Understanding the biological responses of nanostructured metals and surfaces

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Abstract. Metals produced by Severe Plastic Deformation ( SPD) offer distinct advantages for medical applications such as orthopedic devices, in part because of their nanostructured surfaces. We examine the current theoretical foundations and state of knowledge for nanostructured biomaterials surface optimization within the contexts that apply to bulk nanostructured metals, differentiating how their microstructures impact osteogenesis, in particular, for Ultrafine Grained (UFG) titanium. Then we identify key gaps in the research to date, pointing out areas which merit additional focus within the scientific community. For example, we highlight the potential of next-generation DNA sequencing techniques (NGS) to reveal gene and non-coding RNA (ncRNA) expression changes induced by nanostructured metals. While our understanding of bio-nano interactions is in its infancy, nanostructured metals are already being marketed or developed for medical devices such as dental implants, spinal devices, and coronary stents. Our ability to characterize and optimize the biological response of cells to SPD metals will have synergistic effects on advances in materials, biological, and medical science.

1. Introduction

The emergence of nanoscale science and technology in the 1990s has produced a wide range of scientific and technological achievements highlighting the virtues of nanostructuring materials for medical applications, especially to enhance osteogenesis in orthopedics [1, 2]. The success of polymeric, ceramic, and metallic materials for orthopedics hinges on the ability of bone and other tissues to adapt to, or integrate with, these xenobiotic materials. Biological optimization of implant materials requires an understanding of the cellular response to the abiotic signals when these unfamiliar substrates are encountered in the human body. While the features of all biomaterials are of interest, the scope of the present paper focuses on biomedical aspects of titanium and its alloys. Titanium is amongst the most biocompatible of metals and is particularly important because of its availability for medical use in unalloyed and alloyed forms, plus nanostructured variants of both. Special interest in titanium has evolved as researchers have demonstrated how commercially pure grades of titanium, most prominently grades 2 and 4, can be nanostructured to attain levels of mechanical properties that exceed those of many titanium alloys [3, 4]. This capability has introduced the prospect of achieving extraordinary properties in metals for biomedical use that are free of the alloying elements that have documented toxicological effects, but must be present in conventional alloys to provide strength and corrosion resistance.
The potential of achieving superior mechanical properties in commercial purity titanium comparable to titanium alloys has motivated substantial interest within the ultrafine grained (UFG) metals research community. Stolyarov et al. [4] prepared one of the first papers in 2000 that recognized the prospect of using ultrafine grain (UFG) titanium in medical device applications. Trauma devices such as bone screws, bone plates, external fixation rods, and spinal rods have been fabricated from UFG titanium produced via Equal Channel Angular Pressing (ECAP) at the Ufa State Aviation Technical University Institute of Physics of Advanced Materials since 1995 [4]. This work established the manufacturability of devices from UFG metals. The prospect of engineering UFG variants of other titanium alloys for medical use was demonstrated by Pushin and Valiev for UFG NiTi [5] and by Semenova, et al. for medical grade (grade 23) Ti-6Al-4V subject to ECAP [6]. As early as 2005 UFG grade 2 titanium was introduced into clinical trials in Europe for a “nanotitanium” dental implant [7]. By 2009 another dental implant system based on UFG titanium was approved for sale in the United States [8]. In 2010 Carpenter Technology Corporation announced its projections for production and sales of $100 million of medical grade UFG titanium after implementing full-scale production SPD technologies. With the availability of UFG titanium, medical device manufacturers will be able to evaluate UFG titanium for adoption in their products.

The growing interest in UFG metals has spawned research on their biological properties. This paper examines the trends and highlights of this research, and identifies gaps and opportunities to enhance the impact of the adoption of nanostructured (NS) and UFG titanium in healthcare. It is important to note that while bulk nanostructured metals and alloys possess nanoscale structure throughout their volume, our emphasis here on biocompatibility focuses most of our attention on the nanoscale characteristics that exist at the surfaces of UFG/NS titanium. These surface characteristics are distinct from conventional course grain (CG) titanium and CG titanium with nanotopographical features imparted by surface treatments.

1.1. On biocompatibility
The subject of biocompatibility of materials with human physiology evokes debate about its precise definition. Jonathan Black offered the alternative phrase “biological performance” to emphasize the two-way nature the interaction between materials and living systems [9]. Two complementary aspects of biological performance are the response of the living host to materials and the material response to the living system. In this paper, we focus only largely on the cellular level host response to bulk ultrafine grained and nanostructured titanium produced by SPD processes such as ECAP or High Pressure Torsion (HPT).

We further restrict our inquiry to biological responses pertinent to orthopedic and dental implant applications. Thus, our primary interest is in cellular level interactions between bone forming cells and titanium as would be found in these medical devices. Other biological environments are also important for titanium, for example in equipment for food handling, processing, and storage where titanium and its alloys are commonly used because of their excellent resistance to corrosion.

1.2. On osteogenesis
Osteogenesis is the three-stage process by which bone forms, beginning with osteoinduction, followed by osteoconduction, and ending with osseointegration. Some of the literature pertaining to biological performance of titanium implants addresses the subject only in terms of the osseointegration even though it is just one of the steps in the overall process of bone formation.

When a metallic implant is placed into tissue, proteins and bone-forming compounds from the blood and other tissue fluids absorb onto the metal surface. Cytokines and growth factors then stimulate osteoinduction, which is the differentiation of mesenchymal stem cells (MSCs) into bone-forming pre-osteoblasts. This stage is followed by osteoconduction, the process by which pre-osteoblasts migrate, and proliferate on a material surface. Osteoblasts develop to form a woven extracellular matrix (ECM) structure, which matures into a lamellar bone structure over time [10]. Osseointegration follows, and includes the development of cell-surface contact through direct
anchorage and biomechanically rigid fixation of bony tissue to a material surface [11]. Understanding the ultimate effects of nanostructured UFG titanium on osseointegration must include consideration of the osteoinductive and osteoconductive stages. Analyses of cell biocompatibility typically encompass some, but rarely all of these stages.

Other cell types influence the osteogenesis processes. Closely related to osteoblasts are fibroblasts. These cells are involved in wound healing and scar tissue formation, which can include encapsulation of rejected implant material. MSCs can also differentiate into chondrocytes that are responsible for the formation of cartilage, which may also be involved in successful implantation. Hematopoietic stem cells (HSCs) circulate in the blood and can give rise to osteoclasts (bone resorbing cells), macrophages, and a wide range of other cell types. Macrophages are important cells of the immune system that function by attaching to foreign material and sending out signals that activate immune responses, which can lead to inflammation and rejection of an implant. For dental implants in particular, osteogenesis can be affected by the saliva in the mouth, which contains over 2000 proteins that may be entrapped during implantation into bone [12]. There are also 600 species of bacteria in the human oral microbiome [13] and the response of bacteria to a dental implant is an important consideration. We are just beginning to understand the fine balance between osteoblasts, fibroblasts, osteoclasts, chondrocytes, macrophages and other factors that contribute to successful implantation [14].

2. Analysis of the literature on biocompatibility of ultrafine grained metals

The earliest research on the biocompatibility of nanostructured materials appears at the beginning of this century, including studies by Galli et al. [1] on protein absorption on nanostructured titanium and Zhu and Chen [2] on osteoblast reactions to submicron-scale porous structures on titanium. Long et al. [15] also examined nanoscale surface morphologies that affect the attachment of proteins and other biomolecules. Mwenifumbo, et al. [16] used lasers to create micro-grooves and grids in silicon substrates coated with titanium to examine the effects of topography on the nature of cellular attachment and adhesion. Other methods of nanostructuring titanium surfaces were introduced for study of biocompatibility of nanostructures, for example sintering of 50-90 nm titanium dioxide into titanium [17] or layer-by-layer Ti-TiO$_2$ assembly of surface films [18], and anodizing [19]. Guillemot, et al. [20] examined the effects of nanostructured $\alpha$ and $\omega$ phase precipitates in $\beta$ titanium alloys. Webster et al. [21-26] were among the first to systematically study osteoblast adhesion to bulk nanophase titanium and nanostructured surfaces, including SPD-processed titanium. They were also among the first to further show that features of nanophase titanium oxide reduces the adhesion of bacteria such as Staphylococcus epidermidis at the same time that it increases osteoblast adhesion and other functions [27]. The literature examining enhanced cell adhesion and proliferation on nanostructured surfaces continues to explore titanium and titania surfaces both within and outside the context of SPD metals.

2.1. Methods for characterizing biocompatibility UFG/NS titanium

Multiple biological assay techniques have been directed toward studies of the potential effects of UFG/NS titanium on cells. While all of these methods provide useful insights, they have differing implications for the extent of biocompatibility. Table 1 summarizes the biological assays that have been applied to UFG/NS titanium produced by SPD. They are categorized as being in vitro, ex vivo, or in vivo. In vitro (literally, “in glass”) and ex vivo (literally, “out of the living”) refer to techniques that are applied outside an intact living organism. The term in vitro is commonly used to describe cell culture experiments since they are done outside of a living organism and are distinct from in vivo experiments, which involve intact living organisms. However, the term ex vivo is increasingly used to distinguish cell culture experiments from in vitro experiments that do not involve the use of any living material, such as simulated body fluids (SBF) experiments.

The effects of SBF on implants provides some clues to surface biocompatibility in vitro. Ex vivo methods involve cell cultures and include measurements of cell viability, cell adhesion, cell
proliferation, cell morphology, protein expression, enzyme activity, gene expression, and (for bacteria) biofilm thickness. Measurements that can only be made as part of *in vivo* experimentation include bone-implant-contact, reverse torque, bone volume, and microtomography. Clinical trials in humans are usually the last to be done, but since titanium is biocompatible, some data for implantation in humans is available [7]. These techniques are categorized in Table 1 under the following headings and associated abbreviations: SBF - Simulated Body Fluid (protein absorption, mineral deposition), CP - Cell Proliferation (MTT, direct cell counts, microscopy), CM - Cell Morphology (by microscopy), HT - Histochemical Techniques (enzymatic assays), MBT - Molecular Biological Techniques (DNA, RNA expression, including PCR & Northern, and protein by Westerns), IV - *in vivo* techniques, and CT - Clinical Trial. High Throughput techniques (microarrays, next generation sequencing) have yet to be applied to UFG/NS titanium, so these are discussed in section 4, Gaps and frontiers.

2.1.1. *Simulated body fluid*. The initial step in the implantation process involves populating the metal surface with proteins and constituents such as calcium and phosphates that may contribute to the formation of bone mineral (apatite group compounds). This process can be studied *in vitro* by introducing fluids comparable to human blood plasma that model the environment found in the body. The presence of specific proteins in these fluids may aid cell attachment. Thus, immersion of implant material into SBF facilitates the determination of phosphate, calcium and protein absorption to the surface, which provides an estimate of bioaffinity. SBF experiments provide only a limited amount of information, but do not require extensive facilities or equipment to undertake and are a useful first step to assess biocompatibility.

Specific measurement techniques used with SBF experiments include colorimetric assays to quantify the amount of specific proteins, such as albumin, that adhere to a material surface and x-ray diffraction, combined with optical microscopy and scanning electron microscopy to quantify the presence of corrosion products and deposition of apatite. SBF techniques have been applied to UFG/NS metals in only a few instances. Zheng *et al.* [28] measured the formation of apatite from SBF on CG titanium, UFG titanium, and surface-modified UFG titanium with a hierarchical porous surface structure. While little difference was noted in the levels of calcium phosphate on the CG and UFG surfaces after 4 days in SBF, x-ray diffraction measurements and scanning electron microscopy observations showed substantially enhanced apatite formation on the UFG titanium with the porous surface. The authors attributed this result to the porous surface providing a much larger surface area for interaction with the SBF. In addition the modification technique included an alkaline treatment that created a sodium titanate layer (Na₂Ti₃O₇) that stimulated the formation of Ti-OH groups on the titanium surface, inducing and accelerating apatite nucleation by increasing the OH⁻ ion concentration. In this case, no significant difference was found between CG and UFG titanium. Furthermore, the relative importance of the roles of surface roughness and near-surface chemistry could not be distinguished. Contrasting results were obtained in experiments conducted by Nie *et al.* [29]. They examined calcification on ECAPed Grade 4 titanium in SBF for up to 14 days. They found very few Ca-P particles on the surface of CG titanium even after 14 days, while on UFG titanium they observed Ca-P nodules after just 3 days, and substantial bone-like apatite formation after 14 days. Nie *et al.* hypothesized that the improved response on UFG titanium was due to a denser layer of TiO₂ at the surface. Nie *et al.* also monitored protein adhesion in the absence of cell cultures. They found that the protein albumin, present in Bovine Serum Albumin (BSA), had significantly higher affinity for UFG titanium. These results provide only an indirect indicator or reference for the bioaffinity of surfaces since albumin has no specific role in osteogenesis.
Table 1. Highlights of published studies of cell interactions with NS/UFG titanium.

| Ref. | Ti Alloy | SPD process | Cell type | Bioassay Technique | Main Result |
|------|----------|-------------|-----------|--------------------|-------------|
| [30] | Gr 2     | ECAP        | Mouse fibroblast | CP                | Enhanced cell proliferation |
| [31] | Gr 2     | HPT         | Mouse pre-osteoblast; Rat fibroblast | CP, CM, SBF, MBT | Enhanced attachment, spreading of pre-osteoblasts over fibroblasts, expression of fibronectin & vinculin proteins |
| [32] | Gr 2     | ECAP        | Rabbit, *In vivo* | IV, Reverse torque | No statistical improvement |
| [7]  | Gr 2     | ECAP        | Mouse fibroblast; Human clinical | CP, CT (N>250) | Enhanced cell colonization, no failures in clinical trials |
| [33] | Gr 2     | ECAP        | Mouse fibroblast | CP                | Enhanced adhesion, proliferation, wettability |
| [34] | Gr 2     | ECAP        | Mouse pre-osteoblast | CP, CM, HT, MBT | Enhanced cell attachment, spreading and mRNA expression |
| [35] | Gr 2     | ECAP        | Mouse pre-osteoblast | CP, CM | Improved cell viability |
| [36] | Gr 2     | ECAP        | Bacteria | CM | Preferential attachment |
| [37] | Gr 2     | ECAP        | Stem cell | CM | Enhanced biovolume of stem cells |
| [38] | Gr 2     | ECAP        | Mouse pre-osteoblast | CP | Increased cell attachment attributed to texture |
| [28] | Gr 4     | ECAP        | Osteoblast-like | CM, SBF, CP | Enhanced osteoblast-like cell attachment and proliferation |
| [39] | Gr 4     | ECAP        | Rabbit, *In Vivo* | IV, Reverse torque | No statistical improvement |
| [29] | Gr 4     | ECAP        | Mouse fibroblast, osteoblast, Dog *in vivo* | SBF, CP, CM, HT, MBT, IV | Improved *in vitro* bioactivity, bioaffinity, cellular functionalization, protein absorption, proliferation, mineralization. *In vivo* suggests possible improvement. |
| [40] | Ti-6Al-4V | HPT         | Mouse pre-osteoblast | CP, CM | Increased cell attachment, spreading attributed to texture |
| [41] | Ti-13Nb-13Zr | Cross rolling | Mouse pre-osteoblast | CP, CM, SBF, HT, MBT | Increased cell differentiation, attachment, spreading |
| [42] | NiTi     | HPT         | Osteoblasts, fibroblasts | CP | Slight enhancement of osteoblast viability |
| [43] | NiTi     | ECAP        | Osteoblast-like | CP, CM | Combination of UFG + sandblasting & acid etching promoted bioactivity & proliferation |

Key to bioassay abbreviations: SBF=Simulated Body Fluid (protein absorption, mineral deposition), CP=Cell Proliferation (MTT, direct cell counts, microscopy), CM=Cell Morphology (by microscopy), HT=Histochemical Techniques (enzymatic assays), MBT=Molecular Biological Techniques (DNA, RNA expression, including PCR & Northern, and protein by Westerns), IV=\textit{in vivo} techniques, CT=Clinical Trial

2.1.2. Cell proliferation. Techniques involving cell cultures facilitate a more thorough examination of biocompatibility but require specialized equipment and facilities to maintain sterility. There are a wide variety of techniques available to assay cell growth, including DNA and protein content, and metabolic assays. Light microscopic techniques are limited to those that use reflected light, but scanning electron microscopy and atomic force microscopy are also useful. Cell attachment and proliferation can be measured by cell counts using a hemocytometer, which is a labor intensive, but direct, technique. The most widely used method for evaluating the interaction of cells with UFG/NS titanium is an \textit{ex vivo} colorimetric assay of cell viability and cytotoxicity after varied amounts of time in culture. This is accomplished by measuring the activity of oxidoreductases, a class of cellular
enzymes that reduce the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) to purple formazan. By quantifying the absorbance of wavelengths between 500 and 600 nm it is possible to establish the metabolic status of living cells on UFG/NS titanium surfaces in contrast to CG titanium surfaces, or an alternative control surface. This approach is fundamentally suited for evaluating toxicity, although MTT assay results are often used as a proxy for cell proliferation. It is critical to keep in mind that MTT results are influenced by the physiological state of the cells cultured and variance in the oxidoreductase activity of different cell types. MTT results are further complicated by the argument that the assay measures enzyme activity in the cytosol, not in the mitochondria [44]. In addition, recent evidence suggests that osteoblasts undergoing differentiation increase the use of cytosolic oxidoreductases for energy production, known as the Warburg effect [45]. These factors make it difficult to contrast results from studies from independent research groups that use different cell cultures, or even different generations of the same cell culture that are undergoing differentiation.

The limitations of MTT assays are apparent from the instances in which they have been applied to UFG/NS titanium. For example, MTT results by Park et al. [34] for mouse pre-osteoblast MC3T3-E1 cells on CG and UFG grade 2 titanium subject to four passes of ECAP via route C at 350 C showed 40% greater living cell density after 48 hours of culturing on the ECAPed material. Estrin et al. [35] also studied the growth of MC3T3-E1 cells on grade 2 CG and UFG titanium produced via four passes of ECAP via route B_C at 350 C. They also used the MTT assay to measure cell viability. However, in distinct contrast, they found phenomenally enhanced cell survival, 19.6 times greater, on UFG titanium compared to CG titanium after 7 and 14 days of culturing. Additional contrast is found in the experiments by Reiss, et al. [46] who compared grade 4 CG titanium to UFG titanium produced via 8 passes of route B_C at temperatures between 200 C and 350 C. Disks 13.5 mm in diameter and 1 mm thick were cut from titanium rods using a low speed cut-off saw. Some samples were polished mechanically for 24 hours using 0.05 µm silica to produce a less than 2 nm average surface roughness R_a. The same cell type used by Park et al. and Estrin et al. was seeded and grown for 3 days before being measured using the MTT assay technique. As shown in Figure 1 below, no statistically significant difference was found between CG or UFG titanium regardless of surface condition. These results have been replicated in follow-on experiments by the same authors. The information available through MTT assays is of itself insufficient to explain the differences in results obtained in these three reasonably similar studies.

As summarized in Table 1, proliferation of cell types other than osteoblasts on UFG/NS titanium surfaces has been reported [7]. For example, Troung et al. [36] examined bacterial growth on UFG titanium surfaces. They found some degree of preference of two strains of bacteria on UFG titanium as compared to conventional titanium. Zheng, et al. [47] conducted similar studies, but included osteoblasts and bacteria together in their ex vivo experiments. They found that when both osteoblasts and bacteria are present together, as would be found in vivo, the osteoblast cells preferentially cover the UFG titanium surfaces in comparison to the bacteria. This result highlights the importance of the oral microbiome to implantation success and the virtue of in vivo experiments.
Figure 1. Preliminary results of MTT assays of MC3T3-E1 pre-osteoblastic mouse cells on conventional coarse grain (CG) and UFG grade 4 titanium surfaces cut using an aluminum oxide blade with a low speed cut-off saw and left unpolished, or polished to be nanometrically smooth (R_a < 2 nm). *Control wells are coated polystyrene.

2.1.3 Cell morphology. Microscopy techniques are important to determine morphology in cell cultures. Direct observation of cell shapes can be obtained from optical microscopy of stained or unstained cultures, scanning electron microscopy, and atomic force microscopy. Optical microscopy is limited to techniques that utilize reflected light due to the opaque nature of titanium. However, by coating glass coverslips with a 35 nm thin film of titanium, Lee et al. [48] were able to use incident light microscopy to track living macrophages. These immune cells exhibited reduced migration on the nanoroughened surface, indicating a reduced immune response. Scanning electron microscopy (SEM) techniques are effective for measuring cell proliferation and cell morphology, but cannot be performed on living cells. Atomic force microscopy (AFM) techniques have potential but are not yet widely used for cell visualization on titanium [49].

Cells display a wide range of morphologies as they interact with surfaces, exhibiting shapes ranging from nearly spherical to extensive flattening and spreading. These morphological changes are mediated by focal adhesions, a complex assembly of proteins that connect the ECM to the internal cytoskeleton. The interaction of focal adhesions with the metal substrates and with the ECM occurs within a size domain of 15 nm around each cell. This defines one size scale at which nanoscale topographical features may influence cell development.

There are numerous antibodies available to label proteins and optically view their involvement in cell attachment and migration. Immunofluorescence labeling of cell proteins followed by fluorescent imaging can detect changes in cell morphology indicative of osteogenesis. For example, immunofluorescence labeling can highlight the morphology of actin, which is present in all eukaryotic cells. Actin is a key element of the cell cytoskeleton microfilaments that play a key role responding to cell signals that require an increase in cell adhesion to enable tissue formation. Vinculin is another cell cytoskeletal protein that links actin to membrane-bound integrin adhesion molecules and is key to cell spreading and movement functions of the focal adhesions. Fibronectin is located in the ECM and also plays a role in cell adhesion, and additionally, influences cell differentiation, growth, migration, and helps bind other elements of the ECM. There are many other proteins that can be detected using antibody probes available to interrogate protein fluxes involved in cell adhesion, migration, and proliferation [50].

Direct observation of cell shapes and densities on UFG/NS titanium alloys has been reported, generally indicating the superior degree to which cells adhere them, as compared to CG titanium surfaces. In the early work on nanophase materials by Webster and co-workers [51] cell attachment was determined by fluorescent light microscopy and scanning electron microscopy. They found that
the density of human osteoblast cells grown on nanophase titanium produced from compacted nano-
sized powders exceeded the density on comparable wrought CG titanium by 58% after one hour of
incubation and by 54% after 3 hours. Using scanning electron microscopy they imaged the adhesion of
nearly spherical cells to the abundant nanoscale interparticle boundaries. Estrin et al. [35] noted
conglobated cell shapes in the culture medium for CG titanium, indicating reduced cell viability, as
compared to the healthier cells in the culture medium with UFG titanium. Valiev, et al. [7] showed
SEM images contrasting extensive fibroblast cell spreading on UFG titanium, as compared to CG
titanium. Faghihi et al. [31] visualized vinculin and actin using immunofluorescent labeling and
demonstrated improved cell adhesion in HPT titanium when compared to undeformed titanium. Park
et al. [41] merged images of blue stained cell nuclei, green stained actin cytoskeleton elements, and
red stained focal adhesions (vinculin) and illustrated more numerous attachments of cells to UFG Ti-
13Nb-13Zr than on CG Ti-13Nb-13Zr. When carefully executed and quantitated, immunofluorescent
imaging provides comparable results.

2.1.4 Histochemical techniques. Histochemical techniques rely on the detection of enzyme activity or
chemical presence by specific staining techniques. These are effective in monitoring enzymes
important for cellular differentiation. For example, alkaline phosphatase (ALP) is produced by
osteoblasts, which form bone. Acid phosphate is produced by osteoclasts, which breakdown bone.
Staining for alkaline and acid phosphatase staining distinguishes between osteoblasts and osteoclasts.
Histochemical techniques were applied by Park et al. [41] to examine on UFG Ti-13Nb-13Zr. They
found that ALP activity was greater at seven days on UFG Ti-13Nb-13Zr than on CG alloys. In
separate work on commercial purity titanium, Park et al. [34] used alizarin red staining, which is
specific for matrix calcification levels, to show the calcification of the ECM as pre-osteoblasts adapt to
UFG titanium substrates more readily than to CG titanium.

2.1.5 Molecular biological techniques. Techniques that assess the abundance of DNA, RNA, or
proteins are collectively categorized as Molecular Biological Techniques (MBT). These include the
estimation of cell numbers from DNA measurements, the use of RNA abundance to determine gene
expression, and the immunodetection of proteins on membranes (Western blot) to directly measure
changes in protein concentration that can confirm gene expression changes detected in RNA. The
amount of DNA in cell cultures can be determined using spectrophotometric techniques and compared
between time points as a proxy for cell growth. This can be effective when cell density measurements
are taken by direct cells counts [31], [40].

More commonly, MBTs are employed to estimate the expression of genes that code for proteins
involved in osteogenetic pathways. The determination of messenger RNA (mRNA) abundance for
specific proteins reveals changes in gene expression associated with cell adaptation to a substrate.
These changes are determined by amplification of mRNA using quantitative polymerase chain
reaction (qPCR). Park et al. demonstrated an increase of ALP mRNA on ECAP titanium when
compared to CG titanium [34], and on UFG Ti–13Nb–13Zr when compared to CG Ti–13Nb–Zr [41].
However, Nie et al. [29] used histochemical techniques to stain for ALP protein activity and noted
little difference between UFG and CG titanium. It is impossible to compare the increase in mRNA, as
measured by qPCR in one experiment to the increase in protein activity in a different experiment,
without validation in the same experimental system.

More comprehensive measurements of biological response of cells to UFG/NS titanium employ
MBT techniques to measure the expression of genes that code for protein constituents of bone and to
measure expression changes of regulatory genes that indicate when a specific biochemical pathway is
activated or inhibited. Osteocalcin (OCN) is a protein found in bone that also acts as a hormone and
osteopontin (OPN) is a protein complexed with carbohydrate that is associated with bone but is also
found in other tissues. RUNX2 (Run-related transcription factor 2) and osterix (SP7) are critical
transcription factors that signal osteoblast differentiation. After seven days in culture, Park et al. [34]
observed that OCN and OPN mRNA levels are greater in UFG titanium than either CP titanium or Ti-
6Al-4V. However, OCN activity showed no significant difference between UFG Ti–13Nb–13Zr and CG Ti–13Nb–Zr after seven days [41]. The differences in OCN expression between CP UFG titanium and alloyed UFG titanium suggest that pure titanium is the preferable substrate. Park, et al. also demonstrated that mouse pre-osteoblasts exhibited increased RUNX2 expression on UFG Ti-13nB-13-Zr compared to CG Ti-6Al-4V or CG Ti–13Nb–13Zr, but SP7 shows no remarkable differences between any of the substrates [41]. Since RUNX2 activity is important in early in osteogenesis and is maintained throughout differentiation and SP7 is involved in later steps [52], one interpretation of the Park et al. results is that the increase in RUNX2 in cells on UFG surfaces may indicate that these were more rapidly proceeding through differentiation, but none of the cells had reached the steps regulated by SP7. These results highlight the utility of qPCR to evaluate the status of cells on different substrates.

Immunodetection on membranes (Immuo- or Western blot) directly measure changes in protein concentration, but detection of specific protein requires the availability of antibodies against the proteins. The antibodies for immunofluorescence techniques (above) can also be used for immunoblotting. Fibronectin is a protein complexed with carbohydrates that is found in the extracellular matrix and vinculin is also found in the ECM but is associated with focal adhesions. Faghini et al. observed that both proteins appear to be more abundant in HPT titanium when compared to CP titanium and coated substrates [31]. The focal adhesion protein zylin expression is decreased in human MSC in response to nanotopography, but has not been tested on UFG/NS titanium [53].

2.1.6 In vivo experiments. Experiments using live animals, such as rabbits or dogs, provide more realistic models of the environmental conditions that would be found throughout the stages of osteogenesis in humans. They have the obvious disadvantage of being most costly and negatively impactful on the lives of animals used in the studies. In cases involving human trials there are the inherent risks of unanticipated negative effects of the implanted materials.

As noted in Table 1, several in vivo studies of UFG/NS titanium have been reported [7, 28, 38]. These experiments integrate the many factors that underlie the biological responses found in simpler in vitro systems. The differentiation between UFG/NS and CG titanium is more difficult to measure and understand in these experiments. For example, improved bone-implant contact was found for UFG titanium when compared to CG titanium implanted in beagles [29], but no differences were apparent between reverse torque measurements in CG and UFG titanium screws transplanted into New Zealand Rabbits [38]. From the perspective of a surgeon the observation of comparable reverse torques to remove implanted CG and UFG titanium could be regarded positively. Clinicians would prefer implant failures to occur within the implant material rather than induce failure in bone tissue. It is important in such tests to document the nature of the failure mechanisms induced during removal of the implants. The differences in in vivo assay techniques may partly account for differences in results. Also, dogs (order Carnivora) and rabbits (order Lagomorpha) are very distantly related and physiological differences are likely to play a role.

The most realistic testing conditions are found in human clinical trials. Studies of UFG/NS dental implants in over 250 human patients described by Valiev et al. [7] found no failures, as compared to the 4% to 8% failure rates that are typical for placement of conventional implants.

3. Discussion
The research contributions summarized in Table 1 provide a substantial basis from which to further build our understanding of the virtues of UFG/NS variants of titanium and other alloys for medical applications. Several key issues have emerged from the work to date, several of which we consider in this section. Then in the final section we consider specific gaps in the work to date and frontier research topics to help fill the gaps.
3.1. Surface topography

Surface topography is amongst the leading substrate characteristics that has been referenced to explain the enhancement of cellular functions on UFG/NS titanium. Researchers have systematically examined this factor, but reported widely varying results. The topic is thoroughly reviewed in the context of conventional materials and engineered nanoscale enhancements by Curtis and Wilkinson [54, 55], Ercan and Webster [56], and Wennerberg and Albrektsson [57, 58]. Specific models of cell-surface interactions have been proposed for UFG metals, for example, Nie et al. [29] suggested that the greater electronegativity of nanostructured titanium surface attracts calcium and other cations, which in turn attract negatively charged biomolecules, proteins, and cells. However, Wennerberg and Albrektsson [57] point out that what is known about the effects of surface topography is limited by the lack of quantitative surface characterization, plus influences of non-topographical surface parameters that are not well documented or controlled in even the most carefully designed studies. There appears to be a need to develop standards by which aspects of surface topography that are important for biomaterial can be quantified.

Patterning of surfaces can impact cell behaviors. Qin et al. [59] used a combination of MTT assays for cell proliferation, SEM to investigate cell morphology, ALP staining for differentiation, and qPCR for gene expression to demonstrate increased preosteoblast response to laser-patterned surface topography of a Co-Cr-Co substrate. The genes investigated by qPCR, Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) and osteoprotegerin (OPG), are involved in the competition between osteoblasts and osteoclasts. RANKL is a cytokine, a signaling molecule that is secreted by many cell types, including osteoblasts and osteoclasts, which is capable of binding to a variety of different proteins. The signal transmitted by the RANKL protein is dependent upon the cell type and the interaction with other proteins, including OPG. When RANKL molecules on the surface of osteoclasts bind to OPG, their proliferation is inhibited, but OPG binding to RANKL on osteoblasts has no effect. PCR results indicate a 9-fold increase in OPG gene expression in preosteoblasts when cells are grown on surfaces with laser ablated 130 µm equilateral triangles patterned on a 315 µm rectangular grid as opposed to square (1.4-fold increase) or circular (0.6-fold decrease) shapes with approximately the same size patterned with the same grid density. The changes in OPG/RANKL expression on the different surface morphologies supports the hypothesis that cells sense and respond to differences in surface features.

3.2. Surface crystallographic texture

Most engineered alloys display distinct crystallographic textures, especially those with limited slip systems such as the α phase of titanium, or Co-Cr alloys which invariably contain mixtures of the elevated temperature α phase and the room temperature hexagonal close-pack ε phase. In several studies of titanium textured by HPT [40] and ECAP [60] the role of texture was found to be more significant than the prospective effect of grain size. Experiments by Faghihi et al. [61] using single crystals of titanium showed the dependence of cell proliferation on surface crystal orientation. The density of cells after 2 hours of culturing was significantly higher on (11\overline{2}0) surfaces by 63% for preosteoblast and 37% for fibroblasts. These increments in cell viability are sufficiently large for one to surmise that surface texture should be considered as a factor to measure or control in all comparative cell-metal surface interaction studies.

3.3. Surface oxide state

Titanium naturally forms a tenacious surface oxide that is several nanometers thick. The properties of this oxide underlie titanium’s corrosion and oxidation resistance. Proteins and cells interact exclusively with titanium oxide TiO_2 on titanium metal, generally in the form of rutile or anatase. The surfaces of many orthopedic implants are anodized to form specific oxide structures.

X-ray Photoelectron Spectroscopy (XPS) has been used to measure the composition, chemical state, and electronic state of SPD-processed titanium. For example, Nie et al. [29] found the expected Ti^4+ peaks in the Ti 2p spectrum associated with the TiO_2 oxide film on samples of CG and UPG
titanium. They detected Ti on CG titanium, but not on ECAPed UFG titanium. They interpreted this result to indicate that a denser, thicker oxide layer forms on UFG/NS titanium. Comparable results were report by Truong et al. [36]. They also reported on the state of oxygen, finding three components: one associated with TiO, another with carbon CO groups, and a third connected with hydrocarbon surface contamination (C-O). They also reported trace amounts of Zn, Cu, Co and Ca, all impurities or possibly cross-contamination from preparation and handling of the samples studied. Attention to hydrocarbon contamination may merit particular attention in view of results reported by Yamada et al. [62] who correlated enhancement of cell adhesion to mirror-polished CG grade 2 titanium with a reduction in the concentration of surface carbon.

Oxygen is particularly important to cellular functions and the biochemical state at cell/substrate interfaces. The presence of non-stoichiometric oxygen concentrations in titanium oxides has been reported, particularly in presence of impurities (from sample preparation, or other sources), surface oxygen vacancies, or other defect states that reduce titanium dioxide to non-stoichiometric forms [63-67]). Botha [63], in particular, notes that the state of the oxide on titanium depends as much on the cleaning and sterilization procedure as it does the titanium substrate. While most XPS analyses agree that the elements most prominently found on titanium surfaces are Ti, O, and C, the characteristics of the oxides and the concentrations of oxygen and carbon vary significantly. This variability underscores the prospective importance of measuring, documenting, and controlling surface oxide states in experiments that study cell/titanium surface interactions.

4. Gaps and frontiers

4.1. High throughput techniques: Microarrays and Next-generation sequencing

The goal of the Human Genome Project, to determine our DNA sequence, was completed early in this century and we are now in the post-genomic era. Advances in engineering have resulted in high throughput techniques that facilitate the collection of massive amounts of data with very small sample size, providing a detailed picture of cellular processes such as osteogenesis.

4.1.1. Microarrays. Prior to the 1990s, assays for gene expression were done one gene at a time, using techniques such as Northers and PCR. Microarray fabrication utilizes photolithography to build up single stranded DNA molecules on a glass surface. Thousands of genes can be represented on a single microarray. These are known as the probe set, which is then interrogated with single stranded nucleic acids extracted from cells under various conditions that are subsequently fluorescently labelled. Once a DNA sequence match is made the fluorescent signal is captured with digital microscopy and analysed. The expression levels of thousands of genes at a time can be measured and used to compare gene expression changes under multiple conditions in ex vivo and in vivo experiments. Microarrays (also known as DNA or gene chips) are now important diagnostic tools in cases where reliable biomarkers are available. Although microarray results indicate altered gene expression profiles in response to surface differences of implants, biomarkers that predict successful osseointegration are not definitively established. For example, Donos et al. [68] detected significant gene expression differences between polished and microrough CG titanium disks implanted into rats. Surprisingly diverse gene expression profiles were observed ex vivo in osteoblasts adhering to five commercially available implant types [69]. Thalji et al. [70] reported that pro-inflammatory genes were down-regulated in cells adhering to nano-roughened surfaces when compared to those on micro-roughened surfaces implanted into rats. This indicates improved osseointegration potential and supports the finding of Lee et al. [48] that macrophages are not activated by these surfaces. Mengatto et al. [71] demonstrated in vivo that osseointegration may be dependent on vitamin D availability, involves genes that produce proteins of the ECM, and includes circadian rhythm gene regulation. Nishimura [14] reviewed results of 12 implant microarray studies and proposed a model for the genetic processes underlying osseointegration of implants that implicate genes already known to regulate the differentiation of MSC into osteoblasts, but also genes that are involved in the MSC to chondrocyte
pathway. However, microarrays have yet to be employed to profile gene expression of cells exposed to titanium produced by SPD and represents an opportunity to further characterize the improved biocompatibility of these materials.

4.1.2. Next Generation Sequencing. Unlike microarray technology, next-generation DNA sequencing (NGS) does not require an a priori knowledge of the genes involved, but a well-annotated genome sequence is necessary. In 2005, Margulies et al. [72] reported on advances that enabled NGS. These included the ability to manufacture flow cells with a density of 480 wells per square mm, resulting in the ability to do 1.6 million sequencing reactions at once, and the development of massively parallel computational techniques. NGS instrumentation became commercially available in 2005 and by 2007 the journal Nature Methods choose NGS as its method of the year. Since then NGS has exceeded expectations as per run capacity continues to increase. NGS studies by Twigg et al. [73] implicate the RUNX2 gene as a major regulator of osteogenesis and identify additional genes in the pathway. Before NGS it was generally accepted that only 2% of a genome is transcribed into RNA. NGS studies reveal that over 90% a genome is transcribed, but only 2% of the RNAs are translated into proteins. The functions of these diverse non-protein-coding RNAs (ncRNAs) are just beginning to be elucidated. MicroRNAs (miRNAs) are 22-24 nucleotide long RNAs that regulate cellular processes, including osteoblast differentiation [74]. Long non-coding RNAs (>200 nucleotides) can regulate chromosome structure, which then modulates gene expression. For example, Zhu et al. [75] describes a long ncRNA responsible for the regulation of RUNX2 expression during osteoblast differentiation. To date, NGS techniques have not been applied to osseointegration studies of any type of titanium implant.

Epigenetics is an emerging discipline that highlights the influence of chromosome organization on gene expression and that is undergoing a period of rapid acceleration due to NGS technology. The methylation of specific regions of DNA and the biochemical modification of histone proteins are responsible for maintaining chromosome configuration and these modifications, in turn, influence RNA expression profiles. DNA methylation is influenced by the environment and plays a major role in the transduction of abiotic signals into the biochemical changes that facilitate gene expression modulation. Eslaminejad et al. [76] confirm that epigenetic processes influence the differentiation of MSCs. The epigenetic modifications necessary for adaptation to a substrate such as UFG/NS titanium are unknown, but this knowledge is accessible with these modern techniques.

The next advance in NGS sequencing is currently under beta-testing, which incorporates graphene nanopores into a solid-state system capable of detecting physical differences between the four DNA bases, eliminating the need for chemical methods to determine DNA sequence [77]. Nanopore sequencing is expected bring us into the era of personalized medicine by making rapid and inexpensive sequencing of individual genomes a reality. Combined with an understanding of optimized implant design that is informed by molecular genetic evidence, there is the potential to design implants based an individual’s genetic make-up, essentially eliminating the possibility of rejection.

5. Conclusion
The research to date that examines the biological properties of ultrafine grained and nanostructured titanium has documented performance that is desirable for a range of biomedical applications. Bioassay techniques that have been applied are most useful within the context of a single study, but less helpful for making comparisons of results across multiple research investigations. This is due in part to the fact that we are still discovering which variables in biomaterials assessments need to be specified, measured, and controlled.

High throughput techniques will provide critical insight into understanding and optimizing the characteristics of the bulk and surfaces of nanostructured and ultrafine grain titanium. Unprecedented opportunities to improve our understanding of cell differentiation are offered by the ability to manipulate substrates at a nanoscale level coupled with the molecular-level characterization of cell
response. Combining nanotechnology with the latest biotechnological techniques that facilitate our understanding of osteogenesis will lead to the optimization of implant material design.

**Acknowledgements**

This project was partially supported by grants from the National Center for Research Resources (5P20RR016480-12) and the National Institute of General Medical Sciences (8 P20 GM103451-12) from the National Institutes of Health. The authors also thank students M. Haber, P. Illescas, M. Glazier, and L. Sanchez for their help on the project, and the Carpenter Technology Corporation for supplying titanium.

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