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A Novel Method for Isolation and Recovery of Ceramic Nanoparticles and Metal Wear Debris from Serum Lubricants at Ultra-low Wear Rates

S.Lal[a,b], R. M. Hall[b], J.L. Tipper[a,b]

[a] *School of Biomedical Sciences, University of Leeds, UK*

[b] *School of Mechanical Engineering, University of Leeds, UK*

Abstract

Ceramics have been used to deliver significant improvements in the wear properties of orthopaedic bearing materials, which has made it challenging to isolate wear debris from simulator lubricants. Ceramics such as silicon nitride, as well as ceramic-like surface coatings on metal substrates have been explored as potential alternatives to conventional implant materials. Current isolation methods were designed for isolating conventional metal, UHMWPE and ceramic wear debris. In this paper, we describe a methodology for isolation and recovery of ceramic or ceramic-like coating particles and metal wear particles from serum lubricants under ultra-low and low wear performance. Enzymatic digestion was used to digest the serum proteins and sodium polytungstate was used as a novel density gradient medium to isolate particles from proteins and other contaminants by ultracentrifugation. This method demonstrated over 80% recovery of particles and did not alter the size or morphology of ceramic and metal particles during the isolation process.

1 Introduction

Modern ceramic-on-ceramic (CoC) bearings generate wear rates which are approximately a hundred-fold lower than the conventional UHMWPE-on-metal bearings in total hip replacements [1]. The motivation for developing low wearing biomaterials and bearing combinations originates from clinical and experimental evidence which has revealed the role that wear debris plays in implant-associated osteolysis and adverse soft tissue reactions in patients implanted with devices [2–5].

Particle concentration and size are considered to be important factors affecting cytotoxicity, inflammatory cytokine release and bone resorption activity in macrophages. Wear particles in a critical size range of 0.1 to 1 μm are believed to be more biologically active in terms of osteolytic cytokine release [2]. The systemic distribution of wear debris in patients is also found to be dependent on particle size [6–8]. Furthermore, a number of studies have demonstrated the effect of particle morphology on phagocytic capacity and release of inflammatory cytokines in macrophages [9–11]. As a consequence, there is a large body of work investigating wear testing of orthopaedic bearing materials and, the subsequent characterisation of wear debris. Newborn calf serum (NCS) diluted with deionised water (protein concentration from 17 g/l to 30 g/l) is the lubricant used within mechanical simulators to simulate physiological wear mechanisms and wear rates with a variety of materials.

Prior to characterisation, wear particles are isolated from the serum proteins together with other contaminants. A number of techniques using acid, base, or enzymatic digestion to break down the proteins, followed by isolation of the wear particles by chemical extraction, density gradients, or direct filtration have been developed. However, these methods were designed for isolation of debris from conventional UHMWPE, metals and alumina ceramic materials [12–18].

The latest generation of ceramic bearing materials such as silicon nitride (Si_3N_4) [19], as well as a number of surface engineered coatings have been explored as potential alternatives to UHMWPE and metal articulations [20–24].

Silicon nitride particles are found to slowly dissolve in aqueous fluids [25] and the wear debris released from silicon nitride articulating surfaces are predicted to slowly dissolve in biological fluids [23]. Current methods are not designed to isolate and recover wear debris in such situations.

As a very low wearing material, Si_3N_4-on- Si_3N_4 bearings have demonstrated wear rates comparable to alumina-on-alumina ceramic bearings [19]. Surface engineered coatings such as diamond-like carbon, chromium nitride, chromium carbonitride and silicon nitride coatings also demonstrated very low wear in comparison to their metal counterparts under standard conditions [20–23].

The amount of wear produced by modern CoC bearings is found to be as low as 0.01 mm^3/million cycles during hip simulator testing [26], whilst the volume of serum lubricant used in current wear simulators is usually several hundreds of millilitres. As a consequence, high sensitivity and high recovery have become critical for isolation of wear particles for these low wearing bearing combinations. For the same reason, it has been difficult to characterise wear debris and test the biocompatibility of very low wearing ceramic materials such as zirconia-toughened alumina (Biolox Delta, Ceramtec) and silicon nitride.

This has created the need for development of methodologies to isolate wear debris from simulator lubricants used in wear testing of ultra-low wearing materials and coatings. In addition, the ability to recover particles for further analysis such as biocompatibility testing would also be beneficial.

We have developed a novel method that is able to isolate any ceramic or metal wear particles denser than 1.6 g/cm^3 (density suitable for separation of serum proteins from metal, ceramic, or ceramic-like coating particles) from serum lubricants. The isolation process maintains the original size and shape of particles and demonstrates high recovery of particles for further analysis. The effectiveness of the method was tested by recovering and characterising Si_3N_4 nanoparticles and Cobalt-chromium alloy (CoCr) wear debris at very low wear rates in the order of 0.01 mm^3 per million cycles and low wear rates in the order of 0.1 mm^3 per million cycles.

2 Materials and methods

2.1 Materials

2.1.1 Particles

Si_3N_4 nanoparticles were chosen to test the isolation and recovery of ceramic particles. This was based on a number of reasons. Firstly, as mentioned previously, silicon nitride has shown potential as a monolithic bearing material and also as ceramic-like coatings for total hip replacements. Secondly, a large consortium of public and private sector European organisations (LifeLongJoints[1]) is currently developing very low wearing silicon nitride coatings for articulating surfaces and total hip replacements. The method developed in the present study will be utilised for the isolation of silicon nitride wear debris produced during the wear testing of these coatings. Lastly, the isolation of silicon nitride particles that slowly dissolve will further test the robustness of the isolation method. Commercially available Si_3N_4 nanoparticles (<50 nm nanopowder, Sigma-Aldrich) were used in the present study to test the isolation and recovery of ceramic particles from serum lubricants. These particles were stored in sealed containers to minimise surface oxidation in the presence of oxygen from air.

CoCr wear debris generated in a multidirectional pin-on-plate reciprocator were chosen to test the isolation and recovery of metal particles, as CoCr has been used consistently as an implant material and has recently been explored as a substrate for ceramic-like coatings [23].

2.1.2 Density gradient medium

Sodium polytungstate (SPT) was used as a novel density gradient medium due to its properties, such as its high solubility in water, the fact that it is nontoxic [27] and acts as a protein denaturant [28], coupled with a large density range of 1.1 - 3.0 g/cm^3 in water.

2.1.3 Particle characterisation instruments

[1] LifeLongJoints, Silicon nitride coatings for improved implant function. http://lifelongjoints.eu

A nanoparticle tracking analysis (NTA) based particle analyser (NanoSight LM10, Malvern Instruments UK) was used to measure particle size distribution and concentration (number of particles per ml) of Si_3N_4 particles dispersed in sterile water. NanoSight has been used to characterise polydisperse samples within the size range of 20 nm to 1000 nm and is able to perform measurements on ultra-low concentrations of particles [29]. In the present study, NanoSight was used to characterise Si_3N_4 nanoparticles at ultra-low (0.023 $\mu g.ml^{-1}$) and low concentrations (0.23 $\mu g.ml^{-1}$).

A dynamic light scattering (DLS) based size analyser (Zetasizer Nano ZS, Malvern Instruments UK) was used to measure overall size range of Si_3N_4 aggregates, owing to its large particle size detection range of 0.3 nm to 10 μm. This equipment required a minimum concentration of 0.1 $mg.ml^{-1}$ of Si_3N_4 nanoparticles in sterile water for accurate results.

An Hitachi SU8230 high resolution cold-field emission scanning electron microscope (CFE-SEM) was used for high resolution imaging of Si_3N_4 nanoparticles and CoCr wear particles. Aztec Energy Energy-Dispersive X-ray (EDX) system integrated in the CFE-SEM, with a high resolution detector (80 mm^2 X-Max SDD, Oxford Instruments), was used for elemental analysis of the samples. Digital image analysis (Image-Pro Plus version 6.1, Media Cybernetics UK) was used to measure the particle size and shape descriptors.

2.2 Preparation of CoCr wear particles

Metal pins and plates were manufactured from medical grade cobalt-chromium alloy (ASTM F1537) with high carbon content (>0.2 % wt) and their contact surfaces were polished to a smooth surface (Ra 0.01 - 0.02 μm). Subsequently, CoCr wear particles were generated in sterile water (Baxter, UK) in a six station multidirectional pin-on-plate tribometer as described previously [30]. The CoCr particle suspensions were collected after 330,000 cycles and frozen at -20°C, before being used for particle characterisation or isolation experiments.

2.3 Characterisation of particles

Aggregates of Si_3N_4 nanoparticles were measured using a Malvern Zetasizer (Section 2.13). The Si_3N_4 nanoparticles were suspended in sterile water (Baxter, UK) at a concentration of 0.1 mg.ml^{-1} and sonicated for 10 min in an ice-cooled ultrasonic bath (USC300T, VWR UK).

Individual Si_3N_4 nanoparticles were assessed using a combination of NTA and SEM. Si_3N_4 nanoparticles were added to sterile water at concentrations of 0.023 µg.ml^{-1} (6.66×10^{-6} mm^3.ml^{-1}) and 0.229 µg.ml^{-1} (6.66×10^{-5} mm^3.ml^{-1}). Based on hip simulator test lubricant volumes of 500 ml per 330,000 cycles, the above concentrations were equivalent to *in-vitro* volumetric wear rates of 0.01 mm^3/million cycles and 0.1 mm^3/million cycles respectively. Particle suspensions were sonicated for 10 min in an ice-cooled ultrasonic bath (USC300T, VWR UK). Particle size distribution and concentration (1×10^8 particles.ml^{-1}) were measured by NTA. For scanning electron microscopy analysis, 20µl of Si_3N_4 nanoparticles suspension (1 mg.ml^{-1}) and 10µl CoCr wear debris suspension (1 mg.ml^{-1}) were re-suspended separately in 10 ml sterile water, sonicated for 10 min in an ice-cooled ultrasonic bath and filtered through 0.015µm pore size polycarbonate membrane filters (Whatman, UK). The filters were then dried under an infrared lamp for two to three hours, mounted on aluminium stubs and sputter-coated with 3nm platinum (Agar Auto Sputter Coater, Agar Scientific UK) to minimise charging and contamination from the electron beam.

Scanning electron micrographs of particles were captured at low magnifications of 1000x and high magnifications of 100,000× to 200,000×. A minimum of 150 particles of each sample were characterised. Major diameter (d_{max}), aspect ratio and roundness measurements were taken for each particle using Image-Pro Plus software in accordance with ASTM F1877-05(2010) [31].

2.4 Preparation of particle doped serum lubricants

Si_3N_4 nanoparticles and CoCr wear particles were added separately to 25% (v/v) calf serum (Seralabs, UK) at volumetric concentrations of 6.66×10^{-6} mm^3.ml^{-1} (equivalent to a wear rate of

0.01 mm^3/million cycles) and 6.66×10^{-5} $mm^3.ml^{-1}$ (equivalent to a wear rate of 0.1 mm^3/million cycles).

2.5 Isolation of particles

Si_3N_4 nanoparticles and CoCr wear debris were isolated from serum lubricants by the newly developed method using SPT gradients. The particle isolation procedure is shown in Figure 1. All solutions except sterile water were filtered using 20nm Whatman® Anodisc membrane filters (GE Whatman, UK) to avoid introduction of any external impurities. All centrifuge tubes were coated with siliconizing fluid (Surfasil, Sigma UK) to minimise sticking of particles to the tube walls. Centrifugation was carried out using an Optima L80 ultracentrifuge (Beckman Coulter, USA). Centrifugation speeds and durations were calculated based on the particle sedimentation equations described previously [32,33].

Prior to the isolation, particle spiked lubricants were gently stirred and sonicated simultaneously for 10 min in an ice-cooled ultrasonic bath to uniformly disperse the particles. Adapted from Billi et al. [12] the first step was to reduce the sample volume by centrifugation at 32,000 rpm (average RCF of 125,755g in SW32Ti rotor, Beckman Coulter) for 3 hours at 20°C using 30ml Thickwall Polyallomer tubes (Beckman Coulter USA). A volume of 27ml of the supernatant was carefully removed by aspiration and examined by NTA to verify that no particles remained in the supernatant. Thereafter, the remaining supernatant and the pellet was re-suspended in HEPES buffer (working concentration 0.1 M) and digested at 50°C by proteinase K (working concentration 0.5 mg/ml) for 18 h in the presence of 0.5% (w/v) sodium dodecyl sulphate (SDS) and 3mM calcium chloride. After digestion, the digest was gently stirred and sonicated simultaneously for 10 minutes in an ice-cooled ultrasonic bath. The enzymatic digestion was repeated by the addition of proteinase K (working concentration 1 $mg.ml^{-1}$) and digestion at 50°C for 22 h. Subsequently, SPT density gradients were prepared by sequentially layering 60% SPT (Density 2.0 $g.cm^{-3}$), 40% SPT (Density 1.6 $g.cm^{-3}$) and 20% SPT (Density 1.2 $g.cm^{-3}$) in a 13 ml tube (Thinwall Polyallomer, Beckman UK). The digest was gently

stirred and sonicated simultaneously for 10 min in an ice-cooled ultrasonic bath and loaded on top of the SPT gradients. The tubes were then filled to the top with sterile water. Particles were isolated from partially hydrolysed proteins and other contaminants by density gradient ultracentrifugation at 40,000 rpm (average RCF of 202,048g in SW40Ti rotor, Beckman Coulter USA) for 4 h. At the end of the centrifugation step, particles were collected at the bottom of the centrifuge tube, leaving protein fragments and other impurities suspended higher up the tube. Supernatant layers were removed by aspiration and isolated particles were washed three times in sterile water at 35,000 rpm (average RCF of 154,693g in SW40Ti rotor, Beckman Coulter USA) for 1 h in the same centrifuge tube. The tube contents were gently stirred and sonicated simultaneously for 10 min in an ice-cooled ultrasonic bath between each washing step. Supernatants from washing steps were examined by NTA to verify that no particles were lost during washing steps.

2.6 Characterisation of particles after isolation

Isolated particles were re-suspended in sterile water and dispersed by sonic agitation for 10 min in an ice-cooled ultrasonic bath. NTA was used for the measurement of Si_3N_4 aggregate size distribution and particle concentration.

For scanning electron microscopy analysis, Si_3N_4 nanoparticles and CoCr wear debris were dispersed in water, sonicated for 10 min in an ice-cooled ultrasonic bath and collected on to the 0.015 μm pore size membrane filters by vacuum filtration in a class II cabinet. Membrane filters were dried under infrared lamps for two to three hours and coated with 5 nm platinum (as described previously in Section 2.3). SEM images of particles were captured at low magnifications of 1000× and high magnifications of 100,000× to 200,000×. A minimum of 150 particles per sample were characterised using Image-Pro Plus software (as described above in Section 2.3).

2.7 Statistics

Particle size and shape distributions were tested for normality using Shapiro-Wilk and Kolmogorov-Smirnov tests. The distributions were not normally distributed. Statistical differences in the particles size distributions before and after isolation were tested using non parametric Kolmogorov-Smirnov Z test (SPSS Statistics Version 22, IBM Corp. USA). The tests were carried out on the original un-binned data for higher accuracy. Statistical differences in the aspect ratio values and roundness values before and after isolation were tested using Mann-Whitney U test (SPSS Statistics Version 22, IBM Corp. USA).

3 Results

3.1 Isolation of Si_3N_4 nanoparticles and CoCr wear debris from serum lubricants

Samples containing Si_3N_4 and CoCr particles were pelleted during the concentration step (Figure 2 A). NTA did not detect any particles in the supernatants collected after the concentration step. The SPT gradients were prepared in centrifuge tubes and the digested Si_3N_4 and CoCr samples were loaded on top of the SPT gradient in each tube (Figure 2 B). At the end of the washing steps, Si_3N_4 and CoCr particles were pelleted at the bottom of the centrifuge tubes as shown in Figure 2 C. NTA did not detect any particles in the supernatants collected during washing steps. Particles doped at ultra-low wear rates were barely visible to the naked eye after washing steps as shown in Figure 2 D.

3.2 Characterisation of Si_3N_4 nanoparticles and CoCr wear debris

Si_3N_4 nanoparticles were observed as aggregates in aqueous solution. Size distributions from DLS and NTA measurements indicated that the maximum size of Si_3N_4 particle aggregates was smaller than 500 nm (Figure 3). The mode size measured by NTA was 150 - 200 nm, while the mode size measured by DLS was 250 - 300 nm. The particle size range measured by NTA was 20 nm - 500 nm (Figure 3 and Figure 4). No statistically significant differences were observed between the size distributions of Si_3N_4 nanoparticle aggregates before and after particle isolation (Kolmogorov-

Smirnov Z test, $p>0.05$) (Figure 4). Therefore, aggregation characteristics of Si_3N_4 nanoparticles were found to be unchanged by the isolation process.

High resolution SEM images of Si_3N_4 and CoCr particles before and after isolation are shown in Figure 5. The morphology of the Si_3N_4 particles was round to oval and was not affected by the isolation process. This was further confirmed by statistical analysis, as no significant differences in the aspect ratio and roundness values of Si_3N_4 nanoparticles before and after isolation were observed (Mann-Whitney U Test, $p>0.05$) (Table 1). A comparison of the frequency (particle size) distributions for Si_3N_4 particles obtained by image analysis of high resolution SEM images before and after the isolation method is shown in Figure 6 A. The mode particle size for Si_3N_4 particles was 30-40 nm and the frequency distributions were statistically similar (Kolmogorov-Smirnov Z test, $p>0.05$), indicating no significant effect of the isolation process on particle size.

Particle morphology of CoCr wear debris was also unaffected by the isolation process (Figure 5). Moreover, no significant differences in the aspect ratio and roundness values were observed for CoCr particles before and after isolation (Mann-Whitney U Test, $p>0.05$) (Table 1). Frequency (particle size) distributions for CoCr particles before and after isolation were also statistically similar (Kolmogorov-Smirnov test, $p>0.05$). Mode size of primary CoCr particles was 10 - 20 nm (Figure 6 B).

3.3 Particle isolation sensitivity and recovery rates

Si_3N_4 nanoparticles isolated from serum lubricants were collected on membrane filters and elemental composition was assessed by EDX analysis. EDX spectra of particles and the background filter are shown in Figure 7. Silicon and nitrogen peaks were observed for nanoparticles, confirming the isolated particles as Si_3N_4 nanoparticles. The background was composed of carbon, oxygen and platinum peaks, originating from the polycarbonate filter and the platinum coating, respectively. No other elements were present, indicating the successful removal of impurities and contaminants. The

percentage recovery of Si_3N_4 nanoparticles from serum lubricants at concentrations equivalent to ultra-low wear rates of 0.01mm^3 per million cycles and low wear rates of 0.1mm^3 per million cycles are shown in Figure 8. Average recovery rates were above 80% for both particle concentrations. This demonstrated the high efficiency and high sensitivity of the novel isolation method.

4 Discussion

This is the first study to demonstrate the recovery of ceramic nanoparticles and metal wear debris from serum lubricants at very low volumetric concentrations of 6.66×10^{-6} mm^3.ml^{-1} and low volumetric concentrations of 6.66×10^{-5} mm^3.ml^{-1}. These concentrations are comparable to wear rates of 0.01 mm^3 per million cycles and 0.1 mm^3 per million cycles in a hip simulator with 500 ml of lubricant used per station, where the lubricant is changed every 330,000 cycles. Such low concentrations of particles are likely to be present in the serum based lubricants collected during hip simulator testing of the latest generation of CoC bearings. Low particle concentrations result from very low wear of the bearing surfaces, coupled with large lubricant volumes used per station in a hip simulator. The majority of modern hip simulators hold 450 to 500 ml of lubricant per station and the lubricant is changed every 330,000 or 500,000 cycles [34–38]. The rationale behind using such large lubricant volumes is to reduce the possibility of overheating of serum-based lubricants. However, this significantly reduces the concentration of particles in serum lubricants and makes it more challenging to isolate them from serum proteins. During the wear of conventional materials in a simulator, despite the fact that wear particles are generated in a large lubricant volume, the amount of wear is sufficient for producing a reasonably higher particle concentration in serum lubricants. Considering the improvements in wear properties of bearing materials, recent studies have reported the sensitivity of novel isolation methods and compared the particle extraction efficiency of existing metal wear debris isolation methods [12,32]. However, current methods only isolated metals such as

CoCr and previous generation alumina ceramics from simulator lubricants [12,32,39], which wear at higher rates compared to the latest ceramics [26] and ceramic-like coatings [23].

The novel wear debris isolation method introduced in this study used enzymatic digestion in combination with novel SPT gradients to isolate the latest generation of ceramic and metal particles. Enzymatic digestion was used to break-down serum proteins, instead of acid or base digestion, to minimise damage to the particles. The digestion was carried out in the presence of sodium dodecyl sulphate (SDS) to increase the efficiency of digestion by unfolding the majority of the secondary, tertiary and quaternary structure of serum proteins. Stabilisation of proteinase K by calcium has been established in the literature [12,40]. The present method stabilised proteinase K during digestion by including 3mM $CaCl_2$ in the digestion buffer. The digestion was carried out at 50°C to maximise the proteolytic activity of the proteinase K, without significantly affecting its stability. The digestion time was chosen to be long enough to maximise proteolysis, without increasing the costs of adding more enzyme. This method also introduced novel SPT gradients for the separation of nanoparticles from protein contaminants. Conventional gradient materials such as isopropanol, sucrose and caesium chloride have a limited density range, which is not suitable for separating metals and ceramics from protein contaminants. Billi et al. [12,13] used custom gradients composed of SDS and UREA for their denaturing properties, and used cesium trifluoroacetate solution as a barrier layer for its high density of 2.0 g/cm^3. However, cesium trifluoroacetate is a toxic compound and for this reason its availability is also limited. Conversely, SPT is a non-toxic compound [27] with low viscosity [27] and a large density range of 1.1 to 3.0 g/cm^3 in water. This allows SPT gradients to effectively separate metal and ceramic nanoparticles from serum proteins. Moreover, SPT is highly soluble in water, which allows its separation from isolated particles by centrifugal washing. Furthermore, SPT is non-flammable [27], odourless [27] and also has protein denaturing properties [28]. Therefore, SPT is a suitable alternative to the previously used gradient materials.

The present method used ultracentrifugation for concentration of the serum lubricants, isolation of particles using SPT density gradients and washing of particles. Previous studies have emphasised the importance of sufficient centrifugal forces and duration of centrifugation in effectively concentrating or separating nanoscale particles [12,32,41,42]. During centrifugation, the rate of sedimentation of particles is dependent on the balance between centrifugal field forces, and opposing buoyancy and friction forces. Based on Newton's second law of motion, the kinetic equation for centrifugal sedimentation has been given as [32]:

$$\frac{d^2r}{dt^2}m_p = F_c + F_b + F_d$$

Eq. 1

where $\frac{d^2r}{dt^2}$ is the acceleration and m_p is the mass of a particle moving through the fluid. F_c is the centrifugal force, F_b is the buoyant force and F_d is the drag force experienced by the particle during motion. For rigid spherical particles, based on Stoke's law, the time of sedimentation has been expressed as [33]:

$$t = \frac{18\eta_f}{\omega^2 {d_p}^2(\rho_p - \rho_f)} \times ln\frac{r_{final}}{r_{initial}}$$

Eq. 2

where η_f is the viscosity of fluid, ω is the angular velocity during centrifugation,d_p is diameter of the particle, ρ_p is density of the particle, ρ_f is the fluid density, $r_{initial}$ is the distance between axis of rotation and the initial position of particle and r_{final} is the distance between axis of rotation and the final position of particle.

This equation was used to calculate the time taken by particles to move from one position to another position in the centrifuge tube. For instance during the washing steps, the serum lubricant was

centrifuged at 35,000rpm using a Beckman Coulter SW40Ti rotor. Based on the centrifugal sedimentation calculations, the time taken to cover the distance of 92.1 mm (maximum displacement of particles in SW40Ti rotor) for 10 nm sized particles in this step would have been 20 min. Centrifugation was carried out for 60 min, which was sufficient to completely sediment the particles larger than 6 nm. This was also verified by analysing supernatants collected at the end of the centrifugation steps using NanoSight for any detectable particles. Within the lower particle detection limit of NanoSight (approximately 10 - 20 nm), no particles were found in the supernatant.

Although the present method uses high centrifugal forces (over 200,000g) to separate particles from serum proteins and other contaminants, SEM analysis confirmed that the particle size and morphology of hard inorganic (Si_3N_4 and CoCr) particles was unchanged during the isolation process. This is in agreement with a previous study by Zolotarevova et al. [43] who demonstrated no change in the morphology of much softer UHMWPE particles by ultracentrifugation. Moreover, ultracentrifugation is a technique routinely used in biology for separation of cell organelles without any significant change to them, as mentioned in the above cited paper.

In addition to the centrifugal forces, the number of steps and transfer of contents from one tube to another could also adversely affect the recovery of particles [12,32,42]. Therefore, the present method combined the concentration and digestion steps in the first tube, followed by isolation and washing steps in a second tube. Adapted from Billi et al. [12] the centrifuge tubes were coated with siliconizing fluid (Surfasil, Sigma UK) to minimise sticking of particles to the tube walls. The loss of particles in the pipette tips while transferring the digest and purified particles in new tubes was minimised by aspirating the samples, while the contents of the tube were constantly shaken and sonicated. This kept the particles in an agitated state and minimised the sticking of particles to surfaces such as centrifuge tube walls or pipette tips. Moreover, the effect of heat on particles during sonication was minimised by carrying out all the sonication steps in an ice-cooled ultrasonic bath.

This was particularly important for Si_3N_4 particles as their dissolution is accelerated by an increase in temperature [25].

Another issue related to isolation of particles from serum lubricants has been the artificial clumping and aggregation of particles by the wear debris isolation methods. Billi et al. [12] has discussed in detail the limitations of previous methods in terms of artificial clumping of particles. No change in the particle size distribution of primary aggregates was found from NTA measurements before and after isolation using the novel method described here. Therefore, the isolation process did not cause any artificial clumping of the particles. The present study also investigated the aggregation characteristics of Si_3N_4 particles using DLS and NTA. Si_3N_4 aggregates were found to be smaller than 500 nm for particle concentrations up to 0.1 $mg.ml^{-1}$. Although DLS was useful for determination of maximum size of aggregates, it did not detect any particles smaller than 142 nm. Conversely, NTA detected a noticeable fraction of particles in the 100 nm - 150 nm size range. This may have been due to the aggregation of Si_3N_4 nanoparticles at particle concentrations required for measuring size distribution by DLS. Both techniques also differ in their measurement accuracy depending on dispersity of the sample. NTA is found to have better peak resolution for polydisperse samples in 20 nm - 1000 nm size range in comparison to DLS [29].

Even though the main purpose of a wear debris isolation method is to characterise particles for size, shape and chemical composition [12], the flexibility to recover particles for further analysis is becoming increasingly important [32,42]. This was demonstrated in the present method by recovering particles in sterile water and further analysing them for size, percentage recovery and aggregation characteristics using commercial particle analysers (NanoSight and Zetasizer).

The present method has adapted a number of steps from previous studies [12,13]. However, implementation of these methods required the use of custom components, which may not be cost effective. Moreover, these methods were primarily developed for characterisation of wear debris

using electron microscopy and the recovery of particles for subsequent experiments is not straightforward. We have developed a highly sensitive method which uses cost effective commercially available reagents and components, and enables the particles to be collected while suspended in a liquid medium, which then could be readily analysed using commercial size analysers, prior to use in cell studies.

5 Conclusions

The new isolation method successfully isolated Si_3N_4 nanoparticles and CoCr wear debris from serum lubricants at low and ultra-low concentrations equivalent to wear rates of 0.01 mm^3/million cycles and 0.01 mm^3/million cycles respectively. The method also demonstrated over 80% recovery of particles and preserved the characteristics of ceramic and metal particles during digestion, isolation and characterisation steps. Future work involves adaptation of the method for isolation of metal and the latest generation ceramic wear debris from peri-prosthetic tissues.

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Figure 1 A scheme for the particle isolation procedure. The method is divided into concentration, enzymatic digestion and isolation steps.

Figure 2 Isolation of Si_3N_4 and CoCr particles from 25% (v/v) bovine serum lubricant. A) Si_3N_4 (top) and CoCr (bottom) lubricant samples pelleted after concentration step as indicated by arrows. Pellets appear yellowish due to the presence of protein contaminants. B) Sodium polytungstate gradients prior to centrifugation (Left: Si_3N_4, Right: CoCr). C) Si_3N_4 (top) and CoCr (bottom) particles pelleted at the end of centrifugal washing as indicated by arrows. The colour of the digested lubricant layer in the right tube was darker due to the presence of CoCr particles. D) Si_3N_4 (top) particles doped at ultra-low wear rates were not visible to the naked eye after washing steps. CoCr (bottom) particles doped at ultra-low wear rates were hardly visible to the naked eye after washing steps as indicated by the arrow.

Figure 3 Size distribution from NTA measurements of Si_3N_4 nanoparticles dispersed in water at ultra-low concentration (dotted line) and low concentration (solid line). Size distribution from DLS measurements of 0.1 $mg.ml^{-1}$ Si_3N_4 nanoparticles (dashed line). Maximum aggregate size for Si_3N_4 nanoparticles was approximately 500 nm with both NTA and DLS measurements.

Figure 4 Particle size distribution of Si_3N_4 aggregates before and after the particle isolation procedure measured by NanoSight at ultra-low wear rates of 0.01mm^3/million cycles. The size distribution of aggregates remained unaffected by the method. Error bars show standard deviation. No significant differences were observed in particle size before and after isolation (Kolmogorov-Smirnov Z test, $P>0.05$).

Figure 5 Scanning electron micrographs of Si_3N_4 and CoCr particles. A) Before the isolation procedure. B) After the isolation procedure. The morphology of particles was similar in both cases indicating no noticeable effect of the novel isolation method on the particles.

Figure 6 A comparison of particle size distributions of primary particles before and after the isolation procedure. A) Si_3N_4 50nm model particles. B) CoCr wear debris. Particle size distributions before and after isolation were not significantly different (Kolmogorov-Smirnov Z test, P>0.05).

Figure 7 Energy-dispersive X-ray analysis of Si_3N_4 particles collected on 0.015 μm Nucleopore membrane filters. High spatial resolution (less than 300 nm) was achieved by using a large area SDD detector (80 mm^2 X-Max SDD, Oxford Instruments) and an accelerating voltage of 5 kV. A) Particle (spectrum 1) and background areas (spectrum 2) used for EDX analysis. B) EDS spectrum of Si_3N_4 particles showing silicon, nitrogen, carbon, oxygen and platinum peaks. C) EDS spectrum of background filter showing carbon, oxygen and platinum peaks. Membrane filters were composed of polycarbonate polymer and therefore showed carbon and oxygen peaks for both areas. Platinum peaks were due to the platinum coating applied to all samples.

Figure 8 Percentage recovery of Si_3N_4 nanoparticles from 25% (v/v) NCS lubricant at ultra-low wear rates of 0.01 mm^3 per million cycles and low wear rates of 0.1 mm^3 per million cycles using the novel method. Error bars show standard deviation.

Table 1 Comparison of major diameter (d_{max}), aspect ratio and roundness of Si_3N_4 nanoparticles and CoCr wear debris before and after isolation.

	Si_3N_4 (Before isolation)	Si_3N_4 (After isolation)	CoCr (Before isolation)	CoCr (After isolation)
Major diameter, d_{max} (nm)	36.813±1.529	36.706±1.356	21.546±1.330	21.725±1.309
Aspect ratio	1.306±0.026	1.319±0.022	1.319±0.030	1.293±0.027
Roundness	0.796±0.016	0.790±0.013	0.677±0.012	0.690±0.012

Note: All values expressed as mean ± 95% Confidence Interval.