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Measuring zinc in biological nanovesicles by multiple analytical approaches

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ABSTRACT

Exosomes are nanovesicles known to mediate intercellular communication. Although it is established that zinc ions can act as intracellular signaling factors, the measurement of zinc in circulating nanovesicles has not yet been attempted. Providing evidence of the existence of this zinc fraction and methods for its measurement might be important to advance our knowledge of zinc status and its relevance in diseases.

Exosomes from 0.5 ml of either fresh or frozen human plasma were isolated by differential centrifugation. A morphological and dimensional evaluation at the nanoscale level was performed by atomic force microscopy (AFM) and Transmission Electron Microscopy (TEM).

Energy Dispersive X-Ray Microanalysis (EDX) revealed the elemental composition of exosomes and their respective total Zinc content on a quantitative basis. The zinc mole fraction (in at%) was correlated to the phosphorous mole fraction, which is indicative for exosomal membrane material. Both fresh (Zn/P 0.09 ± 0.01) and frozen exosomes (Zn/P 0.08 ± 0.02) had a significant zinc content, which increased up to 1.09 ± 0.12 for frozen exosomes when treated with increasing amounts of zinc (100–500 μM; each $p < 0.05$). Interestingly, after zinc addition, the Calcium mole fractions decreased accordingly suggesting a possible exchange by zinc.

In order to estimate the intra-exosomal labile zinc content, an Imaging Flow Cytometry approach was developed by using the specific membrane permeable zinc-probe Fluozin-3AM. A labile zinc content of 0.59 ± 0.27 nM was calculated but it is likely that the measurement may be affected by purification and isolation conditions. This study suggests that circulating nano-vesicular-zinc can represent a newly discovered zinc fraction in the blood plasma whose functional and biological properties will have to be further investigated in future studies.

1. Introduction

Exosomes are cell-derived nanovesicles (20–100 nm) that are present in all eukaryotic fluids, including blood and urine, but also in the medium of cell cultures [1,2]. The significance of these extracellular vesicles lies in their capacity to transfer information between cells thereby influencing the recipient cell's function [1,2]. Extracellular vesicle-mediated signals can be transmitted by all the different biomolecule categories such as protein, lipids, nucleic acids and sugars and the unique package of this information provides both protection and the option of simultaneous delivery of multiple different messengers even to sites remote to the vesicular origin [1,2].

Among all the proteins, the database ExoCarta counts more than 40 zinc finger proteins [3]. All these evidences suggest a potential role of exosomes as novel zinc carriers, as well as a potential zinc function in mediating the biological activity of these nanovesicles. Considering the importance of zinc in most cellular signalling pathways [4,5], it would be interesting to evaluate the amount of zinc in the exosomes and whether the exosomal zinc concentration changes under different environmental conditions. The biological fingerprints of exosomes

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practically mirror those of the parental cells they are originated from [2]. Therefore, measuring exosomal zinc can be a proper method to detect zinc deficiency in patient care. This could have a deep impact for both researchers and clinicians considering the lack of a reliable, responsive, and specific indicator of zinc status [6].

However, the ultra-small size of exosomes poses significant challenges to the accuracy and reliability of their identification, characterization and quantification. To date, the most widespread method applied for exosome quantification is the immunoaffinity capture [7]. This method uses proteins on exosomal membranes (i.e. CD63, CD81) as recognition sites for exosome isolation and quantification [7–9]. Other analytical approaches such as Dynamic light scattering [10], Nanoparticle Tracking Analysis [11,12], Electron Microscopy [12,13], Surface plasmon resonance [14,15] and Atomic Force Microscopy [16–18] have also shown their potential application on exosomes characterization and quantification due to their high lateral resolution in the nanometer range. Beyond that, fluorescent labeling of nanovesicles by high-resolution and imaging flow cytometry [19,20] has been receiving much attention owing to the increasing development of fluorescent probes. The opportunities offered by the commercial availability of membrane permeant specific probes, provide the potential to extend these applications to the measurement of nanovesicular zinc.

The aim of this work was to characterise and measure for the first time the amount of zinc in isolated human circulating exosomes through multiple analytical approaches in order to set up a reliable method of zinc quantification for further research and clinical purposes.

The quantitative nanoscale morphological, biomechanical and surface biomolecular properties of exosomes were analyzed by atomic force microscopy (AFM) and transmission electron microscopy (TEM). Energy dispersive x-ray microanalysis (EDX) yielded the elemental composition of exosomes on a quantitative basis whereas imaging flow cytometry evaluated the intra-exosomal zinc content. In order to evaluate the possibility of exosomes to interact with extra-exosomal zinc-loaded molecules, serial treatment with different zinc concentrations was performed. ELISA assay was performed in order to evaluate the effect of the storage temperature on exosomes integrity.

2. Material and methods

2.1. Study sample

The study was approved by the Ethics Committee of the INRCA Institute and was performed in accordance with the 1964 Declaration of Helsinki. In this study, zinc determination was developed in exosomes obtained from human plasma of a healthy young donor. Plasma was isolated from LiHep-whole blood (obtained by phlebotomy after overnight fasting), by centrifugation at 300*g* for 15 min at RT. Half of the plasma sample was immediately processed (exosome isolation) in order to obtain “exosomes from fresh plasma”. The other half was frozen 1 week at −20 °C and processed later on in order to obtain “exosomes from frozen plasma”.

2.2. Exosome isolation and sample preparation

Exosome isolation was performed following the method published by Thery et al. in 2006 [21]. In brief, the human plasma sample was centrifuged a) at 2000*g*, 30 min at 4 °C and b) at 12,000 g, 45 min at 4 °C in order to discard dead cells and cell debris. The supernatant was recovered after each centrifugation and finally ultracentrifuged at 110,000*g* for 2 h at 4 °C in order to obtain the pellet containing exosomes. The pellet was then suspended in PBS (Carlo Erba; w/o Ca and Mg) and the solution was filtered through a 0.22 μM filter. Finally, two more ultracentrifugations at 110000*g*, 70 min, 4 °C were performed in order to obtain purified exosomes.

All the pellets were resuspended in 300 μl of PBS in order to prepare the samples (n = 3 each one) for the following determinations:

a) ELISA protein determination

ELISA was performed on:

- whole plasma samples: a) kept 1 week at +4 °C and b) kept 1 week at at −20 °C
- purified exosomes: c) kept 1 week at at +4 °C and d) kept 1 week at −20 °C

in order to:

- compare exosomes recovery between whole plasma and purified exosomes
- evaluate the effect of the storage temperature on exosomes integrity.

b) AFM assay

AFM was performed in exosomes from frozen plasma samples. In order to obtain a uniform exosome dispersion for the AFM assay, only 5 μl of the suspension (purified exosomes) were plated on a highly oriented pyrolytic graphite (HOPG) layer and left to dry.

c) TEM − EDX assay

TEM-EDX was performed in exosomes isolated from both fresh and frozen plasma samples. Purified exosomes diluted in PBS (1:50) were dried on formvar-coated Al-grids (100 mesh) and washed three times with ddH_2O prior to a final drying step. Three additional samples were prepared by incubating exosomes with 100 μM, 200 μM or 500 μM $ZnSO_4$. Pyrithione (100 μM) was also added in order to induce zinc loading into exosomes. Afterwards, exosomes were washed in PBS (70 min at 100000 g, 4 °C) to remove excess zinc-loaded buffer. Finally, exosomes were dried to TEM grids, washed again 3 times with water and dried again for EDX evaluation in the TEM.

d) Imaging Flow Cytometry

Imaging Flow Cytometry was performed in exosomes from frozen plasma samples. Purified exosomes from plasma were assessed alone (without probes) and in presence of the following membrane permeable probes:

Carboxyfluorescein succinimidyl ester (CFSE) (Thermofischer Scientific) was used to gate the exosome area. The gates obtained from the samples treated with CFSE were also used in the samples labelled with the membrane permeant zinc specific probe Fluozin-3-AM (Sigma Life Science) in order to measure the labile intra-exosomal zinc [22]. Each sample treated with Fluozin-3-AM was acquired at baseline. After this first acquisition, the sample was divided in two aliquots:

Aliquot 1

a) EDTA (Ethylenediaminetetraacetic acid, Fluka) (1 mM) was added 1 min before the acquisition in order to measure the extra-exosomal zinc level (1 st acquisition).
b) In the same vial, TPEN (*N*,*N*,*N*′,*N*′-Tetrakis(2-pyridinylmethyl)-1,2-ethanediamine, Calbiochem) (200 μM) was successively added 1 min before the acquisition in order to measure the baseline intra-exosomal zinc level (2nd acquisition).

Aliquot 2

a) $ZnSO_4$ (Sigma-Aldrich) (100 μM) was added 1 min before the acquisition in order to measure the maximum intra-exosomal zinc level after active transport via membrane carrier proteins or after passive transport (1 st acquisition).
b) In the same vial, Pyrithione (2-Mercaptopyridine *N*-oxide, Sigma-Aldrich) (50 μM) was successively added v1 min before the acquisition in order to measure the maximum intra-exosomal zinc level (2nd acquisition).

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