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# FABRICATION OF CLAY BEADS LOADED WITH OPTIMISED GENTAMICIN-NIGELLA SATIVA EMULSION INTENDED FOR LOCAL TREATMENT OF OSTEOMYELITIS

BY

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# ABSTRACT

Local treatment of osteomyelitis using gentamicin-loaded PMMA beads has several challenges such as (i) requires invasive surgery to remove the beads from the implant site, (ii) can become the site of adherence for biofilm producing bacteria and (iii) has reduced effectiveness against biofilm bacteria. Therefore, in this study, gentamicin was fused with *Nigella sativa* oil (NSO) to be formulated as an optimised emulsion. The emulsion was then combined with clay-chitosan beads to improve gentamicin's efficacy and reduce the risk of toxicity. During the pre-formulation phase, the physical mixtures of gentamicin, NSO, and other excipients were subjected to compatibility test using differential scanning calorimetry (DSC) and attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR). The excipients used were Span 20, Span 80, Tween 20, Tween 40, Tween 80, Triton X-100 (TX100), polyvinyl alcohol (PVA), and polyethylene glycol 400 (PEG400). Design of experiment (DOE) was employed to develop the formulation of gentamicin-*N. sativa* oil emulsion (GNE). Plackett-Burman design (PBD) was used to identify the significant factor(s) and understand the effect of several factor(s) on emulsion droplet size ($Y_1$), and polydispersity index (PDI) ($Y_2$). These factors include (i) NSO concentration ($X_1$), (ii) emulsifier concentration ($X_2$-$X_{12}$), (iii) type of machine ($X_{13}$), (iv) homogenisation time ($X_{14}$) and (v) rate ($X_{15}$). Central composite design (CCD) was utilised to predict the best formulation of GNE. The stability of GNE was further characterised based on droplet size, PDI, and zeta potential after centrifugation 4000 rpm for 20 min. Antimicrobial test of the emulsion was conducted against osteomyelitic *P. aeruginosa*, *S. aureus*, and *S. epidermidis* strains. GNE-loaded clay-chitosan beads were then fabricated. Furthermore, scanning electron microscope (SEM) was utilised to observe the morphology of the beads. To quantify gentamicin in the beads, a quantification method validation was carried out using ATR-FTIR following ICH Q2 (R1) guideline. From the compatibility test, the result indicated that GS interacted physically with NSO and the excipients, but lacked in the chemical interactions that may compromise the efficacy and safety of GS in further formulation. The main effect analysis of each significant factors showed that adding NSO into the emulsion would significantly increase the droplet size, whereas the presence of Tween 40 and TX100 was able to decrease the droplet size. Stable emulsion with low PDI value (<0.5) was achieved by using sonicator and increasing the concentration of Tween 20, 40, and 80. NSO and Tween 80 were selected to be further investigated during GNE optimisation. The formulation with NSO concentration of 50% v/v and Tween 80 6% v/v was selected to be the optimum formulation with 292.00 ± 36.52 nm, 0.21 ± 0.04, and -31.23 ± 1.24 mv on droplet size, PDI, and zeta potential, respectively. Antimicrobial test showed that the combination of gentamicin and NSO in the optimised emulsion was able to enhance its efficacy of antimicrobial effect. The optimised GNE was successfully incorporated with clay-chitosan to form beads with the average diameter size of 4.27 ± 0.14 mm. SEM image showed the morphology of the beads appeared as a nonporous surface. The method validation for quantification of gentamicin using ATR-FTIR successfully met the acceptance criteria underlined by ICH Q2 (R1) guideline. These findings will serve as baseline research to develop new strategy in treating osteomyelitis.

# خلاصة البحث


يواجه العلاج الموضعي باستخدام خرز البولي مثيل ميثاكريلات المحملة بالجنتامايسين العديد من الصعوبات مثل (أ) الحاجة إلى عمليات جراحية كبيرة لإزالة الخرز في موقع الطعم، (ب) وإمكانية التصاق البيوفيلم البكتيري على الخرز، (ج) وتأثيرها الضعيف ضد بكتيريا البيوفيلم. وبالتالي تم في هذه الدراسة جمع الجنتامايسين مع زيت الحبة السوداء (NSO) لصياغتها كمستحلب محسَّن. بعد ذلك تم دمج المستحلب مع خرز الصلصال والتشيتوسان لتحسين أداء الجنتامايسين وللحد من السمية. في مرحلة ماقبل عملية الصياغة، تم اختبار المزائج المادية للجنتامايسين، وزيت الحبة السوداء، والسواغات الأخرى لتقييم التوافق باستخدام المسح الكالوري التفاضلي (DSC) ومطيافية تحويل فورييه ما فوق الحمراء ذي الانعكاس الكلي الموهَن (ATR-FTIR). السواغات التي تم استعمالها كانت سبان 20، وسبان 80، وتوين 20، وتوين 40، وتوين 80، وتريتون إكس-100 (TX100)، وكحول البوليفينيل (PVA)، وبولي إيثيلين جليكول 400 (PEG400). تم اتباع تصميم التجربة (DOE) لتطوير صياغة مستحلب الجنتامايسين وزيت الحبة السوداء (GNE). تم اتباع تصميم بلاكيت-بورمان (PBD) لتحديد العوامل الهامة ولفهم آثار العوامل المتعددة على حجم قطيرات المستحلب ($Y_1$) ومؤشر التشتت المتعدد (PDI) (Y2). تتضمن هذه العوامل على: (أ) تركيز الـ NSO (X1)، و (ب) تركيز المستحلب (X2-X12)، و (ج) نوع الآلة (X13)، و (د) وقت التجانس (X14)، و (هـ) المعدل (X15). تم استعمال تصميم المركب المركزي لتنبؤ أفضل الصياغات لـ GNE. تم بعدها توصيف استقرارية الـ GNE بناء على حجم القطيرات، والـ PDI، و جهود زيتا بعد الطرد المركزي بقوة 4000 دورة في الدقيقة لمدة 20 دقيقة. تم اختبار الخواص المضادة للمكروبات للمستحلب ضد مكروبات الزائفة الزنجارية (*P. aeruginosa*) المسببة لالتهاب العظام، والمكورات العنقودية الذهبية (*S. aureus*)، والعنقودية البشروية (*S. epidermidis*). بعد ذلك تم تشكيل خرز الصلصال والتشيتوسان المحملة بالـ GNE. بالإضافة إلى ذلك، تم استعمال المجهر الإلكتروني الماسح (SEM) لمعاينة تشكل الخرز. لقياس كمية الجنتامايسين في الخرز تم القيام بالتحقق من صحة طريقة التحديد الكمي باستخدام الـ ATR-FTIR وباتباع إرشادات الـ ICH Q2 (R1). أظهرت نتائج اختبار التوافق أن الـ GS اختلط فيزيائيا بشكل جيد مع الـ NSO ومع السواغات، ولكنه لم يكن كذلك في الاختلاط الكيميائي. أظهر تحليل المؤثر الهام لكل من العوامل الهامة أن إضافة الـ NSO إلى المستحلب يزيد من حجم القطيرة بشكل كبير، بينما صغَّر وجود التوين 40 و TX100 حجم القطيرات. تم انتاج مستحلب مثبَّت بكمية PDI منخفضة (<0.5) باستخدام جهاز الموجات فوق الصوتية، وزيادة تركيز توين 20، و40، و80. تم اخيار NSO و توين 80 للمزيد من الاختبارات في تجارب تحسين الـ GNE. تم اختيار صياغة الـ NSO بتركيز 50% v/v وتوين 6.80% v/v كالصياغة ذي الأداء الأعلى بـ 292.00 ± 36.52 نانومتر لحجم القطيرة، و0.21 ± 0.04 للـ PDI ، -31.23±1.24 مل فولت لجهد زيتا. أظهر تقييم النشاط المضاد للمكروبات أن بإمكان مزيج الجنتامايسين وزيت الحبة السوداء في المستحلب المحسَّن تقوية التأثير ضد المكروبات. تم دمج الـ GNE المحسن مع الصلصال والتشيتوسان لتكوين خرز بقطر طوله حوالي 4.27 ± 0.14 مم. أظهرت صور الـ SEM لتشكيل الخزر أن سطحها خال من المسامات. أوفى التحقق من صحة طريقة التحديد الكمي باستخدام ATR-FTIR معايير القبول المذكورة في إرشادات ICH Q2 (R1). نتائج هذا الدراسة ستكون بمثابة الخط الأساسي في الأبحاث التي تهدف لتطوير خطط جديدة في معالجة التهاب العظام والنخاع.

# APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Health Sciences (Biomedical Science).

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# DECLARATION

I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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## FABRICATION OF CLAY BEADS LOADED WITH OPTIMISED GENTAMICIN-NIGELLA SATIVA EMULSION INTENDED FOR LOCAL TREATMENT OF OSTEOMYELITIS

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*To my beloved family for the supports and trust.*

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Indeed, all the praises is due to Allah, we praise him and we seek his assistance and forgiveness, and we seek refuge in Allah from the evil of ourselves and our bad deeds, whoever Allah guides there is none that can misguide him and whoever is leads astray there is none that can guide him, and I bear witness there is no God other than Allah, He has no partners, and I bear witness that Muhammad (PBUH) is his servant and messenger.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| API | Active Pharmaceutical Ingredient |
| ATR-FTIR | Attenuated Total Reflectance Fourier-transform infrared spectroscopy |
| BBD | Box-Behnken Design |
| CB | Clay Beads |
| CCD | Central Composite Design |
| CCFD | Central Composite Face-Centred Design |
| CoA | Certificate of Analysis |
| DOE | Design of Experiment |
| DSC | Differential Scanning Calorimetry |
| EINECS | European Inventory of Existing Commercial Chemical Substances |
| EPS | Extra Polymeric Substance |
| FCC | Food Chemicals Codex |
| FD | Factorial Design |
| FFD | Fractional Factorial Design |
| GM | Gentamicin |
| GNE | Gentamicin-*Nigella sativa* oil Emulsion |
| GS | Gentamicin Sulphate |
| IR | Infrared |
| MIC | Minimum Inhibition Concentration |
| NS | *Nigella sativa* |
| NSO | *Nigella sativa* oil |
| OVAT | One Variable at a Time |
| PBD | Plackett-Burman Design |
| PDI | Polydispersity index |
| PEG400 | Polyethylene glycol 400 |
| PMMA | Polymethylmethacrylate |
| PVA | Polyvinyl alcohol |
| QTPP | Quality Target Product Profile |
| RSM | Response Surface Method |
| SEM | Scanning Electron Microscope |

# CHAPTER ONE

# INTRODUCTION

## 1.1 BACKGROUND OF THE STUDY

Osteomyelitis refers to an inflammatory process of bone that is usually caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Lew & Waldvogel, 2004; Brin et al., 2008; Gomes, Pereira, & Bettencourt, 2013). The main method of treatment is surgical debridement of affected bone with or without antibiotic. In chronic osteomyelitis, for example, parenteral antibiotic is given for 4-6 weeks but may result in toxicity (Gogia, Meehan, Di Cesare, & Jamali, 2009). Acute osteomyelitis on the other hand would require surgical treatment alone with parenteral antibiotic treatment as an option as need arises (Hatzenbuehler & Pulling, 2011).

In recent decades, the use of local antibiotics to prevent musculoskeletal infection has been on the increase in osteomyelitis. Local antibiotic is believed to have a great impact delivering antibiotic on the site of action. It has some advantages, for example, it requires low serum concentration, has a selective target of action, and benefits the patient economically (Gomes et al., 2013).

The right selection of antibiotic to be used in osteomyelitis treatment not only depends on the microbes involved in infections but also on the ability of the drug to maintain stability and delivered as safe doses in the infected area. Various antibiotics have been tested for local administration in osteomyelitis. Gentamicin as an aminoglycoside meets the above criteria. It has a concentration-dependent bactericidal characteristic and is effective against a wide range of aerobic gram-negative bacteria (Chen, Chen, Wu, & Chen, 2014). It also shows a synergistic effect in a combination therapy against gram-positive bacteria (Nandi et al., 2009). Its thermo-stability prevents gentamicin from being degraded by high temperature during fabrication and application in affected areas. This is the single reason why gentamicin remains as the preferred antibiotic these days for treating osteomyelitis.

Gentamicin-loaded PMMA (polymethylmethacrylate) beads has been used as local treatment of osteomyelitis until recently. It has been approved in Europe as there was evidence that it can positively support the surgical treatment in chronic osteomyelitis (Klemm, 2001). However, PMMA is non-biodegradable. A second invasive surgery is required to remove the beads after the antibiotic has been completely released. This adds to the cost of treatment with the increased number of surgeries.

A number of materials have been studied to replace PMMA as the drug carrier (Nandi et al., 2009). Most of the carriers depend on biodegradable materials, such as: collagen sponge, lactic acid polymers, and calcium phosphate based ceramics. However, some of the materials e.g. lactic acid polymers are expensive. A few studies have tried to develop a low cost carrier as a substitute to PMMA by using clay (Rodrigues et al., 2013; Yang et al., 2015).

Clay has been used in pharmaceutical field as an excipient or the active substances (M.I. Carretero, Gomes, & Tateo, 2013). For many years, it was reported that clay may be used to (1) improve bioavailability, (2) reduce the toxic effect, and (3) control the release of the drug (Zhou, Zhao, Wang, Chen, & He, 2016). Clay could also sustain the release of drug when coated with polymers such as chitosan (Wang, Du, & Luo, 2008). Clay minerals e.g. hydroxyapatite can be used as implant material due to their biocompatibility with bone structures. This can be used to treat osteomyelitis by hybridization with antibiotic e.g. gentamicin. In addition, the use of natural compound such as Nigella sativa oil (NSO) combined with gentamicin to provide synergistic effect has also been explored, with preliminary studies suggesting evidence of possible use as osteomyelitis treatment (Yusof, Shafri, Yaakob, Yusof, & Mohamed, 2014).

## 1.2 LITERATURE REVIEW

### 1.2.1 Osteomyelitis

In 1852, Chassaignac described osteomyelitis by the words osteon and muelinos that mean bone marrow infections (Lindfors, 2011). The infection can be caused by any microorganisms that can reach the soft tissue inside bone. Even though normal bone is relatively resistant to infection, traumatic bone damage and surgery may predispose an individual to the infection. Other conditions such as, decubitus ulcers, intravenous drug users, immune diseases, immunosuppression, smoking, malnutrition, chronic hypoxia, and diabetes mellitus may also increase the risk of osteomyelitis (Eid & Berbari, 2012). Early detection of non-specific symptoms e.g. fevers, fatigue, chills, irritability, or malaise combined with physical examination and imaging as well as laboratory studies will increase the chance of successful treatment in osteomyelitis (Chihara & Segreti, 2010).

The prevalence of osteomyelitis ranges from 2 to 16% based on the level of trauma and the correct selection of antibiotic (Browner, Jupiter, Trafton, Levine, & Neil E. Green, 2002). In the early 70's, a review stated that the percentage of haematogenous, contiguous, and vascular insufficiency osteomyelitis were 19, 47, and 34%, respectively (Waldvogel, Medoff, & Swartz, 1970). Various types of osteomyelitis can be grouped according to Waldvogel and Cierny & Mader classification. Waldvogel divide osteomyelitis according to its physiopathology (haematogenous, contiguous, and vascular insufficiency) and duration of infection (acute and chronic) (Table 1.1) (Lima et al., 2014). Acute haematogenous osteomyelitis mainly affects children and involves the area of highly vascular long bones (tibia and femur) and vertebral with back pain as the main symptoms. Contiguous infection is usually a chronic osteomyelitis associated with traumatic incidence which multiple surgeries are needed to undergo prosthetic implantation. Vascular insufficiency related to osteomyelitis is caused by inadequate flow of blood through the veins, causing the blood to pool in the legs or feet, which is commonly associated with diabetes patients (Lew & Waldvogel, 2004).

Haematogenous osteomyelitis develops mostly in children (age 1-10) and in elderly patients (age 51-60) (Kremers et al., 2015). In addition, a population-based study from 1969-2009 showed an important data that the incidence of osteomyelitis was higher for men than women annually (Kremers et al., 2015). The study also reported that diabetes mellitus is a major risk factor for patient older than sixty years.

On the other hand, Cierny and Mader categorized osteomyelitis according to the bone involvement, and three types of host, depending on the patient's clinical conditions (Table 1.2) (Lima et al., 2014). The types of osteomyelitis notwithstanding, once pathogens are able to reach the bone, pathogens can cause infection. The most common pathogens responsible for osteomyelitis in humans are *Staphylococcus* species, followed by Enterobacteriaceae and *Pseudomonas* species (Gogia et al., 2009). A summary of different aetiology related to age and the predisposing conditions is shown in Table 1.3.

Table 1.1 Waldvogel classification of osteomyelitis (Lima et al., 2014)

| | Characteristics |
|---|---|
| *Mechanism of bone infection* | |
| Haematogenous | Secondary to bacterial transport through the blood. Majority of infections in children. |
| Contiguous | Bacterial inoculation from an adjacent focus e.g. Post-traumatic Osteomyelitis. |
| Associated with vascular insufficiency | Infection affecting the feet in patients with diabetes, hanseniasis or peripheral vascular insufficiency. |
| *Duration of infection* | |
| Acute | Initial episodes of osteomyelitis. Oedema, formation of pus, vascular congestion, thrombosis of the small vessels. |
| Chronic | Recurrence of acute cases. Large areas of ischemia, necrosis and bone sequestra. |

Table 1.2 Cierny and Mader classification of osteomyelitis (Lima et al., 2014)

| | Characteristics |
|---|---|
| *Anatomical stage* | |
| 1. Medullary | Infection restricted to the bone marrow. |
| 2. Superficial | Infection restricted to cortical bone. |
| 3. Localised | Infection with clearly defined edges and bone stability preserved. |
| 4. Diffuse | Infection spread to the entire bone circumference, with instability before or after debridement. |
| *Classification of the host* | |
| A – Host healthy | Patient without comorbidities. |
| Bl – Local compromise | Smoking, chronic lymphedema, venous stasis, arthritis, large scars, fibrosis by radiotherapy. |
| Bs- Systemic compromise | Diabetes mellitus, malnutrition, renal or hepatic failure, chronic hypoxia, neoplasms, extremes of age. |
| C – Poor clinical conditions | Surgical treatment will have higher risk than the osteomyelitis itself. |