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Molecular imprinted specific sorbents based on multiwalled carbon nanotube for the detection of progesterone through chromatographic urine analysis

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Abstract

Molecular imprinting approach was used to synthesize vinyl functionalized carbon nanotube supported progesterone imprinted polymer. The imprinted and non-imprinted polymers were characterized using infrared spectrophotometer, X-ray diffractometer and scanning electron microscopy. The structural superiority of synthesized polymer was reflected by the above measurements. The adsorption behaviour of polymer was evaluated using batch static adsorption experiments by the effect of concentration, temperature, mass, and solvent effect. Meanwhile, it was further demonstrated that the prepared adsorbent has high selectivity towards structurally similar analogues like testosterone and cholesterol. From urine analysis it is very clearly demonstrated the detection of progesterone in urine samples even in low circulating level. The approach we described would provide a new opportunity in the design of polymers with selective recognition properties.

Keywords: molecular imprinting, binding studies, progesterone, MWCNT

Introduction

"Molecularly imprinted polymers are artificially synthesized polymeric materials, each of which contains a large number of cavities that are complementary in terms of shape, size, and functional groups to bind a specific target molecule"[1]. Therefore, imprinted polymers show the ability to recognize specific molecules, and high binding affinities for these target molecules. "Despite wide applications and numerous fabrication methods of the conventional bulk molecularly imprinted polymers, it still suffers some intrinsic limitations, such as the heterogeneous distribution of the binding sites, the deep buried binding sites in bulk, and poor site accessibility for the template molecule"[2]. "To resolve these problems, scientists have made efforts to prepare molecular imprinted polymers based on various novel assisted matrices including silica and multiwalled carbon nanotubes"[3,4]. In response to such limitations, surface imprinting has been proposed as a viable strategy for protein imprinting. "Multiwalled carbon nanotubes (MWCNTs), which were first discovered in 1991"[5], are widely considered the quintessential nano material. Due to their high strength, extremely large surface areas, and unique chemical properties, MWCNTs can serve as a reinforcing element or the core when fabricating core-shell structural imprinted polymers. A layer of molecular imprinted polymers can be polymerized onto the surfaces of MWCNTs. Thus, "binding cavities in the thin imprinted polymer outer layer can greatly improve the

accessibility to template molecules"[6]. The vast majority of molecularly imprinted media are based on the use of organic acrylate or acrylic type polymers.

"Multiwalled carbon nanotubes are ideal support materials because they have strong interactions, stable under acidic conditions, no swelling and large surface area"[4]. "When the MWCNTs were used as a backbone for the polymerization of molecular imprinted polymers layer, the composite material has mechanical strength and chemical stability"[7]. Thus, the binding sites in the outer layer of the composite will improve the accessibility of the template molecule and reduce the binding time. "MWCNTs as the support matrix to prepare MWCNTs-MIPs have attracted great attentions in recent years"[8]. Among many methods for preparation of MWCNTs-MIPs, "vinyl group functionalized MWCNTs selective polymerization of polymers by covalent bonds on the MWCNTs surface reported by Kan et al. using MWCNTs as the reinforcement material in the imprinted polymer matrix"[2]. The unique properties of carbon nanotube make it desirable for different applications. For most of these applications "nanotubes require functionalization, such as changing some of the graphite properties to make nanotubes soluble in different media, or attaching different groups or even inorganic particles for future utilization of modified nanotubes"[9].

Experimental

Materials and methods

MWCNTs (internal diameter 2-6 nm, outer diameter 10-15 nm, length 0.1-10 m, and purity > 90%), ethylene dimethacrylate (EGDMA), 2,2 azoisobutyronitrile (AIBN), were purchased from Sigma-Aldrich Germany). Thionyl chloride ($SOCl_2$), dimethlysulfoxide (DMSO), dimethylformamide (DMF), tetrahydrofuran (THF) and triethylamine (TEA) were obtained from Merck, Germany. Triethylamine (TEA), acrylamide (AM) and acetic acid (HOAc) were obtained from SRL (India). Progesterone, testosterone and cholesterol were purchased from Sigma-Aldrich (Germany) and used as received.

The polymer morphology was investigated by scanning electron microscopy (SEM) using a JEOL-JSM-6390 A microscope. Binding studies were carried out using Shimadzhu UV-vis. Spectrophotometer. The FT-IR studies were carried out using Perkin-Elmer spectrum 400 FTIR spectrophotometer. Crystalline nature was studied using X- Ray diffractogram which was recorded by PAN analytic XPERT - PRO.

Modification of MWCNTs

The modification of MWCNTs is based on the protocols suggested in Scheme I.

i) Acid treatment of MWCNTs

"The carboxylic acid functionalized MWCNT (MWCNT-COOH) were synthesized as reported method"[10]. MWCNTs (0.5 g) were oxidized with 60 mL of concentrated nitric acid at 100°C for 12 h. After cooling to room temperature, the mixture was filtered and washed thoroughly with distilled water for several times until the pH of the final wash came down to neutral. The filtered solid was dried under vacuum to obtain carboxylic acid incorparated MWCNTs (MWCNTs-COOH).

ii) Acylation of MWCNTs-COOH

Functionalized MWCNTs (0.4 g) were suspended in the mixture of 10 mL $SOCl_2$ and 30 mL chloroform for 24 h reflux condition at 60°C. Excess $SOCl_2$ was removed by multiple washings of the solid with THF and then dried under vacuum to obtain MWCNTs-COCl.

iii) Vinylation of MWCNTs-COCl

MWCNTs-COCl (0.2 g) was dispersed in 30 mL THF and then 20 mL allyl alcohol dissolved in 10 mL of DMF was added drop wise to the mixture. The mixture was stirred at 60°C for 24 h and the solid collected after centrifugation was washed with THF. Finally the resulting solid was vacuum dried to obtain vinyl group incorporated MWCNTs (MWCNTs-$CH=CH_2$).

Estimation of carboxyl capacity of functionalised MWCNT

The extent of incorporation of carboxyl group on MWCNTs were followed by acid-alkali titration. The carboxyl capacity was found to be 2.4656 mmol/g.

Synthesis of progesterone imprinted polymer on MWCNTs

Development of imprinted polymer onto the vinyl group functionalized MWCNTs surface is depicted in Scheme II. Accordingly, the monomer, template, and the cross-linker were mixed together in a test tube followed by the addition of 60 mg MWCNTs-$CH=CH_2$ (dispersed in methanol). To this pre-polymerization mixture, AIBN (0.1 g) was added and the mixture was purged with N_2 gas for 10 min. Then the test tube was sealed and cured at 37°C for 10 h. Non-imprinted polymer was prepared following the above procedure but in the absence of template. In order to reveal the specialty of MWCNTs, an imprinted and non-imprinted polymer was also prepared without carbon nanotube by following the similar procedure as described above.

Binding studies

In order to evaluate the recognition properties of the polymer towards the target molecule, binding studies were carried out by batch experiments. Progesterone sample solution (7 mL) was allowed to contact with the imprinted and control polymer of same dimension. Binding conditions were optimised by varying the concentration of PGN solution, solvent and time. Selectivity of the imprinted polymer was investigated towards cholesterol and testosterone in addition to progesterone.

Column preparation

A total of 150 mg of the sorbent (molecular imprinted polymer) was poured into the SPE column. A PTFE frit was placed at both ends to prevent loss of the sorbents during the sample loading. Sample solution was delivered into the column by a programmable syringe pump (New Era Pump System). Before loading the sample, SPE cartridges were conditioned by passing 1 mL methanol and 1 mL deionised water. Then, 1 mL of urine sample or progesterone standard solution in deionised water was passed through the column at flow rate of 0.15 mL/min. The column was washed with 1 mL water/methanol (95:5, v/v) and then eluted with methanol/dichloromethane (80:20, v/v) at flow rate of 0.15 mL/min. The final extract was placed in a water bath (40°C) and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 mL of methanol.

Urine samples

Urine of a 2.5 year old girl was chosen as the blank throughout this study. Urine samples of a girl child were collected and stored at -20^{o}C in a freezer until analysis. The spiked urine samples were prepared by adding appropriate amount of progesterone standards in the blank urine. Urine samples were filtered through a 0.2 mm syringe filter before analysis.

Results and discussion

Synthesis of MWCNT based progesterone imprinted polymer

Development of MIP onto the vinyl group functionalized MWCNTs surface is depicted in Scheme 2. Accordingly, the monomer, template and the cross-linker were mixed together in a test tube followed by the addition of MWCNTs-CH=CH_2 (table 1.). To this pre-polymerization mixture, AIBN was added and the polymerisation was done. Non-imprinted polymer (NIP) was prepared following the above procedure but in the absence of template. In order to reveal the specialty of MWCNTs, an imprinted and non-imprinted polymer was also prepared without carbon nano tube by following the similar procedure as described earlier.

Characterization of progesterone imprinted and non-imprinted polymers on functionalised carbon nanotubes

i) Fourier transform infrared spectroscopy (FT-IR)

In order to confirm the presence of the co-monomer in the synthesised polymers, the FT-IR spectra were recorded. FT-IR spectrum of carboxyl group incorporated MWCNT showed various peaks corresponding to the carboxyl group as 3560 cm^{-1} (-COOH), 2920 cm^{-1} (-CH) and 1710 cm^{-1} (-C=O). The incorporation of chlorine on thionyl chloride treatment is confirmed by peaks at 2923 cm^{-1} (-CH), 1780 cm^{-1}(-C=O), 850 cm^{-1}(-C-Cl). MWCNT-CH=CH_2, 1740 cm^{-1} (C=O), 1624 cm^{-1}(C-C). The imprinted polymer showed carbonyl stretching at 1681 cm^{-1} characteristics of the amide groups and in addition, acrylamide co-polymer showed typical broad NH stretching at 3368 and 3469 cm^{-1}.

ii) Scanning electron microscopy (SEM)

SEM images of the progesterone imprinted and non-imprinted polymers with functionalised carbon nanotube are shown in the Fig. 1. The change in surface morphology of the imprinted and non-imprinted polymers has been investigated using this method.

iii) X-ray diffractogram (X-RD)

For the study of the crystallanity of the polymers X-ray diffractogram was taken. From the data (Fig 2) it is clearly understood the crystalline nature of the functionalised carbon nanotube impregnated polymers.

The MWCNT has three peaks corresponds to (002), (100) and (004) plane. On PGN imprinting, the two peaks remains corresponding to the MWCNT part. In MIP a broad peak is obtained which indicates the polymer part itself.

Investigation of the specificity of progesterone imprinted polymers

The binding of progesterone by the imprinted polymer is higher than the non-imprinted polymer (Fig. 3). This arises from the specific binding of PGN at the imprinted sites. During imprinting process, the binding

sites of the functional monomers undergo some rearrangement for optimum configuration and this is imprinted during polymerisation. Thus these sites retain memory of the shape and geometry of the progesterone and resulted in its specific binding. The imprinted polymer incorporated on MWCNTs showed high binding than the conventional imprinted polymer.

Among the two systems, the polymers with methacrylic acid have high binding affinity. This can be attributed to the effective interaction between the progesterone and the carboxyl group of the methacrylic acid units in the imprinted polymer host system.

Optimisation of the condition of PGN binding

From the above two studies we can conclude that the PGN imprinted EGDMA-crosslinked methacrylic acid based polymer on MWCNTs showed good binding capacity than those with EGDMA-crosslinked acrylamide based MWCNT-MIPs. Further studies were carried out using the PGN imprinted EGDMA-crosslinked methacrylic acid based polymer on multiwalled carbon nanotubes.

i) Effect of concentration

To evaluate the effect of concentration of progesterone solution on its binding, progesterone solution of different concentrations range from 1 -6 x 10^{-3} M were equilibrated with MWCNT imprinted and non-imprinted polymers and concentration of progesterone was followed spectrophotometrically at λ_{max} 242 nm.

From Fig. 4 we can infer that the solution with concentration 6 x 10^{-3} M optimum binding of progesterone. In particular non-imprinted polymers also showed a similar retention, which is non-specific due to the interaction of the progesterone with the nitrile groups of the base polymer and the functional group of the monomer. MWCNT-MIP exhibited much higher retention. The different sorptions between each imprinted and corresponding non-imprinted polymer represent the specific binding capacity. In addition, also the specific binding capacity of the MWCNT-MIP resulted the highest. This behaviour can be ascribed to the acid-base interaction between progesterone and methacrylic acid. The specific binding results from the formation of recognition sites complimentary to the progesterone in the shape and positioning of functional groups during the imprinting process.

Selectivity study

To compare the efficiency of the PGN imprinted polymer, specific binding studies were performed and compared with structurally similar compounds such as testosterone and cholesterol.

It should be mentioned that the imprinted polymer is a selective sorbent because, in the process of formation of imprinted polymer, the cavities created in the polymeric network are complementary in size and shape to the template for the subsequent rebinding process. These cavities are able to bind the template. Only molecules that are same in size and conformational structure can trap in the cavity. Therefore, the main problem that should be checked for imprinted polymer application is the adsorption of species consisting of functional groups at the surface of the polymer and non-imprinted polymer. The amounts of these analogues that adsorbed on the imprinted polymers were measured spectrophotometrically and compared with those on the non-imprinted polymers. The results show that the affinity and specificity of the MWCNT-imprinted polymer for

PGN were greater than those of other examined structure analogues (Fig.5). This fact could have been due to the high selectivity of the prepared imprinted polymer (MWCNT imprinted polymer). The increase of adsorption capacity is due to the physico-chemical properties, such as pore size and surface area.

Analysis of progesterone in urine using MWCNT-MIP

Urine of a 2.5 year old girl and 3 year old boy were spiked with three different amounts of progesterone to reach final concentrations of 100, 150 and 200 ng/mL. Spiked urine samples were processed under the proposed extraction conditions. The amount of template in the supernatant subtracted from the amount initially in the solution gave the amount of template adsorbed by the polymer.

In this study, standard addition was used for the determination of progesterone in spiked urine samples. The spectra of blank urine and spiked urine samples are presented in Fig. 6. In Table 2, the determined values of progesterone in urine samples are shown. The recoveries were 96-98.9 %, indicating that accuracy of this method is satisfactory for the analysis of progesterone. The recovery (R %) was calculated by the following equation:

$$R = \frac{Cdetected}{Cspiked} \text{ X } 100$$

where $C_{detected}$ and C_{spiked} are detected and spiked testosterone concentrations, respectively.

From the above studies it is clear that, using the molecular imprinted polymers we can easily identify the excess amount of hormones- progesterone in human urine. The method is simple and effective in the case of hormonal diseased people.

Conclusion

A molecular imprinted polymer impregnated with functionalised MWCNT was prepared. All the imprinted and non-imprinted polymers were characterised by FT-IR, XRD and SEM techniques. Binding studies were conducted. The results emphasised the choice of functionalised MWCNT as the functional monomer appropriate for the imprinting of progesterone and more effective than the conventional polymers. Urine analyses using the prepared polymers were done. The discussed method was highly sensitive and capable of quantifying low circulating levels of progesterone in urine specimens.

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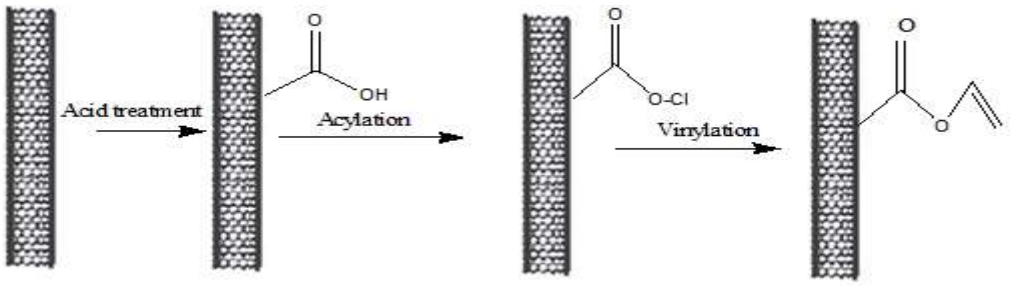
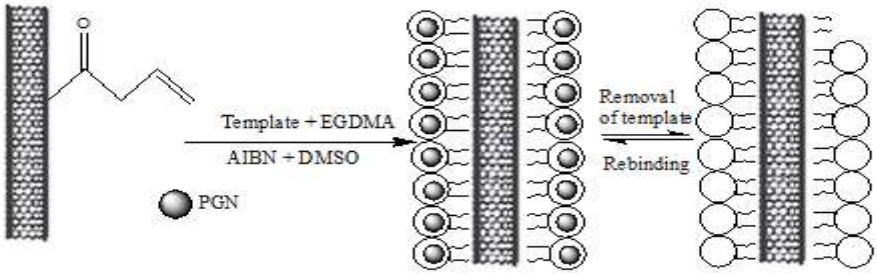


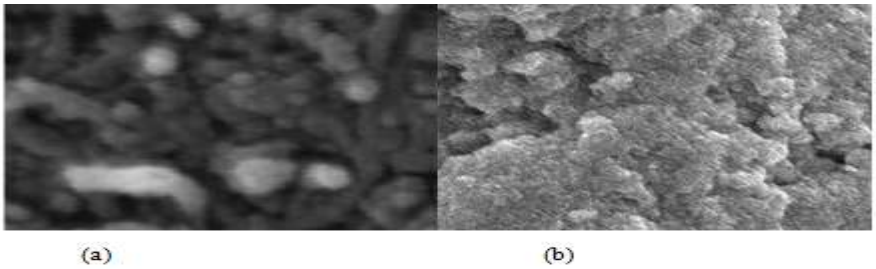
Scheme I. Functionalization of MWCNT



Scheme II. Schematic representation for the synthesis of progesterone imprinted polymer on MWCNTs

Table. 1. Various components for the preparation of MWNTs-MIPs

Polymer	MWCNTCH=CH_2 (g)	EGDMA (mmol)	AIBN (mg)	PGN (mmol)
MIP	0.02	1.25	10	0.05
NIP	0.02	1.25	10	0



(a) (b)

Figure 1. **Surface micrographs of PGN imprinted polymer(a) MWCNT-MIP and (b) without MWCNT|**

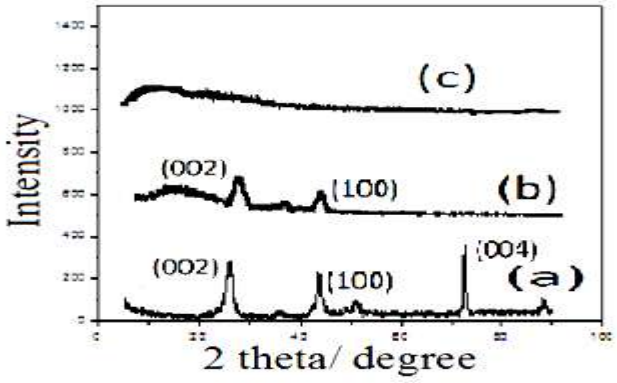


Figure 2. **XRD patterns of (a) MWCNT, PGN imprinted polymer (b) with MWCNT and (c) MIP**

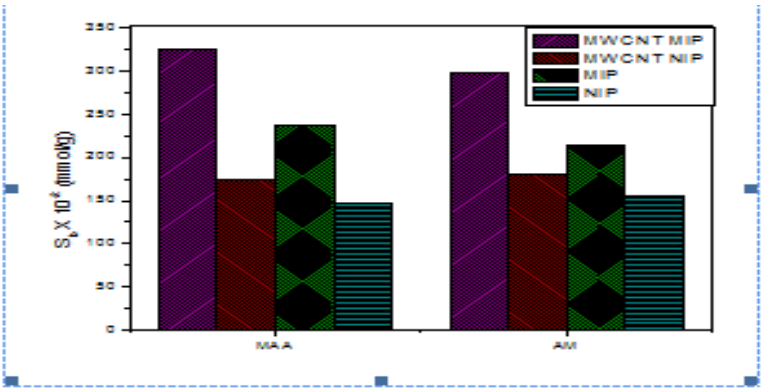


Figure 3. **Specific rebinding of PGN by imprinted and non-imprinted polymers**

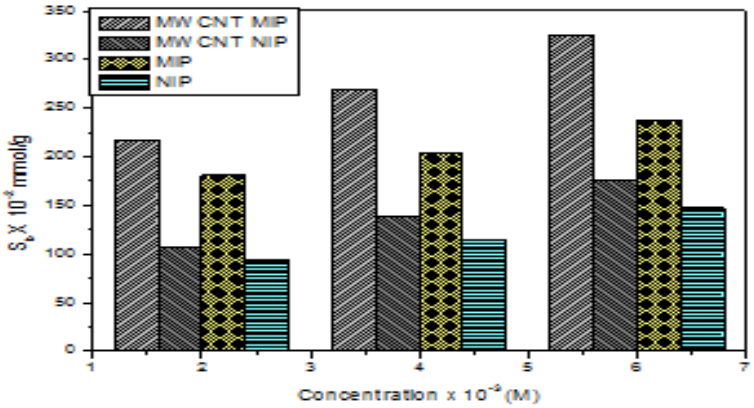


Figure 4. Effect of concentration of PGN on its rebinding by various polymers

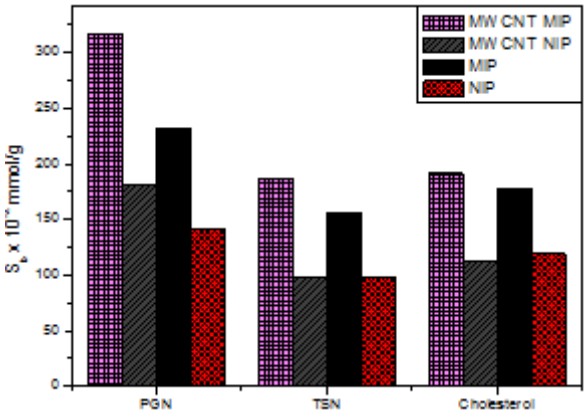


Figure 5. Selectivity study with cholesterol and testosterone

Table 2. Recovery (%) of PGN and TSN after extraction of spiked artificial urine by applying the optimized MISPE protocol

Samples	Spiked sample (ng/mL)	Detected sample PGN (ng/mL)	Recovery (%)	Relative standard deviation (%) (n=3)
1	100	97.6	97.6	3.8
2	150	148.8	98.67	4.9
3	200	198.4	99.2	6.8

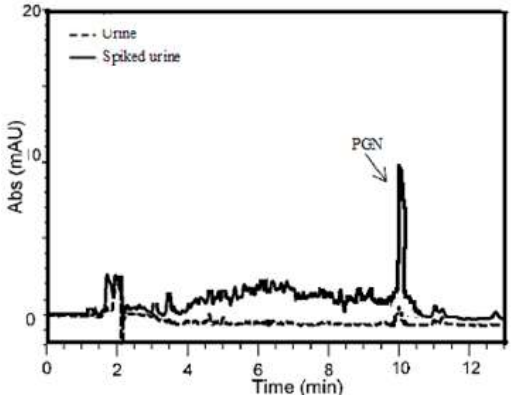


Figure 6. Representative chromatogram obtained from extract of the urine and standard mixture of progesterone