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The Imprinted Mechanism of Metallothionein-imprinted Polymers

Zhen-Feng Cai* and Feng-Lian Ren

College of Chemistry and Chemical Engineering, Central South University
Changsha-410083, P.R. China

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ABSTRACT

Metallothioneins (MTs) are a super-family of low molecular weight cysteine-rich proteins. Although many research works have been reported on their structure, application, etc., there is not any article reported on imprinted mechanism of MT-imprinted polymers, yet. These polymers are prepared by using acrylic acid (AA) as a functional monomer, ethylene glycol dimethacrylate (EGDMA) as a cross-linker, 2,2'-azobis(2-methylpropionitrile) (AIBN) as an initiator, and chloroform as a solvent. The imprinted mechanism is first studied by infrared spectroscopy analysis, Scatchard analysis, and TEM technique. Infrared spectrograms indicate the functional groups of SH in thiolate sulphurs have lone pair electrons which can form π-π bond with C=O in AA. Scatchard plot shows at least three kinds of binding sites exist in MT-imprinted polymer and TEM technique indicates that most terminal thiolate sulphurs in MT are polymerized mostly as binding sites during the polymerization. The study will offer experimental foundation for further research and application of MT-imprinted polymers.

Key Words:

metallothionein;
imprinted mechanism;
imprinted polymer;
Scatchard analysis;
thiolate sulphur.

INTRODUCTION

Metallothioneins (MTs) as a super-family of low molecular weight cysteine-rich proteins was described at first in equine kidney by Margoshes et al. [1]. The designation "metallothionein" reflects the extremely high thiolate sulphur and metal content, both of the order of 10% (w/w). MTs occur not only throughout the animal kingdom, but also in eukaryotic and prokaryotic microorganisms and higher plants.

Despite many decades of intensive research, the precise physiological function of MTs has not yet been identified. In view of their unusual metal binding properties,

(*) To whom correspondence to be addressed.
E-mail: chfhl77@hotmail.com

physiological functions such as transport and storage of essential d^{10} metal ions (zinc and copper) and detoxification of non-essential ones (cadmium and mercury) have been proposed. The recent advances in our knowledge regarding the function and biology of MTs have been described in recent reviews [2-7]. MT is an emergent protein, but it is not applied universally in clinic which is greatly related to the purity of MT.

The molecular imprinting technique creates specific recognition sites using template molecules. In addition to small molecules, proteins can also be used as templates [8-12]. The reports by Braco [13] and Dabulis [14] which involved the lyophilization of an excess of a template molecule with a protein such as bovine serum albumin (BSA) have claimed the formation of binding sites in the protein that are specific for the original template.

When this is considered together with the observations that enzyme activity [15], substrate specificity [16,17] and enantioselectivity can be markedly affected by lyophilizing or precipitating an enzyme with a given substrate, it suggests that a new approach to "synthetic enzymes" or complementary, to catalytic antibodies is possible. The constraining point of this technique, now called bio-imprinting [18] is that the protein or enzyme must remain in an anhydrous or nearly anhydrous solvent during the reactions otherwise the acquired specificity is lost. It was previously suggested that the technique of bio-imprinting of BSA generated specific binding cavities in the protein [13,14].

However, it has not been conclusively shown that the results are due directly to conformational changes. Only a limited set of compounds have been studied and other possible explanations have not been explored [19]. The protein prepared in anhydrous organic solvents should exhibit a greater capacity to bind the original ligand than in water or than the protein freeze-dried in the absence of the ligand [13,20]. Enzymes have been determined to be catalytically active in anhydrous organic solvents [21].

The imprinted mechanism of polymer is usually studied by Scatchard curve [22] or FTIR technique [23]. The imprinted mechanism of MT-imprinted polymer is for the first time investigated in this article, by using infrared spectroscopy analysis, static adsorption isotherm, Scatchard analysis, and transmission electron microscopy technique. Infrared spectrograms indicate that MT can shift the absorbance of functional groups of AA. Scatchard figure shows at least three kinds of binding sites exist in MT-imprinted polymer and TEM indicates most terminal thiolate sulphurs in MT are polymerized first as binding sites in the process of polymerization.

EXPERIMENTAL

Materials

MTs were provided by Lugu Biotechnology Ltd. Co. (Hunan, China) and used as imprinted molecule. Ethylene glycol dimethacrylate (EGDMA), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma, acrylic acid (AA), 2,2′-azobis(2-methylpropionitrile) (AIBN), chloroform, and other chemicals were analytical grade. Deionized water was used in the whole experiment.

Apparatus

Infrared spectroscopies of AA alone and MT and AA in chloroform were carried out by 360AVATAR FT-IR spectrometer (Nicolet, USA). Scanning electron microscopy (SEM) imaging was carried out on a Jeol JSM-6360LV scanning electron microscope (Tokyo, Japan). Transmission electron microscopy (TEM) analyses were performed on a Jeol JEM-3010 apparatus (Tokyo, Japan).

Prepartion of MT-imprinted Polymer

MT (4.7 mg), AA (2.0 mL), EGDMA (2.0 mL) and chloroform (2.0 mL) were mixed with AIBN (30.0 mg), dissolved 5 min by ultrasonic irradiation. The mixtures were deoxygenated with a stream of argon for 15 min, then sealed and irradiated at 366 nm for 24 h in 0ºC. Finally a stiff transparent polymer was obtained.

The polymers were grinded in an end runner mill and passed the sieve (75 μm), the particles were collected and put in an alkaline solution (NaOH 0.2 mol/L) with oscillating for 24 h, then they were washed repeatedly using deionized water until the washing water was neutral. In the end, the particles were dried on a sintered glass funnel.

The non-imprinted polymer was prepared using the same procedure without addition of MT and worked up by the same procedure.

Determination of MT

The polymer particles were put in a series of MT solutions, which were shaken 12 h, then centrifugalized using CR-21G high-speed refrigerated centrifuge (Hitachi, Japan). The sulphydryl method [24] was adopted in the experiment to determine the concentration of MT. According to the following simplified eqn (1), the concentration of MT labeled C could be obtained.

$$C = \frac{9.56A}{20} \quad (1)$$

Adsorption Isotherm of Imprinted Polymer

The adsorption capacity was calculated according to eqn (2) based on the difference of MT concentration before and after adsorption, the volume of aqueous solution, and the weight of MT-imprinted polymer.

$$Q = \frac{(C_i - C_f)V}{W_{MIP}} \quad (2)$$

where, Q is the balance adsorption capacity (mg/g), C_i and C_f are the primal and ultimate concentrations of MT (mg/mL), V is the volume of MT solution (mL), M_{MIP} is the weight of sorbent (g).

RESULTS AND DISCUSSION

Structure of MT

MT used in the experiment is mostly extracted from rabbit liver, the most complete summary of the primary sequence of a wide range of metallothioneins that fall into the classes I and II has been recently compiled by Kägi [25]. The absence of disulphide bonds means that the three-dimensional structure in metal-free metallothionein is essentially that of a random chain. In the mammalian protein 20 cysteinyl sulphurs (S_{cys}) out of a total of between 60 and 62 amino acids in the peptide are available for coordination to metals. Figure 1 shows the sequence of the 62-amino acids of the rabbit liver MT 2a as described by Kägi [25]. The formation of S_{cys}-M-S_{cys} bonds cross-links the peptide, much like disulphide groups, resulting in formation of a metal binding site enclosed by the hydrophilic envelope of the peptide chain.

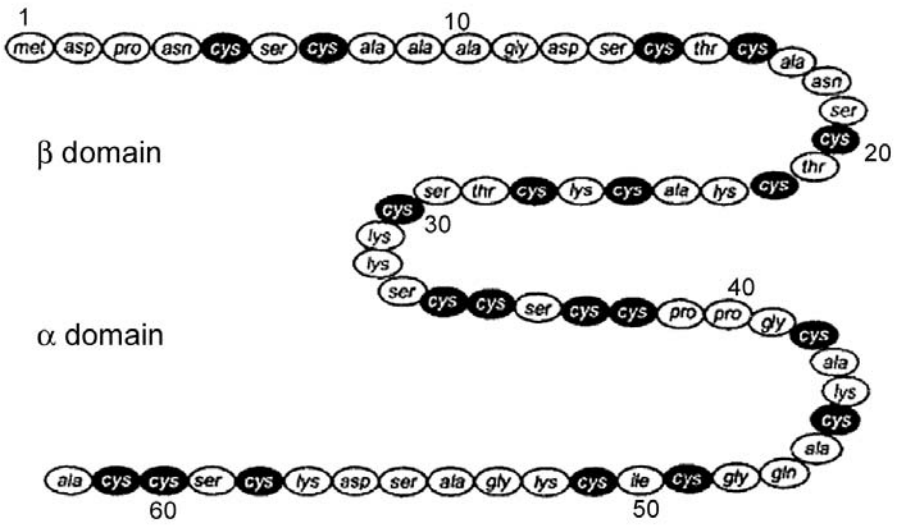


Figure 1. Amino acid sequence of rabbit liver metallothionein isoform 2a. Zn(II) binds in two domains, named α and β, that are associated with 31 residues of the *N*-terminal and, for isoform 2a, also 31 residues of the C-terminal, respectively. The diagram shows the distribution of the nine cysteines in the β domain and the 11 cysteines in α domain. Drawing is used by data from Kägi [25].

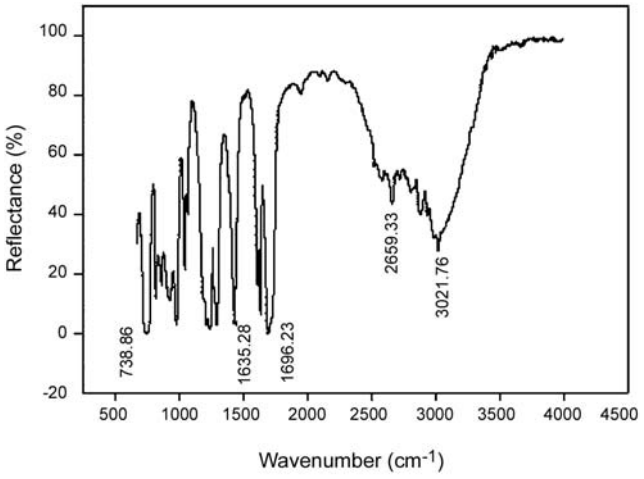


Figure 2. Infrared spectrogram of AA in chloroform.

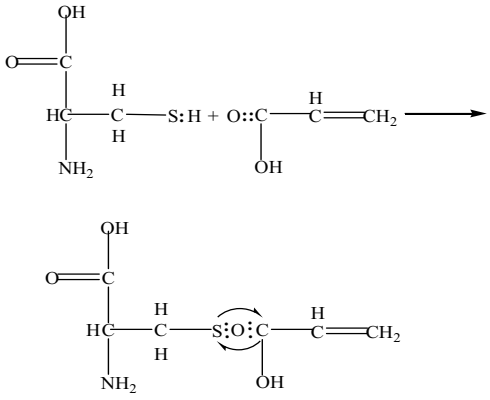
Infrared Spectroscopy Analysis

In order to confirm the reaction of MT and AA in chloroform, the infrared spectrograms of AA in chloroform and MT and AA in chloroform are presented in Figures 2 and 3.

Since MT is very expensive, it is impossible to use too much, thus, the characteristic peaks of MT cannot be detected in Figure 3. But changes can be found from functional groups of acrylic acid. From Figures 2 and 3, it is found that, stretching vibration of C=O in AA shifts from 1696.23 cm^{-1} to 1697.31 cm^{-1} stretching vibration of OH in AA shifts from 3021.76 cm^{-1} to 3022.53 cm^{-1}, and stretching vibration of C=C in AA shifts from 1635.28 cm^{-1} to 1635.23 cm^{-1}. The twenty thiolate sulphurs of MT function as bridging and terminal ligands for the coordination of seven divalent metal ions, but when MT is under acidic condition, it will lose metal ions to become apoMT as follows:

$$MT \underset{M^{2+}}{\overset{H^{+}}{\rightleftharpoons}} apoMT$$

The functional groups of SH in thiolate sulphurs have lone pair electrons which can form π-π bond with C=O in AA as reaction model (2). Thus, wavenumber of C=O in AA shift from 1696.23 cm^{-1} to 1697.31 cm^{-1} which is considered as binding sites. A small quantity of MT was added in the analytical sample which can bind to C=O groups in AA partially and leads to small change in the wavenumber of C=O.



Static Adsorption Isotherm of Imprinted Polymer

Some imprinted polymer (0.1000 g) was put in a series of MT prepared solutions (0 to 0.4529 mg/mL),

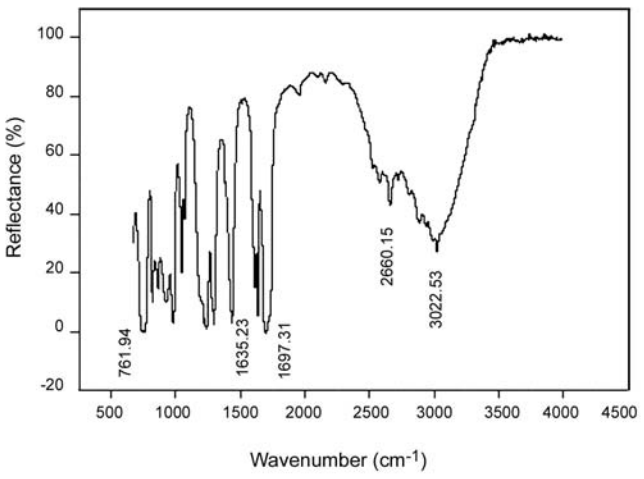


Figure 3. Infrared spectrogram of MT and AA in chloroform.

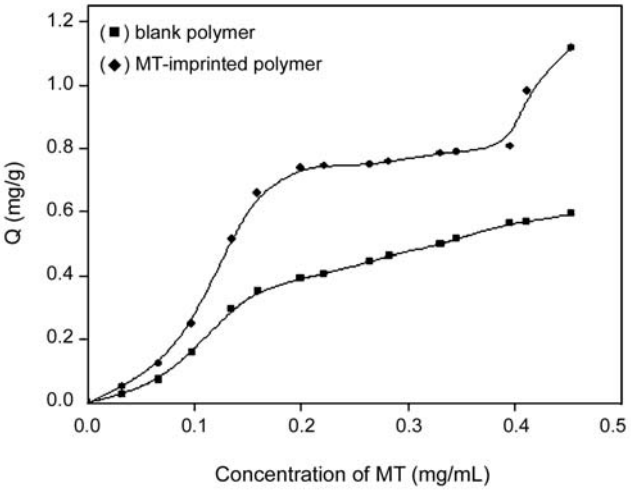


Figure 4. Static adsorption isotherm of MT-imprinted polymer.

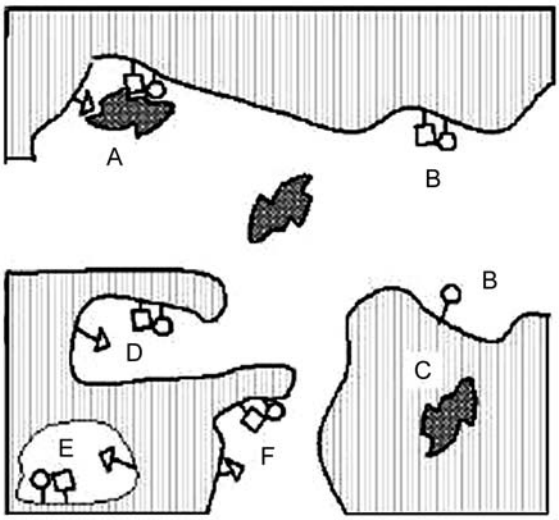


Figure 5. The schematic of the resulting gel emphasizes the heterogeneity of binding sites: high affinity site in macropore (A) and micropore (F), and lower affinity sites (B) in macropore, (C) trapped template, (E) embedded site, (D) highest affinity site with shape selectivity from polymer.

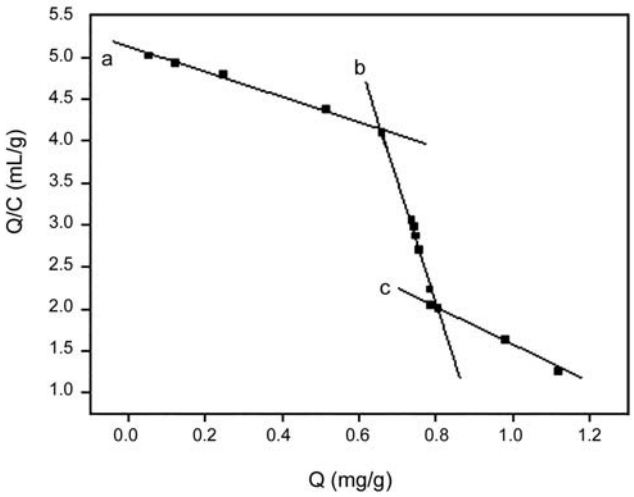


Figure 6. Scatchard curve of MT-imprinted polymer.

other procedures are the same as the previous step. From Figure 4, it is found that MT-imprinted polymer has obvious adsorption onto MT relative to blank imprinted polymer. This may confirm that the imprinting method creates a microenvironment based on shape selection and position of functional groups that recognizes MT molecule.

Since the bulk of MT is rather big, when imprinted molecule is washed out of imprinted polymer, many more cavities suitable with imprinted molecule are left in polymer, which can adsorb MT quickly because of the big cavities. When all cavities in surface are filled with MT, the transfer resistance will increase gradually which leads to transfer rate reduction, and still the adsorption of polymer to MT will increase continuously.

During the polymerization, different binding sites can be informed because of non-control of free radical (Figure 5), some extraordinarily tight binding sites are present, but most sites have considerably lower affinity and presumably lower selectivity [26]. Static adsorption isotherms indicate the presence of few tight binding sites that are rapidly occupied and the overall properties of the MIP are then dominated by a large number of lower affinity sites. When, the concentration of MT increases, however, the adsorption capacity changes by a slight increase in the mass transfer rate with non-specific adsorption.

Scatchard Analysis of Imprinted Polymer

In analysis of molecular recognition, Scatchard model is commonly used to estimate binding characteristic of molecular imprinted polymer [27]. The Scatchard equation is as following.

$$Q/C = (Q_{max} - Q)/K_d \quad (3)$$

where, Q_{max} is the most adsorption capacity (mg/g), is the balanceable concentration of MT (mg/mL), and K_d is the dissociate equilibrium constant of binding sites (mg/mL). Figure 6 is drawn using Q as abscissa and Q/C as ordinate according to Figure 4.

Table 1. The three linear regression equations.

	Linear regression equation	R	K_d (mg/mL)	Q_{max} (mg/g)
a	$Y_1=5.11323-1.50851X_1$	0.99703	0.6629	3.3896
b	$Y_2=14.0622-15.02102X_2$	0.99139	0.06657	0.9362
c	$Y_3=3.91616-2.3616X_3$	0.99667	0.4234	1.6583

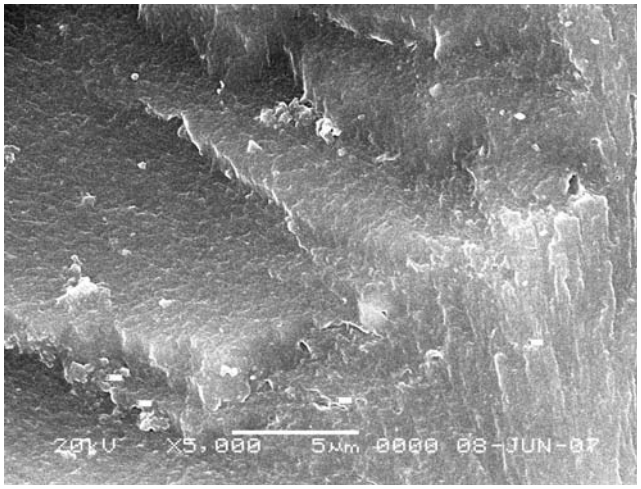


Figure 7. SEM Microphotograph of MT-imprinted polymer (×5000).

Since the places of twenty thiolate sulphurs are different in MT [2], binding strength is different because of spatial hindrance. It is found from Figure 6 that the Scatchard plot consists of three distinct straight lines, and the three lines have good linear relationship, which indicates that three binding sites exist when MT-imprinted polymer is formed. Based on the existence of three binding sites in MT-imprinted polymer and the structure of MT, we infer that the first kind of binding site is formed by thiolate sulphurs (located at 4, 6, 58, 60, and 61), the third kind of binding site is formed by thiolate sulphurs (located at 20, 22, 42, and 45), the other thiolate sulphurs form the second kind of binding site.

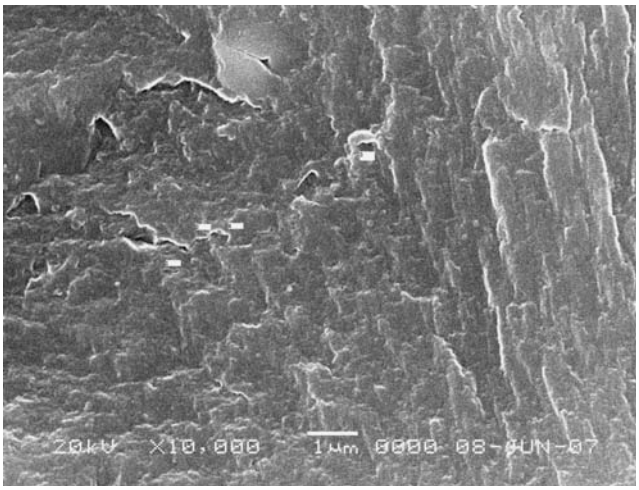
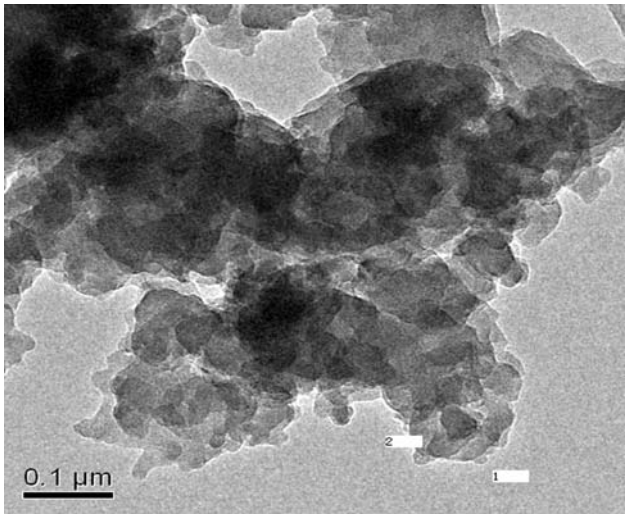


Figure 8. SEM microphotograph of MT-imprinted polymer (×10000).

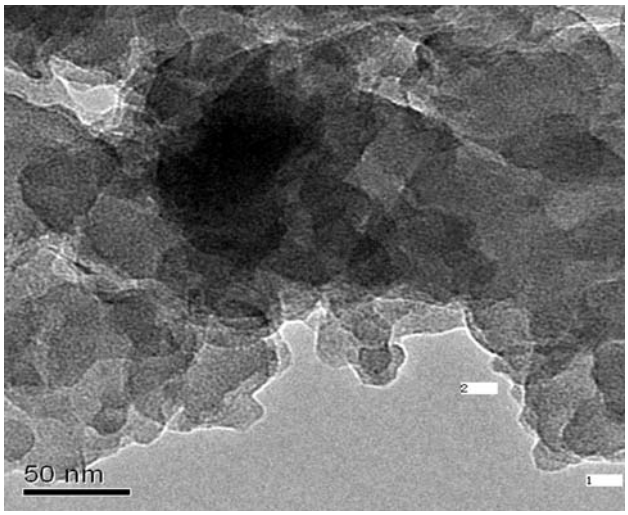
Correlation coefficients (R), balance constants of binding sites (K_d) and the most adsorption capacities (Q_{max}) of the three linear regression equations (labeled as a, b, and c) in Figure 6 are listed in Table 1. From Table 1, it can be found that K_1 is 10 times greater than K_2 and 1.5 times greater than K_3, which indicates that the binding capability of the first binding sites is the strongest among the binding sites during polymerization.

Analysis by SEM and TEM

Further characterizations are obtained by carrying out SEM analysis for the surface frame of MT-imprinted



(a)



(b)

Figure 9. TEM Microphotographs (a: magnification ×25000 and b: magnification ×60000) of MT-imprinted polymer.

polymer. As described in Figure 7 and Figure 8, the labeled places are estimated as cavities that are left by MTs washed out of imprinted polymer.

SEM can only be used to observe the surface structure of polymer, the inner structure of imprinted polymer is studied by TEM in Figure 9. Preparation of the imprinted polymer particles for TEM examinations are carried out using three methods: (1) Crushing the imprinted polymer particle, dispersion in ethanol and transfer onto a copper grid. (2) Drying the copper grid quickly through heating to avoid the configuration of polymer change. (3) Putting the copper grid in the transmission electron microscope, making the environment vacuum, and obtain the TEM images.

The figures indicate that the terminal thiolate sulphurs play an important role in the process of polymerization, which can form strong binding sites. The binding capability of other thiolate sulphurs is weak since spatial hindrance exists. Through Scatchard analysis, three kinds of binding sites have been deduced. Therefore, it can be concluded from TEM images that the binding sites formed by the terminal thiolate sulphurs are the fastest among the other binding sites.

CONCLUSION

The imprinted mechanism of MT-imprinted polymer is studied for the first time through investigating infrared spectrograms, Scatchard analysis, and TEM technique. Infrared spectrograms indicate the functional groups of SH in thiolate sulphurs have lone pair electrons which can form π-π bond with C=O group in AA. Static adsorption isotherm indicates MT-imprinted polymer has obvious adsorption to MT. Through studying Scatchard analysis, it is deduced that at least three kinds of binding sites are in the imprinted polymer. TEM indicates most terminal thiolate sulphurs in MT are polymerized as strong binding sites in the process of polymerization.

Though molecular imprinted polymer is first prepared in 1973 [28], many problems still exist up to the present and need to be solved especially the imprinted mechanism of protein. Since many binding sites possibly exist in the protein including MT, the imprinted mechanism is fairly complicated, MT-imprinted mechanism is under further studying.

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