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Pre-clinical (in-vitro) biological and biocompatibility responses of the wear particles of the newly developed nano-composite

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Contents

Executive Summary

This report presents the predicted biocompatibility and biological responses of wear particles generated from 3D-printed polyetheretherketone (PEEK), SLS polyetherketoneketone (PEKK) and a 3D-printed PEEK composite variant with hexagonal boron nitride (hBN), with conventionally manufactured neat PEEK used as a baseline control. The study aims to evaluate the viability, cytotoxicity, and inflammatory potential of these particles through standardized in vitro assays.

PEEK and PEKK are increasingly being explored as alternatives to traditional materials for orthopaedic applications due to their favourable mechanical properties and biocompatibility. Recent advancements in additive manufacturing, including fused filament fabrication (FFF) and selective laser sintering (SLS), enable the development of customized PEEK-based components. The benefits of bespoke medical parts include precise size customization to meet individual patient needs, enhanced sustainability through reduced material waste, and the potential for on-site manufacturing, streamlining production and delivery. However, the impact of wear particles generated from these manufacturing processes remains underexplored, particularly regarding biological viability and inflammatory responses.

A screening tribological test was conducted using a reciprocating tribometer in a ball-on-flat configuration, with a zirconia (ZrO_2) ceramic ball and 3D-printed PEEK composite samples as the reciprocating surface. Testing parameters included a 5 N load, 5 mm stroke length, and 2 Hz frequency, with phosphate-buffered saline (PBS) as the lubricant. Results demonstrated that PEEK-hBN composites exhibited significantly lower coefficients of friction compared to neat PEEK, with 3mBN-PEEK achieving the most stable and lowest friction throughout the tests.

Tribological testing under controlled conditions using roughened CoCrMo to produce wear particles. These particles were subsequently isolated, cleaned, and sterilized for biological evaluation. The inclusion of hBN in the PEEK composite is hypothesized to improve wear resistance and friction properties, potentially leading to particles with more favourable morphologies and biocompatibility profiles.

Key biological assays, including the MTT assay (CyQUANT™) for cell viability, Reactive Oxygen Species (ROS) analysis for oxidative stress, and TNF-α cytokine measurements for inflammatory response, will provide insights into the cellular response to wear particles. It is hypothesized that 3D-printed PEEK and the hBN composite will demonstrate comparable or improved biocompatibility relative to conventionally manufactured PEEK. Challenges such as particle aggregation, autofluorescence, and potential surface chemistry changes induced by sterilization are acknowledged and addressed with appropriate controls.

The outcomes of this study will contribute to the understanding of newly developed advanced polymer composites as biomaterials for joint replacements and pave the way for further in vivo validations, providing essential data for clinical adoption of 3D-printed PEEK and its composites in orthopaedic applications.

1) Introduction

Total joint replacements are among the most successful medical interventions, [1], significantly improving the quality of life for individuals suffering from degenerative joint diseases. However, the longevity of these implants is often compromised by wear particle-induced osteolysis, a major cause of implant failure [2]. The generation of wear particles from bearing materials leads to adverse biological responses, in the local and surrounding tissue, including inflammation and bone resorption, ultimately resulting in implant loosening and revision surgery.

Polyetheretherketone (PEEK) and its variant Polyetherketoneketone (PEKK) have emerged as potential alternatives to traditional metallic and polymeric materials used in joint replacements [3], [4], [5], [6]. These thermoplastics offer advantages such as biocompatibility, radiolucency, and mechanical properties closer to human bone. Recently, advances in additive manufacturing have enabled the production of 3D-printed PEEK, PEKK and PEEK composite components, which promise greater design flexibility and customisation. However, the biological impact of wear particles generated from these materials, particularly in their 3D-printed forms, remains underexplored.

Previous studies have demonstrated the biocompatibility of conventionally manufactured PEEKs and PEEK composites [7], but the unique characteristics of 3D-printed PEEK, including surface finishing and particle morphology, necessitate dedicated investigation. This study aims to address the gap by evaluating the biological viability of wear particles generated from in-house 3D-printed neat PEEK and PEKK as well as newly developed PEEK-hBN nanocomposites. Specifically, we will compare the biocompatibility of these particles to those from conventional PEEK.

This investigation focuses on critical biological parameters, including cell viability, cytotoxicity, and inflammatory response, using standardized in vitro assays [8]. These assays will allow us to assess whether the wear particles from 3D-printed PEEK elicit comparable or lesser adverse responses than those from conventional materials. By leveraging tribological tests, particle characterization, and advanced biological assays, this work seeks to provide foundational insights into the feasibility of adopting 3D-printed PEEK for joint replacement applications.

The outcomes of this study will not only contribute to the growing body of knowledge on the biological performance of advanced materials but also have direct implications for the design and implementation of next-generation joint replacements. Furthermore, they will serve as a stepping stone for future in vivo studies to validate the newly developed PEEK composite materials, and 3D printed PEEK and PEKK under physiological conditions.

1) Materials and Methods

2.1 Materials

Conventionally manufactured, via extrusion, medical grade PEEK (Evonik, Germany) is to be used as the control material in this study, providing the baseline biocompatibility from which comparative biocompatibility can be observed from the additively manufactured wear particles. CreatBot PEEK-300 high-temperature printer (Henan Suwei, China) was used to produce FFF printed PEEK and hexagonal boron nitride (hBN) PEEK composite, using medical grade PEEK pellet (Evonik, Germany). The PEKK was produced via selective laser sintering (SLS) (OPM, Connecticut, USA). Medical grade CoCrMo (Oracle, UK) was used as the countersurface.

2.2 Tribological testing

The FFF and SLS 3D printed parts were formed directly into pins, 15mm in length with a 9mm diameter, including a shouldered top side reducing to a 7mm diameter face. The shoulder is to reduce the impact of edge loading during the tribological testing.

Wear particles were generated using a 3 station multi-directional pin-on-disk tribometer (SimSol, UK), using a roughened countersurface of 0.1 µm to increase the volume of wear in the system, with the CoCrMo material simulating the femoral head of a typical hip joint articulation. Testing was conducted under controlled conditions with a load of 80N, corresponding to a contact pressure of 2MPa, a speed of 1 Hz, and a total of 200,000 cycles to produce particles sufficient for biological analysis. Deionized (DI) water served as the lubricant during testing. The solution is then to be frozen at -20°C after testing is complete.

2.3 Tribological Testing of 3D Printed PEEK composite

Tribological experiments were performed using a reciprocating tribometer (TRB, Anton Paar, Graz, Austria) configured in a ball-on-flat arrangement. A 6 mm zirconia (ZrO_2) ceramic ball was used as the stationary counterface, while the reciprocating surface consisted of polished 3D-printed PEEK composite samples. The polishing process was carried out in three stages: initial grinding with 180-grit SiC paper, followed by refinement using 500-grit and 800-grit papers to achieve a smooth surface. Before testing, the samples were subjected to ultrasonic cleaning in ethanol for 20 minutes and allowed to air dry.

Phosphate Buffered Solution (PBS) was used as the lubricant during testing. The parameters included a 5N load, a 5mm stroke length, and a frequency of 2Hz, resulting in an initial apparent contact pressure of 37MPa. Each test was conducted for a duration of 1 hour, with three repetitions performed for consistent and reliable friction data collection**.**

2.4 Ultracentrifugation and cleaning

Following testing, the collected lubricant containing wear particles was subjected to ultracentrifugation, enabling effective particle isolation and cleaning. This step will remove proteins and impurities. A Sodium Poly Tungstate (SPT) density gradient is used. This allows the lighter PEEK/PEKK materials to move up the gradient whilst more dense metallic debris will remain at the bottom.

First the particle solution was defrosted and sonicated in an ultrasonic bath for 10 minutes. In an ultracentrifuge tube, layer 2 ml of each of the following: 1.8 g/ml SPT, 1.6 g/ml SPT, 1.2 g/ml SPT.

Once the layers have been carefully established, 4.4 ml of the particle suspension is to be added to the layers without disturbing the gradient. Then top up each tube, using ultrapure water to ensure that they are all of equal mass ± 0.01g. ultracentrifuge the samples at 200,000g at 20°C for 4 hours. Resulting in separation of the separate materials.
The particles must now be extracted and cleaned to remove impurities and SPT. The polymer particles will be suspended in a band between the 1.2 g/ml and 1.6 g/ml SPT gradient layers, this is to be extracted via pipetting. Add the extracted polymer particles to a new centrifuge tube and top up with Ultrapure water so that the masses are equal ± 0.01g. Ultracentrifuge for 1 hr at 30°C at 125,000g. Repeat this process three times, to ensure that all contaminants are removed from the samples. The particles are subsequently sterilized in a hot-air oven at 190°C for four hours to ensure the removal of endotoxins. And then covered in sterlised foil and frozen at -80 to prevent any bacterial or endotoxin contamination.

2.5 Particle Characterization

The size and morphology of the isolated wear particles are to be characterized using scanning electron microscopy (SEM). Images were acquired at magnifications of 60k, 75k, and 90k, with particle dimensions (e.g., area, perimeter, aspect ratio, roundness) analysed using image J analysis software. Particles were classified across all size ranges, encompassing both nanometre and micron scales. Morphological data are to be exported and compiled into particle size distribution graphs for comprehensive evaluation.

2.6 Biological Testing

To evaluate biocompatibility, L929 mouse fibroblast cells were selected, given their established use in ISO standard cytotoxicity assays [8]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin, and 2 mM glutamine, under standard conditions of 37°C and 5% CO_2. Cells were seeded at a density of 5×10^4 cells per well in 96-well plates, setup shown below in Figure 1, and allowed to adhere for 24 hours before exposure to wear particles.

Wear particle suspensions were prepared at concentrations calculated based on cell-particle interaction ratios, specifically 100 μm^3 particles per cell shown in Figure 1. The suspensions were introduced into wells containing cells, followed by incubation for 1, 3, and 6 days. Controls included untreated cells in complete media, particle-only suspensions, and cells exposed to dimethyl sulfoxide (DMSO) as a positive control.

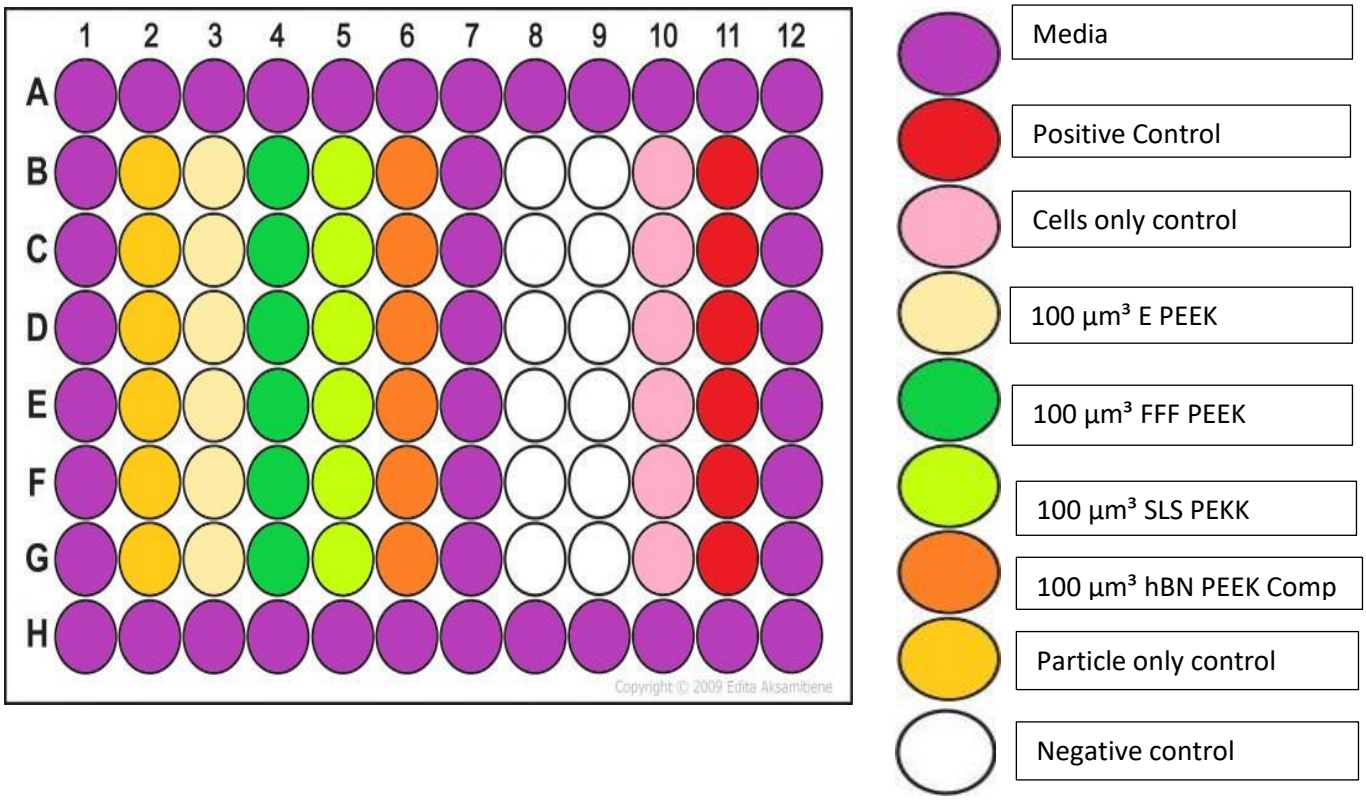

Figure 1 – 96 well plate set up for an MTT biocompatibility assay

2.7 Biological Assays

The metabolic activity of L929 cells exposed to wear particles quantified using the MTT assay. Following each incubation period, the supernatant is removed, and 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline) is added to each well, along with 100 µL of fresh media. Plates were incubated for four hours, after which the supernatant was replaced with 100 µL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. Absorbance at 570 nm is to be measured using a plate reader.

Further biological assays will be carried out, following the toolkit, CWA 17253-2, [8]. To assess oxidative stress, reactive oxygen species (ROS) levels are to be evaluated using a commercial ROS detection kit. Fluorescence imaging provided qualitative data on ROS production, while quantitative readings are to be obtained via fluorescence plate reader analysis.

Cell cytotoxicity will be assessed using the trypan blue exclusion assay. Cell viability was calculated based on live and dead cell counts using a haemocytometer. Parallel fluorescence-based cytotoxicity assays will be performed to validate results.

2) Preliminary MTT viability Assay Results

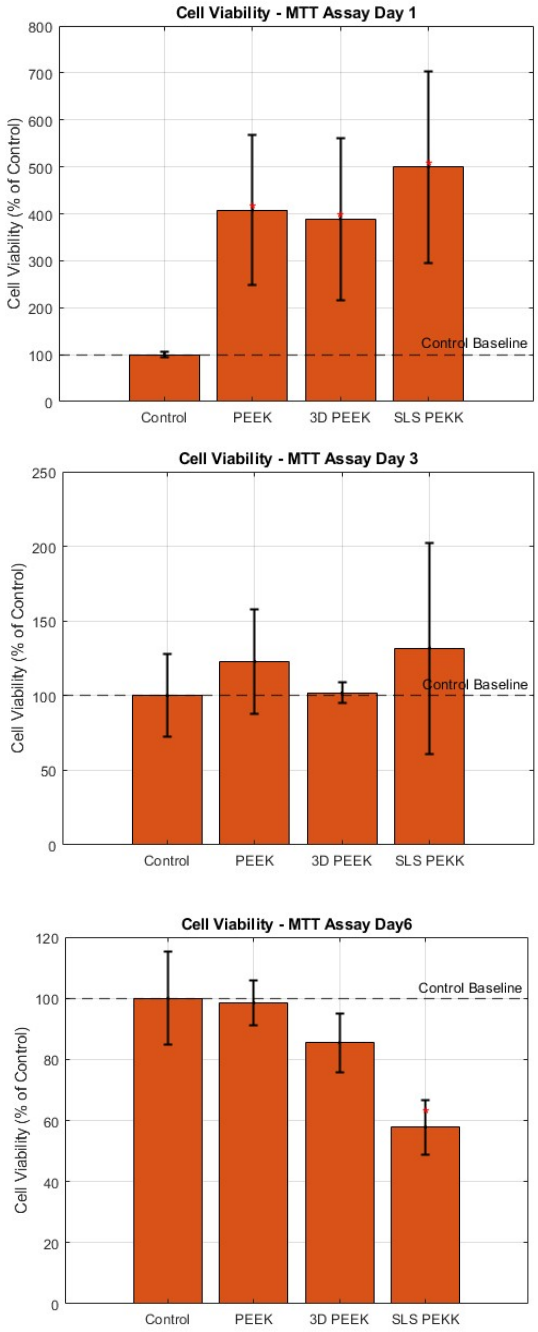

Figure 2 – Percentage viability of L929 fibroblasts cultured with Conventional PEEK, FFF PEEK, SLS PEKK wear particles at a particle volume of 100 µm3 / cell ratios compared to cell only controls. * Indicates significant reduction in cell viability compared to negative cell only control (p<0.05 ANOVA)

3) Preliminary results discussion

The report would like to focus on results from day 3 and day 6. It is the authors understanding that a manual error occurred during pipetting that led to the cell only control not producing the correct outcome. The data is present to show the full process of the assay

By Day 3, cell viability appeared to stabilize across all groups. The control group maintained its baseline level at 100% viability, while cells exposed to wear particles from PEEK and SLS PEKK displayed viability levels between 100-130%. The viability for 3D PEEK remained close to 100%. While variability persisted, particularly for SLS PEKK, the error bars were reduced compared to Day 1.

The stabilization of metabolic activity by Day 3 suggests that the initial cellular stress observed on Day 1 had subsided as the cells adapted to the presence of wear particles. This result confirms the low cytotoxicity of PEEK and 3D PEEK, aligning with the document's predictions regarding the biocompatibility of PEEK-based materials.
Day 6, the results for the test materials were more varied. PEEK retained a viability level close to 100%, demonstrating consistent biocompatibility over the testing period. 3D PEEK showed a slight decline in cell viability to approximately 85%, which remains within acceptable thresholds for biocompatibility. SLS PEKK exhibited a significant reduction in viability to approximately 60%, with noticeably reduced variability compared to earlier time points. The decline in viability for SLS PEKK on Day 6 may suggest the presence of delayed cytotoxic responses or cellular stress induced by the wear particles.

Overall, both PEEK and 3D PEEK exhibited consistent cell viability across the testing period. These results support the hypothesis that 3D-printed PEEK materials display comparable biocompatibility to conventionally manufactured PEEK.

The decline in cell viability for SLS PEKK highlights the potential challenges associated with the material. Repeated testing and subsequent further analysis of reactive oxygen species (ROS) levels and cytokine release (e.g., TNF-α) will be necessary to determine the mechanisms underlying this response.

4) Predicted Results & Analysis

The biocompatibility study of PEEK/PEKK and PEEK-hBN composites is currently ongoing, with the first set of data expected in early January. In the meantime, this section presents relevant biocompatibility data from previous studies on newly developed nanocomposites for use in total joint replacements.

The mechanical and tribological testing data has been carried out as a preliminary study, by ESR4 in deliverable 5.6. to validate the improved ultimate tensile strength, impact strength, and coefficient of friction.

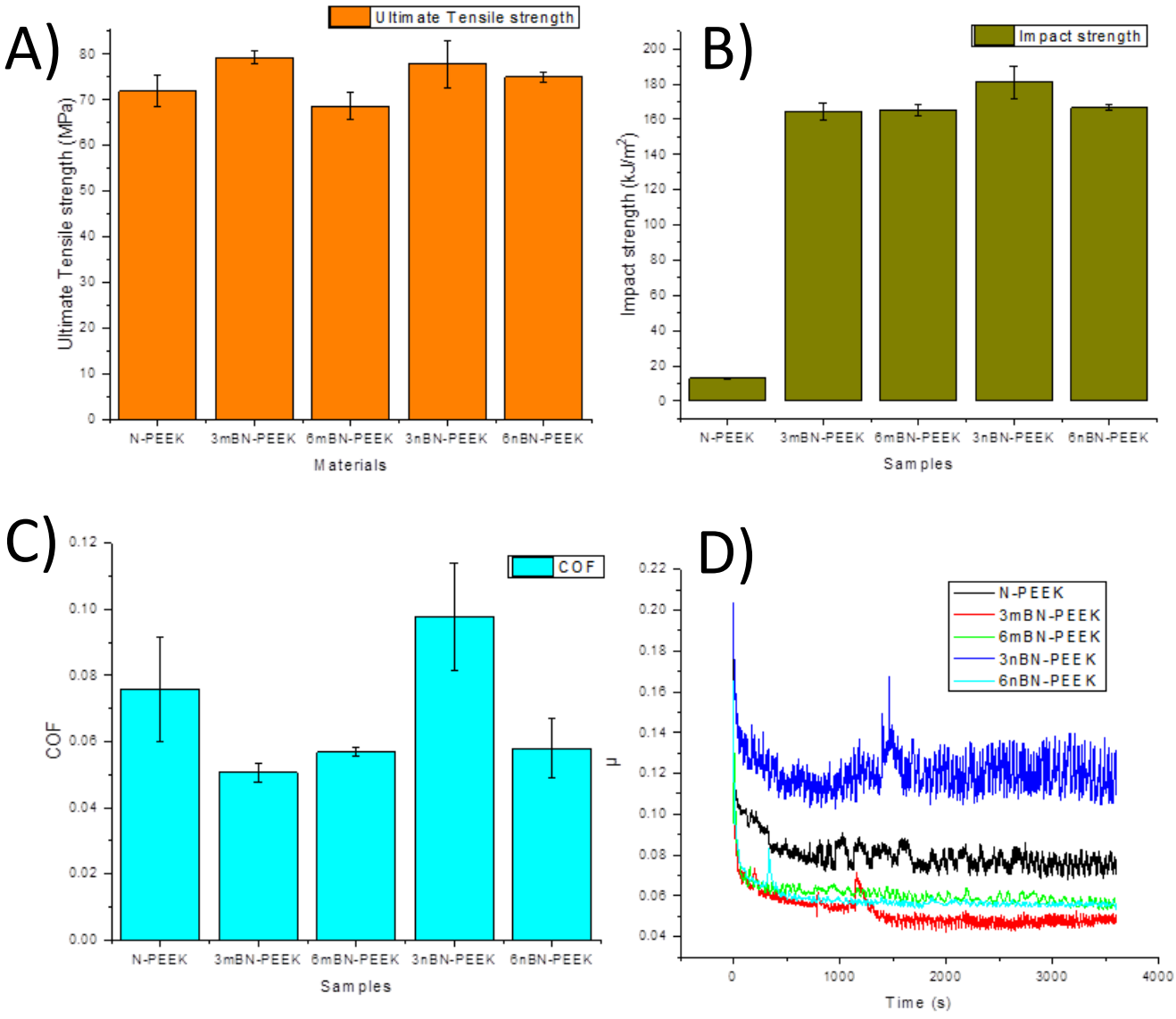

Figure 3 A)Ultimate Tensile strength of neat PEEK compared to various composites B) Comparison of Impact Strength C) Average coefficient of Friction D) In situ Co efficient of Friction across the wear test

5.1. Tribological Testing of 3D printed PEEK composite

The mechanical and tribological evaluation of neat PEEK and its composites with hexagonal boron nitride (hBN) revealed significant enhancements in the material properties of the composites shon in Figure 3. In terms of ultimate tensile strength, neat PEEK exhibited a baseline performance that was surpassed by both the 3mBN-PEEK and 6mBN-PEEK composites. This suggests that the addition of hBN did not compromise the mechanical integrity of the material and has contributed to improvements in structural performance. A similar trend was observed in impact strength, where neat PEEK demonstrated the lowest performance among the samples. The inclusion of hBN in the composites resulted in significantly higher impact strength, with both 3mBN-PEEK and 6mBN-PEEK achieving greater toughness and energy absorption upon impact.

The tribological performance, as assessed by the coefficient of friction (COF), further underscores the benefits of hBN inclusion. Neat PEEK showed a higher average COF compared to the composites, with 3mBN-PEEK exhibiting the lowest values. This trend was consistent in the time-dependent COF data, where neat PEEK started with a relatively high friction coefficient that stabilized over time, while the composites maintained lower and more stable COF values throughout the testing period. Notably, the 3mBN-PEEK composite demonstrated superior frictional properties, indicating that higher hBN content enhances lubrication and wear resistance**.**

5.2. Biocompatibility of Wear Particles

Based on established biocompatibility of conventional neat PEEK [7], it is expected that the wear particles generated from both FFF-printed PEEK and SLS PEKK exhibit comparable, if not superior biological responses considering the and the PEEK-hBN composite. Literature on polymer composites with biocompatible nanoparticles, such as the PE composites studied in prior work, suggests that the inclusion of hBN may reduce cytotoxicity and oxidative stress, leading to improved cell viability and reduced inflammatory responses Figure 4 and Figure 5

Viability assays, such as MTT, are expected to show L929 cell metabolic activity close to or exceeding 90% of untreated controls for both FFF-printed PEEK and the PEEK-hBN composite. The composite is hypothesized to increase cell viability due to its superior wear and friction properties, leading to more favourable particle morphologies and the inherent biocompatibility of the reinforcement.

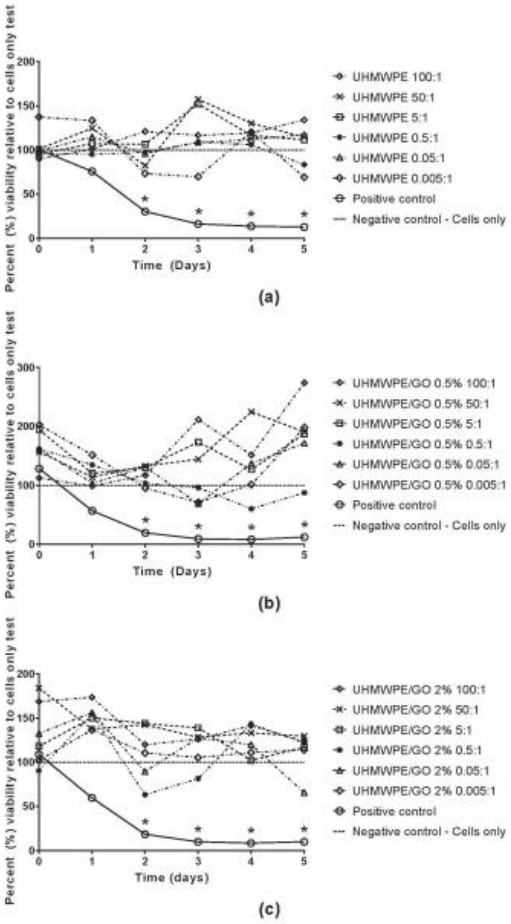

Figure 4 Percentage viability of L929 Fibroblasts cultured with (a) UHMWPE GUT 1020, (b) UHMWPE/GO 0.5wt% and (c) UHMWPE/GO 2wt% wear particles at the indicated particles volume (μm^3) to cell number ratios compared to cells only controls. *Indicates significant reduction in cell viability compared to negative cell only control (p<0.05 ANOVA) [9]

5.3. Reactive Oxygen Species (ROS) and Cytotoxicity

The oxidative stress induced by wear particles will likely be within acceptable thresholds [7], as indicated by similar studies using biocompatible polymeric composites. The ROS levels are anticipated to be slightly higher in the neat FFF-printed PEEK and SLS PEKK particles compared to the composite, given that hBN may mitigate oxidative stress. Trypan blue exclusion and fluorescence-based cytotoxicity assays are predicted to confirm minimal cytotoxicity, with live/dead cell ratios indicating >85% viable cells across all samples.

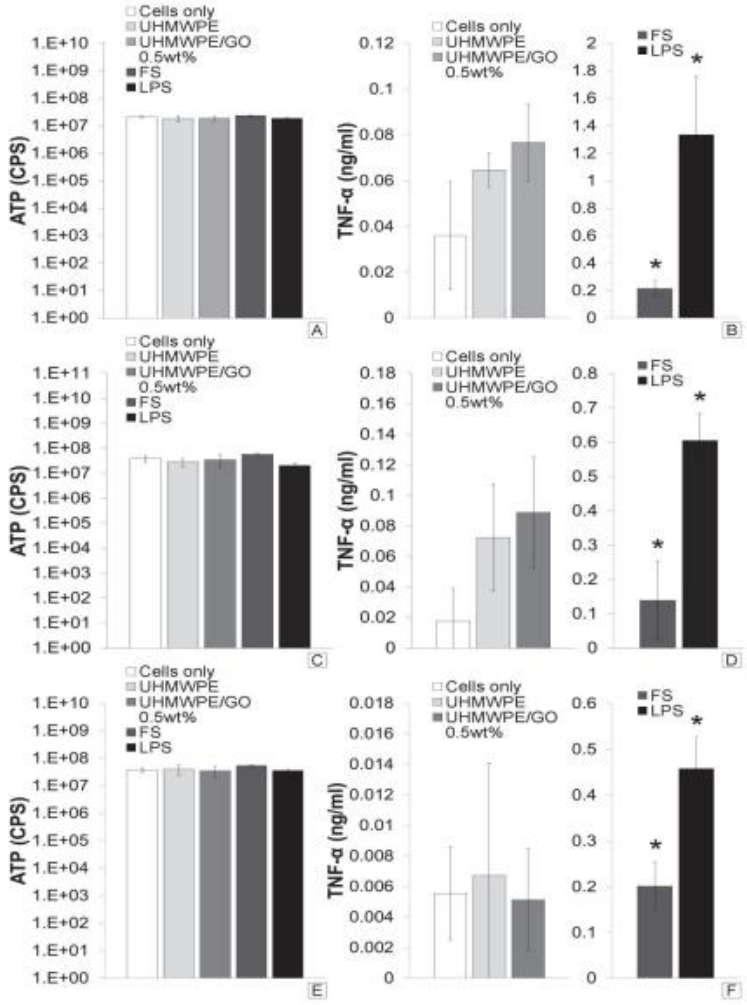

Figure 5 Viability of PBMNC's and TNF-α release from PBMNC's (mean ± std. dev.) from (a, b) Donor 2, (c, d) 3 and (e, f) 15 cultured with UHMWPE GUR 1020 and UHMWPE/GO 0.5 wt% wear particles, 0.2 µm FluroSpheres at a ratio of 300 µm³/cell and LPS (200 ng/ml).

*** Indicates significant reduction in cell viability (a, c, e) and significantly higher TNF-α production (b, d, f) compared to negative cell-only control ($p < 0.05$ ANOVA).** [9]

5.4. Influence of Particle Morphology

Particle morphology, influenced by the tribological test configurations, is a critical factor in cellular responses. Surfaces with increased roughness are commonly employed [9], [10]for bio-tribological studies. The increased roughness of the countersurface (0.1 μm) is expected to generate large volume of wear particles in an acceptable period of time. Although these particles are likely varied in shapes and sizes compared to smoother articulating surfaces. While the CoCrMo setup simulates worse-case scenarios for wear debris, clinical PEEK articulations against smooth ceramic or metallic counter surfaces are anticipated to produce fewer particles with more uniform morphologies, potentially yielding even more favourable biocompatibility results.

The use of 190°C sterilization, while above PEEK's glass transition temperature (143°C), does not exceed its melting point and is unlikely to alter the particle shape significantly. However, changes in surface chemistry, such as oxidation, could variably affect the cellular responses. This will be assessed in subsequent ROS and viability analyses.

5.5. Comparative Insights

When compared to conventional neat PEEK and polyethylene wear particles, which are known to induce adverse inflammatory responses at higher concentrations [7], [10], 3D printed PEEK and PEKK wear particles—particularly the PEEK composite—are expected to generate similar and lower levels, respectively, of inflammatory cytokines such as TNF-α. These predictions align with observations in previous studies on polyethylene composites shown in Figure 5. The presence of hBN in the PEEK composite may further suppress cytokine release, enhancing its potential as a biomaterial.

5.6. Anticipated Challenges

Potential challenges include particle aggregation, which could skew dosimetry in biological assays. Additionally, autofluorescence of PEEK particles, particularly under fluorescence-based ROS assays, may introduce variability in data interpretation. Careful inclusion of particle-only controls will mitigate these issues.

5.7. Statistical Analysis

All experimental data will be analysed using ANOVA to identify significant differences between groups, with post hoc tests used to evaluate specific comparisons. Results will be presented as mean ± standard deviation, with significance set at $p < 0.05$. Graphs will visualize time-dependent cell viability, ROS levels, and cytokine release trends

5) Discussion

6.1. Tribological Testing of 3D printed PEEK composite

The results indicate that PEEK composites containing hBN exhibit significant improvements in both mechanical and tribological properties compared to neat PEEK, reinforcing their potential for orthopaedic applications. The enhanced tensile and impact strengths observed in the composites highlight their suitability for load-bearing applications, as these properties are critical for maintaining the structural integrity of joint replacements under physiological loads.

Tribological performance plays a crucial role in the long-term success of joint replacements, as lower friction coefficients are often associated with reduced wear, consequently, reducing the risk of wear-induced osteolysis. The 3mBN-PEEK composite demonstrated the lowest average and time-dependent COF, suggesting that the self-lubricating properties of hBN significantly contribute to reducing friction. This performance stability over time further emphasizes the composite's resistance to wear under dynamic conditions, making composites particularly well-suited for articulating surfaces in orthopaedic implants [6].

The combination of enhanced mechanical strength and reduced frictional resistance indicates a synergistic effect of hBN within the PEEK matrix. These results suggest that the incorporation of hBN improves both the durability and the tribological performance of the composite without compromising its structural properties. Additionally, the testing conditions subject the contacting surfaces to high contact pressure providing a worst-case scenario for the articulating bearing.

Overall, the findings support the hypothesis that PEEK-hBN composites, particularly the 6mBN variant, exhibit superior performance compared to neat PEEK. These materials hold great promise for next-generation orthopaedic implants, where reduced friction, enhanced wear resistance, and improved toughness are critical for minimizing complications and ensuring long-term success.

6.2. Biological Testing

The preliminary hypotheses and framework established in sections 3 and Section 4 predict that the biocompatibility of wear particles generated from 3D-printed PEEK an SLS PEKK and the PEEK-hBN composite will match or surpass that of conventionally manufactured PEEK. This expectation is grounded in the biocompatibility of conventional PEEK and evidence from prior studies demonstrating the favourable biological performance of polymer composites incorporating biocompatible nanoparticles.

The predicted favourable biological response is likely attributed to the inclusion of hBN, which may mitigate oxidative stress and inflammatory responses. The properties of hBN may include increased mechanical performance and self-lubrication, which can not only enhance wear resistance but also reduce friction at the articulating surface, potentially altering wear particle morphology in a manner that minimizes cellular stress and inflammation.

Despite reasonable expectations of biocompatibility for these materials, several challenges may emerge during testing. The CoCrMo counterface used in tribological testing, with a roughened surface of 1.0 µm, is anticipated to generate wear particles with greater variability in shape and size compared to smoother ceramic articulations, due to the increased friction within a PEEK and CoCrMo bearing. While this setup provides a worst-case scenario for wear generation allowing to collect sufficient particles for examination, clinical applications involving zirconia toughened alumina (ZTA) result in less

wear, potentially different wear particle creation mechanisms, whether this would affect the individual biocompatibility of the particles is unknown, but it is a worthy observation to take into consideration.

Additionally, the sterilization of particles at 190°C—although necessary for endotoxin removal—may induce subtle changes in particle surface chemistry, such as oxidation, which could variably impact cellular responses.

From a biological perspective, the study will particularly focus on inflammatory cytokines, specifically TNF-α, as a marker of cellular immune response. Literature suggests that conventional PEEK wear particles elicit acceptable levels of TNF-α production, a benchmark that the 3D-printed materials and composites are expected to meet or improve upon. The reduced inflammatory response anticipated from the composite particles aligns with the trend observed in prior work on polymeric composites [9], [11], [12], [13]

Potential limitations include challenges with particle aggregation during biological testing, experience with previous studies on polyethylene [10], which could skew particle dosimetry and affect assay reproducibility. Similarly, the autofluorescence of PEEK particles may complicate fluorescence-based ROS assays, underscoring the importance of particle-only controls to ensure accurate data interpretation. These limitations highlight areas for methodological refinement in future studies.

6) Conclusion

This study aims to address a critical gap in the understanding of wear particle biocompatibility from 3D-printed PEEK and PEKK and in-house newly developed PEEK composites with improved tribological performance, offering insights into their potential application in joint replacements.

Even with the lack of data on the particle similarity of the materials. It is predicted that both materials will exhibit biocompatibility comparable to or exceeding that of conventional PEEK, with the hBN composite demonstrating enhanced performance due to its superior wear and friction properties.

The anticipated reduction in inflammatory markers such as TNF-α further underscores the promise of these materials as next-generation solutions for load-bearing orthopaedic implants.

The findings from this work will contribute to the growing body of knowledge on novel advanced biomaterials, laying the groundwork for subsequent in vivo studies to validate these materials under physiological conditions. Additionally, this study will provide a basis for the development of standardized protocols for evaluating PEEK and AM PEEK wear particle biocompatibility in emerging polymer composites.

7) Timescale

The ongoing research on the biocompatibility of 3D Printed PEEK, PEKK and PEEK composites will continue into the new year.
The project will undertake some repeats of the initial viability screening tests to ensure that the data is reliable and repeatable, correctly representing the viability of the wear particles.
During January, the PEEK composite will be formed and tribologically tested, using the protocol defined in this report, to generate sterile, endotoxin free wear particles, which will then be tested in a variety of concentrations to determine the overall viability of the material compared to a control, as laid out in the tiered toolkit [8].
The project aims to have carried out the tribological testing and 1st phase of biological testing by February 2025. Which will then lead onto the ROS and cytotoxicity testing and analysis.

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