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A comparison of the binding of urinary calcium oxalate monohydrate and dihydrate crystals to human kidney cells in urine

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Abstract

Objective—To compare the binding kinetics of urinary calcium oxalate monohydrate (COM) and dihydrate (COD) crystals to human kidney (HK-2) cells in ultra-filtered (UF), and centrifuged and filtered (CF) human urine; and to quantify the binding of COM and COD crystals to cultured HK-2 cells in UF and CF urine samples collected from different individuals.

Materials and methods—Urine was collected from healthy subjects, pooled, centrifuged and filtered. ^{14}C -oxalate-labelled COM and COD crystals were precipitated from the urine by adding oxalate after adjustment of two aliquots of the urine to 2 and 8 mM Ca^{2+} , respectively. For the kinetic study, the crystals were incubated with HK-2 cells for up to 120 min in pooled CF urine adjusted to 2 and 8 mM Ca^{2+} . Identical experiments were also carried out in UF urine samples collected from the same individuals. For the quantitative study, the same radioactively labelled COM and COD crystals were incubated with HK-2 cells for 50 min in separate CF and UF urines collected from eight healthy individuals at the native Ca^{2+} concentrations of the urines. Field emission electron microscopy and Fourier transform-infrared spectroscopy were used to confirm crystal morphology.

Results—Binding of both COM and COD crystals generally bound more strongly at 8 mM than at 2 mM Ca^{2+} . The kinetic binding curves of COM were smooth, while those of COD were consistently biphasic, suggesting that the two crystal types induce different cellular metabolic responses: HK-2 cells crystals appear to possess a transitory mechanism for detaching COD, but not COM crystals. In UF urine, COM binding was significantly greater than that of COD at 2 mM Ca^{2+} , but at 8 mM Ca^{2+} the binding of COD was greater at early and late time points. COD also bound significantly more strongly at early time points in CF urine at both 2 and 8 mM Ca^{2+} . In both CF and UF urine, there was no difference between the binding affinity of urinary COM and COD crystals.

Conclusion—Binding of both COM and COD crystals to cultured human renal epithelial cells is influenced by urinary macromolecules and ambient Ca^{2+} concentration. HK-2 cells appear to possess a mechanism for the rapid detachment of bound COD crystals, making it difficult to show any unambiguous overall difference between the binding affinity of COM and COD crystals.

Keywords

calcium oxalate monohydrate; calcium oxalate dihydrate; HK-2 cells; crystal attachment

Introduction

The most common mineral in human renal calculi, calcium oxalate (CaOx), exists under physiological conditions as two crystalline polymorphs: monoclinic CaOx monohydrate (COM) and tetragonal CaOx dihydrate (COD). The relative amounts of COM and COD in stones are important, because COM calculi are more difficult to disintegrate by percutaneous and extracorporeal lithotripsy [1,2], and because individuals who form COD stones are more likely to have further episodes than those who form COM stones [1,3]. Thus, several clinical studies have been undertaken in stone-formers with the aim of correlating urine composition with the occurrence of COM and COD in their calculi, to estimate the force required to achieve complete stone disruption and clearance during lithotripsy, or to predict the likelihood of stone recurrences. A strong relationship has been reported to occur between COD stones and hypercalciuria [1,4–7], reduced citrate levels [1,7] and higher pH [5]. On the other hand, COM calculi occur more frequently in association with normocalciuria [8].

Although COM predominates in kidney stones [9], COD crystals are more commonly excreted in urine [4,10], which implies that differences between the structures of the two crystal types may determine whether they are harmlessly excreted or become attached to renal epithelial cells and induce stone formation. Consequently, it has been proposed that selective formation of COD crystals would protect against stone pathogenesis [11,12]. Although an obligatory forerunner to CaOx urolithiasis, crystal nucleation within the urinary collecting system does not inevitably proceed to stone pathogenesis. COM and COD crystals are occasionally passed by both stone-formers and healthy subjects, and it is now widely accepted that the development of renal stones must require crystals to nucleate upon, or attach to epithelial cell membranes.

Heralded by the early work of Mandel *et al.* [13] and Lieske *et al.* [14], numerous studies have investigated the attachment of CaOx crystals to, and phagocytosis by renal epithelial cells. Exposure to COM was reported to initiate DNA synthesis and stimulate cell proliferation [14], which in turn increased production of hyaluronic acid [15]. The crystals also caused cellular injury [16], characterized by changes in markers of inflammation, apoptosis and necrosis [17,18], which were accompanied by a significant increase in the generation of hydroxyl and superoxide radicals [19]. In BSC-1 cells, COM crystals induced the expression of genes encoding transcriptional activators, one regulator of extracellular matrix, and growth factors, including the early genes *c-myc*, *EGR-1*, and *Nur-77*, plasminogen activator inhibitor and platelet-derived growth factor-A chain [20]. Crystal adherence and internalization also caused phosphorylation and activation of p38 mitogen-activated protein kinase [21], increased the expression of monocyte chemoattractant protein-1 mRNA and protein [22–24], and elevated the activities of N-acetyl- α , D-hexosaminidase, γ -glutamyl transpeptidase and leucine aminopeptidase [17]. Osteopontin mRNA, as well as expression of the protein itself, was significantly elevated in BSC-1 [25] and NRK 49F [22,23] cells, the abundance of fibronectin increased [26], while cultured macrophages released TNF- α and interleukin 6 in a time-dependent manner [27]. More recently, more than 35 genes were reported to be up- or down-regulated at least two-fold in NRK-52E cells by exposure to COM crystals [28].

Although not as extensively investigated, hyperoxaluric animal models have confirmed the occurrence of similar metabolic responses to COM crystal formation *in vivo* [15,29,30]. Using microarray analysis, the expressions of a large number of genes associated with tubule function and regulation, oxidative damage, and inflammation were shown to be altered in the kidneys of hyperoxaluric rats [31]. The expressions of a staggering 173 genes were regulated at least two-fold in the same rat model [32], while those of 31 genes were altered during the progression of nephrolithiasis in mice [33].

Compared with COM, only a few studies have examined the interaction between cultured epithelial cells and COD crystals [34–36], or directly compared the metabolic sequelae of COM and COD crystal attachment. Exogenous COM [37] and COD [34] crystals have been reported to bind to the surfaces of BSC-1 renal cells with different, face-selective affinities, and 50% more COM than COD crystals bind to primary explants of rat inner medullary collecting ducts [11]. More recently, Sheng *et al.* [38] reported that COD crystal faces attach less avidly than COM faces to atomic force microscopy tips coated with biologically relevant functional groups. Another investigation showed that inner medullary collecting duct cells bound 50% more COM than COD crystals of comparable size [11]. Although those studies collectively appear to support the hypothesis that selective formation of COD crystals would provide relative protection against stone formation, they were all performed using an inorganic binding medium, and the COM and COD crystals were prepared from inorganic solutions. However, *in vivo* crystals form and bind to cells in the presence of urinary macromolecules, which alter their ability to attach to cells by covering their surfaces and becoming trapped within the mineral phase [39,40]. Differences between the binding affinities of COM and COD crystals formed in urine may thus hold one key to why stones form, and it is vital that they be explored. Thus the aims of the present study were: (i) to examine the binding kinetics of urinary COM and COD crystals to human kidney (HK-2) cells in ultra-filtered (UF), and centrifuged and filtered (CF) human urine and (ii) to quantify the binding to HK-2 cells of COM and COD crystals in UF and CF human urine samples collected from different individuals.

Materials and methods

This study was reviewed and approved by the Flinders Clinical Research Ethics Committee. To make radiolabelled COM and COD urinary crystals from CF urine, 24-h urine specimens were collected from four healthy laboratory colleagues (two men, two women) and shown to be free of blood by dipstick analysis (Combur-8 Test®, Roche Diagnostics GmbH, Mannheim, Germany). The urine was pooled, centrifuged (10 000 *g*) for 20 min at 20 °C to remove debris and Tamm-Horsfall glycoprotein, filtered (0.22 µm; GVWP14250, Millipore Corporation, Billerica, MA, USA) as described earlier [41], and the pH was adjusted to 6.1: this sample was designated CF urine. The Ca²⁺ concentration was determined by the o-cresolphthalein complexone technique, using an automated biochemical analyser, at a wavelength of 546 nm. The sample was then divided into two portions, and the final Ca²⁺ concentrations were adjusted to either 2 or 8 mM by dropwise addition of filtered (0.22 µm) 1 M CaCl₂ solution.

Radiolabelled COM and COD crystals, respectively, were precipitated from the CF urine samples containing 2 and 8 mM Ca²⁺ by adding two standard oxalate loads above an experimentally determined metastable limit [42] in the presence of ¹⁴C-labelled oxalic acid (Sigma-Aldrich Co., St Louis, MO, USA), at a concentration of 115.625 kBq per 100 mL, before addition of the oxalate load. After incubation for 1 h in a 37 °C water bath with shaking at 90 oscillations per minute, the crystals were harvested, washed, lyophilized and stored as described previously [41]. Immediately before use, crystals were sonicated for 10 min in the urine samples, which were to be used in the binding experiments.

Crystal morphology was assessed using a Philips XL30 field-emission scanning electron microscope (FESEM) as described previously [41,43]. Crystals were also analysed using Fourier transform-infrared spectroscopy (FTIR), which confirmed their chemical compositions as COM and COD.

For SDS-PAGE, the crystals were demineralized in EDTA (pH 8.0) as described previously [44] and the resulting solution, was desalted by passage through a Bio-Gel P-6 DG column

(Bio-Rad Laboratories, Hercules, CA, USA). The protein fraction of each sample was lyophilized and stored at -70°C for later analysis. The lyophilized samples were mixed with reducing sample buffer, separated using SDS-PAGE, and the gel was stained with silver as described previously [39,41].

For the cell culture, the immortalized human kidney proximal cell line (HK-2) was obtained from American Type Culture Collection. Experiments were performed using high-density quiescent cultures in 35 mm plastic plates according to the method of Lieske *et al.* [45].

To assess crystal binding kinetics, urine samples were collected from healthy volunteers (two men; two women), pooled, and processed as described above to give two separate CF urine samples with final Ca^{2+} concentrations of 2 and 8 mM. Additional samples were obtained from the same individuals on a later occasion and treated as before, except that they were also UF through a regenerated cellulose cartridge (Prep Scale TM TFF, cat# CDUF001LC, Millipore Corporation) with a nominal relative molecular weight threshold of 10 kDa. These were divided and the Ca^{2+} concentrations adjusted to give two UF samples with concentrations of 2 and 8 mM Ca^{2+} .

Cells were rinsed three times with 2 mL of the urine in which the binding experiment was to be performed and then 2 mL of the CF or UF urine sample was added in which had been suspended 400 μg of ^{14}C -labelled COM or COD crystals: thus, identical masses of COM and COD crystals were added to the cells. Experiments were performed as previously described [39], in CF and UF urines at final Ca^{2+} concentrations of both 2 and 8 mM. These concentrations were used because COM and COD crystals typically precipitate from urine at low and high Ca^{2+} concentrations, respectively [41]. The plates were gently agitated to distribute the crystals uniformly and incubated at room temperature for periods up to 120 min. The urine was then aspirated and the cells were washed three times with the same urine in which binding had been performed. The crystals remaining on the cells were dissolved by adding an additional 1 mL of urine followed by 0.5 mL of concentrated HCl, and the cells were removed with a cell scraper. The suspension was clarified by centrifugation, and 600 μL of the supernatant was counted for 1 min in 4 mL of Ready Safe scintillation fluid (Beckman) in a Beckman LS 3801 Liquid Scintillation System scintillation counter. Crystal binding affinity was expressed as counts per minute remaining attached to the cells as a percentage of those applied (see next section).

All other factors being equal, adhesion of crystals to a planar surface such as a cell monolayer must be a direct function of crystal surface area available for contact. In the present study, binding was quantified as radioactivity remaining attached to the cells, relative to the total radioactivity applied. Given the large disparity between the sizes of the COM and COD crystals used in the study (Fig. 1), *prima facie*, it would seem intuitively logical to correct raw counts per minute for differences in surface area between the two hydromorphs. However, identical amounts (400 μg) of COM and COD crystals were applied to the cells. For both crystal types therefore, the amount of radioactivity that remained associated with the cells must have reflected not only the fraction of attached crystal mass, but also that of crystal volume, number and surface area. Thus, for a population of uniformly shaped crystals, binding affinity can be validly calculated simply as percentage radioactivity bound to the cells, relative to that applied to them, without correcting for any differences in surface area.

To compare COM and COD binding in individual urine samples, additional individual 24-h urine specimens were collected from eight healthy individuals (four women; four men) and processed as described above to give eight separate CF urine samples. Further samples were also collected from the same individuals, CF and UF as before, to give eight individual UF

urines. The pH of each urine was adjusted to 6.1, and cell attachment experiments were performed using the individual CF and UF urines as the binding medium, as detailed above. Binding was carried out for 50 min, since the kinetic study had shown that binding of both COM and COD crystals had stabilized by that time. These studies were performed at the native Ca^{2+} concentrations of the individual urine samples.

All binding experiments were performed in quadruplicate and two acidified aliquots were withdrawn from each culture dish for counting, giving eight values at each time point. For statistical analysis, the Mann–Whitney *U*-test was used to compare kinetic data at specific time points. For comparisons of binding data for the eight individual CF and UF urine samples, the Wilcoxon signed-rank sum test was used, with $P < 0.05$ considered to indicate statistical significance.

Results

FESEM and FTIR

COM crystals were exclusively precipitated at 2 mM Ca^{2+} and only COD crystals, which were considerably larger, were deposited at 8 mM Ca^{2+} . These results (not shown) were confirmed by FTIR analysis.

SDS-PAGE analysis

The SDS-PAGE profile of proteins present in the demineralized extracts of the COM and COD crystals showed that the intracrystalline protein content of the two crystal types was different, as we have previously reported using both one-dimensional [41] and two-dimensional [46] SDS-PAGE. Results are therefore not presented.

Crystal binding kinetics

Figure 2 shows the time course of crystal binding of the COM and COD crystals in the CF and UF urine samples collected from the same individuals at 2 mM Ca^{2+} . Binding curves of both crystal types were biphasic, the effect being more marked with COD. Initial binding of COM and COD was rapid in both CF and UF urine, with the dihydrate binding significantly more avidly than the monohydrate, but slowed by 5 min, COM attachment then increased again after 10 min and reached a plateau by 40 min. However, binding of COD crystals decreased between 10 and 20 min, but then rose slightly and remained relatively stable thereafter. Except for the first 10 min, COM binding in CF urine was consistently and significantly greater than that of COD. There was a similar pattern in UF urine, except that COD attachment was virtually identical to that of COM at 1 min. From 10 min, binding of COM was always significantly greater than that of COD. Generally, crystal binding of both COM and COD appeared to be slightly greater in UF urine than in CF urine; however, direct comparison was not performed because although the samples were derived from the same donors, they were collected on different occasions.

Binding data for COM and COD crystals in CF urine at a Ca^{2+} concentration of 8 mM are shown in Fig. 3. In CF urine, the COM binding curve was smooth, reaching a maximum between 60 and 80 min and declining to 120 min. In contrast, COD crystal binding was obviously biphasic, rapidly reaching a maximum at 5 min, followed by a decline between 10 and 20 min and then attaining a plateau after 40 min. As occurred at 2 mM Ca^{2+} , binding of COD crystals exceeded that of COM crystals before 10 min, but relative binding values switched between 20 and 80 min. At all time points, differences between the binding values were significantly different. In UF urine, both COM and COD crystals showed strong initial binding followed by a decline. COD crystals initially bound more strongly than COM in UF urine after 1 min, but attached less avidly between 10 and 20 min. After that time, COD

binding consistently exceeded that of COM. As at 2 mM Ca^{2+} , all differences between the binding values of COM and COD were statistically significant.

Unlike the apparent tendency for the crystals to bind more avidly in UF urine at 2 mM Ca^{2+} , at 8 mM there was no clear difference: at some time points binding was greater in UF than in CF urine, while at others the pattern was reversed.

Quantitative study: comparison of COM and COD binding in individual urine samples

Binding data for the same COM and COD crystals in separate CF and UF urine samples from eight individuals are shown in Fig. 4. As would be expected from the kinetic binding patterns of COM and COD crystals shown in Figs 2 and 3, together with the dependence of binding on urine Ca^{2+} concentration, there was no consistent difference between the binding data of COM and COD crystals in either the CF or UF urine samples, whose Ca^{2+} concentrations had not been adjusted. Direct comparison of binding in UF and CF urine was not possible because the samples, although from the same individuals, were collected on separate occasions.

Discussion

Despite their structural similarities [34,35,47], COM and COD crystals possess significantly different characteristics. COM crystals are seven times more injurious to red blood cell membranes than are COD crystals [48,49] and bind 15 times more polyphosphate [50], properties that have been ascribed to differences in their ability to coordinate with adsorbents [50], such as the ordering of water molecules within their crystalline lattices [49] or differences in surface ζ -potential [51]. Collectively, such features probably also contribute to the fact that the two polymorphs preferentially bind and incarcerate markedly distinct urinary proteins [41,46], as confirmed in the present study, which may also account for the observation that COM [37] and COD [34] crystals attach to cultured renal cells with different, face-selective affinities. The same distinctions could also explain the observation that exposure of Madin-Darby canine kidney (MDCK) cells to COM crystals altered the abundance of 53 proteins [52], while COD crystals caused changes in only 11 [53], which is consistent with the greater injurious effect of COM on red blood cell membranes [48,49]. While the cited studies indicate that COM and COD crystals either have different binding affinities for, and/or evoke different reactions in renal epithelial cells, they all used pure synthetic crystals rather than urinary crystals formed in urine, and were performed in aqueous inorganic media. Such factors are important, because inorganic COM crystals (i) bind more avidly to cultured cells than do COM crystals formed in human urine [39,40]; (ii) cause greater cellular injury than crystals formed *in vivo* [54] and (iii) bind more readily in inorganic media and in urine fractions containing low concentrations of macromolecules [39].

The first aim of the present study was to compare directly the binding kinetics of COM and COD crystals derived from the same pooled urine specimen to HK-2 cells in the same urine after either CF or UF: CF urine contains most of its macromolecular complement other than Tamm-Horsfall glycoprotein, while UF urine contains no macromolecules > 10 kDa [43]. Because COM tends to precipitate at lower, and COD at higher urinary Ca^{2+} concentrations [41] the experiments were also performed at Ca^{2+} concentrations of 2 and 8 mM. The second aim of the present study was to compare quantitatively the binding of the same COM and COD crystals in CF and UF urines from eight different individuals.

Adherence of both hydromorphs was greater at a Ca^{2+} concentration of 8 mM than at 2 mM. This is consistent with the findings of a recent study, which showed that adhesive forces between COM crystals and MDCK cells increased proportionately with controlled changes

in Ca^{2+} concentration up to 100 mM [55]. It is also possible that the ambient Ca^{2+} concentration could influence binding by determining the type and abundance of proteins attached to the crystal surfaces. For instance, in urine, osteopontin does not bind irreversibly to COM crystals, but does attach to COD crystals, and in greater quantities at higher Ca^{2+} concentrations [41,56]. Because osteopontin promotes crystal–cell binding [57,58], a higher Ca^{2+} concentration could encourage binding by increasing the amount of osteopontin bound to the crystal surface. The binding of prothrombin fragment 1 to COM [41] and matrix Gla protein to hydroxyapatite [59] is also profoundly affected by the ambient concentration of Ca^{2+} . Although Western blotting was not performed in the present study to identify individual proteins, the protein content of the COM and COD crystals used was markedly different, as has been shown in previous studies [41,46]. That difference may have contributed to the difference in binding, as even intracrystalline proteins can affect the attachment of crystals to MDCK II cells [40].

Perhaps the most unexpected findings of the present study were the different kinetic binding patterns of the COM and COD crystals. In CF and UF urine, and at 2 and 8 mM Ca^{2+} , COD crystals consistently exhibited biphasic binding curves, which were not as obvious with the COM crystals. Furthermore, at 1 min, adherence of the COD crystals was invariably greater than that of COM. The initial effect may have resulted principally from the larger size of the COD crystals, which would have caused them to settle faster than COM. However, although the time varied slightly with experimental conditions, COD binding then declined to reach a minimum by 20 min, after which it increased once more, although to lower levels than those of the COM crystals. Such COD binding curves are reproducible: we have observed biphasic kinetics with COD crystals precipitated from a range of different urine samples (unpublished data). The decrease in bound radioactivity between ≈ 1 and 20 min is unlikely to result from crystal dissolution, which is considerably slower [39]. It is therefore more probable that the biphasic COD binding curves reflect a rapid metabolic response of the cells, which enables them to release some of the crystals from their surfaces, but which is later overridden, as the detached crystals appear to re-bind later. The binding of a cell to a substrate, such as a crystal surface, is a complex, multistage process [60] influenced by a large number of environmental and cellular properties [40] and which, as discussed in the Introduction, elicits *in vitro* and *in vivo* a battery of cellular responses whose physiological significance and physical consequences have yet to be fully elucidated. It is therefore not possible even to speculate on the reason why COD crystals appear to detach from the cells, however temporarily, while COM crystals do not. Nonetheless, it could be argued that even fleeting disengagement of even some attached crystals might be sufficient *in vivo* to explain the preponderance of COD crystals in urine, which could reduce the likelihood of permanent crystal retention and stone pathogenesis.

The biphasic pattern of the COD kinetics caused the binding curves of the COM and COD crystals to intersect, making it impossible to discern a consistent, clear-cut difference in binding affinity between the two polymorphs. The same lack of overall consistency occurred in the present study that compared the binding of the two hydromorphs in separate CF and UF urine samples from eight different individuals, where percentage attachment values of COM and COD crystals to the cells in the same urine samples were statistically indistinguishable in both CF and UF urine.

To our knowledge, this is the first study that has directly compared the interaction between urinary COM and COD crystals and human renal epithelial cells under conditions similar to those that would be expected to occur *in vivo*. Attachment of both hydromorphs was dependent upon the ambient concentration of Ca^{2+} , with greater binding occurring at 8 mM than at 2 mM. Binding of both COM and COD crystals to cultured human renal epithelial

cells is therefore influenced by environmental conditions, particularly the concentration of Ca^{2+} .

Binding data showed that the adhesion kinetics of the two polymorphs were obviously dissimilar, and revealed no overall difference between COM and COD crystals across the entire binding period: at some times points COM bound more strongly than COD, while at others, the relative binding was reversed. This effect was most marked in the initial stages of attachment, where some COD crystals apparently detached temporarily from the cells, suggesting that HK-2 cells possess a mechanism for dislodging them from their surface as a possible form of protection against urolithiasis.

In vivo, the relative likelihoods that crystals will be retained within the kidney will depend upon a combination of surface binding affinity, ionic conditions, macromolecular content and the total amount (bulk) of mineral precipitated. HK-2 cells appear to possess a mechanism for the rapid detachment of bound COD crystals. It is possible therefore, that COD crystals would be less likely than COM crystals to be retained within the renal collecting system. However, the detachment response is only transitory and urinary COD crystals are typically bigger than COM crystals. It is possible therefore that *in vivo*, any advantage conferred by the detachment of COD crystals might be countervailed by their larger size, which would be more likely to cause tubular occlusion.

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Abbreviations

COM	monoclinic CaOx monohydrate
COD	tetragonal CaOx dihydrate
UF	ultra-filtered (urine)
CF	centrifuged and filtered (urine)
HK-2	human kidney cells
FESEM	field-emission scanning electron microscope
FTIR	Fourier transform-infrared spectroscopy
MDCK	Madin-Darby canine kidney

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Fig. 1. Typical COM (left) and COD (right) crystals used in the binding studies. Bars, 50 μm.

Fig. 2.

The time courses of binding of COM and COD crystals to HK-2 cells in CF (top graph) and UF (bottom graph) urine at a Ca^{2+} concentration of 2 mM. Curves were drawn by eye. Symbols show significant differences between the curves at the designated time points. ‡ $P = 0.001$; † $P = 0.005$; * $P = 0.05$.

Fig. 3.

The time courses of binding of COM and COD crystals to HK-2 cells in CF (top graph) and UF (bottom graph) urine at a Ca^{2+} concentration of 8 mM. Curves were drawn by eye. Symbols show significant differences between the curves at the designated time points. ‡ $P = 0.001$; † $P = 0.005$; * $P = 0.05$.

Fig. 4. Binding of COM and COD crystals in paired CF and UF urine samples obtained from eight different healthy volunteers. Studies were performed at native Ca^{2+} concentrations.