Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytica Chimica Acta 504 (2004) 37–41
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Rate-enhancement of hydrolysis of long-chain amino acid ester by cross-linked polymers imprinted with a transition-state analogue: evaluation of imprinting effect in kinetic analysis

Takashi Sagawa [a,*], Keita Togo [b], Chie Miyahara [b], Hirotaka Ihara [b], Katsutoshi Ohkubo [a]

[a] *Institute of Advanced Energy, Kyoto University, Gokasho, Uji 611-0011, Japan*
[b] *Department of Applied Chemistry and Biochemistry, Faculty of Engineering, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan*

Received 6 January 2003; received in revised form 14 July 2003; accepted 14 July 2003

Abstract

Acceleration property of a cross-linked polymer catalyst for the hydrolysis of *N*-dodecanoyl leucine-*p*-nitrophenyl ester is attributed to the molecularly imprinting of phenyl-1-undecylcarbonylamino-3-methylbutyl phosphonate as the template of transition-state analogue (TSA) and 4-[(3′-methacryloylamino)ethyl]imidazole as the binding site of the polymer catalyst. Equimolar complex of the template of the TSA and the binding site of imidazole-containing monomer through the electrostatic interaction and hydrophobic effect was confirmed by ^{1}H NMR measurements and the complex was copolymerized with hydrophobic styrene monomer and 10% divinylbenzene cross-linker. A control polymer without the template, but with the cross-linker, was also prepared. After removal of the template, imidazole-containing imprinted polymer gave a ca. two-fold rate-enhancement in comparison to the non-imprinted polymer.

Keywords: Molecularly imprinted polymer; Transition-state analogue; Esterase; Histidine; Amino acid esters

1. Introduction

Molecular imprinting has been applied for creating transition state imprinted enzymes as artificial "catalytic antibodies" [1–17]. The resulting molecularly imprinted polymer catalysts can selectively recognize the template molecule of the transition-state analogue (TSA) used in the imprinting process, so that the catalytic polymers are applicable to stable synthetic media for highly selective reactors, sensors, drug assay tools, and functionally separating chromatographic stationary phases. In order to evaluate the imprinting effect of the polymer catalysts, kinetic analysis is often adopted [3] instead of measuring the equilibrations of the polymer and the template analyzed by LC, GC, microplate reading, QCM, and so on [18–20]. We previously reported polymer catalysts, which were imprinted with a TSA of phenyl-1-benzyloxycarbonylamino-3-methylbutyl phosphonate for the hydrolysis of amino acid *p*-nitrophenyl esters such as *N*-(benzyloxycarbonyl) leucine-*p*-nitrophenyl ester [21–26]. In our previous work, using a control polymer without the template had not performed, although acceleration factors were evaluated by the ratios of the pseudo-first-order reaction rate constants obtained with and without the catalyst (k_{cat} and k_{uncat}, respectively). Therefore, further investigations of the control experiments with the control polymer are necessary to evaluate a real imprinting effect. We describe here our improvement on the acceleration properties for the hydrolysis of *N*-dodecanoyl leucine-*p*-nitrophenyl ester by using an imprinted polymer that was prepared from phenyl-1-undecylcarbonylamino-3-methylbutyl phosphonate as the template of TSA for the hydrolysis of long-chain ester–substrate and 4-[(3′-methacryloylamino)ethyl]imidazole as the nucleophilic binding site.

In the membrane–molecular aggregate system containing an imidazole unit as esterolytic nucleophile, the long-chain catalyst and/or the long-chain ester–substrate increased hydrophobicity and resulted to facilitate the catalyst–substrate complex formation in the ester hydrolysis [27]. This specific intermolecular force between the catalyst and the substrate in the membrane play an essential role in the enhancement

* Corresponding author. Tel.: +81-774-38-3511; fax: +81-774-38-3516.
E-mail address: t-sagawa@iae.kyoto-u.ac.jp (T. Sagawa).

0003-2670/$ – see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/S0003-2670(03)00945-0

of the hydrolysis. In order to introduce the similar additional cooperative effect in the imprinted polymer catalyst system, we investigated the long-chain substrate (or TSA).

2. Experimental

2.1. Materials

Phenyl 1-aminobutylphosphonate was prepared by the reductive elimination of benzyloxycarbonyl group from 8.5 g (22.5 mmol) of phenyl 1-(benzyloxycarbonylamino)-isobutylphosphonate [28] by using Pd/C in methanol (120 ml) under H_2 at 25 °C for 5 h. TSA was synthesized by the amide linkage reaction of phenyl 1-aminoisobutylphosphonate (1.0 g, 4.1 mmol) with dodecanoylchloride (1.8 g, 8.2 mmol) and triethylamine (1.1 ml, 7.9 mmol) with stirring in chloroform (20 ml) at 0 °C for 3 h. Purification of the crude product by chromatography on silica-gel (Wako-gel C-200) column eluting with 100:1 chloroform/methanol afforded phenyl-1-undecylcarbonylamino-3-methylbutyl phosphonate (21% (0.44 g) yield based on the initial amount of phenyl 1-(benzyloxycarbonylamino)-isobutylphosphonate). ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 3H, CH_3), 0.95 (d, 6H, $2CH_3$), 1.27 (m, 16H, $(CH_2)_8$), 1.55 (m, 1H, CH), 1.65 (m, 4H, $(CH_2)_2$), 2.09 (m, 2H, CH_2), 4.75 (m, 1H, CH), 5.71 (br, 1H, NH), 7.18 (q, 3H, *m*- and *p*-ph), 7.30 (q, 2H, *o*-ph).

Imidazole containing monomer of 4-[(3′-methacryloylamino)ethyl]imidazole was synthesized by the amide linkage reaction of histamine dihydrochloride (1.0 g, 5.4 mmol dissolved in 20 cm^3 of water in the presence of $KHCO_3$ (1.7 g, 16.7 mmol)) and methacryloyl chloride (0.61 ml, 6.2 mmol dissolved in 10 cm^3 of ether) with stirring the mixture at 0 °C for 30 min and at 25 °C for 3 h. After the acidification of the mixture to pH 2 with HCl, methacrylic acid was extracted to remove with ether and the aqueous layer was alkalized to pH 8–9 with NaOH. Hydroquinone as the inhibitor of polymerization was added to the alkaline solution and 10 ml of methanol was poured into ca. 5 ml of condensed mixture. The resulting white precipitation was removed by filtration and the filtrate was evaporated. The residue was washed with ether–hexane (2:1, 20 ml) resulted crude powder. After re-dissolution of the crude powder in chloroform, insoluble histamine dihydrochloride was removed by filtration. The filtrate was evaporated again and the residue was washed with ether–hexane (2:1) produced white powder (19% (0.18 g) yield). Melting point 102–108 °C. ^{1}H NMR ($CDCl_3$) δ: 2.00 (s, 3H, CH_3), 2.84 (d, 2H, CH_2), 3.61 (d, 2H, –C(=O)–N–CH_2), 4.85 (br, 1H, NH), 5.36 (s, 1H, =CH), 5.75 (s, 1H, =CH), 6.82 (s, 1H, imidazole-5-CH), 7.60 (s. 1H, imidazole-2-CH). Anal. Calcd. for $C_9H_{13}ON_3$: C, 60.32; H, 7.31; N, 23.45. Found: C, 59.15; H, 7.02; N, 22.82.

Long-chain substrate of 4-nitrophenyl-*N*-dodecanoylleucinate was obtained by the similar way as described previously [3] with 4-nitrophenyl-L-leucinate (1.3 mmol) and dodecanoylchloride (1.9 mmol). Yield 44% (0.25 g), mp 53–54 °C. ^{1}H NMR (DMSO-d_6) δ: 0.86 (t, 3H, CH_3), 1.00 (d, 6H, $2CH_3$), 1.23 (br, 16H, $(CH_2)_8$), 1.60 (br, 3H, CH_2 and CH), 1.81 (d, 2H, CH_2), 2.25 (t, 2H, CH_2-C=O), 4.62 (br, 1H, CH), 7.39 (d, 2H, *o*-ph), 8.30 (d, 2H, *m*-ph). Anal. Calcd. for $C_{24}H_{38}O_5N_2$: C, 66.33; H, 8.81; N, 6.45. Found: C, 66.13; H, 8.78; N, 6.55.

Cross-linked polymers were fabricated by the radical polymerization. Equivalent amounts (29 μmol) of imidazole containing monomer (5.2 mg) and TSA (12 mg) were mixed in styrene (2.0 ml, 17 mmol) followed by the addition of divinylbenzene (0.23 ml, 1.7 mmol) and AIBN (36 mg, 0.22 mmol). After thorough degassing of the reaction mixture under vacuum by successive freeze–pump–thaw cycles, the reactants were stirred at 60 °C for 20 h. Upon cooling to −196 °C, the product crystallized out. This crude polymer was ground to powder in a mortar with a pestle at −196 °C. Stirring the suspension in methanol (and in ethanol) at 25 °C for 30 min for five times (respectively) performed to remove the template of TSA from the polymer. Recover of the template from the cross-linked polymer was 22.2 mol% estimated by HPLC analysis. We utilized JASCO Finepack SIL C_{18} at UV 260 nm eluted with 90 vol.% MeOH–H_2O (retention time of the TSA was 5.5 min) and measured the amounts of the eluted TSA in the washings. Another imprinted polymer without cross-linker was prepared by the similar way. In this case, the removal of TSA from the polymer was 20.2 mol%. A control polymer without template, but with cross-linker, was also prepared.

2.2. Measurements

Hydrolysis of 4-nitrophenyl-*N*-dodecanoylleucinate (30 μM) by the polymer catalyst (imidazole unit concentration = 10–60 μM) was carried out in 30 vol.% ethanol–HEPES buffer (pH 7.45) at 25 °C. The pseudo-first-order reaction rate constants obtained with and without the catalyst (k_{cat} and k_{uncat}, respectively) were determined by monitoring the produced amount of 4-nitrophenolate anion spectrophotometrically at 400 nm.

3. Results and discussion

3.1. Molecular interaction of TSA and binding site

We examined the analogy between the transition-state (TS) and TSA for ester hydrolysis in their electronic structures calculated by PM3-ESP of MOPAC version 6.0 as shown in Fig. 1.

The tetrahedral configurations of TS and TSA were approximately the same, though the net charges were apparently different, and the ester C–O bond (1.48 Å) in TS was appreciably lengthened as compared with that (1.38 Å) in the ester–substrate ground state [28]. Thus, polymer catalyst imprinted with a phosphonic ester as TSA for alkaline

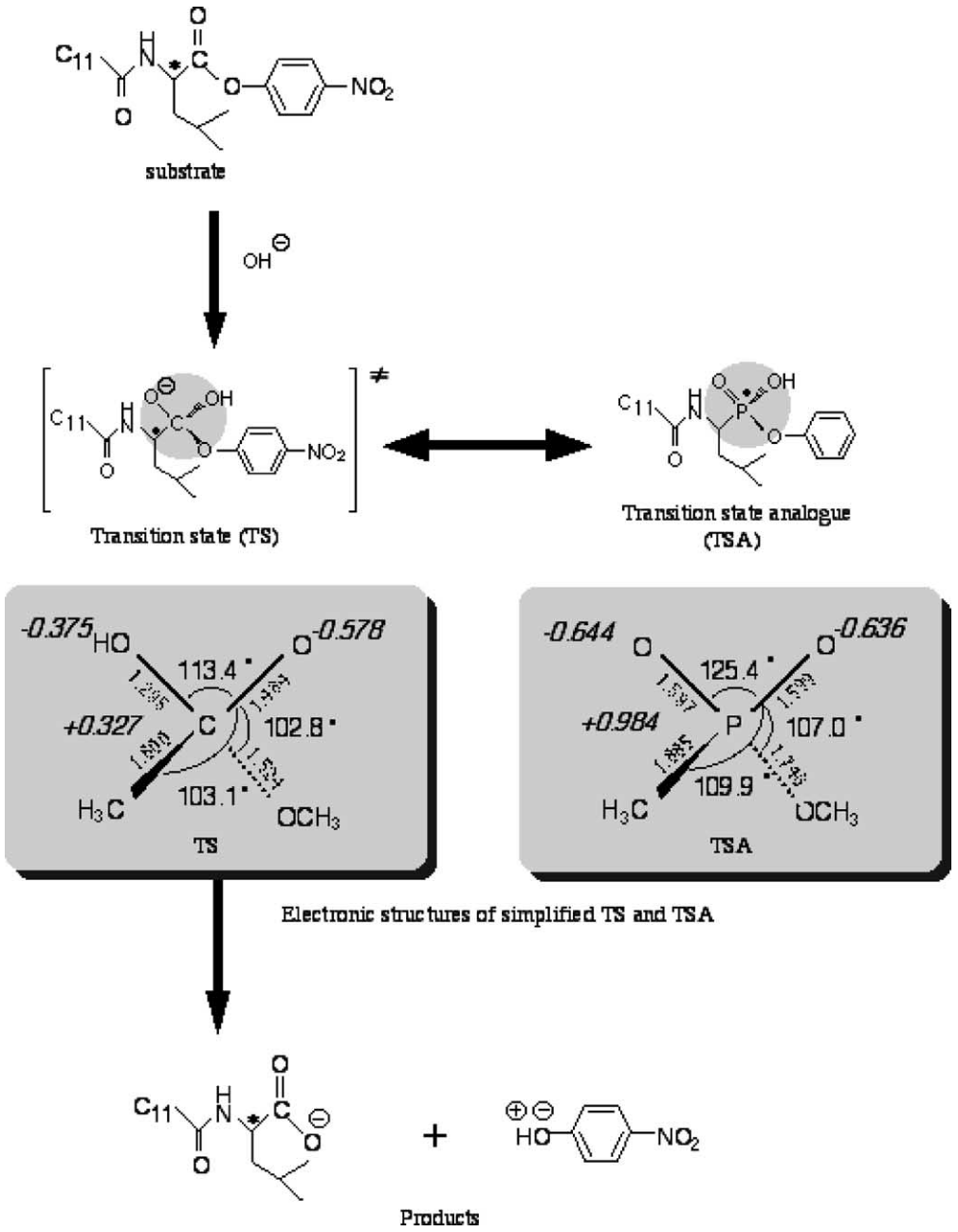

Fig. 1. Transition-state (TS) and TSA of ester hydrolysis.

ester hydrolysis has a possibility to enhance the rate of ester hydrolysis.

In order to unveil the modes of multi-point interactions between the TSA and the imidazole-containing monomer before the polymerization, the 400 MHz ^{1}H NMR nuclear overhauser effect spectroscopy (NOESY) measurement was performed as shown in Fig. 2.

As Fig. 2 shows, the NOESY cross-peaks of the interaction between the phenyl ring of TSA and the imidazole-5-CH proton or –C(=O)–N–CH_2 proton of the imidazole-containing monomer were observed. Another cross-peak of the interaction between the isovaler methyl protons of TSA and the imidazole-5-CH proton of the imidazole-containing monomer was also seen. While, the cross-peak of the interaction between the alkyl protons ($(CH_2)_8$) of TSA and the terminal methyl proton of the imidazole-containing monomer was observed. Such a mode of multi-point interaction makes the distance between the reaction positions of the nucleophile and P=O site of TSA closer, so as to bring about the rate-enhancement of ester hydrolysis through making a suitable ester binding sites. The interamide hydrogen bond depicted in Fig. 2 might be present in the hydrophobic region of the polystyrene, but it was not clearly detected.

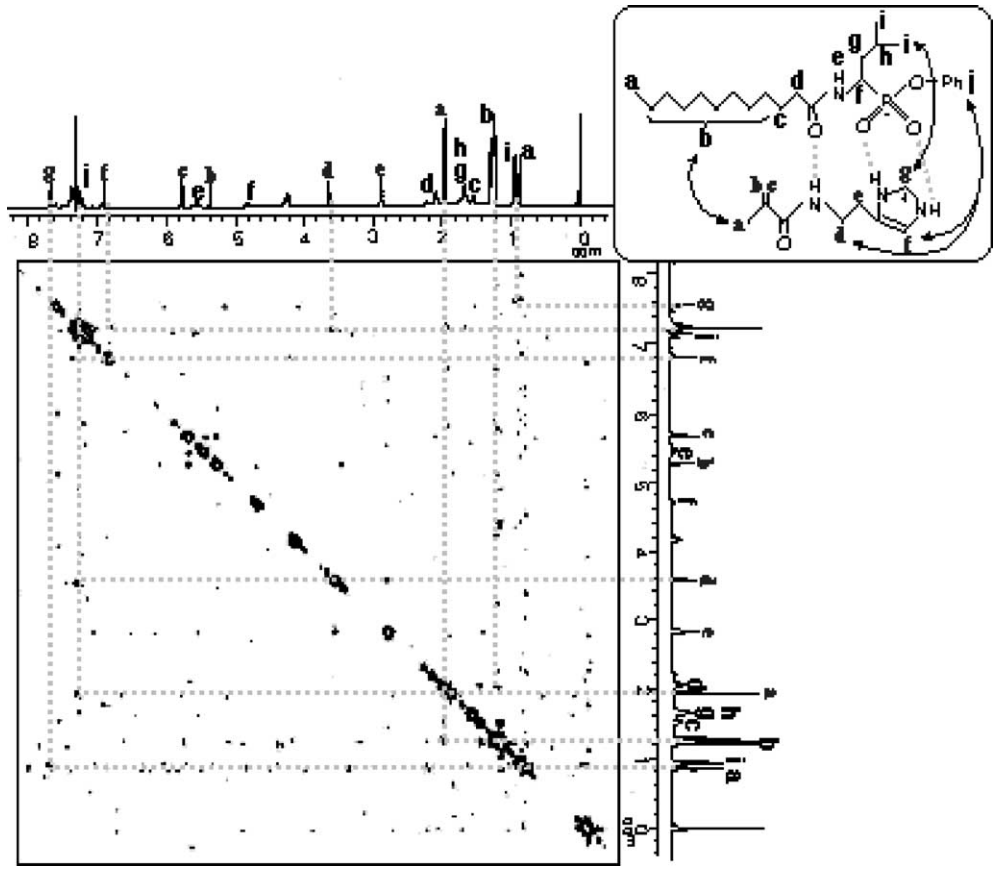

Fig. 2. 400 MHz ^{1}H NMR NOESY spectrum of the imidazole containing monomer and TSA in $CDCl_3$.

3.2. Kinetic analysis of imprinting effect

Kinetic parameters (k_{cat} and $k_{cat}k_{uncat}^{-1}$) of polymer catalyst for hydrolysis of 4-nitrophenyl-*N*-dodecanoylleucinate were summarized in Table 1.

As increasing the concentration of polymer catalyst, the pseudo-first-order reaction rate constants (k_{cat}) and the ratios of rate-enhancement ($k_{cat}k_{uncat}^{-1}$) increased remarkably. In particular, TSA-imprinted polymer showed a ca. two-fold enhancement (from 1.31 to 2.56) in the rate compared to the control polymer when [imidazole] was 60 μM. This reflected the TSA-imprinting effect. On the other hand, addition of divinylbenzene slightly enhanced the hydrolysis from 1.05 ([imidazole] = 60 μM) to 1.18 ([imidazole] = 15 μM), and 1.21 ([imidazole] = 10 μM) when the cross-linker contents were 10 mol%.

Table 1
Kinetic parameters (k_{cat} and $k_{cat}k_{uncat}^{-1}$) of polymer catalyst (10–60 μM) for hydrolysis of 4-nitrophenyl-*N*-dodecanoylleucinate (30 μM) in 30 vol.% EtOH–HEPES buffer (pH 7.45) at 25 °C

Polymer catalyst	$10^5 k_{cat}$ (s^{-1}) ($k_{cat}k_{uncat}^{-1}$)		
	10[a]	15[a]	60[a]
TSA-imprinted polymer			
Cross-linked polymer	4.37 (1.52)	4.87 (1.70)	7.35 (2.56)
Without cross-linker	3.62 (1.26)	4.13 (1.44)	7.03 (2.45)
Without TSA			
Cross-linked polymer	3.01 (1.05)	3.68 (1.28)	3.75 (1.31)

[a] Concentration of the containing imidazole unit (in μM) in the polymer catalyst.

3.3. Esterolysis activities of TSA-imprinted polymer catalysts during the processes of substrate-binding and polymer catalyst–substrate complex reaction

There are two possible acceleration processes in the present reaction; the substrate binding process (viz. the catalyst–substrate complex formation pathway) and the reaction step of the catalyst–substrate complex to form the product (Fig. 3).

The substrate-dissociation constant (K_m) and the rate constant (k_2) for the esterolysis were obtained from the linear relation of $(k_{cat}-k_{uncat})^{-1} = K_m(k_2-k_{uncat})^{-1}[\text{imidazole}]^{-1} + (k_2 - k_{uncat})^{-1}$. Comparison of the K_m and k_2 values

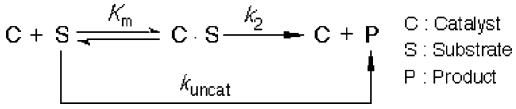

Fig. 3. Simplified reaction process of esterolysis with TSA-imprinted polymer catalyst.

Table 2
Kinetic parameters (K_m, k_2, and $k_2K_m^{-1}$) of TSA-imprinted polymer catalyst for hydrolysis of 4-nitrophenyl-*N*-dodecanoylleucinate[a]

TSA-imprinted polymer catalyst	10^3K_m (M)	10^4k_2 (s^{-1})	$k_2K_m^{-1}$ ($s^{-1}\,M^{-1}$)
Cross-linked polymer	0.0392	1.02	2.60
Without cross-linker	1.61	13.1	0.814

[a] [Substrate] = 30 μM in 30 vol.% EtOH–HEPES buffer (pH 7.45) at 25 °C. The containing imidazole groups in the polymer catalyst were 10, 15, or 60 μM in the reaction.

of the cross-linked polymer with another polymer without cross-linker was shown in Table 2.

It is worth emphasizing that the cross-linked polymer catalyst actually incorporates the ester–substrate to form the catalyst–substrate complex with 41-fold enhancement of the K_m^{-1} value of non-cross-linked polymer. In regard to the reaction step of the catalyst–substrate complex to form the product, kinetic hindrance caused by the addition of divinylbenzene resulted only 13^{-1} from 1.31×10^{-3} to $1.02 \times 10^{-4}\,s^{-1}$ of k_2 value. This result probably came from the addition of the cross-linker into the TSA imprinted polymer which caused the depression of the diffusion of *p*-nitrophenolate anion (product) from the inner reaction cavity to the outside of the polymer catalyst. The $k_2K_m^{-1}$ values of TSA imprinted polymers were reflected affinity of the catalyst for its substrate in the similar manner to the K_m^{-1} value. Although systematic investigation of the contents effect of cross-linker has not been performed, these kinetic results for evaluation of imprinting effect suggested the presence of three-dimensional networks with a "memorized cavity" of the shape and functional group positions of the template molecule.

4. Conclusions

Consequently, the origin of the enhancement of the hydrolytic activity of the present polymer catalyst is the molecular binding process owing to the hydrophobic effect and electrostatic forces of the memorized cavity of the shape and functional group positions of the template molecule of TSA.

References

[1] G. Wulff, T. Gross, R. Schönfeld, Angew. Chem. Int. Ed. Engl. 36 (1997) 1961.
[2] A.G. Strikovsky, D. Kasper, M. Grün, B.S. Green, J. Hradil, G. Wulff, J. Am. Chem. Soc. 122 (2000) 6295.
[3] G. Wulff, Chem. Rev. 102 (2002) 1.
[4] D.K. Robinson, K. Mosbach, Chem. Commun. (1989) 969.
[5] R. Müller, L.I. Andersson, K. Mosbach, Macromol. Rapid Commun. 14 (1993) 637.
[6] B. Sellergren, K.J. Shea, Tetrahedron: Asymmetry 5 (1994) 1403.
[7] J.V. Beach, K.J. Shea, J. Am. Chem. Soc. 116 (1994) 379.
[8] B. Sellergren, R.N. Karmalkar, K.J. Shea, J. Org. Chem. 65 (2000) 4009.
[9] R.N. Karmalkar, M.G. Kulkarni, R.A. Mashelkar, Macromolecules 29 (1996) 1366.
[10] B.S. Lele, M.G. Kulkarni, R.A. Mashelkar, React. Funct. Polym. 39 (1999) 37.
[11] Y. Kawanami, T. Yunoki, A. Nakamura, K. Fujii, K. Umano, H. Yamauchi, K. Masuda, J. Mol. Catal. A 145 (1999) 107.
[12] K. Polborn, K. Severin, Chem. Commun. (1999) 2481.
[13] K. Polborn, K. Severin, Chem. Eur. J. 6 (2000) 4604.
[14] K. Severin, Curr. Opin. Chem. Biol. 4 (2000) 710.
[15] T. Shimada, K. Nakanishi, K. Morihara, Bull. Chem. Soc. Jpn. 65 (1992) 954.
[16] J. Heilmann, W.F. Maier, Angew. Chem. Int. Ed. Engl. 33 (1994) 471.
[17] Y. Ohya, J. Miyaoka, T. Ouchi, Macromol. Rapid Commun. 17 (1996) 871.
[18] K. Haupt, K. Mosbach, Chem. Rev. 100 (2000) 2495.
[19] K. Haupt, Chem. Commun. (2003) 171.
[20] A. Patel, S. Fouace, J.H.G. Steinke, Chem. Commun. (2003) 88.
[21] K. Ohkubo, Y. Funakoshi, Y. Urata, S. Hirota, S. Usui, T. Sagawa, Chem. Commun. (1995) 2143.
[22] K. Ohkubo, Y. Urata, S. Hirota, Y. Funakoshi, T. Sagawa, S. Usui, K. Yoshinaga, J. Mol. Catal. A 101 (1995) L111.
[23] K. Ohkubo, Y. Funakoshi, T. Sagawa, Polymer 37 (1996) 3993.
[24] K. Ohkubo, K. Sawakuma, T. Sagawa, Polymer 42 (2001) 2263.
[25] K. Ohkubo, K. Sawakuma, T. Sagawa, J. Mol. Catal. A 165 (2001) 1.
[26] T. Sagawa, H. Ihara, K. Ohkubo, Rec. Res. Dev. Pure Appl. Chem. 3 (1999) 57.
[27] T. Sagawa, K. Urabe, H. Ihara, K. Ohkubo, Rec. Res. Dev. Pure Appl. Chem. 4 (2000) 1.
[28] K. Ohkubo, Y. Urata, S. Hirota, Y. Honda, Y. Fujishita, T. Sagawa, J. Mol. Catal. 93 (1994) 189.