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Polymer inclusion membrane to access Zn speciation: comparison with root uptake

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Abstract

Metal speciation studies can be performed with a new technique based on a functionalized membrane. The estimation of not only the total amount of metal, but also the metal available to living organisms is very important. In this context, we have investigated the use of a polymer inclusion membrane (PIM) in a new tool for the determination of free metal ion concentration. In order to check the usefulness of PIM devices in metal speciation studies and metal availability to potato plants (*Solanum tuberosum*), Zn has been chosen as a case study. The PIM designed for Zn transport uses polyvinyl chloride (PVC) as polymer and di-(2-ethylhexyl) phosphoric acid (D2EHPA) as carrier, with 0.01M nitric acid in the receiving solution. The stability of the PIM has been demonstrated and good linearity of PIM-device fluxes (J_{PIM}) with free metal concentration was observed for total metal concentrations ranging from 3 μM up to 70 μM. The presence of different ligands, such as ethylenediaminetetraacetic acid (EDTA), humic acid (HA) and citrate, greatly influences the measured J_{PIM} because the formation of metal complexes in the donor phase decreases the free Zn concentration in the sample. Good correlation has been found when comparing PIM fluxes and metal accumulation in potato plants roots in the presence of EDTA. But, the root uptake did not change when adding citrate and HA to the hydroponic medium, so the uptake does not always follows the Free Ion Activity Model (FIAM). These ligands might induce physiological changes in the roots and enhance metal uptake.

Keywords: Polymer inclusion membranes (PIMs); metal speciation; di-2-(ethylhexyl) phosphoric acid (D2EHPA); zinc; roots.

1 Introduction

Dissolved trace metals in environmental water or in pore water of soils and sediments can be found in numerous chemical forms or species, such as free ions, and inorganic or organic complexes. Metal partitioning among the different forms is a dynamic process that depends on different parameters such as type and concentration of ligands, temperature, pH or redox conditions of the medium. It has been established that for cationic metals, the concentration of the free, uncomplexed, metal ion is usually the best predictor for both metal bioaccumulation and toxicity in most aquatic systems. This general consensus has led to the predominance of the free ion activity model (FIAM), which postulates that the metal uptake by an organism is proportional to the free ion concentration of the metal in the surrounding solution. FIAM relies on the internalisation flux being limiting, rather than the supply of the metal from the solution to the surface of the organism (Wilkinson et al., 2004).

The biotic ligand model (BLM) is an extension of the FIAM taking into account the competitive binding of cations and protons at the surface of the biological membrane (i.e. the biotic ligand) (Di Toro et al., 2001). Despite being extensively accepted, exemptions to these models have also been reported, and some studies point out that in the case of higher plants, the uptake might be favoured by the presence of organic ligands, which would facilitate the metal uptake at the roots (Gramlich et al., 2013; Samson and Visser, 1989; Wang et al., 2009). Indeed, apart from internalisation of the free cation (Zhao et al., 2016; Degryse et al., 2006), other mechanisms can be contribute to the uptake such as the direct internalisation of intact metal complexes by the organism (simple diffusion across the lipid bilayer or permeation across the lipid bilayer via a ligand transport site).

Different techniques have been developed to measure, not only metal speciation, but also to estimate labile fractions available for organisms (Sierra et al., 2017; Jones et al., 2016). This is the case of diffusive gradients in thin films (DGT) technique, for example, consisting of a porous gel matrix attached to a layer of complexing cation exchange beads (Chelex 100). DGT is nowadays widely accepted as a tool to monitor metal availability and to calculate the so-called DGT concentrations (Davison, 2016; Davison and Zhang, 1994).

Permeation liquid membrane (PLM) techniques are used for the separation and preconcentration of target elements, which are complexed by an organic complexing agent supported in a membrane. This membrane separates two different aqueous solutions: the donor (also called source or feed) phase, containing the sample with the analyte, and the acceptor (also called receiving or stripping) phase, where the analyte is accumulated. The transport is based on liquid-liquid extraction coupled with diffusion. In the nineties, Buffle and Parthasarathy firstly reported PLM for speciation studies as an approach to mimic the processes of metal transport across biological membranes (Parthasarathy and Buffle, 1994). The metal flux across the membrane is evaluated from the variation of the metal concentration in the acceptor solution as a function of time. Under certain conditions, PLMs measure the free metal ion fraction (Pesavento et al., 2009), and PLM has been used as a reliable sensor for free Ni concentrations down to 10^{-7} M as reported by Bayen et al. (Bayen et al., 2007). Pb availability to freshwater algae was studied by comparing PLM fluxes and Pb biouptake fluxes (Slaveykova et al., 2004). Both, the simplicity of this technique and the large number of available complexing agents render this methodology very attractive. However, a weak point is the poor stability of the membrane due to the leaching of the organic extracting compound into the aqueous adjacent phases (Buffle et al., 2000). This fact has seriously limited a wider use of PLM.

In this work, we explore for the first time the use of a simple device based on a polymer inclusion membrane (PIM) to determine free ion concentrations in aqueous samples (*i.e.* specifically targeting one relevant fraction of the total concentration). The principles of this system are similar to those of PLM, since the analyte is transported across the membrane, by means of the carrier, from the donor to the acceptor phase. However, to increase the stability of the membrane, this carrier is entrapped within a polymeric matrix, normally made of cellulose triacetate (CTA) or polyvinyl chloride (PVC). The role of the polymer is to provide mechanical strength to the membrane, and sometimes, besides these components, the addition of a plasticizer is also necessary to improve the elasticity as well as to modify the diffusion characteristics of the membrane (Almeida et al., 2012). This improved stability, in addition to their easy preparation, low cost, versatility, good chemical resistance and high efficiency, had led to the use of these membranes for separation or preconcentration purposes of different metals or organic compounds (Garcia-Rodríguez et al., 2015; Güell et al., 2011). PIMs made of PVC and the carrier di-

(2-ethylhexyl) phosphoric acid (D2EHPA) have been applied for the determination of the time-weighted average total concentration of Zn (Almeida et al., 2014), but its use as sensor for Zn speciation has - as far as we know- not been described yet. The environmental relevance of this new sensor stems from the crucial role, as stated by FIAM and BLM, of the free ion concentration on the ecotoxicological impact of the considered element.

Zn is one of the essential trace metal micronutrients, required by plants in small amounts for various biochemical reactions and physiological functions such as formation of chlorophyll, photosynthesis and respiration (Humayan Kabir et al., 2014; Remans et al., 2012). Even though Zn deficiency is more widespread than Zn toxicity, excessive Zn accumulation affects the capacity to maintain homeostasis and can induce oxidative stress. In this work, PIM fluxes for Zn in the absence and presence of organic ligands forming complexes with different binding strength and charge, such as EDTA, citrate, and humic acids, have been determined using as a donor phase a plant nutrient solution (*i.e.* a hydroponic medium). Moreover, PIM fluxes have been compared with Zn uptake by potato (*Solanum tuberosum*) roots under the same experimental conditions. Potato plants were chosen for this study because (i) potato is the fifth largest staple crop consumed worldwide, fulfilling essential needs in human nutrition and health (FAO, 2015), and (ii) potato plantlets were reproduced asexually using cuttings, reducing the the overall variability among samples and contributing to increase the reproducibility of the experiment. Special attention is devoted to elucidate whether the the PIM-based sensor (in the current conditions) are determining the free Zn concentration, rather than the total concentration or any other fraction.

2 Experimental

2.1 Reagents and solution

All reagents and solvents used in this study were of analytical grade. The polymer PVC, and the acid 2-(N-morpholino) ethanesulfonic (MES) were obtained from Fluka (Bern, Switzerland). The organic solvent, tetrahydrofurane (THF), was purchased from Panreac (Barcelona, Spain). The carrier, D2EHPA, was provided by Sigma-Aldrich (St Louis, Missouri, USA). For the preparation of the hydroponic solution the following reagents, purchased from Panreac (Barcelona, Spain), were used: ammonium nitrate (NH_4NO_3), boric acid (H_3BO_3), calcium nitrate ($Ca(NO_3)_2.4H_2O$), copper sulphate ($CuSO_4.5H_2O$),

potassium nitrate (KNO_3), potassium dihydrogen phosphate (KH_2PO_4), sodium molybdate ($Na_2MoO_4.2H_2O$), sodium hydroxide (NaOH), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), potassium hydroxide (KOH), manganese chloride ($MnCl_2.4H_2O$) and zinc sulphate seven hydrate ($ZnSO_4.7H_2O$). As iron source the commercial product Kelamix Fe was used (Sicosa, Girona, Spain). Hoagland solution at half strength, used as a nutrient solution and donor medium, is described in Table 1. The pH of the solution was adjusted at 6.0 ± 0.1 using 2.5 mM MES. Suprapur grade nitric acid (HNO_3) was obtained from Panreac (Barcelona, Spain). Organic ligands such as L-histidine monohydrochloride monohydrate, humic acids sodium salt (HA, used as received), ethylenediaminetetraacetic acid (EDTA) were provided by Sigma-Aldrich (St Louis, Missouri, USA) and sodium citrate ($Na_3C_6H_5O_7 \cdot H_2O$) was obtained from Scharlau (Barcelona, Spain). Propidium iodide (PI) and Fluorescein di-acetate (FDA) stains were obtained from Sigma and Invitrogen (Thermo Fisher, Waltham, Massachusetts, USA).

Table 1. Chemical composition of the nutrient solution corresponding to a half-strength Hoagland solution, used as donor medium in all the experiments performed throughout the study.

Chemical compound	Concentration (μM)
KNO_3	2500
$Ca(NO_3)_2.4H_2O$	2500
Fe (in Kelamix)	12
$MgSO_4.7H_2O$	1000
NH_4NO_3	500
KH_2PO_4	250
MES	2500
H_3BO_3	23
$MnCl_2.4H_2O$	4.57
$ZnSO_4.7H_2O$	0.38
$CuSO_4.5H_2O$	0.10
$Na_2MoO_4.2H_2O$	0.25

Calibration standards of Zn were prepared using a 1000 mg L^{-1} stock solution for atomic spectroscopy (Sigma-Aldrich, St Louis, Missouri, USA). The working range was from

0.05 mg L^{-1} to 2.5 mg L^{-1} in the donor solution and 0.3 mg L^{-1} to 15 mg L^{-1} in 0.01M nitric acid for the analysis of the receiving phase.

Ultrapure water from a MilliQ Plus water purification system (Millipore Ibérica S.A., Madrid, Spain) was used to prepare all solutions.

2.2 Instrument and apparatus

An Ethos Plus Milestone microwave with HPR-1000/10S high-pressure rotor and temperature sensor (Sorisole, Bergamo, Italy) was employed for acid digestion of samples. A sequential inductively coupled plasma atomic emission spectrometer, ICP-OES, (Liberty RL, Varian, Mulgrave, Vic., Australia) was used to determine the total concentration of Zn (λ= 213.81 nm) in aqueous solutions and acid plant digestions. The pH values of the aqueous samples were determined with a Crison Model GLP 22 pH meter (Barcelona, Spain).

Fluorescent root plant images were obtained using an epifluorescence BX-41 Olympus microscope that was connected to a DP-73 Olympus digital camera (Olympus, Tokyo, Japan).

2.3 Membrane preparation

PIMs were prepared by dissolving 400 mg of PVC in 12 mL of THF and the resulting mixture was stirred for 2 hours. Later on, the appropriate amount of carrier D2EHPA (from a 0.5 M D2EHPA solution in THF) was added to the corresponding polymeric solution and stirred again for 1 hour in order to obtain the final membrane composition of 70% PVC-30% D2EHPA or 60% PVC-40% D2EHPA (% in mass). Finally, the resulting mixture was poured out into a 9.0 cm diameter flat bottom glass petri dish, which was set horizontally and covered loosely. The organic solvent was allowed to evaporate during 24 h at room temperature, and the resulting film (of an approximate thickness of 55 μm) was afterward carefully peeled off from the bottom of the petri dish. Reproducibility of the PIM preparation was routinely checked by means of total weight, IR spectroscopy and thickness measurements.

2.4 PIM-device experiments

The designed device (see Fig. 1), with similarities to those previously reported (Almeida, et al., 2014; Almeida et al., 2016) consists of a glass tube with two openings, one at the top (0.9 cm diameter) and another one at the bottom (1.8 cm diameter). The PIM was placed at the bottom opening (1.75 cm^2) and fixed with a screw cap. The opening at the top was closed with a standard screw cap and was used to fill the device with 5 mL of receiving solution. To carry out the experiments using this device, 250 mL of donor nutrient solution spiked with zinc (and organic ligands when indicated) were poured into a glass beaker placed on a magnetic stirrer. The device incorporating the membrane and receiving solution (5 mL 0.01M HNO_3) was immersed 1 cm in the donor nutrient solution in a vertical position under stirring conditions. After the deployment period, the device was removed from the solution and the receiving solution was taken for analysis. The final concentration in the donor solution was also checked in order to verify that no depletion took place.

Fig. 1

2.5 Plant growth

Potato plants (*Solanum tuberosum* cv Desireé) were grown *in vitro* in Murashige and Skoog solid medium (MS) supplemented with 2% sucrose (Serra et al., 2008). 1-node explants without leaves, using only axial gems of four week-old plants, were cultured *in vitro* in solid MS for three weeks in order to ensure the homogeneity amongst the different biological replicates. Then, they were transferred to the hydroponic media. Plants *in vitro* and plants in hydroponics were grown under light/dark photoperiod cycle of 12/12 h at 67 μmol m^{-2} s^{-1} and 25 ºC and 22 ºC respectively

2.6 Plant exposure and analysis

Plant exposure to Zn and to the different organic ligands was studied following the experimental design described in Fig. S1 (in the Supporting Information). From the *in vitro* plants, fourteen plants (for each treatment) were grown in ten-litter buckets of nutrient solution containing Hoagland's half strength solution (Table 1) during two weeks before plant exposure to the metal or the ligand. The treatments include (i) no metal addition (control experiment), (ii) fortified donor medium with 35 μM Zn (no ligand) and

(iii) three Zn-fortified media each with an added specific organic ligand at a final concentration of 20 μM of EDTA, 240 μM citrate or 60 mg L^{-1} of HA. The ligand concentrations for this study were selected so that, for a total Zn concentration of 35 μM, similar free Zn concentrations were reached in all treatments. Plants were exposed for a period of 48 h. After that, plants were removed from buckets and carefully dipped into cold water for 30 seconds prior to rinsing them with a 5 mM $Pb(NO_3)_2$ solution (Panreac, Barcelona, Spain) to remove Zn from the surface of the roots. Finally, plants were dried in an oven at 60ºC for 5 days (Cuypers et al., 2002; Gramlich et al., 2013). Pools of roots from 4 plants were cut into small pieces, dried and weighted before microwave acid digestion. Then, they were transferred to PTFE vessels and digested with 8 mL of nitric acid of suprapure grade (69%) and 2 mL of hydrogen peroxide (30%). The vessels were closed and heated into the microwave following a digestion program consisting of a first step of 5 min to reach 180 °C and then 10 min at 180 °C. After cooling, digested sample solutions were transferred to a 30 mL flask and brought to volume with ultrapure de-ionized water prior to ICP-OES analysis.

2.7 Vital staining of roots

Once the different exposures were finished, a pool of three plants were removed from the buckets and their roots were cut and transferred directly to an aqueous solution containing 5 μg mL^{-1} of PI and 2 μg mL^{-1} of FDA for 15-30 min. The roots were then rinsed twice with water, and mounted on a microscope slide which was subsequently submerged in water (John et al., 1995). The samples were viewed within 15 min from the staining with a BX-41 Olympus microscope that was connected to a DP-73 Olympus digital camera. For FDA detection, we used the excitation filter B (excitation at 475 nm and emission at 515 nm using a long path filter). For PI detection, we used the excitation filter G (excitation at 530 nm and emission at 610 nm). The same specimen was analysed separately at wavelengths specific to each stain and the two images were merged to give the final image.

3 Results and discussion

3.1 Evaluation of the PIM system

When speciation is of interest, the test sample composition should not be modified, and, in particular, the decrease in analyte concentration in the source solution due to membrane extraction should be negligible (*i.e.* no depletion of the analyte test sample). These conditions are usually achieved by using a volume of the test solution much larger than the volume of the acceptor phase, e.g. using a hollow fibre geometry (Tomaszewski et al., 2003; Slaveykova et al., 2009) or with ”flow-through” approaches (Almeida et al., 2014). Apart from the donor and acceptor volumes, the membrane composition and the acceptor phase acidity can be modulated to ensure no depletion of the analyte in the donor phase. So, we fixed an aqueous volume of 250 mL for the donor solution and of 5 mL for the acceptor solution. We evaluated the effect on metal transport and depletion when the nutrient solution (without MES added) was supplemented with 35 μM Zn(II), and tested two nitric acid concentrations of 0.01 and 0.1 M in the acceptor phase and two PIM compositions. Results are presented in Table 2, in terms of metal concentration in both the donor and in the receiving solutions after 24 h experiment in each condition. As expected, the better the transport to the receiving solution (i.e. higher final concentrations in the acceptor), the larger the variation from the initial Zn concentration in the donor solution is obtained. Given that the use of a PIM made of 70% PVC- 30% D2EHPA and a 0.01 M HNO_3 receiving solution ensures the negligible depletion conditions required to perform our studies, we fixed these conditions for further experiments.

Table 2. Effect of PIM and receiving phase compositions on Zn transport and depletion. Contact time: 24 h.

PIM	HNO_3 (M)	Initial [Zn] donor solution (μM)	Final [Zn] donor solution (μM)	[Zn] receiving solution (μM)
70% PVC- 30% D2EHPA	0.01	35.92	35.4	60.1
	0.1		31.6	120.7
60% PVC- 40% D2EHPA	0.01		30.1	131.7
	0.1		20.8	417.7

3.2 Stability studies

It has been extensively reported that the lack of stability is the main drawback of PLM (Buffle et al., 2000). Gramlich et al. (2012) described the use of a PLM in hollow fibre configuration consisting of a mixture of a Kryptofix 22DD and lauric acid as carriers dissolved in phenylhexane and toluene (1:1 v/v) as solvents for the transport of free Zn. They found a decrease in the transport capacity of the membrane down to 63 % within 8 h after impregnation. Therefore, it was necessary to use freshly impregnated membranes in all experiments (which lasted 2 hours).

To study the stability of the PIM system, we evaluated the variation of Zn flux (J_{PIM} in mol m^{-2} s^{-1}) with time. For that, three experiments were performed contacting the PIM-devices with the corresponding donor solution and retrieved them at different times, from 24 to 48 h. The average flux can be calculated from the number of moles found in the acceptor phase and considering the membrane area and the deployment time. Results are collected in Fig. 2, where it can be seen that there is not a significant variation in Zn flux for the time period studied, so one can assume the system to be under a steady-state regime (Galceran and Puy, 2015). Moreover, the stability of the PIM was also evaluated in terms of membrane mass loss, and it was found only a decline of 2.2± 0.4% of total weight after 24 h of immersion (this corresponds to 7% carrier loss).

Fig. 2.

Hence, these results support the use of PIMs as an attractive alternative to PLM, since they maintain the important features of PLMs such as simplicity, low cost and the possibility to prepare selective membranes, while providing the necessary stability to perform long-term experiments.

3.3 Measurement of diffusional J_{PIM}

Several experiments were performed to measure J_{PIM} in the absence of organic ligands. The initial total metal concentration in the nutrient solution was varied from 3 μM up to 70 μM. J_{PIM} was calculated as before and, as can be seen in Fig. 3, it is proportional to the free Zn^{2+} concentration in the donor phase (computed using visual MINTEQ with its standard database, and neglecting the contribution of the complex Kelamix Fe (Yunta et al., 2003)). The equation for a lineal model of J_{PIM} (nmol cm^{-1} h^{-1}) vs. [Zn] (μM) is:

$$J_{PIM} = 0.092(\pm 0.004)\,[Zn]_{PIM} - 0.27(\pm 0.15) \qquad (1)$$

and $R^2 = 0.9905$

Fig. 3.

To test the real possibilities of PIM as a tool to measure the free ion concentrations, different organic ligands have been used in this work: EDTA, citrate and humic acid. EDTA is a polydentate ligand which forms strong complexes with metals and is used as a ligand to buffer Zn^{2+}, as Zn-EDTA is assumed to barely contribute to Zn uptake (Bell et al., 2003; Hart et al., 1998; Stacey et al., 2008). Citrate is involved in the storage of Zn in plant vacuoles (White et al., 2009). Finally, humic acid is a part of the natural organic matter which plays an important role in aquatic ecosystems by binding trace metals and, thus, influencing their speciation, bioavailability, and toxic effects. Thus, 20 μM of EDTA, 60 mg L^{-1} of HA or 240 μM of citrate were added to the growth medium. J_{PIM} was evaluated in the presence of these ligands at two different total metal concentrations, 35 and 70 μM. We could observe that the addition of organic ligands produced a decrease in all J_{PIM}, which correlates with the reduction of free Zn according to visual MINTEQ calculations. $[Zn^{2+}]_{PIM}$ was determined (using the calibration provided by Fig. 3 and plotted in Fig. 4 together with $[Zn^{2+}]_{MINTEQ}$ and the 1:1 line on which both values should match. The good agreement found supports the claim that the sensor is measuring free Zn concentrations. Moreover, the use of PIMs as a new speciation technique (as an alternative to PLM) appears as a promising possibility.

Fig. 4.

3.4 Modelling the flux of Zn

Separation by means of functionalized membranes is based on the chemical affinity of the target compound towards the ligand or functional group existing inside the membrane. In both, PLMs and PIMs, an appropriate carrier is added to the membrane to interact with the analyte, Zn^{2+} in this study. For this work, the extraction reaction of the metal by D2EHPA (denoted as HL) can be described with the following equilibrium reaction (Kolev et al., 2009):

$$Zn^{2+}_{(aq)} + 3/2\ (HL)_{2(mem)} \rightleftharpoons ZnL_2 \cdot HL_{(mem)} + 2H^+_{(aq)} \qquad (2)$$

329 Taking into account our current data and similarly to existing literature on PLM (Buffle
330 et al., 2000), a simple model can be derived from the following hypotheses:

331 A) equilibrium at both interfaces.

332 For the Donor/membrane (indicated as superscript D/m) interface

333 $$K = \frac{\left[\mathrm{ZnL_2{\cdot}HL}\right]^{\mathrm{D/m}}\left(\left[\mathrm{H^+}\right]^{\mathrm{D/m}}\right)^2}{\left(\left[\left(\mathrm{HL}\right)_2\right]^{\mathrm{D/m}}\right)^{3/2}\left[\mathrm{Zn^{2+}}\right]^{\mathrm{D/m}}} \quad (3)$$

334 while for the membrane/Acceptor (indicated as superscript m/A) interface

335 $$K = \frac{\left[\mathrm{ZnL_2{\cdot}HL}\right]^{\mathrm{m/A}}\left(\left[\mathrm{H^+}\right]^{\mathrm{m/A}}\right)^2}{\left(\left[\left(\mathrm{HL}\right)_2\right]^{\mathrm{m/A}}\right)^{3/2}\left[\mathrm{Zn^{2+}}\right]^{\mathrm{m/A}}} \quad (4)$$

336 All concentrations in these section are expressed in mol m^{-3}.

337

338 B) diffusion co-limitation of the fluxes, both in the membrane

339 $$J^{\mathrm{m}} = \frac{D^{\mathrm{m}}_{\mathrm{ZnL_2{\cdot}HL}}}{\ell}\left(\left[\mathrm{ZnL_2{\cdot}HL}\right]^{\mathrm{D/m}} - \left[\mathrm{ZnL_2{\cdot}HL}\right]^{\mathrm{m/A}}\right) \quad (5)$$

340 (where ℓ is the thickness of the membrane, in m, and $D^{\mathrm{m}}_{\mathrm{ZnL_2{\cdot}HL}}$ is the diffusion
341 coefficient, in $m^2\ s^{-1}$, of the complexed carrier inside the membrane) and in the Acceptor
342 solution

343 $$J^{\mathrm{A}} = \frac{D_{\mathrm{Zn^{2+}}}}{\delta^{\mathrm{A}}}\left(\left[\mathrm{Zn^{+}}\right]^{\mathrm{m/A}} - 0\right) \quad (6)$$

344 (where δ^{A} is the thickness of the diffusion layer in the Acceptor and $D_{\mathrm{Zn^{2+}}}$ is the
345 diffusion coefficient of free Zn ion there).

346 C) Given that the presence of labile ligands (see Fig 4) does not relevantly increase J_{PIM}
347 (but rather J_{PIM} is proportional to the free Zn concentration), we assume that there is no
348 limitation by diffusion in the Donor solution

349 $$\left[\mathrm{Zn^{2+}}\right]^{\mathrm{D/m}} \approx \left[\mathrm{Zn^{2+}}\right]^{*} \quad (7)$$

350 where superscript * indicate the bulk concentration of the sample. This also leads to

$$\left[\mathrm{H^+}\right]^{\mathrm{D/m}} \approx \left[\mathrm{H^+}\right]^{*} \tag{8}$$

D) Steady state is assumed taking into account the lack of variation of J_{PIM} seen in Fig 2. In steady-state regime, all fluxes of Zn (be it complexed or free) have to be equal to the resulting flux

$$J^{\mathrm{m}} = J^{\mathrm{A}} = J_{\mathrm{PIM}} \tag{9}$$

Simple algebra using previous assumptions leads to

$$J_{\mathrm{PIM}} = \frac{\dfrac{K\left(\left[\left(\mathrm{HL}\right)_2\right]^{\mathrm{D/m}}\right)^{3/2}}{\left(\left[\mathrm{H^+}\right]^{*}\right)^{2}}}{\dfrac{\ell}{D^{\mathrm{m}}_{\mathrm{ZnL_2\cdot HL}}} + \dfrac{K\left(\left[\left(\mathrm{HL}\right)_2\right]^{\mathrm{m/A}}\right)^{3/2}}{\left(\left[\mathrm{H^+}\right]^{\mathrm{m/A}}\right)^{2}}\dfrac{D^{\mathrm{A}}_{\mathrm{Zn^{2+}}}}{\delta^{\mathrm{A}}}}\left[\mathrm{Zn^{2+}}\right]^{*} \tag{10}$$

In general, $\left[\left(\mathrm{HL}\right)_2\right]^{\mathrm{D/m}}$ and $\left[\left(\mathrm{HL}\right)_2\right]^{\mathrm{m/A}}$ are not constant, but depend on the loading of Zn, because of the balance of ligand, e.g. at interface Donor/membrane,

$$2\times\left[\left(\mathrm{HL}\right)_2\right]^{\mathrm{D/m}} + 3\times\left[\mathrm{ZnL_2\cdot HL}\right]^{\mathrm{D/m}} = c_{\mathrm{T,L}} \tag{11}$$

where $c_{\mathrm{T,L}}$ stands for the total D2EHPA (monomer) concentration inside the membrane.

Given the observed linear relationship between flux and free concentrations, we consider "excess of ligand" conditions, i.e. the amount of Zn bound to the carrier is approximately constant:

$$\left[\left(\mathrm{HL}\right)_2\right]^{\mathrm{D/m}} \approx \frac{c_{\mathrm{T,L}}}{2} \approx \left[\left(\mathrm{HL}\right)_2\right]^{\mathrm{m/A}} \tag{12}$$

Considering the fast diffusion of protons and their huge concentration (in comparison to Zn)-, one can take

373 $$\left[\mathrm{H^+}\right]^{\mathrm{m/A}} \approx \left[\mathrm{H^+}\right]^{\mathrm{A}}$$

374

375 With these considerations, eqn (10) simplifies to

376 $$J_{\mathrm{PIM}} \approx \frac{\dfrac{K\left(c_{\mathrm{T,L}}/2\right)^{3/2}}{\left(\left[\mathrm{H^+}\right]^{*}\right)^{2}}}{\dfrac{\ell}{D^{\mathrm{m}}_{\mathrm{ZnL_2\cdot HL}}} + \dfrac{K\left(c_{\mathrm{T,L}}/2\right)^{3/2}}{\left(\left[\mathrm{H^+}\right]^{\mathrm{A}}\right)^{2}} \dfrac{D^{\mathrm{A}}_{\mathrm{Zn^{2+}}}}{\delta^{\mathrm{A}}}} \left[\mathrm{Zn^{2+}}\right]^{*} \qquad (13)$$

377

378 This expression justifies:

379 i) the linearity of the steady-state flux with free Zn concentration (Figs. 3 and 4) and the
380 lack of impact of labile complexes present in the donor solution. This proportionality is
381 reflected in eqn.(1), where the non-null intercept can be attributed to experimental
382 uncertainty as well as to the system having an initial transient regime not considered in
383 eqn. (13);

384 ii) higher fluxes when the acceptor is more acidic, *i.e.* higher $[H^+]^A$ (in Table 2, for
385 instance, in the case of 30% D2EHPA, compare the final accumulated $[Zn^{2+}]$ in the
386 acceptor around 121 μM for 0.1 M HNO_3 with $[Zn^{2+}]$ around 60 μM for 0.01 M HNO_3)

387 and iii) higher fluxes are expected for higher total carrier concentrations, i.e. higher $c_{T,L}$
388 (in Table 2, for instance, in the case of 0.01 M HNO_3, compare the final accumulated
389 $[Zn^{2+}]$ around 132 μM for 40% D2EHPA with [Zn] around 60 μM for 30% D2EHPA)

390

391 Apart from limitation by diffusion in both the membrane and in the acceptor solutions (as
392 underlying eqn (13)), the accumulation rate of the analyte might be affected by other
393 processes such as interfacial kinetics (which could become critical for low concentrations
394 of carrier), but this is not considered here given some previous reported results with PIM
395 (Fontàs et al., 2007; Kolev et al., 2013) and given that the model agrees with our current
396 data.

397

Further experiments varying the membrane thickness and the carrier ligand concentration over a range of values (Van Leeuwen et al., 2005), can confirm or discard the present model and experimentally tune the PIM to probe the free fraction or others (such a labile one).

3.5 Toxicity of the ligands for the roots

According to the FIAM paradigm, biological fluxes are directly proportional to free metal ion concentrations rather than to the total ones (Campbell, 1995). This assumption has been tested using living organisms such as freshwater algae (Bayen et al., 2006; Slaveykova et al., 2004), microalgae (Rodríguez-Morales et al., 2015) or wheat plants in hydroponics media (Gramlich et al., 2014). Alternatively, fluxes measured with the different speciation techniques (PLM, DGT) have also been correlated with metal bioavailability in some systems (Bradac et al., 2009). However, it is essential to take into account the intrinsic metal and ligand toxicity before biological fluxes are measured. It has been described that metal chelates at higher concentrations may damage roots, which in turn affects metal uptake (Bell et al., 2003).

So, we performed viability analyses of the roots of potato plants growing in the different donor solution media with 35 μM Zn and supplemented with the different ligands. Distinction between viable and inviable cells was performed through a double fluorescent staining procedure. FDA is cleaved by esterases present in living cells and fluoresces green after it is cleaved. On the other hand, PI fluoresces red in the nuclei of dead cells. PI also stains most of the cell walls, viable or inviable, which means that PI staining sometimes overlaps FDA staining and appears yellow. In potato plants, young lateral roots are viable when no treatment is applied (Fig. 5 a) and all the root tips assessed presented green colour. When 35 μM metal content was supplemented in the donor solution, roots succeeded in being stained with FDA and a green colour was obtained in root tips (Fig. 5 b). Likewise, in treatments where 35 μM metal content and either EDTA, HA or citrate were applied as ligands, cells were metabolically active and thus, green colour was also predominant (Fig. 5 c-e). Lateral roots treated with the same amount of Zn and histidine ligand, failed to stain with FDA, but were stained with PI (red), suggesting that the cells die as a consequence of the treatment (see Fig. 5 f). Therefore, measurements with histidine as a ligand were not reported in previous section 3.3, and also not considered in what follows.

Fig. 5.

3.6 Plant metal uptake and comparison with J_{PIM}

Predicting availability of metals to plants is a challenge that has been addressed using different approaches. For example, in the case of soil fertility evaluation and risk assessment of soil contaminants, diethylentriaminepentaacetic acid (DTPA)-extracts and $CaCl_2$ (0.01 M) extractable concentrations are commonly used (Lindsay and Norvell, 1978; Meers et al., 2007; Menzies et al., 2007). Moreover, models based on a mechanistic approach have been employed to describe uptake of major nutrients and micro-nutrients, such as Mn and Zn (Adhikari and Rattan, 2000; Sadana and Claassen, 2000). Additionally, DGT and PLM techniques have been suggested as tools to predict metal bioavailability and different studies have been reported. The limiting transport steps in PLMs and biological plasma membranes have been compared (Buffle et al., 2000; Degryse et al., 2009). Despite being the transport processes in the test solution almost identical for living organisms and PLM setup, the size and geometry of the systems clearly differ. Also, relevant differences may arise in the transport steps at the water-membrane interface or inside the membrane, despite being conceptually similar processes: the nature of the binding sites is different, multistep processes usually account for the transport in biological membranes, and passive diffusion of lipophilic complexes is differently affected by the viscosity and other physical parameters. In any case, PLM has been used to test the FIAM model, and considering the advantages of PIMs compared to PLM, we have performed a series of experiments devoted to compare J_{PIM} with fluxes in plant roots. Thus, the accumulated Zn in the roots was measured and "normalized" fluxes (J_{root}) were computed by using the dry weight of the roots instead of an area. Zn normalized fluxes obtained were in the range 0.56 ± 0.05 - 1.10 ± 0.05 nmol mg^{-1} h^{-1}. A control experiment with no Zn supplementation, showed also some metal uptake (0.12 ± 0.02 nmol mg^{-1} h^{-1}) due to the presence of just a trace amount of Zn in the donor solution.

The normalized biological fluxes are shown in figure 6 for the different treatments. An ANOVA test was performed and significant differences ($p = 0.05$) were found among the treatments. As can be seen, in the treatment with EDTA, the obtained root plant uptake was lower compared to the treatment without ligand, which supports that the total concentration of Zn in the solution is not the best indicator for uptake. This tendency can also be observed in Fig. S2 (Supporting Information), where normalized J_{root}-values are represented in front of J_{PIM}.

In the presence of humic acid and citrate, the found root fluxes are practically identical to the fluxes without ligand. Similar results for citrate were found by other authors (Gramlich et al., 2013) and were attributed to the high lability and mobility of these metal-organic complexes that dissociate in the vicinity of the root surface. The direct uptake of undissociated complexes has also been reported. In these situations, FIAM would not apply (i.e. the uptake is not proportional to the free concentration).

In the case of HA, we have observed the formation of lateral roots (Fig. S3 in the Supporting Information), which in turn would increase the exchange area and would yield a metal uptake higher than expected. Moreover, the adsorption of humic substances into biological surfaces can increase their negative surface charge and, thus, enhance their overall electrostatic attraction for cations (Zhao et al., 2016). The increase of cell permeability in the presence of humic acids can contribute to metal internalization as described in (Slaveykova et al., 2003; Vigneault et al., 2000;Samson and Visser, 1989). For higher plants as spinach or wheat, deviations from the FIAM have repeatedly been reported (Degryse et al., 2009; Gramlich et al., 2013) pointing out that metal uptake in roots is highly dependent on many factors, such as the plant species, metal concentration and the rate of dissociation of metal complexes, among others.

Fig. 6.

4 Conclusions

In this study we have developed a simple technique based on a polymer inclusion membrane to assess Zn speciation in a complex nutrient solution. The stability of this type of membrane is substantially improved, rendering this technique suitable for (relatively) long speciation experiments. When a PIM made of 70% CTA and 30% D2EHPA is incorporated in a special device and a 0.01 M HNO_3 solution is used as an acceptor phase, a correlation is found with the free metal, both in absence and in presence of ligands (EDTA, citrate and humic acids). This correlation is the basis for PIM to determine $[Zn^{2+}]$ and has been justified with a model consistent with existent evidences. We have also evidenced the need to consider viability and physiological changes of the roots prior to study metal internalization. Additionally, when comparing J_{PIM} and metal uptake by potato plant roots, different behaviours arise depending on the ligand. In the case of Zn-citrate and Zn-HA complexes we have observed a direct proportionality between total metal and root uptake. However, in the case of EDTA, this relationship is observed for free metal.

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Figure captions

Fig 1. Schematics of the PIM device and the whole setup used in the experiments

Fig 2. Evolution of PIM flux in front of the device deployment time. The initial concentration was 35 μM of Zn. The values show the mean ± SD (n = 2).

Fig 3. J_{PIM} at different free Zn concentrations in the donor phase added to the half-strength Hoagland medium. PIM deployment time was 24 h. The values show the mean ± SD (n=3).

Fig 4. Comparison of $[Zn^{2+}]$ determined with the PIM device and the free concentration calculated with MINTEQ for several source solutions consisting of half strength Hoagland medium without and with added ligands (EDTA, HA and citrate). Deployment time was 48 h. Full symbols, 70 μM total Zn; empty symbols, 35 μM total Zn. The values show the mean ± SD (n=3). The solid line is the 1:1 along which $[Zn^{2+}]_{MINTEQ}$ would exactly match $[Zn^{2+}]_{PIM}$.

Fig 5. Representative epifluorescent micrographs of roots stained with FDA (green nuclei) showing the living cells and PI (red nuclei) showing the dead cells after different treatments: control with no Zn added (a) and 35 μM of Zn content (b) supplemented with 20 μM EDTA (c), 60 mg L^{-1} of HA (d), 240 μM citrate (e) and 20 μM of histidine(f).

Fig 6. Comparison of normalized root fluxes (35 μM Zn treatment and supplemented with EDTA, HA and citrate as ligands). Deployment time was 48 h. The values show the mean ± SD (n = 3).