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## **Microwave Enhanced Synthesis of Chitosan-Graft-Polyacrylamide Molecular Imprinting Polymer for Selective Removal of 17 $\beta$ -Estradiol at Trace Concentration**

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### **ABSTRACT**

Polymers have been molecularly imprinted for the purpose of binding specifically to 17 $\beta$ -estradiol. A molecularly imprinted polymer (MIP) material was prepared using 17 $\beta$ -estradiol as the imprinted molecule, acrylamide as functional monomer and macroporous chitosan beads as functional matrix. Chitosan-graft-polyacrylamide was synthesized without any radical initiator or catalyst using microwave (MW) irradiation, which allowed MIP production in about 1 h compared to conventional method which requires 8-10 h. The representative microwave synthesized graft copolymer was characterized by Fourier transform-infrared spectroscopy, taking chitosan as a reference. The parameters studied included MIP synthesis optimization, using microwave, adsorption kinetics, adsorption isotherm, selectivity and reusability. Batch as well as column flow studies were performed. Removal experiments in batch were carried out by applying the chitosan-graft-polyacrylamide Molecular Imprinting Polymer (MIP) to various 17 $\beta$ -estradiol aqueous solutions. The chitosan coated polyacrylamide MIP was more rigid with better stability and enhanced adsorption capacity. The maximum adsorption capacity was observed to be 5.01 mg g<sup>-1</sup> of MIP, which gave removal efficiency of above 91%. After 60 min, the change of adsorption capacities for 17 $\beta$ -estradiol did not show notable effects. The Langmuir and Freundlich adsorption models were also applied to describe the equilibrium isotherms. Equilibrium adsorption data showed that the adsorption of 17  $\beta$ -estradiol onto the MIP fitted well to the Langmuir equation. The MIPs were easily regenerated after each experiment with Methanol:Acetic acid (4:1 v/v) solvent and reused.

**Key words:** Micropollution, wastewater, adsorbent, langmuir isotherm, freundlich isotherms

### **INTRODUCTION**

The increased use of pharmaceutical drugs for human and veterinary applications provides an important clinical solution to the treatment of disease; however, there are technical challenges involved in monitoring these drugs in biological and environmental matrices. Quantification of pharmaceutically active compounds and their metabolites in blood, serum, or urine is difficult, often requiring tedious analytical procedures. One area of particular concern is the release of active pharmaceuticals into the environment. Endocrine disruption, a specific problem of micropollution, is induced by Endocrine Disrupting Compounds (EDCs) in water and wastewater streams. Various

adverse health effects of endocrine disrupting compounds (EDCs) have been reported in recent years (Sonnenschein and Soto, 1998; Kim *et al.*, 2007; Mendes, 2002; Auriol *et al.*, 2006). Among the EDCs, estrogenic compounds, mainly natural steroids and synthetic estrogenic compounds, are the main concerns. The adverse effects of EDCs became an important issue and have received public attention, since the link between synthetic birth-control pharmaceuticals (e.g., ethynylestradiol) and their toxicological impact on fish had been reported (Purdom *et al.*, 1994; Jobling *et al.*, 1998; Kramer *et al.*, 1998). Their persistence in various environmental matrices is not always mitigated by the standard wastewater treatment processes. Many EDCs are natural hormones or consumer products such as pharmaceuticals and personal care products (Daughton, 2002; Daughton and Ternes, 1999). It is precisely their potency at extremely low level combined with the large number at which they can be simultaneously found that make EDCs so worrisome and requires the development of highly efficient water treatment methods. The US EPA tried to establish the Endocrine Disruptor Screening Program (EDSP) to develop official screening methods and toxicity testing strategies. The European Organization for Economic Cooperation and Development (OECD) also has made an effort to develop a reliable method to confirm the significance of EDCs (Hecker *et al.*, 2007). However, the proposed methods have not yet fully accepted within scientific communities (Snyder *et al.*, 2003). Despite great efforts to prepare official guidelines of EDCs, their definition and terms are still quite ambiguous. The trace level concentration of EDCs creates a challenge for both the detection and removal processes. Conventional wastewater and drinking water treatment processes are partially inefficient in removing EDCs (Jones *et al.*, 2005), explaining why contaminants such as drug metabolites or human estrogens are frequently found in water resources (Stackelberg *et al.*, 2004; Ternes *et al.*, 2003). Although, ozonation and advanced oxidation processes are efficient in removing many pollutants (Nakagawa *et al.*, 2002; Ternes *et al.*, 2002), they are also limited by the competitive removal of interfering organic matter as one study, for instance, it was reported that 5 mg L<sup>-1</sup> of O<sub>3</sub> were necessary to remove 2 mg L<sup>-1</sup> of nonylphenol in river water (Zwiener and Frimmel, 2000), which more than 800 times the amount theoretically required to fully mineralize this compound. To date, most of the adsorption research on the removal of estrogenic compounds involves broad-spectrum adsorption such as activated carbon adsorption. However, the adsorption of trace estrogenic compounds might be affected by competing substances in water stream, such as Natural Organic Matters (NOMs). It is well known that NOM can be easily adsorbed by activated carbon. Consequently, NOM may compete with trace estrogenic compounds for the adsorption sites, thus consuming most of the adsorption capacity of activated carbon. Selective removal of estrogenic compounds is a promising idea and studies regarding the selective removal of estrogenic compounds using Molecularly Imprinted Polymer (MIP) have intensified in the last decade. MIP, an artificial receptor, is a kind of adsorbent with the function of selective adsorption of molecules with similar molecular structure. Basically, MIPs are extensively cross-linked polymers containing specific recognition sites with a predetermined selectivity for compound of interest (Ye and Mosbach, 2001). The imprinting of small molecules has been well-established and considered almost routine. However, the imprinting of biomacromolecules such as proteins continues to be a significant challenge due to the difficulties with large molecular sizes, structural complexity, environmental sensitivity of the templates and significantly reduced non-covalent template-monomer interactions in aqueous media (Shiomi *et al.*, 2005; Li *et al.*, 2006; Hawkin *et al.*, 2005). Since, the conventional approaches were proven to be non-effective for the protein imprinting, many artifices have been adopted to overcome above problems, for example, surface imprinting (Bossi *et al.*, 2001) and epitope imprinting (Nishino *et al.*, 2006). Meng *et al.* (2005) suggested that MIP with

17 $\alpha$ -estradiol as template might be used to remove estrogenic compounds while Le Noir *et al.* (2006, 2007) employed MIP, based on bulk polymerization approach, to remove trace 17 $\beta$ -estradiol from aqueous solution using MIP adsorbent column.

Polyacrylamides and their derivatives are often used as polymeric materials for bulk protein imprinting with a low degree of cross-linking to ensure the mobility of large molecules (Ou *et al.*, 2004; Pang *et al.*, 2006), but difficulty in removing the templates and stability of the gel still seem to be limitations. Guo *et al.* (2004) prepared haemoglobin-imprinted polymers by physically entrapping or chemically grafting of selective polyacrylamide (PAM) gels onto porous chitosan beads and the MIPs obtained showed significantly enhanced template rebinding capacity. These results suggest that incorporating chitosan, a natural biopolymer bearing abundant -NH<sub>2</sub> and -OH groups, into the PAM gels may produce homogeneous MIPs for proteins with a large binding capacity. However difficulty in removing the template and the stability of the gel was still a problem. Considering the previous work of Guo *et al.* (2004), there is no chemical bonds between the chitosan matrix and the polyacrylamide gel, the polyacrylamide gel could be washed out of the pores of the chitosan beads after multiple using cycles. In this work we have further prepared a novel chitosan matrix by introducing double bonds to the chitosan beads first and then the polyacrylamide gel was grafted to the modified chitosan beads. Thus, the interaction forces of the selective polyacrylamide gel and chitosan matrix could be strengthened by chemical bonds. Microwave irradiation, is yet another efficient source of thermal energy that is used in a variety of modern synthetic reactions. Besides its heating effect, microwave radiation has been shown to be an effective tool to separate emulsified water from oil in water-in-oil emulsion (Saifuddin and Chua, 2006; Nour *et al.*, 2007). The microwave irradiation has been successfully used in the synthesis of chitosan-graft-poly (acrylonitrile) without any radical initiator in a very short time of 1.5 min (Vandana *et al.*, 2005). Microwave irradiation has been extended for use in the chemical modification of polysaccharides such as starch and chitosan. Ge and Luo (2005) reported the carboxymethylation of chitosan in aqueous solution under microwave irradiation, which because no organic solvents are required, represents a more environmentally-friendly method for modification of chitosan. In this study rapid process to synthesize chemically modified chitosan beads using microwave irradiation for molecularly imprinted polymer attachment is attempted.

In this study, the novel MIP adsorbents using chitosan crosslinked with epichlorohydrin (ECH) as the matrix for the entrapment of the soft polyacrylamide gel in the pores of a rigid matrix by letting the monomers and the protein diffuse into the pores of the modified chitosan nanoparticles before the start the polymerization. The present study focuses on a novel synthetic route for the generation of imprinted micro and nanospheres for selective removal 17 $\beta$ -estradiol focusing on optimization of the governing parameters for polymerization, including the polymerization temperature and the cross-linker, yielding a one-step synthetic approach with superior control on the bead diameter, shape, monodispersity and imprinting efficiency. The MIP beads thus obtained were characterized by IR, ESEM and UV spectrophotometer measurement. The sorption behaviours of the MIP adsorbents including kinetics, isotherms, effect of pH and ionic strength were investigated in detail. Finally, the adsorption selectivity and the regeneration of the MIP adsorbents were also studied.

## **MATERIALS AND METHODS**

**Chemical and reagents:** Chitosan was purchased from Fluka as a flaked material, with a deacetylation percentage of approximately 87%. Glutaraldehyde (GLA), epichlorohydrin (ECH) and ethylene glycol diglycidyl ether (EGDE) purchased from Fluka were analytical-reagent grade.

Acrylamide (Merck) and potassium persulfate (BDH, Analar Grade) were used without further purification. All the reagents used were of analytical-reagent grade and used as received. Ultrapure deionized water was obtained using the ELGA LabWater (UK) water purification system in our laboratory and was used to prepare all the solutions.

In Malaysia, the riverine ecosystem is of particular interest since river water provides about 98% of the country's water requirements. This study was carried out using water samples collected in 2009 from the Selangor River, in Malaysia.

**Preparation of macroporous chitosan beads:** Chitosan beads were prepared as reported elsewhere with some modification (Chandy and Sharma, 1993; Shu and Zhu, 2001; Guo *et al.*, 2004). In this study, 3.0 g chitosan dissolved in 100 mL, 2% (v/v) acetic acid. The solution was dropped through a seven-gauge needle into 2.0 M sodium hydroxide solution and the gelled spheres formed instantaneously. This process was accomplished by using a model 100 push-pull syringe pump. The formed chitosan beads were kept in the sodium hydroxide solution for 24 h and washed with distilled water until the washing solution became neutral. After filtering the beads (hereafter called chitosan beads) were stored in distilled water for later use.

**Preparation of cross-linked chitosan with microwave irradiation:** Cross-linked chitosan beads were prepared using epichlorohydrin (ECH) as the cross-linking agents following a novel microwave irradiation method modified from the previously mentioned method (Wei *et al.*, 1992; Guo *et al.*, 2004). A solution of 0.04 M epichlorohydrin solution containing 0.067 M sodium hydroxide was prepared (pH = 10). Freshly prepared, 20 g of wet chitosan beads (using filter to absorb the surface water) were put into a flask with 100 mL of the epichlorohydrin solution and stirred to allow proper mixing. The mixture was subjected to several (about 12) short burst of microwave irradiation using a microwave oven at frequency of 2.45 GHz at power output of 200 W. Each short burst of irradiation lasted for 1 min. The mixture was cooled (35-40 sec at room temperature) between each irradiation. This ramp/cool cycle was repeated 12 times. After the 12th cycle (total time required about 25-30 min), the cross-linked beads were filtered and washed extensively with distilled water to remove any unreacted epichlorohydrin until the washing solution became neutral (the neutrality measured by universal indicator paper) and air dried. The diameter of the wet cross-linked bead was approximately  $1.75 \pm 0.05$  mm. The newly formed cross-linked beads (hereafter called chitosan-ECH beads) obtained were confirmed by a Perkin-Elmer FT-IR System 2000 Model spectrometer.

**Characterization of cross-linked chitosan beads:** The amine content of the cross-linked chitosan beads was determined titrimetrically. Cross-linked chitosan beads (1.0 g) (filter paper used to absorb the surface water) were added to 0.1 M hydrochloric acid (20 mL); the mixture was left to stand for 16 h in a tightly closed bottle. The mixture was filtered and the filtrate (5 mL) was titrated with a 0.1 M sodium hydroxide. The surface morphology of the wet beads was studied using an environmental scanning electron microscope (ESEM Philips XI30). Samples containing water without drying were mounted on metal stubs and at low vacuum degree ( $\sim 10^{-3}$  atm) and relatively low temperature (near 0°C) observed.

**Dissolution and swelling test of chitosan:** Chitosan and cross-linked chitosan beads were tested with regard to their solubility in each of 5% (v/v) acetic acid, distilled water and 0.10 M

sodium hydroxide solution by adding 0.10 g of chitosan and cross-linked chitosan beads in each of the dilute acid, distilled water and dilute alkaline solutions for a period of 24 h with stirring.

The swelling studies of chitosan and cross-linked chitosan beads were carried out in distilled water at room temperature for a period of 24 h. The percentages of swelling of these beads were calculated by using the following Eq. 1:

$$\text{Percentage of swelling} = \frac{W_s - W}{W} \times 100 \quad (1)$$

where,  $W_s$  is the weight of swollen beads (g) and  $W$  is the weight of dry beads (g).

**Preparation of 17 $\beta$ -estradiol imprinted polymer by graft copolymerization of acrylamide under microwave irradiation:**

The preparation of 17 $\beta$ -estradiol imprinted polymer was achieved by the combine use of acrylamide as the functional monomer and cross-linked chitosan particles (chitosan-ECH beads) as supporting matrix. The imprinted polymer was prepared by homogeneous chemical graft copolymerization of acrylamide into the porous cross-linked chitosan beads before starting the polymerization. Typically, the template, 17 $\beta$ -estradiol (0.272 g, 1 mmol) was dissolved in acetonitrile (30 mL) in a flask. Then 16.0 g wet chemically-modified cross-linked chitosan beads (as supporting matrix) (use filter to absorb the surface water), 26.5 mmol (1.9 g) acrylamide (functional monomer), 0.1 mmol (15.4 mg) N, N'-methylene-bis-acrylamide (as cross linker) and 40 mL 0.01 M sodium dihydrogen phosphate buffer (pH 6.8) were added to the flask (four-necked flask equipped with a nitrogen inlet). After degassing and nitrogen purging for 15 min, the flask was sealed and the contents were irradiated in microwave oven at frequency of 2.45 GHz at power output of 200 W for 5 min to allow the polymerization to take place. After the polymerization, the formed polyacrylamide-LACC composite beads were freed from the surround polyacrylamide gel by pressing out the surrounding polyacrylamide gel using a nylon stocking. The freed polyacrylamide-LACC beads were wet grained in a ball mill and then sieved to a size between 100 mesh (10  $\mu$ m) and 500 mesh (2  $\mu$ m). The resulting MIP particles were collected by centrifugation. The template molecules were leached out by washing with methanol-acetic acid solution (3:1, v/v) four times each at duration of 1.5 h. Then it was followed by ten washings with 15 mL of methanol at 1 h for each washing. The particles were then extensively washed with water and dried at 40°C. The corresponding Non-Imprinted Polymer (NIP) was prepared in parallel in the absence of template and treated in the same manner.

**The morphologies of the MIP beads:** The surface morphologies of the beads were observed using a scanning electron microscope (SEM, model S-3500 N, HITACHI, Japan). The beads were freeze-dried prior to be coated with a gold layer.

**Adsorption experiments of the MIP:** To evaluate the binding capacity of the MIP beads obtained, static adsorption test and dynamic adsorption test were carried out in acetonitrile solutions. For the dynamic adsorption test, a series of tubes containing 1.0 g of the wet MIP beads, placed in a 35 mL polypropylene centrifugal tubes containing 15.0 mL 17  $\beta$ -estradiol of 0.5 mmol L<sup>-1</sup> (0.14 mg mL<sup>-1</sup>) were prepared. The tubes were shaken in the water bath at 25°C for different time intervals (10, 20, 30, 40, 50, 60 and 70 min). A blank was prepared with only  $\beta$ -estradiol solution to account for any potential binding of  $\beta$ -estradiol onto the centrifuge tube.

Similarly, in a separate tube, non-imprinted particles were also incubated and were treated in the similar way as the MIP beads. After incubation at the appropriate time interval the samples were centrifuged at 15000 rpm for 5 min and filtered. The concentration of free 17 $\beta$ -estradiol after the adsorption, was recorded by UV spectrometry at 280.0 nm. Meanwhile, reference pure solutions of 17 $\beta$ -estradiol were prepared with varying concentrations (0.05 to 1.0 mmol L) and their absorbances at 280 nm were determined for the construction of the calibration graph. The adsorbed quantity was calculated by subtracting the free concentrations from the initial concentrations.

The adsorption capacity  $Q_t$  was calculated based on the difference of 17 $\beta$ -estradiol concentration before and after adsorption, the volume of aqueous solution and the weight of the beads according to Eq. 2:

$$\text{Adsorption capacity } Q = (C_0 - C_t) \frac{V}{W} \quad (2)$$

where  $C_0$  is the initial 17  $\beta$ -estradiol concentration (mg mL<sup>-1</sup>),  $C_t$  is the 17  $\beta$ -estradiol concentration (mg mL<sup>-1</sup>) of different time,  $V$  is the volume of 17  $\beta$ -estradiol solution (mL) and  $W$  is the weight of the MIP beads (g).

The adsorption removal efficiency of 17 $\beta$ -estradiol from aqueous solution was calculated as follows (Eq. 3):

$$\text{Removal efficiency (\%)} = \frac{C_0 - C_f}{C_0} \times 100 \quad (3)$$

where,  $C_0$  is the initial 17 $\beta$ -estradiol concentration in the solution and  $C_f$  is the final 17 $\beta$ -estradiol concentration in the solution.

For the static adsorption test, similar test was performed as above. The wet MIP beads, 1.0 g, (using filter to absorb the surface water) were placed in a 35 mL polypropylene centrifugal tubes containing 15.0 mL of 17 $\beta$ -estradiol of various concentrations (0.10-0.80 mmol L<sup>-1</sup> or 0.027 to 0.217 mg mL<sup>-1</sup>). A blank was prepared with only  $\beta$ -estradiol solution to account for any potential binding of  $\beta$ -estradiol unto the centrifuge tube. Similarly, in a separate tube, non-imprinted particles were also incubated and were treated in the similar way as the MIP beads. After shaking in a water bath for 60 min at 25°C, the samples were centrifuged at 15000 rpm for 5 min and filtered. The concentration of free 17 $\beta$ -estradiol after the adsorption, was recorded by UV spectrometry at 280.0 nm. The concentration of free 17 $\beta$ -estradiol was estimated from the absorbance ( $A_{280}$ ) versus 17 $\beta$ -estradiol concentration calibration graph. The adsorbed quantity was calculated by subtracting the free concentrations from the initial concentrations.

**Adsorption isotherm:** The wet MIP beads (1.0 g approximately) (the beads were gently dabbed dry with a filter paper to remove surface water) was placed in a 35 mL polypropylene centrifugal tubes and mixed with 15 mL of a known concentration of 17 $\beta$ -estradiol solution (increasing the initial concentration of 17 $\beta$ -estradiol from 0.03 to 0.30 mg mL<sup>-1</sup>). The polypropylene centrifugal tubes were oscillated in an oscillator with constant temperature bath at 25°C for 1 h. The concentration of 17 $\beta$ -estradiol in the solution was determined using the spectrophotometric method at 280 nm as mentioned above. The amount of adsorption at equilibrium time  $t$ ,  $q_e$  (mg g<sup>-1</sup>), was calculated by Eq. 4:

$$q_e = \frac{(C_o - C_e)V}{w} \quad (4)$$

where,  $C_o$  and  $C_e$  are the liquid-phase concentrations of 17 $\beta$ -estradiol at initial and equilibrium time, respectively;  $V$  the volume of the solution (L);  $W$  is the mass of dry adsorbent (MIP) used (g).

**Regeneration of MIP beads:** The MIPs were easily regenerated after each experiment with Methanol:Acetic acid (4:1 v/v) solvent and reused. During the regeneration step, 500 mL of methanol: Acetic acid solution (4:1 v/v) was added to the fully adsorbed MIP beads and allowed to stirrer for 30 min at 200 rpm. After that the solution was drained off in a beaker and the beads were washed again using smaller portions of the solution (250 mL) in the same manner. Each time after washing the absorbance at 280 nm of the effluent was measured and the washing was repeated until the absorbance drop to almost zero.

## RESULTS AND DISCUSSION

The characterization of the chitosan beads and cross-linked chitosan beads. As observed from our previous work, the Fig. 1a and b show the surface morphologies of the chitosan beads where pores with different size were observed and the average pore size of the beads were calculated to be 5.4  $\mu\text{m}$  (Saifuddin and Nur Yasumira, 2010) . The SEM images of cross-linked chitosan-ECH beads after ground and sieved are depicted in Fig. 2. It can be seen that there is a significant difference in surface morphology of the two forms of chitosan beads. A striking feature of the chitosan-ECH beads image was that the appearance were noted to have much asperity and to be more coarsely grained, more porous and rough internal structure. This may offer more adsorption sites for adsorbate (acrylamide binding for MIP preparation). Site accessibility, mass transport and hydrodynamic properties are significantly improved by producing porous beads (Dai *et al.*, 2004). The cross-linked chitosan beads had average pore size of 4.1  $\mu\text{m}$  (Saifuddin and Nur Yasumira, 2010). This result also indicates that chitosan has been chemically modified (Kamari *et al.*, 2009).

Epichlorohydrin was selected as a convenient base catalyzed cross-linking agent. The advantage of using ECH as a cross-linking agent is that it does not eliminate the cationic amine function of chitosan, most notably, the crosslinking with ECH can considerably improve the

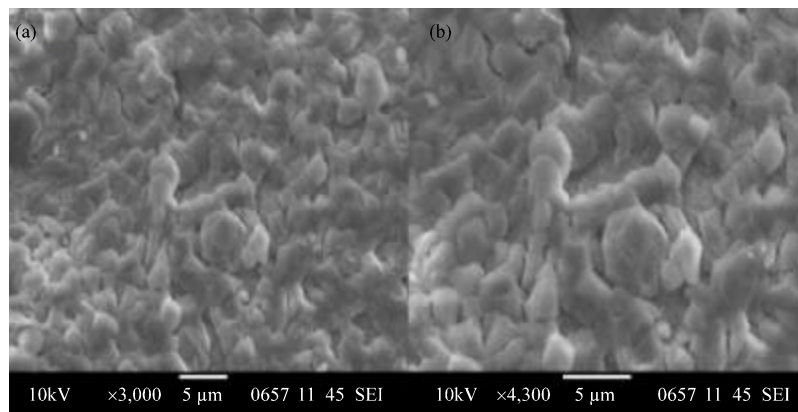


Fig. 1: SEM images showing the surface morphologies of the chitosan beads; (a) at 3000 $\times$  and (b) 4300 $\times$  magnifications



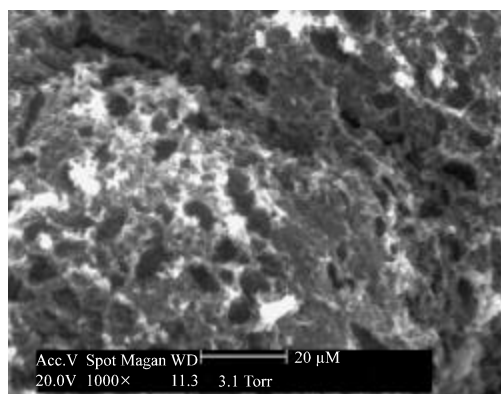


Fig. 2: ESEM microphotograph of cross-linked chitosan bead (X 1000)

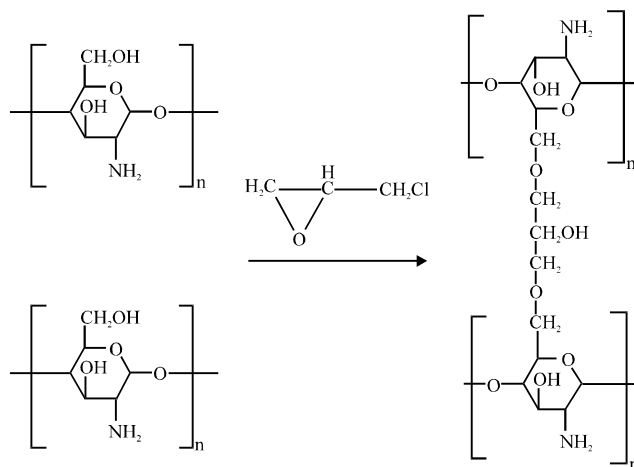


Fig. 3: Schematic representation for the cross-linking reaction of chitosan with epichlorohydrin

wet strength of the chitosan beads (Wan Ngah *et al.*, 2002; Lee *et al.*, 2004). An analysis of the cross-linked chitosan beads gave 0.18 mmol of amine content per gram wet beads (the water content was 93.5%); that means about 18% amine groups were cross-linked with epichlorohydrin. Obviously, this analytical result indicates that there are enough amine groups in the accessible positions of the cross-linked matrix. The analysis of Chitosan-ECH gave 0.25 mmol of carboxyl content per gram wet beads (water content was 85.3%). That means that the vinyl group content was above 0.25 mmol g<sup>-1</sup> and that most amine had reacted with epichlorohydrin. The reaction of chitosan with epichlorohydrin in an acidic condition might be cross-linked at hydroxyl groups to form the epichlorohydrin cross-linked chitosan product, as shown in Fig. 3, which is similar to the scheme that has been reported previously (Wan Ngah *et al.*, 2002; Lee *et al.*, 2004).

**Solubility and swelling test of chitosan:** After cross linking with epichlorohydrin, the cross-linked chitosan (Chitosan-ECH) was found to be insoluble in acidic and alkaline medium as well as distilled water. It is well known that the high hydrophilicity of chitosan beads or raw chitosan are due to primary amine groups, which makes chitosan easily soluble in dilute acetic or formic acid solutions to yield a hydrogel in water. Therefore, the crosslinking treatment of chitosan reinforces

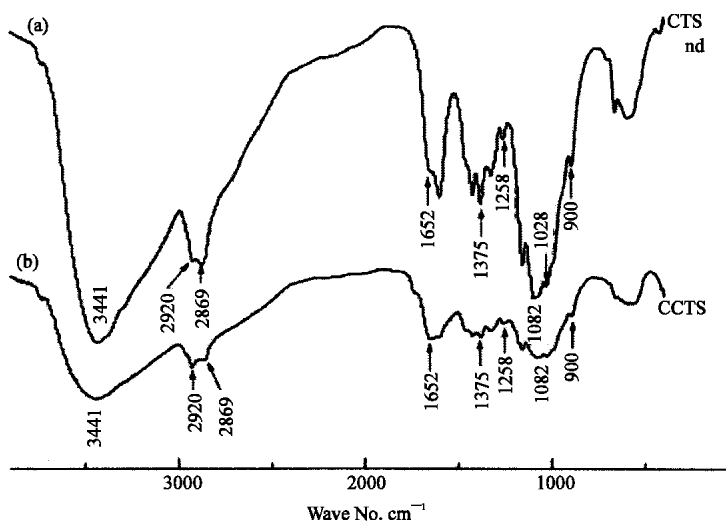


Fig. 4: FT-IR spectra (a) Chitosan (labelled as CTS) and (b) epichlorohydrine cross- linked chitosan (labelled as CCTS), using KBr pellets. The range from 4000 to 400  $\text{cm}^{-1}$  was scanned

its chemical stability in organic acidic media, making it useful for the removal of chemical pollutants from wastewaters in acidic solution.

The swelling behaviour of chitosan improved after cross-linking. It was observed from our earlier studies that chitosan beads had 37.5 and 32.6% swelling when allowed to remain in distilled water and 0.1 M NaOH solution, respectively for 24 h at room temperature. However, the swelling for cross-linked chitosan beads was only 15.3% in distilled water and 11.8% in NaOH under similar conditions (Saifuddin and Nur Yasumira, 2010). Hence, the change in the swelling percentages between chitosan beads and cross-linked chitosan beads gives a preliminary indication that cross-linking enhances the physical strength of the chitosan beads. Therefore, cross-linking modification is not only able to increase the surface area and reinforce the chemical strength of the chitosan beads but also reduces the swelling of the beads. Thus, cross-linking treatment increases the rigidity and also the chemical stability of the beads. It is interesting to note that, the less swelling behaviour of cross-linked chitosan beads is important in order for it to be used in an adsorption column.

**Infra red spectra of chitosan derivatives:** The characteristic IR absorption peaks of chitosan were observed (Fig. 4a), which included a broad and strong band ranging from 3200-3700  $\text{cm}^{-1}$  (stretching vibration of O-H and extension vibration of N-H). The peaks located at 2920 and 2869  $\text{cm}^{-1}$  can be assigned to asymmetric and symmetric  $-\text{CH}_2$  groups. The peak located at 1652 is characteristic of amine deformation. The prominent peak at observed at 1375  $\text{cm}^{-1}$  represents C-N stretching. The peak at 1258  $\text{cm}^{-1}$  can be attributed to the C-O-C stretching. The peak at 1082  $\text{cm}^{-1}$  is characteristics of C-O stretching vibration. The absorption band at 900  $\text{cm}^{-1}$ , corresponds to the characteristic absorption of  $\beta$ -D-glucose unit. The infrared spectrum of epichlorohydrin cross-linked chitosan was rather similar to that of chitosan (Fig. 4b), since the functional groups of epichlorohydrin were also present in chitosan. Therefore, the same vibrations were observed but with different relative intensities. The absorption intensity of  $-\text{NH}_2$  group and  $-\text{OH}$  group (peak 3200-3700  $\text{cm}^{-1}$ ) from cross-linked chitosan is obviously lower than that of  $-\text{NH}_2$  group and

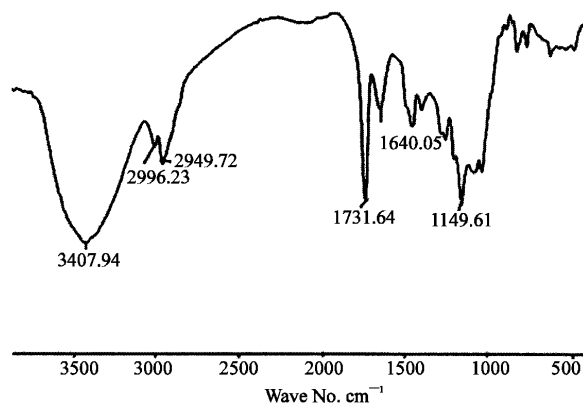


Fig. 5: IR spectra of microwave synthesized grafted polyacrylamide-cross-linked chitosan beads

-OH group from chitosan, which indicates cross-linked reaction occurred between chitosan and epichlorohydrin. Moreover, the reduction in the intensities at  $1418\text{ cm}^{-1}$  peak (primary amino group,  $-\text{NH}_2$ ) showed that most of the primary amino groups were involved in the cross-linking process.

Graft copolymer based on chitosan has been synthesized by grafting acrylamide onto modified chitosan (polysaccharide molecule) in aqueous medium using microwave irradiation. The grafting was confirmed by comparing the IR spectra of cross-linked chitosan (Fig. 4b) with that of the grafted product (Fig. 5). The IR spectrum of the cross-linked chitosan has strong peak around  $3441\text{ cm}^{-1}$  due to the stretching vibration of O-H, the extension vibration of N-H and inter hydrogen bonds of the polysaccharide. In graft copolymer the strong peak around  $3400\text{ cm}^{-1}$  could be assigned to the stretching vibration of O-H, the extension vibration of N-H and inter hydrogen bonds of the polysaccharide (Fig. 5). The IR spectrum of a polyacrylamide grafted cross-linked chitosan (Fig. 5) has additional sharp absorption peaks at  $1731$  and  $2966\text{ cm}^{-1}$  (due to carbonyl stretching and symmetrical stretching of the methyl group, respectively). Peak around  $1432\text{ cm}^{-1}$  due to C-N stretching in graft copolymer further supports grafting. This result provides a substantial evidence of grafting of polyacrylamide on to the cross-linked chitosan.

Singh *et al.* (2006), reported grafting of acrylamide and methyl methacrylate onto chitosan using microwave irradiation. They have shown that under microwave irradiation, grafting could be achieved in the absence of this initiating system (potassium persulfate and ascorbic acid), whereas control experiments with thermal heating at  $100^\circ\text{C}$  without initiator did not show any grafting, indicating the presence of non-thermal microwave effects. Grafting of both acrylamide and methyl methacrylate was demonstrated to improve the solubility of the chitosan at neutral pH. Moreover, these grafted copolymers showed increased zinc(II) binding (methyl methacrylate and acrylamide) and/or calcium(II) binding (acrylamide) making them suitable candidates for the removal of these ions from waste water. Thus microwave can be exploited for polyacrylamide grafting on the cross-linked chitosan beads to produce MIP, where both time as well as chemicals can be saved. It can be assumed that the chitosan coated polyacrylamide MIP should have better pH stability and enhanced adsorption capacity.

**Dynamic adsorption of  $17\beta$ -estradiol on cross-linked chitosan-polyacrylamide MIP:** The imprinting effect was initially evaluated by performing dynamic adsorption tests. The Fig. 6 shows

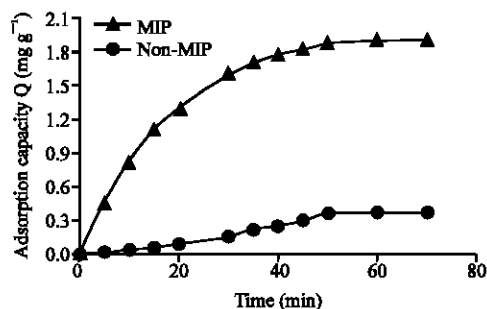


Fig. 6: Adsorption dynamics of 17 $\beta$ -estradiol on the cross-linked chitosan-polyacrylamide MIP beads at various times. V of analyte = 15 mL; Concentration of analyte = 0.14 mg mL<sup>-1</sup>; MIP or Non MIP quantity = 1.0 g; T = 25°C

the rate of adsorption of 17 $\beta$ -estradiol by MIP measured as function of time in which the imprinted polymer always adsorbed larger amount of 17 $\beta$ -estradiol than that of non-imprinted polymer. It was noted that the adsorption of 17 $\beta$ -estradiol increased quickly with time and then reached equilibrium. The contact time to reach equilibrium was 60 min. The adsorption capacity Q, was calculated based on the difference of 17 $\beta$ -estradiol concentration before and after adsorption, the volume of aqueous solution and the weight of the beads according to Eq. 2.

The Fig. 7 shows that the amount of 17 $\beta$ -estradiol adsorbed from aqueous solution increased with time and equilibrium being achieved within 60 min. The maximum adsorption capacity was observed to be 1.91 mg g<sup>-1</sup> of MIP, which gave removal efficiency of 91%. It also displayed a higher affinity for template molecule than non-imprinted polymer. The difference in the 17 $\beta$ -estradiol removal efficiency between MIP and non-MIP was up to about 6 fold. This is relatively larger than that obtained by Le Noir *et al.* (2007) and Zhang and Zhou (2008) which reported the difference in removal efficiency between MIP and non-MIP in the range of 2-3 fold. After 60 min, the change of adsorption capacities for 17 $\beta$ -estradiol did not show notable effects. As a consequence, the adsorption equilibrium time considered for the further work was taken as 60 min.

The static adsorption data as depicted in Fig. 8 showed that the binding capacity of imprinted polymer increased with the increasing of the initial concentration of 17 $\beta$ -estradiol and displayed a higher affinity for template molecule than non-imprinted polymer. The adsorption experiments were conducted in a series of 17 $\beta$ -estradiol solutions (15 mL) with different initial concentrations (0.10-0.80 mmol L<sup>-1</sup>). The adsorption capacity of 17 $\beta$ -estradiol increased from 1.75 to 4.38 mg g<sup>-1</sup> by increasing the initial concentration of 17 $\beta$ -estradiol from 30  $\mu$ g to 300  $\mu$ g mL<sup>-1</sup> (Fig. 8). In these experiments the fixed amounts of imprinted polymer was incubated with different concentrations of 17 $\beta$ -estradiol. This high affinity of imprinted polymers was also demonstrated by dynamic adsorption tests.

**Adsorption isotherm:** The adsorption isotherm is the most important information, which indicates how the adsorbent molecules distribute between the liquid and the solid phase when the adsorption process reaches an equilibrium state (Hasan *et al.*, 2008). Using the data from the adsorption experiments conducted in a series of 17  $\beta$ -estradiol solutions (15 mL) with different initial concentrations (Fig. 8), the adsorption capacity and adsorption behaviour of 17 $\beta$ -estradiol were

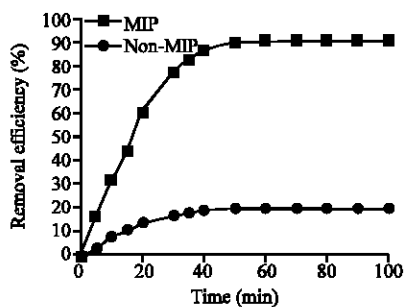


Fig. 7: Adsorption dynamics of 17β-estradiol on the cross-linked chitosan-polyacrylamide, MIP beads and Non-MIP beads. V of analyte = 15 mL; Concentration of analyte = 0.14 mg mL<sup>-1</sup>; MIP or Non MIP quantity = 1.0 g; T = 25°C

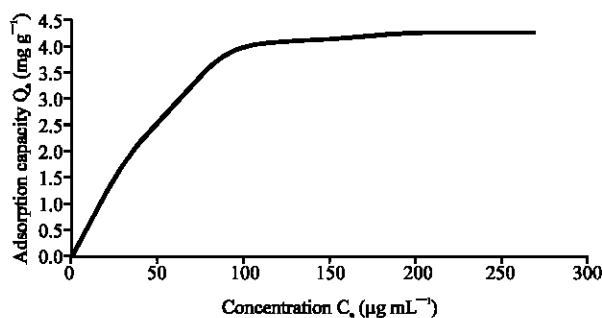


Fig. 8: Adsorption isotherms of 17β-estradiol on the cross-linked chitosan-polyacrylamide, MIP beads with varying concentration of analyte. Volume of analyte = 15 mL; Concentration of analyte = 50-300 μg mL<sup>-1</sup>; MIP quantity: 1.0 g; T = 25°C; adsorption time = 60 min

analysed by adsorption isotherm. The Langmuir and Freundlich models are often used to describe equilibrium adsorption isotherms.

The Langmuir isotherm considers the adsorbent surface as homogeneous and all adsorption sites equivalent. There is no interaction between adsorbed molecules and at the maximum adsorption, only a monolayer of adsorbate is formed on the surface of the adsorbent. Equation 5 represents the Langmuir isotherm:

$$\frac{C_e}{q_e} = \frac{C_e}{Q} + \frac{1}{Q_b} \quad (5)$$

where, Q is the maximum adsorption at monolayer (mg g<sup>-1</sup>), C<sub>e</sub> is the equilibrium concentration of 17β-estradiol (μg mL<sup>-1</sup>), q<sub>e</sub> is the amount of 17β-estradiol adsorbed per unit weight of the MIP at equilibrium concentration (mg g<sup>-1</sup>) and b is the Langmuir constant related to the affinity of binding sites (mL mg<sup>-1</sup>) and is a measure of the energy of adsorption. A linearized plot of C<sub>e</sub>/q<sub>e</sub> against C<sub>e</sub> gives Q and b. The plot of specific sorption, C<sub>e</sub>/q<sub>e</sub>, against the equilibrium concentration, C<sub>e</sub>, for MIP is shown in Fig. 3.10.

As seen in Fig. 9, 17β-estradiol adsorption onto the MIP was well described by the Langmuir model with correlation coefficients of R<sup>2</sup> = 0.9906. The fact that the Langmuir isotherm is in good

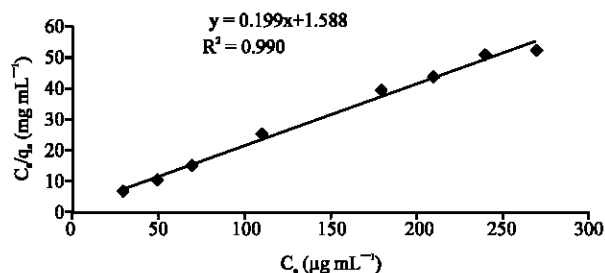


Fig. 9: Adsorption isotherms of 17β-estradiol cross-linked chitosan-polyacrylamide MIP beads, linearized according to the Langmuir equation

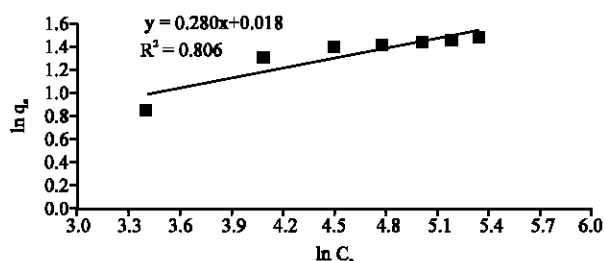


Fig. 10: Adsorption isotherms of 17β-estradiol cross-linked chitosan-polyacrylamide MIP beads, plotted according to the Freundlich equation

agreement with the experimental data may be due to homogenous distribution of caves on the MIP surface.

The empirical Freundlich Eq. 6 based on adsorption on a heterogeneous surface is given as follows (Bhatnagar and Jain, 2005):

$$q_e = K_p \times C_e^{1/n} \quad (6)$$

This expression can be linearized to give Eq. 7:

$$\ln = \ln K_p + \frac{1}{n} \ln C_e \quad (7)$$

where,  $K_p$  and  $n$  are the Freundlich constants, which represent adsorption capacity and adsorption intensity, respectively.  $K_p$  and  $n$  was determined from a linear plot of  $\ln q_e$  against  $\ln C_e$  (Fig. 10). The calculated results of the Langmuir and Freundlich isotherm constants are given in Table 1. Compared to Freundlich, the Langmuir plots have a higher correlation coefficient of 0.9906. It can be confirmed that 17β-estradiol adsorption by MIP follow the Langmuir Model with a maximum adsorption capacity of 5.01 mg g<sup>-1</sup> at 25°C under the range studied.

The essential characteristics of the Langmuir isotherm can be expressed in terms of dimensionless constant separation factor,  $R_L$  which is used to predict if an adsorption system is favourable or unfavourable. The separation factor,  $R_L$  is given by Eq. 8:

Table 1: Langmuir and freundlich isotherm model constants and correlation coefficient

Langmuir isotherm			Freundlich isotherm		
Q (mg g <sup>-1</sup> )	b (L mg <sup>-1</sup> )	R <sup>2</sup>	K <sub>p</sub> (mg g <sup>-1</sup> )	n	R <sup>2</sup>
5.01	0.126	0.9906	1.018	3.565	0.8069

Table 2: R<sub>L</sub> values based on the Langmuir equation

Initial 17β-estradiol concentration (mg L <sup>-1</sup> )	R <sub>L</sub> value
30	0.212
50	0.143
70	0.102
110	0.071
180	0.043
240	0.030

$$R_L = \frac{1}{1 + bC_0} \quad (8)$$

where, C<sub>0</sub> is the initial 17β-estradiol concentration (mg L<sup>-1</sup>) and b is the Langmuir adsorption constant (L mg<sup>-1</sup>). Table 2 lists the calculated results. Based on the effect of separation factor on isotherm shape, the R<sub>L</sub> values are in the range of 0<R<sub>L</sub><1, which indicates that the adsorption of 17β-estradiol on crosslinked chitosan-polyacrylamide MIP beads are favourable (Bhatnagar and Jain, 2005). Thus, this MIP is favourable adsorbent.

## CONCLUSIONS

The potential use of a molecular imprinted polymer prepared from acrylamide as functional monomer for the effective extraction of 17β-estradiol was investigated. It was shown that the rate of adsorption 17β-estradiol increased rapidly in the initial stage and then reduced until it reached equilibrium. The adsorption capacity of 17β-estradiol increased with increasing concentration of 17β-estradiol and the maximum adsorption was 5.01 mg g<sup>-1</sup> of the MIP beads.

These results good removal capacity of up to 91% which in close to that obtained by Celiz *et al.* (2009). The results showed that the adsorption process obeys a Langmuir adsorption isotherm. Important aspects of this work include the use of macroporous chitosan beads as functional matrix to offer functional groups and bead shape for the imprinted polymer. The prepared imprinted beads exhibit good characteristics, such as fast adsorption dynamics and high adsorption capacity. The fabricated MIP has potential use in a pre-concentration process in trace analysis of and also in development of stationary phase in liquid chromatography columns. Furthermore the MIP developed from cross-linked chitosan-polyacrylamide is inexpensive, non-toxic and easy to prepare.

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