Isolation of calcium phosphate crystals from complex biological fluids using bisphosphonate-modified superparamagnetic beads†

Aaron Hernandez-Santana,[a] Alexander Yavorskyy,[a] Adedayo Olinyole,[a] Geraldine M. McCarthy[b] and Gillian P. McMahon*[a]

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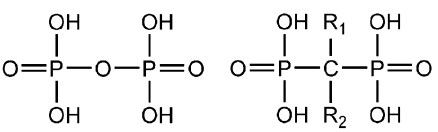
The surface of superparamagnetic magnetic beads was modified with bisphosphonates to selectively capture calcium phosphate crystals from complex biological fluids (*i.e.* synovial fluid).

Calcium phosphate crystals such as hydroxyapatite (HA), basic calcium phosphate (BCP) and calcium pyrophosphate dihydrate (CPPD) are known to occur in intra-articular synovial fluid and may be linked to a number of destructive joint conditions.[1] For instance, BCP crystals are found in up to 60% of synovial fluid samples from patients with knee joint osteoarthritis (OA) and CPPD crystal deposition is observed in a variety of clinical manifestations including acute pseudogout, which suggest that calcium phosphate crystals may represent a potential therapeutic target.[2] Progress in understanding the role of calcium crystals in different disease states is hindered by our limited ability to detect them accurately.[3] Hence, the development of potential therapies is restricted. Routine diagnosis of intra-articular crystal arthropathies relies on the use of polarised microscopy to detect the presence of crystals from a sample aspirated from the affected joint.[4] However, these methods fail to detect submicron sized crystals and suffer from a large degree of bias and inconsistency, as described in a number of different reports.[5] Previous studies have used enzymatic[6] and chemical degradation methods[7] in an attempt to isolate inorganic crystals from the inherently complex synovial fluid matrix, but these approaches are very time consuming and again, prove ineffective when the crystals are present in low abundance.

The use of superparamagnetic microparticles as solid supports for the selective capture of analytes is extraordinarily broad, and has been widely exploited in biomedicine.[8] Amongst their many advantages, microparticles have a large surface area which is potentially available for sites of adsorption and desorption and chemical reactions. The microparticles are magnetic only when placed in a magnetic field, with no magnetic remanence when the magnetic field is removed, which allows manipulation and separation of the particles from the rest of the sample. They are commercially available in a range of sizes and the surface chemistry may be tailored to suit a particular application, such as the conjugation of antibodies, proteins and synthetic oligonucleotides to microparticles to provide the specific capture of a desired analyte.[9]

In this respect, we have identified the potential of using surface bound bisphosphonates to trap calcium crystals dispersed in biological fluids, based on their known affinity for solid-phase calcium phosphate. Bisphosphonates (BPs) are an important class of drugs used in the treatment of osteoporosis and other conditions that involve bone fragility.[10] Their success as therapeutic drugs relies on their exceptional selectivity and strong affinity for solid-phase calcium phosphate as found in bone mineral (mainly carbonated HA) but they are also known to bind strongly to BCP and CPPD crystals.[11] BPs are analogues of naturally occurring pyrophosphate in which the linking oxygen is replaced with a carbon atom, resulting in resistance to hydrolysis (Fig. 1). Binding is thought to occur through optimised chelation of the bisphosphonic moiety with superficial Ca^{2+} ions on the bone surface.[12] The α-carbon in bisphosphonates typically carries two separate substituents, R_1 and R_2, which may significantly affect the mineral affinity and the pharmacological activity.[13] BPs with an –OH group in the R_1 position have increased affinity for bone mineral while amino-containing BPs typically exhibit a higher potency in antiresorptive effects.[14] Not surprisingly, these unique bone-seeking properties have been used to synthesise medicinal agents for a number of applications, including bone imaging and selective delivery of macromolecular therapeutics to the bone surface.[15]

In this work, we have combined the chemical specificity of BPs towards solid-phase calcium phosphate with magnetic separation technology to selectively extract crystals from synovial fluid. Fig. 2A shows the synthetic route for preparing bisphosphonate-modified superparamagnetic magnetic beads (BPSM beads). Amongst the commercially available BPs, the alkyl amine containing analogues such as neridronate **4** (Sigma Aldrich, UK) provide suitable anchoring groups for



1 **2**

Fig. 1 Structure of pyrophosphate **1** and its synthetic analogue **2**, the bisphosphonate.

[a] *Bioanalytical Chemistry & Diagnostics Group, National Centre for Sensor Research, School of Chemical Sciences, Dublin City University, Dublin 9, Ireland. E-mail: gillian.mcmahon@dcu.ie; Fax: (+353) 1700 5503; Tel: (+353) 1700 5914*
[b] *Mater Misericordiae University Hospital, Dublin 7, Ireland*

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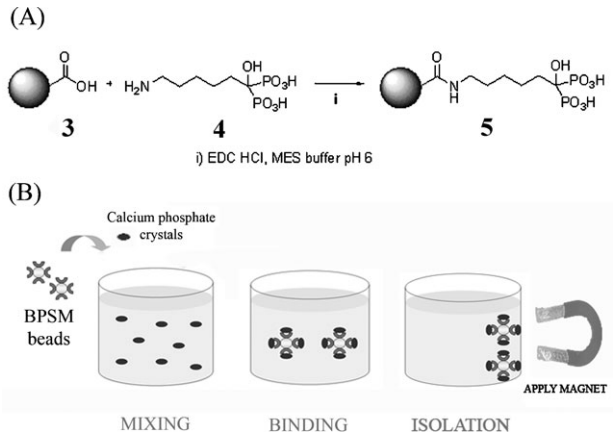

Fig. 2 (A) Modification of the surface of superparamagnetic beads with bisphosphonate and (B) procedure for the isolation of calcium phosphate crystals from synovial fluid.

further derivatisation and conjugation *via* the free amine, while leaving the bisphosphonic moiety intact. The superparamagnetic beads used in this experiment were commercially available (Dynabeads® My One™, Dynal Biotech Ltd.) and approximately 1 μm in size. Carboxylated superparamagnetic beads **3** (300 μl, 10 mg ml^{-1}) were reacted with compound **4** (225 μl, 10 mg ml^{-1} in MES buffer, pH 6) to yield the BPSM beads **5** using a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC HCl, 75 μl, 10 mg ml^{-1}) as a coupling mediator. Carbodiimide chemistry was found to be the most suitable for this purpose since these coupling reagents are generally cheap, show high conversions at room temperature and can be used to make amide bonds efficiently. The coupling reaction was carried out at 4 °C for 18 h with occasional mixing on a vortexer and the amino-bisphosphonate and carboxylated magnetic beads were pre-mixed prior to the addition to ensure immediate availability of the amine ligand for conjugation to the activated carboxyl group. A magnet was used to pull the beads to the side of the reaction vessel during the washing steps, allowing the supernatant to be removed using a pipette. The highly polar groups on the bead surface provide excellent dispersion properties in aqueous media at neutral pH and are hence suitable for use directly in synovial fluid.

We tested the ability of the BPSM beads to bind calcium phosphate crystals by mixing them with synovial fluid spiked with HA, BCP or CPPD synthetic crystals (1 mg ml^{-1}). Synovial fluid (SF) aspirated from the knees or shoulders of patients with rheumatoid arthritis (RA) was used as a diluent since BCP crystals are rarely found in RA joint fluids.[16] Aspirated SF samples were stored in plastic syringes at 4 °C and used within 3 months. The isolation of calcium phosphate crystals from SF was carried out in three steps: mixing, binding and isolation (Fig. 2B). Firstly, BPSM beads (10 μl, 10 mg ml^{-1}) were mixed and incubated under rotary mixing with the SF samples (100 μl) and chromatography grade water (400 μl) for 1 h to promote crystal capture. Removal of residual biological material (which can be used for further examination of other biomarkers of interest) was achieved by using a small permanent magnet to pull the beads to the side of the vessel. Washing steps were introduced after the initial binding step to remove any unwanted material. The whole extraction process takes less than 90 min and it may be adapted to a 96-well format for higher sample throughput.

Crystal capture was assessed using scanning electron microscopy (SEM). Fig. 3 shows SEM images of the BPSM beads (3A) and of the different calcium crystals captured after selective isolation (3B–D). The commercially available magnetic beads were approximately one micron in diameter while the synthetic crystals used in this study ranged from submicron to ~100 μm in size. SEM images clearly showed attachment of the derivatised beads to the crystal surfaces, presumably through the binding of the bisphosphonate moiety to free Ca^{2+} sites. To test the applicability of this separation technique to real samples (un-spiked), we carried out the extraction protocol described above on samples from patients diagnosed with CPPD deposition disease and/or OA. The SEM images confirmed the presence of crystals as expected (Fig. 3E). The composition of the crystals isolated from one of the test patients was probed using energy dispersive X-ray (EDX) spectroscopy, confirming the presence of calcium, phosphorus and oxygen within the crystals (Fig. 3F).

Fig. 4 shows an SEM image of a group of crystals isolated from the synovial fluid of a patient with suspected OA. Multiple EDX point spectra were carried out on this sample to create compositional maps showing the relative distribution of the three main elements of interest in the sample, namely calcium, phosphorus and oxygen (Ca, P, O). The white colour over the black background shows the presence of the three

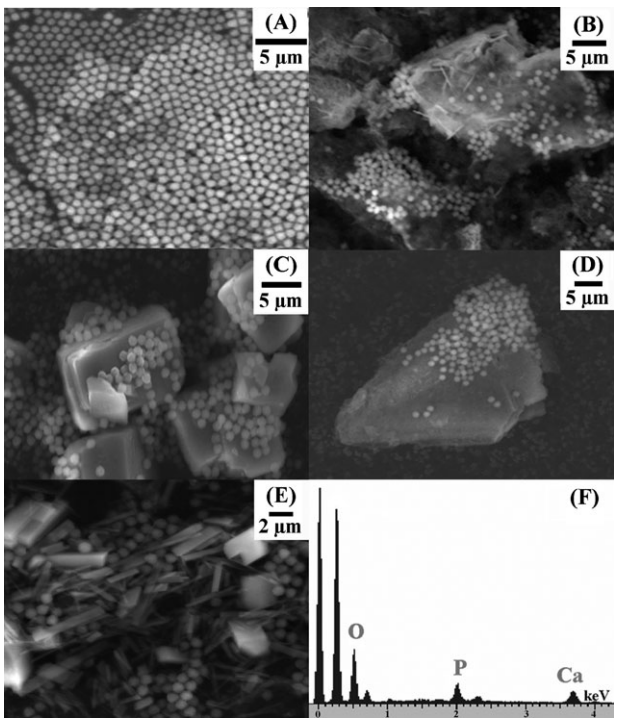

Fig. 3 Scanning electron micrograph (SEM) images of (A) BPSM beads, and captured (B) BCP, (C) CPPD and (D) HA synthetic crystals. (E) SEM image of crystals isolated from the synovial fluid of a patient with suspected OA. (F) EDX spectrum of crystals isolated found in (E).

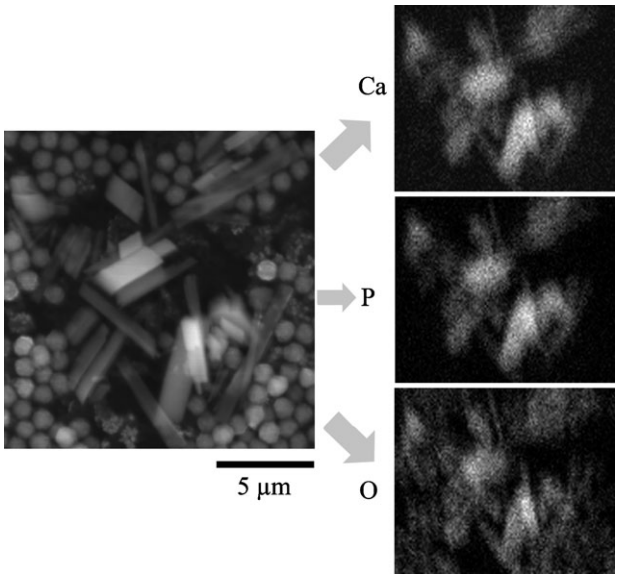

Fig. 4 Scanning electron micrograph (SEM) of crystals isolated from the synovial fluid of a patient with suspected OA and EDX maps for the elements Ca, P and O of the same region.

different elements within the scanned image, which may be correlated to the crystals observed on the SEM image. Ca and P signals were found almost exclusively in regions where crystals were present, whereas O was found throughout the sample since the beads also contain this element in their magnetic core and polymer shell.

In summary, we have employed the chemical specificity of bisphosphonates for solid-state calcium phosphate to create an extraction platform which can isolate crystals from complex synovial fluid samples in a simple and selective manner. This was demonstrated by the extraction of crystals from spiked samples and ultimately from fluid from patients diagnosed with crystal arthropathies. The extraction method is non-destructive, which allows further tests (such as pH, hyaluronic acid content, cell count, *etc*.) to be carried out on the synovial fluid samples to complement the diagnosis. We are currently investigating the combination of this extraction method with simple detection techniques based on colorimetry and Raman microspectroscopy. Isolation and subsequent detection of calcium phosphate crystals in synovial fluid could serve as a tool for improved diagnosis and characterisation of OA and other calcium phosphate crystal deposition diseases.

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