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Polyion-Sensitive Polymeric Membrane-Based Pulstrode as a Potentiometric Detector in Liquid Chromatography

Xuewei Wang,[a] Anant S. Balijepalli,[a] and Mark E. Meyerhoff*[a]

Abstract: Potentiometric polyion-sensitive polymeric membrane electrodes are capable of detecting a wide variety of polyionic macromolecules. Herein, we utilize this lack of selectivity to report the first application of this sensor technology as a detector in liquid chromatography (LC). A reversible polycation pulstrode based on tridodecylmethylammonium-dinonylnaphthalene sulfonate doped within a polymeric membrane is employed as the LC detector. Poly-arginines/protamine mixtures are separated by cation-exchange/affinity chromatography on an immobilized heparin column, with eluted polycation peptide bands clearly observed via the pulstrode detector. The LC-pulstrode system is further applied to follow the production of different polycation peptides derived from thermolysin catalyzed protamine digestion.

Keywords: Detector · Liquid chromatography · Polyion · Potentiometry · Pulstrode

1 Introduction

Many important macromolecules such as highly sulfated polysaccharides (e.g., heparin, chondroitin sulfate, and fucoidan), arginine/lysine-rich proteins (e.g., protamine), amine-terminated dendrimers, polyquaternaryammonium species, and polyphosphates are polyionic. Owing to the lack of any innate chromophores, (for wavelength detection > 260 nm), fluorophores, or redox active groups within their structures, many of these species are difficult to detect by traditional optical or electrochemical measurement techniques. Potentiometric polymeric membrane electrodes have proven to be very useful tools for label-free detection of these polyionic species [1–5]. In such sensors, the polyions can be extracted into polymeric membranes doped with appropriate lipophilic ion-exchangers and quasi-steady state potentiometric responses at the membrane/sample interface are obtained that are proportional to the concentration of target polyions [6]. Traditional potentiometric polyion electrodes, however, are intrinsically irreversible, as the extracted polyions form very strong ion-pairs with the ion-exchangers in the membrane phase and therefore are difficult to be fully ejected from the membrane by typical chemical methods (e.g., pH change, high salt concentration, etc.) [6–8].

Conventional single-use polyion sensors cannot be used as reversible detectors in flowing solutions (e.g., automatic analysis of polyions by polyion sensitive electrodes), including flow-injection systems, microfluidic devices or chromatographic techniques. In 2003, Bakker's group proposed the concept of a polycation-sensitive pulstrode by using a polymeric membrane doped with a lipophilic salt that did not exhibit spontaneous ion-exchange properties [9]. The pulstrode scheme employs a series of sequential electrochemical pulses to control the extraction and then ejection of polyions (polycationic protamine in the original work) into and out of the sensing membrane. By using a modified lipophilic salt and improved electrochemical pulse sequence, a pulstrode for reversible detection of polyanions was recently proposed by our group [10]. We further applied, for the first time, this new polyion pulstrode technology as a detector in conventional flow-injection analysis (FIA) systems [11]. Multiple polycation and polyanion samples can be analyzed sequentially using such a FIA system with the potentiometric membrane electrode detector. Further, concentrations of both polycations and polyanions can be monitored with the same device simply by reversing the polarity of the current used to polarize the lipophilic ion-exchanger species within the polymeric membrane [11]. In related work, Qin's group proposed a reversible and sensitive polyion sensing strategy based on controlled release of the polyion from a polymeric membrane [12–14], and further applied this technique in flow-injection analysis of aptamers and heparin [15,16].

For all types of polyion sensitive polymeric membrane electrodes, their selectivity toward polyions is based on the cooperative ion-pairing interaction between target polyions and the corresponding lipophilic counter-ion within the organic membrane phase. Depending on the properties of the target polyion species, such as charge density and lipophilicity, the EMF sensitivities toward different polyions vary. Thus, for samples containing more than one polycation or polyanion species (e.g., heparin products may contain oversulfated chondroitin sulfate and dermatan sulfate; mixtures of different polycationic peptides during digestion of protamine [17,18]), detection of each polyion would require a separation step, since the sensors will respond to all the polyions of the same

[a] X. Wang, A. S. Balijepalli, M. E. Meyerhoff
Department of Chemistry, University of Michigan
930 N. University, Ann Arbor, MI 48109-1055, USA
*e-mail: mmeyerho@umich.edu

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charge type. However, the lack of polyion selectivity should make such pulstrode type polyion sensitive electrodes well-suited for use as universal detectors in liquid chromatography. Herein, we provide the first examples of using this type of detector in the polycation sensing mode for monitoring the separation of arginine-rich peptides on an immobilized heparin column.

2 Experimental

2.1 Reagents and Materials

Protamine sulfate salt from salmon, thermolysin from *Geobacillus stearothermophilus*, high molecular weight poly(vinyl chloride) (PVC), 2-nitrophenyloctyl ether (*o*-NPOE), tridodecylmethylammonium chloride (TDMAC), Trizma base, and tetrahydrofuran (THF) were purchased from Sigma-Aldrich (St. Louis, MO). Dinonylnaphthalene sulfonic acid (DNNSH) as a 49 % solution in xylenes was a gift from King Industries (Norwalk, CT). HiTrap Heparin HP column was purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Hexa-arginine trifluoroacetate salt (R6) and hepta-arginine trifluoroacetate salt (R7) were purchased from Bachem Americas, Inc (Torrance, CA). Nona-arginine (R9) was obtained from American Peptide Company (Sunnyvale, CA).

2.2 Pulstrode Electrode Preparation and Electrochemical Measurements

The TDMA/DNNS lipophilic salt was synthesized according to a previously reported procedure [11]. Polymeric membranes containing TDMA/DNNS, PVC and *o*-NPOE in a weight ratio of 10 : 30 : 60 were prepared by a solvent-casting technique with THF as the casting solvent. After transferring the cocktail into a glass ring on a glass plate and letting the THF evaporate overnight, a uniform membrane was obtained. Disks of 8-mm diameter were punched from the parent membrane and held in a Philips electrode body with an inner Ag/AgCl reference electrode. The inner filling solution of the electrode consisted of 10 mM Tris-HCl buffer, pH 8.0, containing 20 mM NaCl and 10 mM $CaCl_2$ (Tris-HCl-NaCl-$CaCl_2$ buffer).

A three electrode electrochemical system (polyion sensitive working electrode in a homemade wall jet configuration, a double junction Ag/AgCl reference electrode, and a counter platinum electrode) were linked to the electrochemical instrument (AFCBI bipotentiostat and NI-DAQPad 6015 interface board), in a manner analogous to that are reported recently for polycation and polyanion sensing in a flow-injection analysis mode [11].

2.3 Chromatographic System Integrated with Polyion Sensors

A schematic diagram of the low pressure chromatographic system used in the present work is shown in Figure 1. A peristaltic pump (MINIPULS3, Gilson, Middleton, WI) was used to induce mobile phase flow and a 6-port manual injection valve (VICI, Houston, TX), equipped with a 100 μL sample loop, was employed to introduce polyion samples. The polymeric membrane-based polyion pulstrode detector was mounted in a wall-jet mode using a custom-made adapter (plastic syringe housing). A HiTrap Heparin HP column (1 mL column volume) was connected between the adapter and the injection valve and the flow rate through this column was controlled at 1 mL/min throughout the experiments. A NaCl gradient was generated by introducing 2 M NaCl at a flow rate of 1 mL/min into a 30 mL well-stirred mobile phase reservoir and the mixed carrier solution was flowed into the column at a rate of 1 mL/min during the entire process.

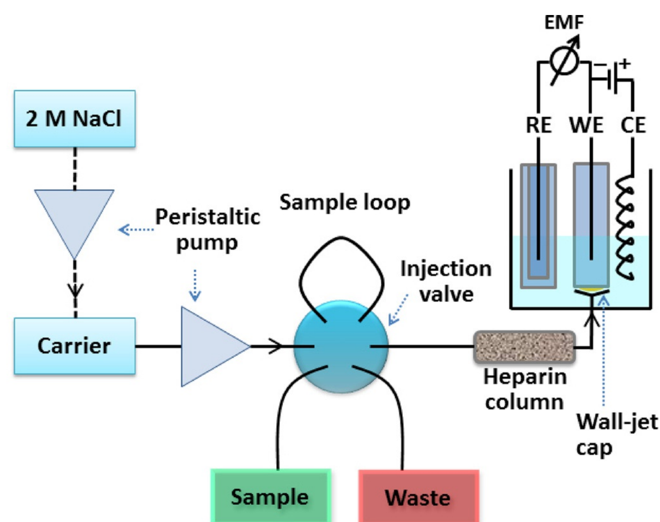

Fig. 1. Schematic representation of the low pressure heparin column-based chromatographic system coupled with a polyion sensitive pulstrode detector. RE, WE, and CE represent reference electrode, working electrode, and counter electrode, respectively.

2.4 Digestion of Protamine

The procedure for column-based protamine digestion has been developed recently by our group [19]. Briefly, thermolysin (5 mg) was immobilized on 3-aminopropyltriethoxysilane-treated silica by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxy-succinimide. Then, the silica gel was filled into a syringe and this thermolysin column was connected to a well-stirred vial containing 40 mL of 25 mg/mL protamine in Tris-HCl-NaCl-$CaCl_2$ buffer. The protamine solution was cycled through the column, submerged in a 60° water bath, via a peristaltic pump at a flow rate of 0.2 mL/min and samples were collected from the vial at different time points after initiating digestion.

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3 Results and Discussion

To eliminate the high iR drop background potential during the initial galvanostatic pulse that polarizes the sensing membrane, the polyion pulstrodes are operated in a triple pulse mode [10,11]. During the initial current pulse (typically 1 s), the applied current forces polyions to be extracted into the sensing membrane from the sample. Then, the EMF values are sampled during the last 10 % of a subsequent zero current pulse phase (typically 0.5 s). In the last step, the membrane is regenerated to the initial chemical conditions by imposing a baseline potential (typically 0 V vs. reference electrode) on the membrane for a period 10–15 times longer than the initial galvanostatic pulse. In our previous FIA work, a 1 s galvanostatic pulse was applied and the total duration for one cycle to complete entire pulse sequence was 17.5 s (1 s galvanostatic pulse, 0.5 s open circuit pulse, 15 s regeneration pulse and 1 s disconnection pulse) [10,11]. Thus, in this earlier work the pulstrode can only record one data point every 17.5 s and such a sampling frequency would be inadequate for chromatographic detector applications.

To explore the possibility of a shorter pulse sequence, we examined the dynamic polyion responses (protamine was used as the sample) in a FIA configuration using the polycation pulstrode with different pulse durations. In a carrier stream of Tris-HCl-NaCl-$CaCl_2$ buffer with a flow rate of 1 mL/min, 100 µL of protamine (at 1 mg/mL) was injected by a 6-port manual injection valve. Figure 2 shows the dynamic potentiometric responses of five consecutive injections of this protamine solution using different pulse sequences. When the galvanostatic pulse is shortened to 0.1 s and the total sequence duration is 2.2 s (the bottom trace in Figure 2), the response peaks for protamine are not decreased significantly compared to those obtained with longer pulses. More importantly, the standard deviation of the EMF peak heights for five consecutive injections is only 0.5 mV when using the 2.2 s total pulstrode sequence, which is significantly better than the reproducibility obtained when employing the longer sampling intervals (SD=7.4 mV for 16.6 s and SD=1.9 mV for 8.6 s). The improved reproducibility suggests the importance of a higher EMF sampling frequency to track the analyte in the flowing phase with a better resolution.

With the optimized pulse sequence in place to achieve potentially better resolution when using the polycation pulstrode as a detector in LC, the detector was examined to monitor the separation of mixed arginine-rich peptides/proteins using a low pressure ion-exchange/affinity chromatography column (see Figure 1, Experimental Section). The heparin-functionalized sepharose column (HiTrap Heparin HP column) was used to separate the polycations based on different affinities of these species toward the immobilized heparin. The starting mobile phase stream was the Tris-HCl-NaCl-$CaCl_2$ buffer. The polyion sample injected from the manual injection valve was then carried into the column by the mobile phase

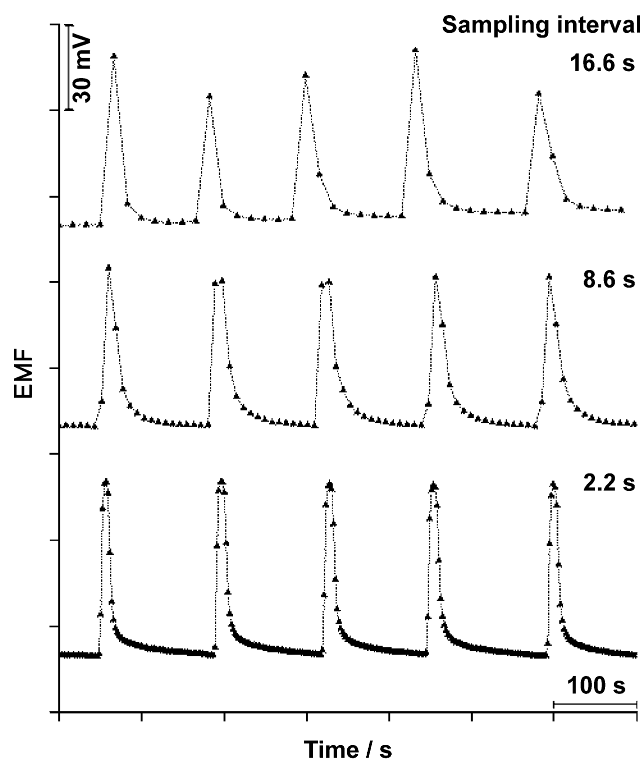

Fig. 2. Effect of different sampling frequencies on dynamic potential responses of 1 mg/mL protamine in Tris-HCl-NaCl-$CaCl_2$ buffer in a flow-injection system (100 µL sample, 1 mL/min flow rate). The sampling interval indicates the duration of one complete cycle of pulse sequence (16.6 s sequence includes 1 s galvanostatic pulse (GP), 0.5 s open circuit pulse (OP), 15 s potentiostatic pulse (PP), and 0.1 s disconnection pulse (DP); 8.6 s sequence includes 0.5 s GP, 0.5 s OP, 7.5 s PP, and 0.1 s DP; 2.2 s sequence includes 0.1 s GP, 0.5 s OP, 1.5 s PP, and 0.1 s DP)). A polymeric membrane doped with TDMA/DNNS was used, and −10 µA current and 0 V potential (vs. Ag/AgCl reference electrode) were applied to the membrane in the galvanostatic and the potentiostatic pulses, respectively.

and forms tight electrostatic complexes with the immobilized heparin. Then, 2 M NaCl was continuously introduced into the carrier solution reservoir to generate a NaCl gradient passing through the column. Polycations with lower number of charges will elute earlier from the column owing to the salt-induced decrease in electrostatic interaction of the peptides, and those peptides with higher total charge and/or charge density will elute later. The eluted polycations reach the sensing membrane of the pulstrode via a homemade wall jet configuration and are detected by the pulstrode in the positive mode. As a proof-of-principle experiment, a mixture of three commercial poly-arginines (R6, R7, and R9) and protamine were separated and detected. As shown in Figure 3, potentiometric signals toward each polycation are obtained as the species elute from the heparin column with a charge number-based elution order. The baseline drifts to a higher EMF values with time are due to increasing

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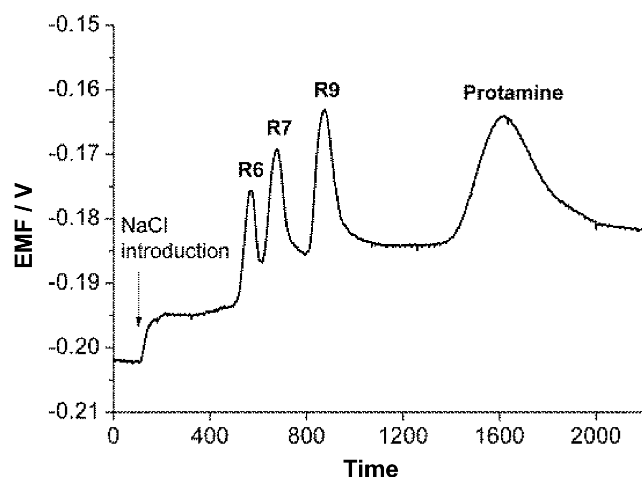

Fig. 3. Potential responses of a polycation mixture (2.5 mg/mL R6, R7, R9, and 5 mg/mL protamine in Tris-HCl-NaCl-$CaCl_2$ buffer) separated and detected via the immobilized heparin column-polyion pulstrode system (Figure 1). The flow rate of the carrier phase (Tris-HCl-NaCl-$CaCl_2$ buffer) was 1 mL/min. The injected sample volume was 100 μL. All pulstrode conditions were identical to those for the bottom trace in Figure 2.

Na^+ concentration within the carrier stream owing to the salt gradient within the mobile phase.

Taking R6 as an example, the EMF responses of the polycation sensor toward 100 μL of different concentrations of R6 injected onto the immobilized heparin column were examined (Figure 4). The areas of the peaks for R6 elution from the column was used for quantification. As shown in Figure 4, the integrated EMF signals are proportional to the logarithm of R6 amount over the range examined (0.002 to 0.25 mg in 100 μL) and a peak for 100 μL of 0.02 mg/mL R6 can clearly be observed. It is known that the dynamic range of pulstrode sensors can be significantly tuned by changing the magnitude of applied current in the galvanostatic pulse or the membrane formulation [20,21], which further facilitates the potential utility of the pulstrode as a detector for various LC applications.

As a further application, the polyion pulstrode-heparin column system was used to analyze peptides generated by thermolysin-catalyzed digestion of protamine. Several thermolysin-digested protamine fragments (so-called "low molecular weight protamine") have been found to exhibit important biomedical functions including serving as a non-toxic heparin antidote, a protein transduction domain, gene carriers, and enhancing the generation of nitric oxide (NO) from NO synthase (NOS) within cells [19,22]. To isolate low molecular weight protamine peptides from coexisting non-functioning fragments and non-digested intact protamine, it is necessary to monitor protamine and its products during the digestion reaction. These digestion fragments have been identified to be rich in arginine by matrix-assisted laser desorption/ionization mass spectrometry [23]. Thus, these species are expected to be polycationic at near-neutral pH and well-suited to

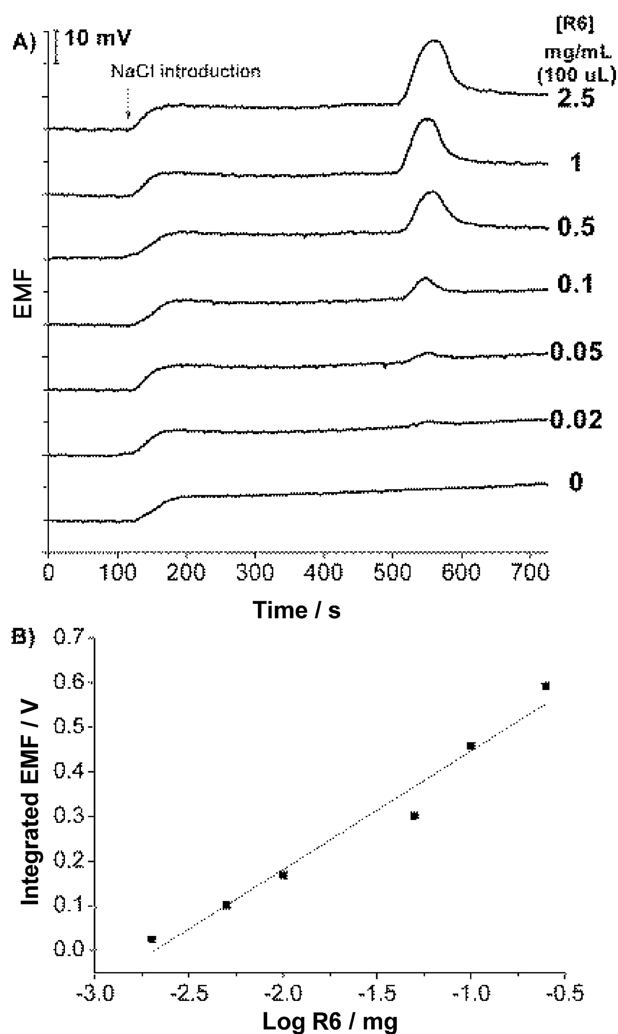

Fig. 4. (A) Potential responses of 100 μL of different concentrations of R6 in the immobilized heparin column-polyion pulstrode system, and (B) the corresponding calibration curve using integrated EMF signals. Chromatography and electrode conditions are identical to those for Figure 3.

be detected by the polyion pulstrode. Therefore, a homemade thermolysin-immobilized silica column was used for the reaction and a protamine solution within a vial was repeatedly cycled through the thermolysin column via a peristaltic pump [19]. The heterogeneous reaction enables the digested protamine to be directly analyzed without addition of enzyme inhibitor and removal of the enzyme and its inhibitor. Samples were then collected from the vial at different time points and injected into the immobilized heparin column-based chromatography system. As is shown in Figure 5, eight peaks are obtained for protamine and the digested protamine. According to reported mass spectrometry results [23,24], two abundant higher MW fragments are biomedically useful "low molecular weight protamine" (VSRRRRRRGGRRR and VSRRRRRRGGRRRR in Figure 5). The generation of other non-functioning fragments and the consumption of protamine are also clearly observed. Therefore, the hepa-

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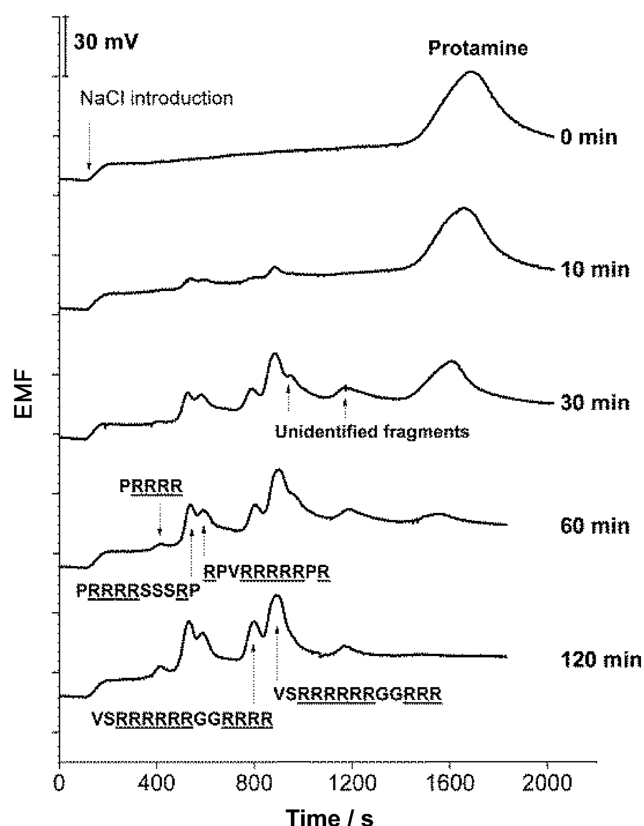

Fig. 5. Chromatograms for thermolysin-digested protamine samples after different reaction times (the sample at 0 min is 25 mg/mL protamine in Tris-HCl-NaCl-$CaCl_2$ buffer). Chromatographic and detector electrode conditions are identical to those used to obtain the chromatograms shown in Figure 3.

rin column-polyion pulstrode system clearly can be used to monitor the process of this enzymatic reaction and facilitate the isolation of functioning peptide fragments that could serve as useful therapeutic agents and also non-toxic species to neutralize the anticoagulant activity of heparin.

It should be noted that the use of more efficient types of cation exchange separation columns with smaller particle size packings and high pressure pumping would likely greatly enhance the resolution of the separated peptides derived from protamine digestion compared to the results shown in Figure 5 for the immobilized heparin column employed in these preliminary studies. At the same time, one potential limitation of the pulstrode detection system would be in separations where the mobile phase requires the use of an organic solvent to elute the polyionic species, in which case the presence of organic solvent will promote the extraction of the lipophilic ion-exchanges within the sensing membrane, decreasing the functional lifetime of the pulstrode detector. Another potential limitation may be lack of ideal sensitivity relative to the use of far UV detection of polyion peptides, polysaccharides and other species that are typically monitored at wavelengths <215 nm. However operation at such wavelengths is often limited by significant background absorbance from buffer components that may be in mobile phase, a problem that would not exist with the pulstrode detector. Further, the pulstrode polyion detector certainly has more than adequate sensitivity to function well for preparative LC applications, especially when high salt gradients are not needed to elute the polyionic species (e.g., size exclusion chromatography, reversed phase LC, etc.).

4 Conclusion

A polymeric membrane-based polyion sensitive pulstrode has been demonstrated to serve as a universal polycation detector in liquid chromatography of arginine-rich peptides. By using an immobilized heparin column as an cation-exchange/ affinity phase coupled with a fully reversible pulstrode detector operated in the polycation sensing mode, mixed polycationic peptides of different charge numbers can be readily separated and detected. Further, the production of lower MW arginine-rich peptides derived from thermolysin digested protamine can be monitored using this same technology. Since polyion pulstrodes can discriminate free and complexed polyions, indirect detection of separated polyions that exhibit low direct pulstrode responses should also be possible (e.g., DNA/RNA, etc.) using post-column reactions (e.g., protamine binding with DNA/RNA, etc.). Further, by combining pulstrode detection with other separation techniques such as size-exclusion chromatography, more classical ion-exchange chromatography, and capillary electrophoresis, the polyion pulstrode detector may prove useful for more types of samples that possess mixtures of polyions such as adulterated heparin products [17] and inorganic polyphosphate samples.

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