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Fetuin is the key for nanon self-propagation

Eric Chabrière [a,1], Daniel Gonzalez [a,1], Said Azza [a], Pierrick Durand [b], Farooq A. Shiekh [a], Valerie Moal [c], Jean-Pierre Baudoin [a], Isabelle Pagnier [a], Didier Raoult [a,*]

[a] Aix-Marseille Université, URMITE, UMR CNRS 7278, IRD 198, INSERM U1095, IHU Méditerranée Infection, Faculté de Médecine, 27 Bd Jean Moulin, 13005 Marseille, France
[b] Université de Lorraine, CRM2, CNRS, UMR 7036, Institut Jean Barriol, Faculté des Sciences et Techniques, 54506 Vandoeuvre-les-Nancy, France
[c] Aix-Marseille Université, Service de Néphrologie, Hôpital de la Conception, Bd Baille, 13005 Marseille, France

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ABSTRACT

"Nanobacteria", also known as nanons or calciprotein particles (CPP), are nano-sized protein mineral complexes which have been isolated from numerous biological sources. Nanons possess self-replication properties and contain only serum proteins (*e.g.* Fetuin-A, Albumin). Herein, we develop a simplified *in vitro* model of nanons propagation composed of only fetuin-A as a protein. Using this model, we demonstrate that fetuin from nanons possesses a different, non-native conformation. Moreover, we show that nanons induce soluble fetuin-A precipitation which could serve as a template for calcification. This phenomenon explains the observed self-propagating properties that mimic infectious behavior. We also demonstrate that renal calculi are capable of inducing a conformational change in fetuin-A, suggesting that the propagation phenomenon of nanons may occur *in vivo*.

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1. Introduction

Nanons or calcifying nanoparticles (CNP) are small coccoid-shaped calciparticles (0.2–0.5 μm in diameter) that were first discovered as blood product contaminants [1]. Since then, they have been reported to be easily isolated from various sources (*e.g.* commercial culture medium, sera from various mammals, renal calculi, placenta, saliva, and coronary artery calcification) [2].

Nanons can be cultivated and multiplied like bacteria and have been considered as the smallest life form on earth [1]. Thus, these particles have been dubbed "nanobes" or "nanobacteria". Nevertheless, evidences demonstrate that nanons cannot be considered as living organisms. First, nanons do not possess nucleic acid or 16sRNA [3–5], and thus their propagation is resistant to both DNase and RNase [3]. Second, nanons are more than a ten times smaller than bacteria and barely ten times bigger than ribosomes, a size incompatible with known metabolic machinery [3]. Finally, nanons can be cultivated after γ-irradiation of serum [1,6] and are resistant to 90 °C heating for 1 h, treatments that would kill typical bacteria. Despite all this evidence, the controversy remains. Indeed, some authors continue to affirm that nanons are living particles [2,7,8].

To explain nanon formation, two theories have been proposed [9]. The first hypothesized a precipitation process of calcium phosphate and a calcium-binding inhibitor factor (such as fetuin-A or albumin) [9]. The second proposes a transmissible conformational change in fetuin-A, reversing its anti-precipitation properties [3]. In both hypotheses, it is proposed that fetuin-A is the key to nanon formation.

Fetuin-A is a ~360 residues glycoprotein that is present in circulating blood and is synthesized by hepatocytes. Fetuin-A is composed of 3 domains, two of which are homologous to cystatin, a protein with affinity towards hydroxyapatite salts that prevents crystal growth [10,11]. Thus, fetuin-A inhibits soft tissue calcification by forming a soluble colloidal microsphere of fetuin A–calcium phosphate complex in the blood stream [12]. Computer-modeled domain structures suggest that a dense array of acidic residues on an extended beta-sheet of the cystatin-like domain mediates efficient inhibition of calcification [13]. Although evidence suggests the involvement of fetuin-A in calcification processes, there are also many discordant findings regarding this molecular relationship [14].

Many studies in the literature report a significant role for nanons in the etiopathogenesis of various diseases, particularly related to

* Corresponding author. URMITE, UMR CNRS 7278, IRD 198, INSERM U1095, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille Cedex 5, France. Tel.: +33 (0)4 91 32 43 75; fax: +33 (0)4 91 38 77 72.
E-mail address: didier.raoult@gmail.com (D. Raoult).
[1] These two authors contributed equally to this work.

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pathological calcification. Nanons serve as nidi for calcification and consequently promote it [15]. Indeed, nanons have been isolated from most kidney stones [15–17], from gallstones [18], from calcification in aortic valves or vessels [19–22], and from placenta [7,23]. These relationships were confirmed *in vivo* using animal models; injection of nanons into an animal induces calcification of tissues such as kidney [22,24] or aorta [25]. Consequently, nanons are considered to be new agents of emerging infectious diseases. In this article, we investigate the role of fetuin-A in nanon propagation using a medium containing fetuin-A as the sole source of protein.

2. Material and methods

2.1. Nanons culture

The "strain" of nanons used in this work was obtained from BioMerieux (Marcy L'étoile, Lyon, France). The nanons were propagated in 0.2 μm sterile-filtered Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Peisley, UK) with high glucose (4.5 g/L) and depleted in sodium pyruvate. This medium was supplemented with 0.7 mM of $CaCl_2$ (Sigma–Aldrich, Lyon, France) and with 1 mg/ml of bovine fetuin-A (Alpha-diagnostics, Texas, USA). These particles were subcultured after 2–3 weeks by diluting the culture 1:10 (v:v) in the same medium. After 5 passages, they were harvested.

2.2. Preparation of nanons samples for 2D SDS-PAGE and Western-blot

Flasks containing nanons were scraped; the resulting suspension was centrifuge at 12,000 rpm during 30 min and washed with Phosphate Buffer Saline (PBS). Nanons were then decalcified with EDTA 0.5 M at pH 8.9 during 4–6 h under shaking, and then dialyzed overnight against 1 L of 10 mM of PBS pH 7.5 at 4 °C using Spectra/por2 Dialysis Membrane (12–14 kDa molecular weight cut-off, Spectrum Laboratories, CA, USA). These decalcified particles containing only proteins were used for structural and biochemical analysis. The same extraction protocol was performed for renal calculi.

2.3. 2D SDS-PAGE and Western-blot

Extracted proteins from nanons were analyzed by 2D SDS-PAGE and Western blotting as previously described [26]. Proteins extracted from renal calculi were analyzed by Western-blot as previously described, using anti-human fetuin (1:10,000) antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) [3].

2.4. Circular dichroism

Native bovine fetuin-A was put in contact with 10 mM of PBS and 10 mM $CaCl_2$ (Sigma–Aldrich, Lyon, France) during 1 h. Then, native bovine fetuin-A was decalcified using the same protocol as fetuin-A extracted from nanons. Both fetuin-A (decalcified and extracted from nanons) were dialyzed overnight at 4 °C against 10 mM of PBS (pH 7.5). CD spectra were recorded at 20 °C on a Jasco 810 dichrograph equipped with a Peltier thermo-regulator, using a 1 mm thick quartz cells [27]. Spectra were measured between 190 and 260 nm with a scanning speed of 20 nm/min and a data pitch of 0.2 nm. Mean residual ellipticity ($[\theta]$) were calculated as ($[\theta]) = m\Delta A/(cn)$, ΔA (difference of absorption between samples and buffer), n (number of residues), m (molecular mass in Daltons), and c (protein concentration in mg/ml). Spectra were performed in triplicate at 0.1 mg/L.

The same protocol was used for fetuin-A extracted from renal calculi; excepting that native bovine fetuin-A (0.05 mg/ml) was incubated with renal calculi for 1 week and pelleted at room temperature (RT). The supernatant was used for circular dichroism. The experimental data (in the 190–260 nm range) were analyzed using DICHROWEB [28]. The CDSSTR deconvolution method was used to estimate the content in α-helical and disordered structure using the reference protein set 7. The reconstructed curves superimposed well on the experimental ones, attesting the reliability of the inferred secondary structure percentages (data not shown).

2.5. Precipitation assay

Native bovine fetuin-A (at 1 mg/ml) was incubated with approximately 25 μg of nanons. The mixture was placed in PBS at RT for 1 week. The concentration of soluble protein was checked each day using a Bradford assay (Bio-Rad, Marnes La Coquette, France); the OD_{595nm} was measured using a microplate reader (Synergy HT, Biotek, USA).

2.6. Transmission electron microscopy tomography

Nanons were sonicated for 10 min on ice and fixed in 3% glutaraldehyde overnight at 4 °C before being placed on formvar carbon film on 400 mesh nickel grids (FCF400-Ni, EMS). Grids were treated with 1% molybdate solution in filtered water at RT. Transmission electron microscopy (TEM) tilt series were acquired with a G^{20} Cryo (FEI) for a tilt range of 100° with 2° increments. The acceleration voltage was 200 keV. The applied defocus was −2 μm. The magnification was 100,000.

2.7. Powder X-ray measurements

A solution of nanons was left to evaporate overnight at 50 °C on a zero-background X-ray holder to obtain a flat and homogeneous film on the surface. Powder X-ray measurements were performed using a Panalytical X'Pert PRO diffractometer on a reflection spinner equipped with a Cu tube (Kα1 ray, 0.15406 nm), a Ge(111) monochromator and an X'Celerator detector. Data collection was conducted in the scattering angle range of 5–70° with a 0.0167° step over 15 h. Crystalline phases present in the sample were identified by searching the powder diffraction file database (pdf4) using the X'Pert High Score Plus version 2.2a PANalytical software.

2.8. Tomographic reconstruction

Tilt series were aligned using ETomo from the IMOD software package (University of Colorado, USA) [29] by cross-correlation. The tomograms were then reconstructed using the weighted-back projection algorithm in ETomo from IMOD. The isosurfaces of the nanons were generated with 3Dmod in IMOD. The images were prepared for presentation with ImageJ (NIH).

2.9. Self-propagation assay

The culture of nanon-like particles (NLPs) was performed as previously described by Young et al. [9]. Briefly, the NLPs were obtained in DMEM supplemented with 10 mM each of NaH_2PO_4, Na_2CO_3, and $CaCl_2$ and 1 mg/ml of protein (lysozyme, fetuin-A or bovine serum albumin). After 24 h of incubation under the same condition as nanons, the NLPs were used to inoculate the same medium as described above (*i.e.* DMEM supplemented with 10 mM of NaH_2PO_4, Na_2CO_3, and $CaCl_2$ and 1 mg/ml of protein). The turbidity (OD_{600nm}) was measured using a microplate reader (Synergy HT, Biotek, USA) during 14 days.

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