# The effects of a composite PEEK-zeolite material upon macrophage phenotype and osteoblast-like cell differentiation

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## Abstract:

Poly-ether-ether-ketone (PEEK) and titanium, commonly used in orthopedic biomaterials, often elicit a pro-inflammatory phenotype among cells of the innate immune system[1]. With the immune system playing a crucial role in modulating inflammation and bone homeostasis, alternative biomaterials that can attenuate the pro-inflammatory environment and promote osseointegration, while retaining load bearing functions are required[2]. The present study compared the *in vitro* response of murine bone marrow-derived macrophages and SAOS-2 cells when cultured on a PEEK-zeolite composite material (ZFUZE$^{TM}$), PEEK and titanium substrates. Primary bone marrow-derived derived macrophages were evaluated in terms of their activation state using RT-qPCR for pro-inflammatory (iNOS, IL1β and IL6) and anti-inflammatory (KLF4, Arg1 and Fizz1) markers, in addition to immunolabelling for iNOS, Arg1 and Fizz1. SAOS-2 cells were evaluated for their osteogenic differentiation using RT-qPCR (BMP2, BMP4 and OCN), ELISAs (BNP2, BMP4, BMP7 and OCN) and alkaline phosphatase (ALP) enzyme activity. The results show that KLF4 and Arginase1 (M2-like activation markers) gene expression in macrophages was enhanced on all substrates compared to tissue culture plastic. Further, the ZFUZE$^{TM}$ and PEEK materials reduced the gene expression of pro-inflammatory markers (IL1β & IL6), with a specifically pronounced (Fizz1$^{+}$) M2-like phenotype in macrophages cultured around ZFUZE$^{TM}$ samples. ZFUZE$^{TM}$ enhanced osteogenic gene expression in SAOS-2 cells compared to Titanium in four out of the six genes tested (*RUNX2*, *BMP2*, *BMP7* and *OCN*), and cells expressed lower levels of *IL6*. ZFUZE$^{TM}$ also exhibited the highest levels of BMP7 and OCN detected in conditioned media and highest levels of alkaline phosphatase enzyme activity in cell lysates when evaluated relative to Titanium 7 d. Taken together, the present study suggests that ZFUZE$^{TM}$, from an immunomodulatory and osteogenic standpoint, elicits a favorable and more biocompatible *in vitro*

immune profile than PEEK or Titanium, and represents a promising alternative orthopedic substrate.

## Introduction:

The biomaterials from which spinal interbody fusion devices are manufactured from, have not changed substantially since the 1990s, but the number of procedures have increased by roughly 220 %, accompanied by the increasing need for revision surgeries[1]. Poly-ether-ether-ketone (PEEK) and titanium alloys have largely met the necessary mechanical requirements for spinal column support[2], but limitations in osseointegration with the PEEK materials and stress-shielding effects with the titanium alloys have impacted clinical success rates, limited the lifespans of devices[2]. There has been recent recognition that cells of the innate immune system play essential roles in not only acute and chronic inflammation, but also in tissue homeostasis, resolution of inflammation, and the proliferation and differentiation of stem cells[3]. Evidence suggests that many commonly used orthopedic biomaterials elicit a pro-inflammatory phenotype among cells of the innate immune system, including macrophages[4]. A sustained foreign body response, coupled with modest osseointegration can result in device micromotion, pain, or worse, delamination requiring surgical reintervention[4]. There is a need and opportunity to develop spinal implant materials that can provide the load bearing function while simultaneously modulating a favorable immune response that mitigates inflammation and leads to effective osseointegration.

In the context of spine surgery, the host immune response to implanted materials has been largely ignored in deference to mechanical and biomaterial properties such as peak load bearing, porosity, and surface characteristics[5,6]. Macrophages actively participate in bone physiology and pathology by secreting factors that regulate osteogenesis (i.e. bone formation) and osteoclastogenesis (i.e. bone breakdown)[7]. Most commonly, it has been shown that a pro-inflammatory (M1-like) macrophages secrete signalling molecules such as IL-6, IL-1β, and TNF-α, all of which are enhancers of osteoclast formation and function, while the pro-regenerative (M2-like) macrophages secrete cytokines such as IL-4, IL-10 and IL-13 that enhance osteoblast activity and bone formation[7]. The *in vivo* macrophage phenotype is dependent upon the surrounding tissue microenvironment and occupies a continuum between the M1 and M2 extremes. It is probable that both macrophage phenotypes play indispensable roles during bone regeneration[8]. Overwhelming

research suggests that commonly used synthetic biomaterials and metals almost always elicit an exclusively pro-inflammatory M1-like macrophage response, while naturally derived biological materials are associated with a more anti-inflammatory M2-like macrophage response[9,10]. Therefore, the macrophage response to biomaterials that are currently being developed for use in spinal interbody fusion devices warrants further investigation.

Biomaterial surface topography and surface chemistry, such as surface roughness, surface charge and hydrophilicity, have the potential to differentially activate macrophage phenotype and modulate downstream osteogenic processes[11,12]. While the underlying biological mechanisms are not fully understood, increasing surface hydrophilicity on titanium and PEEK implants have been shown to promote osteogenesis at multiple levels, by modulating the implant-serum protein interactions during the early stages of wound healing, and subsequently promoting an earlier expression of pathways involved in cell proliferation, osteoblast precursor differentiation[13–15]. Studies have shown the attenuation of pro-inflammatory cytokine gene and protein expression in response to increased surface hydrophilicity[16,17]. It is plausible that biomaterial surface charge may also influence the overall macrophage and osteogenic response, by mediating the interfacial proteomic profile and subsequent cell-biomaterial interactions[10,11]. For instance, the release of cationic particles associated with implant wear, especially in metals, represents one of the most common contributors to implant failure, and plays a significant role in causing a sustained M1-like chronic inflammatory macrophage response[10]. The ability to polarize this response toward a predominantly M2 macrophage response could help to mitigate these adverse events, possibly promoting a healthy tissue repair response and prolonging implant longevity.

The present study compared the biological response of murine bone marrow macrophages and an SAOS-2 osteoblast-like cell line when cultured on a PEEK-zeolite composite material (ZFUZE™) versus PEEK and titanium based substrates. Zeolites are crystalline aluminosilicates, widely used as ion exchangers in the context of biological modification, can serve as sites that incorporate various bioactive cations (*e.g.*, Ca, Si, Zn, Sr, and Mg) by ion exchange, and these ions can slowly get released into the extracellular space via ion-exchange, exhibiting long-lasting biological effects[18]. We hypothesized that the zeolite loading of PEEK will encourage a more favourable immune response and promote osteogenesis compared to PEEK and Titanium. Macrophage

biological response was evaluated using gene and cytokine expression of common M1-like and M2-like markers of macrophage polarization. Further, the osteogenic gene expression and protein secretion from the SAOS-2 cells was evaluated when cultured on the substrates. The development of improved spinal fusion devices that can attenuate the pro-inflammatory environment and promote osseointegration, while retaining the required mechanical properties can be beneficial in reducing revision surgery and improving patient outcomes,

**Materials and Methods**:

*Implant fabrication*

Medical grade Titanium was purchased from TMS titanium, Zeniva medical grade PEEK (poly-ether-ether- ketone) was procured from Solvay and PEEK-zeolite composite (ZFUZE$^{TM}$) tiles were fabricated through a proprietary manufacturing process (US Patent:8821912); developed by DiFusion Technologies (Texas, Austin, USA). To create a roughened surface texture on the Titanium, samples were treated post-machining using a proprietary etching process (DiFusion Technologies, Texas, USA). The ZFUZE$^{TM}$ is a composite of PEEK (base material) and 10 wt% 4A sodium aluminosilicate (zeolite), a ceramic that enhances hydrophilicity and surface potential. All the implants were sterilized using gamma irradiation prior to all cellular experiments. The square tile dimensions (1.5 cm (L) x 1.5 cm (W) x 3 mm (H) were designed in order to ensure a snug fit into the well of a 12-well plate.

*Implant physical characterization*

The loading of zeolite within the PEEK devices were confirmed using energy-dispersive X-ray spectroscopy. The surface topography of the tiles was evaluated using Scanning Electron Microscopy (Instrument name, CBI, Pittsburgh, PA). Scanning electron microscopy images were recorded using a 5 kV accelerating voltage and 6 mm working distance. The surface hydrophilicity of the tiles was evaluated with contact angle measurements from a goniometer, equipped with a digital camera. Ultrapure water was used as a wetting liquid, with a drop volume of 20 uL.

*Isolation, culture and seeding of bone marrow derived macrophages*

Bone marrow-derived macrophages were isolated from the femurs and tibias of C57bl/6 mice as previously published. For immunolabeling experiments, bone marrow derived macrophages were cultured in the wells of a 12-well plate around the tiles at a density of 2 x $10^6$ cell per well suspended in 1mL of macrophage growth media. This technique enabled easy visualization of cells

post immunolabeling. For all other experiments, 2 x $10^6$ cells suspended in 250 uL of macrophage growth media were cultured directly onto the tiles and allowed to attach overnight, followed by the addition of 2 mL of growth media the following day. The macrophage growth media included Dulbecco's Modified Eagles High Glucose Medium (Hyclone, Cat # SH30022.01), 10% fetal bovine serum (FBS), 10% L929 supernatant, 0.1% beta-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM nonessential amino acids, and 10 mM HEPES buffer for a total of 7 days with media changes every 2 days. Assay controls included macrophages cultured on tissue culture plastic in (i) growth media for 7 d (M0 naïve macrophages), (ii) growth media for 7 d followed by an additional 24 h treatment with inflammatory cytokines (20 ng/ml IFN-γ and 100 ng/mL lipopolysaccharide LPS) (M1 control) and (iii) growth media for 7 d followed by anti-inflammatory cytokines (20 ng/mL interleukin IL-4) (M2 control).

*Culture and seeding of SAOS-2 osteoblast-like cells*

SAOS-2 cells, a clonal cell line derived from a human osteosarcoma, was purchased from American Type Culture Collection (ATCC) (Rockville, MD). SAOS-2 cells are widely used as a model for bone cell metabolism and differentiation. The cells were maintained in osteogenic growth medium containing McCoy's 5A Medium, supplemented with 15% fetal bovine serum, 1.5 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 pg/ ml), with media changes every 2-3 days. Cells from passages 3-6 were utilized for all osteogenic assays and were repeated in three independent experiments. For all osteogenic experiments, SAOS-2 cells were detached using 0.025% Trypsin/EDTA, suspended in 250 uL osteogenic growth media and seeded directly onto the tiles at a density of 10,000 cells/$cm^2$. The following day, 2 mL of media was added and media changes made every 2-3 days for a total of 7 days.

*Immunophenotype and osteogenic gene expression analysis*

For immunophenotype gene expression, total RNA was isolated from the macrophages at 7 days using Trizol-Reagent (Molecular Research Center Inc., Cincinnati, OH) using published methods[19]. For osteogenic gene expression, total RNA was isolated from the SAOS-2 cells at 7 days and 14 days. cDNA was synthesized from 500 ng of total RNA in a volume of 20 µL using a Superscript RT III kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For the immunophenotype assessment, quantitative real-time PCR was performed using the PowerUp™ SYBR® Green Master Mix (Cat. No. A25778; Applied Biosystems) with primers designed to be specific for genes known to be strong indicators of either an M1 type (inflammatory)

or an M2 type (anti-inflammatory) macrophage response. (Supplementary Table. 1) The levels of expression were normalized to the housekeeping gene, *HPRT1*. For the osteogenic assessment, primers designed to be specific for genes that regulate osteogenesis were used and normalized to *PGK13* as the housekeeping gene. (Supplementary Table. 1) Results were expressed as fold change log ($2^{-\Delta\Delta Ct}$) relative to non-treated macrophages.

*Bone-marrow macrophage immunolabeling*

At 7 days after culture, the cells were washed and fixed in 2% paraformaldehyde. Following two additional PBS washes, the cells were incubated in a blocking solution consisting of 0.1% Triton-X 100, 0.1% Tween 20, 4% normal goat serum, and 2% bovine serum albumin (BSA) for 1 h at room temperature to prevent non-specific antibody binding. Following blocking, cells were incubated in one of the following primary antibodies, each diluted in the blocking solution: (1) rat monoclonal anti-F4/80 (Abcam, Cambridge, MA) at 1:200 dilution for a pan-macrophage marker, (2) rabbit polyclonal anti-iNOS (Abcam, Cambridge, MA) at 1:200 dilution for an M1 marker and finally, (3) rabbit polyclonal anti-Fizz1 (Peprotech, Rocky Hill, NJ) and rabbit polyclonal anti-Arginase1 (Abcam, Cambridge, MA) at 1:200 dilution for the M2 markers. Following incubation overnight at 4$^{o}$C, the cells were washed two times in PBS and incubated in the corresponding fluorophore-conjugated secondary antibodies, Alexa Fluor goat anti-rat 488 (Fisher Scientific, A11006) or Goat anti-rabbit 488 (Fisher Scientific, A11034) for 1 hour. After PBS washes, the nuclei were counterstained with 4'6'diamidino-2- phenylindole (DAPI) and imaged using a fluorescent live-cell microscope (Zeiss AXIO Observer Z1). Light exposure times were standardized to a negative isotype control and kept constant across images. Images were quantified utilizing CellProfiler Image Analysis software to obtain positive F4/80, iNOS, Arginase and Fizz1 percentages.

*Quantification of osteogenic secreted proteins through ELISA:*

At 6 days after culture, the SAOS-2 cells were incubated with fresh media for an additional 24 h, and their conditioned media was extracted. The media was then centrifuged at 2,000 rpm at 4$^{o}$C for 10 minutes to remove any cell debris, supernatant collected and used for subsequent immunoassay analyses. Levels of BMP2 (R&D Systems, Minneapolis), BMP4 (R&D Systems, Minneapolis), BMP7 (R&D Systems, Minneapolis) and Osteocalcin (R&D Systems, Minneapolis) were assayed using the enzyme-linked immunosorbent (ELISA) assay.

*Alkaline phosphatase enzyme activity:*

As an additional measure of osteogenesis, intracellular alkaline phosphatase activity was measured in the SAOS-2 cells cell lysates using the alkaline phosphate colorimetric kit (Abcam, ab83369). At either 7 days and 14 days after culture, the cells were rinsed in PBS and supplemented with 0.5 mL of enzyme extracting reagent (5 mM Tris, 20 mM Tricine and 1 mM EDTA, pH 7.4). Following cell lysis using an ultrasonic homogenizer (Model-100, Fisher Scientific), the samples were centrifuged at 12,000 xg for 10 min to obtain the cytosolic fraction containing the intracellular ALP enzyme. The Alkaline phosphatase activity was normalized to total protein content measure by the Pierce BCA Protein Assay Kit (Rockford, IL).

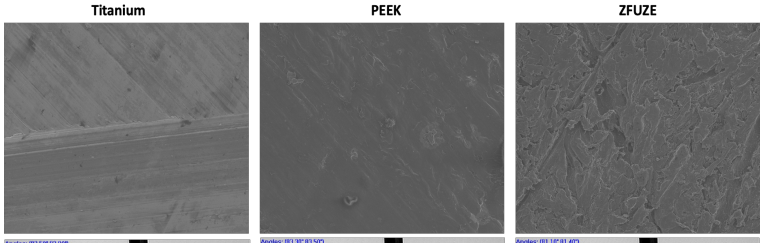
*Statistical Analysis:*

All numerical data are depicted as mean ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA) by two-way ANOVA with a Tukey's post-hoc comparison of the means. Differences were considered statistically significant at $p < 0.05$ unless otherwise noted.

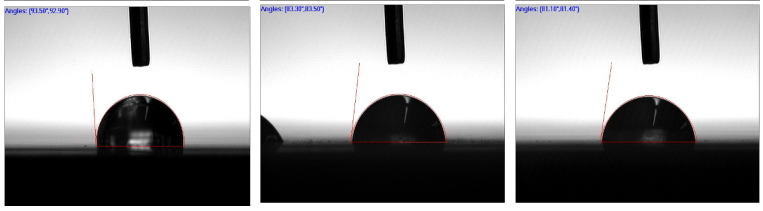
**Results:**

*Characterizing the surface properties of the spinal fusion devices*

A




B



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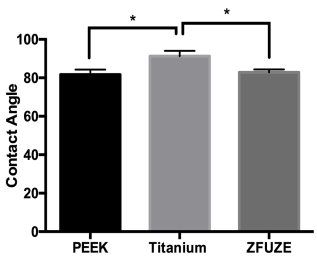


D

| | PEEK | ZFUZE | Aluminum |
|---|---|---|---|
| Element (Symbol) | Atomic % | Atomic % | Atomic % |
| Na | 0 | 14 | 0 |
| Al | 0 | 33 | 6 |
| Si | 0 | 53 | 0 |
| C | 89 | 0 | 0 |
| O | 11 | 0 | 0 |
| Ti | 0 | 0 | 91 |
| V | 0 | 0 | 3 |

E

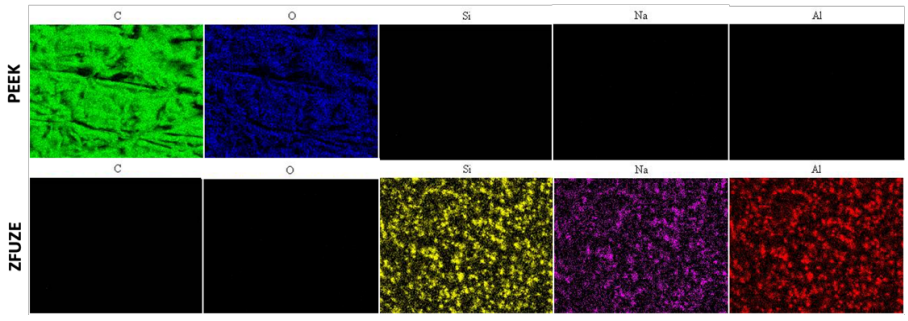


**Figure 1:** Physical characterization of the three substrates in terms of (A) surface morphology, (B&C) hydrophilicity and (D&E) elemental analysis. The PEEK, Titanium and ZFUZE$^{TM}$ samples were unique in terms of surface topography, hydrophilicity and surface charge. The zeolite loading of PEEK resulted in a ZFUZE$^{TM}$ substrate that was characterized by increased hydrophilicity, an overall negative charge, and an uneven topography.

The three spinal fusion substrates evaluated were distinct in terms of their elemental composition, surface topography, hydrophobicity and surface charge. Scanning electron microscopy of the substrates clearly show that the Titanium and PEEK samples exhibited an even, smooth surface, while the ZFUZE samples had an irregular topography (Fig. 1A). The topography observed corresponds to the zeolite loaded onto the base PEEK samples. A surface is classified as hydrophobic or hydrophilic based on the water contact angle measurements (WCA), with a $WCA > 90°$ and $WCA < 90°$ corresponding to a hydrophobic and hydrophilic material respectively[20]. Contact angle analysis performed on the substrates showed that the ZFUZE (WCA=81°) and PEEK (WCA=83°) samples were hydrophilic and the Titanium (WCA=93°) samples were hydrophobic (Fig. 1B&C). The elemental analysis of the devices showed a distinctive composition of devices for each device; the Titanium consisted of predominantly Titanium (~91%), with trace amounts of Aluminum (~6%), and Vanadium (~3%), the PEEK samples consisted of predominantly carbon (~90%) and oxygen (~10%), and the ZFUZE samples consisted of mostly Sodium (~14%), Aluminum(~33%) and Silicon (~53%) (Fig. 1D). Elemental analysis further confirmed the loading of zeolite within the PEEK devices, and demonstrated the even distribution of the zeolite throughout the samples. The detailed EDX spectra for each sample can be found in Supporting Fig. 2.

*PEEK and zeolite-loaded devices reduced the pro-inflammatory gene expression in primary bone-derived macrophages compared to Titanium*

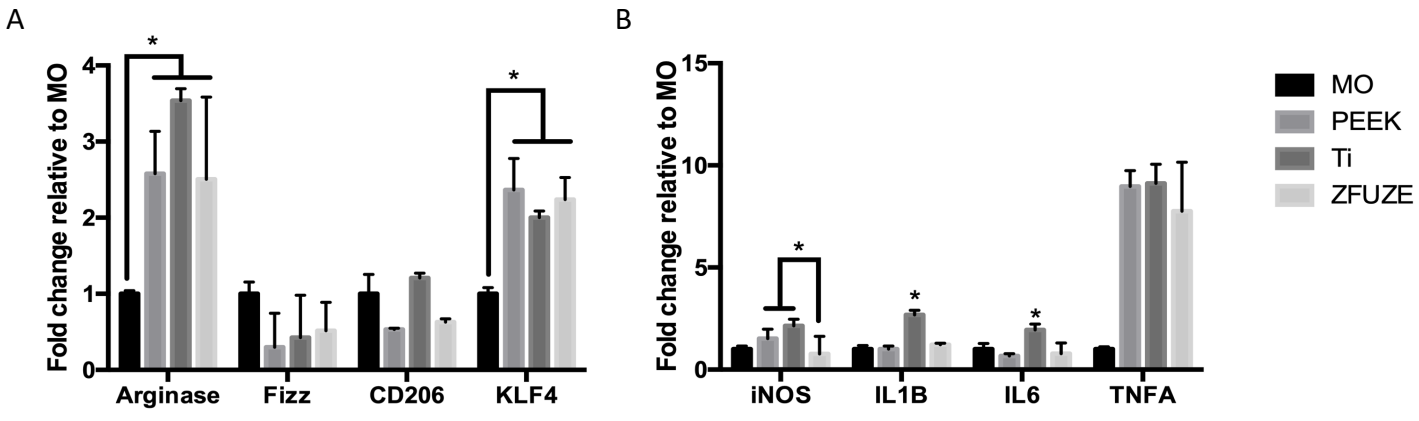


**Figure 2:** Gene expression of macrophages cultured on the three substrates relative to the media only control. Culturing the macrophages on either of the substrates upregulated the expression of Arginase and KLF4 as compared to TCP. The macrophages cultured on ZFUZE expressed lower levels of iNOS, IL1B and IL6 compared to Titanium substrates. *Represents values significant compared to all groups unless otherwise stated; *$p<0.05$

The gene expression of common M1-like (pro inflammatory) (Fig 2A) and M2-like (anti-inflammatory) markers (Fig 2B) was evaluated in three independent experiments, in order to determine the activation state of the macrophages when exposed to the substrates. Of the four M2-like genes evaluated, culturing the bone marrow macrophages on all the substrates enhanced the gene expression of *KLF4* and Arginase as compared to tissue culture plastic. However, no significant differences were observed in gene expression between substrate types within a given gene. Analyses of the pro-inflammatory genes showed that the ZFUZE reduced the *iNOS* gene expression when compared to both Titanium and PEEK. Of note, the macrophages cultured on the ZFUZE and PEEK substrates also showed a significantly lower expression of *IL1B* and *IL6*, both of which were enhanced on Titanium substrates.

*The zeolite-loaded substrates enhanced the M2-like protein expression in primary bone-derived macrophages*

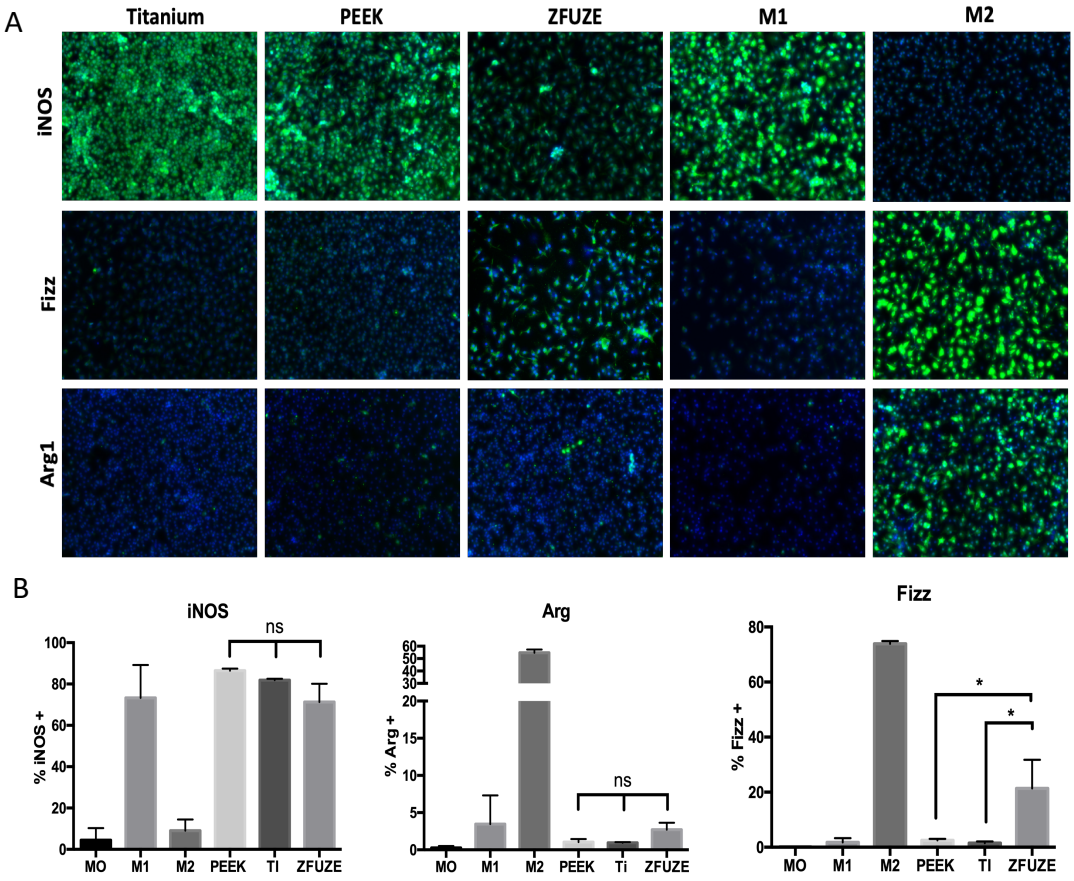


**Figure 3:** (A) Macrophages immunolabelled to visualize M1-like macrophages (iNOS) and M2-like macrophages (Fizz and Arginase). (B) Quantitative analysis of immunolabelled cells depicting percentage of positively stained nuclei. Qualitatively, ZFUZE$^{TM}$ seemed to decrease the iNOS expression in comparison to PEEK and Titanium, and quantitatively resulted in a concurrent increase in Fizz1 expression. *p<0.05

To complement the gene expression data, the protein expression of common M1-like (pro inflammatory) and M2-like (anti-inflammatory) markers was evaluated using fluorescent staining techniques (Fig. 3A). ZFUZE decreased the iNOS expressed by the macrophages in comparison to PEEK and Titanium, while causing a concurrent increase in Fizz1 expression, suggesting that the ZFUZE elicited a more "M2-like" macrophage phenotype. The quantitative assessment of relative protein expression levels (Fig. 3B) showed that the ZFUZE enhanced the expression of Fizz as compared to both PEEK and Titanium. There was a trend suggesting that the ZFUZE reduced the iNOS protein expression and increase the Arginase expression, but these results were not statistically significant.

*The zeolite-loaded substrates enhanced the osteogenic gene expression of SAOS-2 cells*

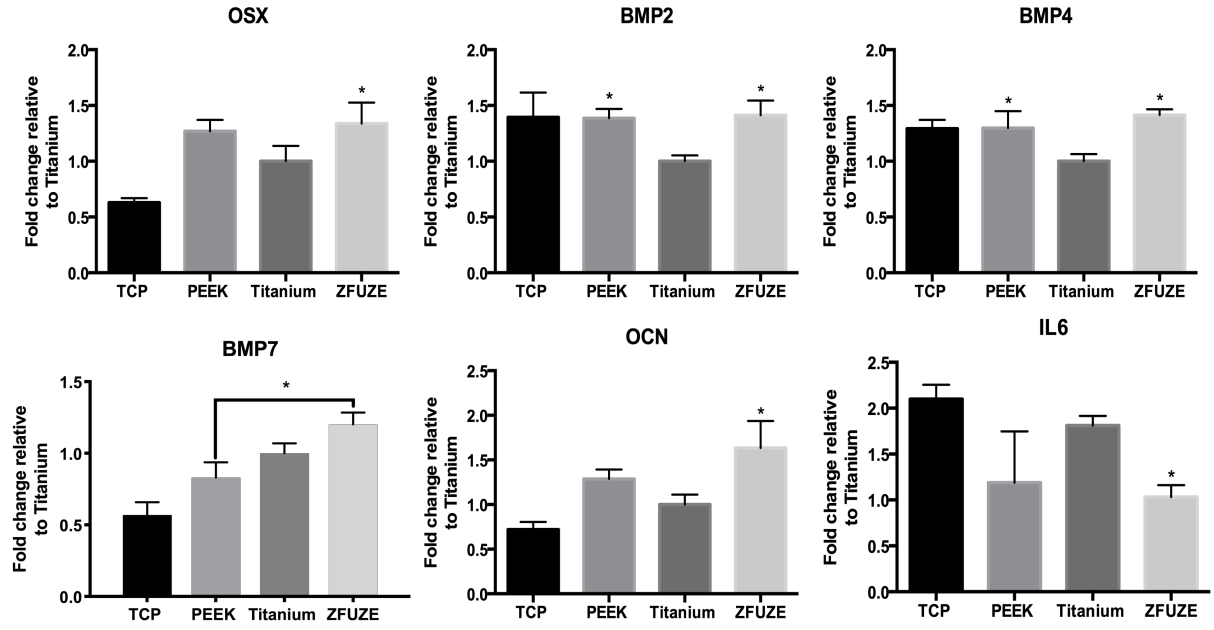


**Figure 4:** Osteogenic gene expression of SAOS-2 cells when cultured on the substrates at 7 d post culture. The ZFUZE$^{TM}$ samples responded better than Titanium for all of the genes sampled. ZFUZE$^{TM}$ enhanced the expression of osteogenic genes OSX, BMP2, BMP4, BMP7 and OCN as compared to Titanium. ZFUZE$^{TM}$ also reduced the expression of IL6 as compared to Titanium. *Represents values significant compared to Titanium; *$p<0.05$

The osteogenic effects of the three substrates on the SAOS-2 cells was determined by quantifying osteogenic gene expression at 7 d, with genes selected from the early (*OSX* and *BMP2*), early-mid (*BMP4 and BMP7*) and late (*OCN*) stages of osteogenic differentiation. ZFUZE showed greater

gene expression than Titanium in four out of the six genes tested (*OSX*, *BMP2*, *BMP7* and *OCN*), while PEEK showed greater gene expression than Titanium in only two out of those genes (*BMP2* and *BMP7*). ZFUZE also showed greater *BMP7* expression than PEEK. SAOS-2 cells cultured on ZFUZE also expressed lower levels of *IL6*, a pro inflammatory cytokine expressed by SAOS-2 cells during bone breakdown. Interestingly, SAOS-2 behaved similarly to the macrophages cultured on the titanium substrates, with the highest gene expression of IL6 observed.

*The zeolite-loaded substrates enhanced the osteogenic protein expression in SAOS-2 cells*

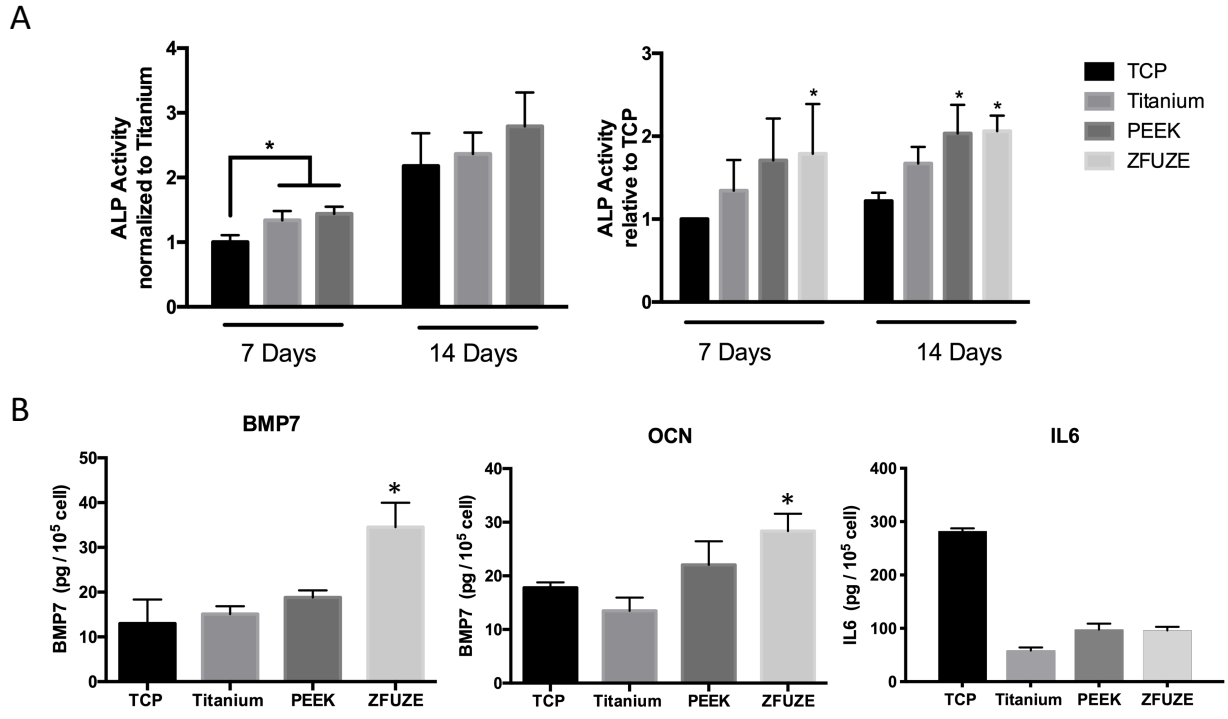


**Figure 5:** Osteogenic protein expression evaluated by (A) intracellular alkaline phosphatase activity and (B) extracellular BMP7 and OCN concentration at 7 d post culture. The PEEK and ZFUZE$^{TM}$ substrates enhanced ALP activity at 7 d post culture compared to Titanium, and SAOS-2 cells cultured on ZFUZE$^{TM}$ samples expressed the highest levels of extracellular BMP7 and OCN at 7 d. Represents values significant compared to Titanium; *$p<0.05$

Alkaline phosphatase activity is required for the mineralization of bone and represents a useful biochemical marker of bone formation. In general, the ALP enzyme activity of SAOS-2 cells cultured on Titanium substrates was similar to cells cultured on tissue culture plastic. In contrast, at 7 days, ALP enzyme activity was significantly enhanced in cells cultured on ZFUZE and

remained consistently higher after 14 days in culture. When measured relative to Titanium at 7 days, cells cultured on PEEK and ZFUZE expressed enhanced levels of ALP enzyme activity. Finally, to complement the gene expression data, ELISAs for BMP7 and OCN were performed. Complementing the gene expression data, cells cultured on ZFUZE expressed higher levels of OCN compared to Titanium, and also outperformed both Titanium and PEEK, with the highest levels of BMP7 detected. The PEEK samples however, performed similar to both Titanium samples as well as cells that were cultured on tissue culture plastic for both proteins tested. The evaluation of IL6 pro-inflammatory cytokine expression in cell culture supernatants however did not differ between the substrates, although all the test articles significantly reduced the pro-inflammatory cytokine secretion as compared to cells cultured on tissue culture plastic.

**Discussion:**

The efficacy of orthopedic biomaterials is often determined by evaluating their capability to induce osteogenic differentiation of cell types such as osteoblasts, osteoblast progenitors and mesenchymal stem cells *in vitro*[21,22]. Osteoimmunology, the interaction between the immune cells and skeletal systems, plays a vital role in regulating bone homeostasis, and immune cell dysregulation has been implicated in pathologies such as osteolysis, osteoporosis and rheumatoid arthritis[5]. Of the immune cells, macrophages in particular are a highly plastic cell type and play multiple roles in wound healing, and are considered a pivotal regulator of bone metabolism[7]. Therefore, the present study evaluated the effect of a zeolite-loaded PEEK spinal fusion device, ZFUZE™, on macrophage activation and osteogenic expression when compared to standard orthopedic biomaterials, Titanium and PEEK. Bone marrow derived macrophages represent a preferred primary cell population for the evaluation of macrophage activation state on both synthetic and naturally-derived substrates[23]. The SAOS-2 cells used in this study are a commonly used model cell type to demonstrate osteogenic differentiation as they display similar expression patterns of osteogenic factors as primary osteoblasts[24]. Evaluating the effects of each substrate on inflammation and osteogenic differentiation independently, confirmed the intricate balance between immunology and osteogenesis, and highlighted the importance of both outcome measures in the development and assessment of orthopedic biomaterials.

ZFUZE™ is a composite polymer of PEEK, that is enriched with negatively charged and hydrophilic aluminum silicate-based zeolite particles. The resulting substrate aims to retain the loading bearing properties of the conventional orthopedic materials such as PEEK and Titanium, while providing modified surface properties known to be favorable for osteogenesis and attenuation of inflammation[25]. The EDS-X ray elemental analysis of the samples confirmed the presence of the aluminum and silicon moieties on the ZFUZE™ surface that was absent in PEEK. Silicate-based ceramics are considered excellent candidates for orthopedic applications, due to their osteoconductive and osteoinductive properties[26,27]. Specifically, silicate-based ceramics have been shown to promote osteoblast proliferation, pre-osteoblast differentiation and accelerate mineral deposition[26,28,29]. Silicon is intrinsically located at the active calcification sites of bone *in vivo* and plays an important role in the mineralization process during bone growth. In particular, zeolite-modified substrates in several studies have enhanced osteoblast cell proliferation, and differentiation that subsequently leads to improved matrix mineralization[18,25,30]. Surface characterization of the substrates in the present study showed that the surface-modification of ZFUZE with zeolite produced a substrate surface that was uneven and exhibited greater hydrophilicity than Titanium. It is accepted that hydrophilicity, surface topography and roughness, can determine the profile and conformation of adsorbed proteins during the Vroman effect[25]. The resulting protein-cell interactions can serve to influence cell density, shape and migration, events that activate intracellular downstream signalling cascades and modulate cell survival, proliferation, and differentiation[12,31]. It would be worthwhile in future studies to investigate the differential kinetic, structural and thermodynamic aspects of protein adsorption on each substrate using Fourier transform infrared spectroscopy (FTIR) to accurately interpret the cellular effects observed. It was expected that the combination of zeolite loading, overall negative surface charge and hydrophilic surface of the ZFUZE™ would work in combination to encourage a more favourable immune response and promote osteogenesis. While the roughness of the resulting substrates was not measured in this study, the uneven surface of the ZFUZE substrates possibly due to the zeolite loading, could have contributed in part to the biological effects observed.

The long-term survival and function of orthopedic biomaterials is highly dependent on nature of the immune response to the material. In particular, the initial macrophage response is known to be a key indicator of downstream tissue repair[23], and in the context of bone plays a crucial role in

determining the implant osseointegration[32]. In this work, although some of the subtle differences between the substrates were masked at the gene level, the results suggested that the ZFUZE™ substrates were able influence the activation state and maturation in primary bone marrow derived macrophages by attenuating inflammation and promoting the expression of a M2-like macrophage phenotype. Lending support to these results, several other studies have tested and supported the role of hydrophilicity and a negatively charged surface in promoting an M2-activated macrophage phenotype in both primary bone marrow derived macrophages and a human macrophage cell line[16,17]. In the context of bone regeneration, while there is no consensus as to which macrophage phenotype (M1 or M2) is most beneficial for osteogenesis, it is imperative that there is an appropriately timed transition from the initial M1-like inflammatory macrophage response, to a M2-like macrophage response ascribed by pro-osteogenic cytokine release and bone formation. In future work, a macrophage challenge assay, evaluating the ability for each substrate to reverse the activation state of M1-like macrophages to a M2-type, could provide further insight into the immunomodulatory response of the materials. Acknowledging that macrophages constitute a heterogenous spectrum of phenotypes[8] and that definitions are continuing to evolve as we develop a better understanding of the macrophage profile during repair, there is a growing need to also expand on the existing pool of standardized biomarkers and improved quantitative analyses for improved interpretation of data.

The osteogenic gene expression data, alkaline phosphatase activity and osteogenic protein expression by the SAOS-2 cells in the present study, clearly show that the ZFUZE™ is more effective in promoting osteogenesis than Titanium and PEEK. Orthopedic biomaterial properties such as surface topography, hydrophilicity and surface charge are being closely evaluated as important determinants of downstream device success. Hydrophilic and negatively charged surfaces, have been shown to encourage osteoblast attachment, proliferation and differentiation, as well as stimulate the local osteogenic factor production that ultimately leads to effective osseointegration[12,13,15,17,33]. The findings presented herein are consistent with several other *in vitro* studies that show that Titanium and PEEK samples that is surface modified to have high hydrophilicity and a negative charge, induced higher levels of osteogenic gene expression (RUNX2, OCN, BSP), enhanced ALP activity and the promotion of subsequent mineral

deposition[12,13,15,17,33]. Interestingly, the combination of both properties (hydrophilicity and surface charge) often result in a synergistic effect.

It is well known that the immune and skeletal systems share cytokines, receptors, signalling molecules and transcription factors, all of which cooperatively regulate osteoclast and osteoblast activity and interaction[6]. Several studies have shown that enhanced osteogenic differentiation in response to materials was accompanied by a reduction in pro-inflammatory gene expression as well as the cytokine secretion of IL6, IL1B, IL9 and IL17 [34]. Similarly, in this present study, the enhanced osteogenic gene expression on ZFUZE™ samples, was accompanied by a reduction in IL6 gene expression in SAOS-2 cells. IL6 has been shown to negatively regulate osteoblast differentiation, activate osteoclast differentiation and induce bone resorption by promoting receptor activator of nuclear factor kappa-B ligand (RANKL) production and reducing osteoprotegrin production in osteoblastic/stromal cells [35].

Overall, the present study lends support to the interdependence of the immune system and skeletal system on osteogenesis and highlights the importance of both outcome measures in the design and development of orthopedic biomaterials. Future work would benefit from evaluation of the osteogenic and immunomodulatory effects of the materials in a single co-culture system. Indeed, a growing body of evidence has indicated the promotion of osteogenesis in bone cells when co-cultured with macrophages potentially through a paracrine mediated mechanism[36–38].

**Conclusion:**

Overall, this study independently evaluated the effects of three spinal fusion devices (ZFUZE™, PEEK and Titanium) on bone-marrow derived macrophages and SAOS-2 cells, cells that play major roles in bone turnover. The collative results in this study show that ZFUZETM promotes a more osteogenic and favorable M2-like immune response that Titanium or PEEK. These findings shed light on the importance of evaluating the immunomodulatory effect of biomaterials in addition to the osteogenic effects for orthopedic applications,and substantiates the role of the inflammatory response as an important mechanism driving osteogenesis. In future work, investigating the paracrine-mediated crosstalk between bone cells and immune cells through co-culture models would provide greater insight and facilitate better interpretation of the data.

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