3. Results and discussion

3.1. The recognition selectivity of TCAA-imprinted polymers

The TCAA-imprinted polymers produced by the copolymerization of 4-vinylpyridine monomer and ethylene glycol dimethacrylate cross-linker in the presence of the TCAA template either by bulk or multi-step swelling polymerization technique were examined for their recognition property. Table 1 lists the microgram adsorption of TCAA or its analogs per gram of the two types of TCAA imprinted- or non-imprinted polymers, in acetonitrile medium. The value of the cross-reactivity, CR (%), which is obtained from the adsorption value of the particular MIP for the analog relative to that for TCAA, is also listed. For the halo acids, the adsorption value for the MIP is considerably larger than that for the non-MIP in both techniques, which this result verifies the selectivity due to the imprint of the MIPs. In general, the MIPs of the two imprinting methods exhibit similar trends of their recognition selectivity to halo acids, in that the adsorption value is the largest for TCAA and is comparable for DCAA, MCAA and DBAA, whereas it is least for TBAA. Except in case of MBAA, only the MIP prepared by the MSP method gives a large value for this. From the result obtained, it is apparent that the MIPs have high cross-reactivity with the structurally closely related TCAA. In addition to above observations, non-specific adsorption of non-halo acids is noticed, from the comparable adsorption of MIP and nonMIP for both techniques. In two classes of TCAA

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analogous compounds, halo acid having halogen substituent group on the side chain shows high binding with the MIPs relative to non-halo acid, suggesting that the TCAA-imprinted polymers prepared have high specificity for the haloacetic acids.

The order of the recognition selectivity of MSP-based MIP for HAAs was TCAA > DCAA > MCAA > DBAA > MBAA > TBAA, while that of BP-based MIP was TCAA > DCAA > DBAA > MCAA > MBAA > TBAA. Typically, hydrogen bonding and proton transfer interactions giving rise to ionic species are central to the non-covalent binding of the template molecule onto the MIP, although other interactions such as hydrophobic, dipole-dipole, van der Waals may play an important role [14]. Due to the presence of the carboxylic group on the TCAA molecule and the amino groups on the functional monomer, two different interactions, hydrogen bonding and ion-ion events can be formed. The rank order in selectivity of both TCAA-imprinted polymers is clear, which suggests that electrostatic interaction via hydrogen bonding is the driving force for the binding of HAAs onto the MIPs. Additionally, through careful analysis of the chemical structure of the lower members of the series, it becomes clear that optimal fit of ligand in the TCAA binding site is also necessary to the selective recognition.

From the result obtained, it appears that both imprinting methods can produce a polymer with binding sites having shape and size selection, and having the orientation of functional groups to recognize the TCAA molecules. The MIPs shows multiple selectivity towards HAAs, implying that their recognition behaviour is group specific rather than ligand specific, and that they are therefore suitable as recognition elements for the screening of the group of HAAs commonly present as mixtures in chlorinated water.

A comparison of cross-reactivity values (Table 1) obtained from the two types of TCAA-imprinted polymers reveals that the MSP-based MIP cross-reacts better with a wide range of HAAs than the BP-based MIP. It is also evident from the adsorption data that the MSP-based MIP shows higher affinity for the TCAA or TCAA analog than the BP-based MIP. Therefore, the TCAA-imprinted polymer prepared by the MSP method was chosen as the sensing element in the proposed sensor. However, in the early stage of developing the sensor, a relatively large amount of polymer is required for this purpose; whilst bulk polymerization method offers an easy and fast synthesis process, leading to a high yield of polymer and a reduction in the cost of the experiments. For these reasons, the MSP-based polymer was replaced by the BP-based polymer for the investigation in the technological parameters.

3.2. The investigation of the technological parameters

3.2.1. Influence of the current frequency on the sensor response

In the present work, the effect of operating frequency on the sensor response was studied to identify the optimum operating frequency. For this purpose, A.C. impedance spectra of the sensor were measured over the frequency range 0.2-30 kHz using the TCAA concentration upto 0-1 mg l^{-1}. Figure 1 shows the effect of the applied frequency on the impedance of the sensor. As can be seen that the impedance response of the membrane sensor initially increases with increasing TCAA concentration but flatters out at high concentrations. The increase of the sensor resistance is attributed to the TCAA molecule preferentially adsorbing on the selective sites in the polymer domain. Moreover, for the same concentration of TCAA, the high frequencies there was no

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proportional increase in the impedance response of sensor for the high frequencies, particularly at high concentration of TCAA. This may be caused by the high A.C. frequency making the membrane pores expand [15] so that the analyte can transport across the membrane. The diffusion of the analyte leads to variation of impedance response of sensor. As is evident in Figure 1, an A.C. frequency of 1.5 kHz gives a reasonable impedance response of sensor over the widest concentration range of TCAA, so that this frequency has been employed in all subsequent experiments.

3.2.2. Influence of the membrane composition on the sensor response

The membrane prepared here gives a stable signal response of sensor within 10 sec, being faster than that of several membranes prepared for other sensors [8,9]. Previous work on the potentiometric detection of organic acids using electrodes modified by PVC membranes incorporating monocyclic hexa-amines demonstrated that phthalate plasticizers could facilitate the penetration of the carboxylic acid into the PVC-based membrane [16]. This may be a reason for the rapid response of our sensor.

In addition, it was found that the quantity of the casting solvent affects the characteristics of the membrane. With a low volume of the casting solvent (much less than 6 ml), the polymer resin was too viscous to spread and form a membrane. By contrast, the specified volume of 6 ml (or even a volume upto 8 ml) yielded not only the good spreading of the solution in forming as membrane but also a good mechanical strength and stability of membrane. With double this volume (12 ml), the electrical

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potential of sensor was not consistent, but gradually increased and eventually reached steady state at the same value as the resting potential. This is because the increased amount of solvent leads to the formation of pores in the immobilised membrane, which allows the diffusion of the analyte to the another chamber, hence the variation of membrane potential with time. This explanation is supported by micrographs from a scanning electron microscope (Jeol Series JSM 5200, CA, USA), showing the formation of large pores in the membrane prepared using 12 ml of casting solvent (see Figure 2a) as contrasted with the smooth skin layer of the membrane prepared using 6 ml casting solvent (see Figure 2b).

The amount of polymer also has a significant influence on the sensor response. As is evident from Figure 3, increasing the amount of MIP promotes increased impedance response of sensor. This can be explained that the numeral binding sites increased with increase in the amount of MIP. However, a low amount of MIP (less than 40 mg) gives a very soft layer membrane. On the other hand, higher amounts of MIP (more than 70 mg) results in a slower response of the sensor and poorer mechanical strength of the membrane due to the reduction in content of the membrane base.

3.2.3. Influence of the NaCl concentration on the sensor response

Generally, the drawback of conductometric sensors is their poor specificity, particularly when the measurement is performed in buffers with a high capacity and high ionic strength. In the present study, the influence of the ionic strength on the sensor response was investigated. It can be seen from Figure 4 that increasing the NaCl concentration up to 35 mM leads to an increased conductivity in both the MIP

sensor and the reference sensor. However, a salt concentration more than 35 mM induces a decrease in conductivity of those sensors, although the conductivity should increase with salt concentration. Indeed, the conductivity behaviour of our sensors is similar to that of the cross-linked polyurethane acrylate electrolytes prepared in previous work [17,18]. It is noteworthy that the sensor fabricated with the MIP-immobilised PVC membrane had an ionic conductivity similar that of polymeric solid electrolytes. It has been established that synthetic polymers such as polyethylene oxide, poly(methyl methacrylate), polyacrylonitrile and polyvinyl chloride exhibit ionic conductivity in the presence of excess electrolytes such as LiCl and NaCl [19-21]. Such behaviour is attributed to the mobility of the ions coupled with the segmental motion of the polymer chains. On the basis of the explanation given in previous work [17,18], the decrease in conductivity of sensor with increasing in salt concentration may be a reflection of two opposing effects, namely an increase in the number of charge carriers and a decrease in the free volume. Although increase in salt concentration leads to increase in the number of charge carriers, increasing the salt concentration also increases the transport of the salt (Na^+) in membrane and the formation of transient cross-link with the PVC polymer, which restricts the segmental motion of the polymer chains. This result leads to a decrease in the mobility of the charge carriers, and hence the conductivity of sensor decreases. In addition, increasing in salt concentration may lead to the formation of charge-neutral contact ion pairs, which do not contribute to conductivity [22,23].

Despite this variation in sensor conductivity with salt concentration there was a significant difference in the conductivity obtained from MIP sensor and that from the

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reference sensor, which is apparent in the NaCl concentration range between 10 and 125 mM (Figure 4), and which indicates the selectivity of the MIP sensor to TCAA. In all cases of that difference, the conductivity response of reference sensor was always larger than that of MIP sensor. It is pertinent to note that the difference of conductivity of the MIP sensor and the reference sensor in the absence of TCAA were very small: G_{eff}-reference sensor/G_{eff}-MIP sensor = 1.05. Moreover, the role of ionic strength on the selective response of sensor was significant since the selectivity of the sensor (G_{eff}-reference sensor/G_{eff}-MIP sensor = 17.5) was the highest at a NaCl concentration of 75 mM.

The present study demonstrates that although there is a minor effect of electrolyte on the sensor response, the sensor comprising of MIP immobilised membrane and the conductometric transducer studied can give high specificity with TCAA and a stable and reasonable signal response throughout the salt concentration range studied.

3.2.4. Influence of the medium pH on the sensor response

The influence of pH of the sample medium on conductivity response of the sensor was examined over the pH range 3-9. It is evident from Figure 5 that increasing in the sample pH leads to a decrease in sensor conductivity. On the other hand, changing the pH did not effect the conductivity of the reference sensor. The difference in conductivity responses of MIP sensor and reference sensor over the pH range studied was apparent and this difference was more pronounced at high buffer pH. It is well known that in aqueous medium the hydrogen bond interaction between MIP and ligand is

suppressed. Also, ionization of TCAA is greater at high pH than at low pH due to its strong acidity. But from the results obtained, the selectivity of MIP sensor was larger at high pH values (pH 7-9). Hence in aqueous environment the main force of binding between the MIP and TCAA molecule is most likely derived from ionic interaction between the deprotonated carboxylic group and functional monomer. It is noted that the value of difference of the conductivity responses obtained from MIP sensor and reference sensor was essentially constant in the interval of pH 7-9, though the maximum selectivity was evident at pH 9. However at very high pH, the mechanical properties of the membrane are poor. In order to have a compromise between the selectivity and mechanical property of the selective membrane, the sensor was measured at pH 7 of sample solution.

3.3. Concentration dependence of the sensors

The dependency of the sensor response upon the concentration of the TCAA was investigated using sensors constructed with MIPs made by either that of the two imprinting methods. As can be seen from Figure 6, MIP sensors give a signal response initially rises with an increase in the concentration of TCAA and then starts to level off. While both reference sensors show very low signal response with much less changes in the sensor responses upon the additional TCAA. The sensor response of reference sensors is apparently caused by weak non-specific adsorption of the polymer materials. The apparently hyperbolic curves shown in case of MIP sensors suggest that the substrate bind to only one active site on the MIPs. Moreover, the sensitivity of the

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sensor derived from MSP-based MIP is better than that of the sensor derived from BP-based MIP. In contrast, the level of the steady-state response of the sensor derived from BP-based MIP was appreciably larger than that of the sensor derived from MSP-based MIP. These results reveal that the imprinted sites of MSP-based MIP are more accessible, but less active than that of BP-based MIP, though the latter conclusion is in conflict with the findings in the adsorption studies. Possibly, MSP-based MIP shows a lower selectivity in the sensor. Ordinarily, the polymer prepared by MSP method has imprints that are close to the particle surface allowing easier diffusion of ligands to binding sites, the aforementioned role of the receptor sites in the case of MSP-based MIP is quite reasonable. For the alteration in activity of the receptor sites of MSP-based MIP when incorporated in sensor this may be explained by the presence of the dibutyl phthalate plasticizer in the membrane, which this is added primarily to improve the mechanical properties of the membrane. Owing to the polarity of the plasticizer, it may solvate the ionized TCAA, leading to a decrease in complexation between TCAA and the MIP, while this additive may promote the penetration of TCAA molecule into the membrane. This view is reasonable in the sense that the plasticizer preferentially disrupts the interaction of TCAA with the easily accessible site and hence increased the extent of the effects for MSP-based MIP.

3.4. The selectivity and sensitivity of the sensors

Most MIP syntheses are performed in an organic medium, and studies on ligand binding of the MIP are then very often conducted using these organic solvents as the incubation medium. However, a number of previous studies have demonstrated that

imprint binding selectivity is different in aqueous medium from that in organic solvents [24-26]. Consequently the applicability of the MIP relies on the assay format. The selectivity of TCAA-imprinted polymers exposed to an aqueous solution of HAAs was examined under optimised sensor conditions. The results revealed the steady state response of the MIP sensors more than 25% for HAAs and the negligible change in signal response of the reference sensors (data not shown). The cross-reactivity values of both MIP sensors responding to TCAA and analogs were shown as histograms, in Figure 7.

In sensor experiments, the MIPs show a high selectivity with TCAA and its analogs similar to the binding experiments. However the selectivity profiles observed from these two assay formats are different and this difference is much more pronounced in the case of MSP-based MIP. This may be interpreted as arising from a difference of the solvents used in the two experiments. For example in the organic solvent (acetonitrile) electrostatic interaction via hydrogen bonding is likely to be the dominating factor governing selective recognition; by contrast, in an aqueous medium, acid-base interactions are likely to play a more important role in the recognition mechanism, as is evident from the pH results. Furthermore, a difference between the selectivity profiles of two imprinted polymers in the sensor measurements was noticed, namely TCAA > DBAA > DCAA > MBAA > TBAA > MCAA in the case of MSP-based MIP and TCAA > DCAA> DBAA > TBAA > MCAA > MBAA in the case of BP-based MIP. This situation suggests that there has been a change of imprint binding selectivity of the MIPs because of their incorporation into the sensor. Also, the differences in the selectivity profiles of the two MIPs may be attributed to the plasticizing effect being different for these polymers. As discussed before, the plasticizing effect is far more pronounced in

the case of MSP-based MIP. Although the selectivity profiles of the MIPs were different, these polymers generally cross-react with chlorinated haloacetic acid better than with brominated haloacetic acid for the same degree of halogen substitution. Also, the tri- or di-substituted HAA cross-reacted greater than the mono-substituted HAA. Thus, it seems that the halogen atoms make a significant contribution to recognition by the MIPs.

The sensor containing MSP-based MIP has a higher cross-reactivity with the HAAs tested (except MBAA) than that containing BP-based MIP. Likewise, the former sensor gives a large cross-reactivity value (more than 65%) for each HAA, which is beneficial for the simultaneous measurement of HAAs. In addition to that this sensor offers the linear dynamic range of HAA concentrations that covers the regulation limit of HAAs.

3.5. Calibration and limits of detection

In this study, the calibration characteristics of sensor fabricating with MSP-based MIP were identified. The calibration graphs were obtained from the plot of logarithm of signal response of the sensor (log $\Delta|Z|/|Z|$) versus the logarithm of the concentrations (log$C$) of TCAA and its analogs. The calibration data are shown in Table 2. The linearity of those was observed over the HAA concentration range of 25 to 1000 μg l^{-1}, depending on the compound. The concentration graphs are linear with a correlation coefficient greater than 0.970 for each HAA. The limits of detection as obtained from extrapolation of linear segments of the calibration graph (IUPAC recommendation) were in the range 0.2-5.0 μg l^{-1}.

3.6. The real analysis of the conductometric sensor

The sensor incorporated with MSP-based MIP was employed for the analysis of bottled water as obtained from a local supplier, either as spiked with or without either TCAA or a series of HAAs in representative quantities. Moreover, a method for analysis of HAAs in water, LLE-GC-ECD (modified US EPA method) was used for the verification the content of HAA in the water samples. Table 3 displays the recovery data obtained from the samples analysed with the sensor and with LLE-GC-ECD. The results show the concentration dependence of the recovery of analysis of either TCAA or mixed HAAs in both analysis methods. At high spiking TCAA concentration, there were differences in the recoveries obtained from the sensor and from LLE-GC-ECD. However, the precision of the sensor was better than LLE-GC-ECD, in that the relative standard deviation for the results from three separately prepared samples of the sensor was the range of 0.1-3% while the maximum relative standard deviation of LLE-GC-ECD was 12%. This is due to the fact that assay with the sensor are done without the need for any separations. In the determination of the bottled water spiked with the various levels of the sum of six HAAs, the sensor gave high recovery and repeatability for the group analysis of six HAAs.

The result for the sum of the six HAAs in the bottled water sample was 4.28 µg l^{-1} when was analysed by LLE-GC-ECD, while this result was 7.22 µg l^{-1} for the analysis with the sensor. Since the US EPA regulations require that the concentrations of the five HAAs added together (total HAAs) equal 60 µg l^{-1} or less in drinking water the

TCAA sensor seems to be suitable for determining HAAs in these samples. Moreover the large reading of the sensor indicates that the sensor has high inherent sensitivity and selectivity for the group analysis of HAAs. This is because the sensor has relatively high cross-reactivity with a wide range of HAAs. In general, the determination with LLE-GC-ECD is applicable only to DCAA, MCAA and TCAA but low sensitivity to TBAA and the other brominated acetic acids. The low sensitivity of LLE-GC-ECD for the brominated haloacetic acids is due to the poor methylation efficiency of these compounds with the acidic methanol used in this procedure [13]. Because the sensor is only semi-quantitative method the quantitative analyses of individual HAAs in the water sample by this means can not be carried out. The relative content of each HAA in the bottled water sample was determined using the LLE-GC-EC method: the result was DCAA 0.5%, MBAA 6.5%, MCAA 72%, TCAA 3% and DBAA 18% (the TBAA content was below LOD).