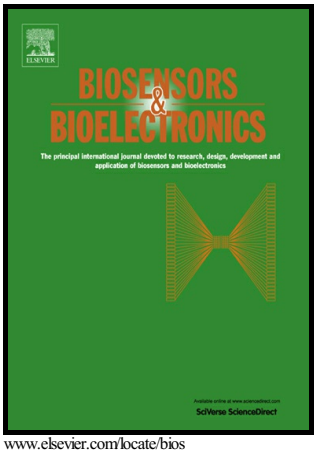

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Meltem Okan, Esma Sari, Memed Duman

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Molecularly imprinted polymer based micromechanical cantilever sensor system for the selective determination of ciprofloxacin

Meltem Okan[1], Esma Sari[2], Memed Duman[1,]*
[1]*Hacettepe University, Institute of Science, Nanotechnology and Nanomedicine Division, Ankara, Turkey*
[2]*Hacettepe University, Faculty of Science, Department of Chemistry, Ankara, Turkey*

*Corresponding Author
E-mail address: memi@hacettepe.edu.tr (M. Duman)

A B S T R A C T

The main objective of this study is to develop molecularly imprinted polymer (MIP) based micromechanical cantilever sensor system that has high specificity, fast response time and is easily applicable by user for the detection of ciprofloxacin (CPX) molecule in water resources. Highly specific CPX imprinted nanoparticles were synthesized by miniemulsion polymerization technique. The average size of the synthesized nanoparticles was measured about 160 nm with high monodispersivity. Covalent and monolayer binding of the MIP nanoparticles on cantilevers was provided by EDC/NHS activation. Validation of the developed cantilever nanosensor was performed in air with dip-and-dry technique by employing the dynamic sensing mode. According to the results obtained, micromechanical cantilever sensor system worked linearly for the concentration range of 1.5–150.9 μM. This concentration range resulted with 18.4-48.9 pg mass load on the MIP modified cantilever. The sensitivity of the developed sensor was calculated as 2.6 Hz/pg. To control the specificity of MIPs, a different antibiotic enrofloxacin (ENF), with a similar physical and chemical structure with CPX, was used, which showed 7 folds low binding affinity. The developed highly specific microcantilever sensor has a response time of approximately 2 minutes and is reusable up to 4 times. The results indicate that the MIP based AFM nanosensor has high sensitivity for the CPX molecule. This combination of MIP nanoparticles with micromechanical sensors is one of the pioneer studies in the mass sensing applications. This fast, low cost and highly sensitive CPX specific MIP nanoparticle based nanosensor developed in this research have the potential to pave the way for further studies.

Keywords: Micromechanical biosensor; Microcantilever; Ciprofloxacin; MIP; Nanoparticle

1. Introduction

Recently, there has been a growing interest in the presence of Pharmaceutical Emerging Contaminants (PECs) in aquatic environment, since they treat drinking water and create serious adverse effect on human health and wildlife. There is a noticeable rising in antibiotic resistance in bacteria that obstructs the treatments of infections. Hence, a great number of hospital-acquired infections are caused by multidrug-resistant bacteria (Acar and Rostel, 2001) (Miranda and Zemelman, 2002). Several pharmaceuticals are now added to the latest contaminant candidate lists of the United State Environmental Protection Agency (US-EPA). Ciprofloxacin (CPX), as a class of antibiotic, is suggested as one of the priority drinking water contaminants at latest European Union Water Framework Directive (EU-WFD). Ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolone carboxylic acid] (CPX) is a third generation fluoroquinolone that show a broad-spectrum of antibacterial activity. CPX is low cost antibiotic with a distinct curative effect (Li et al., 2008) (Wang et al., 2014). Due to this fact its prevalent use made it become one of the most commonly used antibiotics (Wang et al., 2014). Nevertheless, CPX residues were found to imperil people's health by affecting mammalian cell replication as well as adverse drug reactions (Wang et al., 2014) (Gao et al., 2014) (Qiao and Sun, 2010) (Torriero et al., 2006b). Like in the case of many antibiotics, CPX is also not completely metabolized in the body and therefore there is high possibility that it enters the environment through urine samples of patients and wastewater.

It is crucial to detect the presence of broad spectrum antibiotics with high efficiency. So far, number of methods have been applied for the detection of CPX including spectrophotometry (C. L. Cazedey et al., 2013), mass spectrophotometry (Caro et al., 2006), liquid chromatography (Urraca et al., 2014) (Desai et al., 2013), solid phase extraction (SPE) (Sun et al., 2014) (Yan et al., 2008), capillary electrophoresis (Barrón et al., 2001) (Zhou et al., 2008) and electrochemical techniques (Torriero et al., 2006a) (Torriero et al., 2006b)(Ionescu et al., 2007). The major drawback of these methods is that they all are time consuming, expensive, rather complicated and requires sophisticated automation and help of experienced users. Throughout the last decade new detection methods in the concept of biosensors have been developed. These new biosensors allow faster and more sensitive real-time measurements. Moreover, the usage of biological molecules, such as antibodies, with these sensors may increase the cost dramatically. Conversely, the Molecularly Imprinted Polymers (MIPs), which are synthetic materials designed to mimic recognition sites, are being studied quite a lot during the last decade for its

potential applications. Above any feature, they selectively bind to the molecule of interest, neglecting other molecules in the environment. The preparation of MIPs is cheap and fast. Moreover, they show high stability in dry state which allows them to remain undegraded (Vasapollo et al., 2011).

The usage of cantilever arrays for the detection of interactions provides an optically label-free analysis down to picogram mass resolution. The detection with this method is based on a surface coverage of the cantilever with a layer specific to compound of interest. The detection is doable both in liquid and in air (Hegner and Arntz, 2004). Yet, high sensitivity can be accomplished by contacting the sensor with the analyte and measuring the resonance frequency in air and naturally by eliminating the liquid damping. For the mass sensor applications of microcantilevers two measurement techniques are usually employed; static deflection and dynamic sensing mode. In the static deflection mode, the mechanical response of the layer on the cantilever surface to the adsorption and desorption of molecules produces a signal (Lang et al., 2007). In the dynamic sensing mode, the cantilever is oscillated at its resonance frequency. Any interaction of molecules with the layers results with a shift in cantilever's frequency, usually by lowering it. By recording this change in frequency, the total adsorbed mass can be calculated (Hegner and Arntz, 2004) (Lang et al., 2007). The equation for this calculation is;

$$\Delta m = k((1/f_1^2) - (1/f_0^2))/(4n\pi) \quad (1)$$

where f_0 is the resonance frequency before and f_1 is the resonance frequency after the binding, k is the spring constant of cantilever and n is a factor related with the geometry of the cantilever and is 0.24 for rectangular shaped cantilevers (Battiston et al., 2001).

Earlier studies in this field gave an opinion about the amount of frequency change in mass sensing experiments. Narducci et al. indicated that a mass sensitivity of 0.07 Hz/pg was recorded for an added mass of polystyrene microspheres on a rectangular cantilever with a natural frequency of 100 kHz. In the same study, when the natural frequency was increased to 400 kHz the mass sensitivity was determined as 1.3 Hz/pg. This showed that the increased frequency properties allowed for better sensitivity (Narducci et al., 2008). In a study of Maute et al. the resonance frequency of the bare cantilever was recorded as 46.21 kHz, and once the cantilever was coated with the polymer, its resonance frequency decreased down to 24.73 kHz. Also, the resonance frequency was decreased from 46.21 kHz to 46.18 kHz as the concentration of *n*-octane was increased from 0 ppm to 12000 ppm. For the concentration of 12000 ppm, the adsorbed *n*-octane mass was calculated as 30 pg. (Maute et al., 1999).

Taking the studies and knowledge provided into account, CPX imprinted polymeric nanoparticles were synthesized, immobilized on the cantilever surface, and the validation studies were performed in air employing the dynamic sensing mode.

2. Materials and Methods

2.1. Reagents

All materials used for the preparation of MIPs; functional monomer Methylacrylic acid (MAA), co-monomer 2-hydroxyethyl methacrylate (HEMA) and cross linker ethylene glycol dimethacrylate (EGDMA), were purchased from Sigma (St. Louis, MO, USA). Ciprofloxacin (CPX) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of CPX was prepared by dissolving 0.005 g of molecule in 50 ml acetate buffer at pH 4.7, which corresponds to 100 ppm (300 µM). Acetic acid (HAc) was purchased from Fluka (St. Gallen, Switzerland). (3-Aminopropyl)triethoxysilane (APTES) and trimethylamine (TEA) used in amination process were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemical used in activation step; *N*-Hydroxysuccinimide (NHS), *N*-(3-Dimethyl aminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) and phosphate buffered saline (PBS) and methanol (MeOH), used in the preparation of desorption solution, were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the desorption solution, a mixture of MeOH:HAc (9:1, v/v) was prepared, which has a pH of 4.7. All solutions were prepared with ultrapure water obtained from Milli-Q water purification system (Millipore Corp., Bedford, MA) with resistivity of 18.2 MΩ cm.

2.2. Instruments and Setup

Dynamic mode experiments were performed by using Nanomagnetics Instruments (Ankara, Turkey). The frequency measurements in air were performed by exciting the cantilever at its resonance frequency. Excitation amplitude kept at 100% during all experiments.

All-In-One-Al-Tipless Budget Sensors AFM chips were purchased from NanoAndMore (Wetzlar, Germany). Those chips have four cantilevers with 15, 80, 150 and 350 kHz frequencies. These cantilevers vary in lengths and spring constants. The cantilever D which has the highest frequency (350 kHz) was chosen for dynamic mode experiments in air. This cantilever yields the highest quality factor, which directly affects the sensitivity of the sensor system.

2.3. Preparation of CPX imprinted polymeric nanoparticles

CPX specific imprinted polymeric nanoparticles were synthesized by two phase miniemulsion polymerization method as described by Sari et. al. (Sari et al., 2016) (see in the supplementary information). Briefly, formation of imprinted cavities is based on the non-covalent interactions between related groups on the template molecule and the carboxyl groups of the functional monomer (Scheme 1). The size and morphology characterization of the nanoparticles were determined by Scanning Electron Microscope (SEM) and Atomic Force Microscopy (AFM).

<<Scheme 1>>

2.4. Preparation of microcantilever sensor system

Before starting the experiments, AFM chips were kept in chloroform solution for 3x5 mins to remove the any unwanted residues on them and were immediately dried with nitrogen. The immobilization of MIP nanoparticles on cantilevers was carried out in three different approaches. For the physical interactions MIP nanoparticles were dropped on the cantilever. In first method they were just incubated there and in the second one they were exposed to UV light. For chemical interaction, cantilevers went through APTES coating and MIP nanoparticles were immobilized on the surface via EDC/NHS activation (see in the supplementary information). Functioning of the cantilevers was controlled by checking their frequency behaviors after the surface coverage.

2.5. Validation of prepared microcantilever sensor system

Validation of microcantilever sensor system was done both in liquid and in air. Moreover, an online immobilization of MIP nanoparticles on the cantilever surface was recorded for the complete reaction. First, the cantilever was oscillated in 1 ml of 0.01 M PBS buffer until the system reached equilibrium. Once the signal was stable, 350 µl of the activation solution (EDC and NHS in 0.01 M PBS buffer) together with 350 µl of MIP solution was injected to the system. The system left untouched for 30 minutes to allow the required time for the activation**.**

For the real time detection of CPX in aqueous media, the chips were placed into the liquid cell of the AFM, which takes up to 2 ml solution. First, the system was introduced with 100 mM acetate buffer (pH 4.7) and left until the frequency became stable. Then, the analyte solution; that is CPX in 100 mM acetate buffer, was injected into the fluid cell at a constant rate of 5 µl/min. After the system reached the equilibrium, the acetate buffer was send to the system, once more, to wash the non-specifically bound molecules away. The final frequency was recorded when the signal was stable again.

For air measurements, first the frequencies of MIP nanoparticle modified cantilevers were measured and recorded as a reference value. Then, these cantilevers were treated with various concentration of 500 µl analyte solution (0.5, 1, 5, 10, 20, 50 ppm) for 10 min. Before their frequencies were checked, the cantilevers were washed with acetate buffer to get rid of the nonspecific interactions. Finally, in order to check the accuracy and reusability of the sensor system, CPX molecules were desorbed from the cavities on the MIP nanoparticles. The cantilevers were treated with the desorption solution for 30 mins and their frequencies were measured. Before measuring the frequencies in air experiments, cantilevers were dried with nitrogen gas after each step.

In all experiments described above, temperature was kept constant at 25.0 ± 0.1 °C. Calibration curve was drawn by calculating the frequency differences before and after the treatments and the limit of detection (LoD) and adsorbed mass were calculated by this curve.

3. Results and Discussion

3.1. Characterization of CPX specific microcantilever sensor system

Three different approaches were applied for the immobilization of MIP nanoparticles on cantilever surfaces. The SEM images of the cantilever prepared by the first method showed that direct incubation of particles does not work properly; after the washing step a vast majority of MIP nanoparticles were removed from the surface (Fig.

1b). Also, when the resonance frequency of the cantilever was checked, no significant shift was observed, which should have resulted from the mass load of MIP nanoparticles. The SEM images of the MIP nanoparticles immobilized on the cantilever surface with the second method showed that the UV drying of particles results in over accumulation and multilayer coverage, which would create additional damping (Fig. 1c). Moreover, when the frequency of the cantilever was checked before and after the immobilization, the frequency shift was much larger than expected. This shows that the multi-layer coverage on the cantilever surface, restrained its resonance frequency properties. These results indicate that the physical immobilization methods cannot be used in further experiments in the validation of the system.

The third method, chemical immobilization via EDC/NHS seemed promising. The characterization of the cantilever prepared with this method was characterized by both SEM and AFM. Figure 1d shows the representative SEM images of the particles, where the size, shape and dispersity properties of particles were revealed. CPX specific MIP nanoparticles were determined to be homogeneous with a spherical shape and a size of around 160 nm by SEM images. The particles showed high monodispersity. According to line profile examination of the AFM image, it is clear that a monolayer surface coverage was accomplished by this activation method (Fig. 1e and f). The RMS roughness of the bare cantilever surface was determined as approximately 2.3±0.1, whereas this value went up only to 7.4±0.5 after the EDC/NHS activation of MIP nanoparticles.

<<Figure 1>>

Before starting the experiments, unmodified cantilever (without any MIP nanoparticles) was tested to record its nominal frequency and quality factor. The exact frequency and the quality factor of the cantilever D in air were determined as 410.5 kHz and 603.43, respectively (Fig. 2a). The frequency range for cantilever D was provided as 200-500 kHz by the producing company. Therefore, it was decided to perform the validation experiments with this cantilever, as its properties were reliable with a high quality factor.

<<Figure 2>

Behavior of the cantilever during the real-time immobilization of MIP nanoparticles on cantilever surface was checked. The binding of MIPs on the cantilever surface was achieved in approximately 20 min. This measurement provided background information about the period of EDC/NHS activation. It took around 12 min. for an observable frequency drop to take place, which was most probably due to the Brownian motion of the particles. Once they reached the surface of the cantilever, a sharp decrease of 453 Hz was observed in frequency, which took place for 4 min (Fig. 3). It was also noticed that the signal to noise ratio was too high during the immobilization of MIP nanoparticles in liquid. The reason for the high fluctuations in frequency even at stable state was either due the large volume of the liquid cell or because the AFM system was not suitable for the liquid measurements. Therefore, the frequency shift resulting from the immobilization of MIP nanoparticles was determined in air. After washing and drying steps, the frequency of cantilever, which was incubated with 50 µl MIP nanoparticle solution, was decreased about 380 Hz (Fig. 2b).

<<Figure 3>>

3.2. Validation of CPX specific microcantilever sensor system

For the validation of the prepared sensor system, first, the binding studies of CPX were tried in liquid, since the liquid experiments was to observe the binding behaviors of the CPX molecules on the MIP nanoparticle cavities such as; how fast they are adsorbed and desorbed. However, as expected, the binding experiments of CPX molecules in liquid resulted with too much noise inflicted from the device and the system (Fig. 3b).

During the binding experiment in liquid using the sensor system, once a stable baseline was obtained, 150.9 µM of CPX solution was introduced to the system. A frequency drop of 150 Hz was observed immediately. However, within 7.5 min an increase of 50 Hz was observed. After this point, in order to wash the nonspecific interaction, acetate buffer at same pH was injected to the system and a frequency increase of approximately 35

Hz was obtained. The noise itself had an effect of 30 Hz in the liquid system as shown in Figure 3b. This high amount of noise hindered the frequency change analysis. Also, since desorption buffer contains MeOH and alcohol dissolves the epoxy of the cantilever holder mechanism, this step could not be performed during online measurements. Therefore, the binding experiments were carried out in air to obtain much convenient results. Moreover, in liquid environment, quality factor of cantilevers decreases dramatically due to the liquid damping. This prevents the possibility to detect small amount of molecules, which in return affects the sensitivity of the system (Rasmussen et al., 2005). High quality factor lowers the minimum detectable resonance shift, increasing the frequency resolution. The liquid experiments, although, provided background information about the amount of time required for the binding which occurred in 5 min. This information allowed us to hold an opinion for the incubation time of cantilever in the CPX solution for the air experiments.

Having determined the period for molecule binding on cavities from the liquid experiments, the dipping period in dip-and-dry technique was designated as 10 min. for the air experiments. The system response for the concentrations of 1.5, 3.0, 15.1, 30.1, 60.4 and 150.9 µM CPX was recorded in air (Fig. 4a). For the given concentrations, frequency changes of 48, 54, 62, 78, 97 and 126 Hz were obtained, respectively. The calibration graph was drawn using the frequency changes (ΔF) versus the concentrations used. As shown in Figure 5a, ΔF value increases with increasing CPX concentration. The calibration curve presents high linearity; the sensor developed shows 94% accuracy for the concentration range of 1.5 – 150.9 µM.

<<Figure 4>>

The total mass of molecules adsorbed on the MIP nanoparticles that are on the surface of the cantilever was also calculated from the frequency shifts obtained in air experiments. To determine the adsorbed mass in each concentration, formula 2 was used. For the frequency changes of 48, 54, 62, 78, 97 and 126 Hz, adsorbed masses (Δm) of 18.4, 20.5, 24.1, 29.6, 37.1 and 48.9 pg were calculated (Fig. 4b). The graph of ΔF versus Δm showed quite high linearity and the system works with 99% accuracy. Using the same graph and data series, the sensitivity of the sensor was calculated as 2.6 pg/Hz. For the determination of limit of detection (LoD), cantilever left oscillating in air, and the frequency changes at equilibrium were recorded. The LoD value was calculated as 0.8 µM. The limit of quantification (LoQ) was determined in a similar manner and was calculated as 260 µM.

<<Figure 5>>

The selectivity and specificity of the microcantilever sensor system was checked both with NIP nanoparticles and a competing agent ENF. The experiments were performed with the same method used in air experiments, using the same cantilever. Single concentration, 3.0 µM, was chosen for these experiments. The frequency response of MIP nanoparticles to 3.0 µM CPX was recorded as 54 Hz, whereas to 3.0 µM ENF molecule the frequency change was only 8.1 Hz. This shows that the affinity of ENF to CPX imprinted nanoparticles were 6.7 folds lower than the affinity of CPX (Fig 5b). When the frequency change was 54 Hz for 3.0 µM CPX using MIP nanoparticles, this value goes down to 11.8 Hz when NIP nanoparticles are used (Fig. 5a). Accordingly, the affinity of CPX molecule to NIP nanoparticles was determined to be 4.6 folds lower when compared to MIP nanoparticles. Even though throughout air experiments, same cantilever was used for the detection of different concentrations of CPX, the reusability studies were performed for a single concentration to observe the stability of the frequency shift after each adsorption and desorption step. In line with this purpose, the same AFM chip was incubated in the same concentration (3.0 µM) of CPX 10 times, followed by desorption after each step. The frequency shift for 3.0 µM CPX was recorded as 54, 53.4, 53.2 and 52.2 Hz in repeated experiments in air. However, in fifth trial, the shift was only 23 Hz. This showed that the sensor system works with high stability up to 4 times (Fig. 5c). After four times, a reliable data cannot be obtained. The relative standard deviation (RSD %) was calculated as 1.41 % after using sensor for four times continuously. When the fifth experiment is added to the calculation this value goes up to 28.67%. One possible explanation for this situation might be that the acid and alcohol that desorption solution contains starts harming the surface of the cantilever after a number of uses. . The aluminum coverage of the cantilever begins to go through corrosion, thus the physical properties of the cantilever were changed which directly affects the resonance frequency features.

4. **Conclusion**

In this study, MIP nanoparticle based microcantilever sensor system was introduced for the first time for the detection of PECs in water resources. For this, CPX imprinted polymeric nanoparticles were prepared and

covalently immobilized on the cantilever surface and the resonance frequency shifts due to molecule adsorptions in air were recorded. During all experiments dip-and dry technique was used and dynamic sensing mode was employed. Results showed that the system works with high accuracy in air. Sensitivity and the LoD of the sensor were determined as 2.6 pg/Hz and 0.8 µM, respectively. However, this LoD can be lowered and the sensitivity of the sensor can be improved by changing the immobilization method of the MIP nanoparticles, as blocking of several groups during EDC/NHS activation might affect the sensitivity of the system. The developed nanosensor for the selective determination of CPX was found to be comparable with existing techniques in terms of selectivity, sensitivity and reusability (Ionescu et al., 2007) (Torriero et al., 2006b) (Urraca et al., 2014) (Caro et al., 2006)(Barrón et al., 2001)(Zhou et al., 2008).

Both static deflection and dynamic sensing modes can be employed in such systems. It is important, however, to coat only one surface of the cantilever while performing the binding studies in deflection mode. Once the issue of the surface coverage is solved, resonance frequency and bending readouts can be traced simultaneously (Battiston et al., 2001).

Developing polymer based electromechanical systems and combining cantilevers with polymers are the new trend towards molecule detection with mechanical systems (Ayela et al., 2014) (Ayela et al., 2007). These systems allow for tailor-made approach for the investigation of any compound of interest in a given environment. The sensor system proposed in this study eliminates the sophisticated system preparation and cantilever fabrication. Instead, the proposed sensor offers a rather easy and cost-effective and most importantly a time efficient approach by direct immobilization of MIP nanoparticles as capture elements on the surface of the cantilever. This sensor system can be used for the detection of various numbers of molecules such as; hormones, viruses, pathogens, toxins and drugs in mass sensing applications.

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Figure and Scheme Captions

Scheme 1. Schematic representation of CPX imprinted polymer.

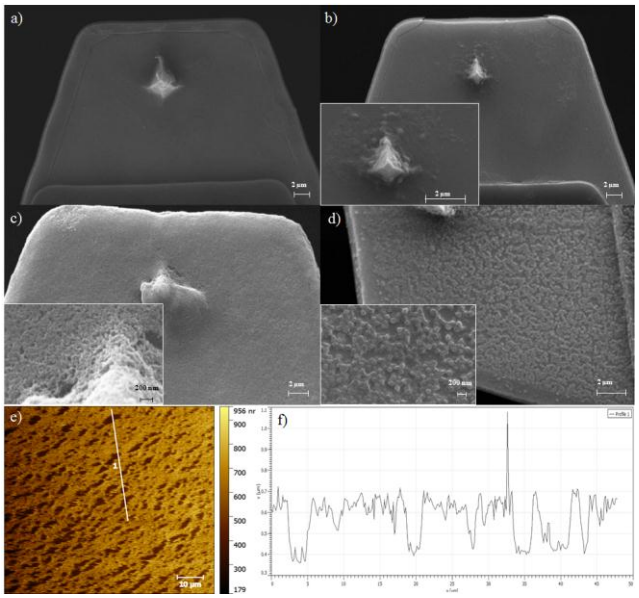
Figure 1. Characterization of MIP nanoparticles by SEM and AFM. a) SEM image of the bare cantilever without any MIP nanoparticles immobilized. SEM images of MIP nanoparticles immobilized b) by direct incubation and washing, c) UV exposure and washing, d) EDC/NHS activation and washing. e) AFM image of MIP nanoparticles immobilized by EDC/NHS activation, f) line profile of the AFM image.

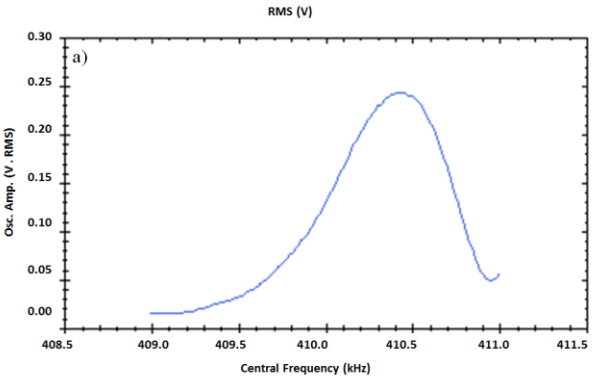
Figure 2. The frequency values of the cantilever in air a) before and b) after the immobilization of MIP nanoparticles.

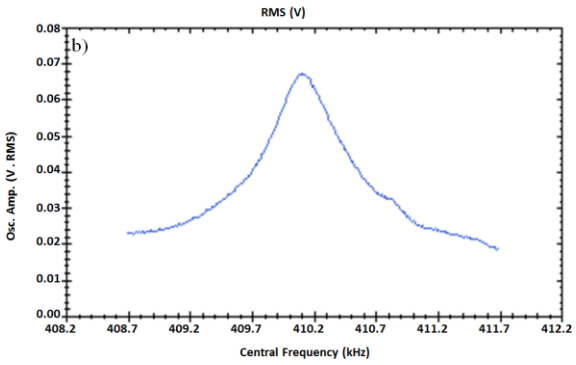
Figure 3. The frequency shifts of the cantilever in liquid. a) The real-time immobilization of MIP nanoparticles on the cantilever surface, b) online monitoring of the binding of 50 ppm CPX molecule.

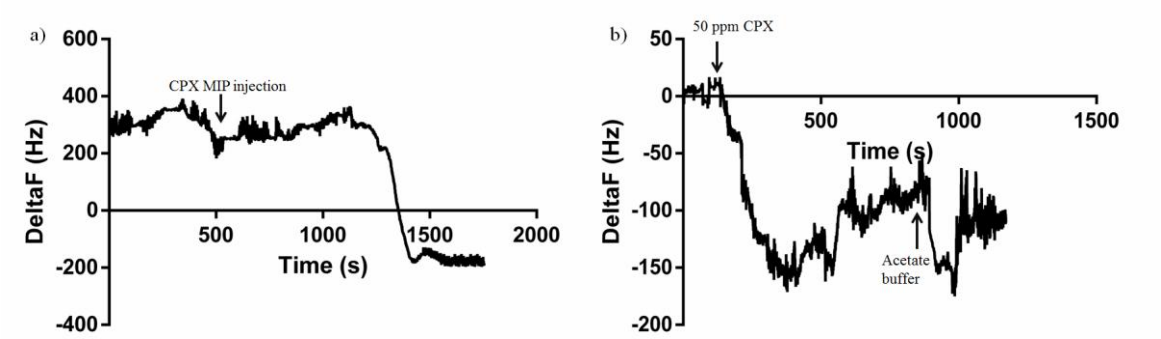
Figure 4. a) Calibration graph of the sensor system in air; frequency shifts (ΔF) vs. concentrations (cMeltem), b) Graph of frequency shifts (ΔF) vs. adsorbed masses (Δm).

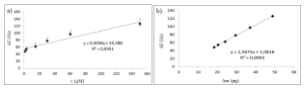
Figure 5. a) The ΔF resulting from binding of CPX molecule to MIP and NIP nanoparticles, b) The ΔF resulting from binding of CPX and ENF molecules to MIP nanoparticles, c) Reusability of the sensor system.

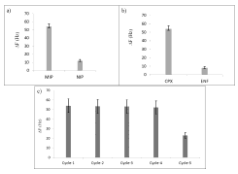


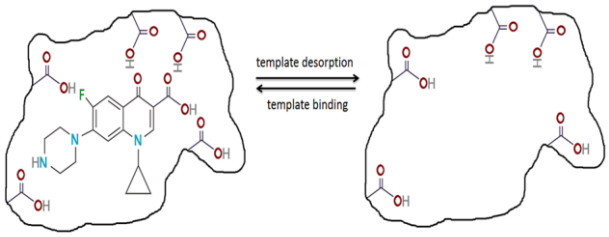












Highlights

- Molecularly imprinted polymeric (MIP) nanoparticles were synthesized using miniemulsion, for the selective determination of ciprofloxacin.
- Immobilization of MIP nanoparticles on the cantilever surface was carried out in three different methods and all prepared cantilevers were characterized by Scanning Electron Microscope and Atomic Force Microscopy.
- This combination of MIP nanoparticles with micromechanical sensors is one of the pioneer studies in the mass sensing applications
- Results showed that this sensor system works with high accuracy in air. Sensitivity and the limit of detection of the sensor were determined as 2.6 pg/Hz and 0.8 µM, respectively.
- The sensor system proposed in this study, eliminates the sophisticated system preparation by the immobilization of MIP nanoparticles as capture elements directly.
- A fast and low-cost method for the detection of CPX by MIP nanoparticles using microcantilever sensor was proposed in this study in a rather easy and reliable technique in the field of mass sensing applications.