Legumain Conjugated Fluorescent Porous Silicon Nanoparticles for Breast Cancer Imaging

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Abstract. Porous silicon (PSi) with a suite of most desirable biomaterial properties has attracted great attention as a multifunctional nanoplatform for bioimaging and drug delivery. Various surface functionalization treatments have been reported for PSi to use as an active tumor cell targeting nanovector. In this study, we investigated surface functionalization treatments with the emerging biomarker legumain peptide as a tumor cellular-specific targeting ligand. The PSi nanoparticles were coated with dextran and subsequently two types of legumain peptide, Y-shaped and linear chain, were conjugated to produce the functionalized PSi. The functionalized (ligand-conjugated) PSi materials were characterized for morphology, size, functional groups, and fluorescence response using electron and fluorescence microscopy and vibrational spectroscopy techniques. Fluorescence microscopy imaging with two excitation wavelengths (450 nm and 600 nm) suggests comparable fluorescence response of the legumain-conjugated PSi to "bare" PSi and the suitability of the legumain conjugated PSi for bioimaging.

Introduction

Nanotechnology is promising improved cancer diagnosis and treatment modalities that can overcome some of the weaknesses associated with the prevalent approaches such as mammography, chemotherapy, and radiotherapy. In particular, unlike the traditional approaches that are characterized by non-localized and less discriminating (tumor cell versus healthy cells) delivery of therapeutics, the anticipated high tumor cell specificity of ligand-conjugated multifunctional nanovectors for *active* targeting [1] of tumor microenvironment promises simultaneously enhanced imaging capability and improved therapeutic efficacy through reductions in drug dosage requirement and side effects.

Porous silicon (PSi) is one of the widely studied materials for imaging and drug delivery owing to its outstanding features such as noncytotoxicity, biocompatibility, and biodegradability [2,3]. Porous silicon in the micro- or nano-particle form can be designed as multifunctional theranostic platforms with appropriately controlled pore size (2-50 nm) making them intrinsically fluorescent and large pore volumes allowing for loading of large drug cargos to be delivered *in vivo*. Moreover, PSi can be made water soluble – an important physiological requirement – with suitable surface functionalization [2].

Various nanoparticle surface functionalization approaches and numerous tumor cellular targeting ligands have been investigated in conjunction with promising nanocarriers including PSi [3,4]. The active targeting ligands include monoclonal antibodies, proteins, peptides, aptamers and other nucleic acids, and small molecules [5]. In the present study, we have investigated functionalization

of PSi for bioimaging purposes using two types of legumain (asparaginyl endopeptidase) peptide: *Y-shaped* legumain and *single chain* legumain. It may be noted that peptide-based ligands with smaller molecular sizes and simple structural conformations have been suggested to have higher stability and relatively lower immunogenicity compared to majority of proteins [4].

Legumain is an intracellular lyosomal cysteine protease expressed in diverse cell types. Recently, legumain has also been reported to occur extracellularly in the tumor microenvironments and tumor cell surfaces of various type of cancers (breast, colon, and prostate) suggesting its potential as a biomarker for cancer targeting due to its overexpression in primary tumor microenvironments [6-8]. The objective of the present study is to assess the performance of legumain-conjugated PSi as a fluorescent bioimaging agent.

Experimental Section

Materials. Nanoparticulate PSi (average particle size ~150 nm; pore size ~16-30 nm) was procured from a commercial supplier (Mawson Institute, University of South Australia). Y-shaped (Ala-Ala-Asn-Leu-His-Lys-(His-Lys)) and single chain (Ala-Ala-Asn-Leu-His-Lys-His-Lys-His-Lys) legumain peptides were purchased from Nextgene. Dextran (mol. wt.: 10,000 g/mol), N-hydroxy succinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Sigma-Aldrich, Germany.

Methods. The as-received as well as functionalized PSi samples were characterized using field emission scanning electron microscopy/energy dispersive x-ray analysis (FE-SEM/EDX), dynamic light scattering, Fourier Transform infrared (FTIR) spectroscopy, and fluorescence microscopy.

The PSi nanoparticles were first coated with dextran by adding 1 ml aliquot of an aqueous dispersion of 0.5 mg PSi nanoparticles in 1 mL aliquot of ultrapure water containing 100 mg of blue dextran. The mixture was stirred overnight and rinsed three times using a centrifugal filter that have 100,000 dalton mol. wt. cut-off. Upon re-suspension in ultrapure water, the dextran coated PSi nanoparticles were filtered through 0.22 µm polyvinylidene fluoride (PVDF) membrane. This was followed by conjugation of fluorescein isothiocyanate (FITC) attached Y-shaped and single chain legumain peptide substrates using NHS-EDC chemistry. To prepare amine reactive dextran coated PSi nanoparticles, the freshly prepared nanoparticles were suspended in solution of 0.1 M NHS and kept at 80°C overnight under constant stirring in a shaking water bath. The solution was filtered through 0.22 µm PVDF membrane and washed with an excessive amount of water to get rid of any free NHS. NHS activated PSi thus obtained was mixed with the Y-legumain or single chain legumain peptide at a mass ratio of 1:2 in an aqueous solution at room temperature, followed by addition of 0.2 M of EDC. The mixture was sonicated in a water bath for 1 h and filtrated through 0.22 µm PVDF membrane. After repeatedly washing with water to remove the unconjugated peptide, the Y-Leg- and single chain peptide conjugated PSi nanoparticles were obtained.

Results and Discussion

Figure 1 shows FE-SEM images of randomly selected “bare” PSi nanoparticles having diameters in the range of 50-60 nm (a) and legumain conjugated PSi with ~700 nm diameter (b). The particle size distributions obtained by dynamic light scattering for the bare and legumain-conjugated PSi are shown in Fig. 2. A median particle size of 122 nm is obtained for the bare PSi while for the legumain-conjugated PSi the median particle size is 458 nm. It may be noted that significant differences were not observed in further characterizations of Y-shaped and single chain legumain, and therefore, they are not treated separately below.

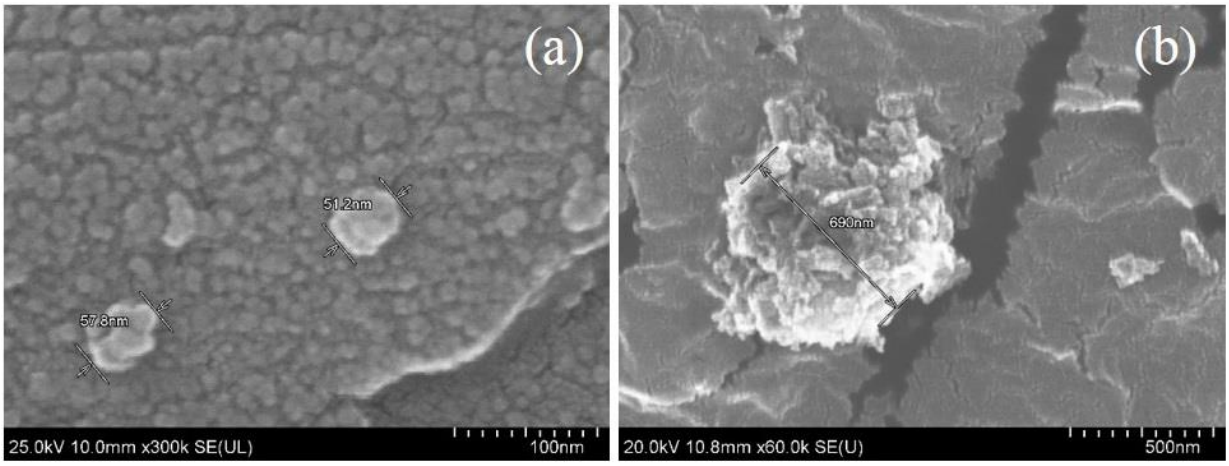


Figure 1. FE-SEM images of (a) "bare" and (b) legumain-conjugated PSi.

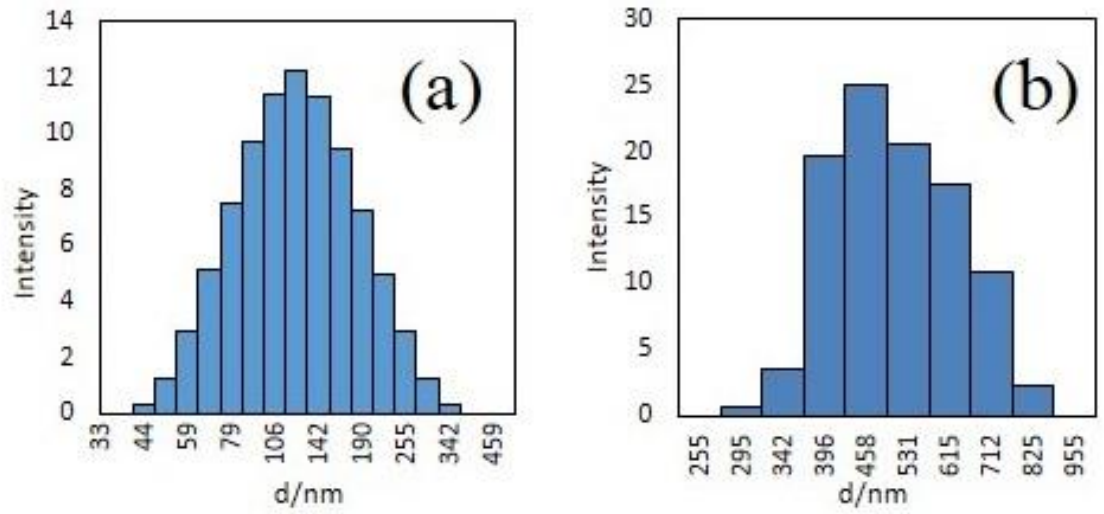


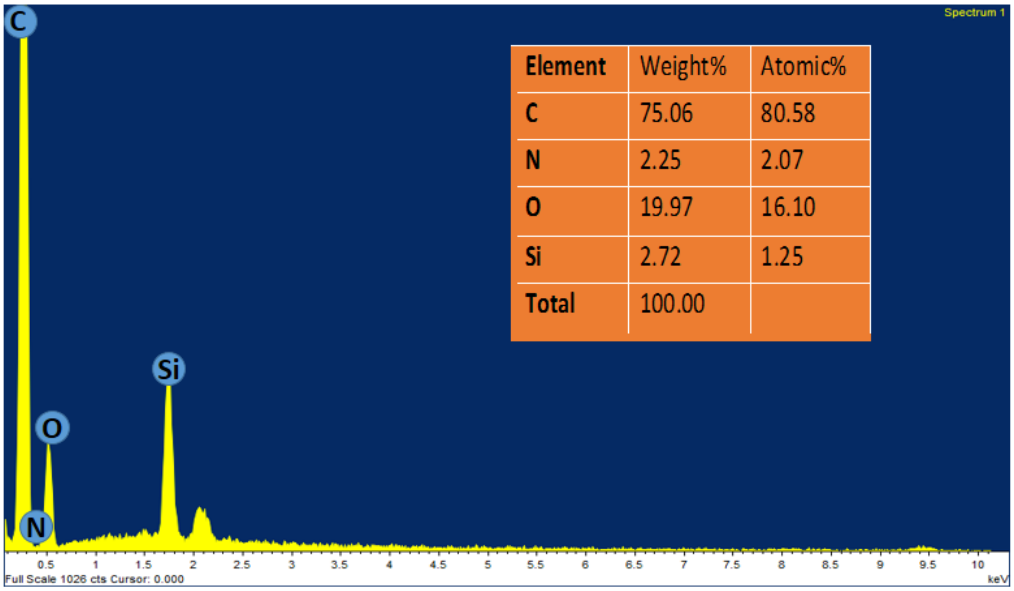
Figure 2. Particle size distribution in (a) "bare" PSi and (b) legumain-conjugated PSi obtained by dynamic light scattering.

Further characterizations of the legumain conjugated PSi nanoparticles were carried out using EDX analysis (Fig. 3) and FTIR spectroscopy (Fig. 4). The chemical analysis confirms the presence of the major constituents in the functionalized nanoparticles with elemental abundances for C, O, Si and N of 75.06%, 19.97%, 2.72%, and 2.25%, respectively.

The changes in the chemical bonding due to the functionalization are shown by the FTIR spectra in Fig. 4. The spectrum of unconjugated PSi shows Si-O stretching vibrations at 1078 cm^{-1}. The peaks between 2129 cm^{-1} and 2331 cm^{-1} represent Si-H vibrations. The distinct absorption peaks at 3380 cm^{-1} and 1633 cm^{-1} can be ascribed to N-H bonds, confirming the presence of amino groups.

The fluorescence spectra of PSi and legumain-conjugated PSi nanoparticles obtained with the excitation wavelengths of λ = 450 nm and 600 nm are shown in Figure 5. With the 450 nm excitation, green emission (λ = 550 nm) and with the 600 nm excitation, orange-red emission (λ = 620 nm) were obtained from the nanoparticles. Both Y-shaped and linear chain legumain yielded

very similar fluorescence responses. The fluorescence images obtained from "bare" PSi were qualitatively similar to those obtained from legumain-conjugated PSi nanoparticles.



Element	Weight%	Atomic%
C	75.06	80.58
N	2.25	2.07
O	19.97	16.10
Si	2.72	1.25
Total	100.00	

Figure 3: Energy-dispersive x-ray spectroscopy analysis of legumain-conjugated PSi.

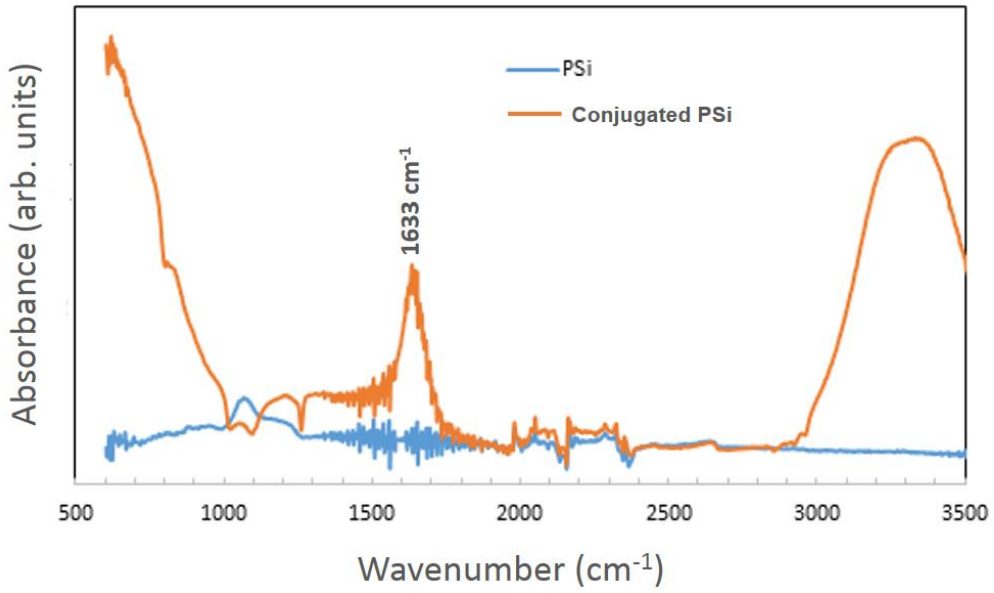


Figure 4: FTIR spectra of PSi and legumain-conjugated PSi. The PSi spectrum is nearly featureless. The sharp absorption band at 1633 cm^{-1} is due to N-H vibration.

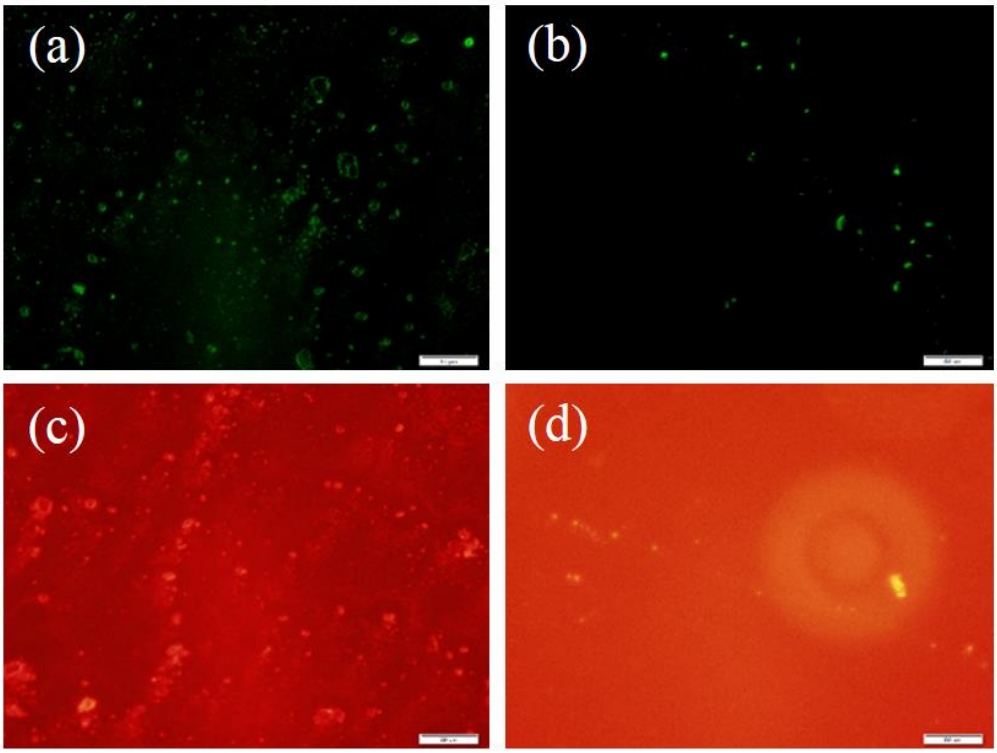


Figure 5: Fluorescence microscopy images obtained from PSi and legumain-conjugated PSi nanoparticles: (a) Pure PSi nanoparticles excited by λ = 450 nm; (b) conjugated nanoparticles excited by λ = 450 nm; (c) PSi excited by λ = 600 nm; and (d) conjugated PSi excited by λ = 600 nm. The scale bar = 50 μm in all images.

Summary

The present study investigated surface functionalization and bioconjugation of PSi nanoparticles for bioimaging of tumor cells. Two types of legumain, namely, Y-shaped and linear chain peptides, were investigated for their effective conjugation and fluorescence responses. It was found that the PSi nanoparticles coated with dextran can be effectively bioconjugated with legumain peptide, yielding similar and efficient fluorescence responses. Further investigations will include *in vitro* breast tumor cell interactions of legumain-conjugated PSi nanoparticles.

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