Formulation Development, Characterization and In Vitro Antibacterial Activity Evaluation of Cefazolin Loaded Mesoporous Silica Nanoparticles

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ABSTRACT: The main aims of this manuscript are to: i) investigate the high drug loading of cefazolin and its characterization, ii), demonstrate the bioactivity of the cefazolin particles in vitro on Staphylococcus aureus. From our results, it is observed that the cefazolin loading into MCM-41 particles is 34 wt %. Furthermore, particles showed the burst release of cefazolin at pH 6.8. At higher concentration, MCM-41 particles are comparatively more cytotoxic as compared to lower concentration. Finally, cefazolin loaded particles showed higher in vitro antibacterial activity against Staphylococcus aureus as compared to cefazolin only.

KEYWORDS: antibiotics, cefazolin, drug delivery, mesoporous silica nanoparticles, MCM-41

1. INTRODUCTION

Infectious diseases are the second major cause of death worldwide [1]. Antibiotic treatment sometimes fails due to emergence of drug resistant strains, or due to insufficient activity or concentration of antibiotics at the site of infection. Different strategies have been adopted to improve antibiotic therapies, such as chemical modification of current antibiotics, development of new antimicrobial agents, or improvement of currently available antibiotics by new formulations [2]. Nanomaterials offer specific advantages to drug formulations, as they can function as antimicrobial agents, while also increasing therapeutic effects of current antibiotics by extended blood circulation, targeted delivery to the site of infection, and controlled release that lessens adverse side effects by minimizing nonspecific interactions and decreasing the required treatment dose [3]. Cefazolin (CEF) is a cephalosporin with increased activity against most gram-positive, many gram-negative, and certain anaerobic bacteria. Cefazolin is mainly used for topical bacterial infections according to FDA with great scope for soft tissue infections preoperatively and postoperatively. It is the most prescribed drug among the patients undergoing orthopaedic surgery because of the increased risk of exposure to Staphylococcus aureus especially in knee arthroplasty [4]. But the drug accounts for its repeated administration due to its shorter half-life (1−2h) as it gets rapidly eliminated from the body thus accounting for bacterial resistance. Antimicrobial agents are decided according to the type of microorganism based on the clinical practice guidelines for post-surgical infections. Cefazolin serves as the standard of care and first drug of choice according to the current practice in antimicrobial prophylaxis [5]. It has been the most widely used agent with confirmed efficacy against S. aureus as well as widely common organisms encountered in surgery such as Escherichia coli and various strains of Streptococci, Proteus mirabilis, and Klebsiella species [6].

Ideally, an antibiotic delivery system should provide sustained and controlled release to nearby tissues, eliminating the need for systemic infusions or repeated injections of the drug [7]. Localized release allows for total dose reduction and minimizes systemic toxicity and resistance, and various biodegradable and bioreparable carriers of antibiotics for the treatment and prevention of prosthetic infections have been studied [8]. Therefore, many materials have been introduced and chemically modified to maintain extended-release properties in the past decade [9]. Simultaneously, with the development of new polymers and inorganic porous matrices [3], nanotechnology has increasingly influenced the field.

In recent decades, mesoporous silica nanoparticles (MSNs) have been one of the most studied drug carriers due to several advantageous properties, such as high surface area, large pore volume, versatile surface chemistry, ease of surface functionalization, high biocompatibility, high chemical and thermal stability, and simple synthesis methods [10]. In this work, we have synthesized, characterized and shown the release kinetics of cefazolin from nanopores of MSNs and bactericidal activity of them on Staphylococcus aureus.
2. EXPERIMENTAL SECTION

2.1. Materials

Cetyltrimethylammonium bromide (CTAB, 99%), tetraethyl orthosilicate (TEOS, reagent grade 98%), (3-Aminopropyl)triethoxysilane (APTES, 99%), 3-(trihydroxy silyl) propyl methyl phosphonate (THMP, 90%), dimethyl sulfoxide (DMSO), Toluene (ACS), Acetone (analytical grade), hydrochloric acid (32%) Acetonitrile (HPLC grade), Trifluoro acetic acid were purchased from MERCK, Frenchs Forest, Australia. Cefazolin Sodium was purchased from Sigma-Aldrich, Australia. All aqueous solutions were prepared using DI water (18 MW) from Millipore Milli-Q system.

2.2. Synthesis of MCM-41

MCM-41 particles were prepared using a previously published protocol with slight modification [11]. Briefly, CTAB (1 g) was dissolved in 480 mL of deionised water to which 3.5 mL of NaOH (2 M) was added, and the temperature raised to 80°C. TEOS (6.7 mL) was slowly added to this mixture and stirred for 2 h at 80°C. The sample suspension was then vacuum filtered and washed with deionised water. The filtrate was dried overnight at room temperature followed by calcination at 550°C for 5 h in a muffle furnace at a temperature ramp rate of 5 °C/min for up-ramp and 10°C/min for down-ramp.

2.3. Loading of cefazolin into MCM-41 using immersion method

To obtain 30 % mass loading, CEF (35 mg) and pristine or functionalized MCM-41 (65 mg) were added in 5 mL of water for the preparation of nanoparticles. The solution was magnetically stirred at 4 °C for 24 h. Drug loaded particles were freeze-dried overnight and stored at 4 °C.

2.4. Characterization of nanoparticles

TEM images of MSNs were acquired by Hitachi 7700 (Hitachi, Japan) electron microscope at acceleration voltage of 100 kV. Nitrogen adsorption -Brunauer-Emmett-Teller (N₂-BET) (Tristar, Micromeritics-II, Norcross, GA, USA) was operated to measure the pore size, volume, and surface area of MSNs. The particle size and surface charge were calculated in water (solvent) by dynamic light scattering (DLS) and zeta potential measurements (Malvern, Nano-ZS, ATA Scientific, Taren Point, Australia). Thermogravimetric analysis (TGA) (Mettler Toledo, TGA/DSC 2, columbus, OH, USA) was performed with a heating rate of 10 °C/min in an airy ambiance. The HPLC analytical (Waters 2695 Separations Module, Waters Corporation, Milford, MA, USA) method for CEF was developed based on different modifications. Briefly, the system consisted of 0.05 M KH₂PO₄: acetone triethylamine (90:10, v/v) pH 5.0 as the mobile phase at an isocratic flow rate of 1.5 mL/min, and detection wavelength 254 nm [12]. Chromatographic separations were performed on Phenomenex® C8 RP-HPLC column (250 mm × 4.6 mm, 5 μ). Standard curve was plotted with n = 3.

2.5. In vitro release study

The CEF-MCM with 500 μg equivalent cefazoline were dispersed in 5 mL of the united states pharmacopoeia (USP) specified simulated gastric fluid test solution without enzymes (pH 1.2) and simulated intestinal fluid test solution without enzymes USP (pH 6.8) and stirred at 150 rpm and 37. Aliquots (200 μL) were taken at 0.08, 0.5, 1, 2, 4, 6, 8, and 24 h and centrifuged at 12000 rpm for 5 h. Equal volume of fresh buffers (pH 1.2 and 6.8) were added to wash the suspension and medium only were used as controls for each plate. After incubation, 25 μL/well of MTT reagent was added for a further 4 h at 37 °C. The cell culture medium was then aspirated, and the formazan crystals were dissolved by adding 100 μL/well DMSO. The absorbance signal of formazan was measured at 595 nm using a microplate reader.

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2.7. In vitro antibacterial assay
2.7.1. Broth microdilution method
This method was used to determine the minimum inhibitory concentration (MIC90) of antimicrobial agent that inhibits visible growth of a microorganism such as S. aureus (ATCC 29213 and clinical strain 22) according to the procedure established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [13, 14]. Briefly, serial two-fold dilutions of antibiotic samples such as CEF and CEF nanoparticles (concentrations of MER in nanoparticles is equivalent ranging from 0.0313 mg/L to 16 mg/L) were prepared in CAMHB (cation adjusted Mueller Hinton Broth) and aliquoted (100 μL) into flat-bottom microtiter plates. McFarland 0.5 standardized inoculum suspension (1×10^6 CFU/mL) prepared in sterile distilled water was added into cation adjusted Mueller Hinton Broth (CAMHB) to give rise to approximately 1×10^6 CFU/mL. Then 100 μL of inoculum suspension prepared in CAMHB was added to the drug samples containing microtiter plates to give rise to approximately 5×10^5 CFU/mL. After that, inoculated plates were incubated at 37 °C for 16 to 20 h and MIC values were determined spectrophotometrically at 620 nm. This experiment for the determination of MIC values was repeated three times.

2.8. Statistical analysis
All experiments were done in triplicate unless otherwise stated. Datasets were analysed using one-way ANOVA and post-hoc Tukey’s test where applicable.

3. RESULTS AND DISCUSSION
3.1. Characterization of the nano-systems
The synthesised particles were first characterised using transmission electron microscopy (TEM) to assess the success of the synthesis. As shown in Figure 1, the MCM-41 particles were mostly spherical in shape with a slightly rough outer surface, and hexagonal pore arrangement. Furthermore, Figure 2 and Figure 3 show dynamic light scattering data in water including particle size and surface potential of these particles before and after CEF loading, respectively. The hydrodynamic size of MCM-41 is 263 nm, which increased to 345 nm after CFE loading (CEF-MCM-41). The MCM-41 and CEFMCM-41 particles had negative zeta potentials of −17.2 and −17.7 mV, respectively. We next performed Nitrogen (N2) sorption isotherm analysis, pore size distribution analysis, and BET surface area plots on MCM-41 and their drug loaded particles. N2 adsorption-desorption analysis demonstrated that all particles displayed typical IUPAC type-IV isotherms, indicating the mesoporous nature of the silica samples (Figure 4). The particles also displayed a steep capillary condensation step at a relative pressure (P/Po) range of 0.2–0.4, characteristic of MCM-41 type mesoporous materials (Figure 4). BET surface area plots demonstrated that the pristine MCM-41 had a surface area of 970 m²/g, which was reduced upon drug loading with CEF to 342 m²/g (Figure 3a). Similarly, the pore size of MCM-41 (2.2 nm) was reduced to 1.7 nm (CEF-MCM). Finally, pore volume of MCM-41 (0.90 cm³/g) was reduced to 0.78 cm³/g for CEF-MCM. These changes in physical properties induced by drug loading of the nanoparticles are consistent with previous reports [10, 15].

Drug loading was also analysed using Fourier transform infrared (FTIR) spectroscopy. As shown in Figure 5, CEF shows a sharp intense peak at 1757 cm⁻¹ (which clearly shows presence of carbonyl group), which confirms the carboxylic group in CEF. Some of the other notable peaks seen in cefazolin containing samples include that at 1647 cm⁻¹ and 1593 cm⁻¹ indicating C=N stretching and the presence of amide [16]. For MCM-41, a peak at 3430 cm⁻¹ is related to the stretching vibration of -OH groups (Si-OH), while other characteristic peaks at 1068 cm⁻¹ and 802 cm⁻¹ are assigned to Si–O and Si–O–Si vibrations of silanol groups [17]. However in case of CEF-MCM-41, the characteristic peak of CEF at 1768 cm⁻¹ can be seen with lower intensity, which confirm the loading of CEF in MCM-41.

TGA was performed to determine the % drug loading. As shown in Figure 6, 34 % CEF was loaded into MCM-41 nanoparticles.
Figure 1. Transmission electron microscope (TEM) images. TEM images of particles; (a) MCM-41 and (b) CEF-MCM

Figure 2. Dynamic light scattering (DLS) data. Hydrodynamic size of particles; MCM-41 and CEF-MCM

Figure 3. Dynamic light scattering (DLS) data. Zeta potential of particles; MCM-41 and CEF-MCM
Figure 4. N$_2$-BET plot for MCM-41 and CRF-MCM-41. The particles exhibit IUPAC-type IV isotherm and retain the porous architecture after drug loading.

Figure 5: (a) Fourier Transform Infrared (FTIR) spectroscopy of CEF, MCM-41 and CEF-MCM-41 particles for range 500 cm$^{-1}$ to 4000 cm$^{-1}$. 
3.2. In Vitro Release Study

As seen from Figure 7, CEF-MCM released over 70% (burst release) of CEF in pH 6.8 in the first 30 min. After that, there was no significant increase in the drug release and total amount of drug release in 8 h was 76%.

3.3. In Vitro cell viability Study

The toxicity of the MCM-41 nanoparticles toward RAW 264.7 macrophages was evaluated using the MTT assay. The results (Figure 8) revealed that toxicity of MCM-41 particles at 100 µg/mL was less than 20% (81% of cells were viable). However, the toxicity of particles was enhanced at high concentration i.e. cell viability was 51% at 1000 µg/mL concentration of MCM-41.
3.4. In vitro antibacterial activity of CEF-MCM

The minimum inhibitory concentration (MIC$_{\text{90}}$) of CEF-loaded nanoparticles was investigated using broth micro-dilution followed by EUCAST guidelines. We used gram-positive (S. aureus) bacteria (both ATCC and clinical strain) to determine the antibacterial effect of MPN formulations. As summarized in Table 1, MIC value for CEF-MCM particles is half as compared to free drug (CEF). Therefore, our nanoparticles showed the potential to significantly decrease the MIC value of the Pure CEF.

### Table 1: Values of MIC (mg/L) of CEF and CEF-MCM against ATCC and clinical isolates S. aureus

| Formulations | S. aureus (mg/L) | ATCC | clinical strain 22 |
|---------------|-----------------|------|--------------------|
| CEF           | 0.125           | 0.0625 |
| CEF-MCM       | 0.0625          | 0.0312 |

**CONCLUSION**

In summary, we demonstrated that encapsulation of the cefazolin within MSNs represents an effective strategy for improving its physicochemical properties. MCM-41 nanoparticles showed good drug loading (34%). Furthermore, drug loaded silica showed improved anti-bacterial activity compared to free drug against both ATCC and clinical strains of S. aureus. Our results demonstrate that mesoporous silica-based carriers have the potential to improve the antibacterial activity of cefazolin.

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