. They used this formulation against *Bacillus cereus* [14] .

A number of different polymers, both synthetic and natural, have been used in formulating biodegradable polymeric nanoparticles. Different synthetic polymers such as aliphatic polyesters (e.g., poly(ε-caprolactone) (PCL), poly(3-hydroxybutyrate) (PHB), poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and its copolymers with glycolic acid i.e., poly(D,L-lactide-co-glycolide) (PLGA) [15-18]) are mostly used in preparation of polymeric nanoparticles. These synthetic polymers are capable of giving sustained release of the encapsulated therapeutic agent for a longer period of time [19] . PLGA provides higher degradation rates from months to years depending upon its composition and molecular weight [15,20] . PCL is an important member of the aliphatic polyester family, and can be used in combination with several polymers to formulate different formulations. It has been shown that the entrapment of hydrophilic drugs inside hydrophobic polymeric nanoparticles is a difficult task [21-23] . This is because the entrapment efficiency of the hydrophilic drug inside the hydrophobic nanoparticle is low due to the low affinity of hydrophilic drugs for hydrophobic polymers. Furthermore, the interaction between the polymer and the entrapped drug is weak and the drug has a tendency to move from the organic phase to the outer aqueous phase during nanoparticle formation [24] . It has been demonstrated that when biodegradable PCL is used in combination with PLGA in different ratios we can vary the size, entrapment efficiency and drug release of different formulations [25] .

As our aim is to develop a controlled release DXY-loaded nanoparticle formulation, the objective of this study is to encapsulate DXY inside PLGA:PCL nanoparticles and to increase the entrapment efficiency by standardizing different parameters, for example polymer ratio (PLGA:PCL ratio), amount of drug loading, solvent selection, pH value of inner aqueous phase and addition of salt to outer aqueous phase. The present study included preparation and characterization of DXY-loaded PLGA:PCL nanoparticles. The DXY-loaded nanoparticles were used against DXY-sensitive bacteria such as *E.coli* (DH5[alpha]) to confirm its antibacterial activity. The stability and sustained activity of the DXY-loaded nanoparticles was observed in the *E. coli* culture and the results were compared with the native form of the drug.

Materials & methods

* Materials

Doxycycline was received as a gift sample from Vetcare R&D Centre, Bangalore, India. PLGA (copolymer ratio 50:50, inherent viscosity [IV] = 0.55-0.75 dl/g, MW = [proportional to]50,000 g/mol) and PCL (IV = 1.08 dl/g, MW = [proportional to]60000 g/mol) were purchased from Birmingham Polymers, Inc. (Birmingham, AL, USA). Polyvinyl alcohol (PVA; average MW = 30,000-70,000) was purchased from Sigma-Aldrich Co (St Louis, MO, USA), and Muller-Hinton broth (MHB), Agar powder and Luria Broth were all purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India). Sodium chloride, disodium hydrogen phosphate and potassium chloride were obtained from Sigma Aldrich Chemicals (Germany). Potassium hydrogen phosphate was purchased from Qualigens Fine Chemicals (Mumbai, India). Chloroform ($CHCl_3$), dichloromethane (DCM), methanol, acetonitrile and acetic acid were purchased from E-merk (India). All

reagents used were of analytical grade from E-merk.

*** Preparation of DXY-loaded nanoparticles**

Doxycycline-loaded PLGA:PCL nanoparticles were prepared by the water-in-oil-in-water double emulsion solvent evaporation method with a few modifications [26] . In this method, DXY (equivalent to 20% w/w dry weight of polymer) was dissolved in 300 µl of phosphate-buffered saline (0.01 M, pH 7.4) to form DXY aqueous solution. The DXY aqueous solution was emulsified in an organic phase consisting of 90 mg of polymer (PLGA:PCL in different ratio) dissolved in 3 ml of organic solvent ($CHCl_3$ /DCM) to form a primary water-in-oil emulsion. The emulsion was further emulsified in an aqueous PVA solution (12 ml, 2% w/v) to form a water-in-oil-in-water emulsion. The emulsification was carried out using a microtip probe sonicator (VC 505, Vibracell Sonics, Newton, MA, USA) set at 55 W of energy output for 2 min over an ice bath. The emulsion was stirred for 2 h on a magnetic stir plate at room temperature to allow the evaporation of organic solvent. Further 1 h vacuum drying was also performed to remove any residual organic solvent present. Any excess amount of PVA was removed by ultra-centrifugation at 40,000 r.p.m. at 4°C for 20 min (Sorvall Ultraspeed Centrifuge, Kendro, USA) followed by washing with double distilled water. The supernatant was collected and kept for an estimation of the amount of drug which was not encapsulated. The recovered nanoparticulate suspension was lyophilized for 2 days (-80°C and <10 µm mercury pressure, LYPHLOCK 12, Labconco, Kansas City, MO, USA) to provide lyophilized powder for further use. To achieve the high entrapment efficiency, we prepared different formulations by varying different parameters:

* The polymer ratio (PLGA:PCL) was varied from 100:0, 80:20, 70:30 and 60:40 while keeping other parameters constant (for further experiments only the polymer ratio that exhibited the lowest size combined with the highest entrapment efficiency will be chosen);

* The solvent was changed from $CHCl_3$ to DCM with a constant polymer ratio (PLGA:PCL, 80:20, the ideal polymer ratio) without varying other parameters;

* pH of inner aqueous phase was changed from 7.4 to 4 and 4% NaCl was added to the outer aqueous phase keeping other parameters such as polymer ratio (PLGA:PCL, 80:20), solvent (DCM) and drug loading (20% w/w) constant;

* Finally the amount of drug loading was varied from 20, 30 and 60% (w/w) dry weight of polymer keeping polymer ratio (PLGA:PCL, 80:20), solvent (DCM), pH (4) and NaCl (4%) concentration constant.

*** Characterization of DXY-loaded nanoparticles**

Particle size analysis & ζ-potential measurement

To determine the particle size and ζ-potential, 1 mg/ml of nanoparticle solution was prepared in double distilled water. A total of 100 µl of the sample was diluted to 1 ml, sonicated in an ice bath for 30 s and subjected to particle size and ζ-potential measurement using a ζ-sizer (Zetasizer Nano, Nano ZS, ZEN3600, Malvern Instrument, UK).

Scanning-electron microscopic studies

The surface morphology of nanoparticles was characterized by scanning-electron microscope (JEOL JSM-T220A scanning electron microscope, JEOL Ltd, Japan) operating at an accelerating voltage of 10-30 kV. The nanoparticles were sputtered with gold to make them conductive and placed on a copper stub prior to the acquisition of scanning-electron microscope images.

Fourier transform infrared spectral studies

To investigate the possible chemical interactions between the drug and the polymer matrix, Fourier transform infrared spectroscopy (FTIR) spectra were taken on the Perkin Elmer spectrometer (Model Spectrum 1, Perkin Elmer, USA). Samples were crushed with KBr to produce pellets by applying a pressure of 300 kg/cm^2 . FTIR spectra of void nanoparticles, native DXY and DXY-loaded nanoparticles were scanned in the 4000-500 cm^{-1} range.

Differential scanning calorimetric studies

The physical state of DXY encapsulated in nanoparticles was characterized using a differential scanning calorimetric (DSC) thermogram analysis (DSC-50, Shimadzu, Kyoto, Japan). Each sample (8 mg of DXY, void nanoparticles and DXY-loaded nanoparticles) was sealed separately in a standard aluminum pan, the samples were purged in DSC with pure dry nitrogen set at a flow rate of 10 ml/min, the temperature speed set at 10°C/min and the heat flow recorded from 0 to 350°C.

High performance liquid chromatography method

The DXY content in nanoparticles was estimated by the reverse-phase isocratic mode of high performance liquid chromatography (HPLC) according to Ruz *et al.* [27] with slight modifications. Agilent 1100 was used (Agilent Technologies, Waldbronn Analytical Division, Germany), which consists of columns (Zorbax Eclipse XDB-C18, 150 × 4.6 mm, internal standard) with an internal standard of dimethylphthalate. A total of 20 µl of the sample was injected manually in the injection port and was analyzed using a mobile phase of 5% acetic acid-acetonitrile-methanol (55:25:20) (v/v/v). Separation was achieved by isocratic solvent elution at a flow rate of 0.5 ml/min with a quaternary pump (Model No G1311A) at 10°C with ThermoStart (Model No G1316A, Agilent Technologies, USA). The DXY level was quantified by UV detection at 347 nm (with DAD, Model No G1315A). The amount of DXY in the sample was determined from the peak area correlated with the standard curve. The standard curve of DXY was prepared under identical conditions.

Entrapment efficiency

The amount of encapsulated drug was calculated indirectly by HPLC. To measure the drug concentration in the supernatant (as collected at the time of centrifugation mentioned during particle preparation), 20 µl of this supernatant was injected manually for analysis. The amount of drug present was calculated by using the standard plot of DXY prepared in HPLC. The amount of drug present in the nanoparticles was calculated by subtracting the amount of drug in the supernatant from the total amount of the drug used in the formulation. The entrapment efficiency was calculated by the following equation:

[Formula omitted]

***In vitro* release of DXY from nanoparticles**

The *in vitro* release kinetics of DXY from the nanoparticles was determined in phosphate-buffer saline (0.01 M, pH 7.4) at 37°C with few modifications [26] . A total of 10 mg of nanoparticles was suspended in 3 ml of phosphate-buffered saline. The nanoparticle suspension was equally divided in three tubes containing 1 ml each. These tubes were kept in a shaker at 37°C and 150 r.p.m. (Wadegati Labequip, India). At particular time intervals these tubes were taken out from the shaker and centrifuged at 13,800 r.p.m. at 4°C for 10 min (SIGMA 3K30, Germany). The supernatants were taken out to estimate the amount of drug released at that particular time. The same amount of fresh phosphate-buffered saline was added to the residue and kept in the shaker for further release kinetics study.

Antibacterial activity studies

The antibacterial activity of DXY on a strain of *E. coil* (DH5[alpha]) was tested by well diffusion. Bacterial suspensions with a cell density equivalent to 0.5 McFarland (1.5×10^8 colony-forming units [CFU]/ml) were transferred individually onto the surface of Muller-Hinton agar plates using sterile cotton swabs. Wells were prepared by punching a sterile cork borer onto the agar plates; 50 µl of DXY (10 µg/ml) in MHB was added to these wells and incubated at 37°C overnight. The following day the inhibition zones around the wells were observed by the naked eye.

Determination of minimum inhibitory concentration & minimum bactericidal concentration of DXY

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by microdilution [28] . Briefly, DH5[alpha] bacterial culture containing 0.5 McFarland (1.5×10^8 CFU/ml) of organisms in Luria broth was added to various concentrations of DXY and DXY-loaded nanoparticles ranging from 0.1 to 8 µg/ml. The MIC concentration of DXY was defined as the lowest concentration inhibiting visible growth of bacteria after overnight incubation of the cultures at 37°C. The MBC is measured by subculturing the broths used for MIC determination onto fresh agar plates. The MBC is the lowest concentration of a drug that results in killing 99.9% of the bacteria being tested.

Comparison of the stability of native DXY & DXY-loaded nanoparticles

The stability of DXY and DXY-loaded nanoparticles in the medium was studied for 10 days. To test this, 1 ml of MHB was taken per tube in two sets (ten tubes each). To one set, 10 µg of native DXY and to another set 10 µg of DXY-loaded nanoparticles were added. On day 1, 20 µl of bacterial culture was added to the first tubes of both sets containing DXY and DXY-loaded nanoparticles and incubated overnight at 37°C in a shaker. The next day both the tubes were plated on Luria agar plates by 10^4 time serial dilution and incubated overnight at 37°C to see bacterial growth in agar plates. This experiment was repeated for all the tubes up to 10 days and each day fresh culture was added and plated the next day to see bacterial growth [4] .

Effect of native DXY & DXY-loaded nanoparticles on bacterial growth kinetics

To compare the effectiveness of native DXY and DXY-loaded nanoparticles on the growth kinetics of bacteria, we have taken two different concentrations (0.1 and 0.2 µg/ml) of native DXY and DXY-loaded nanoparticles in different tubes. MHB and void nanoparticles were taken as a control for this experiment. On the first day, 20 µl of DH5[alpha] bacterial culture containing 0.5 McFarland (1.5×10^8 CFU/ml) of organisms was added to all the tubes. The tubes were incubated at 37°C in an incubator (150 r.p.m.) for 24 h. The following day serial dilution followed by spread plating was performed on Luria agar plates and they were incubated at 37°C overnight. After 24 h the colonies were counted by the naked eye. On the second day, the tubes were again plated to see the bacterial growth kinetics. The experiment continued in this way for 5 days. Growth graphs were plotted by taking the number of colonies versus the number of days for comparing the action of native DXY with DXY-loaded nanoparticles on the growth of *E. coli* [29] .

Statistical analysis

One-way analysis of variance was performed for comparison of the results. P-values less than 0.05 were considered significant.

Results & discussion

*** Physiochemical characterization of nanoparticles**

Particle size is an important parameter as it has a direct effect on the stability, cellular uptake, drug release and biodistribution. The mean particle size of the nanoparticle is a function of the polymer composition. The

results of the mean particle size, as measured by laser light diffraction, was found to range from 230 to 360 nm (Figure 2). The ζ-potential values for different formulations of nanoparticles ranged from -10 to -20 mV. The nanoparticles were negatively charged owing to the presence of ionized carboxyl groups on their surface [26] . The particles were spherical with a smooth exterior as demonstrated by scanning-electron microscopy (Figure 3). Studies conducted by Mundargi *et al.* have shown that microparticles prepared with only PCL-loaded DXY had a very low entrapment efficiency ([proportional to]9%) [1] . Zhu *et al.* showed that ibuprofen when encapsulated in PCL microspheres had an entrapment efficiency of 25.4% [30] . The fact that the entrapment efficiency of the hydrophilic drug loaded in PCL nanoparticles/microparticles is low was also supported by Hombreiro *et al.* who reported that propranolol HCl-loaded PCL microparticles exhibited a low entrapment efficiency of 37% [31] . Therefore, we prepared different formulations by taking PLGA and PCL in different ratios (100:0. 80:20, 70:30 and 60:40) and compared their size and entrapment efficiency. It was observed that with increasing concentration of PCL in the formulation (80:20, 70:30 and 60:40), the size of the nanoparticles decreases along with a considerable decrease in entrapment efficiency (Table 1). The size of the particle decreases because of the higher flexibility of the PCL chains and the carbonyl groups present in PCL may form hydrogen bonds with the PLGA chain, forming smaller-sized particles [1] . The low entrapment efficiency of DXY in the formulation (as we increase the concentration of PCL) may be due to the high diffusion rate of DXY in PCL during solvent evaporation [1] . Smaller particle size may also be the reason for the lower entrapment efficiency of DXY in nanoparticles. As shown previously, the 80:20 ratio of PLGA:PCL produced the smallest size nanoparticles and highest entrapment efficiency, and thus was selected for further studies.

The polarity of the organic solvent used in the emulsion formation during the nanoparticle formulation might affect the entrapment efficiency. Therefore, nanoparticles with two different organic solvents, $CHCl_3$ and DCM, were formulated under identical conditions. With $CHCl_3$ the particles were round in shape but the entrapment efficiency was slightly lower than with DCM, as shown in Table 1. In the solvent $CHCl_3$, the entrapment efficiency of PLGA: PCL (80:20) was found to be 32%, while when the solvent was changed to DCM the entrapment efficiency increased to 47%. The main reason for this difference was probably due to fewer $CHCl_3$ particles diffusing to the aqueous phase as compared with DCM. It has been reported that DCM is slightly more hydrophilic than $CHCl_3$, as DCM is soluble in approximately 50 parts of water while $CHCl_3$ is soluble in approximately 200 parts of water [26] , and the polymer precipitates faster in DCM compared with $CHCl_3$. Thus, DCM is the more appropriate solvent to use when encapsulating hydrophilic drugs in a hydrophobic polymer [32,33] .

The choice of polymer ratio (PLGA:PCL 80:20) and solvent (DCM) helped to increase the entrapment efficiency of DXY from 32 to 47% (Table 1). To improve the entrapment efficiency of the nanoparticles further, three more additional parameters were taken into consideration. Previously, Peltonen *et al.* have studied all the three parameters:

* Changing the pH of the inner aqueous phase (in which the drug is dissolved);

* Electrolyte addition to the outer aqueous phase in the double emulsion;

* Amount of drug loading (w/w). The entrapment efficiency of nanoparticles was increased substantially in PLA nanoparticles loaded with the hydrophilic drug sodium cromoglycate [24] .

In our formulations (PLGA:PCL ratio of 80:20 and DCM as the solvent), by changing the pH of the inner aqueous phase from 7.4 to 4 and by adding 4% w/v NaCl to the outer aqueous phase the entrapment efficiency increased from 47 to 70% (Table 1). The pH of the water phase affects the ionization of the drug substance and, hence, the solubility. An ionic drug substance is likely to stay in the water phase, while the

molecular form is more likely to be attached to the hydrophobic polymer phase, and, in this case, the drug substance is more efficiently encapsulated [24] . Based on this finding, by simply adjusting and controlling the pH value, the entrapment efficiency of DXY inside nanoparticles can be increased. By changing the pH of the inner aqueous phase from 7.4 to 4 the drug is dissolved in this acidic aqueous solution and does not diffuse to the outer aqueous phase. As a result the drug may be more easily entrapped in the polymeric matrix leading to a higher entrapment efficiency of nanoparticles [23,34] . Addition of an electrolyte affects the osmotic gradient between the inner and outer aqueous phases; this may have an impact on drug entrapment. With the addition of salt, the concentration of the outer aqueous phase (PVA solution) increases and becomes hypertonic; therefore, the drug does not diffuse into the outer aqueous phase and remains in the polymeric matrix [17,35] .

For enhanced drug entrapment, varying concentrations of DXY were incorporated into nanoparticles. The percentage of drug loading was varied from 20 to 60% (w/w), corresponding to the amount of polymer dry weight (Table 1). The entrapment efficiency of the nanoparticles was found to be highest with 20% DXY loading. With an increase in drug loading up to 60% the entrapment efficiency of nanoparticles slightly decreases. This may be due to the saturation level of DXY inside the nanoparticles after 20% drug loading. As the amount of drug loading increases, a more porous polymeric matrix structure may be formed with a large number of channels and hollow spaces, through which the drug could easily escape to the outer phase thereby decreasing the content of drug inside the polymeric matrix [36] . Furthermore, owing to the increased concentration of drug inside the polymer, a difference in osmotic pressure between the outer and inner aqueous phase results which may cause the drug to escape from the inner aqueous phase [37] . Thus, by changing the pH and by adding an electrolyte, the entrapment efficiency of the formulation consisting of 20% drug loading, a PLGA:PCL ratio of 80:20 with DCM as the organic solvent can be enhanced to a maximum value of 70%. In summary, Mundargi *et al.* have prepared DXY-loaded PLGA:PCL microspheres with an entrapment efficiency of up to 24.7% [1] but by changing different parameters we successfully incorporated the hydrophilic drug DXY into a hydrophobic polymer with a high entrapment efficiency of 70%.

*** Fourier transform infrared spectroscopy studies**

Fourier transform infrared spectroscopy results confirmed the chemical stability of DXY in the nanoparticles. Figure 4 shows the FTIR spectra of void nanoparticles, native DXY and DXY-loaded nanoparticles. Native DXY showed characteristi c bands due to different functional groups. The band at 3388 cm^{-1} is due to O-H/N-H stretching vibrations, while those observed at 2924 and 2855 cm^{-1} are due to the C-H stretching vibrations. Bands at 1673 and 1583 cm^{-1} are due to primary amide (N-H) bending and aromatic N-H bending vibrations, respectively. Carbonyl (C=O) stretching vibrations are seen at 1615 cm^{-1} , while the bands at 1458 and 1328 cm^{-1} are due to $-CH_2$ bending and C-H bending vibrations, respectively. The bands at 1219 and 1171 cm^{-1} belong to C-N stretching vibrations. The bands that occurred in void nanoparticles are almost identical to the bands in DXY-loaded nanoparticles, in addition to some extra bands due to DXY. The bands that appeared at 2853, 2923, 1628 and 1131 cm^{-1} for native DXY appeared in DXY-loaded nanoparticles (with minor shifting), indicating the chemical stability of DXY inside the nanoparticles.

*** Differential scanning calorimetry studies**

Using differential scanning calorimetry the nature of the drug encapsulated in the nanoparticles was analyzed. This analysis was performed on native PLGA, native PCL, native DXY, void nanoparticles and DXY-loaded nanoparticles (Figure 5). Different compounds show their characteristic peaks in DSC. The endothermic peaks of the PLGA and PCL polymers were found at approximately 48 and 58°C, respectively, due to the glass transition temperature (Tg) of PLGA and melting temperature of PCL [1] . The peaks of PLGA and PCL were slightly shifted in drug-loaded nanoparticles, as compared with that of native PLGA and PCL. The

endothermic peak of native DXY was found to be at approximately 198°C. DSC thermogram of DXY-loaded nanoparticles did not show the native DXY peak at 198°C, while the endothermic peaks of PLGA and PCL were present distinctly in the formulation. The absence of detectable crystalline domains of DXY in the drug-loaded nanoparticles clearly indicates that DXY-loaded nanoparticles are in the amorphous or disordered-crystalline phase or in the solid-state solubilized form in the polymer matrix [38] . The DSC curves of void nanoparticles are almost identical to PLGA and PCL polymer curves, indicating no influence of the organic solvents on the thermal properties of PLGA and PCL. Thus, the displacement of the peaks of drug-loaded nanoparticles is mainly caused by the presence of DXY in the nanoparticles.

*** *In vitro* release behavior**

The *in vitro* release behavior of DXY-loaded nanoparticles is summarized as the cumulative percentage release of DXY, as shown in Figure 6. An initial burst release of the drug was observed during the first 24 h where more than 50% of the encapsulated drug was released. This was followed by a slower release of DXY leading to a release of 90% of entrapped drug after 15 days. Release of a drug from nanoparticles involves three phases. The initial burst release or the fast release of the drug may be due to the diffusion of DXY adsorbed at or just beneath the surface of the nanoparticles [39] . Studies conducted by Esmaeili *et al.* have shown that release of rifampicin from PLGA nanoparticles was rapid, with an initial burst release of 14% followed by a slower and constant release thereafter. The slow and constant release of DXY in our study after the initial burst release is mainly due to the slow diffusion of drug molecules through the polymeric matrix of the nanoparticles [40] . Finally, the slower and sustained release of the drug at later stages can be attributed to the diffusion/erosion of the polymeric matrix that releases the encapsulated drug [41] .

*** Antibacterial activity studies**

Doxycycline is a bactericidal antibiotic, with a wide spectrum of activity. It inhibits bacterial protein synthesis by disrupting tRNA and mRNA at the ribosomal sites. The antibacterial property of DXY was tested on the DH5[alpha] strain of *E. coli* by well diffusion. It was found that the DH5[alpha] strain was susceptible to DXY because a distinct inhibition zone was formed around the wells. The MIC of bacteria was found to be 6 µg/ml for native DXY and 4 µg/ml for DXY-loaded nanoparticles in our study. The MBC is measured by subculturing the broths used for the MIC determinations onto fresh agar plates. MBC was 8 µg/ml for native DXY and 6 µg/ml for DXY-loaded nanoparticles. It is observed that MBC values are higher than the MIC values, suggesting that a higher concentration of drug is required to kill the bacteria completely. However, it is noteworthy that even though a 8 µg/ml concentration of native DXY killed 99.9% of bacteria in our study, it was shown that microbes multiplied when transferred to a fresh medium, thus indicating that even at a higher dose the antibiotics are not able to inhibit growth of bacteria completely. The native drug gradually loses its effect after 24 h and bacteria that escaped drug action can multiply at a faster rate when given suitable conditions. Therefore, a sustained release formulation is required that can control the growth of bacteria for a longer period of time [29] . However, it is worth mentioning that MIC and MBC values in nanoparticles were less than that of native DXY in our study. This result is similar to results obtained by Esmaeili *et al.* where the MIC was found to be 0.008 µg/ml for free rifampicin and 0.002 µg/ml for rifampicin-loaded nanoparticles. The reason may be due to better penetration of smaller nanoparticles into the bacterial cells and better delivery of DXY to its site of action [40] .

To prove that native drugs lose their potency over time a comparative stability experiment was performed in which the stability of DXY and DXY-loaded nanoparticles in the medium was studied for 10 days and the drug was tested on bacterial culture (Figure 7). It was observed that no bacterial growth was found in the medium of both native DXY and DXY-loaded nanoparticles on the first day. On the third day, results showed growth in native DXY but no growth in the DXY-loaded nanoparticles. On the fifth day numerous colonies were found with native DXY but DXY-loaded nanoparticles showed no growth. On the tenth day, very few

colonies were found in the DXY-loaded nanoparticles, while there was profuse growth with native DXY. This experiment showed that native DXY is effective for only 2 days where as the nanoparticle formulation is effective for up to 10 days. This shows that drugs in their native form lose their stability and hence their antibacterial property reduces after 2 days, which is confirmed by a higher number of bacterial colonies. As the DXY-loaded nanoparticles provide a controlled release of the encapsulated drug for a longer period of time these are more effective than native DXY for up to 10 days when used against the *E.coli* bacterial strain (Figure 7).

To further confirm the fact that a sustained release formulation of DXY has a more profound bactericidal effect than native DXY on the growth kinetics of *E. coli* , a viable colony count method was performed. Figure 8A shows that on the first day 0.1 μg/ml of native DXY is more effective than the same concentration of drug in the nanoparticulate formulation, as evident from colony numbers (shown in Table 2). On the second day, the colony number increased by 2.4-fold in native DXY whereas in the DXY-loaded nanoparticles there was a decrease in colony number (by 1.9-fold) when compared with the plate count results from the first day. A similar plate count of the third day samples showed a steep increase in the number of bacterial colonies with native DXY while there was a decrease in number of colonies with DXY-loaded nanoparticles. On the fifth day a decrease in the number of colonies was observed in all the tubes including both the controls. This may have been due to completion of the bacterial lifecycle. A difference in viable bacterial number was observed because 0.1 μg of native DXY was able to kill a maximum number of bacteria in the medium on the first day. However, DXY-loaded NPs were not able to release the entire encapsulated drug on the first day, thus a higher number of colonies were observed on the agar plates. Following the sustained release pattern, the drug which was released from the nanoparticle (<0.1 μg) was effective but not as much as the native drug. By the second day the native drug had started losing its activity, which was indicated by an increase in the number of bacteria, but the NP was able to control the multiplication of the bacterial strain as the amount of drug released from the nanoparticle was enough to prevent further growth of the bacteria. On the third day the native drug had completely lost its antibacterial activity but the drug-loaded nanoparticle was able to prevent the bacteria from further multiplication. To confirm these results we have repeated the aforementioned experiment using a higher concentration of DXY and DXY-loaded NPs (0.2 μg/ml) and found similar results (Figure 8B). The noncytotoxic nature of void nanoparticles, which served as a control, was confirmed by Figure 8C. It was observed that the colony numbers are approximately the same in both the controls (MHB only and void nanoparticles). Thus, the antibacterial studies conducted on *E. coli* proved that DXY-loaded nanoparticles have a higher antibacterial activity than native DXY. Drugs in their native form may become less effective over a short period of time, however, nanoparticles remain effective for a longer period of time owing to the sustained release of the drug.

Conclusion

This study demonstrates that the entrapment efficiency of hydrophilic drugs in hydrophobic polymeric nanoparticles can be increased by varying the different formulation parameters. Varying parameters such as the polymer ratio of PLGA:PCL (from 100:0 to 80:20), organic solvent used (from $CHCL_3$ to DCM), pH (from 7.4 to 4), drug loading (from 60 to 20%), and by adding an electrolyte (NaCl 4%) enhanced the entrapment efficiency of the model hydrophilic drug DXY by up to 70%. The antibacterial properties of free DXY and DXY-loaded nanoparticles were evaluated using the DH5[alpha] strain of *E.coli*. DXY-loaded nanoparticles were stable for 10 days while native DXY loses its stability and its antibacterial property after 2 days; hence, DXY-loaded nanoparticles were more effective in controlling the growth kinetics of bacteria following their sustained release. Thus, DXY-loaded nanoparticles can be used efficiently for antibacterial therapy against a large number of bacterial species.

Summary

The study emphasizes the use of PLGA:PCL blends in nanoparticular formulations for increasing the entrapment efficiency of hydrophilic drugs by varying different parameters such as polymer ratio, solvent

choice, pH, use of electrolyte and drug loading. Change of the aforementioned parameters resulted in an increase in entrapment efficiency of DXY up to 70%. The antibacterial property of DXY was tested on a strain of *E.coli*. The study confirms the fact that native drugs are less stable in solution form compared with drugs loaded in nanoparticles and hence less effective in controlling the growth of bacteria. DXY-loaded nanoparticles are able to control the growth kinetics of bacteria more efficiently than native DXY owing to sustained release of the drug from the nanoparticle and thus, have a more profound antibacterial effect.

Table 1. Results of the entrapment efficiency and particle size of different formulations by varying different parameters.

Formulation	Ratio of PLGA:PCL	Solvent	PVA (%, w/v)	4% (w/v) NaCl	pH of inner aqueous phase	Drug loading (%, w/w)	Size (nm)*	Entrapment efficiency (%)
1	100:0	$CHCl_3$	2		7.4	20	360 ± 1.1	40
2	80:20	$CHCl_3$	2		7.4	20	290 ± 2.9	32
3	70:30	$CHCl_3$	2		7.4	20	256 ± 1.7	28
4	60:40	$CHCl_3$	2		7.4	20	237 ± 1.2	25
5	80:20	DCM	2	-	7.4	20	276 ± 2.7	47
6	80:20	DCM	2	+	4	20	285 ± 1.3	70
7	80:20	DCM	2	+	4	30	298 ± 1.0	60
8	80:20	DCM	2	+	4	60	310 ± 1.1	49

* *Mean hydrodynamic diameter measured by photon correlation spectroscopy.*

+ indicates addition of NaCl to the outer aqueous phase (PVA solution); - indicates absence of NaCl in the outer aqueous phase.

DCM: Dichloromethane; PCL: Poly(ε-caprolactone); PLGA: Poly(D,L-lactide-co-glycolide); PVA: Polyvinyl alcohol.

Table 2. Comparative analysis of native DXY with DXY-loaded PLGA:PCL nanoparticles in bacterial culture.

Days	**0.1 µg/ml**				**0.2 µg/ml**			
	DXY	***DXY NP***	***Void***	***MHB***	***DXY***	***DXY NP***	***Void***	***MHB***
1	35 ± 2.7	57 ± 5.5	452 ± 3.3	445 ± 4.7	24 ± 4.3	47 ± 5.9	457 ± 4.6	405 ± 3.9
2	85 ± 3.3	112 ± 7.7	523 ± 5.1	564 ± 5.6	9 ± 3.9	96 ± 6	502 ± 5.1	537 ± 4.3
3	167 ± 4.1	93 ± 6.5	621 ± 5	655 ± 6.1	106 ± 3.8	24 ± 5.7	635 ± 6.6	674 ± 4.1
5	59 ± 4.9	40 ± 8	502 ± 5.1	534 ± 4.2	54 ± 4.2	12 ± 6.8	513 ± 6.2	546 ± 4.9

The colony number is the average of three independent experiments.

DXY: Doxycycline; MHB: Muller-Hinton broth; NP: Nanoparticle; PCL: Poly(ε-caprolactone); PLGA: Poly(D,L-lactide-co-glycolide).

Executive summary

* Poly(D,L-lactide-co-glycolide):poly(ε-caprolactone) blends in nanoparticular formulations can be used to increase the entrapment efficiency of the hydrophilic drug, doxycycline (DXY).

* Different parameters such as polymer ratio, solvent choice, pH, use of electrolyte and drug loading can affect the entrapment efficiency of DXY in the formulation.

* It is evident that the entrapment efficiency of DXY can be enhanced by up to 70% if the aforementioned parameters are changed accordingly.

* DXY has antibacterial properties; this has been confirmed by the use of DXY on the DH5[alpha] strain of *Escherichia coli* .

* The native drug is less effective compared with DXY-loaded nanoparticles in controlling the growth of DH5[alpha].

* DXY-loaded nanoparticles are able to control the growth kinetics of bacteria more efficiently than native DXY following sustained release.

* DXY-loaded nanoparticles have a more profound antibacterial effect compared with native DXY and hence can be used for antibacterial therapy.

CAPTION(S):

Figure 1. Doxycycline.

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