Research Journal of Recent Sciences _____ ISSN 2277-2502
Vol. 3**(ISC-2013),** 50-55 **(2014)** *Res. J. Recent. Sci.*

Molecular Imprinted Membranes as Synthetic Receptors for the Analysis of Progesterone in Human Urine

Anju Augustine and Beena Mathew
School of Chemical Sciences, Mahatma Gandhi University Kottayam 686560, Kerala, INDIA

Available online at: www.isca.in, www.isca.me
Received 26th November 2013, revised 31st December 2013, accepted 8th January 2014

Abstract

Progesterone imprinted copolymer membranes of acrylonitrile with acrylic acid, methacrylic acid and acrylamide were synthesised by phase inversion technique. The developed membranes were characterised by FT-IR and SEM techniques. Imprinted membranes showed specificity towards the template progesterone. Among the various copolymers, the acrylamide incorporated copolymer showed high binding towards the used template. Investigation of the selectivity characteristics revealed that the developed membranes showed selectivity toward the template progesterone than similar compounds. The bound template could be totally recovered and regenerated membranes maintain their recognition property after repeated use. On the basis of the results, the imprinted polymer can be applied for the direct extraction of progesterone in clinical analysis.

Keywords: Molecular imprinting, acrylic copolymers, membranes, progesterone, binding capacity, urine analysis.

Introduction

Molecular imprinting technology has become an effective way to prepare polymer materials that show a "memory effect" towards the selected templates[1]. Compared to other recognition systems, molecular imprinted polymers possess many promising characteristics and properties such as low cost and easy synthesis, high stability to harsh chemical and physical conditions, and excellent reusability[2]. The molecular imprinting technique, first proposed by Wulff in 1972[3], is one of promising and facile methods to impart molecular recognition sites in synthetic polymers. Molecular imprinted polymers have been used for molecular recognition ranging from small molecules[4-6] to macromolecules[7-9]. The improper or illegal use of hormones as veterinary drugs may result in unwanted residues in food products derived from live stock breeding. However the direct detection of target corticosteroids in complex biological matrices can be a difficult task, and sample clean-up treatments are frequently necessary before performing the instrumental analysis. To extract synthetic corticosteroids from biological samples using the MIP approach efficiently, an imprinted polymer should be able to selectively recognise the main analytical target by making use of several non covalent interactions. The preparation of membranes using imprinted polymers opened a new way in the field of separation[10]. Membrane technology has been already applied in many industrial fields but the possibility to introduce specific recognition sites in a synthetic membrane plays an important role for the transport of specific substances[11, 12].

Progesterone (PGN) is a naturally occurring estronic compound, and a product of cholesterol by a long biosynthetic pathway. It is produced mainly in ovaries, also in adrenal cortex, brain, testes and during pregnancy in placenta. In humans, PGN produced naturally in both sexes, but women have much higher concentration[13]. Progesterone is required for the normal functioning and native circulation in the human body but carcinogenic effects are possible at enhanced levels [14]. Progesterone presents several binding sites capable to interact via H-bond with the amide and carboxyl groups of the synthesised polymers. At proper level PGN is necessary for normal functioning and at elevated levels they can be toxic and carcinogenic because they are growth promoters[13]. The present paper deals with the investigation of the influence of the nature of functional monomers on the binding capacity of progesterone imprinted membranes. Imprinted membranes were prepared via wet phase inversion technique from acrylonitrile copolymers. Three kinds of polyacrylonitrile based membranes were developed. A functional monomer like acrylamide, methacrylic acid or acrylic acid was used to create binding site in the polyacrylonitrile based membranes. The PGN imprinted and non-imprinted polymers were investigated for their specific and selective binding of PGN.

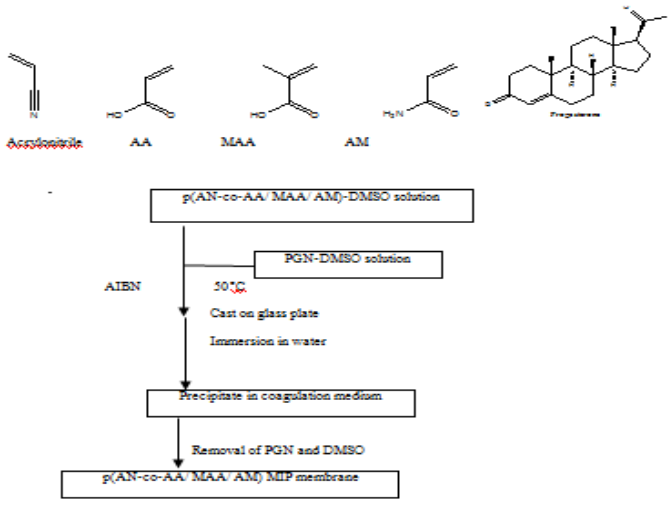
Material and Methods

Acrylonitrile (AN), acrylic acid (AA), dimethyl formamide and dimethylsulphoxide (DMSO) were of purity grade reagents purchased from Merck, Germany. Acrylonitrile and acrylic acid were distilled under reduced pressure 78^{o}C. DMSO was purified by distillation at 80^{o}C under reduced pressure (18 mm Hg). Methacrylic acid (MAA) was purchased from Aldrich and is distilled in vacuum prior to use in order to remove stabilizers. Acrylamide was purchased from SRL (India). All other reagent

grade products were used without further distillation. 2, 2'-Azobis (isobutyrilo nitrile) (AIBN; Fluka, purity >98%) was used as the initiator and kept in a freezer until use. Progesterone, testosterone and cholesterol were purchased from Sigma-Aldrich (Germany) and used as received.

The membrane 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.

Membrane preparation: General procedure: For the preparation of MIP membranes, the co-polymer contains acrylonitrile and the co-monomer in the ratio 95:5 (6.2 mL of AN and 0.34 mL of AA, 0.36 g of AM, 0.42 mL of MAA) and 97.5:2.5 (6.38 mL of AN and 0.17 mL of AA, 0.178 g of AM, 0.21 mL of MAA) were dissolved in the solvent (DMSO 15 mL). 0.01 g of AIBN as initiator was also added and kept in a thermostatic bath at 50^{o}C. The reaction was conducted under nitrogen atmosphere for 4 h.



Scheme-1
Schematic representation of the synthesis of PGN imprinted polymers

For the preparation of MIP, progesterone (0.16 g for 95:5 and 0.078 g for 97.5:2.5 ratios) was added to the casting solution. The membranes were cast onto a glass plates (70 x 100 x 4 mm) with a film thickness of 250 µm. After casting, the membrane forming solutions were immersed in water coagulation bath of 2 L for 30 min at room temperature. The membranes were then transferred in fresh water. In the case of MIP membranes, the progesterone was washed out using methanol-acetic acid solution and complete removal of progesterone was confirmed spectrophotometricaly at λ_{max} 242 nm. All membranes were stored in fresh water. NIP membranes were also prepared as described earlier excluding progesterone.

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

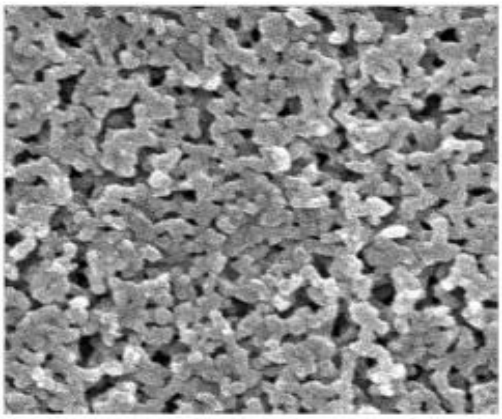
Column preparation: A total of 150 mg of the sorbent (molecular imprinted membranes) was poured into the SPE column in connection with a UV detector. A PTFE frit was placed at both ends to prevent loss of the sorbents during the sample loading. Sample solution was delivered into the column by a programmable 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^{o}C) and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 mL of methanol.

Urine samples: Urine of a 3 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.

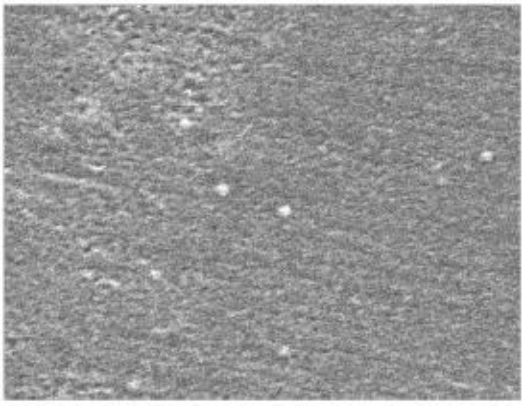
Results and Discussion

Synthesis of polyacrylonitile based progesterone imprinted membranes: Polyacrylonitrile membranes with co-monomers as acrylic acid, methacrylic acid and acrylamide were prepared by water-phase precipitation copolymerisation at 95:5 and 97.5: 2.5 polyacrylonitrile to functional monomer ratio with a thickness of 250 µm. The membranes were insoluble in water, and precipitated in the aqueous phase (Scheme 1). The phase inversion method used for membrane preparation obtained flat sheet membranes. The prepared membranes were stored in fresh water after removing the template completely.

Characterisation: In order to confirm the presence of the co-monomer in the synthesised copolymers, the FTIR spectra were recorded. There is a band at 1736 cm^{-1} due to C=O stretching of the acrylic and methacrylic carboxyl groups. p(AN-co-AM) copolymer shows a similar carbonyl stretching at 1681 cm^{-1} characteristics of the amide compounds. In addition, acrylamide co-polymer shows typical broad NH stretching at 3368 and 3469 cm^{-1}.



(a)



(b)

Figure-1
Surface micrographs of PGN (a) imprinted and (b) nonimprinted p(AN-co-AM)s

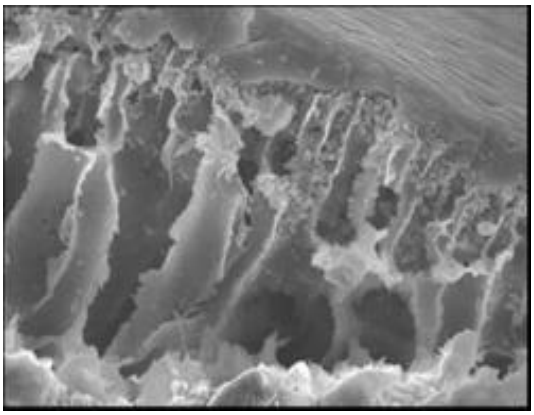


Figure-2
Cross section morphology of p(AN-co-AM) MIP

SEM images of the progesterone imprinted and non imprinted membranes of poly (acrylonitrile-co-acrylamide) are shown in the Figure-1. MIP is characterised by the finger like macro voids and a sponge like layer near the top surface Figure-2. But in NIP it is just like a flat sheet without any cavities on the surface.

Binding studies: Investigation of the specificity of PGN imprinted membranes: The binding of PGN by the imprinted membranes is higher than the non imprinted membranes figure-3. This arises from the specific binding of PGN at the imprinted sites which were sculpt during imprinting with size and shape complementary to the PGN molecule. During imprinting process, the binding sites of the functional monomer and PGN undergo some rearrangement for optimum configuration and this is sculptured during polymerisation. Thus these sites retain memory of the shape and geometry of the PGN and resulted in the specific binding.

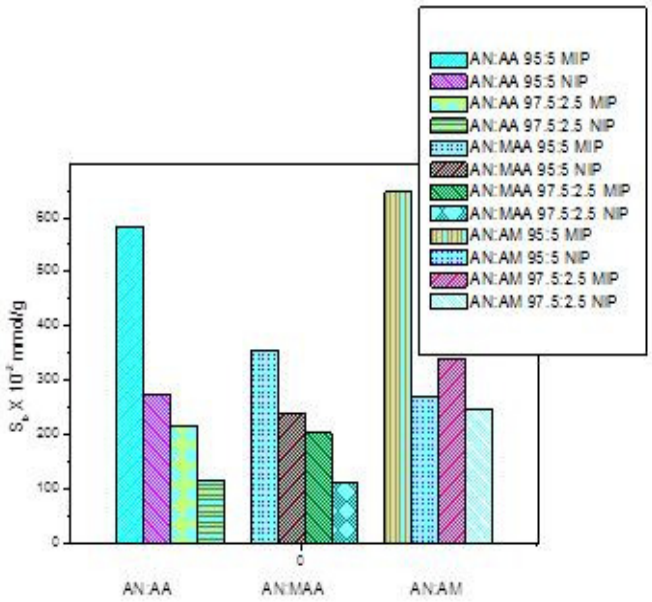


Figure-3
Specificity of PGN by imprinted and non-imprinted polymers

Among the three systems with varying composition of functional monomers, the 95:5 systems has high binding affinity. This can be attributed to the increased composition of functional monomer which resulted in effective interaction between the PGN and the host system. Among the co-monomers, acrylic acid, methacrylic acid and acrylamide, the acrylamide system has high binding. This can arise from the effective H-bonding affinity of the acrylamide with PGN.

Dependence of functional monomer - PGN ratio: From the specificity study, we understood that the AN: AM system shows high specificity. So inorder to get an idea about the dependence of the composition between the functional monomers AM and template PGN, we prepared three different ratios such as 1:5, 1:10 and 1:15. In all these systems, the extend of PGN binding increased with increasing composition of the functional co monomer and the 1:15 ratio shows high binding.

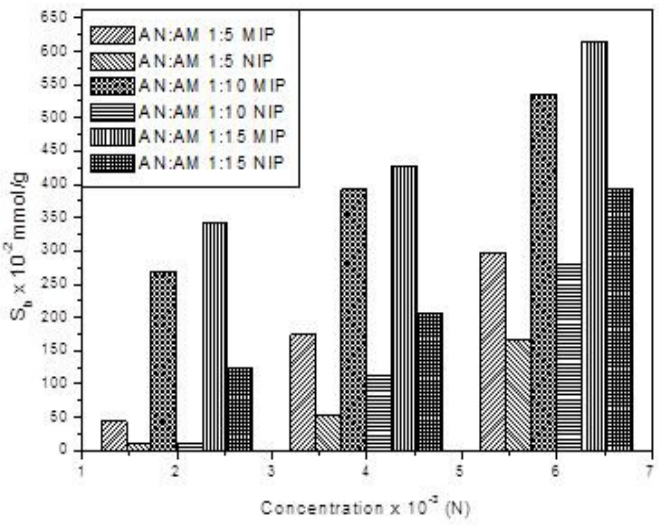
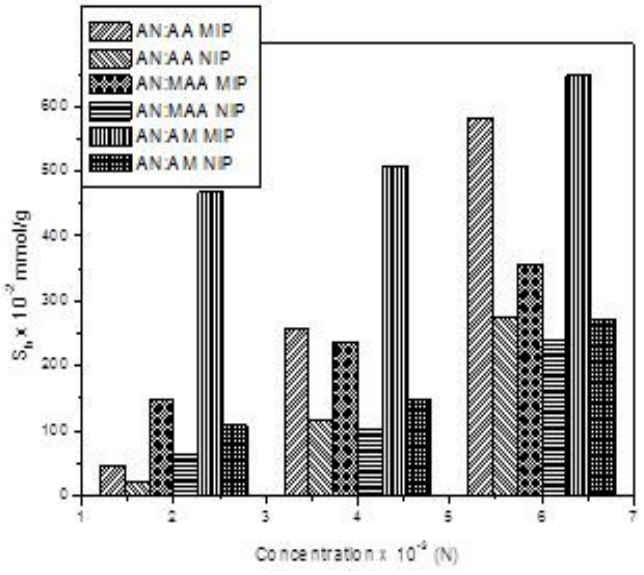


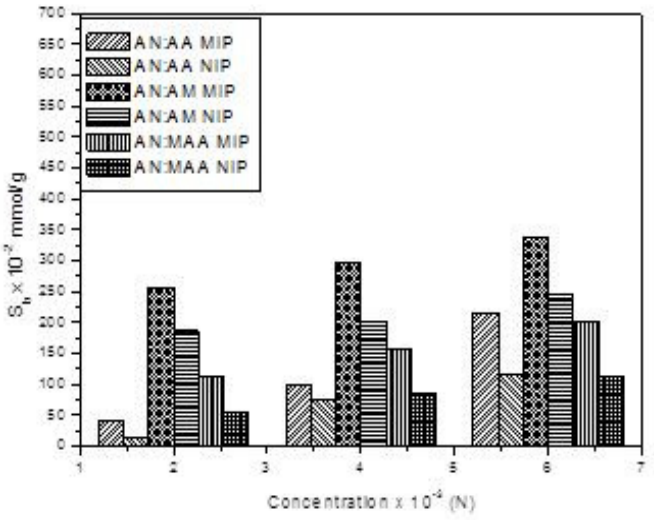
Figure-4
PGN rebinding by p (AN-co-AM) based systems with 1:5, 1:10 and 1:15 AM to PGN ratio

From the figure-4 it is clear that the 1:15 system shows maximum binding towards PGN. This is because as the amount of functional monomer increases, the number of binding sites also increases. So as the binding also.

Optimisation of the conditions of PGN binding: Effect of concentration: To evaluate the effect of concentration of PGN on its binding, progesterone solution of different concentrations ranging from 1 - 7 x 10^{-3} N were equilibrated with imprinted and non imprinted membranes and concentration of PGN was followed spectrometrically at λ_{max} 242 nm. Among three co-monomers, the acrylamide co-monomers showed high affinity.



(a)



(b)

Figure-5
Effect of concentration of PGN on the polymer ratios (a) 95:5 and (b) 97.5:2.5

From figure-5. we can infer that 6 x 10^{-3} N is the concentration for optimum binding of PGN. In particular NIP membranes 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 co-monomer. All MIP membranes exhibited much higher retention. The different sorption between each MIP membrane and corresponding NIP represent the specific binding capacity.

Imprinted membrane with 95:5 AN to AM ratio showed the highest overall binding capacity. In addition, also the specific binding capacity of this membrane resulted the highest. This behaviour can be ascribed to the acid-base interaction between the weakly acidic phenolic hydrogen of progesterone and the amide group of acrylamide, the nature of which is rather basic. 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.

Effect of solvent: The effect of solvent on PGN binding was studied using DMSO and DMF.

From the figure-6 it is clear that the polymer prepared with DMSO is the best for the binding of PGN. Specific binding of PGN was also high for the DMSO imprinted polymer. This is mainly due to the porous nature of the polymer.

Effect of time: Imprinted membrane reached the saturation limit after 80 min. Whereas for the control polymer this limit is 60 min. This different behaviour can be attributed to the effective specific recognition properties of the MIP membranes. In the beginning the amount of progesterone bound by the MIP was much higher than the NIP. This difference in time increased until template molecule saturates the complimentary saturation

points. Also the PGN took more time to penetrate to the pre-arranged imprinted sites. But in the control polymer, there are no such pre-arrangements.

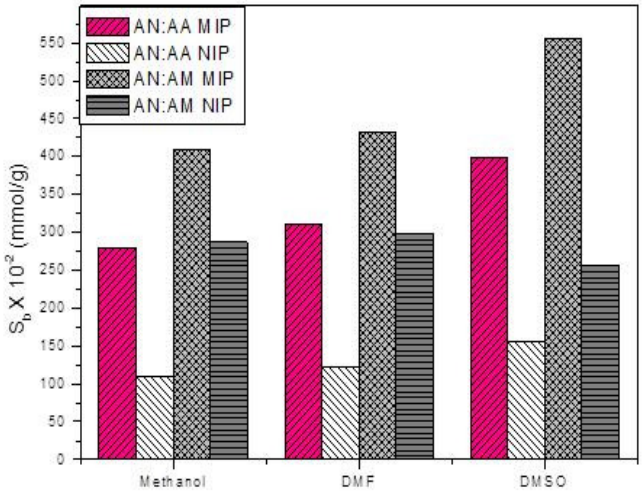


Figure-6
Solvent effect on the binding of MIP and NIP

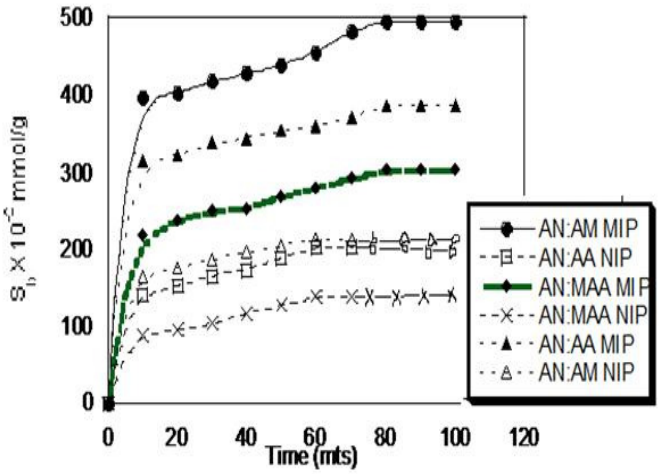


Figure-7
Effect of time on the binding of the MIP and NIP

Desorption and Regeneration Studies: To test repeatedly using the imprinted membranes and to recover the template, the recovery and regeneration tests were conducted. The imprinting process is to be considered as an economical process, it is necessary that the spent imprinted membranes to be regenerated. This experiment investigates the extent of PGN desorption from the membranes. Desorption and regeneration studies were carried out using methanol-acetic acid solution. Imprinted membrane having a binding quantity of 95.3 % of PGN, 90.8 % was desorbed in methanol-acetic acid solution. The recovery of PGN was decreased 90.8 % in the first cycle to 87.3 % in the third cycle. The result shows we can regenerate the spent membranes using methanol-acetic acid solution. The regenerated membranes showed uptake efficiency comparable to that of the fresh one over three cycles.

Selectivity: 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.

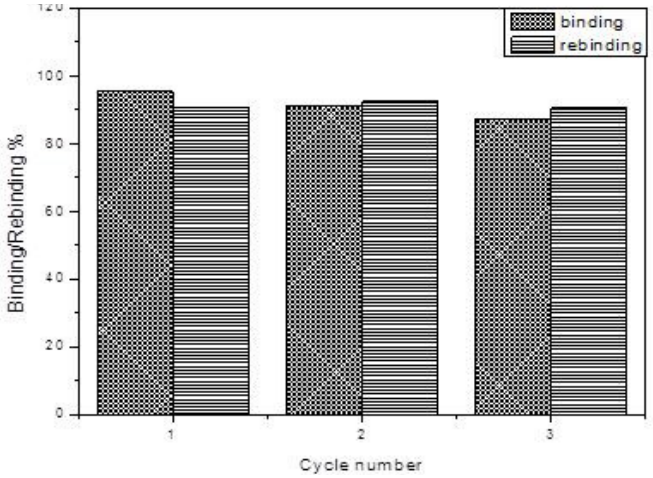


Figure-8
Three cycles of PGN binding-rebinding

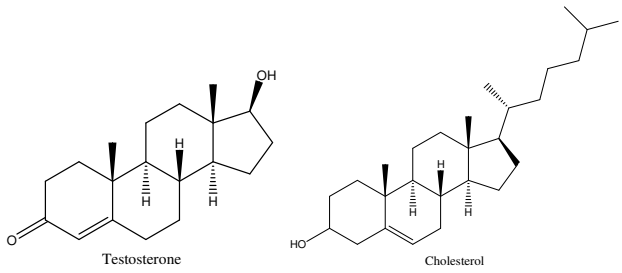


Figure-9
Structures of the competing molecules

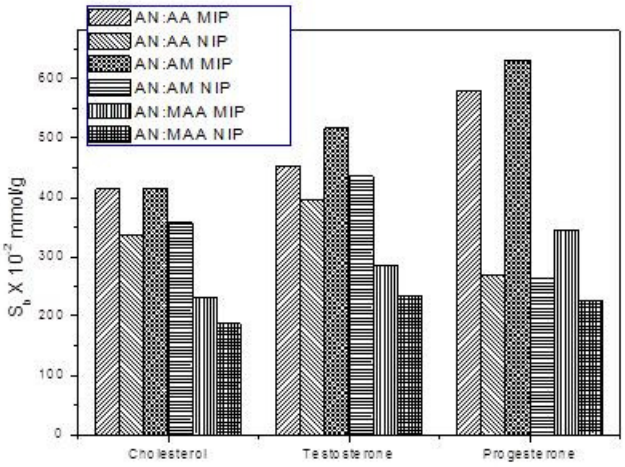


Figure-10
Selectivity study with cholesterol and testosterone

From the figure-10 it is clear that, the imprinted polymers strongly identify progesterone than testosterone and cholesterol. This is due to the complementary binding site available in the membrane, which was created in the time of polymerisation. This strongly suggests that the imprinted membranes can be effectively used as an absorber for progesterone. Also the acrylonitrile – acrylamide based polymer system showed higher selectivity to the template PGN.

Analysis of urine samples: Urine of a 3 year old girl was spiked with three different amounts of progesterone to reach final concentrations of 120, 150 and 200 ng/mL. Spiked urine samples were processed under the proposed extraction conditions.

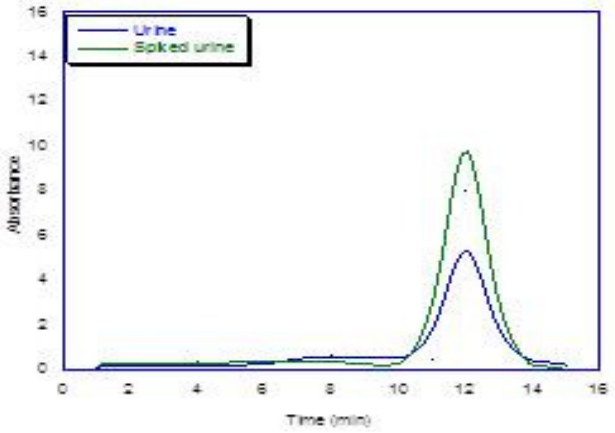


Figure-11
The comparison of recovery values between MWCNT MIP and other polymers in elution step

Table-1
Recovery (%) of PGN after extraction of spiked artificial urine by applying the optimized MISPE protocol

Samples	Spiked sample (ng/mL)	Detected sample (ng/mL)	Recovery (%)
1	120	115.6	96.33
2	150	147.3	98.2
3	200	197.8	98.9

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 figure-11. In table-1, the determined values of progesterone in urine samples are shown. The recoveries were 97-109%, indicating that accuracy of this method is satisfactory for the analysis of progesterone. The recovery (R%) was calculated by the following equation:

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

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

Conclusion

Different functional monomers such as AA, MAA, AM were copolymerised with AN by water phase precipitation polymerisation process. PGN imprinted and non imprinted films were synthesised in varying composition. But the efficiency in specific rebinding followed the order : p(AN-co-AM) > p(AN-co-MAA) > p(AN-co-AA). Among the various compositions studied acrylonitrile to acrylamide ratio 95:5 is having high binding properties. All the imprinted and non imprinted membranes were characterised by FT-IR and SEM techniques. The results emphasised the choice of acrylamide as the functional monomer appropriate for the imprinting of progesterone and more effective than those functional monomers with carboxyl groups.

Reference

1. Ovidiudima S., Sarbu A., Dobre T., Bradu C., Antohe N., Lauraradu A. and Nicolescu T.V., A. Lungu Materiale Plastics **46,** 4 **(2009)**

2. Chen L., Xuab S. and Lia J. Chem. Soc. Rev., **40,** 2922 **(2011)**

3. Wulff G., A. Sarhan Angew Chem., **84,** 364 **(1972)**

4. P. Wang, H. Zhu, W. Zhang, Z. Ye, R. Zhu and X. Su, *J. Sep. Sci.,* **36,** 1455 **(2013)**

5. L. Hillberg and M. Tabrizian, ITBM-RBM, **29,** 89 **(2008)**

6. L. Ye and K. Mosbach, *Chem. Mater.,* **20,** 859 **(2008)**

7. F. Trotta, M. Biasizzo and F. Caldera, *Membranes,* **2,** 440 **(2012)**

8. N.A. Yusof, N.D. Zakaria, N.A.M. Maamor, A.H. Abdullah and Md. J. Haron, *Int. J. Mol. Sci.*, **14,** 3993 **(2013)**

9. X.W. Kan, Y. Zhao, Z.R. Geng, Z.L. Wang and J.J. Zhu, *J. Phys. Chem. C,* **112,** 4849 **(2008)**

10. R. Thoelen, R. Vansweevelt, J. Dughateau, F. Horemans, J. D'Haen, L. Lutsen, D. Vanderzande, M. Ameloot, M. vandeVen, T.J. Cleij and P. Wagner, Biosens. *Bioelectron.,* **23,** 913 **(2008)**

11. Alenus, P. Galar, A. Ethirajan, F. Horemans, A. Weustenraed and T.J.C.P. Wagner Phys. Status Solid A 209, 5, 905 **(2012)**

12. T. Alizadeh, N. Memarbashi Separation and Purification Technology, **90,** 83 **(2012)**

13. J. Ricanyová, R. Gadzała-Kopciuch, K. Reiffova , Y. Baze and B. Buszewski Adsorption, **16,** 473 **(2010)**

14. K.P. Singh, R.K. Prajapati, S. Ahlawat, S. Ahlawat, M. Mungali and S. Kumar, *Open J. Appl. Biosensor*, **2,** 20 **(2013)**