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Determination of copper in composite biomaterials by capillary electrophoresis: an UV-direct method based on *in situ* complex formation

Juan P. Cattalini, Viviana S. Mouriño and Silvia E. Lucangioli*

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This work describes the optimization and validation of an UV-direct capillary electrophoresis method for the analysis of copper ions in samples obtained from release studies of bone-tissue-engineering composite biomaterials. Ethylenediaminetetraacetic acid was used as a complexing agent added to the background electrolyte to modulate the selectivity of the separation and quantitation of free copper ions. The separations were performed under reverse polarity and the parameters of validation such as specificity, linearity, limits of detection and quantitation, precision, accuracy and robustness were evaluated. The method resulted to be suitable for copper determination in biomaterials and showed advantages such as rapidness, specificity and suitable sensibility, achieving a limit of detection and quantitation of 0.05 and 0.16 μg mL^{-1}, respectively.

Introduction

The demand for engineered bone tissue has been increasing during the last few decades due to the rise of human population and their life expectancy.[41–44] In common tissue engineering (TE) strategies, a three-dimensional structure (scaffold) made from suitable composite biomaterials is used.[45–49] It is desirable that these scaffolds should not only provide mechanical support for growing bone cells, but they also should conveniently stimulate osteoblast proliferation and differentiation.[49] In this sense, bioactive glasses (BGs) are considered 3rd generation biomaterials[50] which have the capability to trigger specific intrinsic cell responses resulting in osteoinductive actions.[46,51] More precisely, it was reported that ionic dissolution products from BGs (*e.g.* Si, Ca, and P) induce expression of osteogenic genes.[52] In addition, in the last few years, specific inorganic ions known for their anabolic effects in bone metabolism (Sr, Cu, Zn or Co) have been incorporated within scaffolds for tissue engineering strategies.[1–3,40,53,54] As the composite biomaterial degrades after exposure to a physiological environment, the subsequent release of its ions in a controlled and sustained manner (*e.g.* Si, Ca, P plus doped Sr, Cu, Zn or Co) could enhance the bioactivity of the scaffolds related to both osteogenesis and angiogenesis.[1] Thus the analyses of the inorganic ion release profile of scaffolds as well as the quantitation of their amount released (in the order of part per million part per billion) are relevant for the evaluation of their therapeutic effects. Particularly, copper ions have an important role in angiogenesis[4–8] and their effects are dose-dependent.[9–12,36] In this sense, there is a clear need to develop efficient methods, with the adequate sensibility and rapidness, to quantify the release of the metallic ions incorporated into scaffolds for tissue engineering approaches. For the particular case of ion release studies of composite biomaterials for tissue engineering like the ones detailed above, ion-exchange chromatography,[13–17] inductively coupled plasma-optical emission spectrophotometry and atomic emission/absorption spectroscopy[18,19] are traditionally performed for the quantitation of small ions with no chromophore groups in their chemical structure. However, these techniques are complex and require extensive sample preparation and high cost per analysis. The reason to use these sophisticated techniques (when compared with conventional spectrophotometry) is that bioactive glass (one of the biodegradable biomaterials present in the studied composite) such as 45S5 Bioglass® (wt%: $45SiO_2$–$25CaO$–$25Na_2O$–$6P_2O_5$) and other silicate based compositions release ionic dissolution products when it comes in contact with biological fluids as they degrade, making quantification of specific ions extremely difficult with simpler techniques (or without using previous separation methods). In this sense, the authors proposed for the first time the optimization and validation of a more sensitive CE method using EDTA as a complexing agent added to the BGE to modulate the selectivity of the separation and quantitation of copper ions released from composite biomaterials in the context of bone tissue engineering when several ionic dissolution products are being produced. Particularly, compared with conventional spectrophotometry, CE is a powerful analytical method with significant importance to assay ions because of its unique separation mechanism, speed, efficiency, and

University of Buenos Aires, Department of Pharmaceutical Technology, Faculty of Pharmacy and Biochemistry, National Research Council (CONICET), 956 Junín 6th Floor, Buenos Aires CP1113, Argentina. E-mail: slucangi@ffyb.uba.ar; Fax: +54-011-4964-8271; Tel: +54-011-4964-8371/8267

versatility.[55] Here we describe the optimization and validation of a sensitive CE method using EDTA as a complexing agent added to the BGE to modulate the selectivity of the separation and quantitation of copper ions released from composite biomaterials in general, and with application in bone tissue engineering in particular.

Materials and methods

Materials

Copper sulphate pentahydrate, sodium phosphate dibasic, sodium hydroxide and trimethyltetradecylammonium bromide (TTAB) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) was from Anedra (Argentina) and acetic acid (glacial) was from MERCK (Darmstadt, Germany). All chemicals were of analytical grade and used without further purification. Ultrapure water was obtained from EASY pure RF equipment (Barnstead, Dudubuque, IA, USA). All solutions were filtered through a 0.45 μm nylon membrane (Micron Separations, Westboro, MA, USA) before use.

Instrumentation

All separations were performed with a P/ACE™ MDQ Capillary Electrophoresis system, equipped with a diode array detector (190–600 nm) and data were processed with Karat V.8 software (Beckman, Fullerton, CA, USA). An uncoated fused-silica capillary of 40 cm length (30 cm to detector) and 75 μm i.d. (MicroSolv Technology, Eatontown, NJ, USA) was used.

Electrophoretic system and capillary conditioning

Copper ion (Cu^{2+}) quantitation was performed using a background electrolyte (BGE) consisting of 5 mM TTAB, 8 mM EDTA and 50 mM acetic/acetate buffer pH 5.5. All samples were introduced into the capillary by pressure at 0.5 psi for 10 s. The instrument was operated in reverse polarity mode with a constant voltage of 15 kV and the detection was performed at 225 nm. Cartridge temperature was maintained at 25 °C.

The capillary was rinsed initially with 0.5 mol L^{-1} potassium hydroxide for 3 min, then with 0.1 mol L^{-1} potassium hydroxide for 4 min, and washed with deionized water for 5 min and then with the BGE for 7 min. Between runs, the capillary was conditioned with 0.1 mol L^{-1} potassium hydroxide for 1 min and washed with deionized water for 2 min and then with the BGE for 3 min. In all cases, 30 psi of pressure was applied.

Quantitation

Preparation of standard solutions. A stock solution containing 1 mg mL^{-1} of Cu^{2+} was prepared in water. Solutions for the calibration curve were obtained by appropriate dilution with 10 mM sodium phosphate buffer at pH 7.4 (diluent) in a range of Cu^{2+} concentration from 0.2 μg mL^{-1} to 20 μg mL^{-1}.

Individual stock solution containing 1 mg mL^{-1} of calcium ions (Ca^{2+}) was prepared in water and diluted to obtain a standard solution of 10 μg mL^{-1} to be used in the specificity assay.

Sample preparation

Cu^{2+} cross-linked alginate films containing bioactive glass nanoparticles (Nbg) intended for BTE applications were similarly prepared as mentioned in a previous study.[3]

Phosphate buffer was prepared at different concentrations (from 5 to 20 mM) for Cu^{2+} release study. Five samples of 10 mm of diameter were tested and aliquots of them were withdrawn at regular time intervals (1, 7, 14 and 30 days) and analysed for the amount of Cu^{2+} released.

Results

CE system

Optimization of the BGE. Cu^{2+} released from matrices was quantified by an UV-direct CE method, which was optimized and validated. EDTA was added to the BGE and used as a complexing agent of Cu^{2+}. The complex formed has a maximum UV response at 225 nm, under acidic conditions (pH 5.5).[26] EDTA is the most widely used chelating agent, forming strong 1 : 1 complexes with metal ions. The possibility to form an *in situ* complex allows avoiding the previous sample derivatization and the dilution of the samples after adding the complexing agent solution. In addition, the fact of using an UV-direct detection mode enhances the sensitivity of the analytical technique, which is higher than that of UV-indirect detection methods.[26] TTAB was incorporated into the BGE to reverse the electroendosmotic flow, so that the negatively charged complex migrates to the cathode direction. Different concentrations of EDTA (5–15 mM) and TTAB (1–10 mM) were tested, and the most suitable conditions during the runs were obtained by using 8 mM and 5 mM, respectively.

The pH 5.5 selected for the BGE was the lowest one able to allow the formation of a stable complex between Cu^{2+}–EDTA. In addition, no other cation, not even Ca^{2+}, complexed with EDTA at pH 5.5, making the CE system more selective for the separation and quantification of Cu^{2+} from biomaterial matrices. For pH 5.5, the most commonly used buffer is acetate/acetic. Different concentrations of the buffer between 100 and 250 mM were tested; the optimum buffer concentration was 50 mM with suitable microamperage, baseline and resolution.

Instrumental parameters. Capillary temperature, applied voltage and hydrodynamic sample introduction were optimized to obtain the best conditions in terms of ion resolution and analysis time. A cartridge temperature of 25 °C was chosen as the optimum value. The applied voltage was found to be 15 kV, under reverse polarity, for better resolution with suitable current inside the capillary. Sample introduction time was tested in the 1 to 10 second range and different pressures were applied, 0.5 psi at 10 seconds being the best condition for obtaining adequate peak symmetry, peak area and resolution of the cation.

Sample preparation. After testing different buffer solution concentrations, 10 mM of sodium phosphate buffer (pH 7.4) was chosen for immersing samples to achieve a suitable ionic strength for ion separations and electrophoretic run conditions together with a good capacity buffer. In addition, the concentration chosen is the most frequently used to assay release profile studies for the samples mentioned.[28–30,39]

Validation

CE system validation was accomplished following the International Conference on Harmonization (ICH) guidelines[31] with respect to the parameters such as specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, and robustness.[32]

Specificity. Ca^{2+} can be present in the samples studied due to the composition of Nbg which are part of the matrix of AlgNbgCu films. Taking this into account, specificity assay was carried out running a standard solution of 10 μg mL^{-1} Ca^{2+} under the same conditions as that of a solution of 10 μg mL^{-1} Cu^{2+}. Fig. 1 shows electropherograms (i) and (ii) referring to Cu^{2+} and Ca^{2+} standard solutions, respectively, where the peak observed in (i) at 2.3 min corresponds to Cu^{2+} and it is not present in (ii) which indicates the specificity of the method for Cu^{2+} where Ca^{2+} does not form any complex with EDTA under the method conditions and has no response at the wavelength used.

Linearity, LOQ and LOD. Linearity was evaluated at five different concentration levels using standard solutions of Cu^{2+} prepared in the diluent (Fig. 2). The linearity was evaluated in a range from 0.2 μg mL^{-1} to 20.0 μg mL^{-1}. The LOD (S/R = 3) and LOQ (S/R = 10) values were 0.05 μg mL^{-1} and 0.16 μg mL^{-1}, respectively (Table 1).

Precision. Precision was evaluated for intraday ($n = 4$) and inter-day assay ($n = 16$) and it was expressed as relative standard deviation (RSD) values for migration times and peak areas (Table 1). The RSD values obtained in the study of precision were less than 2.

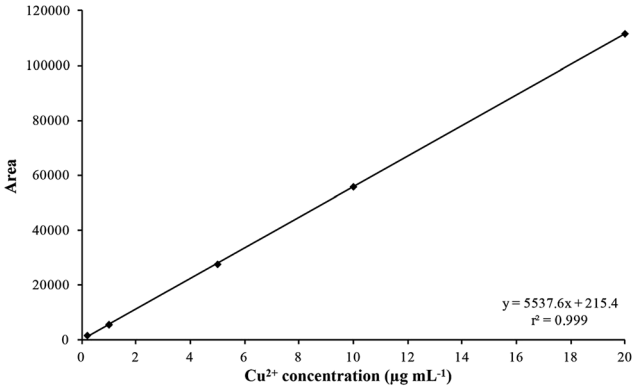

Fig. 2 Linearity range of the quantitation method of Cu^{2+}. Suitable values for r^2 were obtained.

Accuracy. Recovery studies were used to test accuracy. Three samples at different times from release study were spiked with enough concentration of the Cu^{2+} standard to obtain a 30% higher response with respect to the sample. Percentages of recovery values above 100 were obtained (Table 1). The recovery values presented were good and they were obtained with high precision.

Robustness. Robustness assay was carried out by making variations in different parameters such as time of injection, cartridge temperature, run voltage and electrolyte pH value. The ability of the methods of not being affected by such variations in electrophoretic parameters was evaluated for a standard

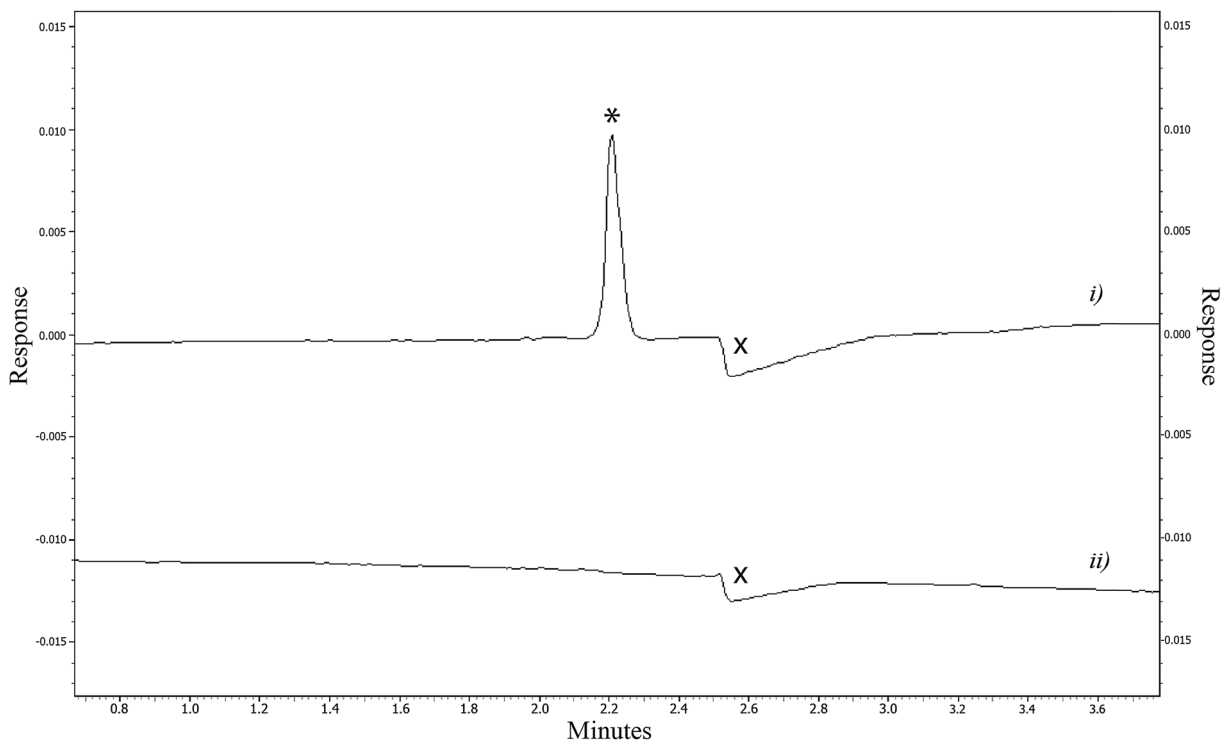

Fig. 1 Electropherogram of a standard solution of Cu^{2+} and a standard solution of Ca^{2+}, containing 10 μg mL^{-1} of each one, referred to as (i) and (ii), respectively. * Cu^{2+} peak, × neutral.

Table 1 Validation method parameters for the Cu^{2+} analytical technique by CE

Parameter		Value	
Linearity range ($\mu g\ mL^{-1}$)		0.2–20.0	$y = 5537.6x + 215.41$
r^2		0.999	
LOD ($\mu g\ mL^{-1}$)		0.05	
LOQ ($\mu g\ mL^{-1}$)		0.16	
Precision			
Intraday ($n = 4$)	RSD of migration time	1.1	
	RSD of peak area	1.9	
Interday ($n = 16$)	RSD of migration time	0.9	
	RSD of peak area	1.7	
Accuracy[a]		98.6 (1.3)	
		104.4 (0.8)	
		103.5 (1.1)	

[a] Percentage recovery mean values obtained from three samples at three different times from release study. RSD values in parenthesis.

solution containing 10 $\mu g\ mL^{-1}$ of Cu^{2+}. The variation levels were chosen as follows: time of injection ± 1 s, cartridge temperature ± 2%, run voltage ± 2%, and electrolyte pH value ± 2%. In all cases, three run replications were made and the effect of the variations was statistically analysed by Student's test[37,38] for migration time, theoretical plates and Cu^{2+} concentration. The results (Table 2) suggested that no significant changes have been found, except for time of injection and voltage, whose variations modify the concentration and time migration, respectively.

Analysis of the release of Cu^{2+} from cross-linked alginate films

Samples were analyzed to study the release rate of Cu^{2+} from AlgNbgCu films. Fig. 3a shows electropherograms of a sample from the release study, the blank used (phosphate buffer) and 5 $\mu g\ mL^{-1}$ Cu^{2+} standard solution. Fig. 3b shows electropherograms of the samples at different times from the release study.

Discussion

CE is well suited for separating and quantifying inorganic ions from complex samples as the ones obtained from release studies of composite biomaterials for BTE by using simple techniques based on UV-direct or UV-indirect detection methods.[20–24,35] When an UV-direct method is envisaged, complexing agents are used to form an UV-absorbing complex with those inorganic ions which have a low response in the UV spectra, in order to aid in the separation and quantitation of the ions.[25,26] In this sense, two possibilities could be considered: either the complexing agent selected is used to derivatize the sample prior to its injection into the system or the complexing agent is added to the background electrolyte (BGE) to form an *in situ* complex in the capillary column. The possibility to form *in situ* complexes makes these methods simpler and avoids the previous sample derivatization.[27] In the novel developed method, the necessity for analyte derivatization has been avoided through employment of a BGE containing EDTA which could facilitate rapid analyses of similar compounds and simplicity. In a previously reported study, ethylenediaminetetraacetic acid (EDTA) was used to form a complex with copper ions for their quantitation by a CE method;[26] however, the possibility to perform *in situ* complexation and the application to quantify copper in samples other than aqueous solutions have not been considered. The method presented in this article was validated to be suitable for copper determination in biomaterials and showed advantages such as rapidness, specificity and suitable sensibility, when compared with the method developed by Baraj *et al.*, achieving a limit of detection and quantitation of 0.05 and 0.16 $\mu g\ mL^{-1}$, respectively.

In terms of sensibility, the method here described has achieved a lower LOD value when compared with the method developed by Baraj *et al.*, which makes this analytical technique applicable to the quantitation of small amounts of copper ions by which they are released from composite biomaterials for BTE. In addition, the present method showed simplicity and low operative cost in comparison with other reported methods

Table 2 Results from the robustness assay for the Cu^{2+} analysis method by CE. *N*: number of theoretical plates. SD: standard deviation

Parameter	Migration time			*N*			Concentration		
Time of injection	**9 s**	**10 s**	**11 s**	**9 s**	**10 s**	**11 s**	**9 s**	**10 s**	**11 s**
Average	2.22	2.23	2.55	18 645	19 069	19 805	9.63	9.92	10.47
SD	0.02	0.09	0.04	467	320	1023	0.15	0.13	0.09
Voltage	**14.7 kV**	**15.0 kV**	**15.3 kV**	**14.7 kV**	**15.0 kV**	**15.3 kV**	**14.7 kV**	**15.0 kV**	**15.3 kV**
Average	2.53	2.23	2.17	19 424	19 069	18 624	10.19	9.92	10.20
SD	0.01	0.09	0.04	227	320	278	0.04	0.13	0.05
Temperature	**24.5 °C**	**25.0 °C**	**25.5 °C**	**24.5 °C**	**25.0 °C**	**25.5 °C**	**24.5 °C**	**25.0 °C**	**25.5 °C**
Average	2.23	2.23	2.26	19 039	19 069	18 169	10.20	9.92	10.33
SD	0.05	0.09	0.00	478	320	790	0.02	0.13	0.21
pH of BGE	**pH 5.4**	**pH 5.5**	**pH 5.6**	**pH 5.4**	**pH 5.5**	**pH 5.6**	**pH 5.4**	**pH 5.5**	**pH 5.6**
Average	2.35	2.23	2.26	19 719	19 069	17 928	9.97	9.92	10.09
SD	0.01	0.09	0.05	1841	320	1172	0.11	0.13	0.04

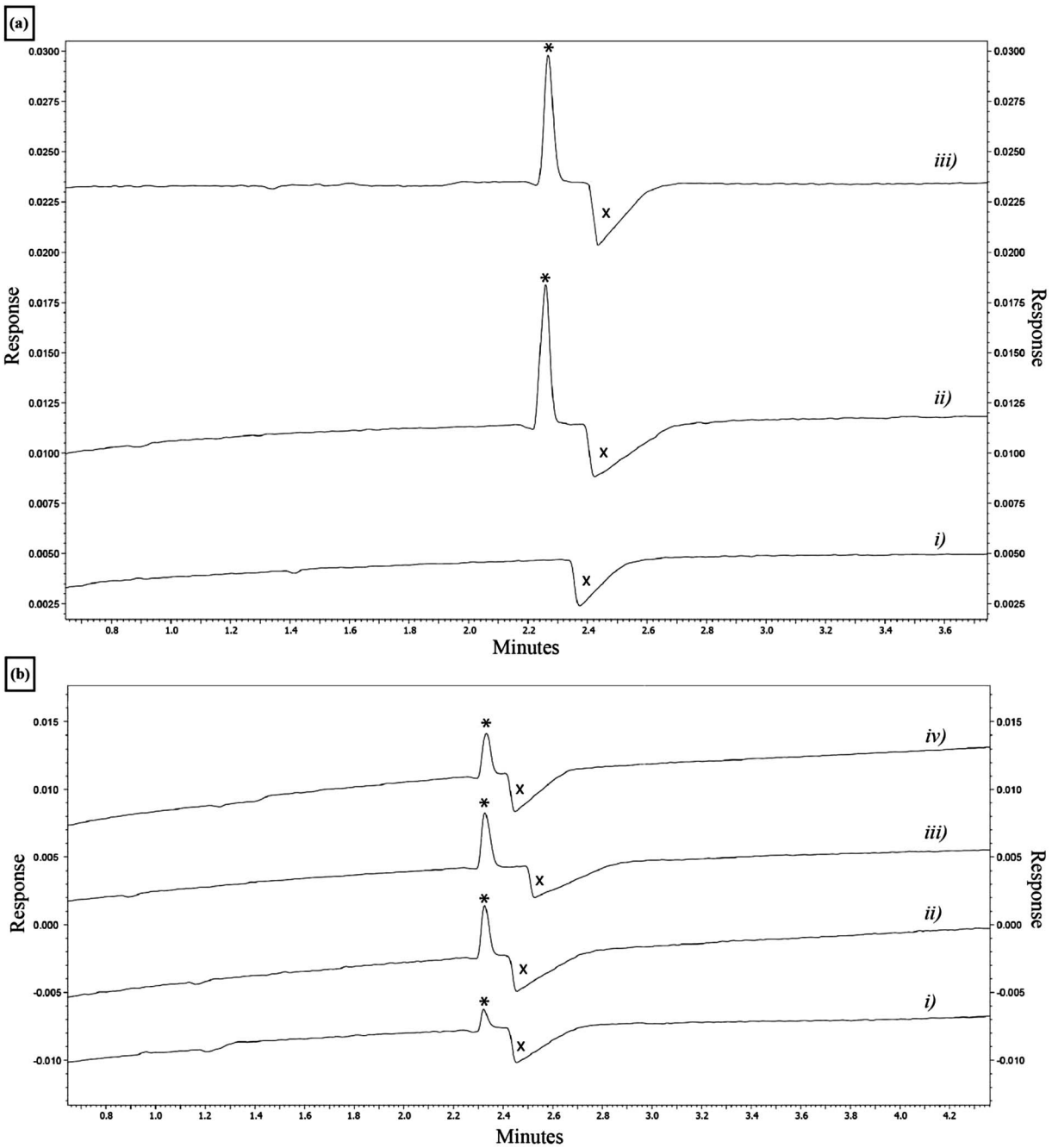

Fig. 3 (a) Electropherogram of (i) phosphate buffer, as the blank; (ii) electropherogram of Cu^{2+} standard solution of 5 μg mL^{-1}; (iii) electropherogram of a sample after 30 days from the release study of copper cross-linked alginate films. (b) Electropherograms of Cu^{2+} release from cross-linked alginate films at 1, 7, 14 and 30 days, referred to as (i), (ii), (iii) and (iv), respectively. * Cu^{2+} peak, × neutral.

to quantify the amount of copper ion release from composite biomaterials in the context of BTE such as atomic emission/absorption spectroscopy,[18] ion chromatography[33] and inductively coupled plasma-mass spectroscopy.[34]

Conclusions

The applicability of an UV-direct CE method has been demonstrated for the analysis of copper ions in samples obtained from release studies of bone-tissue-engineering composite biomaterials achieving a limit of detection and quantitation of 0.05 and 0.16 μg mL^{-1}, respectively. The combination of the use of a complexing agent such as ethylenediaminetetraacetic acid to the background electrolyte under reverse polarity and acidic conditions, and the detection at a specific wavelength modulated the selectivity of the method to allow the quantitation of free copper ions released from composite biomaterials. The obtained results suggest the suitability of this technique for the precise analysis of copper ions released from biomaterials, which could be applied in quality control and stability studies.

Thus, we described, for the first time, the optimization and validation of a sensitive CE method using EDTA as a complexing agent added to the BGE to modulate the selectivity of the separation and quantitation of copper ions released from composite biomaterials in general, and with application in bone tissue engineering in particular.

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