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Silaffin Peptides as a Novel Signal Enhancer for Gravimetric Biosensors

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Abstract Application of biomimetic silica formation to gravimetric biosensors has been conducted for the first time. As a model system, silaffin peptides fused with green fluorescent protein (GFP) were immobilized on a gold quartz crystal resonator for quartz crystal microbalances using a self-assembled monolayer. When a solution of silicic acid was supplied, silica particles were successfully deposited on the Au surface, resulting in a significant change in resonance frequency (i.e., signal enhancement) with the silaffin–GFP. However, frequency was not altered when bare GFP was used as a control. The novel peptide enhancer is advantageous because it can be readily and quantitatively conjugated with sensing proteins using recombinant DNA technology. As a proof of concept, this study shows that the silaffin domains can be employed as a novel and efficient biomolecular signal enhancer for gravimetric biosensors.

Keywords Silaffin peptides · Biomimetic silica formation · R5 peptide · Quartz crystal microbalances · Signal enhancer

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Introduction

Diatoms, which are aquatic, photosynthetic, unicellular algae, have gained much attention owing to their intricately and hierarchically nanostructured silica cell walls. The silaffin polypeptides derived from *Cylindrotheca fusiformis* are major components of the intricate silica cell wall. They are heavily post-translationally modified proteins responsible for silica deposition at ambient temperature and pressure [1, 2]. Recently, it was discovered that an unmodified silaffin peptide, the R5 peptide (H_2N-SSKKSGSYSGSKGSKRRIL-COOH) identified as a repeat unit of the silaffin polypeptide showed the same silica precipitation activity when added to a silicic acid solution under ambient conditions [1, 3]. In previous research, the R5 peptide was successfully applied to immobilize biomolecules in silica materials [4, 5]. In addition, they were fused with proteins for novel nanocomposite materials [6–9] and for immobilization [10, 11]. Recently, we constructed a fusion protein consisting of R5 peptide and glucose oxidase for biosensors [12].

A gravimetric biosensor such as a quartz crystal microbalance (QCM) is a simple, cost-effective mass sensing technique and is highly effective at determining the affinity of molecules (proteins, in particular) to surfaces functionalized with recognition sites. The sensing principle of QCM is to measure the frequency decrease corresponding to the mass increase on the functionalized surface through molecular recognition [13, 14]. It has been used for real-time monitoring of interactions between biomolecules [15, 16]. However, the gravimetric biosensor frequently suffers from its limited sensitivity in liquid, particularly for small biomolecules. For instance, the mass sensitivity of a QCM with 5 MHz crystal is typically ~0.1 Hz/ng/cm^2, which is not sufficient for most applications [17, 18]. Therefore, various methods have been devised in order to improve the mass sensitivity of QCMs [19–21].

In this study, we have applied the silaffin peptides as a signal enhancer for gravimetric biosensors for the first time. When recognition proteins tagged with the peptides are bound to analyte molecules on the surface in a sandwich assay, they may be able to induce silica deposition when a silica precursor is added, consequently increasing the mass and thus amplifying the signal. As a proof of concept, we have employed green fluorescent proteins (GFP)–silaffin chimeric proteins as a model system and immobilized them on gold quartz crystals. When a silicic acid solution was supplied, the biomimetic silicification and the resulting frequency changes in a QCM were investigated in the present study.

Materials and Methods

Materials

Plasmid pGFP including the GFP gene was kindly provided from Korea Research Institute of Chemical Technology (KRICT, Korea); pET28(+) was purchased as an expression vector from Novagen, EMD Chemicals, Inc. (USA); the expression host BL21 (λDE3) was from RBC Bioscience (Taiwan); Amicon Ultra-4 (MWCO-10) for concentration of samples was purchased from Millipore. Sense and antisense primers for polymerase chain reaction to generate chimeric genes were synthesized by Bioneer, Inc. (Korea). PD-10 desalting columns for buffer exchange were purchased from GE healthcare, Inc. (USA). All other reagents were from Sigma-Aldrich (USA).

Immobilization of GFP–Silaffin Chimeric Proteins and QCM Measurement

Quartz crystal microbalance with dissipation monitoring (QCM-D) system (Q-sense, Sweden) was employed. AT-cut 5 MHz gold quartz crystals (diameter of 12 mm) were pretreated with piranha solution (75 % H_2SO_4/25 % H_2O_2, *v*/*v*). After mounting the crystal in the cell, a small amount of 50 mM HEPES buffer was introduced into the cell. When the frequency became stable, 1.5 mM disulfide-NTA in 50 mM HEPES was injected into the cell. The working temperature in the chamber was kept constant at 25 °C and the flow rate was 100 μl/min. After self assembly of disulfide-NTA on Au surface, the surfaces were washed with injection of 50 mM HEPES buffer to remove unbound disulfide-NTA. To immobilize histidine tagged proteins, Ni^{2+} was bound to NTA group by injecting 25 mM $NiSO_4$ in 50 mM HEPES, and then the sensor chips were washed with injection of the same buffer. Silaffin chimeric proteins (GFP-R1 to GFP-R7) with a histidine-tag at the *N*-terminus were expressed in *E. coli* as previously reported [11]. The proteins purified through Ni-NTA column (Qiagen, USA) were eluted by 50 mM HEPES buffer (pH7.0) instead of 50 mM sodium phosphate buffer through PD-10 desalting column. The protein solution (10 μg/ml) in 50 mM HEPES buffer was introduced to the Ni^{2+} bound self-assembled monolayers (SAMs). Biosilicification was carried out in situ by introducing 100 mM tetramethoxysilane (prehydrolyzed in 1 mM HCl).

Scanning Electron Microscopy

Deposited silica on the Au chip was analyzed using scanning electron microscopy (SEM). The silica samples were aspirated onto graphite adhesive aluminum stubs, sputter coated to 100 Å with Au/Pb, and imaged using a Philips XL-30S FEG SEM operating at 10 kV. Concurrently, SEM-assisted energy dispersive X-ray spectroscopy (EDS) was also performed.

Results and Discussion

Biosilicification for Signal Enhancement

Silaffin chimeric proteins (GFP-Rx) with a histidine-tag were expressed in *Escherichia coli* as previously performed [11]. The amino acid sequence of each silaffin domain to be tethered to GFP is summarized in Table 1. In order to immobilize the chimeric silaffin proteins on the Au surface, a SAM of disulfide-NTA (Fig. 1a) was constructed and further chelated with Ni^{2+} ions to allow the recombinant proteins to bind with a histidine-tag. The

Table 1 Amino acid sequence of each silaffin domain to be tethered to model GFP protein (R1–R7)

Silaffin domains	Amino acid sequence
R5 (=R3=R7)	SSKKSGSYSGSKGSKRRIL
R1	SSKKSGSYYSYGTKKSGSYSGYSTKKSASRRIL
R2	SSKKSGSYSGYSTKKSGSRRIL
R4 (=R6)	SSKKSGSTSGSKGSKRRNL

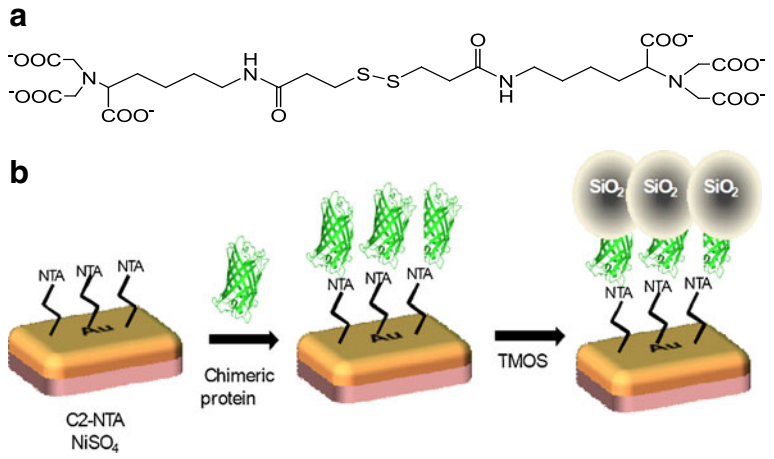

Fig. 1 **a** The structure of 3,3′-dithiobis [*N*-(5-amino-5-carboxypentyl) propionamide-*N*′,*N*′-diacetic acid] dihydrochloride (disulfide–NTA). **b** Scheme of stepwise architecture of immobilization of the chimeric protein using disulfide-NTA and silica formation by the silaffin domains

stepwise architecture of the self-assembled monolayer and immobilization of the chimeric proteins are shown in Fig. 1b.

For biomimetic silica formation, tetramethylorthosilicate prehydrolyzed in 1 mM HCl (final 100 mM) was injected. The variations in the frequencies of the quartz crystals for GFP-R5 and bare-GFP (without silaffin domains as a control) as a function of time are shown in Fig. 2a. A substantial decrease of the resonance frequency was observed on the Au quartz crystals where the chimeric silaffin protein was immobilized. However, the resonance frequency was not shifted on the Au surface covered with bare-GFP as shown in Fig. 2a. Even though the silaffin domain occupies only about 6 % of the chimeric protein by mass, the immobilized fusion protein successfully formed silica particles on the quartz crystals. This clearly demonstrates that the R5 peptide can act as a biomolecular signal enhancer for QCM sensors as we intended. To date, inorganic nanoparticles such as gold have been widely used as a signal enhancer for gravimetric immunosensors [18, 22]. They must be attached to sensing proteins (i.e., antibodies); this conjugation process requires chemical cross-linkers and harsh conditions, which may be detrimental to proteins, and lacks reproducibility generally owing to involved chemical reactions. In this respect, the peptide enhancer in this work is advantageous because it can be readily and quantitatively fused with target proteins using recombinant DNA technology. In addition, the silaffin peptide can produce silica particles at neutral pH and at ambient temperatures and pressures.

The other silaffin fusion proteins (GFP-R1, GFP-R2, and GFP-R4) were also tested as signal enhancers for QCM. The frequency shift ($-\Delta f$) for each fusion protein after 5 min is shown in Fig. 2b with that for GFP-R5. As expected, the other silaffin domains tethered to GFP all exhibited silicification activity; silicification induced by GFP-R1 led to the largest change (67 Hz), whereas silicification by the other chimeric proteins caused a shift of about 40 Hz. It is known that lysine with a positively charged amine group is one of the important amino acids in the primary sequence of the silaffin proteins, catalysts for structured silica synthesis of diatoms [1, 23, 24]. Since the silaffin R1 domain (with six lysine (K) residues) has more lysine residues

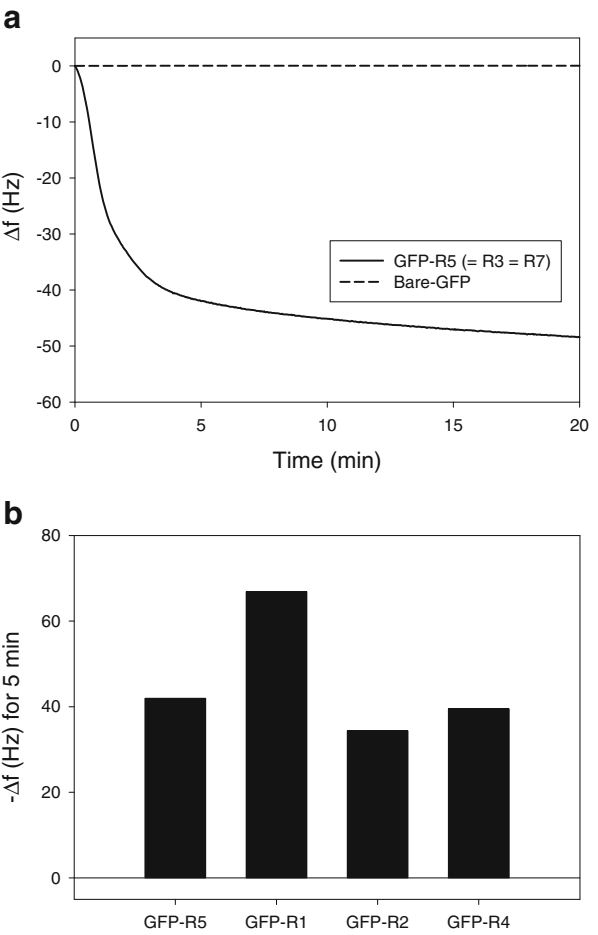

Fig. 2 **a** The resonance frequency shift as a function of time on the Au surface: GFP-R5 (=R3, R7) (*solid line*); bare-GFP (*dash line*); **b** the resonance frequency shift for 5 min biosilicification by silaffin fusion proteins

than the other siliaffin domains (with four lysine residues), the GFP-R1 chimera may be more efficient in silicification than the others.

Surface Analysis

Silica particles induced by the GFP-R1 chimeric protein on the Au surface were analyzed using SEM and EDS. As shown in Fig. 3a and b, silica nanoparticles precipitated on the entire Au surface. Small isolated particles of ~20 nm diameter as well as larger slabs of silica coexisted on the Au surface. Such heterogeneity made it difficult to determine the amount of silica formed on the quartz crystals in this study, even if QCMs can indicate a mass per unit area by measuring the change in frequency of a quartz crystal resonator. For reference, in our previous study in which the silica was formed with free (i.e., not immobilized) GFP-Rx chimeric proteins, the amount of silica precipitates was measured using ICP-OES (inductively coupled plasma-optical emission spectroscopy). We found out that the molar ratio of silica to the silaffin chimeric protein ranged from 250 to 350 [11]. EDS analysis showed Si

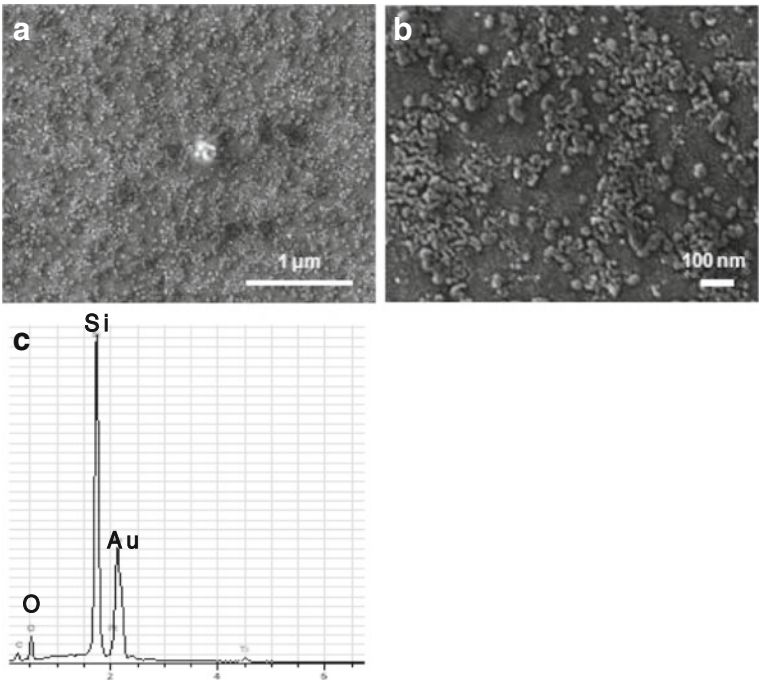

Fig. 3 **a** and **b** SEM image of silica nanoparticles induced by the silaffin R1 chimeric protein on the Au surfaces. **c** Energy dispersive spectroscopy (EDS) analysis of silica nanoparticles deposited on the Au surface

and O atoms from the sample, confirming that the precipitates formed on the Au surface were indeed silica (Fig. 3c).

Conclusion

As a proof of concept, we have successfully demonstrated the application of biosilicification induced by peptides as a signal enhancer for QCM for the first time. A substantial change of the resonance frequency was observed on the Au quartz crystals where the chimeric silaffin proteins were immobilized. However, any frequency shift was not observed on the Au surface covered with bare GFP (without silaffin domains). The silaffin peptide enhancer is advantageous because it can be readily and quantitatively conjugated with sensing proteins using recombinant DNA technology. Combining silaffin peptide domains with antibodies is expected to provide a novel and efficient signal enhancement system for gravimetric immunosensors.

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