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Recognition and Release of Nalidixic Acid Using Uniformly Sized -Imprinted Nanospheres: Methacrylic Acid to Methyl Methacrylate Different Mole Ratios

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Abstract: In the presence of imprinting molecules of Nalidixic acid, uniformly sized molecularly imprinted polymers (MIPs) in nanometer range were synthesized. The MIPs were successfully prepared by precipitation polymerization using methacrylic acid (MAA) and methyl methacrylate (MMA) as functional monomers at different mole ratios. The effect of combination of MAA-to-MMA on the morphology, binding, recognition and release behaviors of the final particles were studied. The produced polymers were characterized by differential scanning calorimetry and their morphology was precisely examined by scanning electron microscopy. We obtained very uniform imprinted nanospheres with diameter of 120- 180 nm. Among the MIP nanospheres the MIPs using combination of MAA and MMA showed nanospheres with lowest mean diameter (120 nm) and the highest selectivity factor (9.7). The adsorption properties of Nalidixic acid in acetonitrile for imprinted nanospheres were evaluated by equilibrium rebinding experiments. Results from binding experiments proved that MIPs exhibit specific affinity to Nalidixic acid in contrast to control polymers and this performance was affected by pH of loading solution and. Moreover, release experiments showed the controlled release of Nalidixic acid in longtime period. The loaded Nalidixic acid was released from the imprinted nanospheres within the 140 h.

Key words: Molecular imprinting · Uniformly sized · Nanospheres · Functional monomers · Nalidixic acid · Recognition · Controlled release.

INTRODUCTION

Molecular imprinting technology provides a way to preparation of new polymer materials containing specific recognition sites for target molecules (template) [1]. This technology is attracting wide spread attention because of its potential to deliver robust molecular recognition elements targeted toward any guest really present in any environment (e.g. drug enantiomers, hormones, toxins, pesticides, peptides, proteins and nucleic acids in matrixes ranging from pure organic solvents to biological fluids) [2]. The imprinting process is performed by copolymerization of functional monomers and cross-linker in the presence of a template molecule. The functional groups on the monomers are then fixed in polymer net work via chemical cross-linking of these monomers [3]. The shape, size and chemical functionality of the recognition sites are complementary to those of the template molecule. Thus molecularly imprinted polymers (MIPs) can be a highly specific rebind of template molecule [4]. Molecularly imprinted polymers (MIPs) are often referred to as ‘‘artificial antibodies’’. Unlike antibodies, MIPs are stable to extremes of pH, organic solvents and temperature, which allows for more flexibility in the analytical methods. MIPs have been prepared in various configurations, including polymer monoliths, membranes, microspheres and nanospheres for different applications. MIPs with well-controlled physical forms in

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different size ranges are highly desirable. For example, MIP nanospheres are very suitable to be used to develop binding assays, microfluidic separations, the stationary phase design for open-tubular capillary electrochro matography and drug delivery systems [5]. The MIP nanospheres are ideal to apply in the well-established nonseparation assay formats, for example, using measurements based on fluorescence polarization-depolarization or fluorescence resonance energy transfer technique [6]. Nanosphere drug delivery carriers are able to protect drugs from degradation and improve the penetration or permeation of drugs across the mucosal surface [7]. Typically, MIPs were prepared using bulk polymerization, where the resultant brittle monoliths had to be ground to create a large surface area which is molecularly imprinted and sieved to the desired particle size. Although this method allows easy preparation of large amount of MIPs, it is time consuming and yields only moderate amounts of useful MIPs, The irregularity of size and shape of such MIP particles also made sample handling difficult and chromatography efficiency reduced. For new analytical applications, the irregular particles are inferior to well-defined polymer beads, especially in developing MIP-based assays, sensor arrays and separation modules. In addition to improving binding performance of MIPs, new physical formats of MIPs and more efficient synthetic method ologies were important research topics in the past years [8]. Recently, novel methods to synthesize MIPs have been reported, including precipitation polymerization, suspension polymerization using fluorocarbon liquid or mineral oil as a continuous phase, swelling polymerization and grafting procedures. A relatively simple method for the preparation of imprinted polymers not requiring mechanical grinding or dispersion polymerization is precipitation polymerization, which yields uniformly sized nanospheres or microspheres [9, 10]. The current study investigates the potential of precipitation polymerization as a promising technique for the synthesis of novel imprinted materials for various applications such as analytical field and drug delivery systems. In this work, the new synthetic conditions are used to obtain very uniformly sized imprinted nanospheres for Nalidixic acid as a model template. Nalidixic acid is Nalidixic acid [1-Ethyl-1, 4-dihydro-7-methyl-4-oxo-1,1,8-naphthyridine-3-carboxylic acid] is a 4-Quinolone antibacterial agent, active against eterobacteriaceae and given by mouth mainly to treat urinary-tract infections. Precipitation polymerization is carried out using methacrylicacid (MAA) and methyl methacrylate (MMA) as functional monomers, trimethylolpropanetrimethacrylate (TRIM) as cross-linker and 2,2'-azobisisobutyronitrile (AIBN) as initiator in acetonitrile asprogenic solvent at 60°C. MAA, MMA and TRIM (acrylate ester) that were the compounds of interest used in this work as the monomers are found to have biocompatibility and nontoxicity. The choice of functional monomer is more critical to the promotion of the imprinting efficiency and other properties of imprinted polymers. Normally, MIPs have been prepared with only a single type of functional monomer. Occasionally, combinations of functional monomers have been used to improve the properties and performance of MIPs with respect to those produced with a single functional monomer [11]. To study and improve the morphology, binding, recognition and release properties of MIPs when the ratio of the acidic monomer MAA and the neutral monomer MMA are used, Nalidixic acid -imprinted polymers were prepared. The three-dimensional structure of binding sites in polymers containing MAA and MMA are improved because of steric hindrance of methyl groups in the side chains. The most successful non-covalent imprinting systems are based on MAA since the carboxylic acid groups of MAA serve as both hydrogen donor and acceptor. It is also a suitable functional monomer for Nalidixic acid, because carboxylic acid group scan form cyclic hydrogen bonds with the functional groups on Nalidixic acid. The neutral monomer of MMA was used to control composition and properties of MIPs. In the current study the binding properties of the MIPs to Nalidixic acid were evaluated by their binding capacity to Nalidixic acid in loading solution with different pH. The molecular recognition ability with respect to template analog(Oxolinic Acid) was also determined. To study Drug release property of particles, nalidixic acid release was carried out using a sodium hydroxide solution.

MATERIALS AND METHODS

Experimental

Materials: Nalidixic acid (99%) and oxolinic acid, was obtained from Sigma-Aldrich (St Louis, MO, USA). Methacrylic acid (MAA)and methyl methacrylate (MMA), trimethylolpropanetrimethacrylate (TRIM, technical grade) and 2, 2'-azobisisobutyronitrile (AIBN, 98%) were purchased from Sigma-Aldrich. Methanol, Acetic acid (glacial, 100%), acetonitrile (99.7%) used for polymer synthesis were purchased from Merck (Darmstadt, Germany). AIBN was re-crystallized from methanol before use.

Table 1: Preparation of Nalidixic acid-Imprinted and non-imprinted Polymers

Polymer	Template (mmol)	MAA (mmol)	MMA (mmol)	TRIM (mmol)
MIP1	0.5	2	-	1.5
NIP1	-	2	-	1.5
MIP2	0.5	1.5	0.5	1.5
NIP2	-	1.5	0.5	1.5

Polymer Syntheses: Molecularly imprinted nanoparticles were synthesized using precipitation polymerization based on mole ratios described in Table 1. For the preparation of the Nalidixic acid -imprinted polymer, the template (Nalidixic acid,
0.5mmol) was dissolved in 10 mL of acetonitrile in a 60-mL borosilicate glass tube equipped with a screw cap and then the functional monomers (MAA and/ or MMA) were added. Then the mixture was sonicated in an ultrasonic bath (XB2, England) for 5 min until clear solution was obtained. The solution was gently mixed for 60min and the initiator (AIBN), the cross-linker (TRIM) and 40 mL of acetonitrile were added to the above solution and the mixture was homogenously dispersed by sonication for 5 min. After sonication it was purged with N_2 gas for 15min to get rid of oxygen in the solution, which would have retarded the synthesis process due to the annihilation of free radicals produced from the decomposition of the initiator. The borosilicate glass tubes were sealed under N_2 atmosphere to prevent air from entering it. The reaction vessel was inserted in a shaker bath (Memmert, WB22, Germany) and shaken horizontally at50 cycles per minute. The temperature was ramped from 25 to 60°C within 40 min, thereafter kept for 20h. After the polymerization, the polymer particles were separated by centrifuging (Hermle, Z36HK, Germany) at 20000 rpm for 30min. To remove template from the polymer matrix, the unleached imprinted polymers were washed with 40 mL of methanol containing 10% acetic acid (v/v) for 6 times for 1 h, until no template could be detected from the washing solvent by spectrometric method (at λ260 nm) (Jenway, 6305, England). The polymer particles were finally washed with same volume of deionized water and the resulted leached imprinted polymers were dried at 50°C overnight. To verify that retention of template was due to molecular recognition and not due to nonspecific binding, a control, non-molecularly imprinted polymer (NIP), was prepared as the same procedure, but with the omission of Nalidixic acid.

Differential Scanning Calorimetry (DSC) and Morphologic Analysis: Thermal properties of polymer particles were investigated by a Mettler DSC 823 (Mettler Toledo, GmbH, Switzerland) equipped with a Julabo The rmocryostate Model FT 100Y (Julabolabortechnik Gm bH, Germany). A Mettler Star software system (version 9.x) was used for the data acquisition. Indium was used to calibrate the instrument. The samples were scanned at a heating rate of 10°C min^{-1} in 20-470°C temperature range.

The shape, size and surface morphology of the polymers was estimated by scanning electron microscopy (TESCAN, VEGAII, Czech).Polymeric particles were sputter coated with gold before the SEM measurement.

Binding Experiments: The 40 mg of imprinted and non-imprinted particles were added to 6 mL of 20 mg/L Nalidixic acid w ater/acetonitrile (4 : 1, v/v) solution (pH = 7). A concentration of 20 mg/L Nalidixic acid was chosen in order to investigate the performance of MIPs in possible biomedicalor biological applications. After the samples were stirred for 2 h, the polymer particles were centrifuged at 20,000 rpm for 30 min. The concentration of free Nalidixic acid in supernatant was measured by UVspectrophotometer. The amount of Nalidixic acid bound to the polymer particles was calculated by subtracting the amount of free Nalidixic acid from the Nalidixic acid initially added.

The effect of pH on the binding efficiency of mg/L was investigated by varying the pH of solutions from 3 to 11. All pH were adjusted with hydrochloric acid and sodium hydroxide solutions. Several batch experiments were performed by incubating 40 mg of MIP or NIP nanospheres with 6 mL of 20 mg/L of Nalidixic acid water/acetonitrile (4: 1, v/v) and applying a gentle mixing under the desired range of pH for 2 h. Then, the amount of Nalidixic acid bound to the nanospheres was measured.

The selectivity of the imprinted polymers was determined by comparing the equilibrium adsorption ability to the template with that to the structural analogue. Fixed amounts of polymer particles was incubated with 20 mg/L of template analog (Oxolinic Acid) in 6 mL of acetonitrile - water (4: 1, v/v) solution (pH = 7) and then proceeding to binding experiments.

***In vitro* Drug Release:** Drug release from CBZ-loaded MIPs was carried out using dissolution method in SDS (5 wt%) aqueous solution as medium. The polymer particles (50 mg) were incubated with 6 mL of 0.5 mmol/ L

Nalidixic acid water/acetonitrile (1 : 6, v/v) solution (pH = 7) for1 h. Unbound Nalidixic acid was separated from the polymer particles by centrifugation at 20,000 rpm for 30 min. The amount of unbound Nalidixic acid by each polymer was determined by UV spectrophotometer. The centrifuged particles were suspended in 2 mL of SDS (5wt %) aqueous solution and placed in a dialysis tube, then sealed at both ends with medicell clips and soaked at 50 mL of SDS (5wt %) aqueous solution. The medium was stirred at 100 rpm. Aliquots of 2 mL were withdrawn from the medium at designed time intervals. An equivalent volume of SDS (5wt %) aqueous solution was added to maintain the volume of the medium at 50 mL. The amount of Nalidixic acid released from the polymer particles was quantified by UV analysis

RESULTS AND DISCUSSION

Thermal Analysis Results: Figure 1 describes differential scanning calorimetric (DSC) plots of NIP1, leached and unleached MIP1 and Nalidixic acid. All polymer particles were thermally stable up to 280°C and the complex processes of decomposition were started at this temperature. NIP1 and leached MIP1 had similar plot. An endothermic transition was observed at 225°C of unleached MIP1, which was due to the melting of Nalidixic acid loaded on the unleached MIP1. The Nalidixic acid has a melting point at 225°C [Fig. 1(a)]. Comparison of peaks area at 225°C demonstrated that drug loading of unleached MIP1 was less than the initial amount used for synthesis.

Morphologic Analysis: The SEM images show spherical and nanometer particles (Fig. 2). There are no considerable differences in the morphology of imprinted and non-imprinted nanospheres. The little difference in size and polydispersity index (index of the dimensional homogeneity of particles) of imprinted and non-imprinted polymers is due to the influence of template compound on the particle growth during the precipitation polymerization. In the absence of Nalidixic acid, functional monomer can form hydrogen-bonded dimers in the non-imprinting system and the pre-polymerization solution contains both functional monomer dimers and free functional monomer. In the imprinting system, there are additional molecular interactions between functional monomer and Nalidixic acid, which might somehow affect the growth of the cross-linked polymer nuclei. The SEM images of imprinted polymers containing only MAA

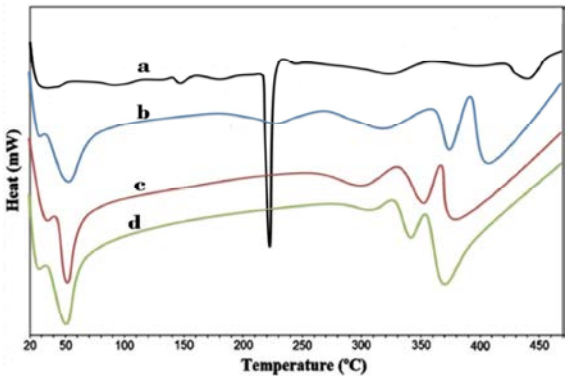

Fig. 1: DSC thermograms of Nalidixic acid (a), unleached MIP1 (b), leached MIP1 (c) and NIP1 (d)

Table 2: Amount of Nalidixic acid Bound by MIP and NIP Nanospheres

Polymer	Nalidixic acid bound by MIP (%)	Nalidixic acid bound by NIP (%)
1	69	54.2
2	37.7	34

(MIP1) clearly show very spherical particles with 182 nm. MIP2 containing MAA and MMA had a smaller average mean diameter (120 nm).The SEM images excellently show the well-defined spherical nanoparticles in narrow size distribution. The results showed that the poly (MAA-co-TRIM) and the poly (MAA-co-MMA-co- TRIM) particles were uniform nanospheres and the use of MMA resulted in small particles.

Nalidixic Acid Binding to Molecularly Imprinted Nanospheres in Water/acetonitrile Solution: Specific binding provided by the imprinted sites can be estimated by measuring the difference of Nalidixic acid uptake between the imprinted and non-imprinted nanospheres. The results from binding experiments showed that all imprinted polymers had more binding capacity than non-imprinted polymers (Table 2), indicating that there were specific binding sites for Nalidixic acid. The template binding by the non-imprinted polymer can be explained with the presence of non-specific binding due to physical adsorption and to random interactions of the template molecules with functional groups in the polymer matrix. Nalidixic acid binding for MIP1 and MIP2 was 69% and 54.2% respectively. When MAA was used as the functional monomer, the imprinted nanospheres (MIP1) displayed higher Nalidixic acid binding than MIP2 containing MAA and MMA as the functional monomer. This phenomenon was most probably due to the fact that the carboxylic acid groups of MAA had additional interactions with the functional groups of Nalidixic acid.

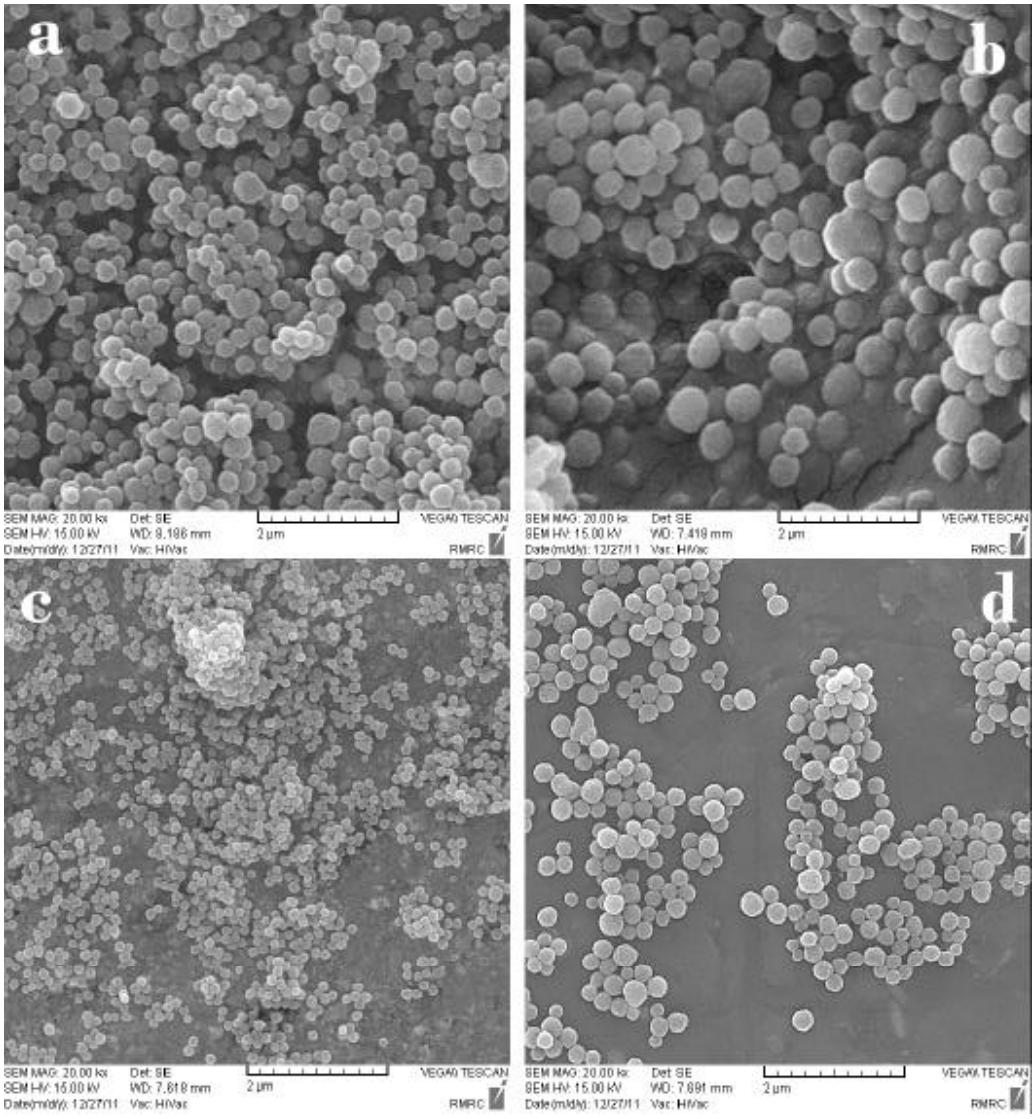

Fig. 2: Scanning electron micrographs of MIP1 (a), NIP1 (b), MIP2 (c), NIP2 (d).

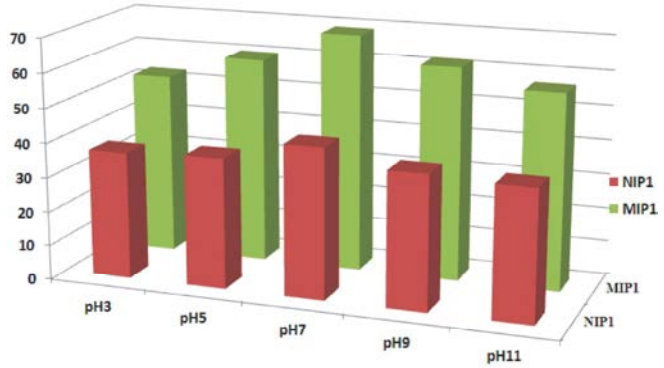

Fig. 3: Nalidixic acid binding of MIP1 and NIP1 nanospheres in loading solution with different pHs

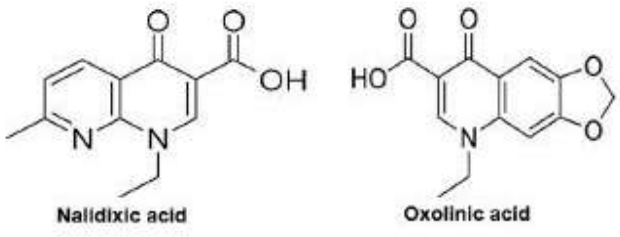

Fig. 4: Chemical structure of template and template analog molecules

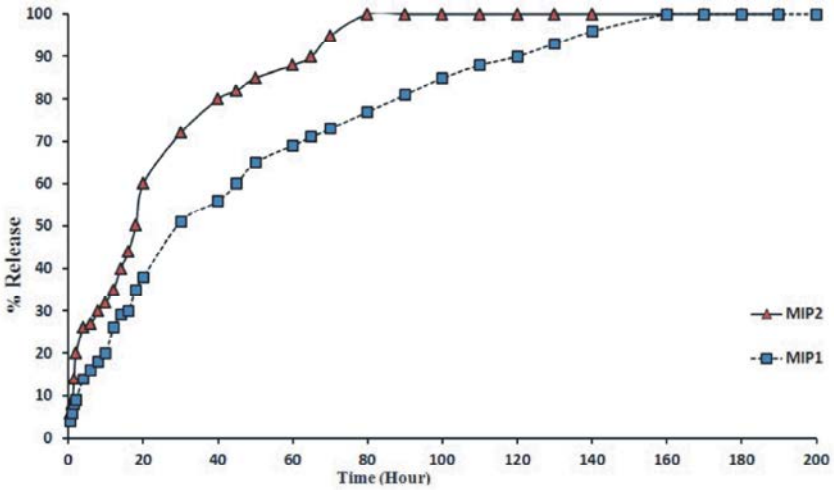

Fig. 5: Release of nalidixic acid in sodium dodecyl sulfate solution (5 wt %) by imprinted polymers.

Table 3: Selectivity Properties of Nalidixic acid -Imprinted Polymers

Polymer	Oxolinic acid bound by MIP (%)	$\alpha_{template/\ analog}$
MIP1	19.17	3.6
MIP2	5.59	9.7

The specific binding was reduced with decrease of MAA percentage in the polymers. The specific binding recovery of Nalidixic acid for MIP1 and MIP2 were 31.3%, 20.2%, respectively.

The effect of pH on the binding of imprinted and non-imprinted polymers is shown in Figure 3. There was a trend of the increase binding with the increase of pH up to 7.0; the maximum binding was observed at pH 7.0 and at higher pH (>7.0), the binding of Nalidixic acid was decreased. These results can be explained as follows: the polymer nanospheres with the carboxylic acid group of MAA bound the template molecule by ionic and hydrogen bonding interaction. At pH 6 or below, which was lower than the pKa values of Nalidixic acid (6), the solute of Nalidixic acid was completely protonated. At acidic or basic conditions, the acidity and basicity of the medium was so strong that its interaction with the template molecule exceeded the imprinting effect. In addition, at higher pH, the negative charge of the polymer was increased, mainly as carboxylic acid groups were deprotonated. At neutral pH, the best conditions existed for the formation of the hydrogen bonding between functional groups of binding sites and template molecules.

Oxolinic Acid has a structure similar to Nalidixic acid (Fig. 4).By comparing Nalidixic acid and Oxolinic Acid binding the selectivity factor α, defined as the binding for the template molecule to the binding for the template analog molecule. Table 3 shows the Oxolinic Acid binding and selectivity factors of Nalidixic acid-imprinted polymers. Oxolinic Acid binding to Nalidixic acid-imprinted polymer is as a result of the non-selective and nonspecific binding. Oxolinic Acid binding to MIPs increased with the increase of MAA percentage. The selectivity factor was obtained9.7 for MIP2 (MAA and MMA). In contrast, the MIP1 gave little selectivity factors

of and 3.6 for Nalidixic. This value was low, because MIP1had excess amount of MAA (led to decrease of imprinting efficiency and increase of non-specific binding).

Drug Release Studies: In vitro release behaviors of Nalidixic acid from the MIP nanospheres were studied in SDS (5wt%) media. Figure 5 shows the percent release of Nalidixic acid from MIPs against incubation time. The quick first release of Nalidixic acid is to be ascribed to weakly adsorbed and available molecules, while in the longer time period; the Nalidixic acid molecules more tightly bound to the polymer networks are released. Many manufacturing parameters determine the drug release behavior from MIP nanospheres. The release of Nalidixic acid from MIP2 was faster than MIP1 and 90% of the loaded Nalidixic acid was released in 65 h. MIP1released about 71% of Nalidixic acid in 65 h. The fast release would be interpreted as weak interactions between the Nalidixic acid and binding points of polymer networks because of lower percentage of MAA and smaller size of MIP2 nanospheres. The small size of MIP2nanospheres increased the solid-liquid contact of polymer particles to solution and, therefore, increased the rate of Nalidixic acid release.MIP1 nanospheres, having only MAA as functional monomer, showed slower rate and released about 90% of Nalidixic acid in 5 days.

CONCLUSION

Imprinted polymers composed of poly (MAA-co-TRIM) and the poly (MAA-co-MMA-co- TRIM), were synthesized by precipitation polymerization allowing direct non-covalent imprinting of Nalidixic acid. Very uniformly sized Nalidixic acid -imprinted nanospheres were prepared with different functional monomer. The morphology, release profiles, affinity and selectivity to Nalidixic acid and the analog Oxolinic Acid were carefully studied by the methods including SEM, PCS, equilibrium experiments and UV spectroscopy. The size of particles was changed by varying the functional monomers in the same precipitation polymerization system. Among the MIP nanospheres prepared, the MIPs using MAA and MMA showed high uniformly sized nanospheres with lower size and high selectivity. The increase in release rate was observed by poly (MAA-co-MMA-co- TRIM) which is related to the decrease in interaction intensity between the Nalidixic acid molecules and binding points of polymer. The combination of different functional monomers in precipitation polymerization opened possibilities of fine adjusting properties of MIPs.

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REFERENCES

1. Pérez-Moral, N. and G. Mayes, 2004. Comparative study of imprinted polymer particles prepared by different polymerisation methods, Analytica Chimica Acta, 504(1): 15-21.
2. Tokonami, S., H. Shiigi and T. Nagaoka, 2009. Review: Micro- and nanosized molecularly imprinted polymers for high-throughput analytical applications, Analytica Chimica Acta, 641(1-2): 7-13.
3. Ye, L. and K. Mosbach, 2001. Molecularly imprinted microspheres as antibody binding mimics. Reactive and Functional Polymers, 48(1-3): 149-157.
4. Gonzαlez, G., P. Hernando and J. Alegría, 2006. A morphological study of molecularly imprinted polymers using the scanning electron microscope. Analytica Chimica Acta, 557(1-2): 179-183.
5. Ye, L., I. Surugiu and K. Haupt, 2002. Scintillation Proximity Assay Using Molecularly Imprinted Microspheres. Analytical Chemistry, 74(5): 959-964.
6. Hunt, C., P. Pasetto, R. Ansell and K. Haupt, 2006. A fluorescence polarisation molecular imprint sorbent assay for 2,4-D: a non-separation pseudo-immunoassay. Chemical Communications: 3(16): 1754-1756.
7. Atyabi, F., F. Moghaddam, R. Dinarvand, M. Zohuriaan-Mehr a nd G. Ponchel, 2008. Thiolated chitosan coated poly hydroxyethyl methacrylate nanoparticles: Synthesis and characterization. Carbohydrate Polymers, 74(1): 59-67.
8. Abouzarzadeh, A., M. Forouzani, M. Jahanshahi and N. Bahramifar, 2012. Synthesis and evaluation of uniformly sized nalidixic acid-imprinted nanospheres based on precipitation polymerization method for analytical and biomedical applications. Journal of Molecular Recognition, 25(7): 404-413.
9. Yoshimatsu, K. K. Reimhult, A. Krozer, K. Mosbacha, K. Sode and L. Ye, 2007. Uniform molecularly imprinted microspheres and nanoparticles prepared by precipitation polymerization: The control of particle size suitable for different analytical applications. Analytica Chimica Acta, 584(1): 112-121.

10. Lai, J.P., M.L. Yang, R. Niessner and D. Knopp, 2007. Molecularly imprinted microspheres and nanospheres for di(2-ethylhexyl)phthalate prepared by precipitation polymerization. Analytical and Bioanalytical Chemistry, 389(2): 405-412.
11. Wu, X., K. Goswami and K. Shimizu, 2008. Comparison of monofunctional and multifunctional monomers in phosphate binding molecularly imprinted polymers. Journal of Molecular Recognition, 21(6): 410-418.