Footprint Catalysis VII.[1—6] Reinvestigation of the Imprinting Procedures for Molecular Footprint Catalytic Cavities: The Effects of Imprinting Procedure Temperature on the Catalytic Characteristics

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The authors' imprinting procedures for "molecular footprint" catalytic cavities on a silica (alumina) gel surface were reinvestigated. The increases of temperature during the incorporation of aluminum ions into the silicate matrix and during the footprint-imprinting with a template remarkably affected the catalytic characteristics of the resulting cavities. A change from room temperature to 75—80 °C increased the catalytic specificities (k_{cat}/K_m) by a factor of nearly 50. The results also provided experimental proof for the speculated mechanism of footprint formation. That is, the imprinting comprises precedent Lewis acid site generation on a silicate surface and subsequent Lewis acid–template complexation. The resulting acid–template complexes give rise to the footprint cavities upon removal of the template.

The present authors have studied the catalytic properties of "molecular footprint" cavities marked on a silica gel surface by the authors' original imprinting procedures.[1—8] The most interesting feature of these catalytic cavities is their tailor-made substrate specificities based on the molecular recognition of the complementary cavities. These complementary structures originate from the template molecules used in the printing. Several kinds of cavities have been designed and prepared merely by replacing templates in the same imprinting procedures. They revealed different catalytic features.[1—8] A template that properly resembles the transition state yielded a much more effective catalytic activity. A chiral template provided chirally imprinted cavities that showed enzyme-like stereoselective catalyses.[6,7] Such tailored catalyses corresponded to those of "abzymes".[9,10] The footprint catalytic cavities can be referred to the chemical version of the abzymes. The conditions of the molecular imprinting, however, have not been defined in detail. They still follow the procedures used for the first successful imprinting.[1]

This paper deals with the reinvestigation of the imprinting procedures with *N*-benzoylbenzenesulfonamide as a template. The conditions for the incorporation of Al^{3+} ions into the silicate matrix, and those for imprinting with the template were examined in detail. The temperature during the incorporation and during the imprinting were found to have remarkable effects on the molecular recognition capabilities of the footprint cavities. These results provide experimental evidence for a speculated hypothetical imprinting mechanism.[1] Additionally, the implication of each step in the imprinting procedures and procedures to gain satisfactory imprintings are described in detail here.

Experimental

Materials. All chemicals were of guaranteed grade from Nacalai Tesque Co., Ltd., if not otherwise specified.

Template: *N*-Benzoylbenzenesufonamide (Ph–SO_2–NH–Bz). Mp 148 °C (lit, 147 °C); (Found: C, 59.51; H, 4.26; N, 5.37%).

Substrate: Benzoic anhydride was of Extra Pure Reagent grade from Nacalai Tesque Co., Ltd., and was recrystallized from benzene–petr. benzine. Mp 42 °C (lit, 43 °C).

Silica Gel: Merck Kieselgel 60, art. no. 7754, particle size 0.06—0.20 mm, mesh 70—230.

Solvent: Acetonitrile, previously dehydrated over calcium chloride, was dried over phosphorous pentoxide overnight. It was distilled using a Hempel fractionating column, Bp 81—82 °C. To the distillate was added calcium hydride, and repeated distillation gave water- and acidic impurity-free acetonitrile.

Nucleophile: Potassium 2,4-dinitrophenolate, prepared by neutralization of 2,4-dinitrophenol in methanol with conc aqueous potassium hydroxide, was recrystallized from hot water and dried at 130 °C in vacuo.

Catalyst Preparation: Imprinting Procedures. Step 1. Activation of Silica Gel Surface. This step is for releasing free silanol groups on the surface.[11,12]

Keiselgel (100 g) was refluxed with conc hydrochloric acid (500 cm^3) for 4—6 h. The activated gel sludge was thoroughly washed with deionized water until the pH of the washing became nearly 6, and then washed repeatedly with very dilute aqueous ammonia (pH 8) until pH 7—8. Many pH adjustments had to be made because the pH changed gradually with time. This acid treatment concurrently removed impurities included in Kieselgel (Fe^{3+}, Zn^{2+}, Cu^{2+}, etc.). The gel was collected by decantation to keep the surface always wet. The wet gel was immediately subjected to the subsequent procedures.

Step 2. Al^{3+} Ion Doping. This step is for Al^{3+} ion incorporation into the silicate matrix by isomorphic substitution of silicate with aluminate. The substitution generates Lewis acid sites on the silica gel surface.[13]

Portions of wet gel, equivalent to 10 g of dry weight, were added to an aqueous solution of aluminum chloride (0.2 mol dm^{-3}, 50 cm^3). The pHs of the supernatants were adjusted to the specified values (6.0, 6.5, 7.0, and 7.5. See text.) with diluted aqueous ammonia. The mixtures were then allowed to stand for 2 d at room temperature (around

20 °C). In the reinvestigation study, they were allowed to stand for specified hours (6 h, 1.5 h, 1h. See text.) at specified temperatures (50 °C, 70 °C, 80 °C. See text.), respectively. The pHs of the mixtures were occasionally checked and adjusted with aqueous ammonia if necessary. Aggregated aluminium hydroxide over the gel sludge was removed by decantation, and the gel was washed three times with dilute hydrochloric acid (pH 4.0). These "superficial" silica (alumina) gel preparations were immediately subjected to the subsequent procedures.

Step 3. Imprinting with a Template. This step is for generation of surface Lewis acid–base complexes, which are the postulated precursors of footprint cavities.

Silica (alumina) gel portions, equivalent to 10 g of dry weight, were suspended in a minimum amount of dilute hydrochloric acid (pH 4.0). To these suspensions, was quickly added the acetone solution of the template of a Lewis base (*N*-benzoylbenzenesulfonamide, 2×10^{-3} mol in a minimum amount of acetone). Vigorous shaking was required to disperse the template as homogeneously as possible onto the gel surface before the template precipitated as aggregate. The pHs of the supernatants were adjusted to 4.0 with dilute hydrochloric acid (pH 1.0).

The pH value of 4.0 originated in our preliminary studies of imprinting with a template of Methyl Red,[14] wherein maximum amounts of footprint cavities were generated at pH 4.0. This is because acidic conditions promote the polymerization of silicate, but too acidic conditions (pH 1—2) might destroy Lewis acid sites. Therefore, weakly acidic conditions around pH 4.0 might give the best results.

The mixtures were allowed to stand for a week at room temperature (around 20 °C). In the reinvestigation study, they were allowed to stand for specified hours (6 h, 1.5 h, and 1 h. See text.) at specified temperatures (50 °C, 70 °C, and 80 °C), respectively. The pHs were occasionally checked and adjusted to pH 4.0 with dilute hydrochloric acid (pH 1.0). The supernatant of the mixtures was discarded, and the gel was transferred portionwise into dilute hydrochloric acid (pH 4.0) for washing. Washing with gentle stirring and decantation were repeated three times to give "template-treated gel". The control catalyst that involved Lewis acid sites but lacked the cavity structures stemmed from the "superficial" silica (alumina) gel preparation. The preparation procedures were the same as those of "template-treated gel" except for the use of only acetone without the template in this step.

All treatments required very careful handling, because the surface structures including postulated Lewis acid-base complexes at this stage seemed extremely delicate and damageable. Therefore, all factors that affected the surface tension of the gel had to be avoided to obtain reproducible imprinting. These factors included, for instance, rapid changes in pH and temperature, contact with air to form bubbles on the surface, and mechanical shock by vigorous stirring.

Step 4. Drying. This step is not only for drying the gel, but for rearrangement of the surface structures. A continual and reversible rearrangement of the silicate matrix occurs during the drying process; depolymerization of silicate and repolymerization of silicic acid take place simultaneously around the Lewis acid–base complexes. Drying gradually increases the concentration of silicic acid, which promotes the polymerization. Such rearrangement under thermodynamic control might stabilize the Lewis acid–base complexes leading to maximum interaction between the complexes and the surrounding silicate matrix.

The "template-treated gel" was collected by careful filtration; the surface of the collected gel was necessarily kept wet. The gel was transferred into Petri dishes and air-dried at constant room temperature (23 °C, relative humidity 37%) until it reached a constant weight. Usually this took several days. Daily plotting of weight vs. time showed the decrease in weight of the "template-treated gel." Its decrease was slower than that of the corresponding control gel; the surface of the template-treated gel might still have been coated with excess template. This delayed decrease in weight could serve as an indication for a successful imprinting. No shrinkage of the gel particles was observed. Usually drying of jelly causes remarkable shrinkage[15] that markedly increases surface tension to destroy the surface structures. In the present drying process, the inner net structures of the bulk silica gel might be rigid and remain unchanged throughout the drying procedures. Only the outer layer of the gel particles might be in a jelly-like state. Drying of the outer jelly-like layer might cause no shrinkage, which enables the gel to retain the complementary surface structures. The dried gel preparation at this stage is considerably stable and suitable for storage.

Step 5. Methanol Extraction. This step is for forced removal of the template. Continual extraction with methanol at its boiling point decomposes the Lewis acid–base (surface) complexes to leave the Lewis acidic counterparts on the surface. They are the "molecular footprint cavities".

The dried gel preparation of sufficient amount for subsequent studies (usually 3 g) was subjected to methanol extraction using a Soxhlet extractor. The optical density at 220 nm was occationally monitored. The extraction was continued until the template was no longer observed, and then for an additional 30 min. The required time for the extraction was usually 5—6 h, though it partly depended on the efficiency of the Soxhlet extractor used. Completion of the extraction in a short time usually results in unsuccessful imprinting. Assumedly unstable Lewis acid–base complexes might undergo degration in a short extraction. It would leave no definite marks on the surface, but only release the template. Whereas, long methanol extraction would gradually decompose stable Lewis acid–base complexes into the template and definite complementary structures (footprints). Some templates have been found to form stable complexes resistant to methanol extraction.[16] In such cases, acid treatments in the course of the extraction are effective for substitutional removal of templates. The gel is immersed in an acid solution (10% acetic acid, fluoroacetic acid, or trifluoroacetic acid in methanol), the supernatant of which is monitored every hour. Then the gel is subjected to further methanol extraction until release of the template is no longer observed.

Step 6. Final Drying. This step is for removal of adsorbed methanol from the gel. Careful handling, i. e., gradual removal of methanol, is always required to avoid rapid evolution of methanol from pores of the gel which can destroy the surface structures of the gel.

The extracted gel was transferred into a desiccator over calcium chloride as quickly as possible to prevent it from collecting moisture. It was dried preliminarily under atmo-

spheric pressure or under very weakly reduced pressure at room temperature overnight, and successively dried under reduced pressure (3 mmHg) at room temperature for 1 d. Finally, the gel was exhaustively dried under reduced pressure (3 mmHg) at 140 °C for 1 h using an Abdelhalden-type glass oven with phosphorous pentoxide. Complete removal of methanol was indispensable, because remaining methanol often caused abnormal rate enhancement during catalytic site titration procedures. Probably it might occur due to general base catalysis with methanol and pyridine. The fully dried gel preparation was stored in a stoppered vial, and usually used within 1 week.

Kinetic Measurements. The catalytic activities of the gel were determined by 2,4-dinitrophenolysis of benzoic anhydride. The procedures were the same as those previously reported.[3] To acetonitrile solutions of benzoic anhydride (49 cm^3, 0.9—4.5×10^{-3} mol dm^{-3}) equilibrated with the gel (50 mg) at 30 °C for 30 min, was added the nucleophile solution (1 cm^3, 1.5×10^{-2} mol dm^{-3}) to initiate the reactions. The catalyzed reactions were followed by triple-wavelength spectrophotometry. The decreases in the optical density of the supernatant of the reaction mixtures at 400, 430, and 500 nm due to 2,4-dinitrophenolate were determined at proper intervals. In inhibition studies, the catalysts were preequilibrated with an acetonitrile solution of an inhibitor for 1 h. Then they were equilibrated with the substrate solutions for an additional 10 min. Subsequent procedures were the same as before. Pseudo-first-order rate constants, k_{obsd}, were calculated from the linear part of semi-log plots of optical density vs. time that usually lasted over at least 1 half life. The k_{obsd}s thus obtained obeyed Michaelis–Menten kinetics with respect to the substrate concentration. Kinetic parameters, K_m, and V_{max} ($k_{obsd\,max}$) were determined from the usual double reciprocal plots (Lineweaver-Burk plots), and k_{cat}s were calculated from V_{max} divided by catalytic sites molarity per gram.

Catalytic Sites Titrations. Catalytic sites molarities were determined by the kinetic titration method previously reported.[3] To acetonitrile solutions of the substrate (benzoic anhydride, 10 cm^3, 1.5×10^{-2} mol dm^{-3}) and the gel catalyst (50 mg), was added specified amounts of pyridine in acetonitrile (5—15 cm^3, 5.0×10^{-5} mol dm^{-3}). The volumes were made up to 49 cm^3 with acetonitrile. The mixtures were incubated at 30 °C for 1 h with gentle stirring to complete irreversible poisoning of the catalytic sites with pyridine. To the mixtures was then added the nucleophile solution (1 cm^3, 1.5×10^{-2} mol dm^{-3}) to initiate the catalyzed reaction by surviving catalytic sites. Subsequent procedures were same as those described above. The k_{obsd}s obtained were plotted against pyridine molarity. Extrapolation of a linear plot gave an intercept on the abscissa, which directly showed the molarity of the catalytic sites per 1 gram of catalyst.

Results and Discussion

Effects of pH on Al^{3+} Ion Incorporation. The optimum pH for Al^{3+} ions incorporation into the silica matrix was examined during the control catalyst preparation. The "activated" gel (Step 1) was subjected to Al^{3+} ion doping (Step 2) at pH 6.0, 6.5, 7.0, and 7.5 at 20 °C for 2 d. The procedures were the same as those described for the usual control catalyst. Apparent catalytic activities (k_{obsd}s) and amounts of the incorporated Al^{3+} ions were determined (Table 1). All the incorporated Al^{3+} ions were assumed to generate the native Lewis acid catalytic sites. They were obtainable by the kinetic titration method with pyridine poisoning.[3] Table 1 shows that doping at pH 6.5 generated the maximum quantities of catalytic sites and catalytic activities. Their k_{obsd}s per catalytic site (efficiency of a catalytic site) were almost identical within experimental error. The efficiencies were independent of the pHs, which suggests that identical catalytic sites were generated. Thus, doping at pH 6.5 is optimum, and all preparations in the present study followed these doping conditions.

Effects of Temperature on Al^{3+} Ion Incorporation. To improve the time-consuming Al^{3+} ion incorporation, the temperature during the doping steps were raised to 50, 70, and 80 °C, and correspondingly doping times were shortened to 6, 1.5, and 1.0 h, respectively. The subsequent imprinting with a templeate was carried out, in the same ways as usual, at room temperature (around 20 °C) for 1 week. The other conditions and procedures were the same as usual. Results are shown in Figs. 1, 2, and 3 and Table 2. Figure 1 shows the Al^{3+} ion incorporation (amounts of acid sites generated) into the "control" and "imprinted" catalysts. The former are always larger than the latter. Such differences in acid site amounts between the "control" and "imprinted" catalysts were usually observed. The imprinting procedures, probably methanol extraction, might destroy a portion of the acid sites. The incorporation into both "control" and "imprinted" catalysts similarly decreased gradually with the rise of temperature. The former, however, decreased more than the latter. Also the catalytic activities (k_{obsd}) displayed distinct differences between them, as Fig. 2 shows. With the rise of doping temperature, the catalytic activities of the "control" catalysts moderately decreased; those of the "imprinted" catalysts remarkably increased. Figure 3 shows that the efficiency of a catalytic site (k_{obsd}/site) on the "con-

Table 1. Effects of pH on Al^{3+} Ions Incorporation into Silicate Matrix

Catalysts	pHs[a]	k_{obsd}[b]	Amounts of acid sites[c]	k_{obsd}/ Acid site[d]
		10^4 M[e] s^{-1}	10^{-2} mol kg^{-1}	10 s^{-1}
Control	6.0	3.54	3.10	1.14
Control	6.5	3.93	3.52	1.12
Control	7.0	2.99	2.65	1.12
Control	7.5	2.82	2.63	1.07

a) pH at Al^{3+} ions doping at 20 °C. b) k_{obsd} at substrate concentration of 3.0×10^{-3} mol dm^{-3}. c) Molarity per gram of the catalyst. d) Efficiency of a catalytic site. e) M=mol dm^{-3}.

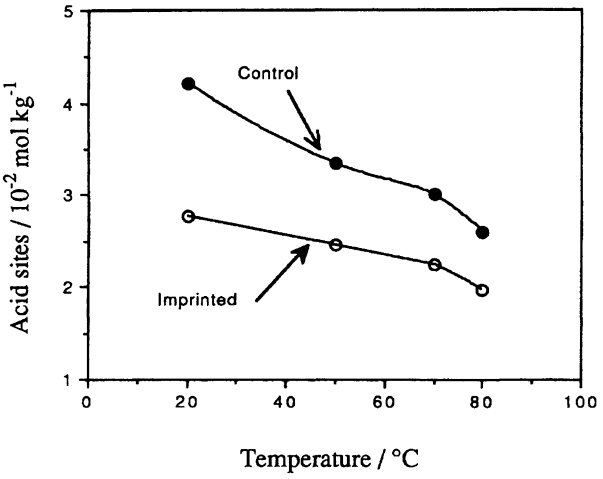

Fig. 1. Temperature effects of Al^{3+} ion doping on catalytic site generation.

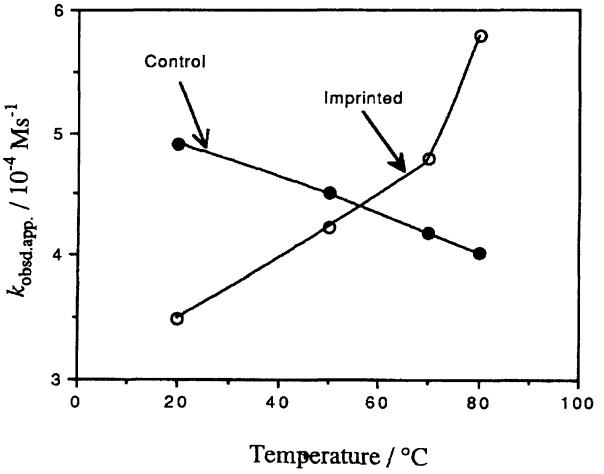

Fig. 2. Effects of temperature during Al^{3+} ion doping on catalytic activity. M=mol dm^{-3}. Apparent k_{obsd}s are the rate constants observed at a substrate concentration of 3.0×10^{-3} mol dm^{-3}.

trol" catalysts slightly increased with temperature increase, but the efficiency of the "imprinted" catalysts distinctly increased. These distinguishable catalytic features suggest that such temperature changes generated different kinds of catalytic sites. The kinetic parameters of the imprinted catalysts, shown in Table 2, supported this assumption. Their K_m and K_i values evidently decreased with an increase of the doping temperatures. These decreases mean increases in the affinities of the catalytic cavities toward the substrate molecules ($1/K_m \approx 1/K_s$) and toward the template molecules ($1/K_i$), respectively. The temperature change from 20 °C to 80 °C, for instance, caused a 2.5-fold increase in $1/K_m$, and a 6.95-fold increase in $1/K_i$. The k_{cat} and k_{cat}/K_m values also increased by factors of 2.5 and 4.0, respectively. The control catalyst doped at 80 °C showed K_m, k_{cat}, and k_{cat}/K_m values that seem less susceptible to temperature effects. The temperature at subsequent imprintings was 20 °C, and other conditions for the "control" and the "imprinted" catalysts were the same. This alteration in quality of catalytic cavities was independent of subsequent imprinting procedures. The effect of increasing temperature during Al^{3+} ion doping remained distinctly until the cavity formation after the imprinting procedures. These findings imply that this molecular footprint imprinting involves two distinct processes. They are the preceding Lewis acid generation and subsequent Lewis acid–base complexation. If footprint imprinting involved only one process, the observed temperature effects (at 20—80 °C) should not remain. In other words, if Lewis acid generation and Lewis acid–base complexation occurred simultaneously, a similar temperature effect at 20 °C during imprinting would be revealed equally in all the preparations. Two possible reasons why the temperature effects remain are as follows. First, labile Lewis acid sites that are susceptible to interaction with template molecules are generated. This is not likely because such Lewis acid sites are hard to prove. Second, the silicate matrix around a Lewis acid site has "activated" and labile structures that can easily depolymerize and repolymerize. So, the Lewis acid sites surrounded with such structures are more susceptible to interaction with template molecules. Continual depolymerization and repolymerization might rearrange the silicate matrix around the Lewis acid sites under thermodynamic control. Such rearrangement might occur spontaneously to stabilize Lewis acid–base surface complexes much more. Then, the complexes might leave finely imprinted footprint cavities on the surface by forced removal of the template molecules. This speculation is more likely.

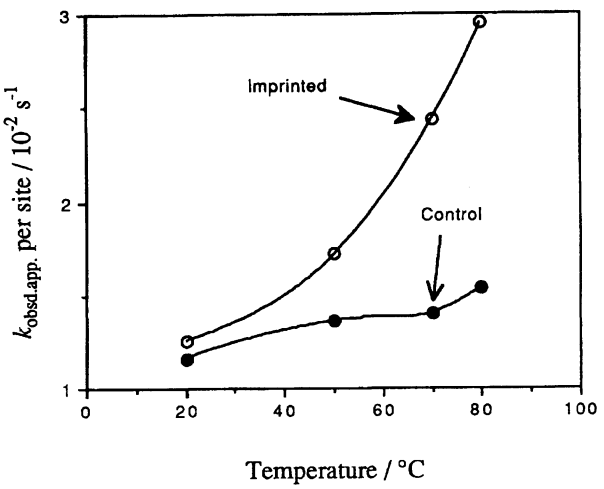

Fig. 3. Effects of temperature during Al^{3+} ion doping on catalytic efficiency. Apparent k_{obsd} divided by amounts of catalytic sites gives catalytic efficiency.

Effects of Temperature on Imprinting with a Template. Such rearrangement of the silicate matrix ought to continue throughout the imprinting process. Therefore, effects similar to increasing temperature

Table 2. Effects of Temperatures at Al^{3+} Ion Doping on Catalytic Properties of Imprinted Catalysts

Temperatures of doping	Time[a] h	K_m 10^{-4} M[c]	V_{max} 10^{-4} M s^{-1}	Acid sites 10^{-2} mol kg^{-1}	K_i[b] 10^{-6} M	k_{cat} s^{-1}	k_{cat}/K_m 10^{-5} M^{-1} s^{-1}	K_m/K_i
Room temp[d]	48	10.80	4.79	2.78	1.32	344	3.19	820
50 °C	6	6.95	5.35	2.46	0.40	434	4.97	1740
70 °C	1.5	6.52	5.38	2.24	0.30	480	6.44	2170
80 °C	1.0	4.33	5.54	1.96	0.19	565	13.05	2280
(Control)[e]								
80 °C	3.0	4.40	3.91	3.15	—[f]	248	5.64	—

a) Time for Al^{3+} ion doping (not for imprinting). b) Competitive inhibition constants for the template molecules. c) mol dm^{-3}. d) Around 20 °C. e) Imprinting was ommitted. f) No competitive inhibition with the template molecules.

Table 3. Effects of Temperature at Imprinting on Catalytic Properties

Temperature of Imprinting[b]	K_m 10^{-4} M	V_{max} 10^{-4} M s^{-1}	Acid sites 10^{-2} mol kg^{-1}	K_i[a] 10^{-6} M	k_{cat} s^{-1}	k_{cat}/K_m 10^{-5} M^{-1} s^{-1}	K_m/K_i
Room temp[c]	8.28	5.45	2.01	0.38	542	6.55	2180
40 °C	3.59	5.81	2.09	0.13	556	15.50	2760
75 °C	0.79	5.90	1.96	0.05	604	76.41	1550
(Control)[d]							
80 °C	4.43	5.52	2.25	—[e]	492	11.00	—
80 °C	4.97	5.50	2.25	—[e]	490	9.86	—

a) Competitive inhibition constants for the template molecules. b) Stand for 1 week. c) Around 15 °C. d) Stand for 1 week. e) No competitive inhibition.

during imprinting should also be revealed during catalysis. Another silica (alumina) gel preparation, obtained by Al^{3+} ion doping at 80 °C for 1 h, was divided into three portions. Each of these was subjected, in parallel, to imprinting with the template at room temperature (around 15 °C), 40 °C, and 75 °C for 1 week. The results were in accord with expectations; evident temperature effects were observed. The kinetic parameters, shown in Table 3, proved that a temperature change from 20 to 75 °C caused a 10.5-fold increase in $1/K_m$ and a 7.5-fold increase in $1/K_i$, and that the k_{cat} and k_{cat}/K_m values also increased by factors of 1.11 and 11.7, respectively. The control catalyst doped and kept at 80 °C for 1 week showed K_m, k_{cat} and k_{cat}/K_m values comparable to those of cavities imprinted 20—40 °C. The native catalytic sites of the control catalyst also seem less susceptible to temperature effects. These temperature effects during the imprinting seem more remarkable but similar in tendency to those during Al^{3+} ion doping. These findings imply that more precisely imprinted catalytic cavities are generated. They support the hypothetical rearrangement mechanism.

Concluding Remarks. The present studies clarified that molecular footprint imprinting comprises two distinct processes, i. e., preceding Lewis acid site generation, and subsequent cavity formation through rearrangement of the surface silicate matrix. Both processes proceed favorably with an increase of temperature. Temperature increase in both processes from 20 °C to 75—80 °C provided finely imprinted catalytic cavities with about 50-fold (4.0×11.7) specificity (k_{cat}/K_m). This finding is practically very significant. If available template molecules are stable enough to withstand such conditions, i. e., contact with a Lewis acid at higher temperatures, a mere temperature increase can effectively improve the molecular recognition capabilities of footprint cavities.

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