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In vivo release of wear debris and corrosion products from the metallic interfaces of total hip replacements is associated with a wide spectrum of adverse body reactions and systemic manifestations. The origin of debris and the electrochemical conditions at the sites of material loss both play a role in determining the physicochemical characteristics of the particles, and thus influence their in vivo reactivity. Debris retrieved from revised CoCrMo tapers and cement-stem interfaces consists of heterogeneous flakes that comprise mechanically mixed metal particles, corrosion products and organic material. Detailed investigation of the size and composition of the metal debris contained within these composites requires the digestion of the flakes to release the small metal particles. Here, we compare alkaline and enzymatic digestion methods that both aim to fragment the flakes and reveal their smallest building blocks. The characterization of debris cleaned with both methods revealed crystalline Cr oxide nanoparticles and clusters. Comparison between the treatments showed that the alkaline method is more efficient in fragmenting the flakes and provided cleaner and generally smaller nanoparticles than exhibited in debris released with the enzymatic treatment. The provision of cleaner nanoparticles from the alkaline method also allows the physicochemical properties of the particles to be more clearly identified. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2018.

Key Words: digestion of wear and corrosion flakes, nanoparticle characterization, electron microscopy, hip replacements, taper and stem debris

INTRODUCTION

With the introduction of modularity to the femoral components of total hip replacements, the issue of adverse local tissue reactions (ALTRs) to metal debris has been extended to include ceramic-on-polyethylene (CoP),1–3 metal-on-polyethylene (MoP),4–7 and metal-on-metal (MoM)8–11 total hip replacements. The wear and corrosion products released from the taper junctions of these implants have been found to be more reactive than the equivalent amount of debris released with the enzymatic treatment. The provision of cleaner nanoparticles from the alkaline method also allows the physicochemical properties of the particles to be more clearly identified. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2018.

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With the introduction of modularity to the femoral components of total hip replacements, the issue of adverse local tissue reactions (ALTRs) to metal debris has been extended to include ceramic-on-polyethylene (CoP),1–3 metal-on-polyethylene (MoP),4–7 and metal-on-metal (MoM)8–11 total hip replacements. The wear and corrosion products released from the taper junctions of these implants have been found to be more reactive than the equivalent amount of debris released from the MoM hip bearings,12,13 and the explanation may reside in the different physicochemical characteristics of the wear and corrosion products. At the crevice, debris is released by “mechanically assisted crevice corrosion” which can result in both wear particles and corrosion products.14 The wear mechanisms and electrochemical conditions at these sites also yield distinct particle sizes, morphologies and chemical compositions. These properties dictate the fate of the particles within the body15–18 and are responsible for the onset of ALTRs even in the absence of metal debris from the bearing surfaces.1–8

Previous studies on damaged tapers of explants have described signs of wear and corrosion, along with loose black and flaky products at the interface and/or around the base of the trunnions.9,20 Other studies investigated taper debris internalized in macrophages or spread in periprosthetic tissue and reported the presence of Cr₂O₃1–23 and CrPO₄.20,23,24 These previous studies, however, provide little information about the size, morphology, and composition of the individual particles released from CoCrMo tapers. It is thus difficult to identify which factors are responsible for their increased body reactivity and to hence find solutions to minimize their release.

Correspondence to: A. M. Crainic; e-mail: A.M.Crainic@soton.ac.uk
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Contract grant sponsor: Osteoplasty for Arthritis Charity
In vivo release of solid and soluble metal may also occur independently from the cement-stem interface, via tribo-corrosion. Again, however, the species generated by this wear mechanism and their physicochemical characteristics have yet to be fully understood. Most previous studies only revealed the morphology and composition of the as-retrieved flakes, which are now believed to be composites of mechanical mixing of Cr$_2$O$_3$ nanoparticles, corrosion products, organic matrix and void spaces. Thus, although these investigations provide evidence of the organo-metallic nature of these flakes, no information has provided about their constituent components.

There is considerably more information concerning the release of debris from the bearing surfaces. For example, various studies have detailed the characteristics of metal particles isolated from periprosthetic tissue and synovial aspirate, and serum lubricants liberated from these environments using protocols which involved chemicals, such as acids or alkaline solutions, enzymes or a mixture of both. These reagents aimed to breakdown the surface active compounds adhered to the particle surface, forming the bio-corona, and provide clean particles suitable for more detailed characterization. An important condition of the treatments is to preserve the characteristics of the original debris and release representative particles. Concerns regarding the dissolution of the particles during chemical treatments have made it difficult to characterize the nano-sized debris. Regardless of the mechanism used to isolate the nanoparticles, technical limitations (principally related to resolution) have made it difficult to characterize the nano-sized debris. Thus, previous reports of particle clusters may have resulted in an overestimation of the particle size. Nevertheless, while previous studies have not always been in agreement with respect to the particle size and composition, it is generally accepted that CoCrMo hip bearings release particles in the nanometer size range, composed mainly of Cr. It is apparent, therefore, that complete characterization of the nanoparticles requires their release from the surrounding matrix, with minimal changes to the morphology and chemical composition, and the use of appropriate imaging and characterization techniques able to resolve nanoparticles.

The present study compares two methods for the digestion of the organic matrix and the release of metal nanoparticles from the wear and corrosion flakes collected from around revised tapers and cement-stem interfaces. The protocols were adapted from previous studies developed to isolate hip-related debris from other biological sources. The main objectives of this study were to: (i) compare an alkaline and an enzymatic treatment with respect to the ability to dissolve the organic matrix, including biomolecules, salts and precipitates, and provide clean particles for the detailed chemical and structural characterization; (ii) compare the characteristics of the particles cleaned with the alkaline and enzymatic protocol. Resolution of these issues is important because a good understanding of the wear and corrosion products released from various wear sites in total hip replacements is essential for obtaining a reliable evaluation of the particles toxicity, using clinically relevant particles in future experimental studies.

**METHODS**

**Debris recovery**

The wear and corrosion flakes were collected from around two CoCrMo taper junctions and from the surface of a cemented CoCrMo stem, explanted at the time of revision from three distinct patients. The surgery followed the onset of patient reported pain and the diagnosis of ALTRs with abnormally high concentrations of Co and Cr ions in blood and/or aspirate samples. The Co and Cr ion levels in blood and/or aspirate, implantation time and details about the revised hip replacements, such as the components size and make, are shown in Table I. After retrieval, the explanted components were sterilized and stored in 10% buffered formalin, until further investigation. The components were visually inspected to localize loose wear and corrosion flakes, which were removed with plastic tweezers and collected in clean plastic tubes. Because the retrievals had been exposed to atmospheric humidity and oxygen prior to the investigation, no additional storage conditions were granted to the recovered flakes.

**Digestion protocols**

**Enzymatic digestion.** The protocol was adapted from those of Doorn et al. and Catelas et al. and consisted of a 24-hr incubation of the flakes (~0.8 mg) with 0.25 mg lyophilized Papain (Sigma-Aldrich, UK) in 2 mL 50 mM Tris-HCl, at 65°C, under continuous stirring at 180 rpm (Stuart, SSM1). After the first incubation, the pellets (metal

**TABLE I. Information About the Implant Types, In Vivo Times and Cr and Co Concentrations in Blood or Synovial Aspirate at the Time of Revision**

<table>
<thead>
<tr>
<th>Implant information</th>
<th>Patient 1 taper debris 1</th>
<th>Patient 2 taper debris 2</th>
<th>Patient 3 stem debris</th>
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</thead>
<tbody>
<tr>
<td><strong>In vivo time (months)</strong></td>
<td>101</td>
<td>113</td>
<td>225</td>
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<tr>
<td><strong>Source of ion levels</strong></td>
<td>Blood</td>
<td>Aspirate</td>
<td>Blood/Aspirate</td>
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<tr>
<td><strong>Co ions (nmol L$^{-1}$)</strong></td>
<td>102</td>
<td>6520</td>
<td>72/8100</td>
</tr>
<tr>
<td><strong>Cr ions (nmol L$^{-1}$)</strong></td>
<td>69.9</td>
<td>1450</td>
<td>30/6930</td>
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<tr>
<td><strong>Head make</strong></td>
<td>BHR</td>
<td>Adept</td>
<td>BHR</td>
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<tr>
<td><strong>Head size and offset (mm)</strong></td>
<td>50 + 4</td>
<td>46 + 0</td>
<td>46-4</td>
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<tr>
<td><strong>Taper make</strong></td>
<td>CPT</td>
<td>CPT</td>
<td>CPT</td>
</tr>
<tr>
<td><strong>Taper size (mm)</strong></td>
<td>12/14</td>
<td>12/14</td>
<td>12/14</td>
</tr>
</tbody>
</table>
debris) were recovered by centrifugation at 50,000g and were subject to a second incubation with 0.8 mg trypsin K (Sigma–Aldrich, UK) in 2 mL 50 mM Tris-HCl, for 24 hr, at 55°C. Before each incubation, the debris was washed with 2 mL 2.5% sodium dodecyl-sulfate (SDS) (Sigma Aldrich, UK) at room temperature and subject to ultrasound for 15 min. To preserve the native characteristics of the metal particles, the boiling steps for 10 min in the original protocols were avoided. The particles were finally recovered by centrifugation at 50,000g (Optima Max-XP, Beckman Coulter, TLA-55 fixed angle rotor), washed twice with 2 mL deionized water (DIW) and stored in 100–200 μL DIW at −20°C, prior to further analysis.

**Alkaline digestion.** The chemical treatment was also adapted from the protocol of Cetelas et al., developed for the isolation of metal particles released from bearing surfaces in bovine serum or peri-prosthetic tissue. The solid debris (0.5–1 mg flakes) was submerged in 1 mL 12 N KOH, mechanically fragmented with a plastic pipette tip and subject to 15 min of ultrasound. The suspensions were incubated at 37°C under continuous stirring (180–200 rpm), for 48 hr. During this time, the samples were subject to 15 min of ultrasound every 8–10 hr, to assist and enhance the digestion process. After 48 hr, the suspensions were centrifuged at 50,000g. The pellets were recovered and washed twice with 1 mL DIW and submersed in 100–200 μL DIW (depending on the amount of debris recovered from the treatment). The particle suspensions were stored at −20°C, until further investigation.

**Electron microscopy**

The as-retrieved flakes were studied for morphology, microstructure and elemental distribution with a FEI Quanta 200 SEM, fitted with EDX and operated at 20 kV. The high resolution characterization of the digested debris was performed with a Cs probe-corrected JEOL ARM200F (cold-FEG) TEM/STEM operated at 200 kV and equipped with a 100 mm² Centurion EDX detector (Thermo-Fisher Scientific, Madison, WI). Prior to investigation, the particles suspensions (100–200 μL) were thawed and set to 15 min of ultrasound to break down the aggregates and homogenize the suspensions. To avoid the particles oxidation, the grids were prepared shortly before the STEM analysis and were stored in a dry and cool place. Small volumes (3 μL) of the particle suspensions were loaded on carbon coated copper grids and blotted with lint free paper to remove excess water. Prior to detailed investigation with STEM, the grids were checked with TEM (FEI Tecnai T12) to ensure a uniform particle loading and the number of suspension aliquots (3 μL each) used to load each grid was adjusted accordingly. At least 75 particles from 15 to 20 micrographs were imaged per sample and cleaning treatment in order to acquire enough data for the statistical analysis. Most of the STEM images were high-resolution micrographs of individual nanoparticles or small clusters of debris. Each sample included at least two low-resolution micrographs of large particle clusters, comprising 20–30 nanoparticles each. The Fast Fourier transform (FFT) images of the high-resolution micrographs were processed with Gatan Microscopy Suite (GMS3) and the measured d-spacing was correlated with the d-spacing of the pristine Cr₂O₃ phase from the XRD database: PDF 00–006–0504 (PDF-2, 2012).

The composition of taper and stem debris was determined from the STEM and EDX analysis of individual nanoparticles and clusters. The EDX maps were processed using the NSS software (Thermo-Fisher Scientific, Madison, WI) to reveal the metal rich regions. The average proportions of Cr, Co, Mo, and other elements in individual particles and clusters were calculated using the Quantify Spectrum function. The comparison between the treatments was made based on the average compositions of all the particles from each sample and cleaning treatment. The composition study included 30 to 50 particles for the sample cleaned with the alkaline treatment (30 particles from taper 1, 50 particles from taper 2 and 34 particles from stem) and 16 to 60 particles for the samples cleaned with the enzymatic treatment (16 particles from taper 1, 60 particles from taper 2 and 32 particles from stem).

**PSD and statistical analysis**

Particle size distribution (PSD) and morphological characterization was performed according to BS ISO 17853/2011. Gatan Microscopy Suite (GMS3) software was used to measure the maximum dimension (dmax or length) and the maximum orthogonal dimension (dmin or width), defined as the longest straight line between two opposite points on the particle outline and the longest orthogonal line, respectively. The maximum diameter (dmax) was used to define the PSD, while dmax/dmin ratio value, hereafter referred to as the aspect ratio (AR), was used for the morphological characterization. The particles were classified as round for 1 ≤ AR < 1.5, oval for 1.5 ≤ AR < 2.5 and needle shaped for AR ≥ 2.5. The statistical significance of the datasets (comparison of the mean sizes) was verified using the null-hypothesis two-sample t test, at a level of confidence of 0.05. None of the size distributions were normally distributed, but the data sets had similar asymmetry which allowed the statistical comparison using t test. The selection of the appropriate t test was based on the results of the f test for each compared datasets (equal or nonequal variance assumed). The repeatability of the PSD was checked by performing three consecutive measurements for one of the three samples. The results of the three independent measurements were no statistically different (p > 0.3).

**RESULTS**

**Electron microscopy**

The morphology and elemental distributions of a representative wear and corrosion flake collected from around a revised CoCrMo taper, are shown in the SEM micrograph and EDX maps in Figure 1. The size, morphology and composition of the as-retrieved debris confirmed the complex nature of these products. The main elements of the CoCrMo alloy are preferentially distributed in the flake and are mixed with Ca and P, which likely originate from the
Biomolecules and salts in the body. Oxygen is distributed across the entire surface of the flake, but its map is more intense in the Cr rich region at the top of the as-retrieved debris and in the P and Co rich areas at the right and bottom of the flake.

The alkaline and enzymatic treatments were used to fragment the flakes and remove parts of the precipitates and organic material from around the metal debris. The micrograph in Figure 2(A) shows a cluster of debris released from taper 1 and cleaned with the enzymatic treatment, which consists of nanoparticles embedded in a less dense matrix (revealed by the HAADF contrast). The low-resolution image allows the measurement of the particle size and morphology, although the matrix around the particulate debris makes the process difficult. The low contrast between the nanoparticles and the matrix means they can only be distinguished by the particle crystallinity that is revealed by the FFT analyses, shown as insets in the high-resolution micrographs. Representative nanoparticles released from taper 1 and characterized with the high-resolution STEM are shown in Figure 2(B,C). The particles are partially embedded in the less electron dense phase, which is visible around the crystalline structures in both micrographs.

The low-resolution image in Figure 2(D) reveals clean particulate debris released from taper 2, although a closer look to the micrographs in Figure 2(E,F) reveals traces of the organic matrix around the crystalline nanoparticles. Debris released from stem 1 also comprises nanoparticles embedded in the less electron dense matrix, visible at the left edge of the cluster in Figure 2(G) and around the crystalline nanoparticles in Figure 2(H,I).

Debris cleaned with the alkaline treatment is shown in Figure 3. The low-resolution micrograph in Figure 3(A) shows debris released from taper 1, which comprises round nanoparticles partially embedded in the organic matrix. The high-resolution micrographs, however, reveal clean and crystalline particles, confirmed by the FFT insets in Figure 3(B,C). Traces of organic matrix are also visible in the top right region of the micrograph in Figure 3(C) and covering the bottom of the elongated, crystalline particle at the center of the image. The two particles on the left and bottom of the crystalline debris in Figure 3(C) may also be crystalline, but the presence of the organic matrix may impede the diffraction of the electron beam.

Figure 3(D) reveals particles released from taper 2, which are brighter and can be easily distinguished from the less electron dense matrix, present in traces. The high-resolution images in Figure 3(E,F) reveal clean and crystalline nanoparticles, which appear free of contaminants. Similarly, the cluster of debris originating from stem 1 and shown in Figure 3(G), consists of clean nanoparticles well released from the surrounding matrix. The high-resolution micrographs in Figure 3(H,I) also show clean, crystalline particles with no signs of the organic matrix.

The FFT insets of the high-resolution micrographs in Figures 2 and 3 were used to measure and compare the d-spacing with those of the pristine Cr$_2$O$_3$. The indexes are shown for each individual FFT pattern in Figures 2 and 3, and suggest the cleaned debris comprises Cr$_2$O$_3$ nanoparticles.

The nature and elemental composition of the round, crystalline nanoparticles were confirmed by EDX mapping. Figure 4 shows the elemental distribution of the main elements in debris released from taper 1 (A), taper 2 (B), and stem 1 (C) cleaned with the alkaline treatment. The EDX analysis of the particles released from taper 1 [Figure 4(A)] show well localized Cr and O maps whereas the Co distribution comprises random spots, which partially overlap the particles region. The cluster released from taper 2 also provided well localized Cr and O maps [Figure 4(B)]. The distribution of Co suggests it is localized mainly outside the Cr and O rich particles, in the right top area which corresponds to a diffuse phase. The maps of the stem debris are shown in Figure 4(C) and consist of well localized Cr and O.
distributions with weak Co signal. Other elements such as P and Ca were also present around the particles from both tapers and stem, but they provided weak maps and therefore were not shown in Figure 4(A–C). The results of the qualitative and quantitative EDX analysis after the exclusion of O, suggest that Cr is the main element of the taper and stem debris (>60%) which is found in association with O, forming Cr oxide with traces of Co, Mo, P, and Ca.

**PSD and morphology**

Figure 5 shows the PSDs for each sample after the enzymatic and alkaline protocols. The particles originating from the same wear site (i.e., taper 1, taper 2, or stem 1) and released from the flakes using either the alkaline or enzymatic treatment, have a similar size range, independently of the treatment. However, for each taper sample, the average particle sizes are smaller after the alkaline treatment [20.3 nm (±13.3) vs. 24.4 nm (±12.6) for taper 1; 15.9 nm (±11.0) vs. 23.5 nm (±14.0) for taper 2] and the difference between the mean sizes after the two treatments is statistically significant (p < 0.03 for taper 1; p < 0.0002 for taper 2). In contrast, the particles released from the stem interface have a smaller average size after the enzymatic treatment [16.9 nm (±6.1) vs. 17.1 nm (±7.1)], but the difference between the two distributions is not statistically significant (p > 0.5). The number of particles used for the size distribution, the corresponding size ranges and the mean and median particle sizes are shown for all samples and treatments in Table T2. For both taper samples the median \(d_{\text{max}}\) and interquartile ranges (IQR) are larger for the particles cleaned with the enzymatic treatment than for those cleaned with the chemical protocol (22.1 nm, IQR: 19.1 nm vs. 16.8 nm, IQR: 17.0 nm for taper 1 and 20.3 nm, IQR: 18.6 nm vs. 11.8 nm, IQR: 11.8 nm for taper 2). For the stem debris, the median \(d_{\text{max}}\) is similar for the particles cleaned with the enzymatic and alkaline treatment (15.2 nm vs. 15.9 nm, respectively), but the IQR range is wider for the particles subject to the alkaline cleaning (12.0 nm vs. 8.7 nm).

The morphological characteristics of the particles, assessed from the AR values, are shown in Table T3. The majority of the particles originating from the taper junctions and released with the enzymatic treatment are oval, followed by round and needle-shaped particles. The debris released from the same wear site and cleaned with the
alkaline protocol are mostly round or oval, with few needle-shaped particles. With both treatments, the particles released from the stem are mainly round or oval, with few needle-shaped debris.

**Particle composition**

The average proportions of Cr, Co, and Mo in the particles cleaned with the enzymatic and alkaline treatments (obtained from the EDX analyses) are shown in Figure 6. After the alkaline treatment, the particles released from tapers [Figure 6(A,B)] showed a significant decrease in the Cr content, concomitant with an increase of the Co proportion, compared to the particles cleaned with the enzymatic treatment ($p < 0.02$ for taper 1, $p < 0.0003$ for taper 2). For taper 1, the proportions of Cr and Co in particles cleaned with the enzymatic treatment were 91.3% (±5.4) and 7.9% (±5.7) respectively, and those in debris cleaned with the alkaline treatment were 73.4% (±3.3) and 25.6% (±3.3), respectively. For taper 2 the concentrations of Cr and Co after the enzymatic treatment were 88.0% (±3.5) and 10.5% (±4.0), respectively, and 79.7% (±3.6) Cr and 19.4% (±3.6) Co after the alkaline treatment.

Debris released from the cement-stem interface [Figure 6(C)] contained more Cr in the particles cleaned with the alkaline treatment [78.4% (±0.9)] than in the particles treated with the enzymatic protocol [76.7% (±1.2)]. The difference was statistically significant, but $p = 0.04$ was close to the level of confidence (i.e., 0.05). In contrast, the P content in the taper and stem samples was lower after the alkaline treatment [Figure 6(D)] [9.8% (±1.0) vs. 20.3% (±6.8), $p > 0.05$ for taper 1; 6.9% (±3.6) vs. 23.2% (±6.9), $p < 10^{-5}$ for taper 2; 2.5% (±1.1) vs. 21.1% (±1.7), $p < 10^{-5}$ for stem].

**DISCUSSION**

The presence of black flaky deposits around revised CoCrMo tapers1–11 and/or at the cement-stem interface25–29 has been reported by several studies, but only a few have investigated the particle morphology and composition. Previous characterizations of the as-retrieved flakes from around cemented stems revealed heterogeneous micron-sized debris, consisting mainly of Cr.25,26,28 Structural analysis of the surface plaque at the cement-stem interface found Cr$_2$O$_3$ nano-sized debris (~50 nm) stacked in an amorphous
organic matrix with voids. The post-mortem analysis of periprosthetic tissue from around a loose stem-cement interface found oval, needle shaped and round CoCr and chromium oxide particles, ranging from 18 to 472 nm. Analyses of the flakes from around CoCrMo taper junctions revealed CrPO₄ debris, but there was a disagreement between the histological studies which reported either CrPO₄ or Cr₂O₃ particles in periprosthetic tissue. Nevertheless, all the studies emphasized the heterogeneous nature of the wear and corrosion flakes, and all were shown to consist of metallic debris and various elements of biological origin. To better characterize the metallic debris released from the secondary interfaces of total hip replacements it is, however, necessary to liberate the nano-sized debris from the composite larger flakes.

The digestion protocols used in this study were adapted from existing methods that have been extensively used to release metal debris from periprosthetic tissue, synovial aspirates or serum lubricants. The alkaline treatment is simpler to perform than the enzymatic treatment, because it requires fewer steps, which is desirable in order to avoid contamination and particles loss. There have been concerns regarding the dissolution of the metal debris during the alkaline treatment, however, which may result in particle size reduction and composition alterations. Catelas et al. compared the effects of the alkaline and enzymatic treatments on the characteristics of CoCrMo particles generated from pin-on-disc tests and hip simulator in various lubricants, and noted a reduction of the particle size after both treatments, with more noticeable changes after the alkaline digestion. Similarly, the effects on the particle composition were more prominent for debris treated with alkaline solutions and the dissolution of Cr ions increased with the reagent concentration. The alteration induced by the alkaline and enzymatic treatments was, however, diminished by the bio-corona formed around the particles in bovine serum, because the surface active biomolecules bound to the particle surface shielded the particles against dissolution by the reagents. The heterogeneous nature of the corrosion flakes, revealed in this study by the SEM/EDX investigation, provides evidence of a similar protection mechanism for the particles against dissolution or corrosion.

The present study has shown that both the alkaline and enzymatic treatment are efficient in breaking down the particle bio-corona and releasing clean nanoparticles. The PSDs showed similar size ranges for the particles released from the same retrievals and cleaned with either of the treatments. The alkaline treatment does, however, result in mean particle sizes of the taper debris which are shifted to the

---

**FIGURE 4.** The HAADF-STEM micrographs and EDX maps of representative particles released from (A) taper 1, (B) taper 2 and (C) cement-stem interface. The composition analysis showed: (A) 74.5% Cr (±0.7), 14.8% Co (±1.1), 0.5% Mo (±0.2%), 6.1% Ca (±0.4) and 4.1% p (±0.9%); (B) 66.9% Cr (±0.2), 21.7% Co (±0.4), 0.4% Mo (±0.1%), 1.9% Ca (±0.1) and 9.1% p (±0.6%); (C) 77.6% Cr (±0.3), 18.8% Co (±0.6), 0.7% Mo (±0.1%), 0.7% Ca (±0.1) and 2.2% p (±0.3%).
lower end of the size ranges, compared to the mean sizes of the particles cleaned with the enzymatic treatment. In contrast, the particles released from the cement-stem interface do not show any significant variation in the size and morphology, regardless of the treatment used to remove the bio-corona. The composition analysis of the taper debris shows lower Cr and higher Co concentrations in the particles treated with the alkaline solution, compared to debris cleaned with the enzymatic treatment. The alkaline protocol also dissolves more of the amorphous organic phase (represented by P) from around the metal particles to yield cleaner debris, whose boundaries are well-delimitated and provide more accurate size measurements. The STEM micrographs show that the particles cleaned with the enzymatic treatment are not fully released from the surrounding matrix, and are thus more difficult to measure. The high-resolution STEM revealed the clean particles localized at the edge of the partially digested flakes, while the particles

**TABLE II. The Number of Particles Characterized for Each Sample and the Corresponding Size Ranges, Mean, and Median \(d_{\max}\), After the Alkaline and Enzymatic Treatments**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>No particles</th>
<th>Size range</th>
<th>Mean (d_{\max}) ((\pm IQR))</th>
<th>Median (d_{\max}) ((\pm IQR))</th>
<th>IQR</th>
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</thead>
<tbody>
<tr>
<td>Taper 1</td>
<td>Enzymatic</td>
<td>107</td>
<td>5–67 nm</td>
<td>24.4 nm ((\pm 12.6))</td>
<td>22.1 nm ((\pm 12.6))</td>
<td>12.6 nm</td>
</tr>
<tr>
<td></td>
<td>Alkaline</td>
<td>110</td>
<td>3–65 nm</td>
<td>20.3 nm ((\pm 14.0))</td>
<td>18.8 nm ((\pm 14.0))</td>
<td>12.8 nm</td>
</tr>
<tr>
<td>Taper 2</td>
<td>Enzymatic</td>
<td>75</td>
<td>4–66 nm</td>
<td>19.6 nm ((\pm 12.0))</td>
<td>18.2 nm ((\pm 12.0))</td>
<td>11.6 nm</td>
</tr>
<tr>
<td></td>
<td>Alkaline</td>
<td>100</td>
<td>3–60 nm</td>
<td>15.9 nm ((\pm 11.0))</td>
<td>14.8 nm ((\pm 11.0))</td>
<td>11.0 nm</td>
</tr>
<tr>
<td>Stem 1</td>
<td>Enzymatic</td>
<td>82</td>
<td>7–32 nm</td>
<td>16.9 nm ((\pm 6.1))</td>
<td>15.2 nm ((\pm 6.1))</td>
<td>7.8 nm</td>
</tr>
<tr>
<td></td>
<td>Alkaline</td>
<td>83</td>
<td>6–37 nm</td>
<td>17.1 nm ((\pm 6.1))</td>
<td>15.9 nm ((\pm 6.1))</td>
<td>12.0 nm</td>
</tr>
</tbody>
</table>

**FIGURE 5.** The particle size distribution of debris released from taper 1, taper 2 and stem 1, after the enzymatic (blue) and alkaline (red) treatments.
embedded in the amorphous phase could not be imaged or measured, and therefore had no contribution to the PSD. It is also possible that the particles are not homogeneously distributed in the flakes and the investigation of particles mainly localized toward the edge of the aggregates can contribute to the different particle size distributions observed for debris cleaned with the alkaline and enzymatic treatment.

The STEM and EDX analyses reveal that the nanoparticles released in vivo from the CoCrMo tapers and cement-stem interfaces are largely composed of Cr, with traces of Co and Mo. The amount of O present cannot be accurately quantified by the EDX technique; hence the composition of debris was estimated from the normalized proportions of Cr, Co and Mo. The analysis showed mainly Cr in all of the investigated particles, but the percentage of Cr, Co and Mo for the taper debris varies with treatment. Because Co and Mo are only present as trace elements, the normalization process applied to the small amounts of Co and Mo has larger associated uncertainties and may have resulted in a significant difference in the composition of debris cleaned with the alkaline or enzymatic protocols. In addition, there were also significant differences between the composition of particles originating from the same wear site (and patient) and cleaned with the same treatment. This observation suggests that the original uncleared particles have different compositions, which may then be reflected in the differences observed after the alkaline and enzymatic treatments. The measured composition of debris can also be influenced by the amorphous biological matrix or precipitates covering the particles, which were more prominent after the enzymatic treatment. The proteins and surface active compounds in the body show an affinity to metal ions, forming organo-metallic structures, believed to facilitate the absorption of the biomolecules at the surface of the implant. Hence, the organic matrix around the metal particles likely contains dissolved Cr, Co, and Mo ions which were then detected by EDX analysis and contributed to different composition totals.

The proportion of P in the debris was used to evaluate the efficiency of the digestion treatments in removing the phosphates from around the oxide particles. The phosphates in taper and stem debris can originate from the hydroxyapatite layer at the back of the implant cup or from bone fragments, resulting in the formation of corrosion products (Co or Cr phosphates) under appropriate electrochemical conditions. Indeed, the presence of CrPO₄ inside macrophages and/or peri-prosthetic tissue after failing hip replacements has been.

### TABLE III. The Morphology of the Particles Originating from Each Wear Site and Cleaned With the Alkaline and Enzymatic Treatments

<table>
<thead>
<tr>
<th>Samples</th>
<th>Treatment</th>
<th>Round</th>
<th>Oval</th>
<th>Needle shaped</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taper 1</td>
<td>Enzymatic</td>
<td>39%</td>
<td>53%</td>
<td>8%</td>
</tr>
<tr>
<td></td>
<td>Alkaline</td>
<td>61%</td>
<td>39%</td>
<td>0%</td>
</tr>
<tr>
<td>Taper 2</td>
<td>Enzymatic</td>
<td>48%</td>
<td>49%</td>
<td>3%</td>
</tr>
<tr>
<td></td>
<td>Alkaline</td>
<td>69%</td>
<td>29%</td>
<td>2%</td>
</tr>
<tr>
<td>Stem 1</td>
<td>Enzymatic</td>
<td>62%</td>
<td>32%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>Alkaline</td>
<td>52%</td>
<td>37%</td>
<td>11%</td>
</tr>
</tbody>
</table>

![FIGURE 6](image-url) The variation of debris composition with treatments for A—taper 1; B—taper 2; C—stem 1. The decrease of P content after the alkaline treatment in comparison to the enzymatic digestion is shown in figure D ($p < 0.05^*; p < 0.0003^{**}; p < 10^{-5}^{***}$).
confirmed by several studies. \cite{20,23,24} In vitro toxicity tests did not report adverse biological effects when cells were treated with commercially available CrPO$_4$.\cite{47} hence other species released from the wear sites may have initiated the adverse reactions observed in these cases. The dissolution of phosphates during the cleaning treatments outlined here allows the physicochemical characteristics of the Cr$_2$O$_3$ particles to be more fully evaluated. Thus, the reduction of the P content after the alkaline treatment indicates this method is more efficient at digesting the phosphates and leads to cleaner particles, which is in agreement with the STEM results for all three samples.

The authors recognize the small number of samples used in this study, but the findings were enough to provide for comparison. Both treatments remove the particle bio-corona and precipitates to reveal clean particles suitable for the high-resolution imaging and in-depth structural characterization. The amorphous matrix or phosphates, however, may have implications to the onset of ALTRs and must be preserved when the scope is to assess debris toxicity. We acknowledge the potential of any chemical and enzymatic treatment to alter the particles physicochemical characteristics, which can result in smaller particles sizes and changed chemistry and morphology. To minimized these effects the boiling steps in SDS, reported in the previous enzymatic protocols,\cite{41,42} were not performed in this study, although they may have resulted in a more efficient dissolution of the particle bio-corona after the enzymatic cleaning.

**CONCLUSION**

To our knowledge this is the first study to provide a direct comparison of chemical and enzymatic techniques to release metal particles from the flakes from around CoCrMo tapers and cement-stem interfaces. The study reveals the smallest building blocks which comprise the wear and corrosion flakes and provides detailed structural and chemical characterization of the metal nanoparticles. Our results show that the nanosized debris consist mainly of Cr$_2$O$_3$, with lower Co and Mo contents, which is in agreement with some previous studies reporting debris released from secondary interfaces in periprosthetic tissue.\cite{7} Depending on the purpose of the study, either of the treatments may be used to reveal the nanoscale debris forming the large flakes generated at tapers or cement-stem interfaces. Detailed structural characterization of the nanosized debris does, however, reveal clean particles, completely released from the organic matrix, which was best achieved in this study by the use of the alkaline treatment.

**CONFLICT OF INTEREST**

The author or one or more of the authors, has received or will receive remuneration or other prerequisites for personal or professional use from a commercial or industrial agent in direct or indirect relationship to their ownership.

**ACKNOWLEDGMENTS**

Data supporting this study are openly available from the University of Southampton repository at https://doi.org/10.5258/SOTON/xxxxx.

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