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# *In vitro* release and antibacterial activity of poly (oleic/linoleic acid dimer: sebacic acid)-gentamicin[1]

YANG Xiu-Fen, ZENG Fan-Dian[2], ZHOU Zhi-Bin[3], HUANG Kai-Xun[3], XU Hui-Bi[3]

*Department of Clinical Pharmacology, Tongji Medical College, Huazhong University of Science & Technology, Wuhan 430030, China;*
*[3]Department of Chemistry, The College of Life Sciences, Huazhong University of Science & Technology, Wuhan 430074, China*

**KEY WORDS** drug delivery systems; polymers; gentamicins; drug carriers; *Staphylococcus aureus*; *Escherichia coli*; osteomyelitis

## ABSTRACT

**AIM:** To investigate whether poly (oleic/linoleic acid dimer: sebacic acid)-getamicin [Poly(OAD/LOAD:SA)-gentamicin] delivery system was useful to treat chronic osteomyelitis. **METHODS**: Drug delivery system consisted of gentamicin sufate dispersed in a copolymer containing oleic/linoleic acid dimer (OAD/LOAD) and sebacic acid (SA) in a 1:1 weight ratio. The gentamicin release from [Poly(OAD/LOAD:SA)-gentamicin] was tested in water, 0.9 % saline, and phosphate buffer 0.1 mol/L. **RESULTS:** The gentamicin concentration peak was found on d 2, then slowly decreased, considerable amount of gentamicin was still released on d 50. From d 2 to d 50, the gentamicin concentration in the releasing fluids was from 59 to 42128-fold and 1.8 to 1314-fold of the MIC for *Staphylococcus aureus* and *Escherichia coli*, respectively. *Staphylococcus aureus* and *Escherichia coli* were strongly inhibited by the releasing fluids for 50 d. The gentamicin release and anti-bacterial activity in the three media were similar, only in 0.1 mol/L phosphate buffer, from d 2 to d 14 it was lower. **CONCLUSION:** Poly(OAD/LOAD: SA)-gentamicin was useful to treat chronic osteomyelitis.

## INTRODUCTION

Managements of chronic osteomyelitis with the local delivery of the antimicrobial agent is a novel therapeutic modality, which achieves elevated antibiotic concentrations at the site of infection without systemic toxicity[1,2]. The following drug beads are such local drug delivery systems (DDS): poly (methyl methacrylate) (PMMA)-gentamicin, calcium hydroxyapatite ceramic (CHAC) -antibiotics and apatite-wollastonite glass ceramic (AWGC)-antibiotics, which are studied and used in animal models[2-4]. A commercial product consisting of PMMA beads loaded with gentamicin has been approved for use in Europe[2]. But these DDS all suffer from the disadvantage that they are not biodegradable and must be removed at a later date[2-4]. Recently, some new biodegradable drug carriers (polymers) were synthesized, such as poly (lactic acid) [poly (LA)][5,6], poly (lactic-co-glycolic acid) [poly(LGA)][7] and poly (erucic acid dimer: sebacic acid) [poly (EAD:SA)][8], which carry antibiotics to treat chronic osteomyelitis are still being studied *in vitro* or in animal models. Lately, we synthesized a novel drug carrier (another biodegrad-

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[2] Correspondence to Prof ZENG Fan-Dian.
Phn 86-27-8363-0652. Fax 86-27-8362-2308.
E-mail xiufenyang@163.net
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able polymer), named poly (oleic/linoleic acid dimer: sebacic acid) [poly (OAD/LOAD:SA)], we mixed it with 20 % gentamicin and got poly (OAD/LOAD:SA)-gentamicin beads[9]. The purpose of this paper is to investigate the *in vitro* release and bacteriocidal activity of poly (OAD/LOAD:SA)-gentamicin:

## MATERIALS AND METHODS

**Materials** Poly (OAD/LOAD:SA) and poly (OAD/LOAD:SA)-gentamicin were synthesized by Department of Chemistry in the College of Life Science of our university. Gentamicin sulfate (standard), antibiotic Medium I (II and IV), *Bacillus pumilus* [CMCC (B) 63202], *Staphylococcus aureus* [CMCC(B)26003], and *Escherichia coli* [CMCC(B)44103] were obtained from National Institute for Control Pharmaceutical and Biological Product. The other chemicals were of analytical grade and obtained from Hubei Province Chemicals Co Ltd.

**Methods** Medium I (I and II), *Bacillus pumilus (B pumilus)* suspension, *Staphylococcus aureus (S aureus)* suspension, and *Escherichia coli (E coli)* suspension were prepared as reported in the literature[10]. The bacteria colony-forming units (CFU) counts and the minimum inhibitory concentration (MIC) determination were described previously[11].

***In vitro* drug release studies**[12] *In vitro* drug release was performed in water, phosphate-buffer saline (PBS) 0.1 mol/L (pH 7.4), and 0.9 % saline.

Twelve drug beads [poly(OAD/LOAD:SA)-gentamicin] and twelve placebo beads [poly(OAD/LOAD:SA)] were used to study. Each bead (4 mm×4 mm×10 mm, weighed 150 mg) was placed in a glass tube containing 5 mL of PBS (water or 0.9 % saline) and stored in a thermostatic chamber at 37 ºC. The elution fluids were replaced at 48-h intervals for 50 d, and the removed elution fluids were preserved at -30 ºC for later determination of the gentamicin concentrations and measurement of their bacteriocidal activities. The placebo beads were studied as controls. All materials and containers were sterile and the experiment procedure was carried out under sterile condition.

**Gentamicin concentration determination** The gentamicin concentration in the elution fluids was measured by a microbiological agar well diffusion assay (M-agar assay) with slight modifications[13]. *Bacillus pumilus* suspension [CMCC(B)63202] was adjusted (1:200 dilution) with PBS (pH 7.8). A total of 0.5 mL of the diluted culture was added to 100 mL of autoclaved molten antibiotic medium I which had been cooled to 56 ºC and mixed gently by inversion. The seeded agar was poured onto glass plates (90-mm diameter) on a horizontal level surface and allowed to harden at room temperature for 30 min. The well in the plates were made by a puncher device (3-mm diameter) in the agar and filled with 0.01 mL samples and standards, 2 wells each plate. The glass plates were covered and incubated at 37 ºC overnight (18 h). Duplicate zone inhibition diameters were averaged and compared with a series of standards. Standard curves were made with known quantities of free gentamicin depending on the sample to be assayed. The concentrations of unknown samples were obtained by extrapolation from the zones of inhibition of standards. Linear regression analysis of standard calibration line was obtained by plotting log gentamicin concentrations versus zone diameters of inhibition. The range of linearity for gentamicin was from 0.5 mg/L to 12.5 mg/L. Final gentamicin concentrations in water, PBS, and 0.9 % saline were calculated by applying an appropriate dilution correction factor to the measured sample concentrations. The lowest sensitivity of the assay was 0.5 mg/L. The correlation coefficients for all standard curves were >0.99.

**Bacteriocidal activity measurement** The bacteriocidal activity was measured by cylinder-plate assay with slight modifications[14]. Each medium (Medium II for *Staphylococcus aureus*, Medium IV for *Escherichia coli*) 21 mL was placed in each of the required number of plates, and was allowed to harden into a smooth base layer of uniform depth. Seed layer inoculum (see above) 4 mL was added, then the plate was tilted back and forth to spread the inoculum evenly over the surface and was allowed to harden. Six assay cylinders (stainless steel) were dropped on the inoculated surface from a height of 12 mm, using a mechanical guide device to insure even spacing on a radius of 2.8 cm, the plates were covered to avoid contamination. After filling the 6 cylinders on each plate with dilutions of samples containing the test levels, the plates were incubated at 35 ºC to 37 ºC for 16 h, finally the cylinders were removed, and the diameter of each zone of growth inhibition was measured and recorded. Each sample was tested in duplicate and the zone inhibition diameters were averaged. Each sample was diluted 10 times from d 2 to d 18, 5 times on d 24, and 2 times on d 28, the original samples were used on d 40 and d 50. The samples from d 2 to d 24 and from d 28 to d 50

were tested at the same day, respectively.

**Data analysis** Data were expressed as mean±SD and analyzed with Microsoft Excel 2002 and SigmaPlot 6.1. A Student's *t* test was used.

## RESULTS

Gentamicin concentrations in the water, PBS, and 0.9 % saline reached their peaks at d 2, then gradually decreased until the end of the experiment at d 50 (Fig 1). On d 4, d 10, and d 18, the gentamicin level in water was higher than that in 0.9 % saline ($P<0.05$, Fig 1); compared with water and 0.9 % saline, the gentamicin level in PBS was lower from d 2 to d 14 ($P<0.01$, Fig 1). The gentamicin concentration in PBS was (2.3±1.1) to (357±20) mg/L in 50 d, (3.0±1.5) to (1643±496) mg/L in 0.9 % saline, and (3.3±1.8) to (1250±446) mg/L in water, which was 1.8 to 285-fold, 2.4 to 1314-fold, 2.6 to 999-fold of the MIC for *Escherichia coli* (1.25 mg/L), and 59.0 to 9146-fold, 76.9 to 42128-fold, 84.6 to 32046-fold of the MIC for *Staphylococcus aureus* (mg/L) (Fig 1).

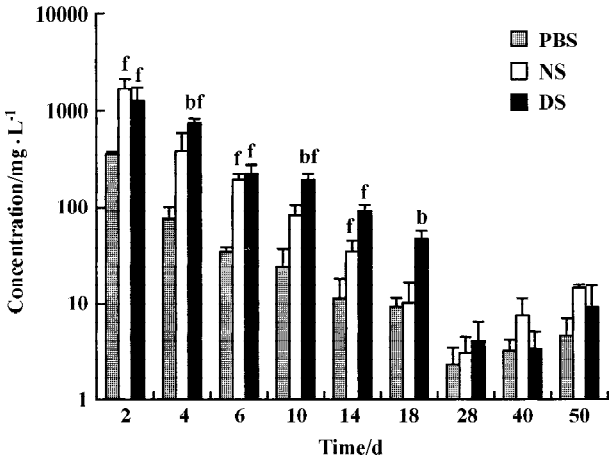


**Fig 1. *In vitro* release of gentamicin from poly [oleic/linoleic acid dimmer: sebacic acid (OAD/LOAD:SA)] drug beads, each bead weighed 150 mg loaded with 20 % gentamicin. PBS: 0.1 mol/L phosphate-buffer saline (pH 7.4). NS: 0.9 % saline. DS: water. $n$=4. Mean±SD. $^bP<0.05$ *vs* NS. $^fP<0.01$ *vs* PBS.**

The elution fluids of the placebo beads in water, 0.9 % saline, and PBS could not inhibit the bacteria *Escherichia coli* and *Staphylococcus aureus* to grow in all 50 days. The elution fluids of the drug beads in water, PBS, and 0.9 % saline all showed a strong inhibitory effect on the common causative bacteria of the chronic osteomyelitis: *Escherichia coli* and *Staphylococcus aureus* in 50 d (Fig 2). The greatest anti-bacterial effect was on d 2, then gradually decreased (Fig 2). Compared with water and 0.9 % saline, the inhibitory effect in PBS was lower from d 2 to d 14 ($P<0.01$, Fig 2). If the concentration of gentamicin standard and gentamicin in the elution fluids was the same, the antibacterial effects were also similar (Tab 1).

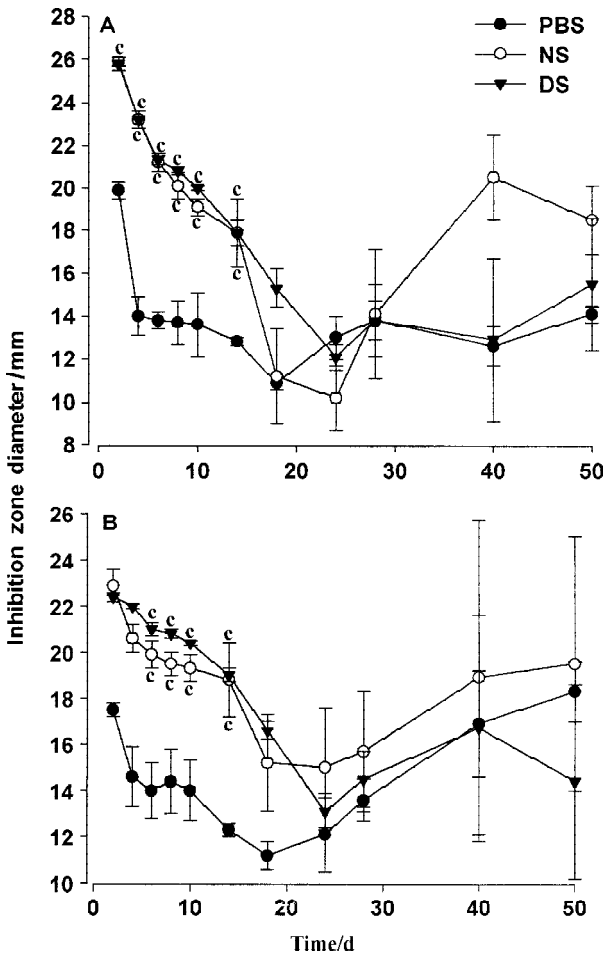


**Fig 2. Antibacterial effects of the elution fluids from drug beads on *E coli* ($2.80\times10^{10}$ CFU/L) (A) and on *S auseus* ($6.82\times10^{10}$ CFU/L) (B). PBS: phosphate-buffer saline (pH 7.4). NS: 0.9 % saline. DS: water. $n$=4. Mean±SD. $^cP<0.01$ *vs* PBS.**

The morphology of the placebo and drug beads remnants were all monitored in water, 0.9 % saline, and PBS. The greater part of the placebo and drug beads were cracked and the morphology and color changed in PBS after 50 d. But these beads only cracked slightly in water and 0.9 % saline, and the morphology and color did not change so apparently (Fig 3).

**Tab 1. *In vitro* antibacterial effects of the gentamicin standard and the elution fluids from drug beads. *n*=4. Mean±SD.**

| Medium | Day | Concentration/ mg·L$^{-1}$ | Diluted time | Inhibition zone diameter/ mm | |
|---|---|---|---|---|---|
| | | | | *E coli* | *S aureus* |
| Gentamicin standard | - | 10 | - | 20.2±0.3 | 16.5±0.4 |
| PBS | d 10 | 24±11 | 3 | 18.7±1.1 | 15.0±2.1 |
| 0.9 % Saline | d 14 | 34±11 | 3 | 21±4 | 17.1±2.8 |
| Water | d 14 | 89±16 | 8 | 20.9±0.1 | 17.2±0.4 |

The *E coli* and *S aureus* suspension used in this study was $2.80\times10^{10}$ CFU/L and $6.82\times10^{10}$ CFU/L, respectively. The final gentamicin concentration of the samples was approximately 10 mg/L.

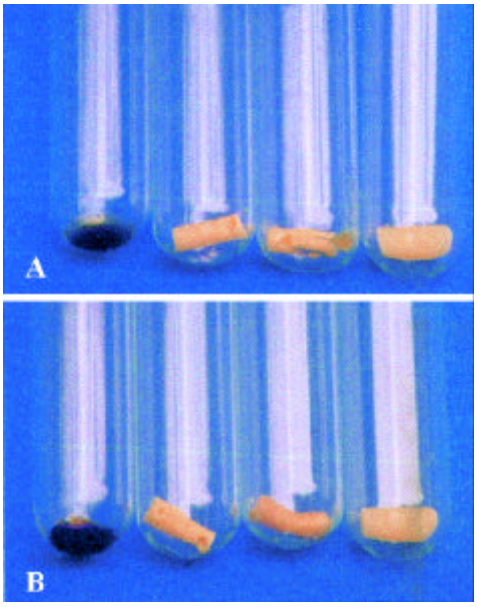


**Fig 3. Morphological evaluation of bead remnants after 50-d *in vitro* dissolution. A: placebo beads; B: drug beads. From right to left: normal placebo bead (drug bead) ® in 0.9 % saline ® in water ® in PBS.**

## DISCUSSION

The surgical removal of necrotic tissue and administration of antibiotic by the intravenous route are the primary methods for treatment of chronic osteomyelitis. But the causative bacteria are known to produce large amounts of extracellular fibrous glycocalyx materials, and necrotic bone provides a surface appropriate for the development of a biofilm, moreover, local bone necrosis and sclerosis can decrease blood flow, only low concentration of antibiotics are achieved in the lesion[1,2]. Even after long-term treatment with higher dose of antibiotics, the causative bacteria in the lesion is not eradicated completely, so the patients suffered from chronic osteomyelitis year after year[1,2].

Since it is impractical to deliver antibiotic to the target site at a sufficiently high concentration by the intravenous route, local administration is considered to be essential[1,2]: (1) The antibiotic can remain higher concentration in the local lesion and keep lower in the blood for a period, and the antibiotic side effects is decreased. (2) Many of the antibiotic carriers are good materials to repair bone defect. Currently, antibiotic-loaded cement beads of PMMA and ceramics (CHAC and AWGC) beads were successfully used as local antibiotic delivery systems, however, these compounds are not degraded or absorbed in the body, they must be removed by further operation and their long-term implantation is difficult[1-4]. The development of a biodegradable or biodegradable drug carrier has been awaited, but there are few reports on such material. Poly(LA), poly(LGA), and poly(EAD:SA) are biodegradable polymers which were studied *in vitro* or in animal models[5-8]. Lately, we synthesized another biodegradable fatty polymer called poly (OAD/LOAD:SA) containing oleic/linoleic acid dimer (OAD/LOAD) and sebacic acid (SA) in a 1:1 weight ratio, we mixed it with 20 % gentamicin to produce poly(OAD/LOAD:SA)-gentamicin beads[9].

In this study we found that poly(OAD/LOAD:SA)-gentamicin could sustainedly release high gentamicin over 50 d in water, PBS, and 0.9 % saline. The gentamicin in the fluids was 59 to 42128-fold of the MIC for *Staphylococcus aureus* and 1.8 to 1314-fold for *Escherichia coli.* The releasing fluids of the poly(OAD/LOAD:SA)-gentamicin beads in water, PBS, and 0.9 % saline all showed a strong antibacterial effects on the common causative bacteria of chronic osteomyelitis: *Escherichia coli* and *Staphylococcus aureas,* in 50 d. The characteristics of the *in vitro* release of poly (OAD/

LOAD:SA)-gentamicin were similar to AWGC-isepamicin[4]. Poly (OAD /LOAD:SA)-gentamicin released more gentamicin than poly (EAD:SA) -gentamicin (another new fatty polymer) *in vitro* and sustained more days[8]. The change of the morphology in the medium was also different from each other[8]. Poly (OAD/LOAD:SA) and poly (OAD/LOAD:SA)-gentamicin only cracked slightly in 50 d in water and 0.9 % saline, but cracked greatly in PBS. Poly (EAD:SA) remained intact in water in 34 d, also cracked greatly in PBS, but poly(EAD:SA)-gentamicin did not crack and appeared to swell over the course of drug release[8].

Poly(OAD/LOAD:SA)-gentamicin can sustainedly release gentamicin in water, PBS, and 0.9 % saline over 50 d, and the elution fluids *in vitro* all show a stong antibacterial effects on *Escherichia coli* and *Staphylococcus aureus* in 50 d. Local antibiotic administration for 4-6 weeks, is normally considered the best way to treat chronic osteomyelitis[12,15]. Poly (OAD/LOAD:SA)-gentemicin sustainedly release gentamicin over 7 weeks, and the characteristics of the *in vitro* release are similar to AWGC-isepamicin. These findings indicated that Poly (OAD/ LOAD:SA)-gentamicin was useful to treat chronic osteomyelitis.

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