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Evaluation on the *Bacillus subtilis* spore in drug delivery

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ABSTRACT

Bacillus subtilis spores were used to study on drug delivery nowadays. However, evaluation on the cisplatin packaging and releasing ability in spores is still limited. In this study, we determined *Bacillus subtilis* spores ($2x10^8$) could be packaged to 54,600,000 drug molecules of cisplatin from 8 to 12 hours. Spores were checked by scanning electron microscope and transmission electron microscope, and then drug release was done. Cisplatin started releasing at 0.5 hours at pH 3.4 (23.8%) that was higher than pH 7.2 (0.5%). Cisplatin released irregularly during 24 hours in pH 3.4. In contrast, the cisplatin release was slow and steady during 24 hours in pH 7.2. The release ability was only 25% that meant that cisplatin packing spores would be used in prolonged-release finished products. The study was the first report of the cisplatin-packaged spore and release ability of cisplatin out of spore that could be used in pharmaceutical field.

INTRODUCTION

Drug delivery is a process of administering an active compound to achieve a therapeutic effect to humans (Tiwari *et al*. 2012). There are many researches on nanoparticle properties in drug delivery. The nanogolds, nanosilvers were used to study in anticancer and antimicrobial activity. The absorption capacity of these nanoparticles to bind or carry drugs and biomolecules gave the investigating molecules-nanoparticles binding events. The absorption process and binding of nanoparticles will explain the pharmacological responses for nanomaterials that could be used as drug delivery systems. Drug target, drug release, drug absorption, side effect are also affected and allowing for immunoisolation (Desai *et al*., 1997). Up to date, thousands of potent drugs have been used and still discovered. However, a medicine with low side effect but high bioavailability is required. The strategies for drug delivery are a result of two main basic properties: small size and use of biodegradable materials. In general, the small sized particles allow the efficient uptake and selective drug accumulation at target sites. Furthermore, the new generations of drug based on the small size of particles to increase the surface area to improve the bioavailability of poorly soluble drug. Recently, the use of bio-nanoparticle has been rapidly grown. The smallest capillaries in the body are 5–6 μm diameter. The size smaller than 5 μm of particle is distributed into the bloodstream, without forming aggregates and do not cause an embolism (Singh and Lillard 2009). Therefore, minicells from bacteria (Nguyen *et al*., 2013; Doan and Nguyen, 2013), spores of *Bacillus subtilis* are currently one of well- studied drug delivery systems used in the treatment of cancer. In addition, killed *Bacillus subtilis* spores had been investigated to express streptavidin delivered paclitaxel (Nguyen, Huynh *et al*., 2013). However, drug package and release of *Bacillus subtilis* spores, especially, cisplatin has been not reported. Cisplatin (304.04 Cl2H6N2Pt), a chemotherapy drug that is given to treat testicular, bladder, lung, gullet (oesophagus), stomach and ovarian cancers. As the above statements, this study developed the encapsulation efficiency**,** drug releasing of *Bacillus subtilis* spore packaged with cisplatin.

MATERIALS AND METHODS

Spore preparation

Bacillus subtilis ATCC 6633 strain was collected from the American-type culture collection that was grown in Luria *broth* in 37°C until OD about 0.08 to 0.1 that was equal to 10^7 cells per milliliter. 100μl inoculum was spread onto sporulation agar plates and incubated for 7 days at 37°C (British pharmacopoeia 2013). Spores were collected by scraping with 5ml sterile water into a sterile centrifuge tube and then heated 70°C into 15 minutes then centrifuged to collect spores at 13000g for 10 minutes.

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The supernatant containing cell debris was discarded. The purity of the spore formation was confirmed by optical microscopic (BioMed Lx400, USA) after staining malachite green spores. The number of spore formation was counted by hemocytometer. The spore suspensions may be stored for long periods at a temperature not exceeding 4 ^{o}C.

Drug Encapsulation

Preparations of purified spores were incubated with chemotherapeutic drugs cisplatin (100µg/ml) in 4, 8, 12, 16, 20, 24 hours at 37°C with rotation. Then, spores were harvested by centrifuged at 13000 g/ 15 min/ 4^{o}C and had been washed thoroughly with three exchanges of sterile water. Finally, drugs were extracted from packaged spores for drug qualification and quantitation using HPLC-UV/Vis Spectroscopy system. HPLC system (Shimadzu, Kyoto, Japan) consisted of LC-8A solvent delivery module and UV-Visible spectrophotometric detector and phenomenax C-18 column (250x4.60 mm-5microns) was used for the analysis. Chromatographic analyses were performed with a mobile phase consisting of sodium octanesulfonate, tetrabutylammonium hydrogen sulfate, potassium dihydrogen phosphate and water (1: 1.5: 2.5: 950) pumped at a flow rate in 1.0 mL/min. The injection volume of sample was 20 µL, and spectrophotometer was set at 210 nm. The working temperature was at 30°C. The concentrations of cisplatin in the total spores were measured according to the standard curve. All experiments were performed in triplicate.

Characterization of spore encapsulated cisplatin

The morphology of the spore was determined using a scanning electron microscope (SEM) Hitachi S-4800 with voltage 10kv applied for all samples. The transmission electron (TEM) micrographs of the spores were collected using JEOL JEM-1400. An accelerating voltage 100kv of TEM was applied to all samples. The spores suspensions ($2x10^{8}$) incubated with cisplatin at 100 µg/ml in 4, 8, 12, 16, 20 hours. Glass bead extraction method was applied to collected cisplatin for HPLC.

The encapsulation efficiency was calculated as:

Encapsulation Efficiency= Drug encapsulated in total spore/total drug x 100

In vitro Release Kinetics

Dialysis bags were immersed in water for one hour to remove the contaminations. The drug encapsulated spores were placed in dissolution media (pH 3.4 and 7.2) and loaded in the dialysis bag. The bag was sealed at both the ends and immersed in 4 mL of dissolution media. Dissolution buffer (pH 3.4) contains 7.507 g of *glycine* and 5.844 g of *sodium chloride* in 800 mL of *water*, adjust the pH to 3.4 with hydrochloric acid and dilute to 1000 mL with water. Dissolution buffer (pH 7.2) consists 9.075 g of *potassium dihydrogen phosphate* in *water* to produce 1000 mL (solution A), 11.87 g of *disodium hydrogen phosphate* in sufficient *water* to produce 1000 mL (solution B). Then, dissolution buffer (pH 7.2) was a mixture of 300 mL of solution A with 700 mL of solution B. The amount of drug released was measured as absorbance using a Multi-mode microplate reader (Winooski, Vermont 05404-0998 USA). The absorbance of each period in different buffers was measured to determine the drug release.

Statistical analysis

All the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed with SPSS (version 18.0) using one- way ANOVA with turkey test for multiple comparision. $P<0.05$ was considered as statically significant.

RESULTS AND DISCUSSION

Spore formation

Bacillus subtilis spores could bring the necessary tools to improve inherent limitations of classical pharmacotherapy. Bacterial spores are hardy and inactive life forms with resistance properties that have potential for drug delivery system (Ricca, E., and Cutting, S. M. 2003). The vegetative cells form single spores or endospores, which are highly resistant to lysozyme, protease digestion, dehydration, antibiotics, solvents and heat up to 80^{o}C (Barnes, et.al, 2007). In this study, *Bacillus subtilis* spores were formation about $2x10^{8}$ spores/ml (79%) from 400 ml starting agar culture medium. The spore size was evaluated by optical microscope, and then by scanning electron microscope and transmission electron microscope (Figure 1). The results showed that the average sizes of spores formation about 0.8 µm to 1.2µm in length and 0.3 µm to 0.7 µm. These sizes allow spores for access into blood stream and cells that is potential beneficial for pharmaceutical requirements. Although the trend of using nanoparticles, the area of drug delivery of large particle may be necessary for loading a sufficient amount of drug onto the particles (De Jong and Borm, 2008).

In summarize, *Bacillus subtilis* spores are biological materials source with characterizations as biodegradable and nano size to micro size that are primary goals in modern drug delivery.

Spore encapsulated cisplatin characterization

In previous reported, particles were convenient for drug delivery with the sizes under 5 µm (Singh and Lillard 2009). As can be seen from the figure 1, the SEM images of spores with their average diameter ranged about 0.7µm of width and 1 µm of length. As the results, spores generated from *Bacillus subtilis* were available for drug delivery. The outer layer of *Bacillus subtilis* spore coat contains a lot of proteins such as CotA, CotB, CotC, CotF (Ricca and Cutting, 2003). Therefore, cisplatin had interacted with coating protein of spore. TEM images show that there were different about structure between spore free cisplatin and spore packaged cisplatin. The cisplatin is not only interaction with outer layer of spore but interaction inside of spore. As a result, spore encapsulated cisplatin as vehicle to everywhere of body.

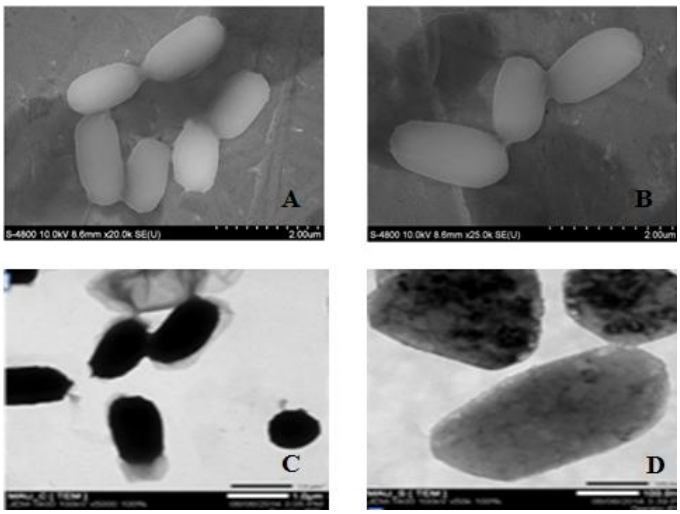

Fig. 1: Electron microscope of Spore encapsulated chemotherapeutic: A) SEM of spore, B) SEM of spore encapsulated cisplatin, C) TEM of spore, D) TEM of spore encapsulated cisplatin.

Encapsulation Efficiency (EE)

Bacillus subtilis spores are very flexible systems because of their abilities delivering different bioactive compounds concluding low and high molecular weight drugs. The *Bacillus subtilis* spores were packaged with cisplatin in different time to test the ability and potency of drug delivery. As shown in figure 1 and 2, cisplatin had abilities to be packaged in *Bacillus subtilis* spores. The drug packaging was dependent on characteristics of molecules and incubation time. The concentrations of cisplatin were detected and quantified by HPLC and whose results were showed in table 1. With the incubation of 2 x 10^8spores in the solution containing 100µg cisplatin for 4, 8, 12, 16, 20, 24 hours, the amount packaged was 12.8 µg, 15.25 µg, 14.81 µg, 11.97 µg, 13.56 µg, 13.63 µg, respectively.

Table 1: Cisplatin quantification in spores (56x107) incubated in the different time.

Time (hour)	Concentration (µg)	NPT (Molecules)[b]	NPS (Molecules)[c]	EE (%)[a]
4	12.8±0.2	25.7	45.8	12.8±0.2
8	15.25±0.25	30.6	54.6	15.3±0.25
12	14.8±0.31	29.7	53	14.8±0.3
16	11.97±0.3	24	42.9	11.9±0.3
20	13.56±0.1	27.2	48.6	13.6±0.1
24	13.63±0.37	27.4	48.9	13.6±0.37

[a] Data are means ± standard deviations of triplicates for all treatments;
[b] Expressed $\times 10^{16}$
[c] Expressed $\times 10^{6}$
NPS: the number of cisplatin molecules present in the loading solution; NPT: the number of cisplatin molecules present in the total spores; NPS: the number of cisplatin molecules per spore; EE%: is encapsulation efficiency.

There are significant difference ($p< 0.05$) of ciplatin concentration packaged in *Bacillus subtilis* spores and different time of incubation. However, the cisplatin were packaged in highest concentration at 8 hours to 12 hours (Figure 2). In the other hand, the highest encapsulation ability of *Bacillus subtilis* spores equate to 54,600,000 drug molecules of cisplatin. Cisplatin has been maintained the structure although it was undergone the encapsulation process.

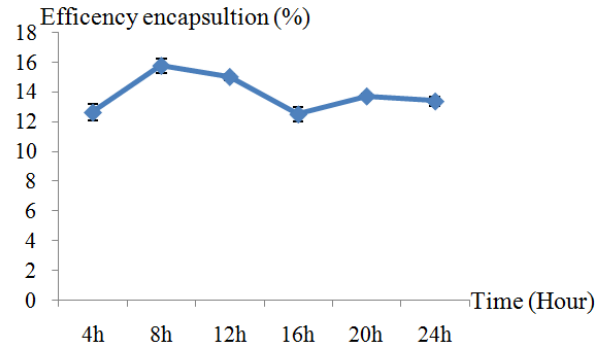

Fig. 2: Efficiency encapsulation of spore packing cisplatin in different time.

In vitro Release Kinetics

Cisplatin can't be formulated in pill, but it can be administered through a vein (intravenously) or an infusion in abdomen cavity. Therefore, *Bacillus subtilis* spore delivered cisplatin were set for a dissolution test in 7.2. However, the dissolution study was also performed at pH 3.4 to understand more about cisplatin can be used in pill in fed patients. In fed patients, pH increases to 3-4. Moreover, when cisplatin bloats in stomach, pH usually increases. Therefore, the study also tried to look for the dissolution test of cisplatin packaging spores in pH 3.4 as a representative pH level in stomach. As a result, figure 3 showed cisplatin was released very fast in the first 2 hours and reach the peak release in 6 hours for dissolution medium pH 3.4, leading to the toxicity highly. In contrast, the releasing cisplatin was slow and steady rising during 24 h and reach the peak in 24 h for dissolution medium pH 7.2. After 0.5 h, cisplatin in medium 3.4 was released faster than medium 7.2 about 7 times (23.8: 3.5). After 24 h, cisplatin releasing in medium pH 7.2 was maximum at 25%, but in medium pH 3.4 was at 55%. In medium pH 3.4, cisplatin released but re-absorbed that made kinetic of cisplatin complicated (Figure 3). In summary, cisplatin packaging spores can be suggested for injection. For orally, many studies should be done and considered.

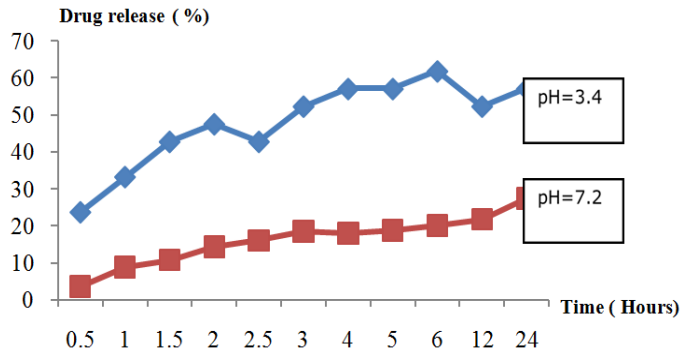

Fig. 3: Dissolution profiles of cisplatin released from spore at pH 3.4 and pH 7.2.

CONCLUSION

Bacillus subtilis spores package an hydrophilic anticancer chemotherapeutic drugs, cisplatin. Drug loading in spore is highest efficiency at 8 to 12 hour. The efficient encapsulation of *Bacillus subtilis* spore depends on time of incubation. Importantly, the spores deliver chemotherapeutic drug and release slowly and regularly in base medium that can be used for prolong-release products. More experiments about stability, pharmacokinetics and clinical trial will be conducted in further study.

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