Progress in developing polymerized crystalline colloidal array sensors for point-of-care detection of myocardial ischemia†

Justin T. Baca,[a] David N. Finegold[b] and Sanford A. Asher*[a]

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The difficulty of rapid, definitive diagnosis of myocardial ischemia leads to unnecessary hospital admissions and treatment delays. Previously, decreased metal binding affinity in human serum was investigated as a marker for myocardial ischemia. Polymerized Crystalline Colloidal Array (PCCA) sensors for Ni^{2+} may be useful in developing a point-of-care test to determine metal binding affinity in plasma and to help rule out myocardial ischemia. PCCA sensors for Ni^{2+}, with 5-amino-8-hydroxyquinoline as a chelating agent, were tested in aqueous solutions and diluted human plasma. The peak wavelength diffracted by the sensors was monitored by reflectance spectrometry and correlated with Ni^{2+} concentration. The PCCA sensors show a linear response to aqueous Ni^{2+} concentrations between 0.2 and 1.0 mmol L^{-1}, and can detect changes in free Ni^{2+} concentration of <60 μmol L^{-1}. The sensors respond at physiologic pH and can be reversibly dehydrated. The PCCA sensors developed here can report on free Ni^{2+} concentration in the presence of human plasma. These sensors can be used to detect a decrease in the Ni^{2+} affinity of plasma proteins, which may indicate recent myocardial ischemia. PCCA sensors offer a practical approach to rapid, point-of-care detection of a proposed biochemical signature of myocardial ischemia.

Introduction

Cardiovascular disease is the leading cause of death in the United States, and approximately 1.2 million Americans will have a coronary attack this year.[1] One of the challenges of treating Acute Coronary Syndrome (ACS) is the difficulty in rapidly and definitively diagnosing the cause of chest pain; only 10% to 15% of patient presenting with chest pain actually have myocardial infarction.[2] The cost of unnecessary admissions to US hospitals for patients with suspected ACS is ~12 billion dollars annually.[2]

Beyond patient history, the electrocardiogram (ECG) is the initial test used to evaluate patients with chest pain.[3,4] The initial ECG, however is non-diagnostic in 45% of cases of acute myocardial infarction[5] and appears completely normal in 20% of cases.[6] Blood markers such as troponins can provide a definitive diagnosis. However, they do not appear in the blood for 3–4 h after a myocardial infarction, after irreversible cardiac damage has occurred.[7] A point-of-care clinical chemistry test that quickly detects myocardial ischemia would help expedite treatment and avoid unnecessary hospital admissions.[8]

A decrease in serum binding affinity for Co^{2+} after ischemia has been described in recent reports and this affinity change can potentially be used to detect ischemia before infarction occurs.[9,10] This change in binding affinity has been termed "Ischemia Modified Albumin" by some investigators, although it is possible that albumin modification is not the deterministic phenomenon causing changes in Co^{2+} affinity during ischemia.[11] The implication of Human Serum Albumin (HSA) as the origin of the affinity change results because HSA is the most abundant metal binding site in human serum; the N-terminus of HSA contains a high-affinity ($K_d \sim 10^{-15}$) binding site for Co^{2+}, Cu^{2+}, or Ni^{2+}.[12,13]

After an ischemic event, the affinity of human serum for these metals is decreased.[14,15] The observed decrease in cobalt binding capacity is due either to the competitive occupation of metal binding sites by Cu^{2+} or degradation of the site by reactive oxygen species, whose formation is catalyzed by copper.[16,17] It should be noted that upon an ischemic event, anaerobic metabolism produces lactic acid, lowering the local pH and leading to the local release of copper and iron complexes.[18] Increases in serum nickel levels of ~20 nmol L^{-1} are also observed after cardiac ischemia.[19]

The decreased Co^{2+} binding affinity disappears 6 h after transient, induced myocardial ischemia,[14] suggesting that competitive binding of locally released Cu^{2+} is the major factor responsible for decreased Co^{2+} binding. Studies of commercial albumin preparations incubated at physiological temperatures for several weeks suggest that the N-terminal albumin binding site for transition metals may also be degraded by a metal-independent, internal cyclization mechanism.[17] This reaction appears to be too slow to account for the rapid decrease in Co^{2+} affinity observed after transient myocardial ischemia.

A reduction in the cobalt binding capacity of human serum has been measured in several clinical trials, and was shown

[a]*Department of Chemistry, Univeristy of Pittsburgh, 219 Parkman Ave, Pittsburgh, PA, 15260, USA. E-mail: asher@pitt.edu; Fax: +1 412 624 0588*
[b]*Department of Human Genetics, University of Pittsburgh School of Medicine, 3705 Fifth Avenue, Pittsburgh, PA, 15213, USA*
† The HTML version of this article has been enhanced with colour images.

to be useful in ruling out myocardial ischemia, especially when combined with other tests.[11,20,21] The cobalt binding assay involves complex sample preparation and access to a central clinical chemistry laboratory.[22] Methods that could detect a change in the Co^{2+}, Ni^{2+}, or Cu^{2+} binding capacity of human serum or plasma at the bedside would improve the utility of this test as an early indicator of ischemia. A point of care assay that does not require specialized instrumentation and sample handling could dramatically improve care for millions of patients presenting with ACS.

Polymerized Crystalline Colloidal Array (PCCA) sensors developed by Asher *et al.* have great potential for point of care sensing applications.[23–25] PCCAs contain a highly ordered array of colloidal nanoparticles embedded in a hydrogel matrix.[26] In low ionic strength solutions, a crystalline colloidal array (CCA) forms when highly charged polystyrene spheres self-assemble into a face centered cubic lattice. The spacing between lattice planes can be selected so that the CCA Bragg-diffracts light in the visible range. A PCCA is formed when the CCA is entrapped in a hydrogel such as polyacrylamide by photopolymerization. The lattice spacing and the resulting diffracted wavelength becomes a function of hydrogel volume, as swelling or shrinking causes a change in the lattice plane spacing. PCCA sensors can be formed by modifying the hydrogel so that a volume change occurs in response to interaction with a specific analyte. At the high ionic strength conditions found in human blood, the most straightforward approach is to design the PCCA sensor so that hydrogel crosslinks are formed or broken in response to the analyte. For example, Alexeev *et al.* recently demonstrated a PCCA sensor that responds to glucose when boronic acid groups attached to the PCCA hydrogel bind glucose at two sites of the molecule.[27]

We present here a PCCA sensor that responds to Ni^{2+} ions at physiological pH and osmolarity. We show that the sensor responds to Ni^{2+} in the presence of human plasma, and that it can be used to measure the Ni^{2+} binding capacity of the constituents of human plasma. We previously demonstrated PCCA sensors that respond to metal ions such as Co^{2+}, Ni^{2+}, and Cu^{2+} in aqueous solutions at low pH.[28] In the work here, we specifically study Ni^{2+} sensing at neutral pH. Ni^{2+} is more soluble that Cu^{2+} at neutral pH and it does not undergo the $M^{2+} \rightarrow M^{3+}$ oxidation that complicates Co ion sensing.[28] The PCCA metal sensors respond rapidly (within minutes) to changes in Ni^{2+} concentration, and this method of detecting a change in metal binding affinity for plasma is potentially faster than the cobalt binding assay. Since the PCCA metal sensors respond in human plasma, it is not necessary to wait for clot formation and to centrifuge the blood sample before analysis. Red blood cells can be rapidly separated from the plasma with size selective membranes or microfluidic techniques.[29,30] The sensor presented here could potentially shorten the analysis time for changes in the metal ion affinity of serum or plasma from ~60 min to 5–10 min.

Experimental

Monodisperse, highly charged polystyrene nanoparticles were prepared as previously described.[31] The particle diameter was measured to be 189 ± 9 nm (mean ± SD) by TEM. After dialysis against deionized water and mixing with AG 501-X8 (D) ion-exchange resin (20 to 50 mesh, mixed bed, Bio-rad), the system self-assembled into a CCA and exhibited a primary diffraction peak at 562 nm at normal incidence. The diffraction preak of the CCA was measured using a 6 around 1 reflectance probe and a fiber-optic diode spectrometer with a tungsten halogen light source (Ocean Optics).

PCCA were formed by photopolymerization of acrylamide (0.10 g, 1.4 mmol, Sigma) and *N*,*N*′-methylenebisacrylamide (0.002 g, 13 μmol, Fluka) dissolved in the CCA (20% w/w dispersion of polystyrene latex spheres) following the procedures of Asher *et al.*[28] Before polymerization, the colloidal suspension and prepolymer solution was degassed, and the evacuation chamber was backfilled with nitrogen. This solution was injected into a polymerization cell (Fig. 1) that was evacuated in order to remove oxygen. A gel support film containing surface vinyl groups (Bio-rad) was used as one face of the polymerization cell, covalently attached to the PCCA during photopolymerization (12 min with two 100 W UV lamps, Black Ray). After polymerization the PCCA was removed from the polymerization cell and cut into rectangular pieces (approximately 8 mm × 12 mm) with a razor blade and stored in deionized water.

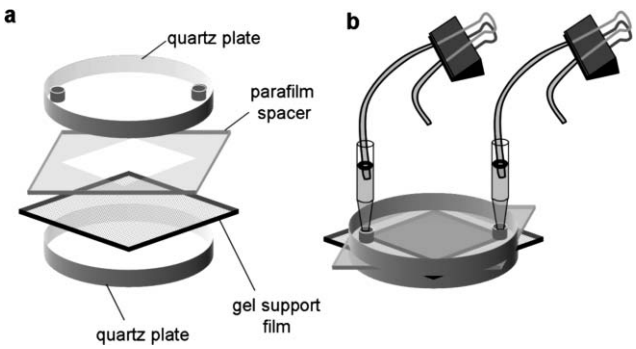

Fig. 1 a: The polymerization flow cell consists of a quartz plate with access holes, a parafilm spacer, a gel support film, and a solid quartz plate. b: The degassed CCA prepolymer suspension is injected through tubing connectors and sealed from the environment in order to exclude oxygen during polymerization.

Metal ion sensors were made from the PCCA by the method of Asher *et al.*[28] The PCCA was first hydrolyzed at room temperature for 2 h in an aqueous NaOH solution (30 mL, 0.1 mol L^{-1}, Sigma) with 10% v/v *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED; Aldrich). The hydrolyzed PCCA was then placed in a 30 mL aqueous solution of 5-amino-8-hydroxyquinoline (0.14 g, 0.60 mmol, Aldrich) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; 0.14 g, 0.73 mmol, Pierce) for 2 h. After rinsing with deionized water, this coupling process was repeated once again.

$NiCl_2$ stock solutions were prepared by dissolving $NiCl_2 \cdot 6H_2O$ (1.189 g, 5.00 mmol, Sigma) in either 100 mL of sodium acetate (50 mmol L^{-1}, Fisher) buffered saline or 100 mL of *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES; 50 mmol L^{-1}, Sigma) buffered saline. The pH was adjusted to 4.2 for the acetate buffered solution and to 7.4 for the HEPES buffered solution by addition of 20% NaOH (Sigma). Solutions with lower Ni^{2+} concentration were prepared by serial dilution.

Before measuring the response of the PCCA sensors to Ni^{2+}, they were first exposed to 50 mmol L^{-1} $NiCl_2$ solutions for at least 30 min, followed by rinsing with the appropriate buffer solution. This was to ensure that the sensors operated in the reversible domain, where the diffracted peak wavelength red-shifts with increasing metal ion concentration.[28] When a metal ion PCCA sensor is first exposed to low concentrations (0–10 μmol L^{-1}) of divalent ions such as Ni^{2+}, Co^{2+}, or Cu^{2+}, there is an initial blue-shift of the peak diffracted wavelength as each ion complexes to two 8-hydroxyquinoline moieties to form hydrogel crosslinks. At higher concentrations (10 μmol L^{-1}–50 mmol L^{-1}), these crosslinks are broken and the diffracted wavelength red-shifts as metal ions are bound by single 8-hydroxyquinoline groups. The sensing response in the red-shifting domain is reversible, whereas the sensor acts as a dosimeter in the initial blue-shifting domain.[28]

For initial studies of the sensor response, the analyte solutions were exchanged before each diffraction peak measurement in order to vary the metal ion concentration. We maintained a volume ratio of approximately 1000 : 1 for the solution volume to the PCCA sensor volume. We measured replicates of the peak diffraction wavelength of a PCCA sensor at different Ni^{2+} concentrations in order to determine the standard deviation of the measurement.

Standard addition studies were conducted by adding small volumes (50–500 μL) of concentrated Ni^{2+} solution to the large (30–50 mL) buffer reservoir bathing the sensor. The diffraction of the PCCA at normal incidence was monitored using a 6 around 1 reflectance probe and a fiber-optic diode spectrometer with a tungsten halogen light source (Ocean Optics).

For PCCA dehydration experiments, the sensor was left in a HEPES buffered saline solution containing a total of ~1.5 mmol $NiCl_2$ and allowed to dehydrate by exposure to room air over 3 days. The sensors were rehydrated by adding deionized water to the dish, and rinsed with HEPES buffer prior to additional sensing experiments.

For the Ni^{2+} binding experiments, 10 mL of human plasma was added to 20 mL of HEPES buffered saline solutions with known Ni^{2+} concentrations. This human plasma was separated from a pooled, heparinized whole blood sample (Bioreclamation) by centrifugation. The sodium heparin had a molecular weight of ~12 kDa and was present at a concentration of 15 USP units per 1 mL of whole blood.

Results

Fig. 2 shows several sequential measurements of the peak diffracted wavelength for a PCCA sensor as the Ni^{2+} concentration is varied. Based on the variance of these measurements, we calculate a maximum standard deviation of 0.6 nm for the peak diffracted wavelength at the different Ni^{2+} concentration and a standard deviation of 0.2 nm at 0.75 mmol L^{-1}. The peak diffracted wavelength shift is roughly linear over Ni^{2+} concentrations between 0.2 mmol L^{-1} and 1.0 mmol L^{-1} (Fig. 3a), which is the relevant pathophysiologic range for monitoring changes in plasma metal binding capacity. We can use the slope of the response curve (8.6 nm $mmol^{-1}$ L^{-2}) to express the standard deviation in terms of Ni^{2+} concentration (0.07 mmol L^{-1} at 7.5 mmol L^{-1} and 0.02 mmol L^{-1} at 0.75 mmol L^{-1}).

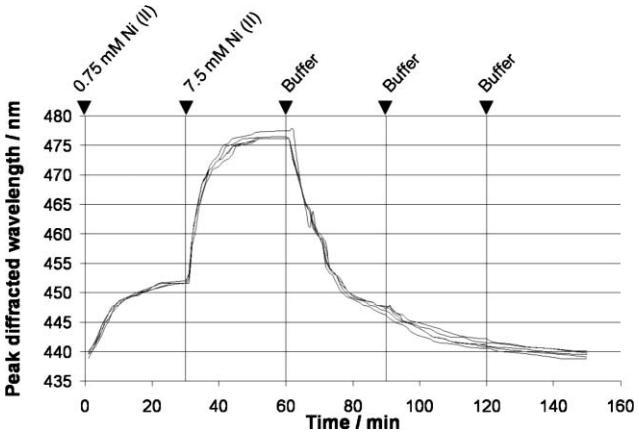

Fig. 2 Time dependence of peak diffracted wavelength of the PCCA sensor upon alteration of Ni^{2+} concentration for 5 consecutive replicates. Solutions containined 50 mmol L^{-1} HEPES buffer and 150 mmol L^{-1} NaCl. Each curve represents a different replicate cycle. Vertical lines indicate times when the analyte solution was replaced. Added solutions contained 0.75 mmol L^{-1} $NiCl_2$ at 0 min and 7.5 mmol L^{-1} $NiCl_2$ at 30 min. At 60, 90, and 120 min, the solution was replaced with HEPES buffered saline. The peak diffracted wavelength was 439.5 ± 0.5 nm at 0 mmol L^{-1} $NiCl_2$, 451.7 ± 0.2 nm at 0.75 mmol L^{-1}, and 476.5 ± 0.6 at 7.5 mmol L^{-1} (average ± SD). This yields an overall standard deviation of ±0.5 nm.

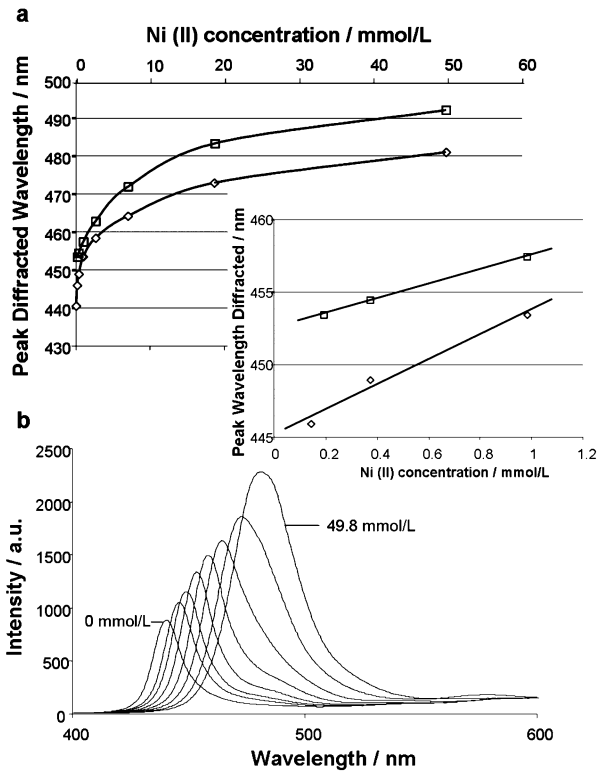

Fig. 3 a: The dependence of peak diffracted wavelength on $NiCl_2$ concentration and pH: pH = 4.2 □ and pH = 7.4 ◇. At pH = 4.2, solutions contain 50 mmol L^{-1} sodium acetate and 150 mmol L^{-1} NaCl. At pH = 7.4, solutions contain 50 mmol L^{-1} HEPES buffer and 150 mmol L^{-1} NaCl. The inset shows the linear response range between 0.2 mmol L^{-1} and 1.0 mmol L^{-1} Ni^{2+}. b: Observed reflectance diffraction spectra for the pH = 7.4 curve points.

Fig. 3 shows the response of a PCCA Ni^{2+} sensor at pH 4.2, and pH 7.4. The response profiles are very similar, except that diffraction is blue-shifted by approximately 10 nm at the higher pH.

Fig. 4 shows the effects of dehydrating the PCCA sensor. The response profiles before dehydration and after rehydration are very similar, except that peak diffraction red-shifts and the sensor sensitivity decreases. For example, the wavelength shift in response to 1 mmol L^{-1} Ni^{2+} was diminished from 12.9 nm to 8.8 nm. The intense Bragg diffraction peak that is indicative of PCCA ordering is recovered after rehydration (Fig. 4 inset).

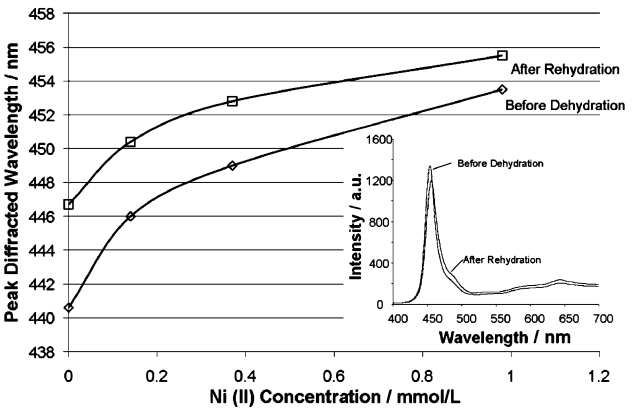

Fig. 4 Dependence of peak diffracted wavelength on $NiCl_2$ concentration before dehydration (◇) and after rehydration (□). The sensitivity of the PCCA sensor is decreased after rehydration as the peak wavelength diffraction shift between 0 to 1 mmol L^{-1} $NiCl_2$ decreases from 12.9 nm before dehydration to 8.8 nm after rehydration. The inset shows the diffraction spectrum at 0.98 mmol L^{-1} before dehydration and after rehydration. The peak diffracted wavelength shifts 2 nm from λ = 453.5 nm (before dehydration) to λ = 455.5 nm (after rehydration). The PCCA sensor was dehydrated and rehydrated *in situ.* Thus, the diffraction spectra are from identical spots of the sensor.

Addition of human plasma to a nickel-free buffer solution surrounding the sensor shifts the peak diffracted wavelength by ~2 nm (Fig. 5). Initially, there is a small, rapid blue-shift, followed by a much smaller and more gradual red-shift. After the removal of plasma, and rinsing with buffer, the peak diffracted wavelength returns to the original value. We observe no evidence of coagulation or clot formation during these experiments.

Fig. 6a shows the effect of adding 10 mL of human plasma to a PCCA sensor equilibrated in 20 mL of a 0.75 mmol L^{-1} $NiCl_2$ solution containing 50 mmol L^{-1} HEPES buffer and 150 mmol L^{-1} NaCl. There is a rapid 12.4 nm blue-shift, which is much larger than in the absence of Ni^{2+}. Fig. 6b shows the effect of adding the same volume of buffer rather than plasma. Accounting for the osmotic effects of plasma addition, we find that the human plasma binds 1.0 mmol Ni^{2+} per L of human plasma (see Discussion below).

We observe a sustained, linear red-shift of 0.5 nm h^{-1} after the addition of plasma and Ni^{2+} (not shown). This small red-shift is within the error of the measurement for a single concentration determination lasting 10–20 min.

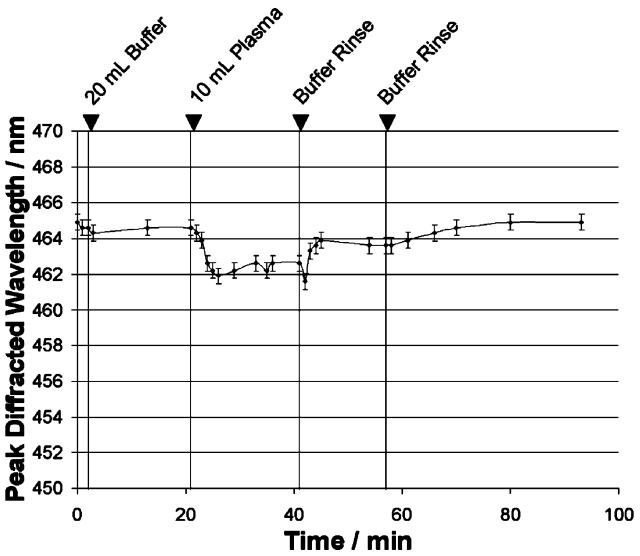

Fig. 5 Response of PCCA sensor to human plasma in the absence of $NiCl_2$. Plasma was added to the analyte solution (50 mmol L^{-1} HEPES and 150 mmol L^{-1} NaCl) at 21 min to form a 2 : 1 (buffer : plasma) solution. After rinsing with HEPES buffer, the peak diffracted wavelength returns to its original value.

Discussion

Based on the standard deviation of 0.02 mmol L^{-1} (at a Ni^{2+} concentration of 0.75 mmol L^{-1}), we can expect to reliably detect changes in plasma binding capacity of 60 μmol L^{-1}. PCCA metal sensors were previously studied at pH 4.2.[28] However, the affinity of the N-terminal metal binding site in HSA for Co^{2+} markedly decreases at pH values below 6.2.[15] The response profiles at pH 4.2 and pH 7.4 are very similar, except that diffraction is blue-shifted by approximately 10 nm at the higher pH (Fig. 3). This difference is likely due to the formation of fewer crosslinks in the hydrogel at pH 4.2, which is caused by the slightly lower affinity of 8-hydroxyquinolines for Ni^{2+} at lower pH.[32]

The ability to maintain PCCA sensors in dry storage before use would greatly aid the development of these sensors for practical clinical applications. However, until now, there has been no demonstration that PCCA sensors can be reversibly dehydrated and rehydrated. Fig. 4 shows that our Ni^{2+} PCCA sensor can be reversibly dehydrated. We believe this to be the first report of a PCCA sensor that can be reversibly dehydrated and rehydrated. (Sanford Asher, Michelle Muscatello and Lee Stunja in our lab are studying the reversible dehydration of PCCA glucose sensors, and are in the process of preparing a manuscript on this topic.) Unconstrained PCCA sensors generally become brittle during the drying process and often crack. Attachment to a gel support film seems to force the sensor to dry more uniformly, which prevents formation of cracks. Polyacrylamide electrophoresis gels are often dried in the presence of concentrated, low molecular weight stabilizers such as glycerol, urea, or ethanol;[33,34] the presence of the HEPES buffer during drying likely acts in a manner similar to other low molecular weight stabilizers, preventing the irreversible collapse of polyacrylamide chains.

While the response of Pb^{2+}, creatinine, and ammonia PCCA sensors in human and bovine serum has been studied

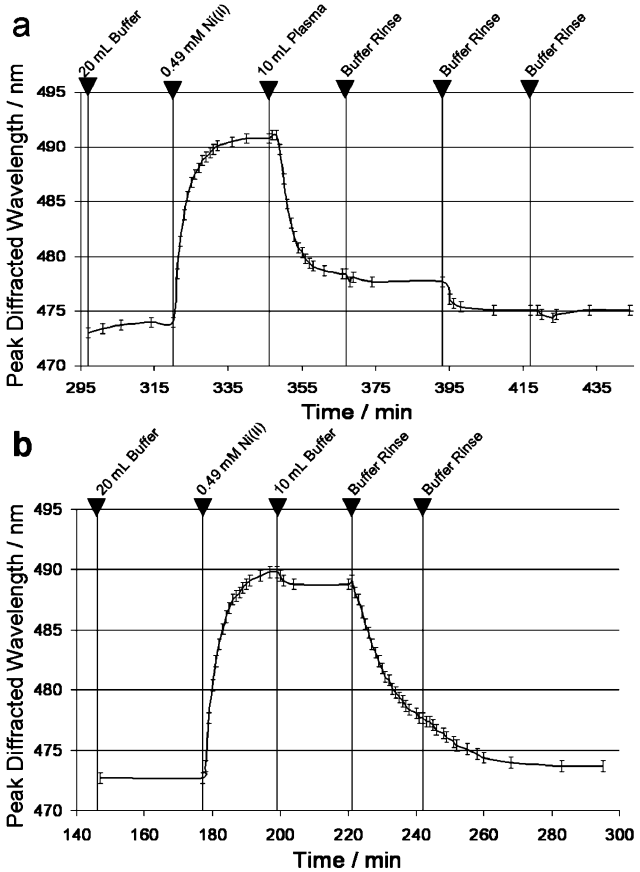

Fig. 6 (a) Response of PCCA sensor to human plasma addition in the presence of $NiCl_2$. The sensor initially red-shifted in response to 0.74 mmol L^{-1} aqueous $NiCl_2$ in 50 mmol L^{-1} HEPES buffer and 150 mmol L^{-1} NaCl. Addition of human plasma at 346 min caused a blue-shift of 12.4 nm. (b) Response of PCCA sensor to HEPES buffer addition in the presence of $NiCl_2$. Addition of HEPES buffer at 199 min caused a blue-shift of 0.7 nm, due to dilution of $NiCl_2$ to a concentration of 0.49 mmol L^{-1}.

previously,[23,24,35] the response of PCCA sensors to human plasma has not been investigated. As shown in Fig. 5, the sensor exhibits a small and rapid initial blue-shift followed by a smaller and more gradual red-shift. This two-phase response is typical of hydrogels placed in a hypertonic solution evolving to osmotic equilibrium.[36] The addition of plasma initially creates hypertonic conditions outside of the hydrogel and water flows out of the hydrogel to equalize the osmotic pressure. At longer time scales, plasma proteins such as albumin diffuse into the hydrogel, diminishing the osmotic pressure. Previous work suggests that the diffusive permeability of albumin in a polyacrylamide hydrogel such as those studied here is approximately 10^{-8} cm^2 s^{-1}.[37] This means that on the time scale of the experiment (20 min), the average albumin molecule will diffuse only about 50 μm into the hydrogel. For this reason, the red-shifting back towards the baseline wavelength is slow.

The large PCCA sensor blue-shift in response to plasma, when Ni^{2+} is present, cannot be explained by osmotic effects of plasma or by dilution effects. Fig. 6b shows the effect of adding the same volume of buffer rather than plasma. We hypothesize that the component proteins of human plasma, especially albumin, sequester Ni^{2+}, leading to a blue-shift in the diffracted wavelength as the effective concentration of Ni^{2+} decreases. Comparing the diffracted wavelength shifts for our sensor after plasma addition with the Ni^{2+} calibration curve allows us to calculate the concentration of bound Ni^{2+}. Accounting for the osmotic effects of plasma addition described above, we find that the human plasma preparation binds 1.0 mmol Ni^{2+} per L of human plasma. The reference interval for human serum albumin in adults is 35–52 g L^{-1} (0.53–0.78 mmol L^{-1}). Hence, other species in the plasma preparation must also have a significant affinity for Ni^{2+}. Nickeloplasmin[38] and histidine[39] have been identified as the major species besides serum albumin that bind nickel in human plasma. Proteins involved in the clotting cascade[40] (factor VIII) and the alternative complement pathway[41] (C-3 convertase) are also known to bind Ni^{2+}. The presence of such species will decrease the total free Ni^{2+} concentration measured by our sensors. However, they are unlikely to interfere with the measurement of the ischemia induced *changes* in plasma Ni^{2+} binding.

As noted above, we observe a small, linear red-shift over time after the addition of plasma and Ni^{2+}. One plausible explanation is that albumin or other plasma proteins are reacting with and/or binding to the hydrogel. Binding of these proteins may alter the free energy of mixing of the hydrogel. This small red shift is within the error of the measurement for a single concentration determination lasting 10–20 min. However, it would become significant during longer experiments, complicating standard addition experiments, where several measurements must be made after the addition of plasma.

Conclusions

We demonstrate here the ability to measure a change in the nickel concentration in the presence of human plasma as it is sequestered by the constituents of plasma. This is the required first step in developing a PCCA sensor for the rapid detection of myocardial ischemia. Future studies will be needed to establish reference intervals and to standardize this assay.

Previous studies suggest that it is possible to make polyacrylamide PCCA metal sensors which undergo greater wavelength shifts in response to Ni^{2+}, Co^{2+}, or Cu^{2+}.[28] This is generally accomplished by modifying the hydrogel volume fraction and initial cross link density. If the sensors showed large wavelength shifts between 0 and 1 mmol L^{-1} a simple visual inspection of the sensor could be used to determine the test result. However, since some end users may be color blind, and for documentation purposes, it may be preferable to use a handheld spectrometer to determine the peak diffracted wavelength.

Financial disclosures

Sanford A. Asher is the scientific founder of Glucose Sensing Technologies LLC, a company developing PCCA sensors for glucose sensing.

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