Molecularly-imprinted Calcium Phosphate/Calcium Alginate Composite Microspheres by Surface Imprinting via Silane Crosslinking

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ABSTRACT: This paper describes the development of a new type of molecularly-imprinted composite microspheres. Composite microspheres of calcium phosphate/calcium alginate (CP/CA CMs) were prepared from calcium phosphate and sodium alginate through the use of calcium chloride (CaCl₂) as a gelling agent in an inverse suspension. Molecularly-imprinted calcium phosphate/calcium alginate composite microspheres (CP/CA MICMs) were then prepared by surface imprinting using CP/CA CMs as the supporting matrix, ethylene tri(β -methoxy)ethoxysilane (A-172) and γ -amidopropyltriethoxysilane (KH-550) as the functional monomers and crosslinking agents, respectively, and Methyl Orange as the template in aqueous solution.

The CP/CA MICMs and non-imprinted composite microspheres (NICMs) were characterized by Fourier-transform infrared spectroscopy and optical microscopy. The adsorption behaviour of the CP/CA MICMs and NICMs were determined by ultraviolet spectrophotometry. The results indicated that molecular imprinting occurs via the crosslinking of silanes on the surface of the CP/CA CMs, and that CP/CA MICMs possess an adsorption capacity which exhibits greater selectivity. A schematic representation of the imprinting and recognition process of CA/CP MICMs by surface imprinting via silane crosslinking was presented.

1. INTRODUCTION

Molecular imprinting is a promising technology that produces a precise chemical architecture involving the binding and differentiation of molecules in a material (Vlatakis *et al.* 1993). In recent years, there has been widespread interest in imprinting in bead form (Yue *et al.* 1992; Mayes and Mosbach 1996; Vorderbruggen *et al.* 1996; Zhang *et al.* 2002, 2003) and organic/inorganic composite microspheres have been introduced as molecular imprinting matrices (Lu *et al.* 2003, 2006). Natural polymers can also be utilized as the supporting matrix for molecular imprinting. Thus, Zhang *et al.* (2006a,b) have reported the preparation of bovine serum albumin-imprinted calcium alginate hydrogel microspheres. Sodium alginate exhibits important gelation features in the presence of divalent cations, such as Ca^{2+} ions. To improve the performance of the hydrogel, a small quantity of hydroxyethylcellulose was introduced to encourage the sodium alginate to form interpenetrating networks.

The surface-imprinting technique can be employed to prepare metal ion-imprinted polymer beads, since the interactions between the functional group and the substance distributed on the surface of the beads is sensitive and fast (Yu *et al.* 1992; Yoshida *et al.* 1998). Organic silanes and their derivatives have been used as functional monomers to prepare surface layers of molecularly-imprinted polymers on solid substrates (Katz and Davis 2000). The solid substrates employed include the surfaces of inorganic materials such as porous silica particles, while the use of polysiloxanes in aqueous solution provides a spontaneous and potentially useful method for imprinting water-soluble biomolecules.

This paper describes the use of artificially synthesized calcium phosphate to improve the properties of composite microspheres. The CP/CA CMs thus produced were utilized as new carriers for surface molecular imprinting in the aqueous phase via silane crosslinking.

2. EXPERIMENTAL

2.1. Materials

Sodium alginate (SA, $\overline{M}_w = 21.8 \times 10^4$, $\overline{M}_n = 3.5 \times 10^4$) was purchased from the Beijing Xudong Chemical Reagent Factory (Beijing, P. R. China), chemical reagent grade ethylene tri-(β -methoxy)ethoxysilane (A-172) and γ -amidopropyltriethoxysilane (KH-550) were supplied by the Tianjin Shengbin Chemical Plant (Tianjin, P. R. China), while analytical reagent grade Methyl Orange (MO) and Methyl Red (MR) were purchased from the Beijing Xinjing Biological Technique Company (Beijing, P. R. China). Analytical grade anhydrous calcium chloride was purchased from the Tianjin Chemical Reagent Institute (Tianjin, P. R. China), while chemical reagent grade chloroform, hexane, methanol, hydrochloric acid and ethyl cellulose were all from the Tianjin No. 2 Chemical Reagent Factory (Tianjin, P. R. China) and were used without any further purification. Calcium phosphate was prepared according to literature methods (Liu *et al.* 2007). The structural formulae of the template molecules and silanes are depicted in Figure 1.



Figure 1. The structural formulae of the template molecules and silanes employed in the present work: MO = Methyl Orange; MR = Methyl Red; A-172 = ethylene tri(β -methoxy)ethoxysilane; KH-550 = γ -amidopropyltriethoxysilane.

2.2. Preparation of CP/CA CMs

A known amount of calcium phosphate (0.75 g) was dissolved in 25 m ℓ of de-ionized water, following which 0.75 g of sodium alginate was added. The resulting mixture was agitated ultrasonically for 1 h to produce a uniform viscous solution exhibiting a milky white colour. This viscous solution was subsequently dispersed at room temperature in a mixture of chloroform and hexane (2:3, v/v) containing a specific amount of ethyl cellulose through the use of a magnetic stirrer. After 10 min, 25 m ℓ of a calcium chloride solution (2.5%, w/v) was introduced into the system and the crosslinking reaction allowed to proceed for 1 h. The resulting microspheres were collected and washed employing large quantities of de-ionized water. The final products were dried at 60°C for 24 h and then passed through sieves of different mesh sizes. Beads with a diameter in the range 75–300 µm were collected for later use.

2.3. Preparation of CP/CA MICMs

A known volume (20 m ℓ) of a 0.5 mmol/ ℓ template solution was mixed in a beaker with 1.00 g of dried CP/CA CMs. The resulting solution was heated to 90°C under gentle stirring and the silanes added. Following this step, an appropriate solution containing 0.01 mol/ ℓ HCl and 0.1 mol/ ℓ CaCl₂ was added in order to adjust the pH value to 4.0. The subsequent interaction was performed by heating the solution at 88–90°C for 2 h. The conditions employed for the preparation of the various samples are listed in Table 1.

The freshly prepared CP/CA MICMs were collected and washed intensively with distilled water. For the removal of the template molecule, a known amount of the wet microspheres was placed in a beaker containing 10 m ℓ of 0.01 mol/ ℓ HCl solution and 90 m ℓ of methanol. The resulting sample was agitated gently for 36 h during which time the elution solution was renewed four times. All of the above procedures were carried out at 25°C. Finally, the CP/CA MICMs were again washed intensively with distilled water and dried *in vacuo* at 60°C for 24 h. The dried CP/CA MICMs were passed through sieves of different mesh sizes and microspheres of 75–300 μ m diameter were collected for the re-adsorption tests.

Non-imprinted composite microspheres (NICMs) were prepared employing the same procedures as above without the addition of template. The same washing procedures were followed as for the preparation and treatment of the CP/CA MICMs described above.

Sample	HCl ^a	A-172 (mℓ)	KH-550 (mℓ)	Template
MICMs1	_	2	2	МО
MICMs2	+	2	2	MO
MICMs3	+	2	0	MO
MICMs4	+	0	2	MO
MICMs5	+	2	2	MR
NICMs	+	2	2	_

TABLE 1. Conditions Employed for Sample Preparation

^a+, HCl was employed in this case; –, HCl was not employed in this case.

2.4. Optical microscopy

The dried CP/CA MICMs were immersed in de-ionized water for 12 h at room temperature. Micrographs of the CP/CA MICMs in the wet form were obtained via an optical microscope (Axiovert 25C, Carl Zeiss Co. Ltd., Germany) connected to a digital camera.

2.5. FT-IR spectra

The FT-IR spectra of MO, MICMs2 (after washing with methanol), MO-CMs2 (MICMs2 before washing with methanol) and NICMs in the dry form were recorded on a Fourier-infrared spectrometer (PerkinElmer 2000 FT-IR) employing diffuse-reflectance Fourier-transform infrared spectroscopy.

2.6. Evaluation of the adsorption and recognition properties of CP/CA MICMs

The adsorption kinetic data for CP/CA MICMs and NICMs were obtained by pouring 25 m ℓ of a 0.025 mmol/ ℓ Methyl Orange solution into a series of 80 m ℓ beakers to which accurately weighed amounts of 0.4 g CP/CA MICMs or NICMs had been added beforehand. After every 5 min, 4 m ℓ volumes of the Methyl Orange solutions were examined using a U-1800 ultraviolet spectrophotometer (Hitachi Co. Ltd., Japan) at 463.5 nm wavelength. After each such examination, the corresponding solutions were immediately poured back into their respective beakers.

The recognition properties of the CP/CA MICMs were evaluated by immersing an accurately weighed amount (0.1 g) of the CP/CA MICM in 10 m ℓ of a solution containing 0.1 mmol/ ℓ Methyl Orange and 0.1 mmol/ ℓ Methyl Red, the latter having been prepared by dissolving the Methyl Red in an ethanol/water (20%, v/v) solution. After a fixed period of time, the concentrations of Methyl Orange and Methyl Red were detected spectroscopically at 463.5 nm and 526.5 nm, respectively. The above detection procedure was continued until the concentration discrimination of the solution was less than 2%.

The adsorbance, Q, was calculated on the basis of the difference between the concentration of template molecules present before and after adsorption, the volume of the aqueous solution and the weight of the microspheres employed according to the relationship:

$$Q = (C_0 - C_t)V/W$$

where $C_0 (mg/m\ell)$ is the initial concentration of the template molecules, $C_t (mg/m\ell)$ is the concentration of the molecules at various time intervals, $V (m\ell)$ is the volume of the template molecule solution and W (g) is the weight of the CP/CA MICMs or NICMs. All the above spectrophotometric measurements were carried out at room temperature and the average value of three such measurements is recorded in Table 2.

3. RESULTS AND DISCUSSION

3.1. Morphology of CP/CA MICMs

Optical micrographs of MICMs1, MICMs2, MICMs3 and MICMs4 are presented in Figure 2, from which it can be seen that most of the composite microspheres were spherical. There is no

(a) MICMs1





Figure 2. Optical micrographs of CP/CA MICMs samples in the wet form.

visible distinction between MICMs1 and MICMs2, indicating that a dilute acid solution had no influence on the optical morphology of the MICMs. However, there was some difference in the optical morphology of MICMs2, MICMs3 and MICMs4 prepared with different silanes.

3.2. FT-IR spectra

The FT-IR spectra of MO, MICMs2, MO-CMs2 and NICMs are presented in Figure 3. Characteristic peaks of MO occurring at 1095 and 1042 cm⁻¹ were assigned to the S=O stretching vibration in SO₂. The spectrum of MO–CMs2 was quite different from those of the NICMs, indicating that MO was bound onto the surface of the CP/CA CMs. The washed product MICMs2 had no characteristic MO spectrum, indicating that the template molecule was removed after washing.

3.3. Influence of hydrochloric acid on the adsorption capacity of CP/CA MICMs

Because of the possibility that CP/CA CMs would be unstable under acidic conditions, dilute hydrochloric acid (0.01 M HCl) was not used in the preparation procedure in our initial work. Figure 4 shows the adsorption dynamics curves for CP/CA MICMs prepared with and without HCl. It will be seen from the figure that CP/CA MICMs prepared in the presence of HCl exhibited higher adsorption capabilities and a more rapid rate of adsorption, so that they took less time to reach equilibrium. It is possible that the dilute acid accelerated the hydrolysis of the silanes so that a greater number of template molecules were attached. Furthermore, an increased number of



Figure 3. FT-IR spectra of MO, MICMs2, MO-CMs2 and NICMs in the dry form.



Figure 4. Adsorption dynamics curves for (■) MICMs1 (without HCl) and (●) MICMs2 (with HCl).

micropores may be formed through the dissolution of Ca^{2+} ions in the CP/CA CMs, although the use of calcium chloride solutions should prevent the dissolution of Ca^{2+} ions.

3.4. Adsorption dynamics of CP/CA MICMs

The adsorption dynamics curves for MICMs2, MICMs3 and MICMs4 were employed to investigate the effect of different silanes on the adsorption capacity of CP/CA MICMs. These curves are depicted in Figure 5, from which it is seen that MICMs3 prepared with only A-172 had

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Figure 5. Adsorption dynamics curves for CP/CA MICMs prepared with different silanes. Data points relate to the following samples: ■, MICMs3 (with A-172); ●, MICMs4 (with KH-550); ▲, MICMs2 (with A-172 and KH-550).

the lowest adsorption capability, MICMs4 prepared with KH-550 alone exhibiting only a slightly enhanced adsorption behaviour. However, sample MICMs2 prepared with both A-172 and KH-550 had a much greater adsorption capability, with the dynamics curve for this sample increasing rapidly during the first 20 min. In the second stage of the adsorption process (from 20 to 35 min), the adsorption rate diminished progressively with the amount adsorbed, Q, attaining a maximum value after 35 min. In the third stage (35–60 min), the amount adsorbed remained virtually constant throughout.

The adsorption is considered to proceed in two steps — sorbate diffusion and attachment. In the first step, the sorbate molecules in solution permeate into the molecularly imprinted cavities, with these molecules combining with the functional groups available in these cavities. In the second stage, the sorbate molecules combine with adsorption groups associated with the sorbent surface; in the present study, the $-NH_2$ group associated with KH-550 can combine with Methyl Orange (the imprinted molecule) mainly via ionic bonding. This latter stage is much faster than the diffusion stage, so that the overall adsorption rate is basically determined by the rate of diffusion. Relative to the first stage, the rate of the second stage is much slower because the permeating sorbate molecules have to overcome the added resistance arising from the pore walls. Adsorption equilibrium is attained when virtually all of the molecular cavities in the CP/CA MICMs are occupied.

The adsorption dynamics curves of NICMs, MICMs2 and MICMs5 in MO solutions shown in Figure 6 illustrate the recognition effect of CP/CA MICMs. Clearly, MICMs2 exhibited greater binding capabilities towards MO than either MICMs5 or NICMs. Adsorption equilibrium was reached within ca. 25 min, which implies that the surface of CP/CA MICMs was favourable towards the adsorption of MO.

3.5. The adsorption thermodynamics of CP/CA MICMs

In the present work, the adsorption thermodynamics were demonstrated by plotting the amount of sorbate adsorbed per g of adsorbent (Q_R) against the initial sorbate concentration (C_R), employing

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Figure 6. Adsorption dynamics curves for NICMs, MICMs2 and MICMs5 in MO solution. Date points relate to the following samples: \blacksquare , NICMs; ⊕, MICMs5 (MR imprinted); ▲, MICMs2 (MO imprinted).

MICMs2 to adsorb MO at room temperature (Zhang *et al.* 2002, 2003). The corresponding plot depicted in Figure 7 shows that the adsorption capacity of MICMs2 (Q_R) towards its corresponding template molecule increased with increasing initial concentration (C_R) of MO. At lower concentrations (< 0.1 mmol/ ℓ), the plot of Q_R versus C_R exhibited good linearity. Accordingly, C_R/Q_R was plotted against C_R and the saturated adsorption capacity of MICMs2 obtained from the slope of the resulting linear plot was determined as 5.732 mg/g. The Langmuir model provided a good fit for the adsorption isotherm of the template molecule onto MICMs2 under the experimental conditions employed.

3.6. The molecular recognition properties of CP/CA MICMs

The static distribution coefficient (K_D), separation factor (α) and the ratio of the separation factor (β) have been utilized to estimate the molecule recognition properties of CP/CA MICMs (Zhang



Figure 7. Adsorption thermodynamics curve of MICMs2 in MO solution.

et al. 2002, 2003). In the present work, MR and MO were respectively used as competitive molecules to evaluate the molecular recognition properties of the CP/CA MICMs. For this purpose, the values of K_D , α and β for CP/CA MICMs and NICMs towards the template and a competitive molecule (MR) were determined under equilibrium conditions. The corresponding data are listed in Table 2 from which it can be seen that the K_D values of CP/CA MICMs towards their template molecule were higher than those towards MR. Both MICMs2 and MICMs5 exhibited better selective recognition properties towards their template molecules than NICMs ($\alpha > 1$). MICMs2 exhibited better recognition properties than MICMs3 or MICMs4. Interestingly, the K_D value of NICMs for MO was higher than for MR. Similarly, the K_D value of MICMs2 for MO was higher than that of MICMs5 for MR. This result may reflect the better solubility of MO in water under the experimental conditions employed.

Figure 8 gives a schematic representation of the imprinting and recognition process of CA/CP MICMs. KH-550 hydrolyzed rapidly in acid or alkali aqueous solutions and generated the corresponding silanols R_{KH-550} SiOH with an $-NH_2$ hydrophilic group. A-172 hydrolyzed more slowly and generated the corresponding silanols R_{A-172} SiOH with a hydrophobic group. During hydrolysis, reversible interactions occurred between the template and the silanols involving

Samples	K _D values	$K_{\rm D}$ values (m ℓ/g)		β
	for MO	for MR		
MICMs1	13.6	7.852	1.732	1.343
MICMs2	17.93	7.02	2.56	2.29
MICMs3	11.2	9.912	1.13	1.02
MICMs4	12.8	10.32	1.24	1.13
MICMs5	8.128	14.386	1.77 ^a	1.58 ^a
NICMs	7.103	6.297	1.12	_

TABLE 2. Values of K_D , α and β Determined for CP/CA MICMs and NICMs

^aThese values were for MR, other values were for MO.



Figure 8. Schematic representation of imprinting and recognition process of CA/CP MICMs prepared by surface imprinting via silane crosslinking. In the figure, the following symbols apply: R_{A-172} SiOH = silanols hydrolyzed from A-172; R_{KH-550} SiOH = silanols hydrolyzed from KH-550; T = template molecule; T-CMs = CMs with template molecule.

different types of non-covalent interactions, viz. ionic, hydrophobic and hydrogen bonds. As a consequence, complementary groups in the silanols or in the structural elements of the template were established. Subsequent crosslinking of these silanols with hydroxy groups on the surface of the CA/CP CMs resulted in the formation of an insoluble matrix in which the template molecules were fixed. After the removal of the template molecule from the matrix, it may be selectively rebound by the sites on the matrix surface. Moreover, during the pre-assembly process, the hydrophobic group in R_{A-172} SiOH is likely to combine with a template molecule through hydrophobic interactions. Interaction between the $-NH_2$ group in R_{KH-550} SiOH and the template molecule occurred via an ionic bond which was more effective (Zhang *et al.* 2003). When A-172 and KH-550 were both employed, the resulting MICMs exhibited the greatest adsorption capacities as well as selection properties. In this case, such enhancement was brought about by cooperation between the two silanes. In addition, A-172 hydrolyzed more slowly and the crosslinking effects of the silanols hydrolyzed from A-172 led to a greater attachment of template molecules onto the surface of the CP/CA CMs.

4. CONCLUSIONS

Molecularly Imprinted calcium phosphate/calcium alginate composite microspheres were prepared using CP/CA CMs as carriers in an aqueous system, with surface-imprinting being achieved via a silane crosslinking reaction. Spectrophotometric detection methods indicated that the CP/CA MICMs exhibited a higher adsorptive capacity than their non-imprinted counterparts. The CP/CA MICMs exhibited good selective recognition properties towards their template molecules, with a corresponding separation factor of ca. 2.56. Adsorption equilibrium was attained within a short time period, thereby implying that the surfaces of the CP/CA MICMs exhibited favourable adsorption capabilities towards the template molecules. The imprinting and recognition processes of CA/CP MICMs by surface imprinting via silane crosslinking were indicated schematically.

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REFERENCES

Katz, A. and Davis, M.E. (2000) Nature (London) 403, 286.
Liu, C., Ji, X.-J., Zhao, K.-Y. and Cheng, G.-X. (2007) J. Appl. Polym. Sci. 104, 2034.
Lu, S.-L., Cheng, G.-X. and Pang, X.-S. (2003) J. Appl. Polym. Sci. 89, 3790.
Lu, S.-L., Cheng, G.-X. and Pang, X.-S. (2006) J. Appl. Polym. Sci. 100, 684.
Mayes, A.G. and Mosbach, K. (1996) Anal. Chem. 68, 3769.
Vlatakis, G., Andersson, L.I., Müller, R. and Mosbach, K. (1993) Nature (London) 361, 645.
Vorderbruggen, M.A., Wu, K. and Breneman, C.M. (1996) Chem. Mater. 8, 1106.

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Yoshida, M., Uezu, K., Nakashio, F. and Goto, M. (1998) J. Polym. Sci. A 36, 2727.

Yu, K.Y., Tsukagoshi, K., Maeda, M. and Takagi, M. (1992) Anal. Sci. 8, 701.

Zhang, F.-G., Cheng, G.-X. and Ying, X.-G. (2006a) React. Funct. Polym. 66, 712.

Zhang, F.-G., Cheng, G.-X., Gao, Z. and Li, C. (2006b) Macromol. Mater. Eng. 291, 485.

Zhang, L.-Y., Cheng, G.-X. and Fu, C. (2002) Polym. Int. 51, 687.

Zhang, L.-Y., Cheng, G.-X., Fu, C., Liu, X.-H. and Pang, X.-S. (2003) Adsorp. Sci. Technol. 21, 775.