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Nanoporous scaffold for DNA polymerase: Pore-size optimisation of mesoporous silica for DNA amplification†

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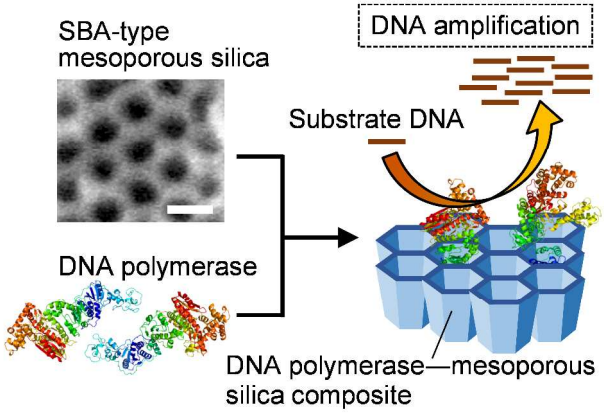
Composites of thermostable DNA polymerase and mesoporous silicas with different pore diameters were used for DNA amplification. The enzyme was selectively immobilised on the mesopores, and DNA amplification activity was retained by regulating the pore size of the mesoporous silicas.

Research into the development of nanopore-containing supporting materials for efficient enzyme immobilisation has energised the fields of biomaterial and biochemical engineering because of their potential application in biocatalytic reactions.[1] The encapsulation of enzymes into the highly ordered pores of mesoporous silica materials, such as the Santa Barbara Amorphous (SBA) series[2] and folded-sheet material (FSM)-type silica,[3] enhances their ability to be reused,[4] and also helps increase their stability against exposure to heat,[5] chemicals,[6] pH changes[7] and organic solvents.[8] However, most previous studies have used low-molecular-weight compounds as reactive substrates for enzymatic catalysis. Because immobilised enzymes are likely to be used in the biochemical reaction field in the future, construction of reaction systems for large biomacromolecules, such as DNA, is an important challenge. Two recent studies immobilising DNA polymerase, the enzyme responsible for DNA synthesis, used mesoporous thin titania films and gold-coated glass as solid supports. These studies highlighted the need for using large mesopores for the smooth diffusion of DNA molecules into the pores[9] and for protecting the active site of DNA polymerase during immobilisation.[10]

The mesoporous silica materials are potentially capable of sustainable enzymatic reactions via the repeated use of enzymes. The reuse of DNA polymerase is extremely useful in DNA synthesis, because commercial DNA polymerases are still very expensive. When mesoporous silica is used as the nanoporous scaffold for DNA polymerase, optimising the pore diameter is vital in order to achieve the appropriate DNA amplification activity in the DNA polymerase-mesoporous silica composites. Therefore, the compatibility between DNA polymerase and mesoporous silica pores, the effect of silica pore diameter on enzyme immobilisation and its reactivity towards enzymes must be clarified.

We developed a nanoporous reaction field using mesoporous silica materials to amplify DNA with the use of thermostable *Thermus aquaticus* DNA polymerase (*Taq* DNA polymerase)[11] as a simple potential immobilising system. In this study, we first investigated the immobilisation state of *Taq* DNA polymerase on seven highly ordered mesoporous silicas from the FSM and SBA series with differing pore diameters ranging from 2.6 to 24.5 nm. We then evaluated the enzymatic activity of the immobilised *Taq* DNA polymerase during the polymerase chain reaction (PCR)-based amplification of substrate DNA. The PCR amplification technique, which is used to amplify and detect a target DNA molecule, can be achieved by repeating cycles of heat denaturation of DNA, annealing two oligonucleotide primers to the denatured DNA, and extension of the annealed primers by DNA polymerase.[11] In this communication, we propose a DNA amplification system using DNA polymerase-mesoporous silica composites, as shown in Scheme 1.



Scheme 1 Schematic representation of DNA amplification by DNA polymerase immobilised on the channels of mesoporous silica. A scanning electron microscope image reveals mesopores captured along the (001) directions of SBA-type mesoporous silica with a 7.1 nm pore diameter (SBA7.1), which corresponds to sample number 4 in Table 1. Scale bar = 10 nm. The structure of DNA polymerase (*Taq* pol) was generated from the Protein Data Bank file (1TAQ) using the PyMOL software.[12]

We successfully prepared two types of FSM (FSM2.6 and FSM4.2) with pore diameters of 2.6 and 4.2 nm, respectively, and five types of SBA (SBA5.4, SBA7.1, SBA10.6, SBA18.5, and SBA24.5) with pore diameters of 5.4, 7.1, 10.6, 18.5, and 24.5 nm, respectively, based on previous publications, with some modifications (Electronic Supplementary Information [ESI]†).[13] The formation of the ordered mesopores of calcined FSMs and SBAs was confirmed by the results of nitrogen adsorption-desorption isotherms and scanning electron microscope images (Figs. S1, S2, S3 and S4, ESI†). The structural properties of the mesoporous silicas obtained are shown in Table 1.

Table 1 Structural properties of FSM and SBA-type mesoporous silicas

Sample no.	Type of mesoporous silica	Pore diameter (nm)	Specific surface area (m^2g^{-1})	Total pore volume (cm^3g^{-1})	Particle morphology
1	FSM2.6	2.6	1057	1.03	Irregular particle
2	FSM4.2	4.2	1086	1.26	Irregular particle
3	SBA5.4	5.4	644	0.61	Rod
4	SBA7.1	7.1	799	0.87	Rod
5	SBA10.6	10.6	671	1.23	Rod
6	SBA18.5	18.5	534	1.56	Sphere
7	SBA24.5	24.5	386	1.93	Sphere

See ESI for the detailed synthesis protocol, Fig. S1 for nitrogen adsorption-desorption isotherms and the corresponding pore-size distribution curves for the mesoporous silicas, and Figs. S2, S3 and S4 for scanning electron microscope images of the mesoporous silicas.

An enzyme adsorption experiment was performed by combining 0.5 mg of each mesoporous silica powder with 42.5 μL of Ex *Taq* buffer solution (pH 8.5) containing 5U *Taq* DNA polymerase (TaKaRa Ex *Taq*), 344 ng of BSA (already present in the buffer solution), 5 ng of substrate DNA (100-bp double-stranded DNA [dsDNA, ESI†]), and 0.4 mM of each dNTP. The *Taq* DNA polymerase was adsorbed onto mesoporous silica by mixing the two together using a vortex mixer for a few seconds, followed by the addition of 7.5 μL of each primer (T7 and SP6 promoter primers, 0.75 μM each). The immobilisation state of the *Taq* DNA polymerase on the mesoporous silica pores before PCR was then evaluated using SDS-PAGE (Fig. 1). As shown in Fig. 1a, *Taq* DNA polymerase was not detected in the supernatants after enzyme immobilisation, while BSA was detected in mesoporous silica, which had a smaller pore size. All of the *Taq* DNA polymerase (5 U) was released from the mesoporous silicas into the boiled SDS-sample buffer, owing to the successful immobilisation of the enzyme in the mesoporous silica pores (Fig. 1b). Therefore, *Taq* DNA polymerase could be very rapidly immobilised on all mesoporous silicas, irrespective of the morphology of the mesoporous silica particles. In addition, the amount of adsorbed BSA was dependent on the pore size.

These data demonstrate that all mesoporous silicas had the potential to preferentially immobilise *Taq* DNA polymerase rather than BSA, although the mole number of *Taq* DNA polymerase in the PCR reaction solution and the molecular weight (6.7 pmol, M_r *ca*. 94,000) are higher than those of BSA (5.1 pmol, M_r *ca*. 68,000). The isoelectric points of *Taq* DNA polymerase and BSA are ~6.03 (theoretical value)[14] and 4.7, respectively. Therefore, both the proteins and the silanol groups of the mesoporous silica are negatively charged under these reaction conditions (pH 8.5), resulting in electrical repulsion between the proteins and/or between the protein and the silica support. It is suggesting that *Taq* DNA polymerase was immobilised preferentially to the mesoporous silicas by the affinity of other interactions between the enzyme and mesoporous silica, such as hydrophobic adsorption.

BSA is more negatively charged than *Taq* DNA polymerase at pH 8.5, since the isoelectric points of BSA is lower than that of *Taq* DNA polymerase, resulting in greater electrical repulsion between the protein and the silica support. Therefore, the silica surface may have preferentially interacted with *Taq* DNA polymerase rather than with BSA, although the hydrodynamic diameter of *Taq* DNA polymerase (*ca*. 9.2 nm)[15] is larger than that of BSA (5.0-7.2 nm)[16] (Table 2).

Table 2 Properties of *Taq* DNA polymerase and BSA

Protein	pI[a]	MW	diameter (nm)	Ref.
Taq DNA polymerase	6.0	94 000	*ca*. 9.2	15
BSA	4.7	68 000	5.0–7.2	16

[a] pI: isoelectric point.

We next performed PCR using *Taq* DNA polymerase conjugated to mesoporous silica to assess the effect of mesoporous silica pore size on DNA amplification activity (Fig. 2 and ESI†). The PCR efficiency depended highly on the pore size (Fig. 2a). For both FSM and SBA, amplification was the most efficient with the smallest pore diameters (*i.e.* FSM2.6 and SBA5.4).

Consistent with this, the efficiency of DNA amplification in the *Taq* DNA polymerase-FSM2.6 and -SBA5.4 composites were 47% and 53% of the efficiency of free enzyme without mesoporous silica, respectively (Fig. 2b). In contrast, SBA18.5 and SBA24.5, with larger pores, were only able to amplify a small amount of substrate DNA. Surprisingly, these results are inconsistent with several

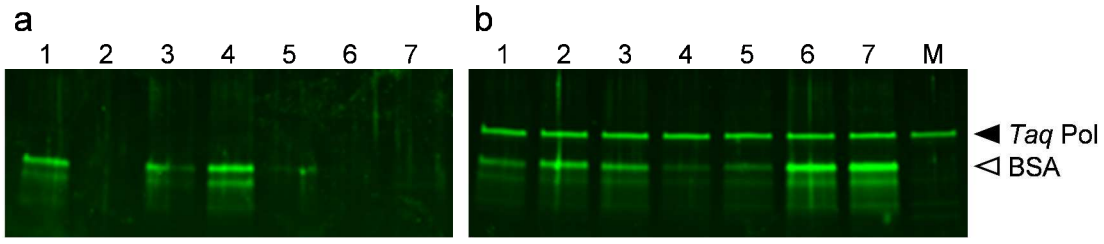


Fig. 1 Analyses of *Taq* DNA polymerase and BSA using SDS-PAGE immediately after immobilisation on mesoporous silicas. (a) Supernatants after enzyme immobilisation; and (b) supernatants after incubating the pellet of (a) with SDS-sample buffer for 10 min at 95 °C. Each sample was separated on 4–15% SDS-polyacrylamide gels and stained with SYPRO Ruby Protein Gel Stain. Numbers above the gels correspond to different mesoporous silicas, which are numbered according to the sample numbers in Table 1. M, *Taq* DNA polymerase marker (*Taq* Pol, 94 kDa, equivalent amount of *Taq* DNA polymerase contained in the PCR reaction solution). Black and white arrowheads indicate the position of *Taq* Pol and BSA (*ca*. 68 kDa), respectively. See also Fig. S1 (ESI†) for SDS-PAGE images of immobilised *Taq* Pol after PCR amplification.

previous reports assessing the relative activity of immobilised industrial enzymes using low-molecular-weight compounds.[17] The results shown in Fig. 2a and b suggest that the enzyme was adsorbed onto the entrance of the mesopores, where it could collide more frequently with the large substrate DNA molecules. This is because *Taq* DNA polymerase, which has a gyration diameter of ~9.2 nm,[15] cannot penetrate beyond the entrance of silica pores that are smaller than the enzyme. Therefore, the increase in DNA amplification efficiency was inversely proportional to the mesoporous silica pore size, as shown in Fig. 2c.

After PCR amplification, the immobilisation state of *Taq* DNA polymerase on the mesoporous silica pores was re-evaluated by SDS-PAGE (Fig. S5, ESI†). The data were consistent with those shown in Fig. 1; the supernatants did not contain *Taq* DNA polymerase, suggesting that the immobilised enzyme was stably and strongly immobilised on the pores and therefore did not leak into the solution, even during PCR amplification. Therefore, it would be reasonable to conclude that the immobilised enzyme is actually involved in DNA amplification.

The abovementioned results demonstrate that mesoporous silica with smaller pores is a suitable platform for DNA amplification reactions using *Taq* DNA polymerase because stable enzyme immobilisation that retains ~50% of the intrinsic enzymatic activity can be achieved. The halved efficiency of DNA amplification shown in Figure 2 may be because of the alternative orientation of *Taq* DNA polymerase with an elongated shape in the smaller pores of the mesoporous silica; *i.e.*, it can be suggested that half the enzyme molecules experience reduced activity because their active centres are oriented towards the deep portion of pores during enzyme immobilisation, whereas the other half can interact with reactants, such as substrate DNA and dNTP.

In addition, mesoporous silica pores can be suitable for the separation of the amplified DNA product from the DNA polymerase-mesoporous silica composites because of the electrical repulsion between the DNA and the silica surface, which are negatively charged in the PCR reaction solution. As shown in Figure S6, it was possible to confirm that scarcely any DNA molecules were adsorbed on the silica surface of unmodified, normal mesoporous silica, owing to the electric repulsion between the DNA and the silica support, *i.e.*, both DNA molecules and the silanol groups of the mesoporous silicas are negatively charged in a pH range of 4 to 8. On the other hand, DNA molecules were completely adsorbed to 3-aminopropyltriethoxysilane (APTES)-modified mesoporous silica, because the amino groups of the mesoporous silicas modified with 3-APTES have net positive charge while DNA molecules are negatively charged, resulting in electrostatic interaction between them. These results suggest that the mesoporous silica will enable the selective amplification of the target DNA by suppressing the contamination of background DNA when reusing immobilised enzyme.

In conclusion, we successfully immobilised *Taq* DNA polymerase on seven types of mesoporous silicas with different pore diameters and retained enzymatic activity sufficient for amplifying 100-bp double-stranded DNA. The mesoporous silica support enabled the simple, selective, and preferential immobilisation of *Taq* DNA polymerase and the regulation of enzymatic activity by optimising pore size. Thus, these data show the great potential of mesoporous silica as enzymatic reaction platforms not only for low-molecular-weight compounds but also for large biomacromolecules, such as DNA.

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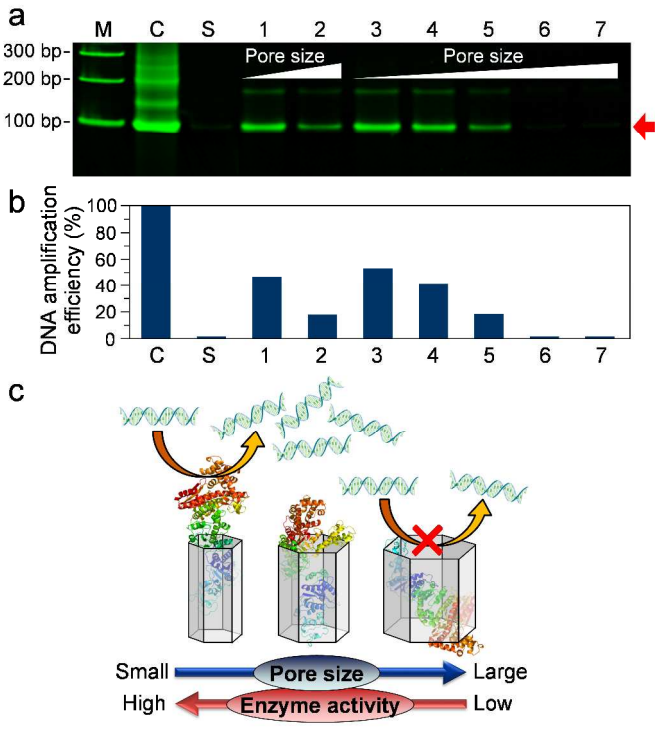

Fig. 2 Effect of mesoporous silica pore size on the DNA amplification activity of *Taq* DNA polymerase–mesoporous silica composites. (a) Analysis of PCR products using TBE-polyacrylamide gel electrophoresis and staining with SYBR Green I Nucleic Acid Gel Stain. The PCR cycling conditions are available in the ESI. Numbers above the gel correspond to different mesoporous silicas, which are numbered according to the sample numbers in Table 1. M, 100-bp DNA ladder marker; C, positive control without mesoporous silica; S, negative control without primers (*i.e.* substrate DNA). A red arrow and white adjustable triangles indicate the position of the PCR products (100 bp) and the pore sizes of the mesoporous silicas, respectively. (b) The estimated corresponding enzymatic activities based on the fluorescence intensity of the bands in (a). The band intensities were quantified using ImageJ software.[18] (c) Schematic illustration of the relationship between mesoporous silica pore size and the enzymatic activity of immobilised *Taq* DNA polymerase.

Notes

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† Electronic Supplementary Information (ESI) available: Experimental details of synthesis and characterisation of mesoporous silicas, preparation of substrate DNA, PCR amplification conditions, and SDS-

PAGE of immobilised *Taq* Pol after PCR amplification. See DOI: 10.1039/c000000x/

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