Quantitative Determination of High Charge Density Polyanion Contaminants in Biomedical Heparin Preparations Using Potentiometric Polyanion Sensors

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Abstract
Quantification of oversulfated chondroitin sulfate (OSCS) in biomedical heparin preparations is achieved using a recently described potentiometric polyanion sensor-based approach operated in a kinetic mode of analysis. This is accomplished by adjusting the concentration of the test sample to a range where the OSCS level is low enough for the sensor not to achieve a full and rapid equilibrium phase boundary potential change at the membrane/sample interface upon exposure to the heparin sample. Using this method, the OSCS wt% determined within heparin samples containing OSCS are shown to be in good agreement with those determined by an accepted NMR method.

Keywords: Potentiometric polyanion sensor, Heparin, Oversulfated chondroitin sulfate (OSCS), ^{1}H nuclear magnetic resonance (NMR), Medicinal chemistry, Polyanions

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Heparin, a highly-sulfated glycosaminoglycan, is widely used as an anticoagulant both intravenously and subcutaneously [1–3]. A variety of medical devices and diagnostic products may also contain or be coated with heparin. Recently, hundreds of cases of an acute, rapid onset allergic type reaction (resulting in a large number of patient deaths) have been reported to the US Food and Drug Administration (FDA) for certain lots of heparin manufactured in China. A specific contaminant, oversulfated chondroitin sulfate (OSCS), has been identified in given lots of heparin preparations that may have caused these adverse events [4, 5]. OSCS possesses a very similar polysaccharide chemical structure and anticoagulant activity as heparin; therefore, typical bioactivity assays [6–8] cannot detect the presence of the OSCS species. To date, only complex and costly nuclear magnetic resonance (NMR) [5, 9] and capillary electrophoresis (CE) [10] techniques have been suggested by FDA for the detection and quantification of the OSCS contaminant.

It has been demonstrated previously in this laboratory that large and reproducible EMF responses toward polyanionic species can be achieved if polymeric ion-selective electrodes (ISEs) are formulated with lipophilic anion-exchangers, such as a tridodecylmethylammonium (TDMA) salt, in plasticized poly(vinyl chloride) (PVC) membranes [11–13]. Further, we have recently shown that by using such simple and inexpensive potentiometric polyanion sensors, it is possible to rapidly detect the presence of OSCS or other high charge density polyanionic structures as contaminants in heparin, since these species, even when present at low levels, cause a greater negative change in the phase boundary equilibrium potential for the contaminated heparin than for the untainted heparin. This is due to the higher charge density of OSCS (or other potential polyanion impurities that might exhibit heparin-like bioactivity) compared to that of porcine heparin, and thus there is a greater equilibrium constant for extraction of the OSCS into the membrane phase to form an ion pair with the TDMA species [14]. Indeed, earlier work has shown that the magnitude of the equilibrium potential change for polyanion sensors is directly related to polyanion charge density [15].

The fundamental response principles of potentiometric polyanion sensors have be described in previous studies [13, 15]. As reported in our most recent work [14], detection of 0.5 wt% OSCS impurity is readily achieved using only 1 mg/mL of final polyion concentration, and if a more concentrated contaminated heparin sample is used, a lower weight percentage of the contaminant polyanion may be detected (i.e., an improved detection limit for contaminant wt% in the heparin sample). Moreover, the overall potentiometric response profile of TDMA-based membrane electrodes toward a heparin preparation containing less than 1 wt% OSCS shows that the measured potential first drops quickly to the EMF change typically observed for pure heparin (ca. −50 mV in background of phosphate buffered saline with 0.141 M chloride), and then drifts down slowly toward the value observed for the pure OSCS or the highly contaminated samples (see Fig. 1). We can take advantage of this kinetic response property to quantitatively determine the

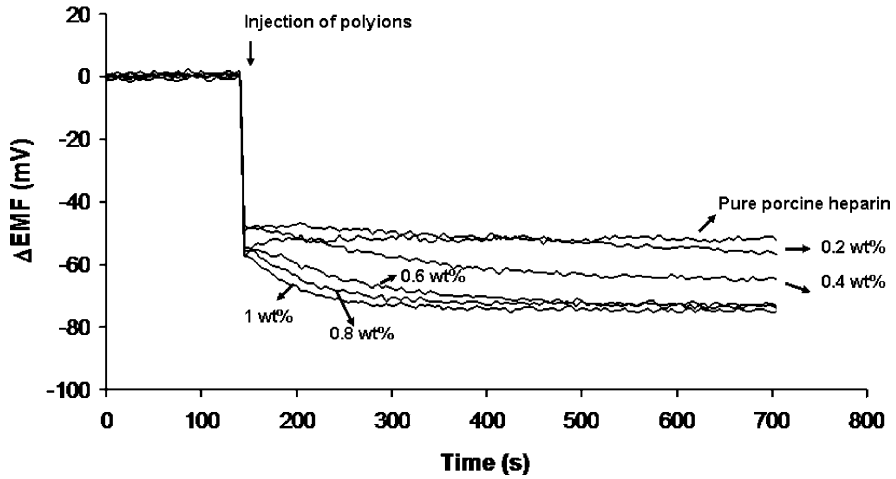

Fig. 1. Response time trace of PVC membranes doped with TDMAC after injection of heparin/polyion preparations (at 1 mg/mL) possessing different degrees of OSCS contamination equal to or less than 1 wt%. For comparison, the response to pure porcine heparin at a final polyanion concentration of 1 mg/mL is also shown.

content of the contaminant if the OSCS weight percentage in the total polyanion preparation is adjusted to be in a range (by dilution of the original heparin sample with pure heparin solution) which is low enough for the electrode not to achieve a rapid equilibrium phase boundary potential change at the membrane/sample interface. Under such conditions, the rate of EMF change of the membrane toward the equilibrium EMF response for the higher charge density OSCS species is proportional to the OSCS concentration.

The critical concentration of OSCS for the potential not to reach equilibrium rapidly has been found to be ≤ 1 wt% when operating the electrodes at room temperature (ca. 22 °C). This operational non-equilibrium range can be raised to higher levels of OSCS if the temperature is increased (see Experimental Section, below). As shown in Figure 1, when a series of standards containing synthesized OSCS mixed in different wt% with pure porcine heparin (with total polyanion concentration of 10 mg/mL) are injected (diluting the sample 1: 10 in the process) into a PBS electrolyte in which the polyanion sensor (and reference electrode) are placed at room temperature, the EMF changes quickly to ca. -50 mV (for pure heparin) and then drifts down slowly toward the -75 to -78 mV change observed for pure OSCS. If the values of ΔEMF are integrated for 5 min after the injection of the polyanion sample preparation, the integrated signal, which is proportional to the rate of change over the 5 min interval, is

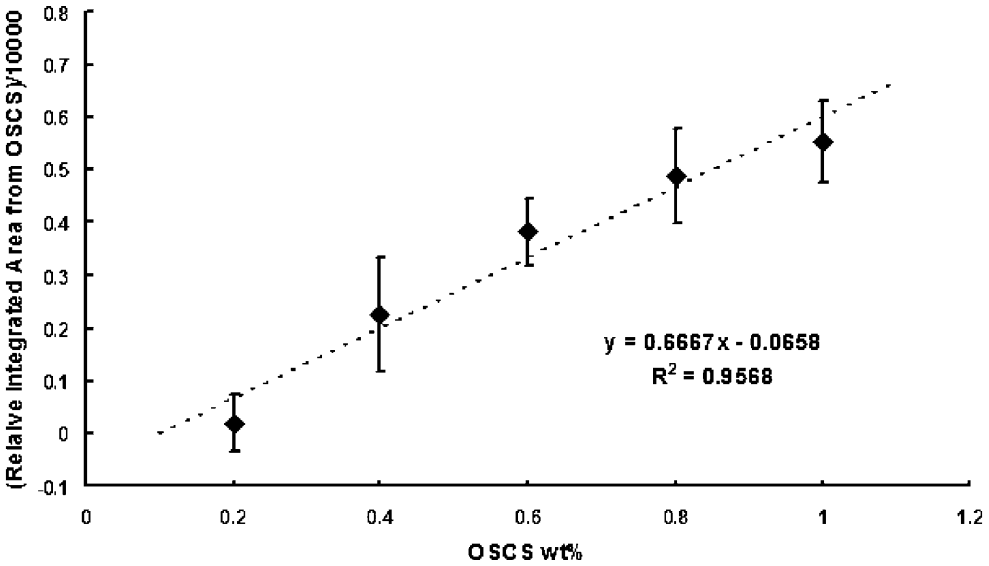

Fig. 2. Calibration curve of the relative integrated area from OSCS for polyanion preparations with different OSCS wt%. The standard deviations were calculated using data collected from 6 electrodes for each concentration. ΔEMF was integrated for 5 min after the injection of polyanion preparation, and the relative integrated area from OSCS was calculated by subtracting the integrated area for the pure heparin response from the polyanion mixture response.

linearly correlated with the weight percentage of OSCS in the polyanion standards (see Fig. 2).

Table 1 summarizes the calculated values of OSCS wt% in several different OSCS test sample solutions prepared with the synthesized OSCS and porcine heparin, after diluting these samples appropriately with pure porcine heparin solutions to fall into the standard calibration range (See Fig. 2). In addition, data is presented in this table for a commercial USP heparin preparation known to be contaminated with OSCS at a value greater than 10 wt%. As shown in Table 1, the calculated OSCS wt% values found by the electrode method are in close agreement to the OSCS wt% known for the three different OSCS synthetic sample solutions containing OSCS. The OSCS wt% in the USP contaminated heparin was found to be 21.3 wt% and 17.7 wt%, respectively, using different dilutions of the original sample, with the more diluted sample showing a much larger standard deviation than the less diluted sample.

Previous studies have shown that NMR spectroscopy can be used to assess and quantify the OSCS impurities in heparin by means of the intensity of the N-acetyl proton resonance signal [16, 17]. Here, using the ratio of acetyl proton signal of OSCS at $\delta = 2.12$ ppm to that of heparin N-acetyl proton at $\delta = 2.02$ ppm (see Fig. 3) enables the preparation of a calibration curve which can be used to determine the wt% OSCS in the given polyanion preparation. As shown in Table 1, the results of OSCS wt% values determined by potentiometric polyanion sensors are in good agreement with the results obtained by the NMR method for the same test samples. Moreover, the potentiometric method to determine the OSCS wt% is reproducible, although the slope and intersect values of the OSCS wt% calibration curves may vary somewhat for experiments conducted on different days owing to variations in temperature and difficulties in obtaining precisely controlled mass transport of polyions to the surface of the sensing membrane. Polyion mass transport and hence reproducibility can be controlled more effectively by using thermostatted water jacket measurement cells, and also by using a rotating electrode version of the polyion sensor [18]. Nonetheless, the results presented clearly indicate that the simple potentiometric polyanion sensor-based method described here, even when operated at room temperature, can be applied to quantify OSCS or other high charge density polyanion contaminants in biomedical heparin preparations with reasonably good accuracy.

It should be noted that although the potentiometric response to OSCS is irreversible using the reported sensor configuration (unless the electrodes are soaked in high concentration sodium chloride solution in order to reverse the ion-exchange process and allow chloride to ion-exchange with the extracted polyanion species), due to their simplicity and the low cost in construction of the electrodes, it is possible to use these sensors as single-use disposable devices.

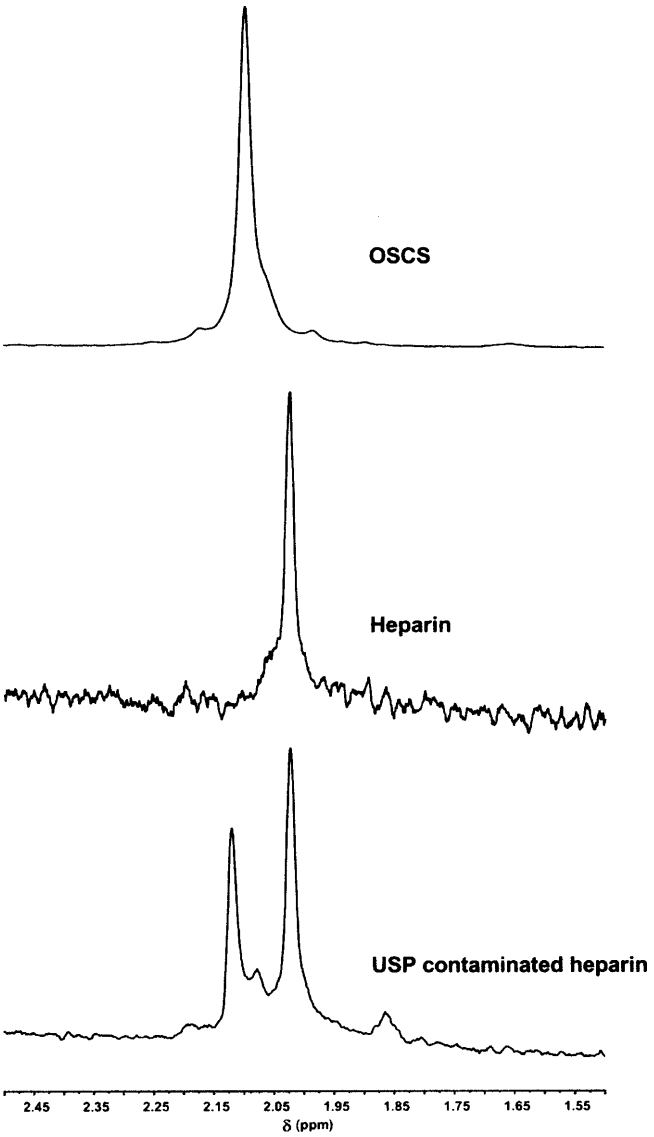

Fig. 3. ^{1}H NMR spectra (400 MHz) of the acetyl region of the OCSC, heparin and the USP contaminated heparin sample.

Experimental

OSCS was synthesized according to a previously reported procedure starting from the sodium salt of chondroitin sulfate (Aldrich, St. Louis, MO) and sulfur trioxide (Aldrich) [5, 9]. A contaminated heparin known to have >10 wt% OSCS (U.S. Pharmacopeia (USP), Rockville, MD) was used as a reference sample. The polyanion sensitive membranes were formulated with 1.5 wt% tridodedylmethylammonium chloride (TDMAC) (Aldrich), 66 wt% poly(vinyl chloride) (PVC) (Fluka, St. Louis, MO) and 32.5 wt% dioctylsebacate (DOS) (Fluka), as reported previously [12]. Membrane components were dissolved in distilled tetrahydrofuran (THF) at 100 mg/mL. Fabrication of the single-use disposable electrochemical sensors was as described previously, using narrow bore Tygon tubing [14]. The sensors were soaked in 0.01 M PBS, pH 7.4, for approximately 1 h before the glass capillaries were carefully

Table 1. Calculated OSCS wt% based on the linear regression of integrated area of ΔEMF for 5 min after the injection of polyanion preparations and the ^{1}H-NMR N-acetyl signal of OSCS and heparin in polyanion preparations with different OSCS wt% [a].

Initial polyanion preparations	Potentiometric method		NMR method 30 mg/mL [c]
	wt% of the initial polyanion preparations in the final diluted sample by adding porcine heparin	Calculated OSCS wt% in the initial sample [b]	
10 wt% synthesized OSCS	5	8.7 ± 2.0	12.6 ± 0.2
20 wt% synthesized OSCS	3.5	19.2 ± 2.7	18.3 ± 1.1
30 wt% synthesized OSCS	3	28.5 ± 2.4	26.2 ± 1.3
USP contaminated heparin	2.5	21.3 ± 3.3	17.9 ± 1.1
	5	17.7 ± 1.5	

[a] The final polyanion concentration for potentiometric method and NMR method are 1 mg/mL and 30 mg/mL respectively.
[b] The confidence intervals (CIs) obtained in potentiometric method were calculated using data collected from 6 electrodes for each concentration using the formula $\mu \pm t\ SD/\sqrt{n}$, where μ is the average of the six electrodes, SD is the standard deviation, $n = 6$, and $t_{0.05,5} = 2.5706$.
[c] CIs obtained in NMR method were calculated using data collected from 3 trials of measurements.

removed. The small Tygon tubing with the sensing membrane at the tip was then filled with the same PBS buffer, and a Ag/AgCl wire covered with heat shrink tubing on the top was inserted. To determine the OSCS wt% in polyanion preparations by the potentiometric method, a 10 mg/mL polyanion solution (in PBS) with OSCS wt% smaller than 1% was tested with the polyanion sensors as described above. The values of EMF change were integrated for 5 min after the injection of polyanion preparations. Other lengths of integration period (e.g., 250 s and 500 s) were also performed, which gives similar results in the calculated value of OSCS wt%. However, longer integration time period should be avoided in order to ensure the potentials do not reach full equilibrium values during the integration period. If this occurred, non-linear calibration curves would likely result. Integrations were performed by Origin Lab 7.5 (Northampton, MA). The maximum EMF change of pure porcine heparin is determined to be -51.2 mV with a standard deviation of 1.6 mV ($n = 6$). The relative signal change from OSCS response was obtained by subtracting the area of heparin response ($51.2 \times 300 = 15360$ mV s) from the area of the polyanion mixture response. All the potentiometric measurements were conducted at room temperature and the data for one calibration curve was collected in one day. Measurements at higher temperature (i.e., 37 °C) were also conducted and showed a similar change in the EMF profile after the injection of the polyanion, but the detectable OSCS concentration range was shifted to a higher concentration range. This can be explained by Equation 1 [13]:

$$\Delta EMF = \frac{RT}{F} \ln\left(1 - \frac{zD_a\delta_m}{R_T^+ D_m \delta_a} C_{poly,sample}\right) \quad (1)$$

where z is charge on polyion; D_a and D_m are the diffusion coefficients of the polyion in the aqueous sample and membrane phases, respectively; δ_a and δ_m are the diffusion layer thicknesses in the aqueous sample and membrane phases, respectively, R_T^+ is the concentration of TOMA in the membran and C_{poly} is the concentration of polyanion in the sample solution. Elevated temperature increases the diffusion coefficient of the ion-pair of the extracted polyion/ion-exchanger in the membrane phase to a greater degree than D_a and thereby shifts the range where initial non-equilibrium *EMF* response occurs to higher concentrations of OSCS. Therefore, the 'critical value' for the potential not to reach full equilibrium is larger than 1 wt% when operating at 37 °C, which means that less dilution of the contaminated sample is required at a higher temperature. To emphasize the lower limits of detection of the proposed method, all electrochemical experiments reported in this manuscript were carried out at room temperature.

For quantification of OSCS by the NMR method, 30 mg/mL OSCS and heparin solutions in D_2O (Cambridge Isotope Laboratories, Inc., Andover, MA) were constantly shaken for 1 h prior to the NMR measurements. Then, the two solutions were mixed to give different wt% of OSCS. ^{1}H NMR spectra were recorded on a Varian 400 MHz spectrometer. For all the polyanion samples, 32 scans were collected over a spectral width of 6400 Hz. The acquisition time was 2.56 s, followed by a relaxation delay of 1 s. All the spectra were recorded at 315 K using a flip angle of 30° and the chemical shifts were reported to the solvent peak at 4.58 ppm (calculate by the equation $\delta = 7.83 - T/96.9$, where T is the absolute temperature in Kelvin [19]) . The data were processed by MestReNova 5.3.2 (Mestrelab Research, Spain). Baseline corrections were mostly done automatically, and phasing was always performed manually.

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