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Sol–gel immobilization of a thermophilic diguanylate cyclase for enzymatic production of cyclic-di-GMP

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ABSTRACT

Efficient enzymatic production of 3′,5′-cyclic diguanylic acid or cyclic-di-GMP (c-di-GMP) by diguanylate cyclases (DGC) is highly desired given the potential use of the cyclic dinucleotide as vaccine adjuvant and antibacterial agent. The immobilization of DGCs for continuous c-di-GMP production is challenging because the dimerization and conformational changes accompanied with the chemical transformation could be impeded by the entrapment of the enzyme. Here we demonstrate that an engineered thermophilic diguanylate cyclase (etDGC) cloned from *Thermotoga maritime* can be effectively encapsulated in both the methyltrimethoxysilane (MTMOS) and tetramethoxysilane (TMOS) gels. However, only the enzyme immobilized in the MTMOS gel through a gradual gelation process exhibited enzymatic activity. The immobilized enzyme exhibits extended lifetime and catalytic activity in comparison to the free enzyme in solution, suggesting that the sol–gel immobilized etDGC could be used as a stable and convenient reagent for long-term storage and *in situ* synthesis of c-di-GMP or ^{32}P-labelled c-di-GMP. The enhanced stability of the immobilized enzyme also demonstrates the feasibility and potential of continuous production of c-di-GMP on a large scale using sol–gel immobilized etDGC.

1. Introduction

The cyclic dinucleotide 3′,5′-cyclic diguanylate or cyclic-di-GMP (c-di-GMP) has emerged as a ubiquitous bacterial second messenger. Recent discoveries highlighted the importance of c-di-GMP in the formation of bacterial biofilm and regulation of bacterial virulence [1–6]. C-di-GMP is also being tested as antibacterial agent and vaccine adjuvant for treating bacterial infection and various forms of cancers [7–11]. The increasing use of c-di-GMP in research laboratories for various purposes calls for the production of this compound at an affordable cost. Currently, c-di-GMP can be synthesized in small quantities chemically or enzymatically. The solution and solid-phase chemical synthesis approaches are expensive and time-consuming given the multi-step nature of the synthetic routes [12–17]. On the other hand, c-di-GMP can be enzymatically synthesized by the bacterial proteins that contain the diguanylate cyclase (DGC) domain [18,19]. DGC domain synthesizes c-di-GMP by catalyzing the formation of the phosphodiester bond between the 3′-OH and α-phosphate group. As a result, two GTP molecules are jointed to form the cyclic dinucleotide c-di-GMP with pyrophosphate released as by-product (Fig. 1) [20]. Recently, we demonstrated the preparation of c-di-GMP by using an engineered thermophilic DGC domain protein with significantly improved thermostability and reduced product inhibition [15]. This engineered thermophilic DGC (etDGC) was derived from a thermophilic protein (TM1788) in *Thermotoga maritime*, with the N-terminus truncated for improving solubility and mutation in the inhibitory I-site for alleviating product inhibition. Since the synthesis of c-di-GMP requires the concerted action of two DGC domain subunits and etDGC was found to exist in solution mainly as monomer, *in situ* dimerization is presumably indispensible for the function of etDGC [15]. The highly efficient enzyme has enabled us to produce hundreds of milligrams of c-di-GMP for enzymatic assay and structural studies in our laboratory [21–25].

Despite being able to produce hundreds of milligram of c-di-GMP, the direct use of etDGC has some disadvantages. Because the enzyme needs to be separated from reaction mixture at the end of the reaction, typically by heat precipitation, and because the soluble protein precipitates over time during the course of the reaction at 55 °C, fresh enzyme needs to be used for each enzymatic reaction for c-di-GMP production [15]. To achieve a more efficient and cost-effective production, a continuous process that recycles the enzyme is desirable, particularly for large-scale production. One solution is to immobilize the enzyme and flow the reactant continuously over the immobilized enzyme. Immobilization of the enzyme in the matrix would circumvent the requirement

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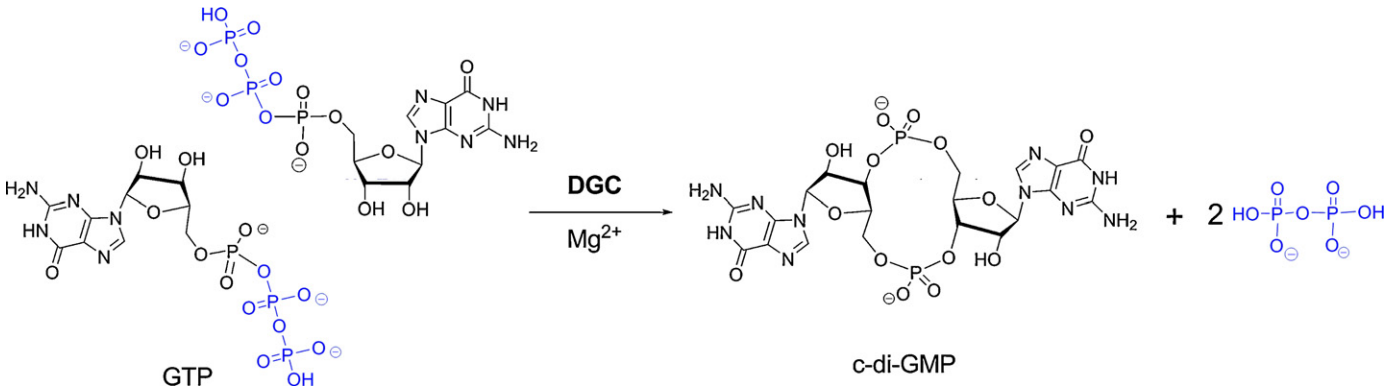

Fig. 1. Enzymatic synthesis of c-di-GMP by the condensation of two molecules of GTP as catalyzed by diguanylate cyclase (DGC). Pyrophosphate is generated as by-product.

for the separation of the enzyme from the product. Moreover, it is expected that the immobilization of the enzyme could prevent the self-aggregation of the enzyme to extend the lifetime of the enzyme. Given the advantages of the immobilized enzyme, we set out to explore the possibility of immobilizing etDGC in various solid supports. Our initial studies showed that the immobilization of etDGC by covalent immobilization methods such as the cross-linking enzyme aggregates (CLEAs) approach rendered the enzyme completely inactive. Considering that the condensation of the two GTP molecules by etDGC proteins is likely to involve *in situ* dimerization and significant conformational change, the loss of activity highlights the challenge of immobilization of such enzyme systems. In this report, we describe the successful immobilization of etDGC in sol–gel, a silica matrix that has been known to be able to encapsulate biological macromolecules via non-covalent entrapment [26–31]. Characterization of the catalytic activity and stability of the entrapped enzyme demonstrates that the sol–gel matrix preserves the enzymatic activity of the enzyme, and thus, could be a viable method for DGC protein immobilization and c-di-GMP production.

2. Experimental

2.1. Materials

Tetramethoxysilane (TMOS) and methyltrimethoxysilane (MTMOS) were purchased from Janssen Chimica (99%) and Merck respectively. Guanosine triphosphate (GTP) was purchased from Sigma–Aldrich (sodium salt hydrate, minimum 95%). The other chemicals that include tryptone (BD, Difco), yeast Extract (BD, Difco), Isopropyl β-D-thiogalactoside (IPTG, Gold Biotechnology), β-mercaptoethanol (Merck) and phenylmethylsulfonyl fluoride (PMSF, Roche) are purchased from the respective suppliers.

2.2. Protein expression and purification

The engineered thermophilic diguanylate cyclase (etDGC) protein originated from *Thermotoga maritime* was cloned and expressed in *E. coli* following the procedure described previously [15]. Briefly, a 2 ml starting culture was grown overnight at 37 °C from 2 μl cell stock. The starting culture was then added to 1 l fresh LB medium and grown at 37 °C. After reaching OD 0.8, protein expression was induced with 0.8 mM IPTG at 25 °C overnight. The cells were harvested by centrifugation at 8,000 rpm and the pellets were lysed by sonication in 20 ml lysis buffer (50 mM Tris–Cl, 300 mM NaCl, 5% glycerol, 1% β-mercaptoethanol, 1 mM PMSF, adjusted to pH 8.0). After centrifugation at 20,000 rpm for 30 min, the supernatant was filtered (0.45 μm Acrodisc syringe filter, Pall) and then incubated with 2 ml Ni^{2+}–NTA resin (Qiagen) for 30 min at 4 °C. The resin was washed with 50 ml of W1 buffer (lysis buffer with 20 mM imidazole) and 20 ml of W2 buffer (lysis buffer with 50 mM imidazole). The proteins were eluted using a stepped gradient method with the elution buffer containing 20 mM Tris (pH 8.0), 300 mM NaCl, 5% glycerol and 200, 300 and 500 mM imidazole. After SDS-PAGE gel analysis, fractions with purity higher than 95% were pooled together and further purified using size-exclusion chromatography. Gel filtration was performed at 4 °C by using AKTA FPLC system equipped with a Superdex 75 HR 16/60 column (Amersham Biosciences). The buffer used for gel filtration contains 50 mM Tris–Cl (pH 8.0), 300 mM NaCl, 5% glycerol, 1 mM DTT. Fractions containing the recombinant protein were concentrated by using Amicon concentrator (10,000 MWCO, Millipore) to a final concentration of ca. 5 mg/ml as measured using Bradford assay. Concentrated proteins were stored at −80 °C degree after flash-freeze using liquid nitrogen (overall yield: 12–15 mg/l).

2.3. Sol–gel preparation

The sol–gel preparation method reported by Savini et al. was used with a slight modification [32]. The stock solutions were prepared by mixing 1.8 ml gel precursor (MTMOS or TMOS), 0.38 ml distilled water, and 0.020 ml of 0.04 M NaOH in a test tube at room temperature. The stock solution was sonicated in a water bath for at least 10 min or until the solution is visually homogeneous. Into the Eppendorf tubes containing 0.36 ml of stock solution, 0.54 ml enzyme solution at various concentrations were added. The mixture was vortexed at low speed briefly and casted into a polystyrene cuvette mold (12.5 mm × 12.5 mm × 45 mm). The cuvettes were covered with parafilm for 24 h at room temperature, and left in the air for aging for 4–6 days. After taken out from the mold, the gels were rinsed with distilled water for an hour to wash off the excess or leached enzyme from the silicate matrix. UV–vis spectra of the supernatant left from the gelation process (if any) and the rinsing solution were taken to determine the presence of unincorporated or leached enzyme. The gels were stored at room temperature in dry condition or immersed in the same buffer used to store the enzyme (50 mM Tris–Cl, 300 mM NaCl, 5% glycerol, adjusted to pH 8.0) for 1–4 weeks before checking its activity along with the free enzyme control.

2.4. Enzymatic assay for the immobilized enzyme

To determine the enzymatic activity of the immobilized etDGC, the assays were performed with gels stored for different period of time (1–4 weeks). The gels were washed with distilled water and soaked in the reaction buffer (50 mM Tris-Cl, 250 mM NaCl, 5 mM $MgCl_2$) for 1 h prior to reaction. The enzymatic assays were initiated by adding GTP to a final concentration of 750 μM followed by

incubation at 55 °C for various length of time. Reverse-phase high performance liquid chromatography (RP-HPLC) was used to monitor the turnover of GTP and formation of c-di-GMP as described previously [15]. At various time points during the incubation, 5 μl aliquots were taken and loaded onto an Eclipse XDB-C18 (4.6 mm × 150 mm) reverse-phase column for quantification. RP-HPLC analysis was performed by using Agilent LC1200 system at 1 ml/min flow rate (mobile phase: 9% methanol, 20 mM triethylammonium bicarbonate, adjusted to pH 7.0 with acetic acid). The turnover of GTP was obtained by comparing the peak area of c-di-GMP to that of GTP.

2.5. Scanning electron microscopy (SEM)

The gel blocks were cut to observe the cross-section surface. Field emission scanning electron microscopy (FESEM, JEOL 7600F) operating at 2 kV was employed to visualize the porosity of the gels. Platinum coating on the sample surface was used to reduce sample charging for high resolution imaging.

3. Results and discussion

Enzymes are usually immobilized onto solid support by covalent or non-covalent attachment methods for continuous production. Cross-linking methods without using solid support have also been employed for enzyme immobilization [33–37]. Immobilization of etDGC by the cross-linking methods such as the cross-linking enzyme aggregates (CLEAs) method with the cross-linker glutaraldehyde resulted in a complete loss of enzymatic activity (data not shown). We suspected that the cross-linking of the enzyme abolishes the catalytic activity of etDGC by impeding the dimerization and conformational change that are critical for substrate binding and GTP condensation. We reasoned that the covalent attachment methods are also likely to cause severe decrease in enzymatic activity, and that non-covalent methods are more suitable for etDGC immobilization. We decided to explore the method of encapsulation rather than adsorption considering that the adsorption methods generally suffer from protein leaching. Immobilization by encapsulation sequesters the enzyme molecules in the cages or pores of solid support to prevent them from leaching or self-aggregating, while at the same time permits diffusion of substrates and products with the high porosity. The cages or compartments formed inside the matrix network may also stabilize the conformation of the enzyme and extend the lifetime of the protein by preventing denaturation [38]. Sol–gel has been investigated extensively for the encapsulation of enzymes such as lipase [39] and lactate oxidase [40]. Various procedures involve the use of a mixture of TMOS and MTMOS, or biocompatible precursors such as poly(glycerylsilicate) have also been explored [41]. Unlike etDGC, most of the immobilized enzymes function as monomeric proteins. The required dimerization and conformational change for etDGC in catalysis present a challenge for immobilization.

3.1. Immobilization of the enzyme

The encapsulating gel was prepared by the acid (HCl) or base (NaOH) catalyzed hydrolysis and condensation of silicon alkoxides tetramethoxysilane (TMOS) or methyltrimethoxysilane (MTMOS). The gels prepared with TMOS as precursor (TMOS gels) undergo sol–gel transition within minutes to form a transparent gel monolith. With 5 mg enzyme used for immobilization, absorption spectroscopy indicates that most of the enzyme was incorporated into the 4 mm × 4 mm × 1.2 mm TMOS dry gel, with only a negligible amount of enzyme leached out when the gel was washed (Fig. 2). In comparison, the complete gelation of the MTMOS gels took from hours to days. The MTMOS gels initially appeared opaque (white)

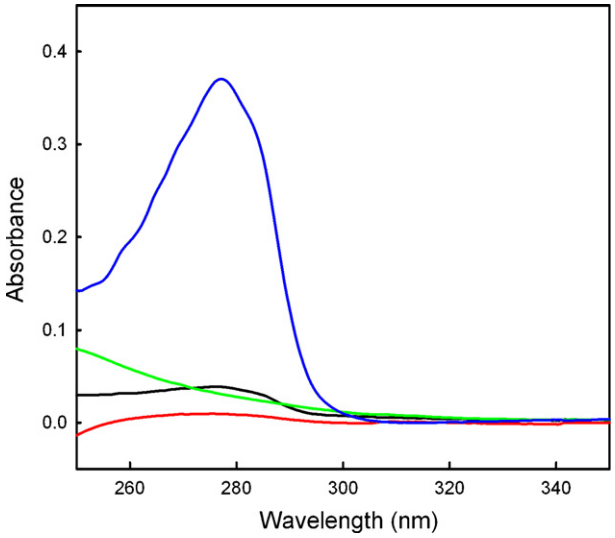

Fig. 2. Analysis of the immobilization of the etDGC by TMOS and MTMOS gels by absorption spectrophotometry. Absorption spectra of the washing solution of the MTMOS (red trace) and TMOS gel (green trace) exhibit negligible absorption peak at 280 nm, indicating that there is no leakage from both gels. The absorption spectrum of the trapped protein (blue trace) released from the gel by crushing the gel blocks and the spectrum of the protein remained in the supernatant after gelation (black trace) demonstrates the effective encapsulation of the enzyme in the gel.

rather than transparent with a small percentage of enzyme left in the supernatant when cast in a cuvette, as evidenced by the absorption spectroscopy of the supernatant. The unincorporated protein could be reduced by decreasing the amount of the enzyme used for immobilization, suggesting that the observation of the free protein is due to over-loading. The entrapment of the enzyme in the gel is evidenced by the absorption spectrum of the protein released from the gel by crushing the gel blocks (Fig. 2, blue trace). These results indicate that both the TMOS and MTMOS gels can effectively immobilize the etDGC protein onto the solid support.

Subsequently, we examined whether the enzyme is entrapped rather than adsorbed by the silica network. After aging for various lengths of time ranging from 1 to 5 weeks, the prepared gels were washed thoroughly with buffer. No protein was detected in the rinsing buffer, indicating there is no significant leaching of the enzyme from the gel (Fig. 2). This conclusion is further supported by the absence of the leached enzyme in the reaction buffer during the production of c-di-GMP, which is described further below.

3.2. Catalytic activity and stability of the immobilized enzyme

The activity assay of the immobilized enzyme was performed by incubating the gel blocks or monoliths with GTP and Mg^{2+} in the reaction buffer at 55 °C, which is the optimal temperature for the production of c-di-GMP by the thermophilic enzyme in solution [15]. Steady-state kinetic measurement has shown that that etDGC catalyzes the GTP condensation with a turnover number k_{cat} of 2.6 min^{-1} at 55 °C [15]. The enzyme immobilized in the TMOS or MTMOS gels prepared using acid catalyst (0.01 M HCl) or NaOH (0.01 M) only exhibited negligible catalytic activity. Meanwhile, the enzyme immobilized in the MTMOS gel formed by using 0.04 M NaOH consistently exhibited much higher activity compared to the one entrapped in TMOS gel. While the MTMOS immobilized enzyme was able to turn over ~95% of the 15 mg GTP substrate, only less than 1% of the GTP was turned over to c-di-GMP with the TMOS gel immobilized enzyme (Fig. 3A). Given the subtle difference in the structure for the TMOS and MTMOS precursors, the observed discrepancy seems to suggest that a hydrophobic gel is more suitable for encapsulating etDGC. Considering that the condensation of

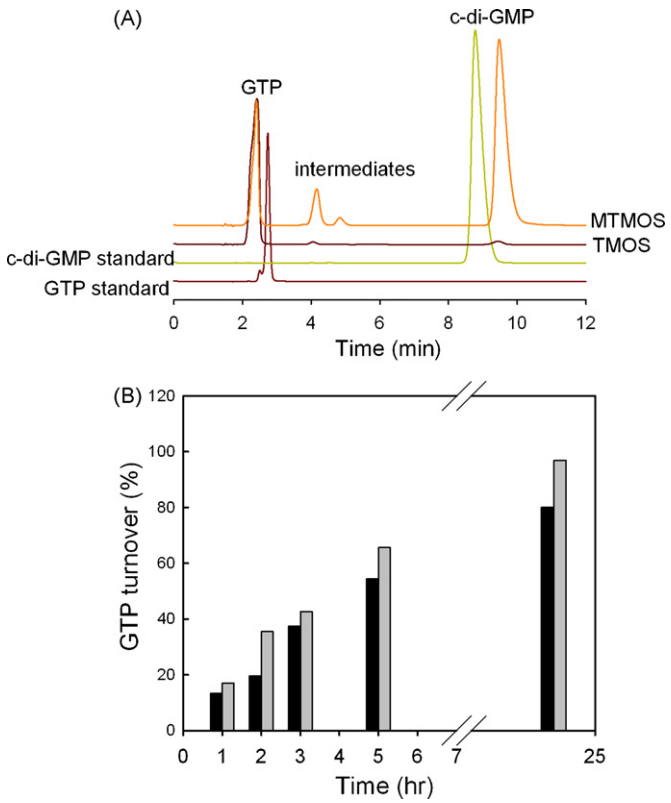

Fig. 3. Catalytic activity of the MTMOS immobilized etDGC. (A) HPLC chromatograms for the turnover of GTP to c-di-GMP by MTMOS and TMOS gel immobilized etDGC. The small peaks between 4 and 5 min correspond to the intermediates formed during the enzymatic reaction. (B) Turnover of GTP (%) is plotted vs. reaction time to show time-dependent progression of the enzymatic reaction. 15 mg of GTP was used as substrate. The immobilized etDGC exhibits enzymatic activity after 3 (black bars) or 5 (grey bars) weeks of aging.

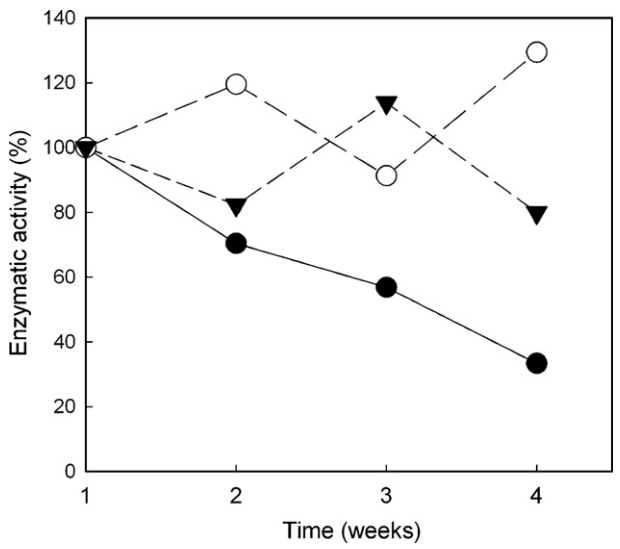

Fig. 4. Comparison of the stability of MTMOS gel immobilized etDGC and free etDGC. Stability of etDGC was examined by comparing the enzymatic activity at various time points of storage. The normalized enzymatic activity (%) was obtained from the measurement of the GTP turnover rate with the free and immobilized enzyme at 55 °C. The enzyme in solution (●) exhibited a half-life of approximately 3 weeks, whereas the immobilized etDGC did not exhibit significant decrease in catalytic activity after 4–6 weeks of storage as dry gel (▼) or wet gel (○).

GTP into c-di-GMP likely requires the *in situ* dimerization of etDGC [15], we reasoned that the hydrophobic microenvironment of the gel matrix and the gradual gelation for the MTMOS gel may in fact promote the formation of dimeric etDGC forms. The facilitation of the dimer formation may also be due to the increase of effective protein concentration as a result of compartmentalization. Considering that both the substrate GTP and product c-di-GMP are highly hydrophilic, the use of hydrophobic precursor may also be advantageous in promoting the diffusion of the substrate GTP and product c-di-GMP in the gel network.

It should be noted that although the substrate turnover for the MTMOS gel is higher than that of the TMOS gel, the activity of MTMOS gel was still lower than the fresh, soluble enzyme. The same amount of fresh, soluble enzyme (5 mg) could turnover up to 95% of the substrate (15 mg GTP) to product (c-di-GMP) in *ca.* 45 min, whereas the MTMOS gel required many hours, with the intriguing observation that the gel with longer aging time exhibits slightly higher catalytic activity (Fig. 3B). The negative effect on enzyme efficiency caused by sol–gel immobilization has been observed for other enzymes [30,31]. It is expected that some of the immobilized enzyme may have lost the activity during the gelation process, presumably because of the unfavorable gelation conditions in the sol–gel process. The unfavorable conditions include the extreme local pH in the microenvironment and liberation of alcohol from the gelation process. Another possible reason for the lower catalytic efficiency of the immobilized enzyme could be the hindered diffusion of the substrates and product in the TMOS gel network. Such possibility is confirmed by the detection of ample amount of c-di-GMP after crushing of the dry gels used for enzymatic reaction. Thus, future optimization of the process may involve the test of sol–gel precursors with hydrophobic chains to prevent the retention of the polar GTP and c-di-GMP.

One important reason to immobilize the etDGC enzyme is to further stabilize the enzyme and thus prolong the lifetime of the enzyme for re-using during the production of c-di-GMP at elevated temperature (55 °C) with the enzyme exhibiting high efficiency for c-di-GMP synthesis. Although the thermophilic enzyme already possesses remarkable thermostability, it gradually loses its catalytic activity in solution with a half-life of 3 weeks at room temperature, and a half-life of 4 days at the temperature (55 °C) used for c-di-GMP production (Fig. 4) [15]. The half-lifetime can be further shortened during enzymatic reaction with constant perturbation and stirring to promote mixing. Thus, a large fraction of the enzyme would have lost the catalytic activity after 2 days of enzyme reaction with repetitive addition of the substrate GTP. The observed decrease in enzymatic activity may also due to the product inhibition at high c-di-GMP concentration after 2 days of enzymatic reaction. The loss of enzyme activity caused by enzyme denaturation and product inhibition can be avoided using a continuous production method with immobilized etDGC. Indeed, we observed that the MTMOS gel immobilized enzyme did not show an apparent drop in catalytic activity even after 5 weeks (Fig. 4). The results clearly demonstrate that the immobilized enzyme can be stabilized by the gel network, and that the loss of the enzymatic activity has been significantly delayed. More importantly, the enzyme immobilized dry gel exhibited similar activity compared to the gel stored in buffer, suggesting that the enzyme can be preserved in the dry gel with little solvent present. Considering that DGC proteins are extensively used by research laboratories for the preparation of small amount of ^{32}P-labelled c-di-GMP, we expect that the sol–gel immobilized etDGC in the powder form can be developed into a stable and convenient reagent for *in situ* preparation of ^{32}P-labelled c-di-GMP. Given the requirement of large quantity of c-di-GMP for animal and clinical trials as antibacterial agent and vaccine adjuvant, the results also highlight the feasibility and potential of immobilizing etDGC for continuous large-scale production of c-di-GMP with further testing

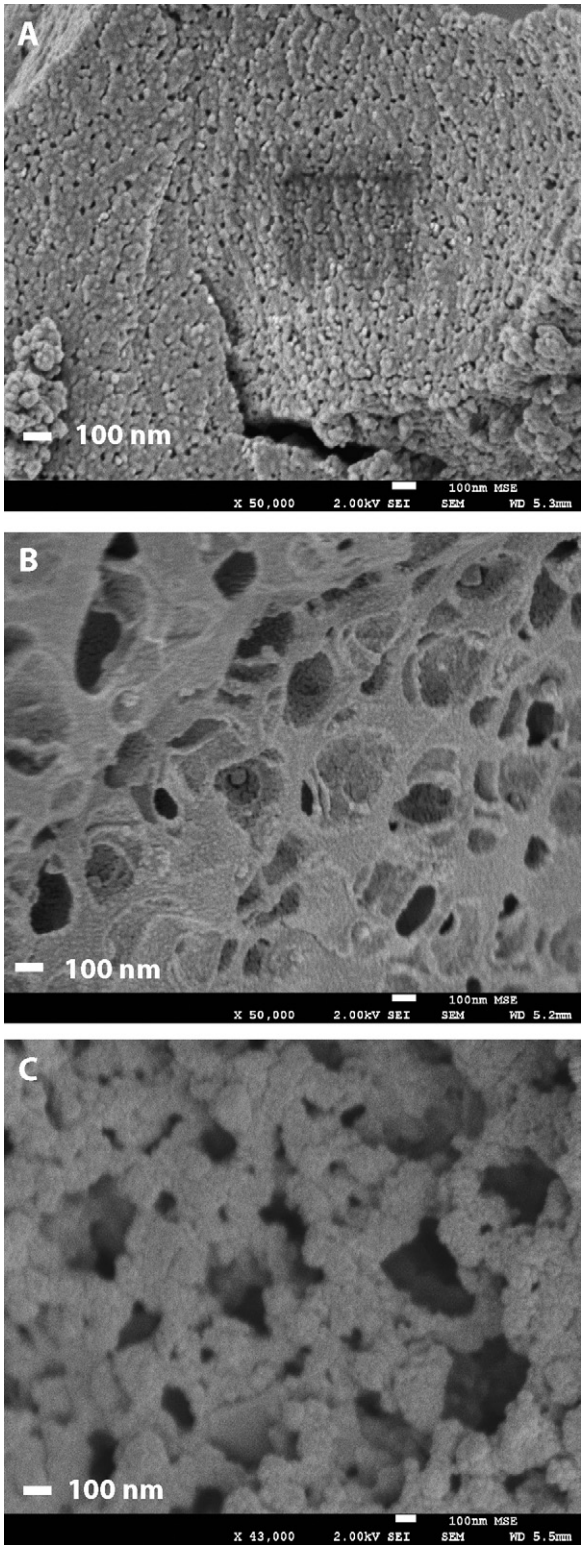

Fig. 5. Scanning electron microscope (SEM) images of the etDGC immobilized MTMOS gel. The images revealed that the porosity of the gel varies with gelation conditions and enzyme concentrations, with the pore size ranging from 10 to 200 nm. Shown here are the representative images of the surface of the MTMOS gel (A) and the cross-section of the MTMOS gel aged for 4 weeks (B, gel stored in air; C, gel stored in buffer).

of various sol–gel precursors and optimization of the gelation process.

3.3. Morphology of the enzyme immobilized sol–gel

The enzyme-immobilized MTMOS gels were examined by scanning electron microscopy (SEM) to identify any correlation between enzymatic activity and porosity. While the surface of the gels aged at 4 °C and room temperature exhibit low porosity and small pores (Fig. 5A), the cross-section of the gels aged at room temperature show an extensive silica network with the diameter of the pores ranging from 20 to 300 nm for the catalytically active gels stored in the air (Fig. 5B) or buffer Fig. 5C). Among the gels aged under room temperature, no apparent correlation could be observed for the catalytic efficiency and porosity. The porosity and pore size of the gels seem to vary considerably with gelation conditions and enzyme concentration. Given the estimated size of the dimeric etDGC (6.5 nm × 4.5 nm), it is expected from the SEM images that most of the prepared gels are able to encapsulate multiple enzyme molecules in a single pore to ensure the dimerization while prevents the leaching of the enzyme at the same time. Considering that the pore sizes may not only affect enzyme dimerization and stability, but also govern the internal diffusion rate of GTP and c-di-GMP, the efficiency of the immobilized etDGC could be further improved by controlling the pore sizes through the optimization of the gelation and aging conditions as well as the use of additives.

4. Conclusion

Immobilization of DGC proteins is critical for continuous production of c-di-GMP on a large scale by saving the time and cost on the expression and purification of the enzymes. Immobilization of DGC proteins by covalent attachment or cross-linking methods is likely to render the enzyme inactive due to the hindrance of dimerization and conformational change during catalysis. The experimental results presented here suggest that the base-catalyzed MTMOS silica gel is a potentially useful solid support for the immobilization of etDGC, a thermophilic DGC protein that has been previously engineered for c-di-GMP production. The observation of the catalytic activity for the immobilized etDGC demonstrates that the immobilization does not hinder the *in situ* dimerization and conformational changes required for catalysis, and thus, highlights the potential of producing c-di-GMP continuously using sol–gel immobilized DGC enzymes. The immobilized etDGC exhibits enzymatic activity for a prolonged period of time in comparison to the free enzyme. The extended life-time of the immobilized etDGC in the dry gels indicates an effective method for long-term storage and use of the etDGC protein in the form of sol–gel powder or particle for *in situ* production of c-di-GMP or ^{32}P-labeled c-di-GMP. The experimental results indicate that the activity of the immobilized enzyme is lower than that of its soluble counterpart. The low efficiency could be due to the partial loss of enzyme activity during the gelatin and the slow diffusion of the substrate and product in the silica gel network. Optimization of the sol–gel conditions and the use of hydrophobic precursors may further improve the catalytic activity of the immobilized etDGC for the continuous production of c-di-GMP.

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