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Proteomic evaluation of biological nanoparticles isolated from human kidney stones and calcified arteries

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ABSTRACT

Calcifying biological nanoparticles (NPs) develop under cell culture conditions from homogenates of diverse tissue samples displaying extraosseous mineralization, including kidney stones and calcified aneurysms. Probes to definitively identify NPs in biological systems are lacking. Therefore, the aim of this study was to begin to establish a proteomic biosignature of NPs in order to facilitate more definitive investigation of their contribution to disease. Biological NPs derived from human kidney stones and calcified aneurysms were completely decalcified by overnight treatment with ethylenediaminetetraacetic acid or brief incubation in HCl, as evidenced by lack of a calcium shell and of Alizarin Red S staining, by transmission electron microscopy and confocal microscopy, respectively. Decalcified NPs contained numerous proteins, including some from bovine serum and others of prokaryotic origin. Most prominent of the latter group was EF-Tu, which appeared to be identical to EF-Tu from *Staphylococcus epidermidis*. A monoclonal antibody against human EF-Tu recognized a protein in Western blots of total NP lysate, as well as in intact NPs by immunofluorescence and immunogold EM. Approximately 8% of NPs were quantitatively recognized by the antibody using flow cytometry. Therefore, we have defined methods to reproducibly decalcify biological NPs, and identified key components of their proteome. These elements, including EF-Tu, can be used as biomarkers to further define the processes that mediate propagation of biological NPs and their contribution to disease.

1. Introduction

Calcifying biological nanoparticles (NPs) have been identified from diverse tissue samples including kidney stones and calcified aneurysms [1]. Whether or not they represent independent biological entities or a form of self-perpetuating biomineralization remains controversial [2–4]. Given the evidence that diverse forms of NPs have biological affects, both possibilities are of interest because of their potential contribution to pathophysiological processes [5].

Biochemical and spectral analysis shows that some NPs are composed of proteins, carbohydrates, lipids and nucleic acids [2–4,6–8]. However, whether NPs contain a unique collection of biomolecules, and whether they possess specific biomolecular and biological activity, is unknown. Probes to definitively identify NPs in biological systems are lacking. Therefore, the aim of this study was to take steps to establish a proteomic biosignature of NPs in order to facilitate more definitive investigations of their contribution in disease.

2. Material and methods

All protocols were approved by the Mayo Clinic Institutional Review Board.

2.1. NP isolation and propagation

All water used for experiments was double-distilled. Both water and phosphate-buffered saline (PBS) used to wash isolated NPs were 0.2 μm-filtered before use. To separate NPs from solution they were centrifuged at 60,000*g* for 1 h at 4 °C followed by pellet resuspension. For propagation, NPs were placed in standard culture medium (Dulbecco's modified Eagle's medium (DMEM) supple-

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mented with 10% γ-irradiated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA)), and maintained in a tissue culture incubator (37 °C, 13% CO_2). NPs were prepared from calcified aneurysms (including strain A2), as previously described [1]. NPs were also prepared from calcium phosphate kidney stones (strains HA399 and AP11) after compositional analysis by infrared spectroscopy performed in the Mayo Clinic Metals Laboratory. Human stones were washed with distilled nanopure water, dried, pulverized using a mortar and pestle, and stored at 4 °C in plastic vials. To extract NPs, pulverized stones were demineralized using 1 N HCl for 10 min with constant stirring, neutralized with 1 N NaOH and centrifuged. The pellet was suspended in DMEM, filtered through a Whatman No. 42 filter, sterile-filtered through a 0.2 μm Millipore filter, inoculated into 250 ml vented tissue culture flasks (Corning; Corning, NY) containing 70 ml of standard culture medium and placed in incubation. NP replication was assessed qualitatively using light microscopy (Olympus BX41 microscope equipped with a CytoViva dark-field adapter and a ×100 UPlanFLN oil lens; CytoViva, Inc., Auburn, AL) and quantitatively by turbidimetry in nephelometric turbidity units (NTU) (Model 2100N Turbidometer Hach Co., Loveland, CO). Every 2–4 weeks flasks containing adherent calcific NP biofilm were scraped with a rubber spatula, diluted 1:10 into fresh standard culture medium and subcultured. Representative flasks were screened for *Mycoplasma* contamination using a sensitive rapid polymerase chain reaction test performed in the Mayo Clinic Microbiology Laboratory, and were always negative.

Flasks were scraped after 30 days incubation to harvest calcified NPs for experiments, and the resulting NPs (free-floating combined with those released by the scraping) were pelleted as described above. Where indicated, NPs in the resulting pellet were decalcified by incubation of the pellet in: (i) 0.5 M ethylenediaminetetraacetic acid (EDTA), 4 °C for 16 h; (ii) PBS, pH 4, 4 °C for 16 h; or (iii) 0.5 N HCl for 5 min. In other experiments performed to define conditions that might favor propagation of NPs lacking a calcium shell, NPs were seeded into medium adjusted to low calcium (0.18 mM) and varied pH (7.5, 6.5 or 5.5). After 4 weeks the presence of free and biofilm-adherent NPs was semi-quantitatively scored (0–3+)

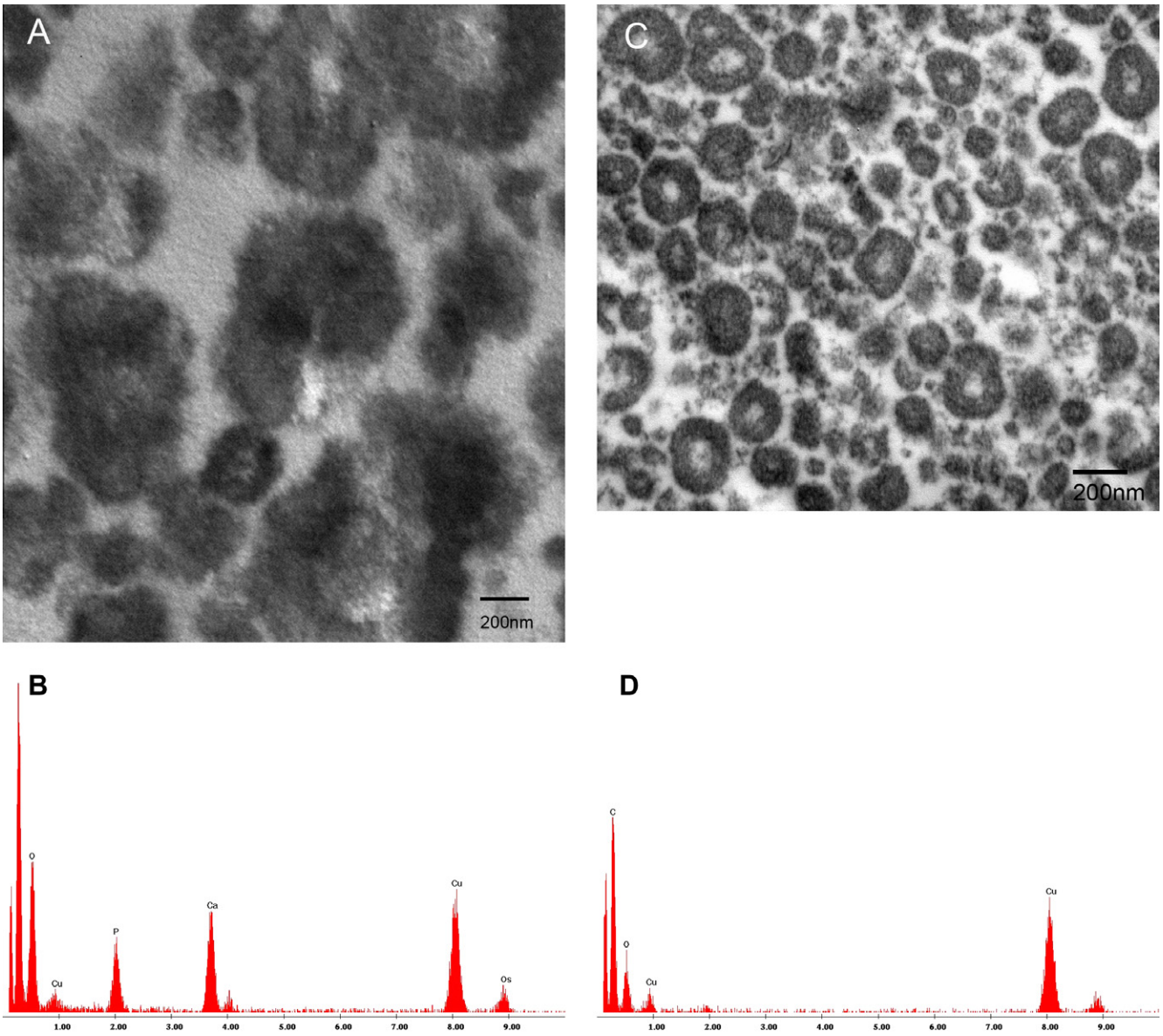

Fig. 1. Transmission electron micrographs of biological NPs derived from a human kidney stone. Prior to demineralization (A), NPs contained a thick calcium phosphate shell, as confirmed by EDS (B). After decalcification with EDTA, an electron dense core remained (C) that lacked calcium, as determined by EDS (D).

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