Anal Bioanal Chem (2007) 389:399–404
DOI 10.1007/s00216-007-1327-7

REVIEW

Molecular imprinting of peptides and proteins in aqueous media

Daniel S. Janiak · Peter Kofinas

Received: 27 December 2006 / Revised: 15 March 2007 / Accepted: 24 April 2007 / Published online: 16 May 2007

Abstract Molecular imprinting has received significant attention in recent years, as it provides a viable method for creating synthetic receptors capable of selectively recognizing specific target molecules. Despite significant growth within the field, the majority of template molecules studied thus far have been characterized by their low molecular weight and insolubility in aqueous systems. In biological systems, molecular recognition events occur in aqueous media. Therefore, in order to create molecularly imprinted polymers capable of mimicking biological processes, it is necessary to synthesize artificial receptors which can selectively recognize their respective target biological macromolecules such as peptides and proteins in aqueous media. In this review, we discuss the challenges associated with the imprinting of peptides and proteins in aqueous media. In addition, we discuss the significant progress which has been made within the field.

Keywords Molecular imprinting · Protein · Peptide · Aqueous · Synthetic receptor

D. S. Janiak
Department of Materials Science and Engineering,
University of Maryland,
College Park, MD 20742, USA

P. Kofinas (✉)
Fischell Department of Bioengineering, University of Maryland,
1120 Jeong H. Kim Engineering Building,
College Park, MD 20742, USA
e-mail: kofinas@umd.edu

Introduction

Molecularly imprinted polymers (MIPs) [1–5] are synthetic materials produced by the cross-linking of functional monomers or polymers in the presence of a template molecule. The template is subsequently removed, leaving cavities possessing size, shape, and functional group orientation which are complementary to the target molecule. The size and shape of the cavity allow the target molecule or similar molecules to occupy the cavity space, while the functional group orientation within the cavity will preferentially bind in specific locations to only the target molecule and not to similar molecules. While MIPs have been prepared for a large number of target molecules and applications [6–9] over the years, the majority of the template molecules studied have been characterized by their low molecular weight and insolubility in aqueous systems.

Much of the pioneering work in the molecular imprinting of peptides and proteins was conducted by Mosbach [10–14] using amino acid derivatives as template molecules to create stationary phases for chromatographic applications. The MIPs synthesized in these early studies were tested for their ability to selectively recognize their respective target molecules in organic solvents. While these experiments laid the groundwork for the synthesis of artificial receptors capable of recognizing peptides and proteins, most molecules of biological importance are water soluble, and many natural recognition events such as antigen-antibody binding occur in aqueous media. Therefore, there exists a strong need to create artificial receptors which are capable of recognizing peptides and proteins in aqueous media in order to create materials and devices capable of mimicking natural processes. For instance, MIPs

which can selectively recognize given proteins or peptide sequences in aqueous media have the potential to be used as substrates in medical diagnostic applications and clinical analysis. In addition, aqueous MIPs could be used as the solid phase for chromatographic analysis and purification of peptides and proteins. Finally, the synthesis of MIPs which exhibit high affinity, selectivity, binding capacity, and low non-specific binding may provide researchers with a low-

Table 1 Summary of MIPs synthesized for recognition of peptides and proteins in aqueous media

Template	Functional monomers	Cross-linker	Ref.
BSA, CEL	DMAEPMA MAA	BAAm	[43]
BHb	Am	BAAm	[39]
BHb	MA	Piperazine diacrylamide	[18]
BSA	Maleic acid NTBA Am	BAAm	[35]
Cyt C hTf Hb	Am	BAAm	[32]
LSZ	Am AA DMAPA	BAAm DHEBAAm	[37]
BSA F IgG LSZ RNAse SAv	Hexafluoropropylene Disaccharide	N/A	[28]
pCyP18	AA	DVB	[16]
T	Am	BAAm	[38]
Bhb	Chitosan Am	BAAm	[44]
GOD	Am MAA	DHEBAAm BAAm	[31]
HRP MP LP Hb	APBA	N/A	[22]
BOC-Gly-OH BOC-Gly-Phe-OH H-Gly-OEt H-Phe-Gly-OH H-Phe-OMe FMOC-Phe-Gly-OH FMOC-Phe-OH	MAA	EGDMA	[30]
Lys-Trp-Asp	2VPy	DVB	[45]
D-Phe	β-Cyclodextrin AMPSA	BAAm	[21]
OT YPLG GLY	MAA	EGDMA	[24, 25]
N-Ac-L-Phe-L-Trp-OMe	MAA	EGDMA	[23]
N-Terminal histidine sequence of dipeptides	Ni-NTA	BAAm	[20]
AYLKKATNE GRYVVDTSK VVSTQTALA	Am PEG-200-diacrylate	EBAAm	[26]
VP OT	AA An N-Benzylacrylamide	N-Acr-L-Cys-NHBn	[15]

BSA bovine serum albumin, *CEL* chicken egg lysozyme, *BHb* bovine hemoglobin, *Cyt C* cytochrome C, *hTf* human transferrin, *Hb* human hemoglobin, *LSZ* lysozyme, *IgG* immunoglobulin G, *F* fibrinogen, *RNAse* ribonuclease, *SAv* streptavadin, *pCyP18* cloned pig cylcophinlin 18, *T* trypsin, *GOD* glucose oxidase, *HRP* horseradish peroxidase, *MP* microperoxidase, *LP* lactoperoxidase, *BOC t*-butyloxycarbonyl, *Phe* phenyl, *OEt* ethyl ester, *FMOC* 9-fluorenyl methoxycarbonyl, *OT* oxytocin, *OMe* methyl ester, *VP* vasopressin, *DMAEPMA N*-[3-(dimethylamino)propyl]methacrylamide, *MAA* methacrylic acid, *AM* acrylamide, *MA* methacrylamide, *NTBA N-tert*-butylacrylamide, *DMAPA N*,*N*-dimethylaminopropylacrylamide, *APBA* 3-aminophenylboronic acid, *2VPy* 2-vinylpyridine, *AMPSA* 2-acryloyl-2-methylpropane sulfonic acid, *Ni-NTA* Ni(II)–nitriloacetic acid complex, *PEG* poly(ethylene glycol), *BAAm N*,*N*′-methylene biscarylamide, *DVB* divinyl benzene, *EGDMA* ethylene glycol dimethacrylate, *DHEBAAm N*,*N*-(1,2-dihydroxyethylene)-bisacrylamide, *EBAAm N*,*N*-ethylene bisacrylamide

cost, easily obtainable method for studying the fundamental interactions which occur during biological recognition processes. In this review, we will discuss some of the advances that have been made in the imprinting of biological macromolecules in aqueous solutions.

A number of studies in which MIPs are used for selective peptide or protein recognition applications in aqueous systems are outlined in Table 1. Some notable recent accomplishments are the detection of peptides using a molecularly imprinted piezoelectric sensor [15], the synthesis of MIPs in the presence of a cloned bacterial protein which are capable of selectively recognizing the authentic protein when incubated in cell extract [16], the development of MIPs with enzyme-like properties [17], and the synthesis of MIPs in the presence of proteins within chromatography columns providing a monolithic bed for the selective recognition of the imprinted protein [18].

There are a number of different strategies for creating polymeric receptors targeting peptides and proteins. Functional groups forming strong template interactions with the target molecules are commonly used. Examples of this include electrostatic and metal-chelating groups [19, 20]. In addition, shape interactions between the template molecule and the synthetic receptor can be exploited. Weak interactions such as hydrophobic and hydrogen bonding have also been employed in numerous cases [21–23].

The epitope approach

Molecular imprinting of macromolecules such as peptides and proteins is complicated by a number of factors. Well-defined recognition sites are more easily formed by the imprinting of small molecules with rigid structures and therefore imprinting small sequences of amino acids (dipeptides, tripeptides, etc.) should be fairly straightforward. Proteins, however, are inherently capable of assuming a large number of conformations depending on a number of factors, including but not limited to, solution temperature, pH, and ionic strength. Steric factors also make molecular recognition of proteins difficult, as it is extremely difficult for their large structures to move freely through highly cross-linked polymer networks. One proposed method of reducing the complications associated with the imprinting of proteins and peptides has been termed the "epitope approach" [24–26] to molecular imprinting (Fig. 1). Traditionally, an epitope refers to the small active site located within the larger protein structure on an antigen, which combines with the antigen-binding site on an antibody or lymphocyte receptor [27]. In this technique, a small sequence of amino acids from the larger protein target molecule is used to create the imprint. When a protein containing this specific amino acid sequence is allowed to incubate in the presence of the MIP, the entire protein can thus be recognized and bound. The epitope approach has been successfully used to bind oxytocin by imprinting the Tyr-Pro-Leu-Gly amino acid sequence [24]. In this study, synthesis of the MIP was performed in an organic environment, but subsequent rebinding experiments were performed using chromatographic methods in both aqueous-rich and aqueous-poor mobile phases. In the aqueous-poor mobile phase, hydrogen bonds and ionic interactions are the dominating factor in creating selective recognition sites. In the aqueous-rich phase, ionic and hydrophobic interactions provide the dominant binding interaction.

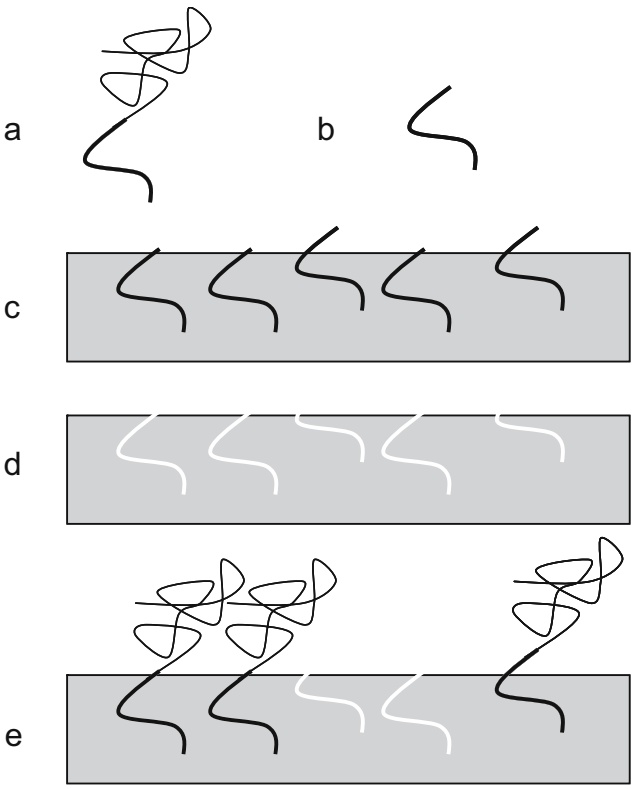

Fig. 1 Schematic of the "epitope approach" utilized for molecular imprinting of proteins and peptides. **a** Target molecule, **b** epitope (imprinted) portion of target molecule, **c** epitope-imprinted polymer before template removal, **d** imprinted polymer after template removal, with cavities that display shape which is complementary to the imprinted portion (epitope) of the target molecule, **e** MIP subsequent to template re-binding. The MIP selectively recognizes the imprinted (epitope) portion of the target molecule, thus, the entire target molecule is successfully bound

Surface imprinting

Confining the recognition sites of MIPs to surfaces is another technique which has been developed to circumvent the steric problems associated with the imprinting of biological macromolecules in aqueous systems. In a classic example of surface imprinting [28], protein-imprinted surfaces were created by first adsorbing a layer of protein onto a mica substrate, followed by the deposition of disaccharide molecules onto the protein layer. Upon

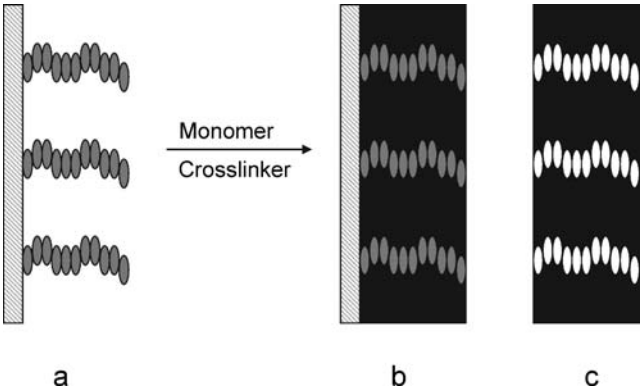

Fig. 2 Schematic of the hierarchical approach to molecular imprinting: **a** target peptide or protein is bound to a sacrificial substrate, **b** cross-linking and functional monomers are polymerized around the target peptide or protein, **c** MIP subsequent to removal of the sacrificial substrate and removal of the template molecules

dehydration, hydrogen bonds were formed between hydroxyl groups on the sugars and functional groups on the protein molecules. Radio-frequency (RF) glow discharge plasma, a commonly used plasma deposition technique for the creation of controlled thin films of fluoropolymers, was then used to deposit hexafluoropropylene onto the protein–disaccharide complexes. Upon removal of the mica substrate and target protein, the hydroxyl groups of the disaccharide molecules, which were spatially complementary to the templated proteins, were available as binding sites. In another example of MIPs in which the recognition sites have been confined to the surface, Sellergren used hierarchical imprinting [29] in an attempt to create well-structured MIPs with homogeneous morphology (Fig. 2). The template molecule is attached to the surface of a porous solid. Polymerization occurs within the pores of a silica bead and subsequent to polymerization, the silica is etched away leaving a porous structure with sites complementary to the structure of the template molecule. The mobile phase in these experiments consisted of a combination of water, acetonitrile, and acetic acid. Solid-phase synthesis products were also used directly as a template [30]. In one case, the surface of microtiter plates was coated with 3-aminophenylboronic acid and polymerized in the presence of various protein templates. All plates showed selectivity for their respective templates [22]. Molecularly imprinted polymer layers were formed around silica beads in the presence of glucose oxidase under conditions which support electrostatic interaction between monomer precursors and the template molecules [31].

Recognition mechanisms in aqueous media

In addition to overcoming the steric and conformational problems associated with molecular imprinting in aqueous media, the exact mechanism by which recognition occurs has also proved difficult to determine. The addition of functional monomers capable of participating in electrostatic interactions with the templated protein was shown, in early studies, to weaken the adsorption characteristics of the MIP, suggesting that electrostatic interactions did not play a large role in the recognition of peptides and proteins within this particular system [32]. On the other hand, molecularly imprinted polymers synthesized for the selective recognition of N-terminal histidine peptides in aqueous solutions most certainly exploit template–receptor complexation through electrostatic interaction [20]. There is strong evidence to suggest that the cooperative interaction between hydrogen bond formation and hydrophobic interaction is the most dominant form of template–receptor complexation in aqueous MIPs. Hydrogen bonds are highly directional, and many artificial receptors rely on the high directionality of hydrogen bonds and shape specificity of target molecules to create receptors capable of strong interaction and molecular recognition [28, 33]. The role of hydrophobic and hydrogen-bonding effects in the recognition of peptides has been studied by chromatographic analysis of MIPs imprinted with various peptide sequences in aqueous/organic mixtures [23]. It was observed that the separation factors of these polymers were significantly lower in acetonitrile/water mixtures than in pure acetonitrile. In addition, it was shown that water significantly lowers the effectiveness of hydrogen bonds between the template and the receptor, as the increased water content weakens the hydrogen-bonding interaction between the template and the receptor [24, 25, 34]. Hydrogen bonds are a major factor in the formation of strong template–receptor interactions in non-polar media [23]. Other studies on MIP hydrogels suggest that a combination of hydrogen bonding and electrostatic effects allow for the selective recognition of target molecules in aqueous media [35]. It has been proposed that binding in aqueous media can be enhanced by exploiting cooperative interaction effects and selecting proper hydrophobic microenvirmonents to create better receptors [33]. In one of the most striking examples of molecular recognition by the cooperative action of hydrophobic and electrostatic interactions, β-cyclodrextin, a cyclic oligosaccharide, was acrylated and co-polymerized with an electrostatic functional polymer, 2-acryoylamido-2,2′-dimethylpropane sulfonic acid, in the presence of D-phenylalanine as the template molecule. β-Cyclodrextin has a hydrophobic core which can bind aromatics groups on the side chains of peptides and protein molecules. While β-cyclodrextin has an inherent selectivity for L-phenylalanine, this natural selectivity is reversed upon imprinting with D-phenylalanine. This MIP was tested in mixtures of acetonitrile and water at various ratios. As is expected, the hydrophobic effect is the dominant factor in imprinting in aqueous-rich mixtures, whereas electrostatic effects dominate template–receptor complexation in aqueous-poor media [21, 36].

MIP microenvironment

In addition to the number of interactions which are possible between the template and the MIP receptor, the microenviroment surrounding the binding site can have a large role in determining how effective the MIP will be in recognizing its target molecule. For this reason, it is extremely difficult to make a quantitative comparison between MIPs that have been synthesized and tested under a number of different conditions. Ionic strength effects were studied in a system in which ionic interactions were the dominant force associated with template–receptor recognition. A number of buffer systems were used to observe the effect of ionic strength and buffer composition on the binding capacity of the MIPs. All buffers examined resulted in a decrease in binding capacity of the MIP hydrogels. Interestingly, the absence of a buffer, with a 60 mM KCl solution showed the largest inhibition of MIP binding capacity. It was therefore concluded that the ionic strength of the binding solution, not the buffer composition itself, plays a large role in determining the effectiveness of the MIP [20]. The inhibition of MIP binding capacity in these experiments is most likely the result of a change in polymer conformation and/or variations in the microenviroment surrounding the template binding sites caused by changes in salt concentration. For example charge-screening effects may effectively block the ionic interaction between the template at high salt concentration, as the salt dissociates into highly mobile anions and cations which can shield sites on both the target and the template. The effect of buffer composition and ionic strength has not been studied in other systems; however, it is highly possible that these two variables will have a large effect on MIP systems, even those which do not use ionic interactions as their primary recognition mechanism. This is because proteins can adopt a wide number of conformations depending on their environment.

MIP composition

The structure and composition of MIPs used for aqueous imprinting can also have a large effect on its ability to recognize its target molecule. Re-binding experiments conducted with MIP hydrogels imprinted in the presence of bovine serum albumin (BSA) were shown to have a strong dependence on the initial BSA concentration used during synthesis. This is most likely related to the fact that high template concentration during synthesis will result in a larger number of available binding sites upon subsequent imprinting experiments. Studies of lysozyme-imprinted silica beads revealed that the amount of lysozyme adsorbed onto the beads depended on the composition of functional monomers used during preparation of the particles. This dependence suggests that there exists some optimum distance between charged groups on the synthetic receptor which will correspond to a maximum in the complexation between receptor and template. The zeta-potential of the lysozyme molecules and the imprinted silica beads was also studied, and results revealed a coincidence between the zeta-potential of the template and that of the imprinted polymer beads at the value where the maximum amount of specifically adsorbed lysozyme was observed. This yields the possibility that zeta-potential matching may play an important role in the design of synthetic receptors [37]. Cross-linking concentration is also a critical factor in creating synthetic receptors with high affinity for their target molecules [20, 38].

Although commonly overlooked, the removal of target molecules from MIPs subsequent to re-binding experiments is a critical factor in the imprinting capability of the MIP in both aqueous and organic systems. Template removal of proteins subsequent to imprinting is complicated by the fact that their high molecular weight makes it difficult for them to navigate the highly cross-linked matrix of the MIP. While very little work has focused solely on the study of template removal strategies, the methods which have been studied have revealed a number of important details. Template removal from polyacrylamide hydrogels synthesized in the presence of bovine hemoglobin was achieved with an aqueous solution consisting of varying ratios of sodium dodecyl sulfate (SDS) and acetic acid (HOAc) [39]. A qualitative model for the interaction between the SDS/HOAc solution and target protein within the cavity was presented and an optimum SDS/HOAc ratio of 10 % (weight/volume) SDS and 10% (volume/volume) HOAc was discovered. Interestingly, while increasing the amount of SDS and HOAC in the wash solution improved template removal, the re-binding effect is clearly compromised. In another MIP system, a solution consisting of acetic acid and Tween 20 was used prior to conducting template re-binding experiments [22]. It is quite possible that the combination of an acidic solution with a detergent is the most effective method of template removal for MIPs imprinted with peptide or proteins. Another obvious choice for template removal in these systems would be the use of proteolytic enzymes (proteases) which are capable of cleaving peptide bonds and subsequently destroying the structure of the imprinted protein or peptide [40]. The peptide fragments could then be removed from the cavity by simply rinsing with buffer solution. The protease trypsin, for example, was investigated as a template removal agent. However, after washing of an MIP with a trypsin solution, the re-binding efficiency suffered as cleaved protein fragments blocked binding sites within the imprint cavity [39].

Future outlook

Despite the recent advancements made in the synthesis and characterization of MIPs which can selectively recognize

proteins and peptides in aqueous media, there is still an enormous amount of work that needs to be conducted in the field. Hydrogen bonds and hydrophobic and electrostatic interactions are the dominant mechanisms through which template–receptor complexation occurs in aqueous systems. However, the exact recognition mechanism, and the way in which the aforementioned forces cooperate to recognize target molecules in many MIP systems is largely unknown. In addition, many fundamental properties of MIPs prepared in aqueous and organic systems, such as the dependence of MIP affinity on cross-linker concentration, functional monomer concentration, solvent, buffer composition, ionic strength, pH, and temperature, have yet to be systematically studied. Finally, the recognition binding of larger macromolecular complexes, such as viruses [41, 42] and cells, has gone largely unstudied. These factors combined with the increasing interest in creating materials which are capable of mimicking biological processes, ensure that the study of MIPs capable of the selective recognition of peptides, proteins, and other biological macromolecules in aqueous media will receive considerable attention in years to come.

Acknowledgements This material is based upon work supported by the US Department of Agriculture National Research Initiative Competitive Grants Program (USDA-NRICGP) Grant # 2005-35603-16278.

References

1. Takeuchi T, Haginaka J (1999) J Chromatogr B 728:1–20
2. Steinke J, Sherrington DC, Dunkin IR (1995) Adv Polym Sci 123:81–125
3. Wulff G (1995) Angew Chem Int Ed Engl 34:1812–1832
4. Shea KJ (1994) Trends Polym Sci 2:166
5. Mosbach K, Ramström O (1996) Biotechnology 14:163–170
6. Haupt K, Mosbach K (2000) Chem Rev 100:2495–2504
7. Sellergren B (2000) Angew Chem Int Ed Engl 39:1031–1037
8. Byrne ME, Park K, Peppas NA (2002) Adv Drug Deliv Rev 54:149–161
9. Sellergren B (1997) Trends Anal Chem 16:310–320
10. Andersson LI, Mosbach K (1990) J Chromatogr 516:313–322
11. Kempe M, Mosbach K (1995) J Chromatogr A 691:317–323
12. Sellergren B, Lepisto M, Mosbach K (1988) J Am Chem Soc 110:5853–5860
13. Andersson LI, O'Shannessy DJ, Mosbach K (1990) J Chromatogr 513:167–179
14. Andersson LI, Miyabayashi A, O'Shannessy DJ, Mosbach K (1990) J Chromatogr 516:323–331
15. Lin C-Y, Tai D-F, Wu T-Z (2003) Chem Eur J 9:5107-5110
16. Zhao Z, Wang C, Guo M, Shi L, Fan Y, Long Y, Mi H (2006) FEBS Lett 580:2750–2754
17. Cheng Z, Zhang L, Li Y (2004) Chem Eur J 10:3555–3561
18. Rezeli M, Kilár F, Hjertén S (2006) J Chromatogr A 1109:100–102
19. Matsui J, Nicholls IA, Takeuchi T, Mosbach K, Karube I (1996) Anal Chim Acta 335:71–77
20. Hart BR, Shea KJ (2002) Macromolecules 35:6192–6201
21. Piletsky SA, Andersson HS, Nicholls IA (1999) Macromolecules 32:633–636
22. Bossi A, Piletsky SA, Piletska EV, Righetti PG, Turner APF (2001) Anal Chem 73:5281–5286
23. Nicholls IA, Ramström O, Mosbach K (1995) J Chromatogr A 691:349–353
24. Rachkov A, Minoura N (2000) J Chromatogr A 889:111–118
25. Rachkov A, Minoura N (2001) Biochim Biophys Acta 1544: 255–266
26. Nishino H, Huang C-S, Shea KJ (2006) Angew Chem Int Ed Engl 45:2392–2396
27. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994) Molecular biology of the cell. Garland, New York
28. Shi H, Tsai W-B, Garrison MD, Ferrari S, Ratner BD (1999) Nature 398:593–597
29. Yilmaz E, Haupt K, Mosbach K (2000) Angew Chem Int Ed Engl 39:2115–2118
30. Titirici MM, Sellergren B (2004) Anal Bioanal Chem 378: 1913–1923
31. Minoura N, Burow M (1996) Biochem Biophys Res Commun 227:419–422
32. Liao JL, Wang Y, Hjertén S (1996) Chromatographia 42:259–262
33. Ariga K, Kunitake T (1998) Acc Chem Res 31:371–378
34. Klein JU, Whitcombe MJ, Mullholland F, Vulfson EN (1999) Angew Chem Int Ed Engl 38:2057–2060
35. Demirel G, Ozcetin G, Turan E, Caykara T (2005) Macromol Biosci 5:1032–1037
36. Piletsky SA, Andersson HS, Nicholls IA (1998) J Mol Recognit 11:94–97
37. Hirayama K, Sakai Y, Kameoka K (2001) J Appl Polym Sci 81:3378–3387
38. Vaidya AA, Lele BS, Kulkarni MG, Mashelkar RA (2001) J Appl Polym Sci 81:1075–1083
39. Hawkins DM, Stevenson D, Reddy SM (2005) Anal Chim Acta 542:61–65
40. Mathews CK, Holde KEV (1995) Biochemistry. Benjamin/ Cummings, Menlo Park
41. Bolisay LD, Culver JN, Kofinas P (2006) Biomaterials 27: 4165–4168
42. Dickert FL, Hayden O, Bindeus R, Mann K-J, Blaas D, Waigmann E (2004) Anal Bioanal Chem 378:1929–1934
43. Huang J-T, Zhang J, Zhang J-Q, Zheng S-H (2005) J Appl Polym Sci 95:358–361
44. Guo TY, Xia YQ, Hao GJ, Zhang BH (2004) Chin Chem Lett 15:1339–1341
45. Klein JU, Whitcombe MJ, Mulholland F, Vulfson EN (1999) Angew Chem Int Ed Engl 38:2057–2060