

Development of Molecularly Imprinted Conducting Polymer Composite Film-Based Electrochemical Sensor for Melamine Detection in Infant Formula

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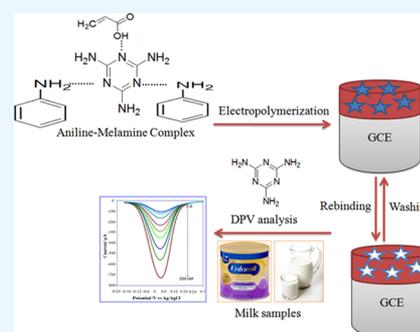
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ABSTRACT: Simple, fast, and sensitive molecularly imprinted composite thin-film-based electrochemical sensor developed by using in situ co-electropolymerization of aniline and acrylic acid in the presence of melamine as a template is described here. The prepolymerization complex formation was studied by using Fourier transform infrared (FTIR) spectrophotometry, while the film formation was performed and characterized by cyclic voltammetry, Fourier transform infrared (FTIR), and scanning electron microscopy (SEM). The optimization of important parameters and removal of melamine generated the binding sites in the polymer matrix, which can recognize melamine specifically. Electrochemical measurements were performed to achieve the linear range, the limit of quantification, and limit of detection of 0.1–180, 0.0573, and 0.0172 nM, respectively. The sensitivity of the sensor was attributed to the synergistic effects of amine from aniline and the carboxylic group from acrylic acid to form multiple noncovalent interactions with the template. Melamine-spiked infant formula and raw milk were analyzed by the developed sensor, and the recovery range of 95.87–105.63% with a relative standard deviation of 1.11–2.23% was obtained. The results showed that the developed sensor using the new composite polymer receptor is promising for the online monitoring of melamine in the food industries in the future.



INTRODUCTION

Melamine (1,3,5-triazine-2,4,6-triamine) is a weak base, nonedible, nonhygroscopic, white crystalline solid, melts with decomposition above 347 °C, sublimates at temperatures below its melting point, and slightly soluble in water (0.38 or 3.7 g/mL of water at 20 or 90 °C, respectively). It is a nitrogen-rich synthetic molecule (66.6% by weight) used in for-profit milk adulteration as the fake protein source and cannot be identified by indirect protein assays based on the total nitrogen content.¹ Powdered infant formula, milk-containing products, and pet food from China are some examples of melamine contamination incident recorded previously. This health issue was due to the acute renal failures in animals and humans related to kidney stone formation associated with the presence of melamine and cyanuric acid in consumed food and feeds.^{1,2} Therefore, online monitoring of the intended addition of melamine into foodstuff during food production is very important by applying relatively cheap, fast, and sensitive analytical methods like chemical sensors.

The high demand of modern society for fast, selective, sensitive, user-friendly, portable, and real-time analysis for food safety, medical, environmental, and security applications makes biochemical sensors most interesting.³ However, preparation, handling, and stability problems of the biorecognition materials used in the sensor fabrication combined with their continuous

increase in price harnessed its competition with other standard analytical techniques.⁴ Synthetic recognition materials such as molecularly imprinted polymers (MIPs) become potential alternative materials because of their broader advantages.⁵ A molecular imprinting technique is the polymerization of a functional monomer and a cross-linker around a template molecule from the prepolymerization complex. Following the template removal, three-dimensional binding sites are generated to recognize the template molecule or its analogue specifically.^{6–8}

Molecularly imprinted conducting polymers (MICPs) provide further opportunities by combining the recognition properties of MIP with the electronic–optical properties and the easy deposition of conducting polymers on to the substrates for modern electronics.^{9–11} The electrical properties of the MICP layer of the chemical sensor change when it rebinds the template and generates the relationship between the concentration of the template molecule and the measured electrical property or signal like current, potential, con-

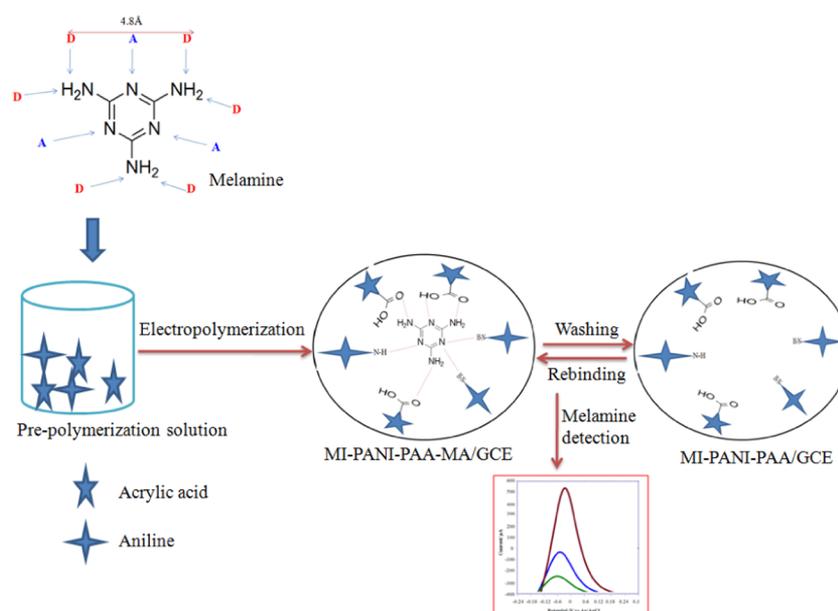
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Scheme 1. Schematic of the General Fabrication Processes of the MI-PANI-PAA Sensor for Melamine Detection (D—Donor and A—Acceptor)



ductivity, or capacitance.¹² Such a kind of approach leads to the development of an MIP-based electrochemical sensor with high specificity, sensitivity, strong adherence of the sensing layer to the electrode, stability, and real-time analysis.⁹

The reversible doping/dedoping process and easily processable and controllable chemical and electrochemical properties of conducting polymers make them promising materials in modern electronic devices. From the conducting polymers, polyaniline (PANI) is the one most studied owing to its high electrical conductivity, excellent electronic and optical properties, good redox behavior, stability, low cost, and simple preparation process.¹³ Electrochemical synthesis of electroactive PANI films on the conducting substrates can be performed using potentiostatic, galvanostatic, or potentiodynamic cyclic voltammetry (CV).^{13,14} Owing to a wide spectrum of properties, PANI is used as a material for sensors and actuators, fuel cells, solar batteries, lithium-ion batteries, supercapacitors, field-effect transistors, and many other purposes.^{15,16} However, the use of pure PANI in electronic devices is limited because it performs well only in acidic media of pH less than or equal to 4 and its conductivity is unstable at neutral pH. Therefore, material modification is important to impart the desired conductivity and stability as well as functionality.¹⁶ A great deal of effort has been made in the improvement of polymer sensing materials for the chemical sensor preparations. This can be done either by amending the polymer molecular structures, altering dopants, and incorporating another component into the conducting polymers.¹⁷ Homma and his co-workers developed a glucose sensor using polyaniline-polyacrylic acid (PANI-PAA) composite film.¹⁸ Sensing material modification with organic acids like acrylic and methacrylic acid increases the binding sites since the carboxyl groups act as a hydrogen bond donor and acceptor simultaneously.¹⁹ This amendment introduces other functional units, with good mechanical and chemical properties, via electrostatic interactions between the amine and carboxylic groups. MIP-based chemical melamine (MA) sensors were reported mainly based on methacrylic acid.^{20–23} Also, very few

reports on pyrrole,²⁴ ionic liquids,^{25,26} 7-(4-vinylbenzyloxy)-4-methyl coumarin-acrylic acid copolymer,²⁷ *o*-aminophenol,²⁸ *p*-aminobenzoic acid,²⁹ and mercaptopropyltrimethoxysilane³⁰ are available. This implies that much work remains on the development of new molecular recognition elements for effective detection of this template. Most importantly, MICPs and their composites are not reported, except polypyrrole, but they are supposed to be promising functional materials for the development of sensitive and selective chemical sensors in the future. Hence, it is interesting and important to introduce organic–organic composite multifunctional materials as a sensing layer through material engineering. For this purpose, we have selected aniline and acrylic acid functional monomers to exploit the synergistic advantages of their composites arising from the PANI conducting polymer with unique properties and acrylic acid, the common hydrogen bond donor and acceptor monomer. The aim of the present work is to develop an electrochemical sensor for melamine determination through the synthesis of conducting polymer composite film sensing material. Our approach focuses on investigating the triplet of hydrogen bond donor–acceptor–donor group (D–A–D) pattern of melamine with a spacing of 4.8 Å³¹ (Scheme 1) to form complementary intermolecular bonds with appropriately spaced functional group triplets comprising hydrogen bond acceptor–donor–acceptor (A–D–A) units of PANI-PAA composite. The hydrogen bond interactions between the template melamine and the functional monomers were studied by Fourier transform infrared (FTIR) to understand the complex formation and facilitate the MIP film preparation and characterization. We use in situ electropolymerization to deposit melamine-imprinted poly-(aniline-*co*-acrylic acid) composite thin film on the glassy carbon electrode (GCE) to develop melamine electrochemical sensors. The fabricated voltammetric sensor was applied for the detection of MA in the laboratory sample and infant formula milk samples with good recoveries.

RESULTS AND DISCUSSION

Functional Monomer–Melamine Interactions. Melamine molecule fulfills the criteria for selecting the template, such as having many functional groups (Scheme 1) accessible for interacting with functional monomers, stable at polymerization conditions, easily available, and of low cost for the preparation of MIP. The amines and imines in the template enable it to form hydrogen bonding with both hydrogen bond donor and acceptor molecules like aniline and acrylic acid. Theoretically, multiple noncovalent interactions are expected to occur between the model template and the monomers. This phenomenon was experimentally confirmed by the FTIR study.

In pure aniline (ANI) solution, the FTIR peaks at 3338 and 1635 cm^{-1} are due to the N–H stretching vibrations and C=N stretching deformation of the quinoid ring. The bands at 3316 and 1638 cm^{-1} are due to the N–H stretching vibrations in secondary amines and C=O stretching in carboxylic groups for the aniline (ANI) and acrylic acid (AA) mixture. The prepolymerization solution containing ANI, AA, and melamine (MA) showed shifted and new peaks at 3304, 1634, 1045, 1016, and 765 cm^{-1} due to N–H stretching vibration, C=N and C=O stretching, C–H bending in the aromatic ring, C–N group vibration, and C–H bending out-of-plane deformation, respectively. The change in absorbance and wavenumber (Figure 1) shows the formation of hydrogen

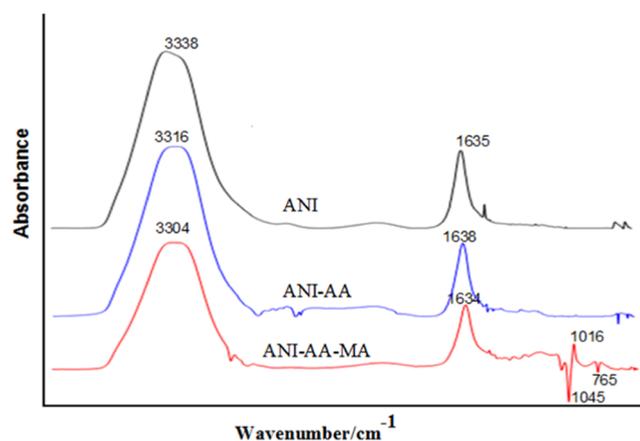


Figure 1. FTIR spectrum of the mixture of ANI and acrylic acid (AA) and the prepolymerization mixture of ANI, AA, and melamine (MA).

bonding and other noncovalent interactions between the monomers and the template molecule. The bands at 3316 and 1638 cm^{-1} are due to the N–H stretching vibrations in secondary amines and C=O stretching in carboxylic groups. The significant shift in both absorbance and wavenumber from pure ANI to the mixture of ANI-AA and then to the mixture of ANI-AA-MA is due to the change in the structure–spectral relationships of the associated molecular vibration. The shift in absorbance is due to the electrostatic attraction and hydrogen bond vibration. The appearance of new peaks around 100 cm^{-1} indicates that there is a change in the complex due to interaction with the template by forming either hydrogen bonding or π – π interactions. The sharp peak observed at about 1000–900 cm^{-1} is due to C–H stretching and C=C bending. The major peaks of ANI, ANI-AA, and ANI-AA-MA and new peaks observed are summarized in Table 1.

Table 1. Absorbances and the Wavenumber Assignments of ANI, ANI-AA, and ANI-AA-MA

material	absorbance	wavenumber (cm^{-1})
ANI	0.2683	3338
	0.11928	1635
ANI-AA	0.24835	3316
	0.100	1638
ANI-AA-MA	0.18008	3304
	0.09212	1634
	−0.04462	980
	0.03733	960
	0.005174	765

Generally, there is a decrease in absorbance and wavenumber from pure ANI to ANI-AA to ANI-AA-MA. This phenomenon is responsible for the formation of a stable complex between the template and functional monomers, which is the main precondition for molecular imprinting technology. The modification of ANI with AA and the interaction of the template with the monomers paved the preparation of the melamine-imprinted composite film on the GCE.

Electrochemical Synthesis and Characterization of the Composite Films. Both MI-PANI-PAA and non-imprinted poly(aniline-co-acrylic acid) (NI-PANI-PAA) films were deposited on the electrode surface using the potentiodynamic method (Figure 2A). The formation of the MI-PANI-PAA film on the dichloroethylene (DCE) was confirmed by using electrochemical, FTIR, and scanning electron microscopy (SEM) techniques. Electrochemical properties of the PANI-PAA films were investigated by CV using Ag/AgCl in acetate buffer solution (ABS) (at pH 7.0 and room temperature), which was purged with nitrogen gas before the actual measurement. The modification of the electrode surface changed the current response of the surface significantly (Figure 2B).

The as-prepared MI-PANI-PAA film has a higher current response than the NI-PANI-PAA film revealing that the imprinting effect arises due to the presence of the template enhanced the mass transfer and electron flow at the interface. The higher current response indicates that a fast reversible system is maintained due to the electroactive nature of the melamine target. The reduction current is highly enhanced when the template is interlocked in the binding cavities created during polymerization and template extraction.

FTIR spectra of the electrodeposited polyaniline-polyacrylic acid composite film were recorded in the wavenumber range of 4000–500 cm^{-1} under a transmission mode (Figure 3). The bands were assigned to different functional groups or bonds.^{34–38} These strong and medium bands observed confirm the formation of MI-PANI-PAA composite films in the presence of the template.

In NI-PANI-PAA, the bands at 3334 and 1636 cm^{-1} are due to the –OH stretching in PAA and –NH bending in amine of PANI. In Figure 3, the strong and broad band at 3334 cm^{-1} was minimized and shifted to 3266 cm^{-1} , while the medium and sharp peak at 1636 cm^{-1} was increased and shifted to 1618 cm^{-1} , indicating that there is a strong interaction between the template and the monomers. For the MI-PANI-PAA film, the peak at 3266 cm^{-1} corresponds to the stretching vibration of –OH, which is shifted due to the formation of hydrogen bonding with the template and the imprinting effect. The band

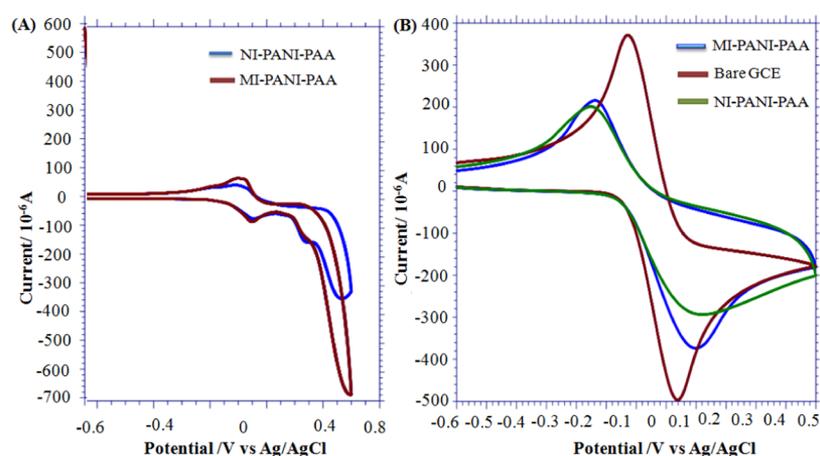


Figure 2. CV curves recorded during polymerization (A) and comparison of different electrodes (B) in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl at 100 mV/s.

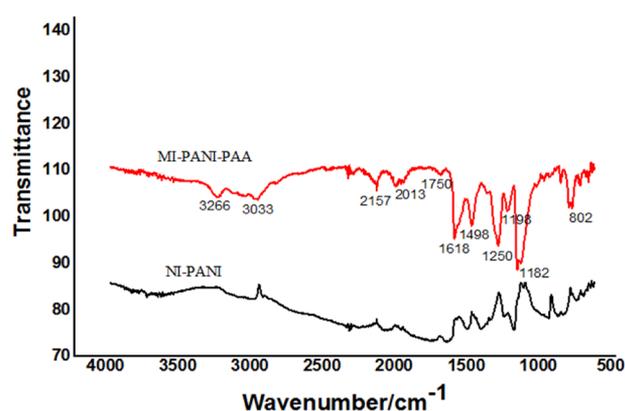


Figure 3. FTIR spectrum of MI-PANI-PAA and NI-PANI-PAA.

at 3000 cm^{-1} belongs to $-\text{NH}$ stretching vibration, confirming that the polymer composite was formed as amine salt based on the electrostatic attraction.

The medium peak observed between 2013 and 1618 cm^{-1} corresponds to the $\text{C}-\text{H}$ bending vibration in the aromatic ring of PANI. The strong and sharp band at 1618 cm^{-1} is due to $\text{C}=\text{C}$ stretching vibration in aromatic amine, while those located at 1314 and 1250 cm^{-1} are due to $\text{C}-\text{N}$ stretching vibrations found at different positions in PANI. The main bands and their assignments are summarized in Table 2.

The sharp peak found at 1182 cm^{-1} accounts for the $-\text{CO}$ stretching vibration and that at 802 cm^{-1} belongs to $\text{C}=\text{C}$ bending vibration. These appearances of new strong and medium bands for MI-PANI-PAA composite films indicate the successful formation of the materials with multiple functional sites.

SEM images of PANI, PAA, and MI-PANI-PAA (Figure 4B–D) composite film surface in acidic media exhibited a porous fibrous structure. PANI and PANI-PAA look smooth while PAA film is highly porous in nature. The coexistence of the imprinted PANI and PAA is due to the electrostatic interaction, which highly assisted the cross-linking of the composite polymer film to exist as an amine salt. As can be observed, the surface of the film looks like smooth and undistinguished. Generally, the SEM images were consistent with the observed roughness and density for each respective polymer/composite film.

Table 2. FTIR Bands of the Imprinted and Nonimprinted Composite Polymer Films and Their Corresponding Functional Units

band (cm^{-1})	expected functional group (s)
3334	$-\text{O}-\text{H}$ stretching vibration in the carboxylic acid
3310	$-\text{N}-\text{H}$ stretching vibration in a secondary amine
3266	$-\text{O}-\text{H}$ stretching vibration in polyacrylic acid
3000–2859	$-\text{CH}$ stretching vibrations
2355–2157	$-\text{carboxylic}$ functional groups in polyacrylic acid
2013	$-\text{C}-\text{H}$ bending in aromatic compound
1636	$-\text{H}$ -bonded COOH stretching vibration
1618	$-\text{C}=\text{C}$ stretching vibration in benzenoid ring
1498	$-\text{O}-\text{H}$ bending in the carboxylic acid
1314	$-\text{C}-\text{O}$ stretching vibration
1182	$-\text{N}-\text{H}$ bending vibration in the aromatic ring
882	$-\text{CH}$ bending out-of-plane deformation
802	$-\text{C}=\text{C}$ bending in the aromatic ring

Optimization Electrochemical Polymerization Parameters.

To achieve MIP films with better rebinding properties, important electrochemical synthesis parameters, such as the functional monomers to template molar ratio, scan cycle, scan rate, and effect of pH on the rebinding capacity of the polymer, were emphasized. The material composition of the sensing layer gave different peak currents after incubation in the melamine solution. Briefly, 0.1:0.2:0.01, 0.1:0.2:0.01, 0.3:0.2:0.01, 0.2:0.3:0.01, 0.2:0.4:0.01, and 0.2:0.1:0.01 molar ratios of (ANI/AA/MA) were used to fabricate different sensors (Figure 5A). From the present data, the high current response was achieved at a molar ratio of 0.2:0.2:0.01 (ANI/AA/MA), and the composition of this material was selected as the optimum molar ratio for the sensor construction.

Different sensors were made by using different scan cycles (5, 10, 15, 20, and 25) while keeping the scan rate constant. The scan cycle of 10 produced the film with high template binding capability (Figure 5B) and was chosen for the preparation of the MI-PANI-PAA/GCE sensor.

To investigate the effect of the scan rate used during polymerization on the final performance of the sensor, five different sensors were fabricated by varying the scan rates (50, 100, 150, 200, and 250 mV/s) using 10 scan cycles to investigate the effect of the scan rate on the final performance of the sensor.

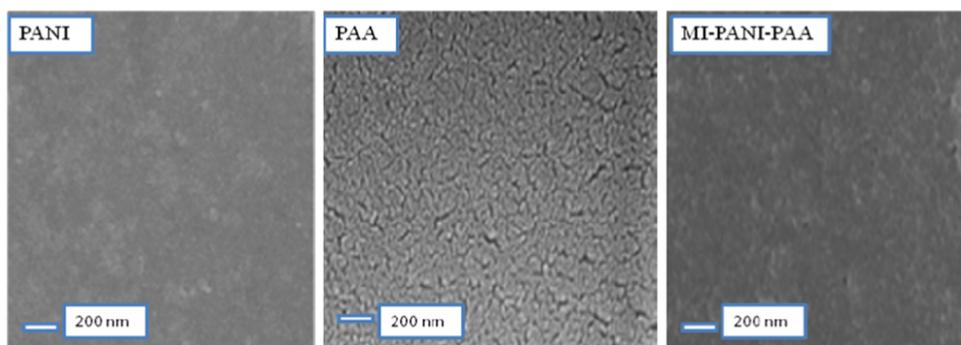


Figure 4. SEM images of PANI, PAA, and MI-PANI-PAA films.

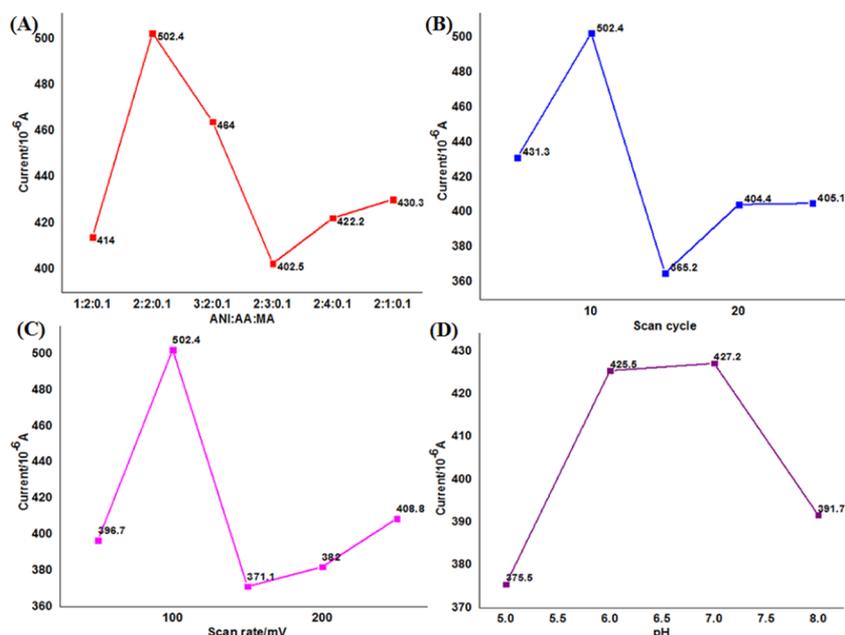


Figure 5. Different molar ratios of ANI–AA–MA (A), scan cycle (B), scan rate (C), and pH (D) and their current responses after incubating in 0.1 mM MA solution and transferring to 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl for CV measurement at 100 mV/s.

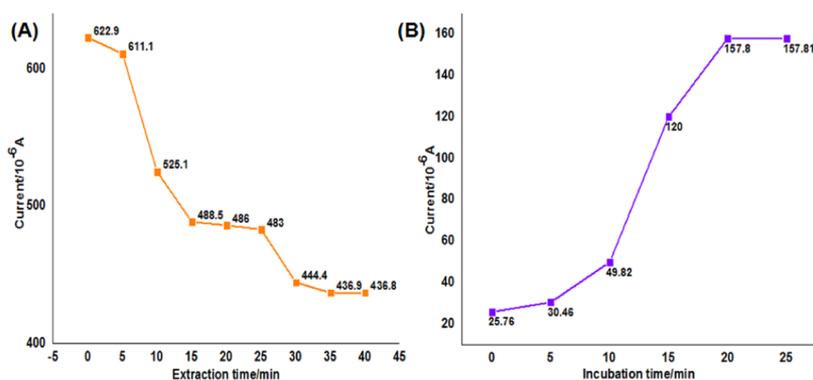


Figure 6. Template extraction (A) and incubation time (B) monitored by CV in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl at 100 mV/s.

The highest current peak corresponding to the high uptake of the analyte was obtained by the sensor prepared using 100 mV/s, and it was selected for the fabrication of the MI-PANI-PAA/GCE sensor in the present work (Figure 5C).

The interaction between the recognition sites generated on the surface of polymer films and the template depends on the acidity or alkalinity of the solution used during the rebinding study. As a result, optimization of pH is very important for

better performance of the sensor. Here, ABS with different pH values (5, 6, 7, and 8) was used for the incubation of the sensor in melamine solution (Figure 5D).

The ABS with pH 7 containing melamine favors the strong interaction between the polymer composite film and melamine that resulted in a high peak current response. The high current value indicates that the labeling solution ($[\text{Fe}(\text{CN})_6]^{3-/4-}$) easily infuses through the polymer films and creates fast

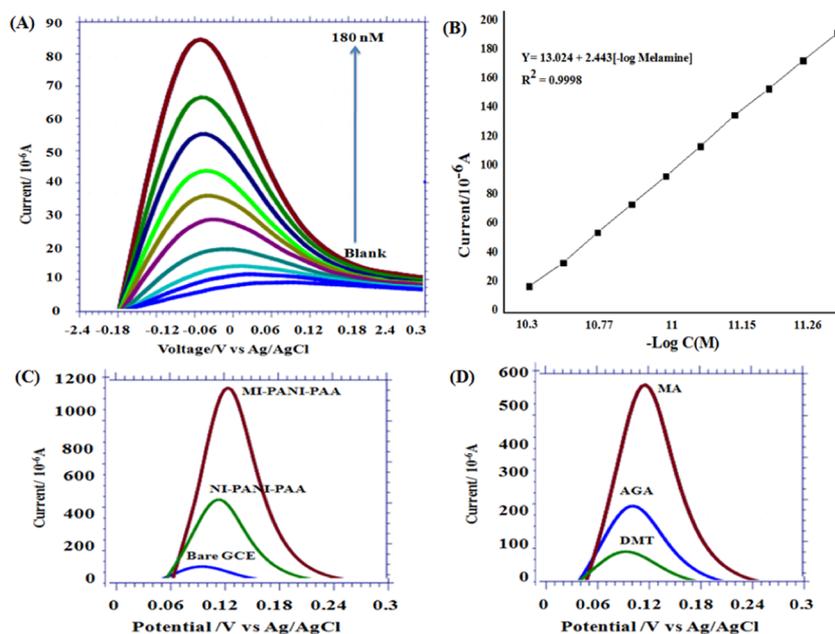


Figure 7. Current response of different concentrations of MA (A), calibration curve (B), specificity (C), and selectivity (D) of the MI-PANI-PAA/GCE sensor monitored by DPV in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl at an amplitude of 0.05 V and pulse width of 0.05 s.

electron flows and reversibility of the system. Hence, pH 7 was selected for all rebinding experiments. The present result shows that the MI-PANI-PAA film works well in neutral solutions as reported before.²⁵

Template Extraction and MI-PANI-PAA Film Incubation Time. After the successful synthesis of the MIP films, the next most important step is effective template removal and rebinding study.³⁶ Complete removal of the imprint molecule leads to the formation of sufficient accessible recognition sites or binding sites.³⁹ Therefore, the optimum removal of the template/imprint molecule is very important in the MIP sensor development. Template extraction was done by applying the solvent extraction method using dimethyl sulfoxide and acetic acid (4:1, v/v) in ABS at neutral pH. The removal of the template was monitored by recording the change in current at each time interval until no significant change in current was observed, as shown in Figure 6A.

Melamine extraction from MI-PANI-PAA/GCE was done by soaking the sensor in extraction solvents through continuous stirring to facilitate the extraction. The optimum time required for the present sensor was 35 min, and this time was used in all experiments. Beyond this time, there is no change in the current response, indicating the complete removal of the template from the polymer matrix. The recognition sites obtained by template extraction need to rebind the template or similar molecule selectively. The sensor was incubated in ABS containing a known amount of melamine at pH 7 for different time intervals to study the rebinding process.

The current responses were monitored by CV until no change in the current response value was observed. As shown in Figure 6B, the incubation of 20 min is the optimum time because, after this, there is no current increase, and this value was selected as an optimum time for rebinding processes.

Effect of MA Concentration on the Performance of the Sensor. Different concentrations of MA (0, 20, 40, 60, 80, 100, 120, 140, 160, and 180 nM) were prepared by serial dilution of the stock solution. The analytical performances of

the sensor were evaluated by applying the differential pulse voltammetry (DPV). The calibration curves for the relationship between changes in the current peak and increasing standard concentration of the template were constructed under optimized conditions for the evaluation of the analytical performance of the MI-PANI-PAA/GCE sensor (Figure 7A). As depicted in Figure 7B, a positive linear relationship between the current response and negative logarithmic concentration of MA was observed from 0.1 to 180 nM. The linear regression equation was the peak current, I_p (μA) = $2.443[-\log \text{Melamine}] + 13.024$ in the concentration range of 0–180 nM with a correlation coefficient $R^2 = 0.9998$. Limit of quantification (LOQ) and limit of detection (LOD) were calculated using the equation $\text{LOQ} = 10S_b/m$ and $\text{LOD} = 3S_b/m$, where S_b and m are the standard deviations of the blank for 10 measurements and the slope of the calibration curve, respectively.⁴⁰ The calculated LOD and LOQ are 1.72×10^{-11} and 5.73×10^{-11} M, respectively.

One of the primary requirements of the MIP-based sensing layer is the selective recognition of the template molecule in the presence of other molecules. This feature is due to the presence of specific binding sites and cavity created in the MIP materials^{41,42} during the polymerization and template extraction processes.

The specificity of the developed sensor was evaluated by comparing the current response of the three different electrodes, namely, MI-PANI-PAA/GCE, NI-PANI-PAA/GCE, and bare GCE, after incubating them in 20 μM MA using DPV. The results showed that there is a great difference between the modified and unmodified electrodes as well as the imprinted and nonimprinted polymer-modified electrodes (Figure 7C). These major differences arise from the imprinting effect, which highly assisted in the recognition of the template, interestingly. To study the selectivity of the MIP sensor, the DPV of MA and structural analogues such as acetoguanamine (AGA) and diaminomethylatrazine (DMT) on MIP sensor were obtained after immersing the sensor into 5 mM ABS (pH

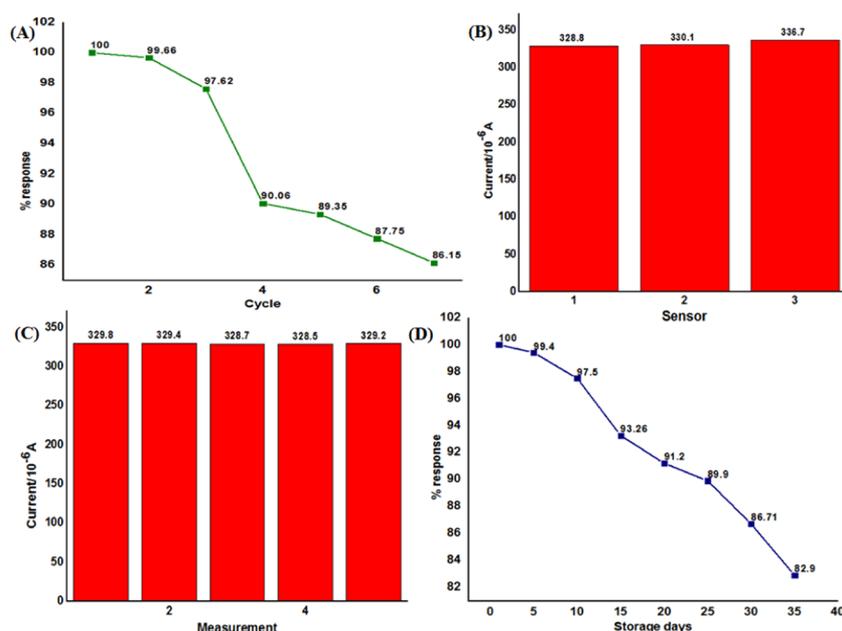


Figure 8. Recycle (A), fabrication and measurement (B, C), and stability (D) of MI-PANI-PAA/GCE sensor and the corresponding current responses monitored by DPV at an amplitude of 0.05 V and pulse width of 0.05 s.

7) containing 10 μM melamine and 30 μM each of the analogues, and the results are shown in Figure 6D.

The presence of interferences does not affect the performance of the sensor significantly. Compared with melamine, the current responses generated by DMT and AGA are very small, and the MI-PANI-PAA/GCE has good selectivity and sensitivity toward the template. This may be attributed to the creation of three-dimensional cavities that are complementary in shape, size, and conformation to the template but not to the interferences. This means the interferences used here do not fit into the binding cavities of the polymer and, thus, their current response is minimal.

The application of target analyte as a template model during polymerization provides an advantage of selectivity. Furthermore, the commonly occurring interferences, such as cyanuric acid and cyromazine, were applied as interference to control the selectivity of the imprinted thin composite film sensor. However, no current signals were observed for both of them. This might be due to the differences in the functional groups and their orientation.

Reusability, Reproducibility, and Stability of MI-PANI-PAA/GCE Sensor. The recycling of MI-PANI-PAA/GCE during repeated utilization was studied by replicate template extraction and incubation processes. The sensor can be used up to five times by maintaining $\geq 90\%$ of its original current response (Figure 8A). After seven cycles, about 86.15% of its response was sustained. Both the fabrication method and measurement reproducibility were investigated for the developed sensor, as indicated in Figure 8B,C. For the repeatability of the fabrication method, three different sensors were fabricated following the same procedure and protocols described in the previous section. The rebinding of the same solution of melamine was observed and current response due to the binding of the template was recorded.

The current values were 328.8, 330.1, and 336.7 μA , respectively, with relative standard deviation (RSD) of 1.28%. This indicates that the fabrication method has good reproducibility with insignificant deviation from one another.

Five different electrodes were prepared by the same method and used to measure the same amount of melamine solution (20 μM) to study the measurement reproducibility. The current responses were 329.83, 329.46, 328.75, 328.59, and 329.28 μA , with RSD of 0.159%, confirming that the sensor has good and acceptable reproducibility to detect melamine. The operational stability of binding sites is the most important factor that affects the performance of MIP-based sensors in addition to the polymer materials' stability itself. The damage of these recognition sites significantly affects both the selectivity and sensitivity of the sensor.^{42,43} For evaluating the long-time stability of the proposed sensor, it was kept in ambient conditions for 35 days.

At different storage periods between 1 and 35 days, the sensor was used to detect the same melamine solution (20 μM) in 50 mM ABS (pH 7) after every 5 days starting from the first day and the current response was measured by DPV. The obtained results indicated that the peak current retained 90% of its first current until 20 days, indicating that the fabricated sensor had good long-term stability, as depicted in Figure 8D. The decline in the current response after 3 weeks might be primarily due to the damage of the recognition cavities or the washing away of the film from the electrode's surface due to degradation or poor adhesion.

Practical Application of the Sensor and Comparison with Other Methods. Melamine from milk samples was treated using the method reported elsewhere.⁴⁴ Briefly, melamine extraction was performed using acetonitrile/water (50:50, v/v) as extracting solvents and filtered by using a filter (0.45 μm) after centrifuging the sample for enough time. The proposed sensor was applied for the determination of melamine in commercial infant formula milk samples to evaluate its practical applications. A known amount of melamine samples was added to the milk sample in three levels and detected by the DPV method. As can be seen (Table 3), the values obtained by the proposed sensor achieved a recovery of 93.2–105.63% with RSD between 1.11 and 4.5%.

Table 3. Determination Results of MA in Infant Formula Milk Sample ($n = 3$)

	amount added (μg)	amount detected (μg)	recovery (%)	RSD (%)
infant formula	100	90.10	95.87	2.23
	200	184.6	92.30	1.42
	300	298.9	105.63	1.11
liquid milk	100	93.2	93.2	4.5
	200	204.8	102.2	3.7
	300	305.2	101.7	3.2

To further study the performance of the MI-PANI-PAA/GCE sensor, the developed sensor's analytical performances were compared with the previously reported results of other MIP-based sensors with different transduction mechanisms (Table 4).

Table 4. Comparison of the Linear Range and Limit of Detection for Various Transduction Mechanisms of MIP Sensors for Melamine Detection

MIP—transduction mechanism	linear range	LOD	ref
<i>o</i> -aminophenol—optical	10–10 mM	5.1 pM	28
silica sol–gel—optical	79.2 μM to 39.6 mM	1.24 μM	45
methacrylic acid—electrochemical	50 μM to 10 mM	6 μM	20
2-mercaptobenzimidazole—impedimetric	100–50 μM	3 nM	46
<i>p</i> -aminobenzoic acid—electrochemical	4 μM to 0.45 mM	36 μM	29
bis(bithiophene)—acoustic	5 nM to 1 mM	5 nM	25
methacrylic acid—electrochemical	100 nM to 10 mM	1.75 pM	23
silica sol–gel—electrochemical	5.1 nM to 1 μM	40 nM	30
methacrylic acid—electrochemical	1 μM to 10 mM	88.6 μM	47
pyrrole/graphene—electrochemical	30 nM to 10 mM	10.2 nM	24
[Bmim]BF ₄ -CNT-IL—electrochemical	40–9.2 μM	11 μM	26
aniline/acrylic acid composite—electrochemical	0.1–180 nM	17.2 pM	present work

The selected parameters compared here are sensitivity, linear range, and detection limits. The proposed sensor showed better affinity, sensitivity, and selectivity toward the target template.

CONCLUSIONS

The current results described a new molecular recognition element based on molecularly imprinted PANI-PAA composite film being successfully electrosynthesized on the GCE for the detection of melamine in infant formula. The presence of PAA successfully modified the electrochemical properties of PANI to work at neutral conditions. The voltammetric sensor developed performs in a neutral solution to recognize the melamine template in both artificial and real samples. The optimization of important parameters and template removal generated melamine-binding cavities in MI-PANI-PAA film with enhanced sensitivity to achieve the calculated detection limit of 0.0172 nM and the recovery range of 95.87–105.63% with a relative standard deviation of 1.11–2.23%. The sensitivity of the proposed sensor may be attributed to the synergistic effect of the amine, carboxylic, and aromatic rings in

the functional monomers/polymer composite. Moreover, the synthesized polymer composite films showed stability and proved as promising molecular recognition elements for the online monitoring of melamine occurrence in the food industry in the future.

EXPERIMENTAL SECTION

Chemicals and Reagents. Melamine (>99%, Sigma-Aldrich, M2659), acrylic acid (anhydrous, >99%, Sigma-Aldrich), aniline ($\geq 99.99\%$, Sigma-Aldrich, 242284), methanol ($\geq 99.9\%$), ethanol (>99%), nitric acid (70%), sulfuric acid (99.98%), dimethyl sulfoxide (AR), acetic acid ($\geq 99.85\%$), sodium acetate ($\geq 99\%$), potassium chloride (AR), hydrochloric acid (AR, 37%), sodium hydroxide (AR), potassium ferrocyanate (AR, >99%) and deionized water (DIW). Prior to use, aniline and acrylic acid were purified by distillation and stored under nitrogen gas at 10 °C. To avoid the self-polymerization of acrylic acid, 80% aqueous solution was prepared by using the distilled acrylic acid. All standard solutions used in this work were prepared by using deionized water (DIW) (18.2 M Ω cm) that was purified by a purity purification system (Inexuxu model 2232, Biotech Pvt Ltd., Simens, Germany). Melamine standard solutions were prepared from melamine standard in dimethyl sulfoxide and then diluted with acetonitrile during experimental works.

Instrumentation and Apparatus. Three electrode system CHI660D electrochemical workstation (CHI instrument) was used for all electrochemical measurements and polymer synthesis. Fourier transform infrared (FTIR) spectra of the samples were recorded on an Avatar 360 spectrophotometer (Thermo Nicolet) using KBr pellet in the wavenumber region of 4000–450 cm^{-1} . Scanning electron microscope (SEM) observations were carried out on a JEOL JSM 6500 F microscope equipped with a field emission source.

Electrode Preparation. GCE, saturated calomel electrode (Ag/AgCl) (saturated with 3 M KCl), and solid platinum were used as a working, reference, and counter electrode, respectively. The working electrode was prepared by mechanically polishing it with 0.3 and 0.05 μm alumina slurry and then sequentially soaked in dilute nitric acid, ethanol, and deionized water for not less than 3 min each. The cleaned electrode was characterized with cyclic voltammetry (CV) in $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl between the sweeping potentials of -0.6 to $+0.6$ V at 100 mV/s. Then the surface of the GCE was activated by CV scanning between -0.2 and 1.0 V in 0.5 M sulfuric acid electrolyte, rinsed with deionized water, blown with nitrogen gas, and air-dried. After this, the electrode was ready for surface modification with the imprinted thin composite film.

Preparation of MI-PANI-PAA Composite Film. There are covalent and noncovalent approaches to synthesize MIPs. However, the latter is more progressive due to advantages such as the availability of various functional monomers for different analytes, simplicity of template extraction, and reversibility of the method.^{32,33} Here, the functional monomers were selected based on the noncovalent interactions with the melamine template, as depicted in Table 5. FTIR was used to evaluate and understand the formation of hydrogen bonds and the prepolymerization complex. The six nitrogen atoms in the structure of melamine act as hydrogen bond acceptor units, while the three amine groups ($-\text{NH}_2$) acts as hydrogen bond donor units. Thus, this molecule has the ability to form hydrogen bonding with both hydrogen bond donor and

Table 5. Hydrogen Bond Donor/Acceptor Counts of Melamine, Aniline, and Acrylic Acid

property	property value		
	acrylic acid	melamine	aniline
hydrogen bond donor unit	1	3	1
hydrogen bond acceptor unit	2	6	1

acceptor molecules simultaneously. Therefore, we have selected aniline that has one hydrogen bond donor unit and acrylic acid, which consists of one hydrogen bond donor unit and two hydrogen acceptor units. The FTIR results confirmed that there is an interaction between the template and the monomers, forming a relatively stable prepolymerization complex.

Then, the molecularly imprinted poly(aniline-co-acrylic acid) (MI-PANI-PAA) film was deposited on GC surface from the solution containing 0.2 M ANI, 0.2 M AA, and 0.01 M MA in acetate buffer solution (ABS, pH 3.5) based on multiple noncovalent interactions between them. The non-imprinted poly(aniline-co-acrylic acid) (NI-PANI-PAA) composite films were fabricated using the same procedure in the absence of MA for comparisons. In this work, an electrochemical sensor for the sensitive and selective detection of melamine, based on an MICP composite receptor is prepared through electropolymerization of aniline and acrylic acid in the presence of the melamine as a temple. The general construction of the MI-PANI-PAA/GCE sensor for melamine detection is presented in Scheme 1. To the best of our knowledge, this is the first MICP organic–organic composite recognition element reported to detect melamine using basic and acidic functional monomers.

The optimized electrochemical parameters used during polymerization steps were: sweeping potential -0.2 to $+1.0$ V, scan rate 100 mV/s, and scan cycles 10, respectively. After rinsing the fabricated electrode with buffer, the template was removed from the polymer films by washing it with a dimethyl sulfoxide and acetic acid (4:1, v/v) mixture in ABS buffer (pH 7.0) for about 35 min. After drying, the prepared sensors were incubated in MA solutions for rebinding investigation for 20 min.

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M.B.R. planned, executed the experiments, and wrote the manuscript; T.R.S. and O.E.F. are scientific advisors; and P.C.R. provided the laboratory facilities for the work and edited the paper.

Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Tolleson, W. H.; Diachenko, G. W.; Folmer, D.; Doell, D.; Heller, D. *Chemistry of Melamine Alone and in Combination with Related Compounds: Toxicological and Health Aspects of Melamine and Cyanuric Acid*; Ottawa, Canada, 2008.
- (2) Satzger, R. D. In *Characterization of a Pet Food Adulteration Problem by Utilizing a Multidisciplinary Analytical Approach*, Proceedings of the 235th National Meeting of the American Chemical Society, New Orleans, LA, Washington, DC, 2008.
- (3) Saylan, Y.; Akgonullu, S.; Yavuz, H.; Unal, S.; Denizli, A. Molecularly Imprinted Polymer-Based Sensors for Medical Applications. *Sensors* **2019**, *19*, No. 1279.
- (4) Pohanka, M. Sensors Based on Molecularly Imprinted Polymers. *Int. J. Electrochem. Sci.* **2017**, *12*, 8082–8094.
- (5) Antuña-Jiménez, D.; Diaz-Diaz, G.; Blanco-Lopez, M. C.; Lobo-Castanon, M. J.; Miranda-Ordieres, A. J.; Tunon-Blanco, P. Molecularly imprinted electrochemical sensors. *Mol. Imprinted Sens.* **2012**, 1–34.
- (6) Algieri, C.; Drioli, E.; Guzzo, L.; Donato, L. Bio-mimetic sensors based on molecularly imprinted membranes. *Sensors* **2014**, *14*, 13863–13912.
- (7) Iskierko, Z.; Sharma, P. S.; Bartold, K.; Pietrzyk-Le, A.; Noworyta, K.; Kutner, W. Molecularly imprinted polymers for separating and sensing of macromolecular compounds and micro-organisms. *Biotechnol. Adv.* **2016**, *34*, 30–46.
- (8) Battista, E.; Scognamiglio, P. L.; Luise, N. D.; Raucci, U.; Donati, G.; Rega, N.; Netti, P. A.; Causa, F. Turn-on fluorescence detection of protein by molecularly imprinted hydrogels based on supramolecular assembly of peptide multi-functional blocks. *J. Mater. Chem. B* **2018**, *6*, 1207–1215.
- (9) Lattach, Y.; Garnier, F.; Remita, S. Influence of chemical and structural properties of functionalized polythiophene-based layers on electrochemical sensing of atrazine. *ChemPhysChem* **2012**, *13*, 281–290.
- (10) Singru, R. N. Electronic applications of 8-HQS-SAOF terpolymer resin. *Pharma Chem.* **2011**, *3*, 123–127.
- (11) Khedkar, K. M.; Kalambe, A. B.; Hiwase, V. V.; Deosarkar, S. D. Synthesis and electrical conductance study of newly synthesized ligands derived from m-cresol, melamine, and formaldehyde. *Arch. Appl. Sci. Res.* **2013**, *5*, 153–158.
- (12) Gonzalez-Vogel, A.; Fogde, A.; Crestini, C.; Sandberg, T.; Huynh, T. P.; Bobacka, J. Molecularly imprinted conducting polymer for determination of a condensed lignin marker. *Sens. Actuators, B* **2019**, *295*, 186–193.
- (13) Bober, P.; Humpolicek, P.; Pachernik, J.; Stejskal, J.; Tom Lindfors, T. Conducting polyaniline based cell culture substrate for embryonic stem cells and embryoid bodies. *RSC Adv.* **2015**, *5*, 50328–50335.
- (14) Janata, J.; Josowicz, M. Conducting polymers in electronic chemical sensors. *Nat. Mater.* **2003**, *2*, 19–24.

- (15) Zhang, K.; Zhang, L. L.; Zhao, X. S.; Wu, J. Graphene/Polyaniline Nanofiber Composites as Supercapacitor Electrodes. *Chem. Mater.* **2010**, *22*, 1392–1401.
- (16) Boeva, Z. A.; Sergeev, V. G. Polyaniline: Synthesis, Properties, and Application. *Polym. Sci., Ser. C* **2014**, *56*, 144–153.
- (17) Bai, H.; Shi, G. Gas Sensors Based on Conducting Polymers. *Sensors* **2007**, *7*, 267–307.
- (18) Homma, T.; Kondo, M.; Kuwahara, T.; Shimomura, M. Electrochemical polymerization of aniline in the presence of poly(acrylic acid) and characterization of the resulting films. *Polymer* **2012**, *53*, 223–228.
- (19) Alexander, C.; Andersson, H. S.; Andersson, L. I.; Ansell, R. J.; Kirsch, N.; Nicholls, I. A.; O'Mahony, J.; Whitcombe, M. J. Molecular imprinting science and technology: A survey of the literature for the years up to and including 2003. *J. Mol. Recognit.* **2006**, *19*, 106–180.
- (20) Liang, R.; Zhang, R.; Qin, W. Potentiometric sensor based on molecularly imprinted polymer for determination of melamine in milk. *Sens. Actuators, B* **2009**, *141*, 544–550.
- (21) Khelif, A.; Gam-Derouich, S.; Jouini, M.; Kalfat, R.; Chehimi, M. M. Melamine-imprinted polymer grafts through surface photopolymerization initiated by aryl layers from diazonium salts. *Food Control* **2013**, *31*, 379–386.
- (22) Hu, Y.; Feng, S.; Gao, F.; Li-Chan, E. C. Y.; Grant, E.; Lu, X. Detection of melamine in milk using molecularly imprinted polymers—surface-enhanced Raman spectroscopy. *Food Chem.* **2015**, *176*, 123–129.
- (23) Bakas, I.; Salmi, Z.; Jouini, M.; Geneste, F.; Mazerie, I.; Floner, D.; Carbonnier, B.; Yagci, Y.; Chehimi, M. M. Picomolar Detection of Melamine Using Molecularly Imprinted Polymer-Based Electrochemical Sensors Prepared by UV-Graft Photopolymerization. *Electroanalysis* **2015**, *27*, 429–439.
- (24) Ji, Z.; Chen, W.; Wang, E.; Deng, R. Electropolymerized Molecular Imprinting & Graphene Modified Electrode for Detection of Melamine. *Int. J. Electrochem. Sci.* **2017**, *12*, 11942–11954.
- (25) Pietrzyk, A.; Kutner, W.; Chitta, R.; Zandler, M. E.; D'Souza, F.; Sannicolo, F.; Mussini, P. R. Melamine Acoustic Chemosensor Based on Molecularly Imprinted Polymer Film. *Anal. Chem.* **2009**, *81*, 10061–10070.
- (26) Liu, B.; Xiao, B.; Cui, L.; Wang, M. Molecularly imprinted electrochemical sensor for the highly selective and sensitive determination of melamine. *Mater. Sci. Eng., C* **2015**, *55*, 457–461.
- (27) Xu, S.; Lin, G. Y.; Zhao, W.; Wu, Q.; Luo, J.; Wei, W.; Liu, X. Y.; Zhu, Y. Necklace-like Molecularly Imprinted Nanohybrids Based on Polymeric Nanoparticles Decorated Multiwalled Carbon Nanotubes for Highly Sensitive and Selective Melamine Detection. *ACS Appl. Mater. Interfaces* **2018**, *10*, 24850–24859.
- (28) Li, W.; Zheng, Y.; Zhang, T.; Wu, S.; Zhang, J.; Fang, J. A Surface Plasmon resonance-based Optical Fiber Probe Fabricated with Electropolymerized Molecular Imprinting Film for Melamine Detection. *Sensors* **2018**, *18*, No. 828.
- (29) Liu, Y. T.; Deng, J.; Xiao, X. L.; Ding, L.; Yuan, Y. L.; Li, H.; Li, X. T.; Yan, X. N.; Wang, L. L. Electrochemical sensor based on a poly(para-aminobenzoic acid) film modified glassy carbon electrode for the determination of melamine in milk. *Electrochim. Acta* **2011**, *56*, 4595–4602.
- (30) Bengamra, M.; Jaoued, N. G.; Riani, A. K.; Chehimi, M. M.; Kalfat, R. Highly Selective Molecularly Imprinted Sol-gel Membrane Grafted to Gold for the Detection of Melamine. *Silicon* **2019**, *11*, 2267–2274.
- (31) Arduini, M.; Calama, M. C.; Timmerman, P.; Reinhoudt, D. A novel type of hydrogen-bonded assemblies based on the melamine–cyanuric acid motif. *J. Org. Chem.* **2003**, *68*, 1097–1106.
- (32) Janotta, M.; Weiss, R.; Mizaikoff, B.; Brüggemann, O.; Ye, L.; Mosbach, K. Molecularly Imprinted Polymers for Nitrophenols - An Advanced Separation Material for Environmental Analysis. *Int. J. Environ. Anal. Chem.* **2001**, *80*, 75–86.
- (33) Zhang, W.; She, X.; Wang, L.; Fan, H.; Zhou, Q.; Huang, X.; Tang, J. Z. Preparation, Characterization and Application of a Molecularly Imprinted Polymer for Selective Recognition of Sulpiride. *Materials* **2017**, *10*, No. 475.
- (34) Bajpai, A. K.; Mishra, A. Preparation and characterization of tetracycline-loaded interpenetrating polymer networks of carboxymethyl cellulose and poly (acrylic acid): Water sorption and drug release study. *Polym. Int.* **2005**, *54*, 1347–1356.
- (35) Kurkuri, M. D.; Lee, J. R.; Han, H.; Lee, I. Electroactive behavior of poly(acrylic acid) grafted poly(vinyl alcohol) samples, their synthesis using a Ce(IV) glucose redox system and their characterization. *Smart Mater. Struct.* **2006**, *15*, 417–423.
- (36) Bajpai, A. K.; Bajpai, J.; Soni, S. N. Preparation and characterization of electrically conductive composites of poly(vinyl alcohol)-g-poly(acrylic acid) hydrogels impregnated with polyaniline (PANI). *eXPRESS Polym. Lett.* **2008**, *2*, 26–39.
- (37) Gupta, B.; Prakash, R. Synthesis of Processible Doped Polyaniline-Polyacrylic Acid Composites. *J. Appl. Polym. Sci.* **2009**, *114*, 874–882.
- (38) Sharma, I. D.; Saini, P. K.; Sharma, V. K. Structural, optical, morphological, and electrical characteristics of polyaniline for device applications. *Indian J. Eng. Mater. Sci.* **2013**, *20*, 145–149.
- (39) Braga, G. S.; Lieberzeit, P. A.; Fonseca, F. J. Molecularly Imprinted Polymer-Based Sensor to Detect Isoborneol in Aqueous Samples. *Procedia Eng.* **2016**, *168*, 448–451.
- (40) Gam-Derouich, S.; Jouini, M.; Ben Hassen, D.; Chehimi, M. M. Aryl diazonium salt surface chemistry and graft photopolymerization for the preparation of molecularly imprinted polymer biomimetic sensor layers. *Electrochim. Acta* **2012**, *73*, 45–52.
- (41) Yan, C.; Li, J.; Meng, T.; Liu, X.; Zhang, R.; Chen, Y.; Wang, G. Selective Recognition of Ciprofloxacin Hydrochloride Based on Molecular Imprinted Sensor via Electrochemical Copolymerization of Pyrrole and o-phenylenediamine. *Int. J. Electrochem. Sci.* **2016**, *11*, 6466–6476.
- (42) Yang, Z.; Liu, X.; Wu, Y. M.; Zhang, C. J. Modification of carbon aerogel electrode with molecularly imprinted polypyrrole for electrochemical determination of dopamine. *Sens. Actuators, B* **2015**, *212*, 457–463.
- (43) Atta, N. F.; Abdel-Mageed, A. M. Smart electrochemical sensor for some neurotransmitters using imprinted sol-gel films. *Talanta* **2009**, *80*, 511–518.
- (44) Filazi, A.; Sireli, U. T.; Ekici, H.; Can, H. Y.; Karagoz, A. Determination of melamine in milk and dairy products by high-performance liquid chromatography. *J. Dairy Sci.* **2012**, *95*, 602–608.
- (45) Yu, J.; Zhang, C.; Dai, P.; Ge, S. Highly selective molecular recognition and high throughput detection of melamine based on the molecularly imprinted sol-gel film. *Anal. Chim. Acta* **2009**, *651*, 209–214.
- (46) Wu, B.; Wang, Z.; Zhao, D.; Lu, X. A novel molecularly imprinted impedimetric sensor for melamine determination. *Talanta* **2012**, *101*, 374–381.
- (47) Harsini, M.; Pari, S. G.; Mandasari, E. Melamine selective electrode based on nanoporous carbon/molecularly imprinted polymer. *J. Chem. Pharm. Res.* **2014**, *6*, 65–70.