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A Highly Sensitive Potentiometric Strip-Test for Detecting High Charge Density Impurities in Heparin

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Abstract

Contamination of heparin with oversulfated chondroitin sulfate (OSCS) became a matter of grave concern in the medical field after many fatal responses to OSCS tainted heparin products occurred during the 2007 – 2008 period. Even though standard lab-based analytical techniques such as nuclear magnetic resonance (NMR) and strong anion-exchange high performance liquid chromatography (SAX-HPLC) have proven useful for monitoring the OSCS content in heparin products, an easy-to-use, quick, portable, and cost-efficient method is still needed for on-site monitoring during and after the heparin production. In this report, a disposable strip-type electrochemical polyion sensor is described for detection of low levels of OSCS contamination in heparin. A magnetic actuator is incorporated into this simple electrode-based microfluidic device in order to create the mixing effect necessary to achieve equilibrium potential changes of the sensor within a microfluidic channel. The planar membrane electrode detector within the sample channel is prepared with a tridodecylmethylammonium chloride (TDMAC)-doped PVC membrane essentially equivalent to previously reported polyanion-sensitive electrodes. When the concentration of heparin applied to the single-use strip device is 57 mg/mL (in only 20 μ L of sample), the same concentration recommended in the NMR analysis protocol for detecting OSCS in heparin, the detection limit is 0.005 wt% of OSCS, which is ca. 20 times lower than the reported detection limit of the NMR method.

Keywords

oversulfated chondroitin sulfate; heparin; polyanion sensitive electrode; ion-selective electrode; disposable electrochemical sensor

Heparin is one of the oldest and most widely used anticoagulant drugs. A sudden increase of severely adverse responses to heparin treatment of patients (including 94 deaths) was reported in U.S. during 2007–2008 period.¹ The U.S. Food and Drug Administration's (FDA) investigation of this problem found that the origin of these fatal side effects was oversulfated chondroitin sulfate (OSCS) that was present in some heparin products.^{2–4} Oversulfate chondroitin sulfate, a highly sulfated polysaccharide that has heparin-like

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anticoagulant activity, can be readily synthesized from chondroitin sulfate, which is obtained from animal cartilage at a much reduced cost compared to heparin isolation from pig intestines.⁵ Inspectors from FDA have tried to determine how this contaminant could have been included in the supply chain; however, the source of the OSCS is still not known.⁴ Despite the enormous effort being made to assure the purity of heparin active pharmaceutical ingredient (API) during production, for economic reasons, the heparin industry is still vulnerable to any accidental or deliberate incorporation of inexpensive OSCS or any other high charge density polyanions that exhibit anticoagulant activity in any level of the heparin supply chain.^{6,7}

The impact of the OSCS contamination on the heparin industry has been considerable. Not only for the providers of both unfractionated heparin and low molecular weight heparin products, but also device manufacturers where heparin is used (e.g., immobilized on catheters or extracorporeal blood loops, etc. to reduce clotting). According to the FDA, the heparin products of some companies were seized, while some had to announce voluntary recalls as preventive measures, and some were given warning letters from authorities.⁸ Since the consequence of OSCS contamination in heparin preparations can have such severe consequences to patients and the heparin industry, rigorous monitoring for the presence of OSCS in all heparin products is now required at many levels of the supply chain. A new United States Pharmacopeia (USP) monograph/guideline for unfractionated heparin production has been published recently (October 1, 2009) to assure the safety of heparin products.⁷ The analytical techniques recommended for detection of OSCS or other impurities in heparin include proton nuclear magnetic resonance (¹H-NMR),^{4,9} strong anion-exchange high-performance liquid chromatography (SAX-HPLC),^{12,13} galactosamine assay and anticoagulation activity measurements with purified coagulation proteases, factors IIa and Xa.^{7,14} Even though these methods are well-established and reliable procedures, the frequent and in-field monitoring capabilities with these lab-based analytical methods are not practically available to most of the API processing sites.

Recently, a simple yet highly sensitive electrochemical technique to detect OSCS within heparin samples was introduced.¹⁵ Potentiometric polyanion-sensitive membrane electrodes were initially studied in the 1990s for the quantification of heparin levels in blood and clinical studies proved that the performance of the polyion sensor is substantially equivalent to the conventional clotting time heparin assay methods.^{16,17} It was also more recently found that if heparin samples contain polyanionic impurities (i.e., OSCS) that have higher charge density than heparin, the electrochemical signal (membrane potential) from such sensors is governed by the more highly charged polyanionic species and exhibits a clearly identifiable response toward the impurity.¹⁵ Indeed, the presence of 5 µg/mL of OSCS in a 1 mg/mL heparin sample (0.5 wt% contamination) can be readily identified within 5 min by the magnitude of the polyion sensor's EMF response when immersed in the sample solution (along with a reference electrode) when sufficient stirring is applied. This new and very simple lab-based detection method has provided a comparable detection limit to the NMR technique with much less time, effort, and cost. However, the electrode-based detection method still uses lab-equipment such as a magnetic stirring device (to control mass transfer of the polyanions to surface of the sensing membrane) and a sample container that limits accessibility of this technique for on-site analysis.

Herein, a very fast and highly sensitive single-use disposable electrochemical OSCS detector with high portability is described. The system is based on fabricating the potentiometric polyanion sensor via screen-printing technology, the method currently employed in the mass-production of the disposable electrochemical glucose sensor strips.^{18,19} This strip-test sensor for OSCS can be plugged into a portable meter containing a simple magnetic vibration actuator. The magnetic actuator generates the convection within a microfluidic channel that is equivalent to stirring of sample solution in the conventional electrochemical experiment. This simple and mass-producible sensor configuration potentially allows fast and multiple tests on any processing site and in any market place. It will be shown that the approach also requires greatly reduced sample volume (less than 20 μL) compared to NMR and the lab-based electrode method. In fact, with only a few tenths of a milligram of heparin powder is more than sufficient to provide a concentrated heparin test solution equivalent in concentration to that needed for NMR analysis, and this high concentration of heparin sample greatly lowers the detection limit of the new electrochemical OSCS monitoring method to levels substantially below that of the NMR method.

EXPERIMENTAL SECTION

Heparin and OSCS tested in this work were kindly provided by Sanofi-Aventis (Paris, France). Tetrahydrofuran (THF), chondroitin sulfate A (CSA), chondroitin sulfate B (CSB) or dermatan sulfate (DS), dextran sulfate (DxS) and 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride was purchased from JUNSEI (Tokyo, Japan). Tridodecylmethylammonium chloride (TDMAC), poly(vinyl chloride) (PVC), and 2-ethylhexyl sebacate (DOS) were products of Fluka (Ronkonkoma, NY). Silver paste was obtained from Asahi chemical laboratory (Tokyo, Japan).

The screen-printed silver electrode was prepared with an epoxy-insulated silver layer on a PET substrate (polyethylene terephthalate, acryl coated, 250 μm thick) using a LS-150 semi-automatic screen-printer (Tokyo, Japan). The configuration of the polyion-sensitive electrode is shown in Fig. 1(a). The exposed silver electrode was oxidized by the treatment with FeCl_3 (0.3 M) for 5 min. followed by washing with deionized water. The resultant Ag/AgCl electrode was covered with a NaCl salt layer by dispensing 0.2 μL of a NaCl solution (1 M NaCl in deionized water) with a pneumatic dispensing instrument (EFD model 1000 XL; Providence, RI, USA). The membrane cocktail consisted of DOS (65.5 mg)/PVC (33 mg)/TDMAC (1.5 mg) in THF (0.5 mL) unless otherwise indicated. The cocktail solution was cast on the dried NaCl layer with the dispensing instrument. A second Ag/AgCl electrode located on a site the next to polyion-sensitive electrode was used as the reference electrode for the EMF measurements.

The polyanion sensor strip was fabricated by assembling a screen-printed electrode, a microfluidic channel made of a double-sided adhesive (300 μm thick), and a transparent PET cover, as illustrated in Fig. 1(b). The double-sided adhesive was mechanically processed in large numbers to have an identical design for every microfluidic channel. The dimension of the resultant sensor strip is ca 30 mm $\times$ 15 mm $\times$ 0.8 mm. Fig. 1(c) illustrates a

completed device design where a sensor strip is plugged into a potentiometric device with a magnetic actuator. The center of the microfluidic channel is sandwiched by a neodymium permanent magnet and an electromagnet, which applies a periodic alternating pressure on the microfluidic channel; the pressure change will create an oscillating bidirectional movement of solution in the channel to mimic the stirring effect.

All experiments were carried out with a TRIS working buffer (0.05 M, pH 7.4, 0.12 M NaCl) containing various wt% of OSCS in heparin (57 mg/mL) at the ambient condition. After a sensor strip was plugged into the device, a sample solution (20 μ L) was introduced at the inlet of the microfluidic channel and the device recorded the EMF value continuously for 120 s after the sample introduction. The alternating current (up to 0.5 A) at 150 Hz was applied to the electromagnet during measurements. Standard deviations (S.D.) for all samples were obtained from three repetitive measurements ($n = 3$) for each sample and are indicated as error bars in figures.

RESULTS AND DISCUSSION

The operational principles of polyion sensors, including the theory and equations that predict the observed potentiometric responses, are well described in the literature.^{20,21} In the case of polyanions such as heparin (average charge (z) = $-60 \sim -70$), the conventional potentiometric response of TDMAC-doped polymer membranes obtained at full equilibrium at the membrane/sample interface is given by the Nernst equation where the response slope ($59.2/z$ mV/decade) is about 1 mV/decade for heparin. Thus, under such conditions, the observed EMF signal is quite insensitive to the concentration change of heparin. However, when a freshly prepared TDMAC-doped polymeric membrane is exposed to the solution containing heparin for the first time, a significant potential change is observed before the membrane/sample interface reaches equilibrium. At a relatively low concentrations of polyanion (e.g., [heparin] < 10^{-6} M), a pseudo-steady-state EMF response is observed where the EMF depends on the bulk concentration of heparin and the stirring rate of solution. However, at high concentrations of heparin, EMF becomes relatively independent of the bulk concentration of heparin since the membrane potential is now governed by the equilibrium response toward the polyanion (i.e., < 1 mV/decade). Interestingly, this EMF at high concentration of polyanion is highly dependent on the nature of a polyanion. A greater negative EMF for true equilibrium response means a more favorable extraction thermodynamics of the polyanion into the polymeric membrane. This is attributed to an increased cooperative binding between the anion exchanger (tridodecylmethylammonium; TDMA⁺) with the polyanion.²² Indeed, it has been found previously that the equilibrium potentiometric response of polymeric membranes to high concentrations of varying polyanions is directly proportional to charge density of the polyanion.²²

In a mixture of polyions that has both lower and higher charge density polyanions, the potential change observed at the equilibrium will be governed by the higher charge density species. It is higher charge density polyanions that are more favorably extracted into the polymeric membrane due to the stronger cooperative binding with the lipophilic anion exchanger. Thus, EMF of the higher charge density polyanion is observed from the mixture of polyions. This is analogous to conventional ionselective membrane electrodes

where selectivity is highly dependent on the ratio of the equilibrium constant of ions binding with the ionophore within the organic membrane phase.²³ Therefore, the EMF observed for a high concentration of polyanion can provide a simple method to distinguish the most highly charged polyanion in a mixture of various polyanions.

To utilize the potentiometric polyanion sensor technology to monitor very low concentrations of a given higher charge density polyanion (i.e., OSCS) in the presence of a high concentration of another polyanion (i.e., heparin), it is necessary to use convection to more rapidly achieve full equilibration at the polymer membrane/sample interface. Indeed, when the concentration of the OSCS polyanion is too low, the membrane first reaches equilibrium with the much higher concentration of heparin present, and the initial potential change observed would be dominated by this species; however, with convection of the sample and the much stronger binding of the higher charge density OSCS species to the TDMA exchanger sites in the membrane, the phase boundary potential of the membrane will quickly begin to move in negative direction and approach the more negative and true equilibrium state EMF value for the trace level of OSCS present in the sample. Hence, some stirring/convection within the sample solution is critical to obtain a distinguishable signal for trace levels of OSCS within heparin, from the signal observed for a pure heparin sample.

In the new microfluidic configuration suggested here, a simple electromagnet-driven actuator is used (see Fig. 1(c)) to provide sample mixing, and this ensures adequate mass transport of the polyanions to the surface of the sensing membrane. The degree of mass transport is controlled by the amplitude of current applied to an electromagnet and the frequency of the alternating current. The alternating current changes the magnetic polarity of the electromagnet periodically which pulls and pushes a permanent magnet on it. The microfluidic channel in the sensing strip is inserted between the electromagnet and the permanent magnet and the solution in the channel is periodically squeezed and released. In order to demonstrate the mass transfer effect of the magnetic actuator, the response of an electrochemical polyanion sensor strip toward 20 $\mu\text{g/mL}$ of heparin in buffer was monitored with various frequencies of electromagnetic movement. At each frequency tested, a new sensor strip is plugged into the meter and the heparin sample is introduced as soon as the EMF recording begins. As shown in Fig. 2(a), the signal observed when using less than 10 Hz vibration is about $-25 \sim -30$ mV, which is the same EMF of buffer solution without heparin added, even though the sample actually contains 20 $\mu\text{g/mL}$ heparin. However, as the frequency of pressurization increases, the change of EMF increases and reaches a plateau at the frequency range from 100 Hz to 400 Hz. The EMFs at these frequencies are about $-50 \sim -55$ mV, which are the typical equilibrium EMF of heparin-containing samples observed with the planar polyanion sensor strip. Under the alternating pressurization with magnets, the resultant oscillation motion of solution in the fluidic channel creates the stirring effect equivalent to the convection generated by the magnetic stirrer in the solution within a beaker. At the given strength of the magnet utilized in this work, a frequency between 100 to 400 Hz provides an optimal mass-transport effect for the sensing membrane. The 150 Hz frequency was chosen for the remainder of experiments, based on the data shown in Fig 2(b).

In contrast to the conventional polyion sensors, which are conditioned in the blank buffer for an hour before measurements, the proposed disposable OSCS sensor strip is designed for use directly without any membrane preconditioning, from a dry state. Usually, preconditioning is required to sufficiently hydrate the sensing membrane and the inner salt layer (internal electrolyte) of planar screen-printed membrane electrodes to avoid an initial drift in the membrane potential. However, when the sensor is to be used directly from the dry state without any preconditioning, the membrane hydration process should reach a steady-state within a reasonable time and sensor strips should have an identical baseline potential between sensors. This is where the screen-printing technology can be used advantageously to prepare the identical electrodes that have nearly identical response properties. Indeed, commercial disposable sensor strips utilize the screen-printing process for mass-production of highly reproducible electrodes.^{18,19} For the polyanion sensors developed here, the given polymer membrane composition on the screen-printed electrodes also exhibits highly reproducible EMF signal changes, as shown in Fig. 3(a). Even though there are fluctuations of potential at the initial period of EMF recording as the sample is applied to the strip, eventually the observed EMF changes reach a characteristic potential for each polyanions after 120 s. The standard deviation of the differences of the average EMF values for each sample is 1.6 mV ($n = 3$ for each polyionic species).

Figure 3(b) summarizes EMF values (membrane electrode vs. Ag/AgCl reference) 120 s after sample application for TRIS buffer (0.05 M, pH 7.4) with NaCl (120 mM), and the same buffer containing CSA, CSB, heparin, OSCS, and DxS. All tested solution contained 1 mg/mL of polyanion in TRIS buffer. The EMF responses to CSA, CSB, heparin, OSCS are -23.4 mV (S.D. = 1.8), -50.3 mV (S.D. = 1.2), -56.5 mV (S.D. = 0.7) and -86.6 mV (S.D. = 2.5), respectively. The difference between response to heparin and OSCS is about 30 mV and it this value is slightly greater than the previously reported value (i.e. ~ -25 mV) for conventional polyanion sensors.¹⁵ The highest EMF response, -124.2 mV (S.D. = 1.9) was obtained from DxS and the EMF difference between OSCS and DxS is 38 mV.¹⁵ Even though EMF differences between polyanions is slightly higher (about 5 ~ 10 mV) with the disposable screen printed polyanion sensors than observed with the conventional polyion-sensitive electrodes, the order of negative EMF changes is DxS > OSCS > heparin > CSB > CSA, which is the exactly the same order as seen in the previous work.^{15, 22} Therefore, the disposable strip-test polyion sensor is essentially equivalent to the conventional polyanion sensors prepared in the tubular configuration.

Notably, the potentials changes obserbed for CSA and CSB are less than that of heparin, as shown in Fig. 3(b). This suggests that in the mixtures of heparin, CSA, and CSB, the response to heparin will defined the overall EMF change that is observed, owing to its higher charge density. The mixture of OSCS and other polyions such as heparin, CSA, and CSB will exhibit the potential change due to the presence of the higher chare density OSCS. Indeed, in Figure 4, the potential change for heparin (1 mg/mL) and OSCS (0.1 mg/mL, 10 wt% to heparin) mixture is -88.2 mV (S.D. = 0.9), which is the EMF change observed for pure OSCS. A mixture of heparin (0.6 mg/mL), CSA (0.2 mg/mL, 33 wt%), and CSB (0.2 mg/mL, 33 wt%) exhibits the a -57.8 mV change (S.D. = 3.1), consistent with that seen for pure heparin. The mixture of heparin (0.5 mg/mL), CSA (0.2 mg/mL, 40 wt%), CSB (0.2 mg/mL, 40 wt%), and OSCS (0.1 mg/mL 20 wt%) exhibits an -88.2 mV change (S.D. =

0.9), which is also similar to the EMF change of pure OSCS. When the heparin is mixed with sufficient amount of polyanions with higher charge density, the observed potential change is dictated by species with the higher charge density. Since OSCS has higher charge density, it induces more negative EMF change at the polymer membrane/sample interface than heparin.¹⁵ Likewise, there is no sensitivity to CSA and CSB in the presence of heparin or OSCS due to the lower charge densities of these species.²¹ Therefore, the proposed polyion sensor is only sensitive to OSCS among the possible impurities in heparin samples. Direct application will be possible for heparin API and commercial heparin drugs. Considering CSA and CSB are common polyanionic contaminant in the crude heparin, the selective monitoring of OSCS is possible at most of heparin production sites using the proposed sensing method.

In order to detect OSCS from heparin mixtures, OSCS should be present in the sample at a concentration that is higher than its detection limit. It should be noted that the detection limit of OSCS in the heparin sample is highly dependent on the concentration of heparin, because the OSCS concentration is proportionally increased for a given wt% contamination in heparin sample (Figure 5). Indeed, what does change with respect to heparin concentration is the sensitivity of being able to measure trace level impurities of OSCS. When samples have only 1 mg/ml of heparin, a 0.005% contamination of OSCS places the OSCS level at too low a concentration to achieve reasonably rapid equilibration at the membrane/sample interface, and hence this level of contamination cannot be observed. However, increasing the heparin concentration some >50 fold, increases the absolute OSCS concentration in the sample to a level where the EMF response is fast enough to be observed (flux into polymer membrane). Therefore, it is beneficial to prepare a more concentrated heparin sample because the concentration of OSCS is also increased proportionally for a given wt% contamination. One of the advantages of using a microfluidic channel sampling approach is that it requires a relatively tiny sample volume ($\sim 20 \mu\text{L}$) for each measurement and the concentrated sample is easily prepared in this volume with a minimal consumption of the solid heparin material. Figure 5 shows the EMF change at three different concentrations of heparin with various levels of OSCS contamination. When the potential response is plotted against the wt% of OSCS in the heparin, increasing heparin concentration clearly lowers the detection limit for wt% OSCS. Increasing the concentration of heparin from 1 mg/mL to 57 mg/mL lowers the detection limit from 0.5 wt% to 0.01 wt% if the detection limit is defined as the lowest concentration where the EMF different than the average EMF response to pure heparin by more than 6 times of the S.D. of the pure heparin responses. The highest heparin concentration examined, 57 mg/mL, is the heparin concentration recommended for the NMR testing of OSCS in heparin and it is also chosen here as the highest concentration of heparin in the polyion sensor method in order to compare the sensor performance to the conventional NMR technique. Considering the detection limit of OSCS in NMR method and SAX-HPLC is about 0.1 wt% and 0.03 wt%, respectively,^{9, 12} the polyion sensor can provide sensitivity 10 times improved compared to the NMR method and ca. 3 times that of the SAX-HPLC method.

It is known from the earlier fundamental studies of polyanion sensors that detection limits can be influenced by the diffusion coefficient of the polyanion-TDMA ions pairs in the polymer membranes of the sensors; lower detection limits can be provided when higher

polymer to plasticizer ratios are utilized in the membrane composition.²⁰ Figure 6 shows the EMF change at various levels of OSCS contamination in 57 mg/mL heparin samples using planar sensor strips prepared using two different membrane compositions (i.e., DOS (65.5 wt%)/PVC (33 wt%)/TDMAC (1.5 wt%) and DOS (38.5 wt%)/PVC (60 wt%)/TDMAC (1.5 wt%)). As can be seen in Fig. 6, the sensing membranes prepared with higher ratio of PVC to DOS provide an improved detection limit; as the amount of the plasticizer (i.e., DOS) decreases, the response curve shifts to the lower contamination level. The best result is obtained with a membrane containing DOS (38.5 mg)/PVC (60 mg)/TDMAC (1.5 mg). The membrane with this higher level of PVC can detect OSCS contamination down to 0.005 wt % in a heparin sample that is 57 mg/mL in TRIS buffer. This detection limit is 20 times more sensitive than the NMR method. Potentially, even higher concentrations of heparin may be employed to improve the detection limit if careful considerations of the solubility of heparin in the solution, viscosity change, and ionic strength change of the solution are taken into account.

CONCLUSION

A disposable OSCS electrochemical sensor strip-test has been developed based on a screen printed potentiometric polyion-sensitive membrane electrode in a microfluidic cartridge platform. The disposable sensor cartridge can quickly detect the existence of OSCS in small volumes of heparin samples within 2 min after sample application, without any need for highly expensive and sophisticated instrumentations. The device only requires a small electromagnet in conjunction with a simple high impedance voltmeter, which is easily implemented into a small portable device. A rapid OSCS screening test with this simple device would be an immediate application of this technology. For example, by setting a threshold of ca. -58 mV (see Fig. 6), concentrations of > 0.005 wt% of OSCS can be quickly detected by this device. Combination of the polyion sensor with other lab-based method will improve not only quality of analysis but also cost and time efficiencies. Therefore, we envision that a commercial version of this strip-test would have immediate applications in the heparin quality control process, ranging from field testing including processing sites supplying raw material heparins, to simple testing in the clinical arena (e.g., hospital), as an extra layer of precaution to better ensure the safety of all heparin products. Indeed, this new method of detecting OSCS in heparin exceeds the detection limits of far more complex NMR method by ca. 20-fold and the SAX-HPLC by ca. 6-fold (when using the optimized membrane composition).

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Figure 1. Schematic diagrams of a polyion-sensitive electrode (a), a disposable sensor strip (b), and a device plugged with a sensor strip (c).

Figure 2.

Response to heparin sample (20 $\mu\text{g/mL}$) with varying pressurizing frequency from 1 to 416 Hz recorded for 120 s from the sample introduction at 0 second (a). Potentials at 120 s for each vibrating frequency (b).

Figure 3.

Polyionic responses to 1 mg/mL of various polyanions from the sample introduction (0 second) to 120 s (a) and EMFs of each polyanion at 120 s (b). Error bars in (b) represents standard deviations.

Figure 4.

Polyionic responses to various mixtures of polyions: Heparin (1 mg/mL), Heparin (0.6 mg/mL) + CSA (0.2 mg/mL) + CSB (0.2 mg/mL), Heparin (1 mg/mL) + OSCS (0.1 mg/mL), Heparin (0.5 mg/mL) + CSA (0.2 mg/mL) + CSB (0.2 mg/mL) + OSCS (0.1 mg/mL). Potential response to heparin does not change by addition of CSA and CSB, while addition of OSCS into heparin or heparin mixture changes the potential about -33 mV.

Figure 5.

Response curves for various contamination level of OSCS (0.001 wt% ~ 10 wt%) in three different heparin concentrations (• 1 mg/mL, ■ 10 mg/mL, and ▲ 57 mg/mL). The membrane composition is DOS (65.5 mg)/PVC (33 mg)/TDMAC (1.5 mg).

Figure 6. Response curves for various contamination level of OSCS (0.001 wt% ~ 10 wt%) in two different membrane compositions (◆ DOS (38.5 mg)/PVC (60 mg)/TDMAC (1.5 mg) and (▲ DOS (65.5 mg)/PVC (33 mg)/TDMAC (1.5 mg)). The concentration of heparin in samples are 57 mg/mL.