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Porous silicon nanoparticle as a stabilizing support for chondroitinase

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ABSTRACT

Chondroitinase ABCI (cABCI) from *Proteus vulgaris* is a drug enzyme that can be used to treat spinal cord injuries. One of the main problems of chondroitinase ABC1 is its low thermal stability. The objective of the current study was to stabilize the enzyme through entrapment within porous silicon (pSi) nanoparticles. pSi was prepared by an electrochemical etch of p-type silicon using hydrofluoric acid/ethanol. The size of nanoparticles were determined 180 nm by dynamic light scattering and the mean pore diameter was in the range of 40–60 nm obtained by scanning electron microscopy. Enzymes were immobilized on porouse silicon nanoparticles by entrapment. The capacity of matrix was 35 μg enzyme per 1 mg of silicon. The immobilized enzyme displayed lower V_{max} values compared to the free enzyme, but Km values were the same for both enzymes. Immobilization significantly increased the enzyme stability at various temperatures (−20, 4, 25 and 37 °C). For example, at 4 °C, the free enzyme (in 10 mM imidazole) retained 20% of its activity after 100 min, while the immobilized one retained 50% of its initial activity. Nanoparticles loading capacity and the enzyme release rate showed that the selected particles could be a pharmaceutically acceptable carrier for chondroitinase.

1. Introduction

One of the many reactive changes that occurs following a spinal cord injury is the formation of a glial scar. One class of inhibitory molecules associated with glial scar extracellular matrix is the chondroitin sulfate proteoglycans (CSPGs) [1,2]. CSPGs are known to suppress axonal regeneration and plasticity following central nervous system injury [3–6]. Bacterial chondroitinase ABC lyase (cABCI, EC 4.2.2.4) could destroy the inhibitory substance by degrading chondroitin sulfate (CS), dermatan sulfate and hyaluronan glycosaminoglycans from the CSPG core protein [7,8]. This makes chondroitinase a very promising candidate for treatment of spinal cord injuries by improving regeneration after spinal cord injury in rodent [9,10]. However, the utilization of cABCI as therapeutics is notably restricted due to its thermal instability [7,11]. Therefore, it necessitates the use of repeated injections or local infusions for a period of days to weeks to provide fresh cABCI to retain its enzymatic activity [9]. Repeated injections could increase the trauma and trigger an immune reaction in the patient. For this reason, thermal stabilization of cABCI has attracted intense interest [12]. To date, several approaches including chemical modification, utilization of stabilizing additives, mutagenesis and immobilization have been employed to increase the stability of enzyme [7]. Upon our previous studies, we have found that imidazole, sucrose and trehalose have the most thermostabilizing effect among various additives [11]. cABCI was also stabilized through release of its conformational strain using site directed mutagenesis [7].

Immobilized enzymes are currently the subject of considerable interest for their benefits over alternative technologies [13–15]. Therefore, recent advances in nanotechnology have provided diverse nanomaterials and approaches for enzyme immobilization and stabilization [16,17]. In particular, mesoporous materials have attracted much attention due to well-defined pore sizes and geometry, good pore connectivity, and mechanical stability. These advantages enable efficient enzyme immobilization into mesoporous materials [18]. In a previous report, subtilisin Carlsberg was immobilized and stabilized on magnetically-separable mesoporous silica (Mag-MSU-F) in the form of nanoscale enzyme reactors (NERs) based on the ship-in-a-bottle mechanism [19]. In addition, the immobilized glucose oxidase on both bulk and porous SiO_2 surfaces preserved and maintained its activity after three months [13].

In the present study, immobilization of chondroitinase ABCI on porous silicon (pSi) was carried out to stabilize and protect it against

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thermal inactivation. porous silicon (pSi) was selected as a carrier because of its favorable properties such as high surface area, biostability, biodegradability, non-toxicity and photoluminescence properties [20–23]. Fabricated pSi was first characterized by scanning electron microscopy (SEM) and atomic force microscopy (AFM). Then the kinetic parameters, storage and thermal stability of both the free and immobilized enzymes were compared. Here, we aimed to investigate the use of pSi nanoparticles as potential chondroitinase carriers; loading capacity and release behavior of nanoparticles were evaluated using UV spectroscopy. The results showed that the selected porous silicon could be a potential candidate matrix for immobilizing chondroitinase through entrapment for in vivo applications.

2. Materials & methods

2.1. Materials

All the reagents were prepared with chemicals of analytical grade. Chondroitin 4-sulfate, HF, KH_2PO_4 and kanamycin were supplied by Sigma-Aldrich (USA). Ni-NTA agarose was provided by Qiagen (USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was provided by Takara (Japan). The p-type Si wafers (0.5 Ω cm, (100) oriented) were purchased from SoCal Nevada (USA).

2.2. Enzyme expression and purification

The enzyme was expressed and purified using the same procedure described by Nazari-Robati et al. [7]. The purity of proteins was assessed by the single band on SDS-PAGE. Protein concentration was estimated according to the method of Bradford [24].

2.3. Enzyme activity

cABCI lyase activity was analyzed by monitoring the product spectrophotometrically at 232 nm in 50 mM phosphate buffer (pH 6.8) at 25 °C. Briefly, 10 μl of purified enzyme (0.66 μg/mL) was added to 290 μl of a solution containing various concentrations of chondroitin-4-sulfate (C4S) in 50 mM phosphate buffer, pH 6.8 [7]. The molar absorption coefficient (ε) of 3800 M^{-1} cm^{-1} was used for the product in the calculations [25]. One unit of cABCI activity is defined as the amount of enzyme that releases 1 μmol of unsaturated oligosaccharides per minute under the assay conditions specified. Data were fitted to Michaelis-Menten equation using nonlinear regression with Prism 5 software (http://www.graphpad.com/scientific-software/Prism).

2.4. Porous silicon formation

The method employed to produce the mesoporous silicon nanoparticles has been previously described in detail [26,27]. In brief, porous silicon (pSi) samples were electrochemically etched from monocrystalline p-type silicon substrates (phosphorus doped, 0.5 Ω cm, (100) oriented, from Silicon Quest Inc.), for 20 min, with different current densities (a) 5 mA/cm^2, (b) 10 mA/cm^2, (c) 20 mA/cm^2 and (d) 30 mA/cm^2, in a 2:3:1 (v/v) mixture of aqueous HF (49% hydrofluoric acid, Fisher Chemicals, Inc.) and ethanol (99.5% absolute ACS reagent, Sigma-Aldrich Inc.) and water. Samples prepared with current density of 20 mA/cm^2 showed optimum results and therefore were used for further measurements. The porous layer was removed from Si substrate using lift-off method with application of 250 mA/cm^2 current density for 30–40 s. Subsequently, the removed layer was converted into microparticles by sonication overnight. The particles were then filtered through a 0.22 μm filtration membrane (Millipore).

Oure previose stadies showed that after piurification, the enzyme activity was remained constant for 20 days after purification in the presence of 300 mM imidazole at 4 °C [7,11]. For this reason the in cubation buffer for the enzyme immobilization contained 300 mM imidazole in the presence of pSi nanoparticles at 4 °C for 7 h. After centrifugation the pellet (immobilized enzyme) was rinsed three times with phosphate buffer and centrifuged at 15000 × g for 60 min and it was resuspended in phosphate buffer [14,27].

2.5. Stability

cABCI lyase stability was analyzed by incubating the free and immobilized enzyme at −20, 4, 25 and 37 °C for 3 h, and after various time intervals, the samples were assyed for their residual activity at 25 °C as mentioned above. Experiments were performed at least in triplicate and the standard deviations were ±5%.

2.6. In vitro release

One mg of silicon which contained 35 μg of immobilized enzyme in phosphate buffer (pH 6.8), was incubated for different times at 25 °C and centrifuged, then the activity of the pellet was detected at 232 nm.The protein release was detected in the supernatant at 220 nm.

2.7. Reusability assay of immobilized enzyme

After each activity measurement, assay reaction containing the immobilized cABCI was centrifuged and washed three times with phosphate buffer (pH 6.8). Then fresh reaction medium was introduced and the next activity measurement (at 25 °C) was carried out [28,29].

2.8. Support characterization

An image of a bare porous silicon wafer was obtained by atomic force microscopy (AFM) (Autoprobe CP microscopy). Morphological properties of virgin and immobilized silicon were observed using a scanning electron microscopy, Philips XL30 scanning electron microscope (Netherlands). Energy dispersive x-ray spectroscopy (EDX), Philips (Netherlands), provided elemental analysis. The hydrodynamic size of pSi nanoparticles were measured by means of dynamic light scattering (DLS) technique using the Brookhaven analyzer, in 50 mM phosphate buffer (pH 6.8) at room temperature

3. Results and discussion

3.1. pSi preparation

Luminescent porous Si nanoparticles (pSi) were prepared by electrochemical etching of wafers in ethanolic HF solution, lift-off of the porous silicon film, ultrasonication and filtration of the formed particles through a 0.22 μm filtration membrane [26,27]. Porosity in pSi samples was prepared with different etching times and current densities (5, 10, 20 and 30 mA/cm^2). Since the samples of pSi obtained with 20 mA/cm^2 for 20 min retained maximum enzyme activity without any leaching, therefore it was appropriate for the immobilization of chondroitinase [22].

The surface morphology was studied by scanning electron microscopy (Fig. 1) and for complementary studies, an image of a bare pSi wafer was obtained using AFM (Fig. 2). Both experiments showed that the pore size lie within the range of 40–60 nm. The hydrodynamic size of the prepared nanoparticles was also analyzed by dynamic light scattering technique. The average sizes of