Polyhedron 101 (2015) 171–178

Contents lists available at ScienceDirect

Polyhedron

journal homepage: www.elsevier.com/locate/poly

Synthesis and characterization of a 3-hydroxy-4-pyridinone chelator functionalized with a polyethylene glycol (PEG) chain aimed at sequential injection determination of iron in natural waters

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ARTICLE INFO

Article history:
Received 9 August 2015
Accepted 6 September 2015
Available online 12 September 2015

Keywords:
3-Hydroxy-4-pyridinone
Iron
PEGylation
Sequential injection
Water solubility

ABSTRACT

The synthesis of a highly water soluble 3-hydroxy-4-pyridinone ligand, functionalized with a hydrophilic ethylene glycol chain (PEG-HPO), was successfully achieved and is reported together with that of its iron (III) complex. The improved hydrophilicity of both the PEGylated 3,4-HPO ligand and its iron(III) complex were fully investigated in an analytical application and, the new chelator is proposed for the spectrophotometric sequential injection determination of iron in waters. The new ligand provided better sensitivity and a lower LOD for iron determination than that obtained for N-alkyl-3-hydroxy-4-pyridinone ligands. The developed sequential injection method presents a dynamic working range of 0.10–1.00 mg Fe/L with a LOD of 48 μg/L. Due to the use of a sequential injection system, the overall effluent production was <2 mL corresponding to the consumption of 44 μg of PEG-HPO, 0.71 mg of $NaHCO_3$, 0.92 mg of HNO_3 and 500 μL of sample. Two reference samples were assessed for accuracy studies and a relative deviation <5% was obtained; eight waters samples were analyzed and the results compared with the reference procedure, and no statistical difference was observed for the two sets of results.

1. Introduction

Diverse chromogenic reagents, such as thiocyanate, 1,10-phenantroline, bathophenanthroline, 2,2-bipyridyl, eriochrome cyanine R, cetyltrimethylammonium, are well known for their analytical usage in the determination of iron [1] but also for their high toxicity.

In a recent work, we tested low toxicity 3-hydroxy-4-pyridinone (3,4-HPO) chelators as chromogenic reagents and successfully applied them for determination of iron in bathing waters [2]. However, its efficiency was limited by the ligand water solubility as the sensitivity for the determination of iron increased with the increase of ligand concentration in the chromogenic solution [2]. In this scenario, it would be rather advantageous to obtain a more water soluble 3,4-HPO ligand. This option is feasible as the structure of 3,4-HPO ligands allows tailoring of their hydrophilic/lipophilic balance (HLB) without significantly changing its chelating properties. The variation in HLB can be achieved by introducing appropriate substituents on the endocyclic nitrogen atom of the pyridinone ring. Therefore, a more hydrophilic ligand is expected to provide a more sensitive method with lower detection limit than a previously used 3,4-HPO ligand [2].

Herein, we reported the synthesis of a 3,4-HPO ligand with improved water solubility that was achieved by introducing an ethylene glycol chain in the endocyclic nitrogen atom of the pyridinone framework. The aim was to increase the sensitivity and decrease the detection limit for the spectrophotometric determination of iron by sequential injection analysis, which was limited by the low concentration of the ligand although a saturated solution was used [2].

Our group has long been interested in the synthesis and solution properties of 3,4-HPO ligands and their complexes with M (II) and M(III) metal ions for several applications [2–5]. To improve the water solubility of the ligands we considered the use of oligo (ethylene glycol)s (OEGs) substituents that have been described to provide high water solubility [6]. Herein we report the synthesis

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http://dx.doi.org/10.1016/j.poly.2015.09.015
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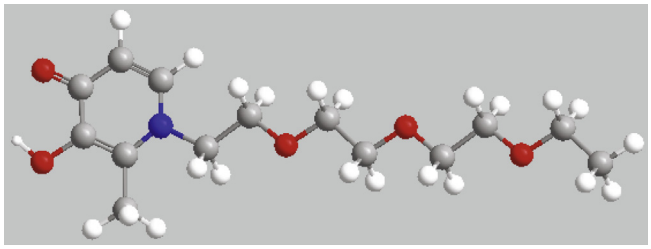


Fig. 1. Formula of 3-hydroxy-4-pyridinone functionalized with a hydrophilic ethylene glycol chain (PEG-HPO).

of an amine-terminated OEGs with a methyl group in the end of the chain and of the corresponding 3-hydroxy-4-pyridinone ligand, PEG-HPO (Fig. 1) obtained through reaction of the amine with 2-methyl-3-hydroxy-4-pyrone (maltol).

The conjugation of these amino-PEGylated chains with 3,4-HPO chelating units improves the hydrophilicity of both the ligand and their metal ion complexes thus allowing their wider use in biological and analytical applications. The iron(III) complex of the new ligand was also synthesized and characterized in order to validate the detection method.

The efficiency of this new compound in solution as a novel chromogenic reagent for determination of iron was tested in a sequential injection system. The complexation reaction of the synthesized ligand and iron(III) was studied in-line and applied to two certified waters samples. The results obtained in terms of sensitivity, selectivity and limit of detection, were critically compared with those previously described using the less hydrophilic 3,4-HPO ligand [2]. The choice of sequential injection (SI) analysis, as flow analysis technique, was based not only on its extensive use as an efficient tool for water analysis [7] but also to attain an appropriate comparison with the previously developed method. In the end, a SI method for the determination of iron is proposed, based on a newly synthesized hydrophilic 3,4-HPO ligand as an alternative reagent for iron determination.

2. Materials and methods

2.1. Synthesis of the 3,4-HPO ligand

2.1.1. Materials and physical measurements for the ligands synthesis

Chemicals were obtained from Sigma–Aldrich (grade puriss, p.a.) or Fluka (p.a.) and were used as received unless otherwise specified.

NMR spectra were recorded on a Bruker Avance III 400 spectrometer, operating at 400.15 MHz for ^{1}H and 100.62 MHz for ^{13}C atoms, equipped with pulse gradient units, capable of producing magnetic field pulsed gradients in the *z*-direction of 50.0 G/cm. Two-dimensional $^{1}H/^{1}H$ correlation spectra (COSY), gradient selected $^{1}H/^{13}C$ heteronuclear single quantum coherence (HSQC) and $^{1}H/^{13}C$ heteronuclear multiple bond coherence (HMBC) spectra were acquired using the standard Bruker software.

High resolution electrospray ionization mass spectra (ESI-MS) were obtained in a Thermo Scientific LTQ-Orbitrap XL mass spectrometer, externally calibrated with a standard kit provided by the manufacturer. The spectrometer was operated in the positive ionization mode setting the capillary voltage to +3.0 kV, sheath gas flow to 6 and the temperature of the ion transfer capillary to 275 °C. Spectra were recorded for *m*/*z* values between 250 and 2000 in the Fourier Transform (FT) mode with resolution (FWHM) set at 60 000. Samples were prepared in water, diluted in a 50% (v/v) water:methanol mixture immediately before analysis and directly infused into the electrospray ion source utilizing the syringe pump in the mass spectrometer at 10 μl min^{-1}.

NMR and Mass spectrometry analyses were performed at Laboratório de Análise Estrutural, Centro de Materiais da Universidade do Porto (CEMUP) (Portugal). Elemental analyses were performed at the analytical services of University of Santiago (Spain).

Microwave-assisted reactions were carried out in a CEM Discovery Labmate circular single-mode cavity instrument (300 W max magnetron power output) from CEM Corporation.

2.1.2. Synthetic procedures

*2.1.2.1. Synthesis of the azide-PEGylated chain: 1-azide-2-(2-(2-ethoxyethoxy)ethoxy)ethane (**b**).* Tri(ethylene glycol)monomethyl ether (**a**) (9.80 mL, 56.1 mmol) was dissolved in anhydrous DCM (40 mL) and the solution was placed in a schlenk tube, which was then closed under argon atmosphere. To the solution was added anhydrous Et_3N (11.7 mL, 84.2 mmol), under magnetic stirring and cooled to 0 °C. To this mixture, a solution of mesyl chloride (5.21 mL, 67.3 mmol), previously prepared by dissolution in 40 mL of anhydrous DCM, was added dropwise, during 90 min. The reaction mixture was kept with stirring, 1 h at 0 °C and the follow 15 h at room temperature. The reaction mixture was then washed with aqueous solutions of HCl (3%) (50 mL) and saturated solution of NaCl (50 mL). The product was purified by liquid/liquid extraction with DCM (3 × 50 mL). The organic phase was dried with Na_2SO_4 and concentrated by evaporation. The next step includes the reaction of the last product obtained in anhydrous DMF (40 mL), in a schlenk tube, with NaN_3 (9.12 g, 140 mmol), at 65 °C during 15 h. The reaction mixture was then washed with water (10 × 30 mL) and with saturated solution of NaCl (40 mL). The product was then extracted from the aqueous phase with AcOEt (3 × 50 mL) and the organic phase was dried with Na_2SO_4 and concentrated by evaporation to afford 10.2 g of product (**b**) as a light brown oil (90% yield) [6] (Fig. 5A).

RMN ^{1}H (400.15 MHz, $CDCl_3$, ppm): δ 3.68–3.62 (m, 8H, $-(OCH_2CH_2)_2O-$), 3.59–3.55 (m, 2H, $-OCH_2CH_2N_3$), 3.51 (q, J = 7.0 Hz, 2H, $-OCH_2CH_3$), 3.37 (t, J = 5.1 Hz, 2H, $-OCH_2CH_2N_3$), 1.19 (t, J = 7.0 Hz, 3H, $-CH_2CH_3$).

*2.1.2.2. Synthesis of the amine-PEGylated chain: 2-(2-(2-ethoxyethoxy)ethoxy)ethylamine (**1**).* A solution of product (**b**) (2.02 g, 9.94 mmol) in methanol (40 mL) was placed into a hydrogenation vessel. The air was removed with N_2, a catalytic amount of 1% Pd/C (w/w) was added and the mixture was stirred at room temperature, with H_2 at 50 PSi for 24 h. The reaction mixture was filtered, washed with methanol and the solvent evaporated in vacuum to give the light brown oil product. The resulting residue was dried under vacuum to give 1.68 g of **1** (97% yield) (Fig. 5A).

RMN ^{1}H (400.15 MHz, $CDCl_3$, ppm): δ 3.65–3.43 (m, 12H, $-CH_2(OCH_2CH_2)_2OCH_2-$), 2.81 (t, J = 5.2 Hz, 2H, $-CH_2NH_2$), 1.50 (s, 2H, $-CH_2NH_2$), 1.16 (t, J = 7.0 Hz, 3H, $-CH_3$).

2.1.2.3. Synthesis of the PEGylated 3,4-HPO. The PEGylated 3,4-HPO was obtained using the typical procedure utilized to prepare 3,4-HPOs in which a 3-hydroxy-4-pyrone reacts with a primary amine [8,9] (Fig. 5B).

*2.1.2.3.1. Synthesis of the protected PEGylated 3,4-HPO (**3**).* A mixture of amine **1** (2.1459 g, 0.01832 mol) dissolved in dried ethanol (6 mL) was placed in a 10 mL reaction vial, which was then closed under argon atmosphere and placed in the cavity of a CEM microwave reactor. The reaction vial was irradiated (1 min ramp to 160 °C and 120 min hold at 160 °C, using 100 W maximum power). The reaction solvent was evaporated and the crude oil resultant was dissolved in 50 mL of water. The product was separated from the starting material **2** by liquid/liquid extraction with 3 × 30 mL of diethyl ether. The organic phase was rejected and the aqueous phase was concentrated and then purified by chromatography,

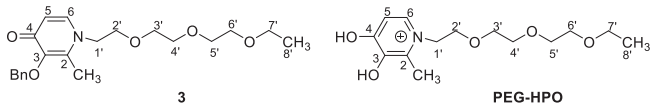


Fig. 2. Formulae and numbering of compound **3** and chelator PEG-HPO.

using a mixture of chloroform/methanol (9:1), affording 1.7520 g of conjugate **3** (49% yield) (Figs. 2 and 5B).

^{1}H NMR (400.15 MHz, MeOD-d_4, ppm): δ 1.55 (t, J = 7.1 Hz, 3H, –CH_2CH_3); 2.22 (s, 3H, 2-CH_3); 3.46–3.61 (m, 10H, H3′–H7′); 3.68 (t, J = 4.6 Hz, 2H, 2′-CH_2); 4.13 (t, J = 4.6 Hz, 2H, 1′-CH_2); 5.07 (s, 2H, –$CH_2C_6H_5$); 6.48 (d, J = 7.4 Hz, 1H, H5); 7.27–7.42 (m, 5H, –$CH_2C_6H_5$); 7.69 (d, J = 7.4 Hz, 1H, H6). ^{13}C NMR (100.62 MHz, MeOD-d_4, ppm): δ 12.9 (2-CH_3); 15.2 (–CH_2CH_3); 54.4 (C1′); 70.5 (C2′); 71.0–71.3 (C3′–C7′); 74.1 (–$CH_2C_6H_5$); 116.4 (C5); 128.9–129.1 (–C_6H_5); 129.8 (Cq, –C_6H_5); 138.1 (C6); 141.9 (C2); 145.1 (C3); 174.1 (C4).

2.1.2.3.2. Synthesis of the PEGylated 3,4-HPO (PEG-HPO). A solution of ligand **3** (2.0117 g, 0.00536 mol) in methanol (40 mL) and HCl (0.01 mL) was placed into a hydrogenation vessel. The air was removed with N2, a catalytic amount of 10% Pd/C (w/w) was added and the mixture was stirred at room temperature, with H_2 at 40 PSi for 6 h. The reaction mixture was filtered, washed with methanol and CH_2Cl_2 and the solvent evaporated in vacuum to give the brown oil product. The resulting residue was dried under vacuum to give 1.6378 g of 4 (95% yield) (Figs. 2 and 5B).

MS: calculated for $C_{14}H_{24}NO_5^+$: 286.16 (monoisotopic molecular weight M^+), found: MS: 286.16261. ^{1}H NMR (400.15 MHz, MeOD-d_4, ppm): δ 1.04 (t, J = 7.1 Hz, 3H, –CH_2CH_3); 2.54 (s, 3H, 2-CH_3); 3.37–3.56 (m, 10H, H3′–H7′); 3.79 (t, J = 4.8 Hz, 2H, 2′-CH_2); 4.48 (t, J = 4.8 Hz, 2H, 1′-CH_2); 7.03 (d, J = 7.4 Hz, 1H, H5); 8.04 (d, J = 7.4 Hz, 1H, H6). ^{13}C NMR (100.62 MHz, MeOD-d_4, ppm): δ 13.2 (2-CH_3); 15.4 (–CH_2CH_3); 57.0 (C1′); 67.5 (C7′); 70.1 (C2′); 70.5–71.5 (C3′–C6′); 111.4 (C5); 140.0 (C6); 143.2 (C2); 144.6 (C3); 160.3 (C4).

2.1.2.4. Iron(III) complex of PEGylated 3,4-HPO ($Fe(PEG\text{-}HPO)_3$). FeL_3 complex was prepared by dissolving stoichiometric amounts of Fe $(NO_3)_3{\cdot}9H_2O$ (0.283 g, 0.7 mmol) and ligand PEG-HPO (0.60 g, 0.0021 mol) in water, adjusting the pH to 8 with a diluted solution of NaOH. The reaction mixture was kept with stirring, for 3 days at room temperature. A precipitate was formed, collected by filtration and washed with water and a red power was isolated (quantitative yield).

MS: $[H_2L]^+$ ($C_{14}H_{24}NO_5^+$), m/z = 286.16499 (Δm = 0.3 ppm); $[NaHL]^+$ ($NaC_{14}H_{23}NO_5^+$), m/z = 308.14664 (Δm = −0.7 ppm); $[Fe(L)_2]^+$ ($FeC_{28}H_{44}N_2O_{10}^+$), m/z = 624.23363 (Δm = −0.6 ppm); $[H(Fe(L)_3)]^+$ ($FeC_{42}H_{67}N_3O_{15}^+$), m/z = 909.39250 (Δm = 1 ppm); $[Na(Fe(L)_3)]^+$ ($NaFeC_{42}H_{66}N_3O_{15}^+$), m/z = 931.37393 (Δm = 0.4 ppm).

2.2. Analytical procedure

2.2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and boiled deionised water (specific conductance less than 0.1 μS cm^{-1}).

Ligand solutions were obtained by dissolution of approximately 9 mg of the synthesized ligand in 50 mL of water corresponding to a concentration of 180 mg/L.

The buffer solutions of hydrogen carbonate 0.25 M were prepared by dissolving 1.05 g of sodium hydrogen carbonate in 50 mL of water. The pH was set to 10.5 with a 0.5 M sodium hydroxide solution. An iron(III) stock solution, 10 mg/L, was prepared by dilution from the atomic absorption standard (Spectrosol, England) of 1000 mg/L. Working standards in the dynamic range 0.1–2.0 mg/L were weekly prepared from dilution of the stock solution in 0.03 M of nitric acid.

2.2.2. Sequential injection manifold and procedure

The sequential injection manifold used for the study of the complexation reaction and for the determination of iron(III) is depicted in Fig. 3.

Solutions were propelled by a Gilson Minipuls 3 peristaltic pump, equipped with PVC pumping tube connected to the central channel of a ten port selection valve (Valco VICI Cheminert C25-3180EUHB). All tubing connecting the different components was made of PTFE (Omnifit), with 0.8 mm i.d. An Ocean Optics USB 4000 charged coupled device detector (CCD) equipped with a pair of 400 μm fiber optic cable and a Mikropack DH-2000 deuterium halogen light source and a Hellma 178.710-OS flow-cell with 10 mm light path and 80 μL inner volume, was used as detection system. Data acquisition was performed through the Ocean Optics – Spectrasuite software at 459 nm.

A personal computer (HP Pavilion zt3000) equipped with a National Instruments DAQcard – DI0 interface card, running a homemade software, was used to control the selection valve position and the peristaltic pump direction and speed.

The protocol sequence used for the iron determination in natural waters using the newly synthesized ligand is described in Table 1.

The first step was the aspiration of 3,4-HPO ligand solution (step A) to the holding coil, followed by the aspiration of the buffer and the standard (steps B and C). Mixing was promoted by the flow reversal while propelling the aspirated plugs towards the detector (step D).

2.3. Accuracy assessment – sample collection

For accuracy assessment, results obtained with the proposed sequential injection method were compared to the certified values of two certified water samples: SPS-SW2 (surface water) from LGC standards and SLRS-4 (river water) from NRC-CNR National Research Council Canada. Furthermore, eight river water samples from recreational areas (ESI Table S1) were collected in polyethylene plastic bottles of 0.5 L capacity at about 30 cm depth. The samples, acidified at collection to pH ≈ 2 (with HCl) according to the collection procedure [10], were introduced directly in the developed system without filtration.

3. Results and discussion

3.1. Synthesis of the new iron chelator

Ligands of the 3-hydroxy-4-pyridinone type are usually synthesized through the reaction of a 3-hydroxy-4-pyrone and a primary amine as depicted in Fig. 4 [11].

The use of the above reaction (Fig. 4) allows the introduction of different R^1 substituents on the heterocyclic nitrogen atom by selection of the appropriate amine. The use of amines with different R^1 substituents allows the synthesis of 3-hydroxy-4-pyridinones with variable physicochemical properties, such as water solubility. Moreover, and as shown in previous studies [4,9,11,12], such alterations on the substituents of the nitrogen atom of the heterocyclic ring of 3,4-HPOs do not significantly change the chelating properties of the 3,4-HPO ligands. Aiming to increase the water solubility of the 3,4-HPOs used in the previous study and considering the water solubility of oligo(ethylene glycol) chains we synthesized an amino-PEGylated chain with a methyl

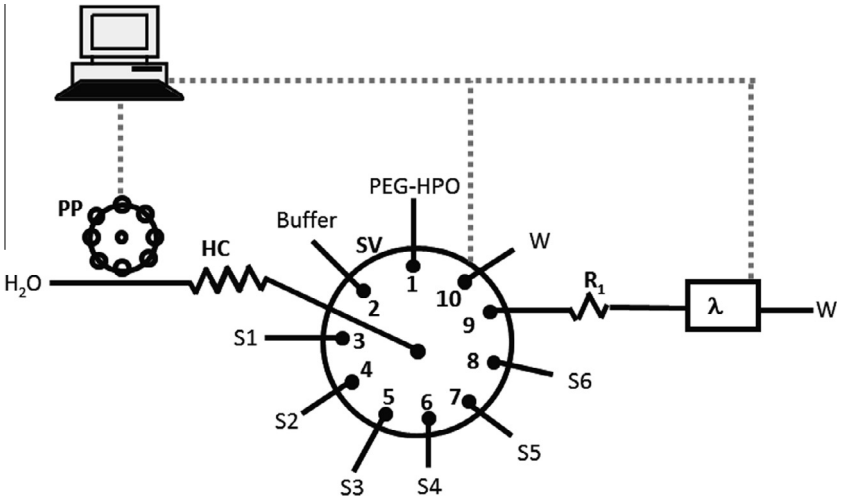


Fig. 3. Sequential injection manifold to study the chromogenic reaction between PEGylated 3,4-HPO ligand and iron; SV, 10 port selection valve; HC, holding coil with 300 cm of length; PP, peristaltic pump; PEG-HPO, 180 mg/L ligand solution; Buffer, 0.25 M hydrogen carbonate solution; S_i, iron(III) standards; R_1, reaction coil with 6 cm; λ, spectrophotometer at 459 nm; W, waste.

Table 1
Protocol sequence for the developed SI methodology for iron determination using a PEG-HPO ligand as a colorimetric reagent.

Step	Port	Time (s)	Flow rate (μL/s)	Volume (μL)	Description
A	1	4	62	248	Aspiration of ligand
B	2	2	17	34	Aspiration of buffer
C	3–8	8	62	496	Aspiration of standard
D	9	30	62	1860	Propelling to detector

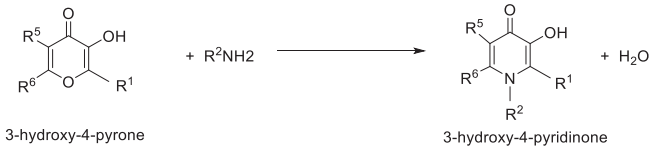


Fig. 4. Scheme of the reaction of the synthesis of 3-hydroxy-4-pyridinones.

group in the end of the chain and the corresponding 3-hydroxy-4-pyridinone ligand as shown in Fig. 5.

3.1.1. Synthesis of amino-PEGylated chain

In order to produce de amino-PEGylated chain, the hydroxyl group of tri(ethylene glycol)monoethyl ether (**a**) was substituted by a mesyl group to react with NaN_3 and to form the azide group (**b**). The azide was subsequently reduced to produce the amine (**1**) by hydrogenation. The products of both reactions were obtained with good yield, 90% and 97%, respectively.

*3.1.2. Optimization of the experimental procedures the synthesis of the protected PEGylated 3,4-HPO (**3**)*

The synthesized amine functionalized with an OGE chain (**1**) reacts with the benzyl protected 3-hydroxy-4-pyrone (**2**) leading to the displacement of the oxygen atom of the ring and its further replacement by the nitrogen of the anime group of the chain producing a benzyl protected 3-hydroxy-4-pyrone (Fig. 5B). Compound **2** was synthesized in our laboratory following the procedures described in the literature [8].

The synthesis of **3** was performed by adding **1** and **2** in ethanol under reflux (oil-bath) for 72 h yielding **3** in 27% yield (a, Table 2). In order to improve the reaction outcome to synthesize **3**, some modifications were performed on the synthetic procedure. Microwave-assisted organic chemistry was used instead of the traditional oil bath in order to obtain the desired compound **3** in a good yield, decreasing the reaction time and consequently more efficient synthetic protocol. Therefore, in a monomode reactor, under closed-vessel conditions the reaction was performed using an excess of 1.5 eq of the amine **1** and 100 mg of **2**, during 2 h. Then, the reacting mixture was analyzed by NMR and the conversion of **2** into **3** was calculated and 38% of the limiting reagent **2** was converted in **3** (b, Table 2). The same conditions were used, except for acidification of the medium with HCl and the percentage of conversion was determined and 24% occurred (c, Table 2). This result shows that the reaction occurs with higher percentage of

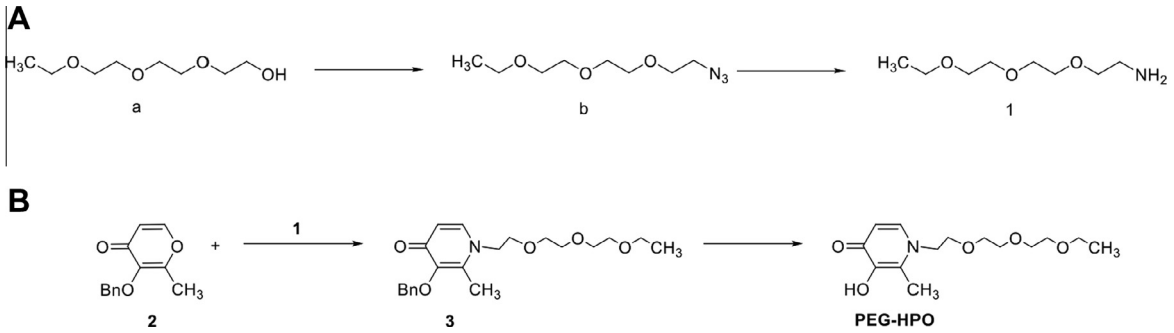


Fig. 5. Synthetic routes of: A, the PEGylated chain; and B, the functionalized 3,4-HPO.

Table 2
Experimental conditions of the synthesis of modified 3,4-HPO (**3**).

Entry	Method	Fold excess of (1)	Amount of (2)	Time (h)	% of conversion in (3) (calculated by NMR)
a	Oil bath	1.5	500 mg	72	27*
b	MW	1.5	100 mg	2	38
c	MW	1.5	100 mg (+HCl)	2	24
d	MW	1.5	500 mg	2	59
e	MW	2	500 mg	2	84

* Yield obtained by product isolation after purification.

conversion of **2** into **3** in non acidified medium and we tested the same excess of 1.5 eq of the amine **1** during 2 h but scaling up the amount of reagents to 500 mg of **2** (d, Table 2) and the conversion obtained was 59%. In the next condition tested (e, Table 2), we increased the excess of the amine **1** to **2** eq and we maintained 500 mg of **2**, and 2 h of reaction leading us to a percentage of conversion of 84%. This modification allowed formation of a higher amount of the ligand **3** and concomitantly we obtained our desired product with lower reaction time. All the experimental conditions performed for the synthesis of **3** are summarized in Table 2.

In order to obtain the final 3-hydroxy-4-pyridinone the benzyl-protecting group of **3** was removed under a hydrogen atmosphere in the presence of Pd/C (10%) and HCl, to obtain the dihydrochloride salt of ligand PEG-HPO.

The modification of 3,4-HPO ligands with amino-PEGylated chains was successfully achieved in this work leading to the synthesis of a new 3,4-HPO ligand with an enhanced solubility in water. The new type of 3,4-HPO ligands will significantly enhance the water solubility of the ligands and its metal ion complexes and for that reason will allow to enlarge the field of application of this class of ligands.

3.1.3. NMR spectroscopy

The structures of the ligands **3**, and PEG-HPO in solution were established by NMR analysis (^{1}H and ^{13}C, 1D and 2D experiments, including COSY, HSQC and HMBC spectra for unequivocal assignment of the most characteristic proton and carbon chemical shifts). The assignment of the resonance signals in ^{13}C NMR spectra of the protected and de-protected compounds was achieved by analysis of ^{1}H/^{13}C HSQC and ^{1}H/^{13}C HMBC spectra, which provide one and multiple bond ^{1}H–^{13}C connectivity.

The ^{1}H NMR spectra of ligand **3** revealed that the resonance signals of the methyl protons from the terminal group (8′-C*H*$_3$) appear at 1.15 ppm and the correspondent carbon (C8′) with HSQC correlation appear at 15.17 ppm. The singlet at 2.22 ppm was attributed to the methyl substituent in the pyridinone ring (2-C*H*$_3$) and HSQC correlation with resonance signal at 12.85 ppm allow us to attribute this pick to 2-C*H*$_3$ carbon. The multiplet in the aliphatic region of the spectra (3.46–3.61 ppm) was assigned to protons of –CH$_2$ groups (3′-C*H*$_2$–7′-C*H*$_2$) of the ethoxyl tail and their respective carbons appear between 71.04 and 71.33 ppm. Signals at 3.68 and 4.13 ppm was assigned as the protons of the –CH$_2$ groups linked to the nitrogen atom of the pyridinone ring (2′-C*H*$_2$ and 1′-C*H*$_2$) and their respective carbons appear at 70.5 and 54.4 ppm. For carbons C1′, HMBC correlations with carbon 2-CH$_3$ was found, confirming the assignment performed.

The resonance singlets at 6.48 and 7.69 ppm were assigned to the aromatic CH protons (H5 and H6, respectively) of the pyridinone residue and show HSQC and HMBC correlation with carbons at 116.4 and 138.1 ppm assigned as C5 and C6, respectively. The signals related with the protecting group are the singlet at 5.07 ppm that corresponds to the protons of the ethyl group and the protons of the benzyl ring appear as a multiplet between 7.27 and 7.42 ppm. The carbon associated to this ethyl group is at 74.1 ppm and the 5 carbons of the benzyl ring appear between 128.9 and 129.0 ppm. The resonance signal at 129.8 ppm was found to give long range coupling to the resonance signals of the protons of the ethyl group and the CH protons of the benzyl ring was attributed to the quaternary carbon of the protecting group.

The last quaternary carbons at 141.9, 145.1 and 174.1 ppm were attributed C2, C3 and C4 due to their correlations in HMBC with the protons of the pyridinone ring and with C1′ for C2 carbon.

After the deprotection (PEG-HPO), significant differences in the ^{1}H and ^{13}C spectra were detected as for example the shift of the protons of methyl substituent in the position 2 of the pyridinone ring (2-C*H*$_3$) that moved from 2.22 to 2.54 ppm and the respective carbon signal, 2-C*H*$_3$, was shifted from 12.85 to 13.21 ppm. The protons of –CH$_2$ groups near to the nitrogen of the pyridinone are also shifted, namely 2′-C*H*$_2$ and 1′-C*H*$_2$, that moved to 3.79 and 4.48 ppm as well as their respective carbons from 70.5 to 70.1 and from 54.4 to 57.0, respectively.

The resonance signals attributed to the –CH protons H5 and H6 revealed the major shift from 6.48 to 7.03 ppm and from 7.69 to 8.04 ppm, respectively. Their respective carbons are also dislocated to low field region, namely from 116.4 to 138.0 ppm in the protected ligand to 111.4 and 140.0 ppm in the deprotected form, respectively for C5 and C6 carbons.

The quaternary carbons were assigned based on their long-range correlations. The resonance signal at 143.2 has shown HMBC correlation with H6, 1′-C*H*$_2$ and 2-C*H*$_3$ protons and was attributed to C2. The carbon at 144.6 shows HMBC correlation with H5, H6 and protons and 2-C*H*$_3$ and was attributed to C3. The pick at 160.3 ppm was attributed as carbons C4 due to their HMBC correlations with H5 and H6.

The observed difference in the chemical shifts of ^{1}H and ^{13}C nucleus in the spectra of the protected and deprotected compounds is primarily due to the deprotection of the hydroxyl group and the acidic pH of the deprotection reaction that lead to isolate the final ligands in the enolic form [13].

3.1.4. Synthesis and characterization of the iron(III) complex of Ligand PEG-HPO

The complex Fe(L)$_3$ was synthesized by mixing the iron salt, Fe(NO$_3$)$_3$·9H$_2$O, and the ligand in water at pH = 8. Upon precipitation, the complex was isolated and its composition was determined by ESI-MS. The mass spectrum of the isolated complex shows the free ligand (m/z = 286.16499 [H$_2$L]$^+$ and m/z = 308.14664 [NaHL]$^+$) and two charged iron complexes, [Fe(L)$_2$]$^+$ (m/z = 624.23363) and Fe(L)$_3$ (m/z = 909.39250 [H(Fe(L)$_3$)]$^+$ and m/z = 931.37393 [Na(Fe(L)$_3$)]$^+$), as observed for the alkylpyridinone iron(III) complexes [11]. The high accuracy mass determination together with the observed isotopic patterns allowed to unequivocally assigning the chemical composition of the ions. As revealed by solution speciation studies, the presence of both iron complexes is to be expected at acidic pH values (pH $\leqslant$ 6), which can be provided by the electrospray plume acidification occurring in positive ionization mode [14,15]. In addition, it should be noted that the Fe(L)$_3$ complex is a neutral species and its ionization requires protonation or sodium adduct formation. However, these reactions are likely to promote ligand dissociation, leading to the detection of the more stable [Fe(L)$_2$]$^+$ cation and the free ligand in its fully protonated ([H$_2$L]$^+$) and sodiated ([NaHL]$^+$) forms. Altogether, MS data provides further evidence for the ability of L to bind ferric ions, forming FeL$_2$ and FeL$_3$ complexes, as expected for a 3,4-HPO bidentate ligand. The UV–Vis spectrum of an aqueous solution of FeL$_3$ at pH $\geqslant$ 7 exhibits a strong charge transfer band (λ_{max} = 460 nm) characteristic of this type of complexes [11].

3.2. Analytical procedure – sequential injection method

The advantageous characteristics of sequential injection (SI) analysis concept namely versatility, extent of automation and low reagent consumption, made it an appropriate choice for automation of the analytical application of 3,4-HPO ligands for iron determination. In this context, it was important to confirm that the complexation reaction was as fast as evidenced in the previous work involving other 3,4-HPO ligands, as this would strongly influence the SI protocol to be designed. To do so, the absorbance of a mixture, placed in a conventional cell, of 1 mL ligand solution 178.5 mg/L, 0.1 mL carbonate buffer 0.25 M and 0.5 mL of 10 mg/L iron(III) standard was measured for 1 min. According to the work of Mesquita et al. [2], the absorbance was monitored at 460 nm, corresponding to the maximum absorbance of the FeL_3 complex. The color was observed almost immediately after mixing, indicating the complex formation. After the observed initial reaction, there was no significant absorbance increase, ⩽9%, during the measured time (1 min). As described before [2], the carbonate buffer was needed to adjust the pH of the solution, obtained by mixing sample/standard, ligand solution and buffer, to about seven. Considering that the standard solutions were acidified to pH ≈ 2, the buffer solution was used at a pH ≈ 9.5. So, the SI protocol involved the sequential aspiration of the following plugs: ligand solution, buffer solution and sample/standard.

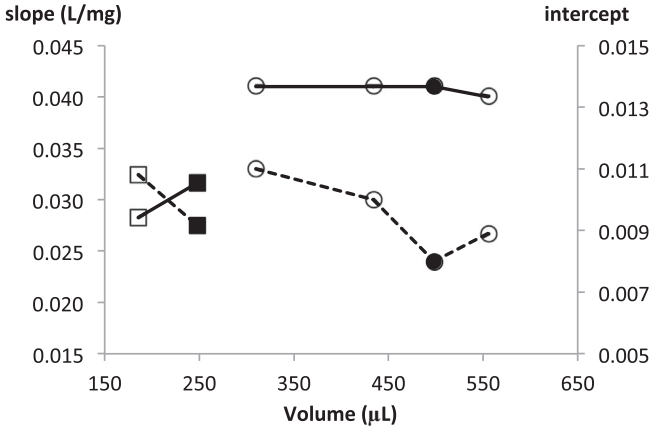


Fig. 6. Study of the influence of the volume of ligand solution (□) and sample (○) on the slope (full lines) and intercept (dashed lines) of the calibration curves for iron determination; the points in full black correspond to the chosen volumes of ligand and sample.

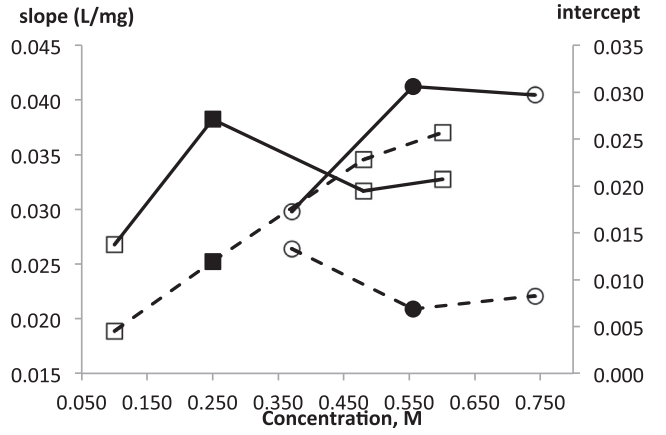


Fig. 7. Study of the influence of the ligand concentration (○) and the buffer concentration (□) in the calibration curves of the iron determination; full lines for the slopes (sensitivity) and dashed lines for the intercept; the points in black represent the slopes and intercept of the chosen concentrations of ligand and buffer.

3.2.1. Physical parameters

The buffer volume was set at 30 µL as it represents the minimal reproducible value to be used in SI methods [16]. The ligand solution volume, of about 250 µL, was also based upon previous studies [2]. However, considering the possibility of a significant increase in the ligand concentration, over 10 times higher than the one used in our previous work, as result of the improved solubility of the newly synthesized ligand, the hypothesis of reducing the ligand volume was evaluated. The previously used volume ensured a ligand excess of 2.5 times above the needed stoichiometry 3:1 (ligand:

Table 4
Assessment of possible interfering ions in the determination of iron(III) according to legislated values, UNFAO, United Nations Food and agriculture Organization [10].

Possible interfering ion	Legislation maximum values UNFAO (mg/L)	Tested concentration (mg/L)	% interference
Al^{3+}	5[a]	5.00	−4.7
Ca^{2+}	15[b]	15.0	1.5
Co^{2+}	0.1[a]	0.10	−2.8
Mg^{2+}	5[b]	20.0	−9.4
Ni^{2+}	Expected – 0.001[b]	0.01	0.9
Zn^{2+}	2[a]	5.00	−3

[a] Irrigation waters.
[b] Stream waters.

Table 5
Results obtained for the determination of iron using the developed SI method and those obtained with the reference procedure, graphite furnace atomic absorption spectrometry (GFAAS); RD, relative deviation.

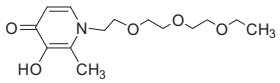
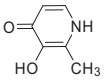
Sample ID	GFAAS		SI		RD (%)
	mg Fe/L	SD	mg Fe/L	SD	
1	0.057	0.001	0.055	0.004	−3.5
2	0.101	0.007	0.098	0.000	−3.0
3	0.031	0.001	<LOD		–
4	0.087	0.009	0.091	0.009	4.6
5	0.049	0.002	<LOD		–
6	0.040	0.001	<LOD		–
7	0.086	0.006	0.091	0.009	5.8
8	0.077	0.001	0.074	0.008	−3.9

Table 3
Features of the developed methodologies for iron(III) determination in water samples using PEG-HPO ligand as a colorimetric reagent.

Dynamic range (mg/L)	Typical calibration curve[a] $A = m\cdot[Fe^{3+}] + b$	LOD (µg/L)	Quantification rate (h^{-1})	Reagent/sample consumption	Effluent production (µL)
0.10–1.00	$y = 0.0392 \pm 0.0017\ [Fe^{3+}] + 0.008 \pm 0.002$ $R^2 = 0.996 \pm 0.005$	48	72	44.3 µg 3,4-HPO 0.71 mg $NaHCO_3$ 0.92 mg HNO_3 500 µL	1860

[a] $n = 5$.

Table 6
Comparison of the developed SI method, using the newly synthesized ligand, PEG-HPO, with the previously described [2] less soluble form of the 3,4-HPO.

Ligand formula	Ligand concentration (mg/L)	Sensitivity (calibration curve slope, L/mg)	LOD (μg Fe/L)	Sample consumption/ determination (μL)	Reference
	178	$S = 0.0392$	48	500	This work
	20	$S = 0.0251$	83	300	[2]

iron) so the reduction of the volume for about 190 μL (the tested valued) still ensured excess of ligand. Two calibration curves were established and the slope and intercepts compared (Fig. 6).

The results showed that decreasing the volume resulted in a decrease in sensitivity (slope) and increase in limit of detection (intercept) so the initial volume of about 250 μL was set. Afterwards, the sample volume was studied within the range 310–558 μL, and the sensitivity increased up to the volume of 496 μL, so that was the chosen value (Fig. 6).

3.2.2. Chemical parameters

Calibration curves with different ligand solution concentrations, 119, 178 and 238 mg/L, were compared, in terms of both the sensitivity (slope) and limit of detection (intercept) (Fig. 7). The ligand solution concentration of 178 mg/L was chosen as the results showed that the sensitivity (slope) increase up to that the concentration together with the intercept decrease (consequently of the limit of detection), proving to be the best choice.

The requirement of buffering the complex formation established in Mesquita et al. [2] work and the use of a carbonate buffer was set. A study of hydrogen carbonate solution concentration was carried out. From the tested concentrations, ranging from 0.10 to 0.60 M, the maximum sensitivity was obtained with a concentration of 0.25 M, so that was the chosen concentration. The intercept increased with the increase of the concentration but as the increase in sensitivity was over 40%, 0.25 M was still the best choice.

3.3. Features of the developed SI methodology

After the detailed studies for the determination of iron based on the colored complex formed with the PEG-HPO bidentate ligand, the characteristics of the developed method were summarized (Table 3).

The limit of detection, LOD, was calculated according to IUPAC recommendations [17]: three times the standard deviation of ten blank signals. A typical calibration curve corresponds to the mean of five calibration curves obtained in consecutive days (with the standard deviation values in brackets). The determination rate was calculated based on the time spent per cycle; a complete analytical cycle took about 0.83 min. An analytical cycle is the sum of the time needed for each step plus the time necessary for the port selection in the selection valve. The presented consumption values, for reagents and sample, and the effluent production were calculated per determination.

The repeatability was assessed by calculation of the relative standard deviation (RSD) obtained by the mean of ten consecutive injections of sample. The calculated RSD was 3.1% (0.225 ± 0.007 mg Fe/L).

3.3.1. Assessment of possible interferences

The possible interference of several bivalent and trivalent cations was assessed, and the tested concentrations were based in the available legislation limits from UNFAO [10]. The concentrations of the tested cations were obtained from proper dilution of atomic absorption standards. Several standards, with 600 ppb of iron(III) and the tested concentration of interfering ions, were prepared and analyzed with the developed SI method. The absorbance values of the standard with and without interfering ion were registered and the interference percentage calculated (Table 4).

Overall, no significant interferences were observed, with almost all the interference percentages being below 5%. Exception was observed for magnesium, with an interference percentage close to 10%. However, the tested concentration was 4 times the expected concentration in water streams, aiming to test the possible application to sea water. So the <10% interference percentage was a good indicator of that possibility.

3.3.2. Method validation

For accuracy assessment of the developed method, two certified water samples were analyzed and the results compared to the certified value. A river water sample NRC-CNR SLRS-4, with an iron content of 103 ± 5 μg/L and the relative deviation obtained was −3% as the concentration calculated with the developed method was 100 ± 5 μg/L. The other certified water, a surface water SPS-SW2 with an iron content of 100 ± 1 μg/L, resulted in a relative deviation of 4% as the calculated concentration with the developed method was 104 ± 8 μg/L.

For further accuracy assessment, several natural waters were analyzed (Table 5) with the developed SI method and the results obtained compared to those obtained with the reference procedure, graphite furnace atomic absorption spectrometry (GFAAS).

A linear regression between the values obtained with the developed SI method (C_{SI}) and the reference procedure, graphite furnace atomic absorption spectrometry (C_{GFAAS}), was established (ESI Fig. S1); the equation found was: $C_{SI} = 1.046\ (\pm 0.441)\ C_{GFAAS} - 0.0036\ (\pm 0.0366)$, where the values in parenthesis are the 95% confidence limits. These figures show that the estimated slope and intercept do not differ from the values 1 and 0, respectively. Thus, there is no evidence for systematic differences between the two set of results [18].

4. Conclusions

The use of the new 3,4-HPO ligand, PEG-HPO, especially designed to possess higher water solubility, proved to meet the aim of improving the detection limit and sensitivity in the determination of iron (Table 6).

In fact, the sensitivity obtained with the developed SI method, using the new ligand, showed an increase close to 60% when compared to the previously described method [2] and a decrease of the detection limit to about half (Table 6).

In that previous work [2], there was also the use of a micro sequential injection format (μSI-LOV), and a comparison within the same conditions, without revisiting the determination parameters, was made. The same sensitivity of the method was

maintained proving the efficiency of the new ligand for iron determination in both flow formats.

The choice of sequential injection as a flow technique proved to be highly appropriated, enabling the detailed study of the complexation reaction with a fast and automatic method. For a flow analysis effective application, contributed the excellent characteristic of the complex formation being almost immediate. Furthermore, the good results obtained with the certified water samples, reinforced the successful application to the iron determination in natural waters.

Finally, and from the point of view of synthesis of new compounds, the newly synthesized ligand is not only important for this particular work but also opens new perspectives for the synthesis of other metal ion complexes with improved water solubility.

Acknowledgments

J.L.A. Miranda thanks to for the Grant PTDC/AAG-MAA/3978/2012_BI_2. T. Moniz and R.B.R. Mesquita thank to Fundação para a Ciência e a Tecnologia (FCT, Portugal) and Fundo Social Europeu (FSE) through the program POPH – QREN for the grants SFRH/BD/79874/2011 and SFRH/BPD/41859/2007, respectively. This work was supported by European Union FEDER funds through COMPETE and by National Funds through FCT, projects PTDC/AAG-MAA/3978/2012, PEst-OE/EQB/LA0016/2013, PEst-OE/EQB/LA006/2013. This work was supported by FCT, Portugal, European Union, QREN, FEDER and COMPETE, projects NORTE-07-0162-FEDER-000048, NORTE-07-0124-FEDER-000066/67. We thank CeNTI, V.N. Famalicão, for making available a CEM Discover microwave reactor. The Bruker Avance III 400 spectrometer is part of the National NMR network and was purchased under the framework of the National Programme for Scientific Re-equipment, contract REDE/1517/RMN/2005, with funds from POCI 2010 (FEDER) and (FCT). This work was also supported under CTQ2013-47461-R project from Ministerio de Ciencia e Innovación (MINCINN, Spain).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.poly.2015.09.015.

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