Investigating dual drug loaded PLGA nanocarriers for improved efficacy in endometritis therapeutics

Rui He\textsuperscript{a*}, Guoping Zhang\textsuperscript{a*}, Jing Yang\textsuperscript{a}, Zhengqiang Bai\textsuperscript{a}, Kun Han\textsuperscript{b} and Hanru Zhang\textsuperscript{c}

\textsuperscript{a}Department of Clinical Laboratory, Gansu Provincial Maternity and Child Care Hospital, Lanzhou, Gansu Province, China; \textsuperscript{b}Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences, Suzhou, Jiangsu Province, China; \textsuperscript{c}Department of Gynecology and Obstetrics, Gansu Provincial Maternity and Child Care Hospital, Lanzhou, Gansu Province, China

ABSTRACT

Modern endometritis therapeutics may require an extremely precise and controlled drug release system. Poly lactic-co-glycolic acid (PLGA) microspheres loaded with two different drugs like amoxicillin and vancomycin were prepared and their efficacy in management of endometritis in murine model was examined. Such biological nanocarriers are need of the hour since they are biodegradable, cytocompatible, hemocompatible and easily formulated. The sizes of the drug loaded PLGA microspheres ranged from 65–100 nm. The electron microscopy images depicted them as uniform spherical structures. The zeta potential and polydispersity index were calculated and the in vitro release investigation indicated steady drug release over a period of 50 h at effective dosage. The drug loaded nanocarriers were found with be cytocompatible and hemolysis test elaborated that they met with hemolysis rate safety requirements. The murine models were induced with endometritis by intravaginal administration of bovine uterine isolates. The mice were sacrificed and histopathological examination of the endometrial slides stained with haematoxylin and eosin revealed that the drug loaded PLGA nanospheres helped in reducing acute endometritis in mice in a very short time. This study thereby demonstrated well described synthesis approach of drug loaded PLGA nanospheres which may be further be transferred to preclinical laboratory studies.

1. Introduction and the concept

Endometritis is a malady which is an incessant and understated recurrence of bacterial infection in the innermost lining of the uterus called endometrium \cite{1}. This is usually asymptomatic with very few subtle indications like pelvic pain, abnormal uterine bleeding,
dyspareunia, and leucorrhoea [2, 3]. But the occurrence of acute endometritis in women of reproductive age varies from 9–75% [4–7]. It has also been seen that chronic endometritis is very much predominant in females with unexplained infertility and females with spontaneous miscarriages [8–10]. Etiology for chronic endometritis was believed to be microorganisms in the uterus which have ascended into the uterus through the cervix from vagina via uterine peristaltic pump [11, 12]. However it has been recently researched that progression of bacterial colonies in uterus in endometritis is independent of flora in vagina once it entered [1]. The causative microorganisms are mainly *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. However, *Streptococcus* spp., *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Staphylococcus* sp., *Corynebacterium* and *Mycoplasma/Ureaplasma* sp. have also been detected in the microbial enculturations [13] and polymerase chain reaction (PCR) studies of chronic endometritis [14–16]. Treatment with antibiotics is usually an effective but transitory one for endometritis since the infection recurs frequently. Hence a more effective and perpetual therapy needs to be identified and researched.

With the advent of nanotechnology drug synthesis, fabrication and detection of novel therapeutic pathways for their improved biological function have been designed for improved therapeutics for several maladies. Our current study is based on the fundamental principle that anticipated consequences of any novel therapeutics may not depend on only pharmacokinetic and vibrant makeup of the drug but significantly on its bioavailability in subjects at the place of occurrence ultimately decided by the method of delivery of the fabricated drug loaded nanocarriers [17–19]. In our study, we have prepared a formulation of a dual drug (amoxicillin and vancomycin) loaded PLGA nanocarrier which was delivered intravaginally in murine models. PLGA was chosen since it was one of the most promising synthetic biomaterial for drug delivery and has exhibited great potential for delivery and therapeutic of drugs [20, 21]. These biopolymers have less detrimental outcome on environment than products based on fossil fuels. The pathway via which PLGA nanomaterials have reforming delivery of drugs has been discussed in several studies [17, 22]. In addition, amoxicillin and vancomycin have been reported to be used for treatment of chronic endometritis although individually [23].

As it is reported recently, endometrium is a complex, dynamic structure which sheds the endometrial cells during menstruation which in itself is anti-pathogenic as it removes the bacteria from uterine cavity to vagina. Still inflammation that perseveres during all the phases of the menstrual cycle leads to chronic endometritis. Therefore, a quick recovery from endometritis is prerequisite to maintain fertility instead of the normal recovery within 15–20 days with antibiotics twice a day [15, 24]. Herein, lays the novelty of our study. Our research elaborated that the fabricated dual drug loaded nanocarriers when delivered intravaginally, helps the endometrium to recover fast from the inflammation. The dual drug loaded PLGA nanocarriers were studied for their morphology, drug loading, in vitro release of drugs, stability, cytotoxicity along with its hemocompatibility and then proceeded for in vivo studies with mice. Hemocompatibility tests were important since we were directly targeting it to the uterus intravaginally. However, however we still do not have the data for the dual drug nanocarrier effectiveness after the shedding of endometrial layer. This is to be noted that this is just a preliminary research involving the production and characterization of the novel dual drug loaded nanocarriers. But since this targeted delivery of drug loaded nanocarriers yielded better and quick results than normal antibiotic therapeutics, we were encouraged to proceed with the experiments.
2. Materials and methods

2.1. Materials

Poly (D, L-lactic-co-glycolic acid) (50:50), amoxicillin (AMOX), vancomycin (VAN), poly vinyl alcohol (PVA 2%), dichloromethane and Milli-Q water were procured from Sigma Aldrich, China. The rest of chemicals utilized were also purchased from Sigma unless otherwise mentioned.

2.2. Preparation of dual drug loaded PLGA nanocarriers

Preparation of the dual drug loaded nanocarriers was done via double emulsion solvent evaporation technique. The method was amended from [25–27] with slight deviations to suit our study. Concisely stating, 4 mL of dichloromethane was utilized to dissolve 6 mg of VAN and 30 mg of poly (D,L-lactic-co-glycolic acid) copolymer. Following which 6 mg of AMOX was added and sonicated for 60 s. Consequently, 2 mL of 2% polyvinyl alcohol (PVA) solution primed in Milli-Q water was supplemented with the prepared solution and further sonicated for 60 s. For a second time, to the above resultant solution, 5 mL of PVA solution was acceded and churned for 4 h continuously to confirm thorough disappearance of the organic solvent. The samples were then washed and centrifuged. Following the same procedure individual drug loaded PLGA nanocarriers were also prepared. The dual drug loaded PLGA nanocarriers were designated as AMOX-PLGA-VAN, the individual drug loaded nanocarriers were designated as VAN-PLGA and AMOX-PLGA respectively. Only PLGA nanocarriers were also prepared for control.

2.3. Morphology of the nanocarriers

Morphological analysis of PLGA nanocarriers, AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA was monitored after centrifugation and redisposing the nanocarriers in distilled water, placing them on a grid, and finally air-dried. They were observed with a JEOL-JSM scanning electron microscope (SEM) at 20 kV.

Transmission electron microscopy (TEM) of the PLGA nanocarriers, AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA was also performed with high resolution JEM-2010HR microscope. A drop of nanocarrier was placed on 300 mesh carbon coated copper grid with the extra sample being removed with an absorbent paper. The nanocarrier samples were stained with 1% sodium phosphotungstate solution and the nanocarriers were observed at magnification up to 1,000,000 X. After the nanocarriers were dried, they were incubated for 12 h after which the observations were made.

Size distribution of the synthesized nanocarriers was done employing a Zetasizer Nano ZS (Malvern Instruments, UK) which utilized dynamic light scattering for the operating technique. The calculation of the mean diameter and polydispersity index (PDI) of the nanocarriers was carried out thrice. The mean diameters were represented as mean ± S.D. (standard deviation).

2.4. Yield of the nanocarriers and encapsulation efficiency

The nanocarrier drug yield or the loading efficiency was estimated by gravimetry according to the process elaborated by Babos et al. [28] with necessary changes. The process was continued after cleansing of known volume of nanocarrier solution followed by drying. The loading capacity (LE) and encapsulation efficiency (EE) were studied in a direct
manner. 10 mg of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA were dissolved in 1 mL DMSO following which the nanocarrier samples were diluted so that it may be evident in the range of linear calibration (1–20 mg/L). The absorbance of the nanocarrier solution was estimated with a spectrophotometer (PG Instruments T80, UK) at the absorbance maxima of amoxicillin (334.5 nm) and vancomycin (280 nm) in DMSO. The encapsulation efficiency of the nanocarriers was calculated according to the following formulae:

\[
EE \% = \frac{\text{Encapsulated drug}}{\text{Total drug}} \times 100\%
\]

\[
LE \% = \frac{\text{Total drug}}{\text{nanocarrier solution}} \times 100\%
\]

### 2.5. Stability of the nanocarrier

The stability of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA was investigated at 37°C. 10 mg of nanocarrier solution were dispersed in 50 mL of distilled water by sonication. Resolving of nanocarrier solution was visually observed after which size distribution, polydispersity index and zeta potential of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA were calculated at regular time interval of 10, 20, 30 days using the Delsa Nano C Zetasizer to note the accretion of nanocarrier solution.

### 2.6. In vitro drug release investigations

The entire in vitro release of drugs was carried out in pH ranging from 3.0 to 7.5, since the uterine pH varies in the range of 3.0–9.0 [29]. In vitro studies were carried out by dialysis bag technique [30]. The release media used in the investigation were phosphate buffer solution (PBS) of pH 7.4, ammonium acetate buffer for pH 5.5, and HCl buffer of pH 3.0 respectively. In the dialysis bag process, 5 mg of the AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA were dissolved in 2 mL of the release medium and kept in a dialysis bag of 12–15 kDa molecular weight cut off with surface area being 22 cm² which was next immersed in flask that had 100 mL of the test media which was retained at physiological temperature and continuously stirred at 100 rpm. At pre-determined time periods of 1, 2, 3, 5, 7 h, 1 mL aliquots were compiled followed by renewal with fresh media. Centrifugation of the aliquots was done @10,000 rpm for 20 min and the supernatants were collected for analysis of amoxicillin and vancomycin. The concentration of amoxicillin and vancomycin present in the supernatant aliquot was determined by spectrophotometric method.

### 2.7. Cytotoxicity studies

The human endometrial receptive cell line, RL95-2 was grown in DMEM F-12 medium augmented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 U/mL streptomycin and 2.5 mM glutamine [31]. After a pre-incubation period of 24 h, cell culture was done at 37°C in a humid environment comprising 5% CO₂. Trypsinization, resuspension and precultured of the cells were carried out before use.

#### 2.7.1. MTT assay

Confluent cell cultures were attained as cited in technique quoted above. In vitro cytotoxicity is generally assessed by performing MTT assay using the particular cell line. Cells
were seeded @ $5 \times 10^3$ cells/well in 96-well plates and gestated for 72 h to confirm that cells are viable when the following samples were added to the confluent cells; Only PLGA nanocarriers (100 µg/mL), free amoxicillin and vancomycin (100 µg/mL each) AMOX-PLGA-VAN (100 µg/mL), AMOX-PLGA (100 µg/mL), VAN-PLGA (100 µg/mL). The drug concentration in the AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA nanocarriers was attuned to the free amoxicillin and vancomycin concentrations which was 100 µg/mL. For two days, further incubation of the cells was done. Thereafter, addition of 20 µL of MTT solution (5 mg/mL) in every well was done and the plates were gestated at 37 °C for 4 h. Cell viability was depicted as per the absorbance measured at 492 nm after the cellular incubation with the aid of a microplate reader.

2.8. Hemocompatibility assay

Hemolysis rate test was performed as per Liu et al. [32] with necessary modifications. Mice blood was procured from other laboratories for regular hemolysis analysis. Anticoagulant was mixed with fresh blood along with sterilized functional saline at a ratio of 4:5. In this study, negative control and positive control were functional saline and distilled water respectively. 0.2 mL of each sample of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA was added to fresh blood with anticoagulant and gestated in a 37 °C water bath for one hour. Subsequently, centrifugation of each tube was done following which the absorbance of the supernatant was noted at a wavelength of 330 nm. Calculation of the hemolysis ratio was done with the following formula:

$$HR(\%) = \frac{D_T - D_{NC}}{D_{PC} - D_{NC}}$$

where HR is hemolysis rate and $D_T$ is the absorbance of samples, $D_{NC}$ & $D_{PC}$ the absorbance of negative controls and positive controls, respectively.

2.9. The mouse model of endometritis

Twenty 6–8 weeks old adult female BALB/c mice weighing 45–50 g were procured from Qingdao Institute for the Control of Drug Products (Qingdao, China). The Ethical Committee on Small Animal Research at the University was approached for the animal studies including Hemocompatibility assay. The Ethical Committee on Small Animal Research approved the study via Approval no. 23/2019/MOU and also suggested some changes in the study which were incorporated. An endometrial swab was designed which consisted of autoclaved ear bud attached to an artificial insemination sheath (length—45 cm). The procedure was followed from [33]. The endometrial swab was checked for sterility by dipping in autoclaved water which did not show any bacterial growth after 48 h. This swab was pushed intravaginally in a buffalo already with confirmed endometritis and the lining of endometrium was scraped. This was then retracted immediately and transferred to laboratory within one hour and immersed in peptone water which was then incubated and streaked on agar for bacterial growth. Briefly, a discrete colony of *E. coli* was picked and further inoculated and cultured till a concentration of $1.5 \times 10^6$ (CFU/mL) was reached. These were then inoculated in the mice uterus.

2.9.1. In vivo histopathological examination

5 mice were allotted into single group and 4 groups were made. After 30 days of incubation with bacterial colony, further studies were taken up. Each uterus was filled with 20 µL of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA and 20 µL of phosphate
buffered saline (PBS) which were inserted intravaginally using a 100 μL syringe which was already patented (201410391820) [33]. The group administered with saline was blank control group. After 48 h of infusion with drug loaded nanocarriers, the mice were sacrificed by CO₂ inhalation and uteri were fixed in 10% formalin. Dehydration of the tissues was done through a series of graded alcohols which were then embedded in paraffin, and sectioned. Staining of the tissues was carried out with hematoxylin and eosin (H & E) following which the pathological variations in the uteri were perceived with a light microscope.

3. Results

3.1. Morphological analysis of the drug loaded PLGA nanocarriers

The images of the synthesized PLGA nanocarriers, AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA were clearly depicted in Figure 1. The microscopic images depicted that the fabricated PLGA nanocarriers were smooth, spherical, uniform in size and without any agglomeration. However upon loading with drugs, the edges were not smooth and appeared a little non-uniform. But nevertheless they did not aggregate and formed masses. They were homogenous in distribution. The TEM images reinforced the findings of the SEM as seen in Figure 2 and also the sizes determined by the Zetasizer. The hydrodynamic diameters were determined by Zetasizer by dynamic light scattering. The mean
diameters were 65.2 ± 3.4 nm, 77.4 ± 3.1 nm, 81.0 ± 3.8 nm and 96.3 ± 4.4 nm of the PLGA nanoparticles, VAN-PLGA, AMOX-PLGA and AMOX-VAN-PLGA respectively. There was an interesting trend observed in zeta potential values of the nanocarriers. The zeta potential values were all negative and values were highest in AMOX-PLGA-VAN (−20.4 ± 1.2 mV) with PLGA nanocarrier being lowest (−34.5 ± 1.5 mV). The values of mean diameter, zeta potential and PDI values were collected in Table 1.

3.2. Yield and entrapment efficiency

The nanocarrier yield or the loading efficiency of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA was found to be 29.8%, 23.2% and 22.8% respectively. The entrapment efficiency was depicted to be 89.21%, 77.35% and 75.22% respectively. The values are clearly mentioned in Table 2. It was observed that the loading efficiency percent and the entrapment efficiency improved with increased number of pores in the nanocarriers as seen in the SEM & TEM micrographs.
3.3. Stability of the nanocarrier

Investigation of the stability of the AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA was carried out by calculating the differences in size, PDI, zeta potential at consistent time periods of 10, 20 and 30 days of preserving at room temperature. The shelf life or the stability of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA was observed through this investigation. The values acquired are collected in Table 3. There was an inconsequential decrease in size of the PLGA nanocarriers and insignificant increase in sizes of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA. A decrease in PDI and zeta potential values was also noted. From the results in Table 3, it was understood that there were no significant variations in the sizes, PDI and zeta potential values of the AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA and the nanocarriers were extremely stable thermodynamically.

3.4. In vitro release kinetics

The in vitro release kinetics of the drug loaded nanocarriers was evaluated at various pH of 3.0, 5.5 and 7.4. The release profiles of the drug loaded nanocarriers were collected in Figure 3. It was seen that the release profile at pH 3.0 was irregular for AMOX-PLGA-VAN. There was an early burst release of drugs (18% of total drug loaded) after which the flow became steady and continued for 35 h. In pH 5.5 and 7.4, the release of drugs from AMOX-PLGA-VAN was steady and continuous for a time period of 50 h. For VAN-PLGA and AMOX-PLGA, the release profiles were of anomalous behaviour in pH 3.0. There was an early burst release (25% of drug) and then continuous release for 26 h. For pH 5.5 and 7.4, the drug release profiles were steady for 40 h. Therefore, it may be concluded that to obtain the best results, one intravaginal dose of drug loaded nanocarrier may be sufficient for a long period of time.

3.5. Cytotoxicity studies

The cytocompatibility of the fabricated AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA, PBS (control), free amoxicillin and free vancomycin were evaluated in human endometrial receptive cell line. It was depicted in Figure 4. Even with a very high dose of 100 μg/mL, VAN-PLGA and AMOX-PLGA caused very small cell toxicities almost comparable to PBS. However, the relative increased cellular toxicity of AMOX-PLGA-VAN was due to the synergistic action of two antibiotics which partially caused the cell death. However the free antibiotics at a similar high dose caused enormous cell death as seen from Figure 4. The percentages of cell viability and concentration of drugs required for cell growth inhibition by 50% (IC50) values were created from the dose response curves. There were represented in Table 4.
|        | AMOX-PLGA-VAN |        | VAN-PLGA |        | AMOX-PLGA |
|--------|---------------|--------|----------|--------|-----------|
|        | Size (nm)     | Zeta potential (mV) | PDI | Size (nm)     | Zeta potential (mV) | PDI | Size (nm)     | Zeta potential (mV) | PDI |
| 10 days| 96.23 ± 1.3   | -20.5 ± 1.1                | 0.312 ± 0.04 | 77.13 ± 1.5   | -24.3 ± 0.1                | 0.261 ± 0.01 | 81.13 ± 1.4   | -25.2 ± 0.4                | 0.275 ± 0.03 |
| 20 days| 94.21 ± 1.5   | -21.1 ± 1.3                | 0.318 ± 0.07 | 77.43 ± 1.7   | -24.9 ± 0.3                | 0.263 ± 0.05 | 83.55 ± 1.1   | -25.6 ± 0.5                | 0.278 ± 0.01 |
| 30 days| 93.13 ± 1.6   | -22.5 ± 0.5                | 0.321 ± 0.03 | 78.15 ± 0.6   | -25.5 ± 0.1                | 0.268 ± 0.07 | 82.13 ± 1.6   | -26.3 ± 1.3                | 0.281 ± 0.04 |

Table 3. Size, zeta potential and PDI of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA after 10, 20 & 30 days during stability studies.
3.6. Hemocompatibility assay

Since the drug was injected vaginally into the uterus, a blood compatibility report was a prerequisite for the synthesized drug loaded nanocarrier. Hence we had performed the hemolysis ratio test. Hemolysis study displayed the stability of red blood cell (RBCs) Figure 3. Different drug release profile of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA at different pH which entirely represented the pH of the uterus from 3.0–9.0.

Figure 4. Cellular viability on being treated with AMOX-PLGA-VAN, VAN-PLGA, AMOX-PLGA, PBS (control), free amoxicillin and free vancomycin. Note that the low cell viability in case of AMOX-PLGA-VAN may be due to the synergistic effect of the antibiotics.

3.6. Hemocompatibility assay

Since the drug was injected vaginally into the uterus, a blood compatibility report was a prerequisite for the synthesized drug loaded nanocarrier. Hence we had performed the hemolysis ratio test. Hemolysis study displayed the stability of red blood cell (RBCs)
when in contact with a foreign body. If the hemolysis rate is below 5%, medical drugs and substances were deliberated as nonhemolytic as per national biological safety prerequisites. The hemolysis ratio of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA and PBS (control) at a very high concentration of 100 \( \mu \)g/mL was investigated in vitro by a straight contact technique (Table 5). The results of AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA and PBS (control) were all under 5%, thereby compliant to the national biological material hemolysis rate security quantified necessities.

**3.7. In vivo mouse model for histopathological investigation**

There was apparently no change in the feed behaviour and general body structure of the mice after the inoculation of the bacterial colony till the time of sacrifice. There were also no signs of depression and the mice were all active upon inoculation. However, typical hallmark changes of endometritis like stromal hyperplasia, sloughing of endometrial epithelial cells and influx of polymorphonuclear leucocytes into the endometrial lumen [33] were seen in the histological images of uterus of mice treated with control as seen in Figure 5(A). But, the uteri of the mice treated with AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA, signs of healed uteri were clearly seen. There was no presence of the polymorphonuclear leucocytes with decidualization of endometrium. The presence of endometrial scars was observed but there was no visible lesions seen. Scars of tissue granulation were also seen. The figures were demonstrated in Figure 5(B)–(D). But the scars varied in their size and length in different exposures to AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA. While the signs of endometrium inflammation were almost diminished in AMOX-PLGA-VAN, they were not completely invisible in treatment with VAN-PLGA or AMOX-PLGA.

**4. Discussion**

Our primary aim was to synthesize nanocarriers which would have the capability to encapsulate two antibiotics with high loading and encapsulation efficiency. These nanocarriers should also be able to deliver the antibiotics in a steady way to the targeted site to as to achieve the desired results within a very short span of time. The nanocarriers would also have to be cell and blood compatible. It was also known that PLGA is very common nanocarriers [28] but we also emphasize on the fact that amoxicillin and vancomycin

| Sl. No. | Optical density (330 nm) | Hemolysis rate (%) |
|--------|--------------------------|--------------------|
| 1.     | Physiological Saline (negative control) | 0.0269 ± 0.0041 | 0 |
| 2.     | Distilled Water (positive control) | 0.576 ± 0.0052 | 100 |
| 3.     | VAN-PLGA | 0.0491 ± 0.0032 | 4.04 |
| 4.     | AMOX-PLGA | 0.0488 ± 0.0051 | 3.98 |
| 5.     | AMOX-PLGA-VAN | 0.0496 ± 0.0047 | 4.13 |

**Table 4. IC50 values free amoxicillin, vancomycin, AMOX-PLGA-VAN, VAN-PLGA and AMOX-PLGA.**

| Drug                  | IC50 value ± S.D. |
|-----------------------|-------------------|
| Amoxicillin           | 55.5 ± 0.02       |
| Vancomycin            | 75.15 ± 0.01      |
| VAN-PLGA              | 95.0 ± 0.01       |
| AMOX-PLGA             | 94.75 ± 0.02      |
| AMOX-PLGA-VAN         | 98.5 ± 0.03       |

**Table 5. Haemolysis rate (%) of the drug loaded nanocarriers.**

| Sl. No. | Optical density (330 nm) | Hemolysis rate (%) |
|--------|--------------------------|--------------------|
| 1.     | Physiological Saline (negative control) | 0.0269 ± 0.0041 | 0 |
| 2.     | Distilled Water (positive control) | 0.576 ± 0.0052 | 100 |
| 3.     | VAN-PLGA | 0.0491 ± 0.0032 | 4.04 |
| 4.     | AMOX-PLGA | 0.0488 ± 0.0051 | 3.98 |
| 5.     | AMOX-PLGA-VAN | 0.0496 ± 0.0047 | 4.13 |
have not been co-encapsulated before for endometritis therapeutics intravaginally to the best of our knowledge. This was not very surprising since co-encapsulation of two different drugs in one matrix comprised of a single polymer was quite difficult to achieve.

We have optimized the process conditions of synthesis of AMOX-PLGA-VAN after undergoing extensive process which involved other methods of preparation like single emulsion, nanoprecipitation and finalized double emulsion solvent evaporation technique. Therefore the other unsuccessful attempts were not discussed in detail since they were inefficient for co-encapsulation of drugs. The mechanism of dual drug loading was by double emulsion evaporation technique. VAN was effectively loaded in outer water phase by microencapsulation technique with dichloromethane and PLGA. Since AMOX is hydrophilic drug, it would be entrapped by PLGA after being mixed with organic phase. Therefore it was used in inner water phase. The double emulsion evaporation technique had an added advantage that the polymer concentration may be kept restricted so that the size of the nanocarriers may be restricted to 300 nm. This was important since nanocarriers greater than 300 nm would be cleared without difficulty by the reticuloendothelial system [34]. Another interesting observation was that in the PLGA nanocarriers, there were white spots visible in the TEM images which clearly revealed that there was formation of empty cavities in the nanocarriers which was necessary for the drug loading. A significant feature which affects the stability of the nanoparticles is the zeta potential which clearly indicated stable nanocarriers. The increased values of zeta potential indicated a small amount of agglomeration for stability of the nanocarriers which was visible in electron microscopy too. The high drug loading and entrapment efficiency may be a result of the increased electrostatic communication between the drug molecules and PLGA polymers [35, 36]. The increased electrostatic interactions may be effective
hydrogen bonding and electrostatic interaction between the hydroxyl, amine and carboxyl groups of drug molecules and carboxyl groups of PLGA. The stable ratio of dual drugs was understood from the encapsulation efficiencies individually. It was seen that VAN-PLGA and AMOX-PLGA had almost same encapsulation efficiency, hence the molar ratio of VAN: AMOX was adjusted as 1:1 in the initial drug content in AMOX-PLGA-VAN which was the optimized ratio for dual drugs.

The stability of the nanoparticles was also due to addition of PVA which acted as stabilizer adhering to the polymer surface not easily removed by washing too.

The fabricated nanocarrier delivery system exhibited a unique property of sustained release of the drugs at pH 5.5 and 7.4. This may be useful as sustained release may be an indication of prevention of active diffusion of the drug into the media. The matrix type microspheres showed diffusion type controlled release which showed an initial high release followed by decreased but steady rate. This was seen exactly in our study which was also pH dependent. The microspheres or nanospheres generally possessed a core surrounded by the polymeric membrane. The drugs were generally dispersed throughout the membrane and core. So, the drugs dissolve first followed by diffusion through the membrane. However, in pH 3.0, there is drug diffusion in the media for small percent (18%). The release of drugs into the media suggested that the co-release of amoxicillin and vancomycin at the targeted site of action may prove to be beneficial for endometritis therapeutics. The burst release of drugs at pH 3.0 indicated the bioresponsive property of the PLGA nanocarriers too. But there was also a hint of anomaly in the release behaviour of the drugs at different pH which may also be due to relaxation of the polymer in the nanocarrier along with drug diffusion [25].

Cellular compatibility or toxicity may be classified as an imperative parameter in deciding the efficiency of a nanocarrier molecule. The prepared VAN-PLGA/AMOX-PLGA exhibited very low cytotoxicity with normal uterus cell line indicating that they would not affect the normal cells. But slightly increased cytotoxicity in case of AMOX-PLGA-VAN may be due to the synergistic antibiotic action of the dual drugs loaded in the nanocarrier. The difference in cell viability demonstrated higher antibiotic efficiency.

Hemocompatibility is an extremely significant pre-requisite in development of any kind of medical devices. Since the surface properties of any nanoparticulate formulation may underlie its capacity to aggregate blood cells, its hemocompatibility needs to be assessed [37]. Although it is indeed understood that nanoparticulate combination may react to blood cells in different ways, the exact mechanism is still unclear. The combination of nanomolecules and polymers may react to blood cells; thereby the hemolysis rate was needed to be determined. When it was clear that the hemolysis rate of novel synthesized drug loaded PLGA nanocarriers was way beneath the hemolysis rate safety security requirements, the study was furthered for in vivo experiments. However there always exists a possibility of betterment of the surface properties of nanocarriers for improved hemocompatibility studies [38].

The histological study revealed that upon treatment with drug loaded nanocarriers the inflamed endometrial layer responded to the released antibiotics at the site of injury and healing began very early. The signs of healing uteri were evident and scars had also diminished. The probable reason may be the synergistic effect of antibiotics on the endometrial microbial profiles. The drug loaded nanocarriers not only reduced the inflammation but did it in a very short time period. This would definitely improve uterine receptivity as chronic endometritis may lead to implantation failure, immune abnormalities and non-functional decidualization of the endometrial layer. Therefore it may be safely suggested that administration of the dual drug loaded nanocarrier through
intravaginal route may be more efficacious than the oral route in women suffering from chronic endometritis. Hence we may also safely assume that the drug loaded PLGA nanocarriers were delivered to the targeted sites. However, additional and new widespread is required to exemplify the exact molecular mechanism and the full side effects of AMOX-PLGA-VAN as an endometritis management procedure.

5. Conclusion

In this study, we have efficaciously demonstrated the co-encapsulation of dual antibiotic drugs in an bioresponsive PLGA nanocarrier which was successful in treating endometritis in mice model within a very short period of time. These were of small size with high efficiency for drug entrapment and were cytocompatible. These were found to be stable too for certain time period. An important aspect of the drug loaded nanocarriers was their path of delivery directly to the site of action which may be one of the reasons for their increased efficiency. Ease of intravaginal insertion, superficial lack of systemic participation and improved release rates are the benefits of this prototype study over others. These nanocarriers can be further suitably optimized for other drugs and also secondary ligands which may ensure quicker and better results for endometritis therapeutics. The time period for the renewal of the drug dosage also needs to considered upon for better results.

Acknowledgements

The authors thank host institute for provision of platform and arrangement of characterization facilities for the research.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

1. Kimura F, Takebayashi A, Ishida M, et al. Chronic endometritis and its effect on reproduction. J Obstet Gynaecol Res. 2019;45(5):951–960.
2. Rotterdam H. Chronic endometritis. A clinicopathologic study. Pathol Annu. 1978;13:209.
3. Yörükoğlu K, Kuyucouğlu F. Chronic nonspecific endometritis. Gener Diagn Pathol. 1998;143:287–290.
4. Michels TC. Chronic endometritis. Am Fam Physician. 1995;52(1):217–222.
5. Wu D, Kimura F, Zheng L, et al. Chronic endometritis modifies decidualization in human endometrial stromal cells. Reprod Biol Endocrinol. 2017;15(1):16.
6. Kasius JC, Fatemi HM, Bourgain C, et al. The impact of chronic endometritis on reproductive outcome. Fertil Steril. 2011;96(6):1451–1456.
7. Knudtson E, Senokozlief M, Ye H, et al. The association of chronic endometritis with preterm birth. Am J Obstet Gynecol. 2003;189(6):S173.
8. Kitaya K, Matsubayashi H, Yamaguchi K, et al. Chronic endometritis: potential cause of infertility and obstetric and neonatal complications. Am J Reprod Immunol. 2016;75(1):13–22.
9. Chen Y-q, Fang R-I, Luo Y-N, et al. Analysis of the diagnostic value of CD138 for chronic endometritis, the risk factors for the pathogenesis of chronic endometritis and the effect of chronic endometritis on pregnancy: a cohort study. BMC Women’s Health. 2016;16(1):60.
10. Bouet P-E, El Hachem H, Monceau E, et al. Chronic endometritis in women with recurrent pregnancy loss and recurrent implantation failure: prevalence and role of office hysteroscopy and immunohistochemistry in diagnosis. Fertil Steril. 2016;105(1):106–110.
11. Chen C, Song X, Wei W, et al. The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. Nat Commun. 2017;8(1):11.
12. Hansen LK, Becher N, Bastholm S, et al. The cervical mucus plug inhibits, but does not block, the passage of ascending bacteria from the vagina during pregnancy. Acta Obstet Gynecol Scand. 2014;93(1):102–108.
13. Fu K, Lv X, Li W, et al. Berberine hydrochloride attenuates lipopolysaccharide-induced endometritis in mice by suppressing activation of NF-κB signal pathway. Int Immunopharmacol. 2015;24(1):128–132.
14. Cicinelli E, De Ziegler D, Nicoletti R, et al. Chronic endometritis: correlation among hysteroscopic, histologic, and bacteriologic findings in a prospective trial with 2190 consecutive office hysteroscopies. Fertil Steril. 2008;89(3):677–684.
15. Kitaya K, Matsubayashi H, Takaya Y, et al. Live birth rate following oral antibiotic treatment for chronic endometritis in infertile women with repeated implantation failure. Am J Reprod Immunol. 2017;78(5):e12719.
16. Cicinelli E, De Ziegler D, Nicoletti R, et al. Poor reliability of vaginal and endocervical cultures for evaluating microbiology of endometrial cavity in women with chronic endometritis. Gynecol Obstet Invest. 2009;68(2):108–115.
17. Mir M, Ahmed N, Ur Rehman A. Recent applications of PLGA based nanostructures in drug delivery. Colloids Surf B BioInterfaces. 2017;159:217–231.
18. Allen TM, Cullis PR. Drug delivery systems: entering the mainstream. Science. 2004;303(5665):1818–1822.
19. Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro-and nanoparticles in drug delivery. J Control Release. 2004;100(1):5–28.
20. Acharya S, Sahoo SK. PLGA nanoparticles containing various anticancer agents and tumour delivery by EPR effect. Adv Drug Deliv Rev. 2011;63(3):170–183.
21. Heggannavar GB, Vijeth S, Kariduraganavar MY. Development of dual drug loaded PLGA based mesoporous silica nanoparticles and their conjugation with Angiopep-2 to treat glioma. J Drug Delivery Sci Technol. 2019;53:101157.
22. Fan D, De Rosa E, Murphy MB, et al. Mesoporous silicon-PLGA composite microspheres for the double controlled release of biomolecules for orthopedic tissue engineering. Adv Funct Mater. 2012;22(2):282–293.
23. Dube A, Reynolds JL, Law W-C, et al. Multimodal nanoparticles that provide immunomodulation and intracellular drug delivery for infectious diseases. Nanomed Nanotechnol Biol Med. 2014;10(4):831–838.
24. Babos G, Biró E, Meiczinger M, et al. Dual drug delivery of sorafenib and doxorubicin from PLGA and PEG-PLGA polymeric nanoparticles. Polymers. 2018;10(8):895.
25. Obradović D, Husar M, Andelković V, et al. Study of the pH values of uterine secretions in women with and without intrauterine devices. Jugosl Ginekol Opstet. 1981;21:7–10.
26. Gao Y, Zuo J, Bou-Chacra N, et al. In vitro release kinetics of antituberculosis drugs from nanoparticles assessed using a modified dissolution apparatus. BioMed Res Int. 2013;2013:1–9.
27. Liu H-Y, Du L, Zhao Y-T, et al. Hemocompatibility and cytotoxicity evaluation of halloysite nanotubes for biomedical application. J Nanomater. 2015;2015:1–10.
28. Dar S, Qureshi S, Palanivelu M, et al. Evaluating a murine model of endometritis using uterine isolates of Escherichia coli from postpartum buffalo. Iran J Vet Res. 2016;17:171.
29. Kobayashi H, Watanabe R, Choyke PL. Improving conventional enhanced permeability and retention (EPR) effects; what is the appropriate target? Theranostics. 2014;4(1):81–89.
30. Ashley CE, Carnes EC, Phillips GK, et al. The targeted delivery of multicomponent cargos to cancer cells by nanoporous particle-supported lipid bilayers. Nat Mater. 2011;10(5):389–397.
36. Meng H, Liong M, Xia T, et al. Engineered design of mesoporous silica nanoparticles to deliver doxorubicin and P-glycoprotein siRNA to overcome drug resistance in a cancer cell line. ACS Nano. 2010;4(8):4539–4550.

37. Radomski A, Jurasz P, Alonso D, et al. Nanoparticle-induced platelet aggregation and vascular thrombosis. Br J Pharmacol. 2005;146(6):882–893.

38. Lvov Y, Abdullayev E. Functional polymer–clay nanotube composites with sustained release of chemical agents. Prog Polym Sci. 2013;38(10–11):1690–1719.