Agarose coating improves protein stability during sustained release

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ABSTRACT

Effectiveness of drug administration has been improved developing many organic systems to target the drug to a specific site and at a desired rate. Nevertheless most of them are rapidly eliminated by the reticulum endothelial system and are poorly stable. Biocompatible and biodegradable porous silicon microparticles can be loaded with bioactive molecules and superficially modified to provide controlled release. However, the stability of the loaded bioactive molecules has not been guaranteed so far.

In this work we describe an agarose coating that provides long term protection of the bioactive molecule prior to its delivery thus assuring an effective protein uptake from the cells.

Keywords: protein stability, porous silicon, coating, drug delivery.

1 INTRODUCTION

The advances in drug-delivery systems over recent decades highly improved the effectiveness of drug administration. Many organic systems (e.g. liposomes, micelles, nanoparticles) have been developed to deliver the drug to a specific site and at a desired rate. Unfortunately the afore mentioned systems are rapidly eliminated by the reticulum endothelial system (RES) and they are poorly stable once injected in the blood stream. Moreover, a sustained release of bioactive molecules is required in many therapeutic applications. Biocompatible, biodegradable porous silicon microparticles can be modified to provide controlled release and enhanced stability of the loaded bioactive molecule. However, the stability of the loaded bioactive molecules has not been guaranteed so far.

Proteins such as growth hormones, cytokines and vaccines become more and more in use as therapeutic agents. Unfortunately they are easily degraded and deactivated by enzymes or other chemical and physical reactions occurring in the body. Growth factors such as bFGF and VEGF, for example, have half-life as short as 3 and 50 minutes respectively. Since protein drugs degrade faster than conventional drugs they often require repeated administrations. The use of a drug delivery system capable to ensure a sustained drug release may reduce the pain and inconvenience of frequent injections although the drug stability may still be an issue, especially when using hydrophobic or biodegradable drug delivery systems (e.g. PLGA may denaturize the protein due to its acidic degradation products).

In this work we propose an agarose coating that improves protein stability within nanoporous silicon microparticles (NSP) designed as drug delivery system for prolonged therapy and tissue engineering application. The protective effect of agarose coating was tested evaluating degradation products and protein amount after enzymatic digestion. Protein cell uptake and microparticle uptake was also evaluated.

2 MATERIALS AND METHODS

2.1 Microparticle- fabrication

The NSP were designed and fabricated in the Microelectronics Research Center at The University of Texas at Austin as described in details in a previous publication [1]. In brief after silicon nitride (SiN) vapor deposition, photoresist was spun cast on the substrate. A pattern consisting of 2 um dark field circles with 2 um pitch on the photoresist was transferred with a mask aligner and developed. Then it was transferred into the SiN layer for 100 nm into the silicon substrate by reactive ion etching. The photoresist was removed from the substrate for anodic etch preparation by piranha clean. The porous particles were formed by anodic etch. The SiN layer was removed by soaking in HF, the substrate was dried and the particles were released in isopropanol by sonication. Particles were then oxidized by piranha washing and modified with aminopropyltriethoxysilane (APTES) to enhance protein loading capacity.

Agarose coating was performed suspending NSP in warm (40°C) agarose solution of the desired concentration for 20 min then cooling the solution at 4°C for 30min and removing the excess of gel by subsequent washing and spin down at 35°C.

2.2 Microparticle characterization

NSP size, shape, porosity, coating quality was observed and their changes analyzed over time during degradation by scanning electron microscope (SEM) (FEI Quanta 400 ESEM FEG) and fluorescence activated cell sorting (FACS) (Becton Dickinson, FACSCalibur).

2.3 Protein loading and release

Bovine serum albumin (BSA) was loaded into NSP by suspending and mixing NSP in 25mg/mL BSA FITC-conjugated aqueous solution for 2 hours.

Release over time was measured with SpectraMax M2 spectrophotometer (Molecular Devices) and by fluorescence activated cell sorting (FACS).

2.4 Released protein stability analysis

Suspensions of coated and not coated NSP were treated for 1 hour with trypsin and the stability of the protein released in solution after 16 hours was evaluated through polyacrylamide gel electrophoresis (SDS page) and compared with the protein released from not treated NSP to asses the effect of the enzymatic digestion.

2.5 Cellular internalization and released protein uptake

NSP internalization and released protein uptake from human umbilical vein endothelial cells (HUVEC) were observed with confocal microscope (Leica MD 6000). Cells were stained with fluorescent phalloidin (actin filaments) and DRAQ5 (nuclei).

3 RESULTS AND DISCUSSION

The quality of the coating was assessed by SEM analysis (Figure 1).

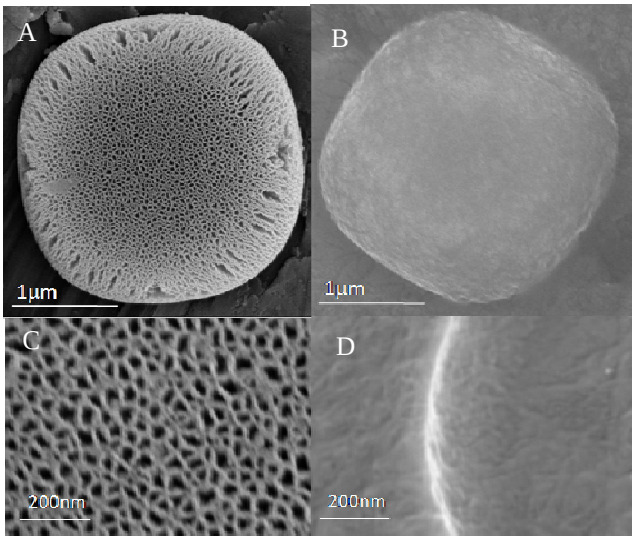

Figure 1: NSP not coated (A, C) and agarose coated (B, D).

Images show a uniform coating that fills and covers the pores.

Fluorescence measurements performed with the spectrofluorimeter and FACS are shown in Figure 2 and 3 respectively.

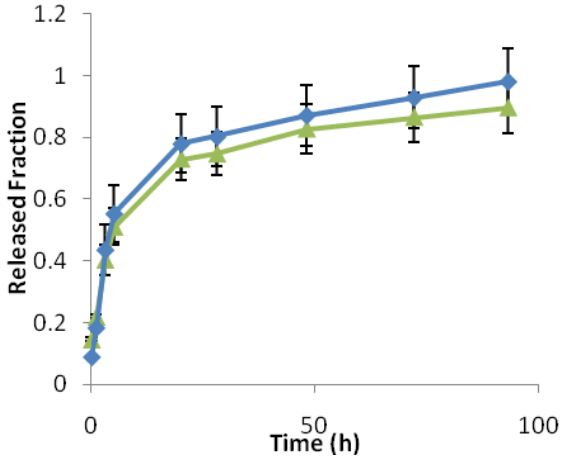

Figure 2: BSA released from not coated (green) and agarose coated (blue) NSP measured over time with spectrophotometer.

NSTI-Nanotech 2010, www.nsti.org, ISBN 978-1-4398-3415-2 Vol. 3, 2010

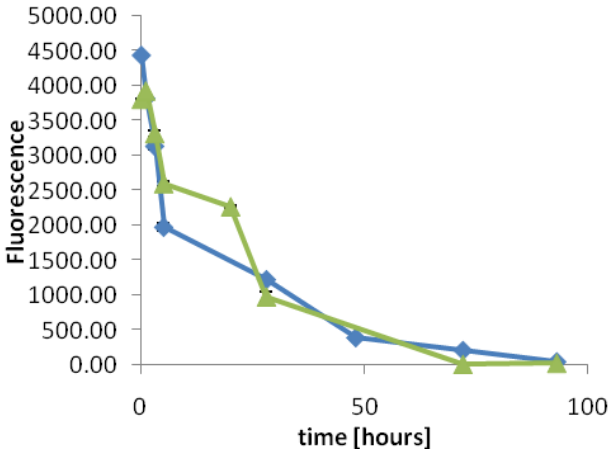

Figure 3: Particle fluorescence decay of not coated (green) and agarose coated (blue) NSP measured over time at FACS.

Graphs show that about 80% of the loaded protein is released in two days and agarose coating does not affect release rate.

In order to asses the protective effect of the agarose coating against degradating agents, the NSPs were treated with trypsin. Figure 4 shows the SDS page of the protein solution released from the NSPs after 16 hours of treatment.

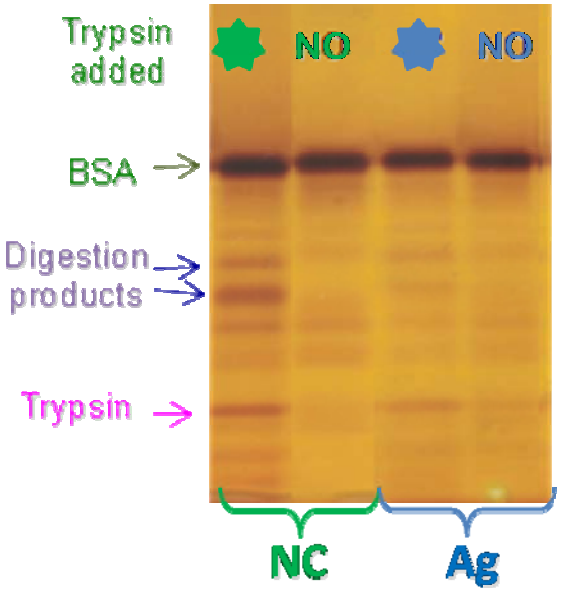

Figure 4: SDS page of BSA released after 16 hours of trypsin treatment from not coated (NC - green) and agarose coated (Ag - blue) NSP.

Image shows a higher amount of BSA degradation products in the protein solution collected from the not coated NSP treated with trypsin. The protein released from the agarose coated particles has a molecular stability similar to the not treated one.

Confocal analysis shows a fast internalization of the particles (Figure 5) and an evident protein release after 24 hours.

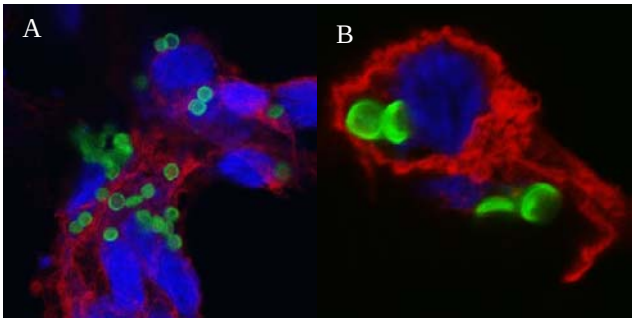

Figure 5: Confocal images of internalized not coated (A) and agarose coated (B) NSP after 6 hours of incubation.

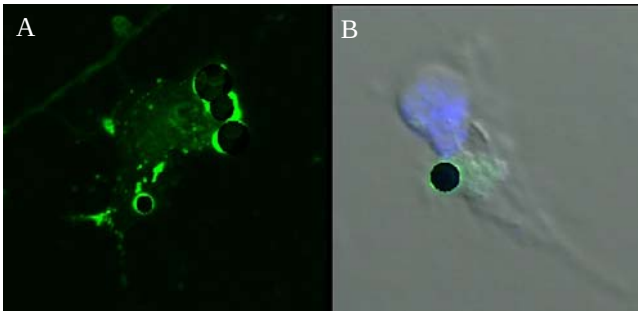

Figure 6: Confocal images of BSA uptake released from not coated (A) and coated (B) NSP after 24 hours of incubation.

4 CONCLUSIONS

The presented data show that agarose coating inhibits enzymatic degradation of the loaded protein and does not affect porous silicon release rate, cellular internalization and protein uptake.

This study demonstrates that agarose coating is a valuable solution against protein instability in porous silicon particles by protecting the loaded molecule from degrading agents while ensuring sustained release.

REFERENCES

[1] E. Tasciotti, et al. Mesoporous silicon particles as a multistage delivery system for imaging and therapeutic applications. Nat Nanotechnology 3, 151, 2008.