Supramolecular Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713649759

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Online publication date: 23 June 2010

To cite this Article Nozawa, K. , Shoji, A. and Sugawara, M.(2010) 'Trypsin-loaded mesoporous silica as a sensing material for amplified detection of ATP^{4-} ions', Supramolecular Chemistry, 22: 7, 389 — 395
To link to this Article: DOI: 10.1080/10610278.2010.483733
URL: http://dx.doi.org/10.1080/10610278.2010.483733

Supramolecular Chemistry
Vol. 22, Nos. 7–8, July–August 2010, 389–395

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Trypsin-loaded mesoporous silica as a sensing material for amplified detection of ATP^{4-} ions

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(*Received 14 January 2010; final version received 12 March 2010*)

In this study, we describe a new method for the detection of an analyte with amplification using enzyme-loaded, polyaminated mesoporous silica. Enzymes with different molecular sizes (trypsin, alkaline phosphatase and glucose oxidase) were tested for loading in the pores of the mesoporous materials. Among the enzymes examined, only trypsin was loaded in the pores (diameter 2.8–5 nm) of the mesoporous silica. The enzymatic activity of trypsin loaded in the pores (2.8 nm) of polyaminated MCM-41(C_{14}) was controlled by an analyte (ATP^{4-}) that binds to the polyamine sites. The formation of *p*-nitroaniline from a substrate, *N*-α-benzoyl-DL-arginine-4-nitroanilide hydrochloride, in the presence of loaded trypsin was decreased by ATP^{4-} in the concentration range from 1.0 to 50 μM in a 2-[4-(2-hydroxyethyl)-1-piperazyl] ethanesulphonic acid buffer solution (pH 7.4). The response was selective to ATP^{4-} over ADP^{3-}, GMP^{2-} and phosphate. The potential use of trypsin-loaded mesoporous silica for the design of a sensing system with amplification ability is discussed in terms of working principle, the effect of pore sizes, sensitivity, selectivity and a signal transduction factor.

Keywords: trypsin-loaded mesoporous material; adenosine 5′-triphosphate; signal amplification

Introduction

Mesoporous silica, MCM-41, is a highly ordered channel that consists of a regular array of uniform, hexagonally shaped pores, whose diameter is adjustable in the range from 2 to 10 nm. In addition to the tunable pore sizes, several interesting features such as large surface areas, large volumes, chemical stability and hydrophilic nature have made MCM-41 and its analogues as an attractive material for a wide range of applications such as drug delivery systems (*1–2*), molecular separation systems (*3*) and enzyme-loaded systems (*4–11*). In addition to these nanopore-based approaches, mesoporous silica have found application in designing new sensing systems for ions and molecules (*12–18*). Glucose sensors have been developed based on enzymes immobilised on mesoporous silica microspheres (*19*), mesoporous MnO_2 (*20*) and mesoporous SBA-15 (*21*). In addition, discrimination of neurotransmitters using poly(lactic acid)-coated mesoporous silica nanospheres that regulate the penetration of neurotransmitters to the inside of the fluorescence probe-modified pores (*22*), optical sensing of a ligand (thymidine) with fluorescent receptor-modified mesoporous silica (*23*) and anion sensing based on the release of marker ions loaded in the pores of mesoporous silica (*24*) have also been reported. We have reported a channel-based sensing system using biotinylated MCM-41 embedded in planar bilayer lipid membranes (*25*).

Considering the possibility of chemically tailoring the pore outlet of MCM-41 with stimuli-responsive moieties (*26*), a molecular gate that can regulate the diffusional transport of ions and molecules through the nanopores of the mesoporous silica will be interesting, because such molecular sensing systems have, in principle, amplification ability (*24*, *25*, *27*, *28*). In the present paper, we describe a new strategy for the detection of an analyte with amplification using the enzyme-loaded MCM-41 (Figure 1). A receptor site is tailored on the pore outlet of MCM-41, which forms a molecular gate upon recognising an analyte and hinders the diffusional penetration of a substrate. By using polyamine as a receptor site, the potential use of trypsin-loaded MCM-41 for amplified detection of ATP^{4-} is discussed in terms of concentration dependence, selectivity and amplification ability.

Experimental

Reagents

Hexadecyltrimethylammonium bromide (C_{16}TMABr), tetraethyl orthosilicate (TEOS; purity > 95%), aqueous ammonia (special grade, 25%), *N*-α-benzoyl-DL-arginine-4-nitroanilide hydrochloride (*N*-α-benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA); purity > 98%), dimethyl sulphoxide (purity > 99.5%), guanosine 5′-monophosphate (GMP) disodium salt (purity > 97.0%) and phosphoric acid (purity > 85.0%) were obtained from

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ISSN 1061-0278 print/ISSN 1029-0478 online

DOI: 10.1080/10610278.2010.483733
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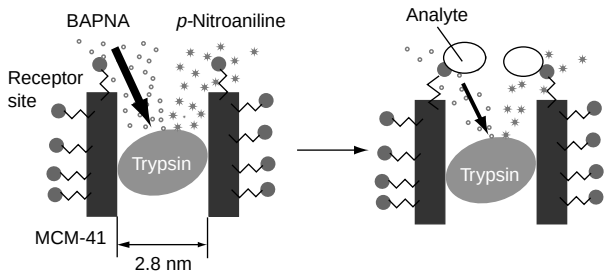

Figure 1. Schematic illustration for the principle of the present sensing system.

Wako Pure Chemicals Co. (Tokyo, Japan). A nanomesoporous material TMPS (pore size 5.4 nm) and an as-synthesised TMPS were kindly provided by Taiyo Chemicals Co. (Tokyo, Japan). Trypsin from bovine pancreas was obtained from Sigma (St Louis, MO, USA). Glucose oxidase (GOD) from *Aspergillus niger*, alkaline phosphatase (ALP) from *Escherichia coli* and 4-methylumbelliferyl phosphate (MUP) were obtained from Wako Pure Chemicals. Adenosine 5′-triphosphate (ATP) disodium salt (purity 99.3%) and adenosine 5′-diphosphate (ADP) disodium salt (purity 94.2%) were obtained from Oriental Yeast Co. (Tokyo, Japan). 3-[2-(2-Aminoethylaminoethylamino)-propyl trimethoxysilane (AEPS) was obtained from Shin-Etsu Chemical Co. (Tokyo, Japan). 2-[4-(2-Hydroxyethyl)-1-piperazyl] ethanesulphonic acid (HEPES) was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals used were of analytical reagent grade. Milli-Q water (Millipore reagent water system, Bedford, MA, USA) was used throughout the experiments.

Apparatus

Absorptiometric measurements were performed with a UV–vis recording spectrophotometer UV-2500 PC (Shimadzu Co., Kyoto, Japan). An Allegra 21R centrifuge (Beckman Coulter, CA, USA) was used for centrifugation. The X-ray diffraction (XRD) patterns for MCM-41 were recorded with an X-ray diffractometer RINT-1100S (Rigaku Co., Tokyo, Japan) using Ni-filtered Cu Kα radiation of 0.1541 nm. Diffraction data were recorded between 0.2° and 8° (2θ) at an interval of 0.01° (2θ) and a scanning speed of 1.0°/min (2θ).

Preparation of MCM-41(C_{16}) and MCM-41(C_{14})

Mesoporous silica, MCM-41(C_{16}) and MCM-41(C_{14}), were synthesised in the same manner as described in our previous paper (*25*). The MCM-41 materials were characterised with XRD. Their pore diameters were calculated to be 3.3 and 2.8 nm, respectively, using the XRD (100) interplanar spacing d_{100} values of 3.74 and 3.26 nm.

The stability of the pore structure of MCM-41(C_{16}) immersed into a 10 mM HEPES/KOH buffer (pH 7.4) solution (denoted as a HEPES solution) was tested using XRD measurements. The intensity of the peak (100) decreased to one-fifth of that of MCM-41 before immersion, probably because the buffer components penetrated into the pores. However, the d_{100} value (3.74) remained constant after immersion into the HEPES solution for 7 days, i.e. $d_{100} = 3.74$, showing that the pore structure of MCM-41 was maintained in the HEPES solution.

Preparation of polyaminated mesoporous silica

Polyamination of MCM-41(C_{16}) and MCM-41(C_{14}) was carried out in a similar manner as described by Casasús et al. (*24*) except that a lower concentration of AEPS (0.17 mmol/g MCM-41) was used, because the loading of trypsin was unsuccessful when polyamination was performed using high concentration of AEPS. Briefly, a mesoporous material (30 mg) was suspended in 1.5 ml of acetonitrile (dehydrated), to which 0.13 ml of 1 (v/v)% AEPS in acetonitrile was added. The mixture was stirred for 1 h. The precipitates obtained by centrifugation (15,000*g*, 3 min) were washed with acetonitrile (dehydrated) and dried at 70°C overnight. Henceforth, polyaminated MCM-41(C_{16}) and MCM-41(C_{14}) will be abbreviated as AEPS-MCM-41(C_{16}) and AEPS-MCM-41(C_{14}), respectively.

Loading of enzymes on mesoporous silica

The loading of GOD, ALP and trypsin on TMPS, MCM-41(C_{16}) and MCM-41(C_{14}) was performed by incubating each material (30 mg) with an enzyme in 12 ml of a buffer solution (pH 7.4) for 24 h at 4°C under stirring. The suspension was centrifuged at 3000*g* for 3 min, and the precipitate was washed five times with a buffer solution and collected each time by centrifugation. The successful loading of the enzyme was judged from the precipitate exhibiting enzymatic activity by the addition of a substrate solution. As-synthesised materials (before template removal) were treated in the same manner as mentioned earlier to know whether the enzyme was loaded in the pores or adsorbed on the outside of the material.

Immobilisation of trypsin

The loading capacity of each mesoporous material was determined from the amount of residual trypsin in the HEPES solution, which was found to be 10 μmol/g for AEPS-MCM-41(C_{14}) and 16 μmol/g for AEPS-MCM-41(C_{16}) (Figure 2). The amount of loaded trypsin on AEPS-MCM-41(C_{14}) and AEPS-MCM-41(C_{16}) from

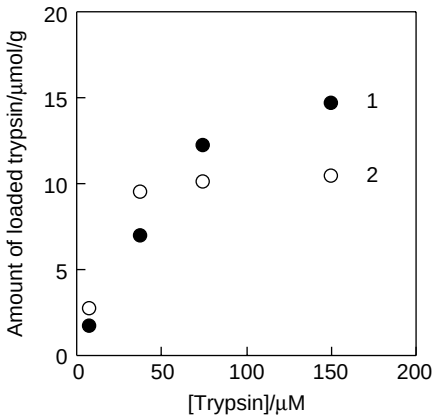

Figure 2. Trypsin loading capacity of (1) AEPS-MCM-41(C_{16}) and (2) AEPS-MCM-41(C_{14}). Trypsin was loaded from a HEPES solution and its amount was determined from absorbance measurements at 280 nm of the supernatant obtained by centrifugation.

7.5 μM trypsin solution was 2.7 ± 0.3 and 2.1 ± 0.4 μmol/g, respectively. The trypsin-loaded material was finally suspended in the HEPES solution to obtain a suspension of 10 mg/ml.

The leakage of loaded trypsin from the pores of AEPS-MCM-41(C_{16}) was tested by incubating trypsin-loaded AEPS-MCM-41(C_{16}) (7.5 mg) with 3 ml of the HEPES solution for 60 min on an ice bath. The supernatant collected from the AEPS-MCM-41(C_{16}) material exhibited no noticeable enzyme activity, i.e. it was less than 2% of that of the precipitate. Thus, the leakage of loaded trypsin was negligible.

Evaluation of enzymatic activity of loaded trypsin

The enzymatic activity of loaded trypsin was evaluated by monitoring the hydrolysis of BAPNA, which proceeds according to (*29*)

$$\text{BAPNA} \rightarrow N\text{-}\alpha\text{-benzoyl-DL-arginine} + p\text{-nitroaniline}.$$

The evaluation of trypsin activity was based on the measurements of absorbance of *p*-nitroaniline. A suspension (0.20 ml) of a trypsin-loaded mesoporous material (12 mg/ml) was mixed with 0.80 ml of the HEPES solution containing ATP in a disposal optical cell. After adding 0.20 ml of 12 mM BAPNA in 50(v/v)% DMSO, the absorption spectra were measured at a given time interval. For correcting the effect of a turbid solution, background absorbance at 420 nm was determined by extrapolating an absorption spectrum between 500 and 550 nm, as illustrated in Figure 3(a). The background absorbance was subtracted from the observed one at 420 nm. At this wavelength, *p*-nitroaniline ($\lambda_{max} = 383$ nm) exhibited strong absorption, while BAPNA ($\lambda_{max} = 315$ nm) showed

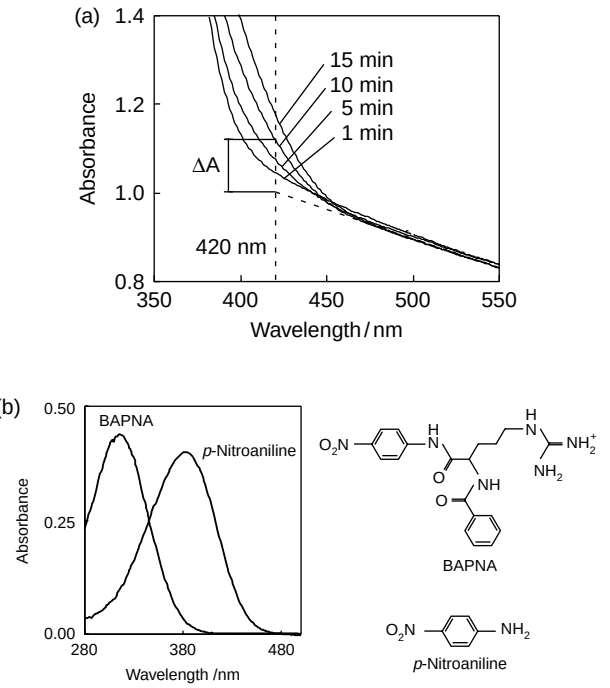

Figure 3. (a) Absorption spectra of *p*-nitroaniline formed by enzymatic activity of trypsin loaded in the pores of 2.0 mg/ml AEPS-MCM-41(C_{14}) in a HEPES solution containing 2.0 mM BAPNA. Before spectrum measurement, 0.20 ml of AEPS-MCM-41(C_{14}) was diluted to 1.2 ml with a HEPES solution to avoid the effect of a turbid solution. An extrapolation method for obtaining background absorbance at 420 nm is shown on the spectrum. (b) Absorption spectra of 33 μM BAPNA and 33 μM *p*-nitroaniline in a HEPES solution (pH 7.4) containing 8.3 (v/v)% DMSO.

negligible absorption (Figure 3(b)). As the absorbance of *p*-nitroaniline, formed 10 min after the start of the enzyme reaction, varied from one AEPS-MCM-41 sample to another, the response to ATP^{4-} was defined as

$$\text{Response} = (A_0 - A)/A_0,$$

where A_0 is the turbid-corrected absorbance in the absence of ATP^{4-} and A is the turbid-corrected absorbance in the presence of ATP^{4-} at a given concentration.

The kinetic constants for BAPNA hydrolysis by free trypsin in the HEPES solution and the apparent kinetic constants for the MCM-41(C_{16})-loaded one were evaluated at room temperature using a range of BAPNA concentrations between 0.42 and 1.7 mM. To obtain the apparent kinetic parameters (K'_m and k'_{cat}) for loaded trypsin, a Lineweaver–Burk plot was constructed. k'_{cat} was calculated by dividing the apparent maximum enzyme reaction rate V'_{max} by the loaded enzyme concentration, which was estimated from the moles of the loaded trypsin and the volume (1.2 ml) of the substrate solution used.

The enzymatic activity of loaded trypsin in the HEPES solution at 4°C dropped to 36% of that of the initial one

after a week. In the present study, AEPS-MCM-41 was used within 1 day after preparation.

Results and discussion

Loading of enzymes on the pores of mesoporous materials

For the design of the present sensing system, the loading of an enzyme in the pores of mesoporous materials is essential. The loading of trypsin (p$I = 10.5$ (*4*)), GOD (p$I = 4.6$ (*4*)) and ALP (p$I = 4.5$ (*30*)) on mesoporous materials with different pore diameters, i.e. TMPS (5.4 nm), MCM-41(C_{16}) (3.3 nm) and MCM-41(C_{14}) (2.8 nm), was investigated by measuring the enzyme activity after addition of a substrate solution to the precipitate. The results obtained are given in Table 1. The pore diameters of the mesoporous materials were slightly larger than or similar to the molecular size (ca. 3.8 nm (*31*)) of trypsin. All the trypsin-treated materials exhibited enzyme activity, which is ascribable to loaded trypsin. On the other hand, none of the as-synthesised materials exhibited enzyme activity, indicating that template-filled pores were unable to accommodate trypsin. By combining these results, it can be concluded that trypsin was loaded in the pores of the mesoporous materials. The loading of GOD on the mesoporous materials was unsuccessful, probably because the molecular size of GOD was much larger than their pore openings. On the other hand, the loading of ALP on the mesoporous materials and as-synthesised ones was observed. These results show that ALP was adsorbed on the outside of the materials. The ALP adsorption is attributable to Zn(II) and Mg(II) sites present in the ALP molecules (*32*), which may interact with negatively charged $Si-O^-$ sites on the mesoporous material.

The enzyme-loading experiments demonstrate that not only the relative size of the pore opening and the enzyme, but also the properties of the enzymes are important for the successful loading of the enzyme. Among the enzymes examined, only trypsin was loaded in the pores of the mesoporous silica. Hence, trypsin-loaded mesoporous materials are used hereafter.

Kinetic properties of loaded trypsin

We measured the kinetic properties of loaded trypsin to know whether its enzymatic activity deteriorated when it was loaded in the pores of the MCM-41 materials. The kinetic constants for BAPNA hydrolysis by trypsin in the HEPES solution and the apparent kinetic constants for the loaded one in the pores of MCM-41(C_{16}) were evaluated. The apparent K_m value (1.2 ± 0.2 mM, $n = 3$) for loaded trypsin was half of the value (2.4 ± 0.4 mM, $n = 3$) determined for trypsin in the HEPES solution. On the other hand, the apparent catalytic constant, $k_{cat} = 0.12 \pm 0.02\,s^{-1}$, was slightly lower than that ($0.72 \pm 0.08\,s^{-1}$) for a free enzyme. Although an explicit comparison of the kinetic parameters between free and loaded trypsin is not attained, the results suggest that the kinetic properties of trypsin are slightly modulated by loading into the nanopores, but the enzyme activity to produce *p*-nitroaniline is not much deteriorated.

Effect of pore sizes on ATP^{4-} recognition

As AEPS sites were modified via silanol groups, the size of the pore openings of the mesoporous materials will affect the density of the receptor sites on the pore inlet, and hence the ability of recognising ATP^{4-}. The effect of the pore diameter of polyaminated mesoporous materials on the recognition of ATP^{4-} was investigated by comparing the extent of blocking of the enzymatic activity of loaded trypsin. The responses of AEPS-MCM-41(C_{14}) and AEPS-MCM-41(C_{16}) in the presence of varying concentrations of ATP^{4-} are shown in Figure 4. Although the response,

Table 1. Loading of enzymes on mesoporous materials.

		Enzyme[a]		
Mesoporous silica	Pore size (nm)	Trypsin[b] ($3.7 \times 3.7 \times 4.2$ nm[c])	ALP[d] ($5.0 \times 5.0 \times 10$ nm[e])	GOD[f] ($7.0 \times 5.5 \times 8.0$ nm[c])
TMPS	5.4	+[g]	+	−[h]
As-synthesised	–	−	+	−
MCM-41(C_{16})	3.3	+	+	−
As-synthesised	–	−	+	−
MCM-41(C_{14})	2.8	+	+	−
As-synthesised	–	−	+	−

[a] Abbreviation for enzyme: ALP, alkaline phosphatase; GOD, glucose oxidase.
[b] Measured in a HEPES solution containing 8.3 (v/v)% DMSO and 1.7 mM BAPNA.
[c] Taken from Ref. (*4*).
[d] Measured in 0.10 M of Tris–HCl (pH 7.4) solution containing 1.0 mM $ZnCl_2$, 1.0 mM $MgCl_2$ and 0.10 mM 4-MUP.
[e] Taken from Ref. (*32*).
[f] Measured in a HEPES solution containing 60 μg/ml HRP, 0.50 mM glucose and 8.0 μM DA-64.
[g] + : enzyme activity was observed.
[h] − : no enzyme activity was observed.

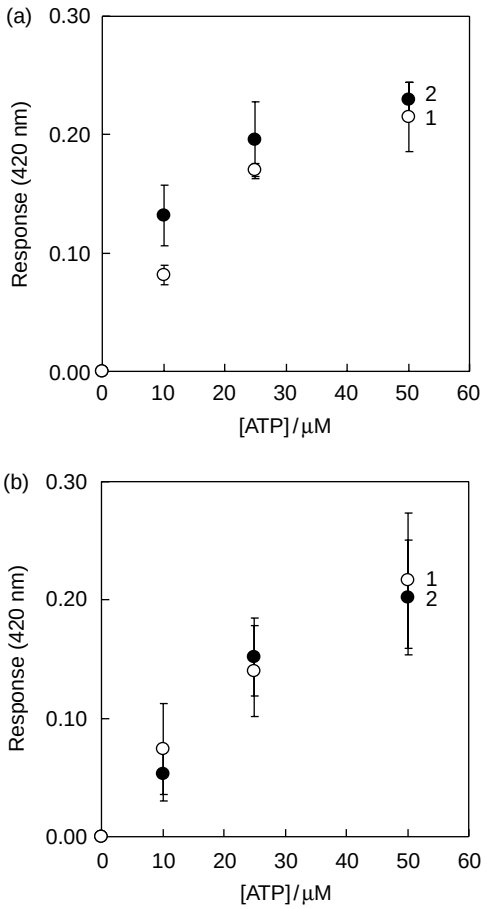

Figure 4. Effect of the pore diameter on the formation of *p*-nitroaniline mediated by loaded trypsin on the mesoporous materials. (a) Curve 1: MCM-41(C_{14}), curve 2: AEPS-MCM-41(C_{14}) and (b) curve 1: MCM-41(C_{16}), curve 2: AEPS-MCM-41(C_{16}). The average of three measurements was plotted as mean ± SD. The amount of the mesoporous materials was 0.42 mg/ml and BAPNA concentration was 1.7 mM.

i.e. the blocking of the enzyme activity by ATP^{4-}, was observed with unmodified MCM-41(C_{14}) (curve 1), an increase in the response was more significant with AEPS-MCM-41(C_{14}) (curve 2), indicating the interaction of ATP^{4-} with polyamine sites. On the other hand, no significant differences in the ATP^{4-}-induced increase in the response between MCM-41(C_{16}) and AEPS-MCM-41(C_{16}) (Figure 4(b)) were observed. This is probably because, irrespective of whether the polyamine sites are present or absent, ATP^{4-} (Stokes–Einstein radius 0.7 nm (*33*)) could have access to the loaded trypsin, owing to its large pore opening. Thus, the role of the polyamine sites in recognising ATP^{4-} becomes more significant in the case of a smaller pore opening.

The blocking of the enzyme reaction with unmodified MCM-41(C_{14}) appeared to be due to the penetration of ATP^{4-} ions into the pores. The comparison of absorbance of the BAPNA solution in the presence and absence of MCM-41(C_{14}) showed the enrichment (28 μmol/g) of BAPNA into the pores. The enrichment was not affected by the presence of ATP^{4-} ions, showing that the potential interaction of ATP^{4-} ions with positively charged BAPNA in the solution is negligible. As the analyte is strongly hydrophilic, i.e. log $D_{cal} = -12.1$ at pH 7.4 (calculated from the chemical structure by the fragment method using the software Pallas 3.0), an electrostatic interaction rather than hydrophobicity may play a role in the penetration of ATP^{4-} ions into the pores, where loaded trypsin (pI = 10.5) is positively charged.

Concentration dependence

Figure 5 shows the concentration dependence for ATP^{4-} ranging from 0.50 to 2.0 μM with trypsin-loaded AEPS-MCM-41(C_{14}), which was obtained after optimisation of quantification conditions, i.e. an increase in the amount of AEPS-MCM-41(C_{14}) and the concentration of BAPNA. The absorbance of *p*-nitroaniline was measured 10 min after the addition of BAPNA under the condition that the initial concentration of BAPNA and the amount of trypsin-loaded AEPS-MCM-41(C_{14}) are constant. The response increased with an increase in the concentration of ATP^{4-} ranging from 1.0 to 2.0 μM. Thus, the formation of *p*-nitroaniline mediated by loaded trypsin was controlled by ATP^{4-} in a concentration-dependent manner. It can be noted that there are some free pathways through which the substrate can reach the trypsin sites in the pores. This may explain the incomplete suppression of *p*-nitroaniline formation, i.e. saturation of the response, even at high concentration of the analyte (Figure 4(a)).

The advantage of the present sensing system is that the method allows us to detect an analyte rather than a

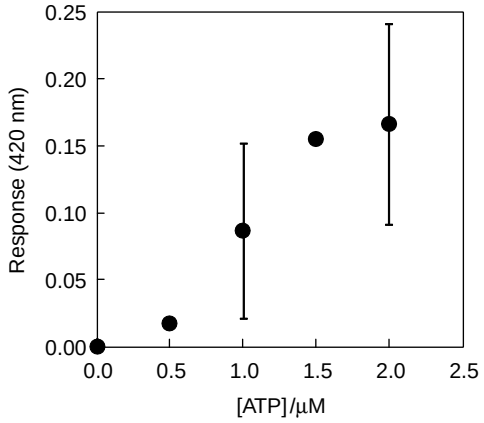

Figure 5. Concentration dependence for ATP^{4-} in a HEPES solution (pH 7.4) with trypsin-loaded AEPS-MCM-41(C_{14}). The average of two to three measurements at 420 nm was plotted. The amount of AEPS-MCM-41(C_{14}) was 2.0 mg/ml and the concentration of BAPBA was 2.0 mM.

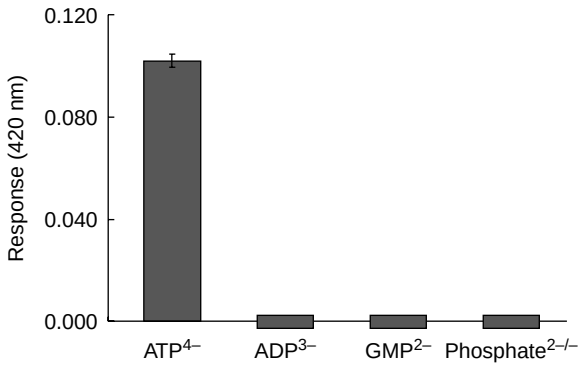

Figure 6. Selectivity among nucleotides and phosphate in a HEPES solution (pH 7.4) with trypsin-loaded AEPS-MCM-41(C_{14}). The amount of AEPS-MCM-41(C_{14}) was 2.0 mg/ml and the concentration of BAPNA was 2.0 mM. The concentration of each nucleotide and phosphate was 2.0 μM. The average of three measurements at 420 nm was plotted.

substrate with signal amplification. We defined a signal transduction factor as the amount (mole) of *p*-nitroaniline formed for a given time with respect to that of an analyte in a sample solution. The factor was calculated from the data given in Figure 5. A signal transduction factor of 3 was obtained at a reaction time of 10 min for 1.0 μM ATP^{4-} (solution volume 1.2 ml). This clearly shows that the amount of *p*-nitroaniline as an analytical signal is an amplified measure of the analyte amount in a solution. Considering that BAPNA is hydrophilic ($\log D_{cal} = -1.22$ at pH 7.4), but could penetrate into the pores, its positive charge may contribute to the amplification ability of the present system, thereby accelerating the approach of the substrate to the MCM-41(C_{14}) pores.

Selectivity

The selectivity among the nucleotides and phosphate was examined with AEPS-MCM-41(C_{14}). The results obtained are shown in Figure 6. The response of AEPS-MCM-41(C_{14}) was selective to ATP^{4-} over ADP^{3-}, GMP^{2-} and $phosphate^{2-/-}$. No discrimination between ADP^{3-} and GMP^{2-} was observed. Casasús et al. (*24*) reported that the extent of suppression of marker release from the pores (diameter 2.5 nm) of polyaminated MCM-41 increased in the order of $ATP^4 > ADP^3 \gg GMP^{2-} \sim phosphate^{2-/-}$. However, when compared with their case, the pore size of our MCM-41(C_{14}) was slightly larger. This may lead to a lower density of the receptor sites on the inlet of the pores, hence the discrimination between the smaller size of the compounds, i.e. ADP^{3-}, GMP^{2-} and $phosphate^{2-/-}$, was not achieved. In addition, the negative charge of the ATP^{4-} ions may contribute to enhancing the blocking of the enzyme reaction, though no further study was performed.

Conclusion

In the present work, we described a new strategy for amplified detection of an analyte rather than a substrate based on the control of the enzymatic activity of loaded trypsin in the pores of the receptor-modified mesoporous silica. The sensitivity and selectivity of molecular recognition can be modified by tuning the pore sizes of the mesoporous silica. The method allows us to amplify an analytical signal by measuring the enzyme reaction product, whose formation is regulated by the binding of an analyte to the receptor sites. Although only ATP^{4-} was examined in the present study, one can extend the approach to various analytes if appropriate receptor sites are introduced on the pore inlet of the enzyme-loaded mesoporous silica. In addition, the immobilisation of an MCM-41 material on solid supports will significantly improve the effect of a turbid solution, and such study is under way.

Acknowledgements

The authors thank Mr J. Sugita and Ms M. Tanaka for their help in the experiments. This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan and Nihon University Research Grant for General Individual Research.

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