ABSTRACT

Porous silicon (pSi) microparticles with pore diameter 15 to 20 nm were fabricated by electrochemical etching of single-crystalline silicon (Si) wafers (n-type) to be used as delivery systems for the anticancer drug mitomycin C (MMC). The in vitro toxicity of the mitomycin-loaded pSi carrier was investigated on human prostate carcinoma (DU145) cells. The cells showed a decrease in viability of ~80% over a 6 hours period, when using mitomycin. Meanwhile, a ~55% decrease in cell viability was observed, when using the pSi carrier to deliver the drug. The drug-loaded carrier showed a sustained release throughout 24 hours, with an 80% decrease in cell viability after 16 hours. This observed controlled release of mitomycin from the pSi carrier suggests a superior therapeutic effect than the direct administration of mitomycin, as it potentially minimizes the drug side effects. Results showed that the strong cytotoxic effect towards the prostate cancer cells was due to the drug and not the carrier since the mitomycin-loaded pSi carrier affected cell viability, but the pSi carrier showed no toxicity. Furthermore, it was observed that with a higher amount of drug-loaded carriers, the toxicity effect was higher, thus, allowing further control of the therapeutic effect of the carrier.

Keywords: Drug delivery, Mitomycin C, Porous Si, Prostate cancer.

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INTRODUCTION

Advances in nanotechnology are playing an important role in medicine and therapeutic areas. It is evident in fields such as nanomedicine, where drugs are delivered utilizing degradation and self-regulation of the carrier as a result of the material properties. Nanoparticles are attractive chemotherapeutic devices, since they have the potential to increase drug levels within tumors by circumventing the tumor vasculature, while decreasing normal tissue toxicities by reducing the level of drug in the systemic circulation.

pSi is a well-known biodegradable and biocompatible material as it degrades in physiological fluids over a period of time into nontoxic orthosilicic acid [Si(OH)₄], which is the natural form of Si found in the body.¹ The pSi nanoparticles have been used in multiple applications, such as, cell culture,² optical biosensing, biomolecular screening, tissue engineering, and drug delivery.³,⁴

A simple fabrication of pSi by electrochemical etching of Si wafers in an electrolyte solution produces Si nanoparticles, whose pore size, porosity, and thickness can be easily controlled depending on various fabrication parameters.⁵,⁶ pSi offers unique advantageous properties, such as, its high specific surface area (200-800 m²/g). Furthermore, surface modification, by oxidation, hydrosilylation, or grafting techniques, increases pSi stability in aqueous solution, making it suitable for drug delivery.⁷ Multiple studies have used loaded pSi particles for in vitro drug release.⁸-¹⁰ The cytotoxicity of pSi microparticles in Caco-2 cells was demonstrated in vitro as a function of concentration, particle size fraction and incubation times,¹¹ and in vivo with the release of peptide and ghrelin.¹²
Cytotoxicity of Mitomycin in Human Prostate Carcinoma Cells

Other payloads, such as, proteins and the anticancer drug doxorubicin have also been investigated. Recently in vitro cytotoxicity of mitoxantrone dihydrochloride loaded pSi thin films was demonstrated on human breast carcinoma. The biocompatibility of pSi has also been demonstrated in various studies, using implants of pSi in rat abdominal wall or as a drug carrier in a mouse model. Rosengren et al. showed the luminescent pSi nanoparticles self-destructed into renal cleared components in a relatively short period with no evidence of toxicity. While in another study, pSi particles were suggested as ideal oral drug delivery devices due to their exhibited excellence in vivo stability, low cytotoxicity, and inflammatory responses in Caco-2 and RAW 264 cells. More recently, methotrexate pSi nanoparticles were used as a conjugated platform for combination chemotherapy by concurrently enhancing the dissolution rate of a hydrophobic drug and sustaining the release of drugs. While, two anticancer drugs, doxorubicin and paclitaxel, were separately loaded into the nanocomposite drug delivery prepared by coating pSi nanoparticles with poly(beta-amino ester) and pluronic F-127.

MMC, a \([6\text{-amino-8a-methoxy-5-methyl-1,4,7-dioxo-1,}
\text{1a, 2, 4, 7, 8, 8a, 8b-octahydroazireno [2',3':3,4] pyrrolo [1,}
\text{2-a] indol-8-yl}] \) methylcarbamate, is a bifunctional alkylating agent that acts as genotoxic antibiotic. MMC also acts as a DNA cross-linking agent, blocking DNA synthesis and inhibiting cell mitosis, which in turns detains the cell cycle. Hence, MMC has been broadly used as a chemotherapeutic agent for over 20 years. Jones et al. published the first study about the investigation of mitomycin activity as a single agent in prostate cancer. Since then, different types of cancers, such as, bladder cancer, and resected gastric cancer, among others, have been treated with MMC. Several studies have pointed to the toxic side effects of MMC and suggested treatment to reduce these undesirable effects. However, avoiding or minimizing these side effects and improving drug efficacy and bioavailability can be accomplished by using drug delivery devices. Several methods have been investigated for the delivery of MMC in nano or micro size. Common methods for MMC delivery consist of encapsulation of the drug in nano- or micro-particles, using biodegradable polymers including albumin, dextran, estradiol, N-succinyl-chitosan, hydrogels, polybutylcyanoacrylate, poly-epsilon-caprolactone, oxidized-dextran microspheres, and polyactic acids (PLA) nanoparticles. However, to our knowledge, MMC has never been loaded into pSi.

In the present study, nanostructured pSi was fabricated as a carrier for delivery of MMC, allowing for a better drug release profile that can potentially prevent the drug side effects. The MMC loading efficacy, release profile, and in vitro cytotoxicity on human prostate carcinoma (DU145) cells were studied and compared to the behavior of empty pSi. Empty and MMC-loaded carriers were characterized by field emission scanning electron microscopy (FESEM), spectroscopy, and FTIR.

MATERIALS AND METHODS

All chemicals, such as, hydrofluoric acid 49%, ethanol absolute 99.8%, ammonium hydroxide 28–30%, hydrochloric acid (AR, 35–37%), and hydrogen peroxide (30 wt.% in H2O) were purchased from Sigma Aldrich, Malaysia. MMC (\textit{Streptomyces caespiatus}) was from Sigma, Germany. Cell culture media, fetal bovine serum, Dulbecco’s modified Eagle’s medium (DMEM) high glucose, L-Glutamine with sodium pyruvate, and Dulbecco’s phosphate buffer saline were purchased from Biowest (Nuaillé, France). Si wafers were obtained from Siltronix SAS (Archamps, France).

Fabrication and Characterization of pSi

pSi samples were prepared from single-crystalline n-type Si wafers. These were first cleaned as per RCA clean procedure, a standard set of wafer cleaning steps, to remove contaminants present on the surface of the wafer. The procedure has three major steps: 1) the removal of organic contaminants is performed with an alkaline solution of 1:1:5 NH4OH:H2O2: H2O for 10 minutes at 70–80ºC, 2) the removal of a thin oxide layer is carried out by immersion of the silicon wafer in a diluted hydrofluoric acid (1:50) solution for 15 seconds, 3) an acidic solution of (1:1:6) HCl:H2O2: H2O for 10 minutes at 70-80ºC is used to remove ionic and heavy metals traces. The pSi samples were prepared through an electrochemical etching of the n-type Si wafer with resistivity 0.008–0.018 cm and orientation (100) in a simple electrochemical Teflon cell, containing electrolyte solution of (1:4) hydrofluoric acid (49%) and ethanol (99.8%). The exposed area of the silicon wafer (0.5 cm2) was in contact with the backside of the aluminum foil. A platinum spiral coil was used as the counter-electrode, and the etching was carried out at a current density of 20 mA/cm2 for 30 minutes. The process was performed with the illumination of incandescent light set 15 cm away from the sample. Following the electrochemical etching, the pores were separated from the substrate by a lift-off method. Freestanding films were obtained by abruptly increasing the current density to 250 mA/cm2. Samples were rinsed first with deionized water, and then in ethanol. The freestanding films were then converted into microparticles with an average size of 36.72 µm by using ultrasonic fracture for 10 minutes in absolute ethanol.

Following the etching procedure, the pSi nanoparticles are typically unstable in aqueous solution, a fact attributable to the oxidation of the reactive surface hydrides. Hence, hydroxylation was used to enhance its stability. The freshly prepared pSi nanoparticles were then placed in a 10 mL Pyrex beaker and 1 mL of undecylenic acid (≥ 95%) was added. This solution was heated in a commercial consumer microwave oven at full power (800 watts) for 5 minutes, and the particle was then rinsed with ethanol to remove any excess undecylenic acid. This surface can resist corrosion in PBS solution, and exhibits a slower release rate than freshly prepared pSi, which is unstable in aqueous solutions at pH 7.4.

The morphology of the pSi was characterized by field emission scanning electron microscopy (FESEM) using Zeiss.
Sterilization of Porous Silicon and Drug Loading Procedure

pSi samples were sterilized with 75% ethanol for 30 minutes, and rinsed with sterile water prior to the loading of the drug. The particles were immersed in an aqueous solution of MMC, and the solvent was allowed to evaporate, leaving the drug packed into the pores via spontaneous adsorption. The particles were rinsed with deionized water in order to remove the drug that had not infiltrated the pores, and hence, remained on the surface of the particles. Drug loading percentage was calculated by the following equation (eq. 1):

$$\text{Drug Loading(%) = \frac{\text{WT MMC-pSi} - \text{WT pSi}}{\text{WT pSi}} \times 100}$$

where,

- WT MMC-pSi = Weight of MMC loaded pSi
- WT pSi = Weight of pSi only

Cell Cytotoxicity Assay

Cell viability was assessed using cells incubated with pSi (negative control), MMC-loaded pSi, and free MMC (positive control) during a period from 2 to 24 hours. Try pan blue assay was used for quantitative analysis of cell viability in order to estimate the functional toxicity of the released MMC after an exposure period from 2 to 24 hours. Cell viability was determined from two independent experiments, in which duplicate measurements were performed for each sample. 1 mL of cells at a concentration of 2 × 10^5 cells/mL were loaded into each well of a 6 well plate and incubated for 24 hours. Three different samples were used in this experiment: 1) pSi (negative control) at a concentration of ~10 mg/mL, 2) MMC-loaded pSi consisting of 10 mg/mL of pSi loaded with 2.99 × 10^{-3} M (~0.3 mg/mL) of MMC, and 3) free MMC (positive control) at a concentration corresponding to the drug released after 24 hours (~0.3 mg/mL). 1 mL of each sample was added into its correspondent well plate. Test plates were incubated at 37°C in a humidified atmosphere of 5% CO₂ for a period of 24 hours. One test plate was measured for its viability at intervals of 2, 4, 6, 16, 18, 20 and 24 hours. All samples were prepared in duplicate.

The effect of the quantity of particles used when preparing MMC-loaded pSi on cell viability was also investigated using a similar method as above. First, 4 mg/mL of MMC were added into solutions containing 3.25, 6.5, 13, and 26 mg of pSi particles. 1 mL of cells at a concentration of 2 × 10^5 cells/mL was loaded into each well in a 6 well plate, as indicated in the ATCC catalog of the cell line. Cells were allowed to attach for 24 hours at 37°C in a humidified atmosphere of 5% CO₂. Then, 1 mL of each of the different MMC-loaded pSi solutions was added to each correspondent well, and incubated with the cells for another 24 hours. Each sample was prepared in duplicate. Cell viability was counted using trypan blue methods.

RESULTS AND DISCUSSION

pSi Fabrication and Characterization

The previously described anodization procedure was optimized to obtain a uniform pSi layer in terms of both pore size and...
pore distribution. The average pore diameter of $24.4 \pm 8.5$ nm was obtained, with a modal group of 15 - 20 nm ($n = 130$, SD).

The reproducibility of the structural features of pSi is an important issue. Hence, in this work we used the optimum experimental parameters [(1:4) volume ratios of 49% hydrofluoric acid and 99.8% ethanol anodizing solution, and a current density of 20 mA/cm$^2$ for 30 minutes] during pSi preparation to obtain the desired reproducibility. Figure 1(a) and (b) show the top view and cross-section of one pSi sample prepared with these optimized etching parameters. When looking at the morphology of pSi, one can see, on a basic level, a crystalline silicon matrix surrounding a network of empty holes. Figure 1(c), which illustrates the cross-section of pSi sample after ultrasonication, clearly shows that all the pores were clean and empty from the residual Si species. These empty pores allow for a drug to be loaded, and then released in physiological solutions based on the degradation rate of the material itself. This is the core idea for the design of a MMC delivery device for the treatment of tumor cells. In this case, the pSi morphology has some advantages since it presents a higher nanoporous exposed surface per unit of volume of the material. Moreover, considering that the free space inside the nanopores has to be loaded with a drug in liquid form, clean pores [observed in Figure 1(c)] ensure that it is easier to load the drug through capillary effect compared with the branched ones in Figure 1(b).

Following the fabrication of pSi substrate, a lift-off method was utilized for removing the porous layer from the Si substrate. This was attained with the application of 250 mA/cm$^2$ current density during 30 seconds. The freestanding films were ultrasonically cleaned (10 minutes in ethanol) to produce particles ranging in size from 10 to 30 µm as shown in Figure 1(d). The particle porosity was determined by gravimetric measurement. 68.97% porosity was obtained by measuring the starting weight of the sample after etching, and after stripping the layer from the bulk. The specific surface area of pSi particles was measured according to Brunauer-Emmett-Teller (BET) theory, which is based on a multilayer adsorption extrapolation of the monolayer molecular adsorption Langmuir theory. This model provides the specific surface area of the material based on the physical adsorption isotherms of gas molecules on a solid surface. The specific surface area of the pSi particles was 460 m$^2$/g, which is considered an appropriate area for loading MMC molecules into those particles.

Mitomycin Loading into pSi Carriers
Simple physical adsorption was employed to load the MMC within freshly etched pSi microparticles. For this, the freshly etched pSi microparticles were immersed in a saturated MMC solution. After evaporation of the drug solution, the particles were washed with deionized water and the presence of MMC in the carriers was confirmed by comparing the FTIR spectra of pSi microparticles before and after loading. The spectrum of fresh pSi (Figure 2) displays bands characteristic of surface hydride species, with a band assigned to Si-Hx at 2,100 cm$^{-1}$, and shows absorption bands near 1056 and 826 cm$^{-1}$ which are associated with Si–O–Si bridges. The absorbance observed at 631, and 813 cm$^{-1}$ peaks are attributed to Si-Si, and different SiH$_2$ deformation modes.

The loading percentage of MMC within the silicon particles was calculated (as per eq. 1) to be 31.8%, which is in accordance with other works. The FTIR spectrum of MMC, according to Z. Hou, et al., shows the characteristic absorption peaks of $\text{–NH}_2$ and $\text{–NH}$ groups at 3,269 and 3,317 cm$^{-1}$, respectively, and the carbonyl C=O stretching vibration peak at 1,725 cm$^{-1}$. The absorption peaks observed at 1,550, 1,447, 1,384 and 1,065 cm$^{-1}$ were attributed to $\text{NH}_2$, C=C, C=N, C=O, respectively.

The spectrum of MMC-loaded pSi particles [Figure 3(a)] illustrates new peaks compared with that of the pSi only.
The absorption bands were characterized and compared with other MMC FTIR analyses. Broadband at 3,448 cm\(^{-1}\) was observed, which refers to the characteristic absorption peaks of the combination of OH, NH, and NH\(_2\) groups. Peaks at 2,860 and 2,926 cm\(^{-1}\) correspond to the CH aromatic band, and the absorption bands at 1,000 to 1,250 cm\(^{-1}\) were assigned to C-O and Si-O. The area between 1,300 and 1,800 cm\(^{-1}\) was enlarged to identify the absorption peaks [Figure 3(b)]. The peaks at 1638 cm\(^{-1}\) correspond to amide II (–N-H) bending. The other absorption bands observed were at 1,335 cm\(^{-1}\) assigned to C-N stretching, at 1,560 cm\(^{-1}\) for the vibration bending of NH\(_2\), at 1,508 cm\(^{-1}\) for the C=C aromatic, at 1,459 cm\(^{-1}\) for C=C stretching, and at 1,715 cm\(^{-1}\) for the carbonyl C=O stretching vibration.

**In Vitro Cytotoxicity Assay**

The human prostate Carcinoma cell line was used for cytotoxicity tests. Following growth profiles initially defined for each cell model, 2 \(\times\) 10\(^5\) cells were plated in each well of a 6-well plate. For analysis of cytotoxicity, cells were then exposed to MMC-loaded pSi carriers for a period between 2 to 24 h to MMC-loaded pSi carriers (diamond symbols, blue trace), empty pSi (square symbols, red trace), negative control cell only (triangle symbols, green trace) and free MMC (cross symbol, purple trace).

Figure 4 shows the cell viability percentage at intervals of 2 hours for 1) the prostate cells, 2) the cells with pSi carrier (negative control), 3) the cells with the MMC-loaded pSi carrier, and finally, 4) the cells with the MMC (positive control). The negative control was performed by incubating the Si particles with cells under the same conditions to prove that the pSi particles themselves did not cause the localized death of cells. This also allowed studying the effect of the carrier on the morphology of the cells.

From Figure 4, it can be observed that the MMC alone causes a decrease of cell viability of 80% within 6 hour, meanwhile, the drug-loaded into the pSi showed a more steady decrease, killing 80% of the cells only after 16 hours. The total viability of the cells in the positive control experiment (MMC alone) displays similar toxicity than pSi loaded with the drug, but the latter presents a controlled release of the drug; hence the toxicity time frame varies. By contrast, the negative control (empty pSi) showed no evident effect on cell viability; no dead cells were counted. The cells exposed to empty pSi carriers showed a 98.92 ± 2.02% viability, a result similar to that found in the case of cells alone 99.02 ± 1.83%, as observed in previous studies on other cell lines.\(^{15,36}\) The morphology of the cell remained unchanged after the incubation with pSi, once more verifying that the localized cells’ death was not caused by the pSi particles themselves.\(^{41}\) This result was accepted as correct, since the cells had been carefully washed with PBS after removing the pSi and before adding the dye. This is a particularly important step when working with cell-based assays and pSi, due to the reaction of the dye with Si and silicic acid, its degradation product.\(^{15}\) The cells’ viability could be sorted from high to low as negative control (empty pSi) > pSi loaded with MMC > positive control (MMC). From these results, it can be concluded that the MMC released from freshly etched pSi particles displays significant toxicity towards the prostate cancer cells.

A series of experiments employing different amounts of MMC-loaded pSi carriers were incubated in separate well plates containing a similar quantity of prostate cancer cells. Figure 5 shows the percentage of dead cells versus the amount of MMC-loaded carrier incubated with the cells. When the drug...
## Table 1: Carriers used for the controlled delivery of MMC

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was incubated directly onto the cells, all the cells were observed dead (100%). Meanwhile, when the drug was loaded into the pSi carrier, much lower dead cells were observed. In this case, the quantity of dead cells was observed to increase with an increasing amount of particles. This can be explained due to the fact that higher amounts of particles contain higher amounts of drug load, as observed with other drugs-loaded pSi carriers.41

Other MMC Carriers
Other carriers have been used for the controlled delivery of MMC. The type of carrier, MMC-loading method, as well as the in vitro monitoring of the drug release and cytotoxicity assays have been described in Table 1.

While other carriers provide significantly long release times, such as, 155 hours37 and 30 days,34 due to the more elaborated drug loading method, either covalent attachment or by lyophilization, these might not be suitable for all types of anticancer treatments. In our work, simple physical adsorption was used to load the drug, and an important therapeutic effect was observed within 16 hours, an increase of 10 hours when using MMC-loaded pSi compared to the drug alone, in the human prostate carcinoma cells. The MMC carriers reported in the literature are based in hydrogels and polymeric particles in general, and have been used to show the therapeutic effect of these on sarcoma, leukemia, bladder, and breast cancer particularly. Considering the intrinsic properties of pSi (nontoxic, biodegradable, and photoluminescence) and hence, its position at the forefront of delivery devices, it is important to study this material individually for different drugs and applications. In light of this, this study has attempted to show the feasibility of using pSi as a MMC carrier for human prostate carcinoma treatment.

CONCLUSION
We have successfully fabricated pSi carriers with adequate pore sizes (15–20 nm) for the loading and controlled release of mitomycin, an anticancer agent with antibiotic activity used for the treatment of prostate cancer. The feasibility of MMC-loaded pSi particles as a chemotherapeutic device was investigated in vitro in a cytotoxicity study on human prostate carcinoma (DU145) cells. The MMC-loaded pSi carriers showed the same level of toxicity as the anticancer drug, but 10 hours later. These results suggest that pSi particles provide a more controlled release compared to the common drug administration method, where a rapid burst was observed only 6 hours after the MMC was added to the cell line. Further, no toxic effect was observed due to the pSi particles. The use of the carriers not only avoids an undesired burst effect, but allows for a more controlled release profile of the drug over an extended period. Additional toxicity was obtained by increasing the amount of drug-loaded particles, hence, increasing the effect of the carrier.

Further control can be attained by coating the particles or by surface modification. In addition, pSi is a known biodegradable material with photoluminescence properties, and thus, its elimination can be monitored via fluorescence microscopy. The controlled release of MMC via pSi carrier has the potential to improve the therapeutic effect of the drug and minimize its side effects by delivering a more precise dose on the tumor site. This work sheds more light on the use of pSi as a regional chemotherapy agent for the treatment of prostate cancer.

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