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REVIEW

Twenty-first century challenges for biomaterials

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During the 1960s and 1970s, a first generation of materials was specially developed for use inside the human body. These developments became the basis for the field of biomaterials. The devices made from biomaterials are called prostheses. Professor Bill Bonfield was one of the first to recognize the importance of understanding the mechanical properties of tissues, especially bone, in order to achieve reliable skeletal prostheses. His research was one of the pioneering efforts to understand the interaction of biomaterials with living tissues. The goal of all early biomaterials was to 'achieve a suitable combination of physical properties to match those of the replaced tissue with a minimal toxic response in the host'. By 1980, there were more than 50 implanted prostheses in clinical use made from 40 different materials. At that time, more than three million prosthetic parts were being implanted in patients worldwide each year. A common feature of most of the 40 materials was biological 'inertness'. Almost all materials used in the body were single-phase materials. Most implant materials were adaptations of already existing commercial materials with higher levels of purity to eliminate release of toxic by-products and minimize corrosion. This article is a tribute to Bill Bonfield's pioneering efforts in the field of bone biomechanics, biomaterials and interdisciplinary research. It is also a brief summary of the evolution of bioactive materials and the opportunities for tailoring the composition, texture and surface chemistry of them to meet five important challenges for the twenty-first century.

Keywords: bone biomechanics; interdisciplinary research; evolution of bioactive materials

1. INTRODUCTION: THE TWENTIETH CENTURY CHALLENGE: FIRST-GENERATION BIOMATERIALS

During the 1960s and 1970s, a first generation of materials was specially developed for use inside the human body. These developments became the basis for the field of biomaterials. The devices made from biomaterials are called prostheses. Professor Bill Bonfield was one of the first to recognize the importance of understanding the mechanical properties of tissues, especially bone, in order to achieve reliable skeletal prostheses. His research was one of the pioneering efforts to understand the interaction of biomaterials with living tissues. The goal of all early biomaterials was to 'achieve a suitable combination of physical properties to match those of the replaced tissue with a minimal toxic response in the host' (Hench 1980). By 1980, there were more than 50 implanted prostheses in clinical use made from 40 different materials. At that time, more than three million prosthetic parts were being implanted in patients worldwide each year. A common feature of most of the 40 materials listed in table 1 of the 1980 article was biological 'inertness'. Almost all materials used in the body were single-phase materials. Most implant materials were adaptations of already existing commercial materials with higher levels of purity to eliminate release of toxic by-products and minimize corrosion.

The principle underlying the bulk of biomaterials development in the decades from the 1960s to early 1980s was to reduce to a minimum the biological response to the foreign body. It is important to note that this engineering design principle is still valid, 40 years later. Tens of millions of individuals have had their quality of life enhanced for up to 25 years or more by use of implants made from 'bioinert' biomaterials. The interface between tissues and bioinert biomaterials is a thin, acellular fibrous capsule with minimal, if any, adhesion between the implant and its

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host tissue. However, Professor Bonfield recognized the need for a new generation of biomaterials. His research group began exploring in the 1980s the concept of designing biocomposite materials that more closely matched the mechanical properties of the host bone (Rea & Bonfield 2004). Such composite materials would minimize, and perhaps even eliminate, stress shielding and bone resorption at the prosthesis–bone interface owing to mismatch of elastic modulus. Higher modulus devices carry almost the entire mechanical load, and this leads to gradual bone resorption, deterioration of strength and eventual failure. The biocomposites developed by Professor Bonfield's laboratory incorporated the new concept of bioactivity, to be discussed below, along with the design concept of tailoring two phases to achieve composite properties that were closer to those of the living composite, bone. This pioneering work led to an innovative composite material composed of a polymeric matrix of polyethylene and a dispersed bioactive phase of hydroxyapatite (HA) particles. The new material, trademarked Hapex, was the first successful biocomposite material specially developed for medical devices. Professor Bonfield continued his pioneering contributions to the field of biomaterials with the development of a new type of bioactive material, Si-substituted HA, which is a successful bone graft material (Thian *et al.* 2006). His breadth of understanding and contributions to interdisciplinary research have made it possible for him to co-found and edit this highly significant *Journal of the Royal Society Interface*.

This article is a tribute to Bill Bonfield's pioneering efforts in the field of bone biomechanics, biomaterials, interdisciplinary research, international leadership and successful commercialization of innovative biomaterials. It is also a brief summary of the evolution of bioactive materials and the opportunities for tailoring the composition, texture and surface chemistry of them to meet five important challenges for the twenty-first century.

2. SECOND-GENERATION BIOMATERIALS

By 1984, the field of biomaterials had begun a shift in emphasis from achieving exclusively a bioinert tissue response (Hench & Wilson 1984). A second generation of biomaterials had been developed to be bioactive. Bioactive materials elicit a controlled action and reaction in the physiological environment. The mechanism of bonding of bioactive glasses (composed of Na_2O–CaO–P_2O_5–SiO_2) to living tissue, established in 1971 (Hench *et al.* 1971), was shown to involve a sequence of 11 reaction steps (Hench 1998). The first five steps occur on the surface of the material (called Bioglass), as summarized in figure 1. The reactions begin by rapid ion exchange of Na^+ with H^+ and H_3O^+. The ion exchange is followed by a polycondensation reaction of surface silanols (Si–OH) to create a very high surface area silica (SiO_2) gel, which provides a large number of sites for heterogeneous nucleation and crystallization of a biologically reactive hydroxyl-carbonate apatite (HCA) layer equivalent to the inorganic mineral phase of bone. The growing HCA layer on the surface of the material is an ideal environment for six cellular reaction stages. The cellular mechanisms include colonization by osteoblast stem cells (stage 8), followed by proliferation (stage 9) and differentiation (stages 10 and 11) of the cells to form new bone that have a mechanically strong bond to the implant surface.

By the mid-1980s, bioactive materials had reached clinical use in a variety of orthopaedic and dental applications. Various compositions of bioactive glasses, ceramics, glass–ceramics and composites were in clinical trials. Synthetic HA ceramics had begun to be routinely used as porous implants, powders and coatings on metallic prostheses to provide bioactive fixation (Yamamuro *et al.* 1990; Klein *et al.* 1993; Hench 1998). Presence of sparingly soluble HA coatings led to a tissue response (termed *osteoconduction*) where bone grew along the coating and formed a mechanically strong interface. Bioactive glasses and glass–ceramics, based upon the original 45S5 Bioglass formulation (Hench *et al.* 1971), were being used as middle ear prostheses to restore the ossicular chain and treat conductive hearing loss and as endosseous ridge maintenance implants to preserve the alveolar ridge from the bone resorption that follows tooth extraction (Hench & Wilson 1996). The mechanically strong and tough bioactive A/W glass–ceramic, developed at Kyoto University, was used for replacement of vertebrae in patients with spinal tumours (Yamamuro 1996). By the 1990s, bioactive composites, such as HA particles in a polyethylene matrix, Hapex, developed by Professor Bonfield in the Interdisciplinary Research Centre in Biomedical Materials, Queen Mary and Westfield College, University of London, had become important in the repair and replacement of bones in the middle ear (Rea & Bonfield 2004). In 1998, a centennial feature article of the American Ceramic Society documented the rapid growth of clinical use of first- and second-generation bioceramics (Hench 1998).

Another category of second-generation, resorbable, biomaterials also became clinically important during this period. The objective of resorbable biomaterials was summarized in 1980 as:

> A second method of manipulating the biomaterials-tissue interface is controlled chemical breakdown, that is resorption of the material. Resorption of biomaterials appears a perfect solution to the interfacial problem because the foreign material is ultimately replaced by regenerating tissues. Ideally, there is eventually no discernible difference between the implant site and the host tissue.
>
> (Hench 1980)

Figs 3 and 4 in the 1980 article showed the progressive breakdown of a suture composed of a copolymer of poly(lactic) and poly(glycolic) (PGA) acids. Hydrolytic decomposition of the copolymer results ultimately in CO_2 and H_2O, which are easily metabolized. By 1984, clinical use of resorbable polymers as sutures was routine. Resorbable fracture fixation plates and screws in orthopaedics and controlled-release drug delivery

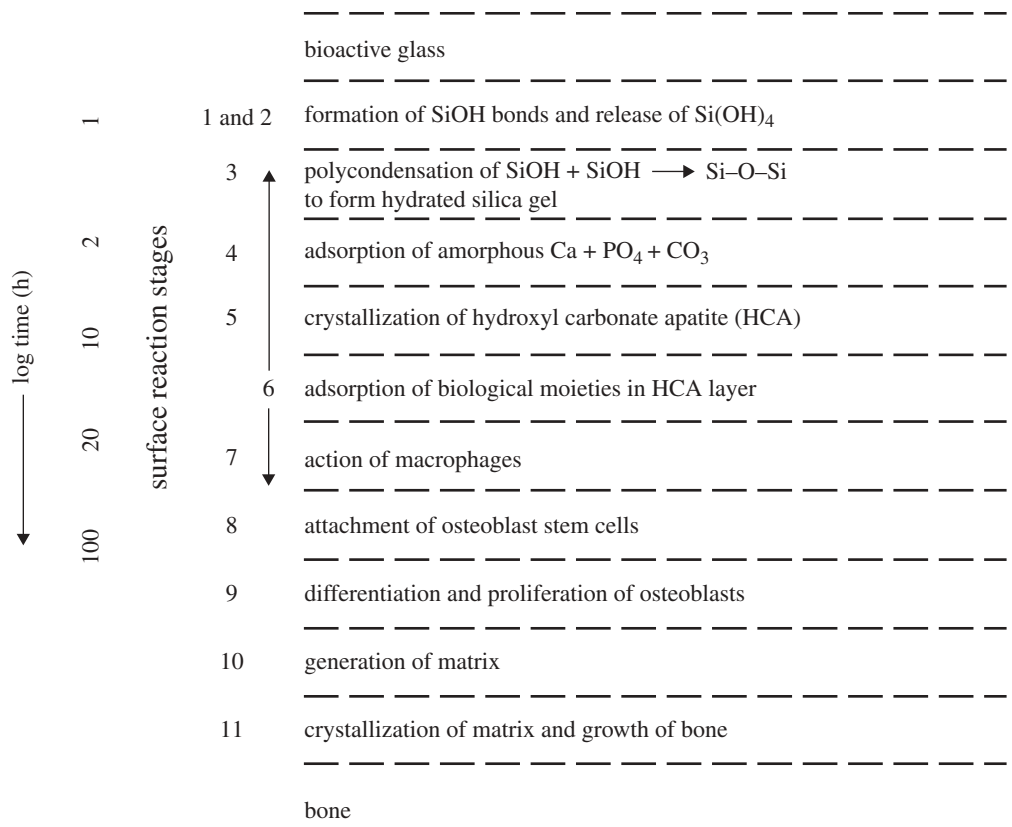

Figure 1. Sequence and rates of reaction stages to grow new bone at the interface with 45S5 bioactive glass.

systems were beginning to be used (Hastings & Ducheyne 1984).

3. TWENTY-FIRST CENTURY BIOMATERIALS CHALLENGE NO. 1: REGENERATION OF TISSUES

The clinical success of bioinert, bioactive and resorbable implants has been a vital response to the medical needs of a rapidly ageing population throughout the developed world. However, survivability analyses of most skeletal prostheses (Hench & Wilson 1996) as well as artificial heart valves (Schoen *et al.* 1992) show that a third to half of prostheses fail within 10–25 years. Failures require patients to have revision surgery. This is costly to the patients and to society and is a significant contribution to the rapidly rising costs of healthcare. Thirty years of research has had relatively small effects on failure rates (Hench & Wilson 1996). Continuing this path, based upon trial and error experiments that require the use of many animals and large numbers of human clinical trials, is prohibitively expensive. Improvements of either first- or second-generation biomaterials are limited in part because 'all man-made biomaterials used for repair or restoration of the body represents a compromise' (Hench 1980). It is essential to recognize that no man-made material can respond to changing physiological loads or biochemical stimuli, as do living tissues. This compromise limits the lifetime of all man-made body parts. Recognizing this fundamental limitation also signals that we have reached a limit to current medical practice that emphasizes *replacement of tissues*. For the twenty-first century, it is critical to emphasize a more biologically based method of repair—*regeneration of tissues*. Bioactive materials with biochemical stimuli provide the starting point for this shift towards a more biologically based approach to repair of diseased or damaged tissues.

4. THIRD-GENERATION BIOMATERIALS: CELL- AND GENE-ACTIVATING MATERIALS

Third-generation biomaterials are being designed to stimulate specific cellular responses at the level of molecular biology (Hench & Polak 2002). During the first decade of the twenty-first century, the concepts of bioactive materials and resorbable materials have converged; bioactive materials are being made resorbable and resorbable polymers are being made bioactive. Molecular modifications are being made to resorbable polymer systems to elicit specific interactions with cell integrins and thereby direct cell proliferation, differentiation and extracellular matrix (ECM) production and organization. Third-generation bioactive glasses and hierarchical porous foams are being designed to activate genes that stimulate regeneration of living tissues.

Two alternative routes of repair use the new molecularly tailored third-generation biomaterials.

Tissue engineering. Progenitor cells are seeded on to molecularly modified resorbable scaffolds outside the body where the cells grow and become differentiated and mimic naturally occurring tissues. These tissue-engineered constructs are then implanted into the patients to replace diseased or damaged tissues. With

time, the scaffolds are resorbed and replaced by host tissues that ideally include a viable blood supply and nerves. The goal is living tissue engineering (TE) constructs that can adapt to the physiological environment and provide repair or replacement that will last as long as the patient. The current status of clinical use of tissue constructs and the companies developing TE products are reviewed in Place *et al.* (2009).

In situ tissue regeneration. This approach involves the use of biomaterials in the form of powders, solutions or doped micro- or nanoparticles to stimulate local tissue repair. We have learned in the last decade that certain formulations of bioactive materials release chemicals in the form of ionic dissolution products, or macromolecular growth factors, at controlled rates that activate the cells in contact with the stimuli. The cells produce additional growth factors, which in turn stimulate multiple generations of growing cells to self-assemble into the required tissues *in situ*, along the biochemical and biomechanical gradients that are present.

The advantage offered by both approaches to regenerative medicine is genetic control of the tissue repair process. The result is equivalent to repaired natural tissue in that the new structure is living and adaptable to the physiological environment.

There is growing evidence to support the hypothesis governing the design of third-generation biomaterials; i.e. generation of specific cell responses to controlled release of biochemical stimuli. For example, when a particulate of bioactive glass is used to fill a bone defect, there is rapid regeneration of bone that matches the architecture and mechanical properties of bone in the site of repair. Both osteoconduction and osteoproduction (Wilson & Low 1992) occur as a consequence of rapid reactions on a bioactive glass surface (Hench *et al.* 1971; Hench 1998). The surface reactions release critical concentrations of soluble Si, Ca, P and Na ions that give rise to both intracellular and extracellular responses at the interface of the glass with its cellular environment. Attachment and synchronized proliferation and differentiation of osteoblasts rapidly occur on the surface of bioactive materials, as summarized in figure 1 (Hench 1998; Xynos *et al.* 2000*a*). Osteoprogenitor cells capable of forming new bone colonize the surface of highly bioactive materials. Slow release of soluble ions from the material stimulates cell division and production of growth factors and ECM proteins. Mineralization of the matrix follows and the mature osteoblast phenotype, encased in a collagen–HCA matrix (osteocytes), is the final product both *in vitro* and *in vivo* (Wilson & Low 1992; Hench *et al.* 2000; Xynos *et al.* 2000*a*,*b*, 2001; Bielby *et al.* 2004, 2005; Gough *et al.* 2004; Christodoulou *et al.* 2005*a*,*b*).

5. GENETIC CONTROL OF CELLULAR RESPONSE

Recent research has established that there is genetic control of the cellular response to the most reactive of the bioactive glasses (45S5 Bioglass). Seven families of genes are upregulated when primary human osteoblasts are exposed to the ionic dissolution products of bioactive glasses (Xynos *et al.* 2001; Hench 2003). The gene expression occurs within 48 h, and includes enhanced expression by more than twofold of the families of genes listed in table 1; see Xynos *et al.* (2001) for a listing of the genes and the extent of their upregulation.

Table 1. Families of genes upregulated or activated by ionic dissolution products from bioactive glass.

transcription factors and cell cycle regulators	two- to five-fold
signal transduction molecules	two- to six-fold
proteins in DNA synthesis, repair, recombination	two- to three-fold
growth factors and cytokines	two- to 3.2-fold
cell surface antigens and receptors	two- to seven-fold
extracellular matrix components	two- to 3.7-fold
apoptosis regulators	1.6- to 4.5-fold

The upregulated genes encode nuclear transcription factors and cell cycle regulators, such as RCL growth-related c-*myc*-responsive gene (fivefold) and G1/S-specific cyclin D1 (CCND1; fourfold). The entry of osteoblasts into the Go/G1 transition of the cell cycle and subsequent commencement of cell division is regulated by these and other transcription factors that are upregulated by the Ca and Si ionic dissolution products, as reviewed in Xynos *et al.* (2001) and Hench (2003). Potent growth factors, especially insulin-like growth factor II (IGF-II), are increased by 3.2-fold along with IGF binding proteins and proteases that cleave IGF-II from their binding proteins. The growth factors are present in a biologically active state, as confirmed by analysis of the upregulation of IGF-II mRNA with quantitative real-time polymerase chain reaction (PCR). This was an important finding because IGF-II is the most abundant growth factor in bone and induces osteoblast proliferation. IGF-II expression is high in developing bone periosteum, growth plates, healing fracture callus tissue and developing ectopic bone tissue. Similar bioactive induction of the transcription of at least five ECM components (2- to 3.7-fold) and their secretion and self-organization into a mineralized matrix is responsible for the rapid formation and growth of bone nodules and differentiation of the mature osteocyte phenotype (Hench *et al.* 2000; Xynos *et al.* 2000*a*,*b*; Jones *et al.* 2007).

Several studies have confirmed the results of the early findings of Xynos *et al.* (2000*a*,*b*, 2001) and extended the generality to include several types of precursor cells and differing sources of biologically active Ca and Si ionic stimuli. Bone biology and gene array analyses of five different *in vitro* models using four different sources of inorganic ions provide the experimental evidence for a genetic theory of osteogenic stimulation (Maroothynaden & Hench 2001; Xynos *et al.* 2001; Hench 2003; Bielby *et al.* 2004, 2005; Gough *et al.* 2004; Christodoulou *et al.* 2005*a*,*b*; Jones *et al.* 2007). The cell and organ culture models used in these studies are summarized in table 2. Sources of the ionic stimuli used in the experiments are given in table 3.

Table 2. Cell and organ culture models used to establish the genetic basis for osteostimulation by bioactive glass ionic dissolution products.

primary human osteoblasts (pHOBs)	Xynos *et al.* (2000, 2001), Gough *et al.* (2004), Jones *et al.* (2007)
foetal human osteoblasts (fHOBs)	Christodoulou *et al.* (2005)
murine embryonic stem cells (mES)	Bielby *et al.* (2004, 2005)
human embryonic stem cells (hES)	Bielby *et al.* (2004)
murine foetal long bones (mFLBs)	Maroothynaden & Hench (2001)

Table 3. Source of ionic dissolution products used in studies of osteostimulation by gene activation.

45S5 bioactive glass culture discs	Xynos *et al.* (2000, 2001)
45S5 bioactive glass particulate (NovaBone)	Xynos *et al.* (2000, 2001)
58S bioactive gel glass	Bielby *et al.* (2004, 2005)
70/30 bioactive gel glass three-dimensional porous tissue engineering scaffolds	Gough *et al.* (2004), Jones *et al.* (2007)

All experiments showed enhanced proliferation and differentiation of osteoblasts towards a mature, mineralizing phenotype without the presence of any added bone growth proteins, such as dexamethasone or bone morphogenic proteins. Shifts in osteoblast cell cycles were observed as early as 6 h for most experiments, with elimination (by apoptosis) of cells incapable of differentiation (Xynos *et al.* 2000*a*). The remaining cells exhibited enhanced synthesis and mitosis. The cells quickly committed to generation of ECM proteins and mineralization of the matrix. Gene array analyses showed early upregulation or activation of seven families of genes (table 1) that favoured both proliferation and differentiation of the mature osteoblast phenotypes, including: transcription factors and cell cycle regulators (six with increases of two- to fivefold); apoptosis regulators (three at 1.6- to 4.5-fold); DNA synthesis, repair and recombination (four at two- to threefold); growth factors (four at two- to threefold) including IGF-I1 and VEG F; cell surface antigens and receptors (four at two- to sevenfold, especially CD44); signal transduction molecules (three at two- to sixfold); and ECM compounds (five at two- to 3.7-fold).

Resorbable bioactive gel glasses offer promise for use as scaffolds in bone TE (Saravanapavan *et al.* 2003; Atwood *et al.* 2004; Jones & Hench 2004; Jones *et al.* 2006*a*,*b*). Under appropriate culture conditions, differentiating foetal and embryonic stem (ES) cells can be induced to form bone nodules (Bielby *et al.* 2004, 2005; Christodoulou *et al.* 2005*a*,*b*); these structures are multi-layers of cells embedded in a mineralized ECM that contains type I collagen and osteocalcin. When a standard osteoblast differentiation medium is conditioned by exposure to resorbable bioactive gel glasses for 24 h and then applied to differentiating ES cells, there is a dose-dependent increase in the numbers of bone nodules formed compared with control cultures (Christodoulou *et al.* 2005*a*,*b*). Bioactive scaffolds have been made that release optimal concentrations (15–30 ppm Si and 60–90 ppm Ca) of the ionic dissolution products as they resorb in the presence of adherent human osteoblasts (Gough *et al.* 2004). Bioactive ceramics can be tailored in many ways, including adding the bioactive phase to polymeric materials, thereby creating bioactive inorganic–organic hybrids that offer potential for molecularly tailoring the mechanical properties and rates of resorbtion to match the increased strength of bone as it regenerates (Pereira *et al.* 2005*a*,*b*).

The cell culture results reviewed above correlate with clinical results using the same bioactive material, 45S5 Bioglass (Wilson *et al.* 1994; Shapoff *et al.* 1997; Lovelace *et al.* 1998; Aitasalo *et al.* 2001; Park *et al.* 2001; Yukna *et al.* 2001; Mengel *et al.* 2003; Ilharreborde *et al.* 2008; Ameri *et al.* 2009). Clinical studies that compare the success of autogeneous bone grafts versus grafts of the gene-activating glasses show equivalent rates of bone regeneration and fewer side effects with the bioactive glasses (Ilharreborde *et al.* 2008; Ameri *et al.* 2009). For example, iliac crest autograft is currently the gold standard for spinal fusion. However, there are disadvantages of an autogeneous graft, including increased blood loss, increased operative time, second-site morbidity and pain. A comparative study of bioactive glass (45S5 Bioglass) versus iliac crest autograft for spinal fusion in adolescent idiopathic scoliosis (AIS) has been reported for a group of 88 consecutive patients (Ilharreborde *et al.* 2008). Forty received iliac crest autograft and 48 received Bioglass with a minimum of 2-year follow-up. The results showed fewer infections (2 versus 5%) and fewer mechanical failures (2 versus 7.5%) in the Bioglass group. Loss of correction of the main thoracic curve was also less for the Bioglass group (11 versus 15.5%). The conclusions for this retrospective study were as follows.

— Bioglass is as effective as iliac crest graft to achieve fusion and maintain correction in AIS.
— Fewer complications were seen in the bioactive glass group of patients.
— The morbidity of iliac crest harvesting can be avoided by use of bioactive glass in spinal fusion.

These are important conclusions for the twenty-first century challenge of affordable healthcare for the aged. Elimination of the need for second-site (iliac crest) surgery in elderly patients that require spinal fusion means less exposure to anaesthesia and potential for infection. It also avoids pain and healing of the second site.

6. TWENTY-FIRST CENTURY BIOCERAMICS CHALLENGE NO. 2: TISSUE ENGINEERING OF SOFT TISSUES

Obtaining and maintaining a blood supply in tissue-engineered constructs is necessary for their long-term

stability following implantation. Studies have used third-generation bioactive, resorbable composites to enhance vascularization of a regenerated soft tissue construct. A novel processing method was developed to impregnate PGA meshes with controlled concentrations of 3–5 μm particles of 45S5 Bioglass (Boccaccini & Maquet 2003; Boccaccini *et al.* 2003*a*,*b*; Day *et al.* 2004; Verrier *et al.* 2004*a*). An optimal concentration of bioactive particles was established by using a rat fibroblast model to monitor cell proliferation and enhance production of vascular endothelial growth factor (VEGF; Hench *et al.* 2003; Day 2005; Day *et al.* 2005). Samples of the bioactive, resorbable composite mesh were implanted subcutaneously in adult rats. The composite meshes were infiltrated by fibroblasts and blood vessels. There were significantly greater number of blood vessels at 28 and 42 days post-implantation within the composite meshes compared with control PGA polymer meshes without the bioactive phase (Day 2005; Day *et al.* 2005). The results indicate that the increased vascularization occurring with meshes coated with 45S5 Bioglass is due to the bioactive phase stimulating release of VEGF, a highly pro angiogenic factor that acts solely on endothelial cells (Hench *et al.* 2003; Day 2005; Day *et al.* 2005).

Findings of Leu & Leach (2008), expanding upon the prior work of Day *et al.* (2005), show that low concentrations of Ca and Si ionic dissolution products released from 45S5 Bioglass particulate are effective in promoting angiogenesis in an endothelial cell module. They also showed that critical concentrations of the inorganic ions stimulate formation and growth of tubules in co-cultures. Their landmark experiments confirm that there is upregulation of VEGF production from human micro-vascular endothelial cells. The stimulation of angiogenesis depends upon the concentration of ions present in the cultures. The concentration of ions available was controlled by using differing quantities of 45S5 Bioglass particles contained within collagen sponges. They found that when there were too few ions, there was no effect; too many ions also had no effect. Leu & Leach also showed that larger concentrations of the ionic dissolution products lead to osteogenesis, as described in the seven experiments on bone stem cells reviewed above. A review article by Boccaccini's group discusses these topics in detail and summarizes the *in vitro* and *in vivo* evidence of the potential to control angiogenesis by use of bioactive glasses (Gorustovich *et al.* 2010). The recently developed ability to prepare nanosized bioactive glass particles is also important in future design of therapeutic treatments using these concepts (Brunner *et al.* 2006; Hong *et al.* 2009).

There are important implications from these findings. Few TE constructs at present produce a stable three-dimensional vascularized bed of tissue. Adding angiogenic stimulating particles could be an effective means to enhance vascularization *in vivo*. These findings are also relevant to a new approach to treatment of chronic wounds that are increasing at an alarming rate owing to the larger number of obese, aged and diabetic patients. At present, most treatment modalities for chronic wounds are at best palliative. There is great need for bioactive wound dressings that can counter the negative stimuli that prevent healing of chronic wounds. It should be possible to combine the anti-inflammatory characteristics of 45S5 Bioglass particles that also have proangiogenic potential at critical dosages, shown above. Adding such a multi-functional phase to wound dressings offers promise to stimulate the keratinogenesis and angiogenesis required to achieve a rapid regeneration of the skin.

These results also show promise for designing minimally invasive micro-injectable particles for stable augmentation of soft tissues. The bioactive particles could serve to stimulate the growth of soft connective tissues that can adhere to the particle surface and increase the mass and elasticity of the regenerated tissue. Such treatments are desperately needed to eliminate stress urinary incontinence in the elderly. This is a societal need and challenge to the biomaterials field that presently costs the NHS billions of pounds per year. Little research or new product development is currently pursued to solve this problem. Another need and challenge is for innovative tissue-engineered tubular constructs to replace segments of the small intestine that have been removed from patients with cancer or chronic inflammatory bowel disease. Such constructs that possess the physiological functions of the intestine are greatly needed to improve the quality of life of this group of patients. This is a particularly difficult challenge for soft TE.

In addition to these approaches using inorganic biomaterials in the engineering of soft tissues, there have been significant advances in the field of organic-based synthetic biomaterials for regenerative medicine. Hydrogels and glycolides are examples of just a few of the many polymers that have been engineered to interact with host tissue as well as the immune system. A recent review article by Hubbell *et al.* (2009) describes how polymeric materials can modulate the immune system by carefully tailoring the surface properties. These materials can also act as adjuvants to mimic certain signals that can alter the cytokine signalling of the host in positive ways. A recent publication by Fisher *et al.* (2010) demonstrates how the manipulation and control of the physical and chemical properties of hydrogels can potentially direct stem cells towards various lineages to create specific therapeutic benefits.

There has also been a significant body of work directed at manipulating the surface and structure of de-cellularized tissues so that they can be engineered for use in the repair and reconstruction of soft tissues. Work by Badylak *et al.* (2009) describes how different processing of these biological tissues for use in soft tissue repair results in unique surface structures and compositions that affect the host response. By controlling these parameters, surfaces are produced that enhance the healing response when implanted for soft tissue repair. Other innovative approaches to soft TE are discussed in the review of Place *et al.* (2009).

All of the above are twenty-first century challenges of an ageing population. There is a need to accelerate emphasis on addressing these challenges by the biomaterials community.

7. TWENTY-FIRST CENTURY BIOCERAMICS CHALLENGE NO. 3: STEM CELL ENGINEERING

Stem cells that exhibit the properties of both self-renewal and multi-lineage potential are a critical part of the foundation for the innovative twenty-first century approach to healthcare called regenerative medicine. It is well established that stem cells can give rise to some or all body tissues. The challenge is to encourage stem cells to differentiate into a required lineage, derive highly purified populations of the required cell phenotype, ensure there is no carcinogenic potential in the cell population, followed by implantation of the specific cell lineage in a manner that ensures that the cells will proliferate and replace, or augment the function of, diseased or degenerating tissues. The starting point is selection of the most appropriate stem cells to form the required tissue. Humans possess their own repository of stem cells that exist in bone marrow, brain, gut and liver, as well as in the circulation (Hubbell *et al.* 2009). These cells can potentially be harvested, propagated, assembled in a tissue construct, if necessary, and be inserted with minimally invasive techniques to a site where repair is needed. The activated stem cells will regenerate the tissues at the local site. For some stem cell types, there are problems with accessibility, low frequency (e.g. in bone marrow, there is one stem cell per 100 000 cells) and reduction in growth potential with age. Molecularly tailored bioactive materials offer promise for controlling the differentiation of stem cells and may provide the technology base for stem cell-based regenerative medicine.

Research has shown that modification of culture conditions can promote the differentiation of a specific cell phenotype, including haematopoietic cells, nerves, muscle, chondrocytes and pancreatic islets (Hubbell *et al.* 2009). Studies in Professor Julia Polak's laboratories at Imperial College London have shown it is possible to derive osteoblasts (Fisher *et al.* 2010) and lung epithelium from murine ES cells (Badylak *et al.* 2009). Initially, osteoblasts were generated by growing them as embryoid bodies that were dispersed 5 days after removal of leukaemia inhibitory factor and grown in a culture medium designed for the maintenance and growth of explanted osteoblasts. Further experimentation revealed that the biochemical composition of the medium promotes differentiation to an osteoblast phenotype. It was also found that the time at which a particular stimulus is administered can enhance differentiation to a specific phenotype. Thus, by providing the cells with dexamethasone 14 days following dispersal of embryoid bodies, it was possible to increase the yield of osteoblasts sevenfold (Fisher *et al.* 2010).

Another important influence on cell differentiation and function is the nature and composition of the surface and ionic environment for cell growth. For example, growth on a bioactive substrate (45S5 Bioglass) enhances derivation of osteogenic cells from ES cells. Bielby *et al.* (2004) showed that soluble ionic extracts containing Si and Ca cations obtained from 58S bioactive gel glasses enhance differentiation of the osteoblast-specific lineage from murine ES cells (Bielby *et al.* 2004). Differentiation of ES cells into osteogenic cells was characterized by the formation of multi-layered, mineralized bone nodules. The nodules contained cells expressing the transcription factor run×2/cbfa-1 and immunostaining showed deposition of osteocalcin and type I collagen in the ECM, both specific markers for the mature osteoblast phenotype. The presence of the soluble bioactive glass extracts increased the formation of mineralized bone nodules by almost 100 per cent. The effect of the bioactive gel glass extracts was dose dependent, with alkaline phosphatase activity and nodule formation increasing with extract concentrations. The gel glass extracts were as effective in inducing osteogenic differentiation as the addition of dexamethasone. Even more important, it was discovered that combining the two stimulants was even more effective. Another study by Beilby *et al.* has extended these results to human ES cells and establishes the potential for designing bioactive materials specifically for stem cell engineering applications (Xynos *et al.* 2001).

Studies by Christodoulou *et al.* enhanced understanding of the genetic effects of the dissolution products of bioactive gel glasses on osteogenesis (Bielby *et al.* 2005; Christodoulou *et al.* 2005*a*). The material studied was 58S bioactive gel glass (Maroothynaden & Hench 2001; Saravanapavan *et al.* 2003; Jones & Hench 2004; Jones *et al.* 2006*b*, 2007). The soluble Si and Ca dissolution products from the gel glass were added to cultures of primary osteoblasts derived from human foetal long bone explants cultures. U133A human GeneChip oligonucleotide arrays were used to examine 22 283 transcripts and variants, which represent over 18 000 well-substantiated human genes. A 24 h treatment with a single dosage of ionic products induced the differential expression of a number of genes important to differentiation of the osteoblast phenotype, including IL-6 signal transducer/gp130, ISGF-3/STAQT1, HF-1 responsive RTP801, ERK1 p44 MAPK (MAPK3), MAPKAPK2, IGF-I and IGFBP-5. The over 200 per cent upregulation of gp130 and MAPK3 and downregulation of IGF-1 were confirmed by real-time reverse transcriptase PCR analysis. These data suggest that 58S ionic dissolution products, Ca and Si, possibly mediate the bioactive effect of the gel glass through components of the IGF system and MAPK signalling pathways. The results from human foetal osteoblasts (Christodoulou *et al.* 2005*a*) confirm many of the findings reviewed above by Xynos *et al.* that used primary human osteoblast cultures derived from excised femoral heads of elderly patients (Hench *et al.* 2000; Xynos *et al.* 2000*b*). The findings thereby demonstrate the generality of the concept of genetic stimulation by the ionic dissolution products of bioactive glasses and gel glasses. The findings are also consistent with prior investigations of the role of ionic dissolution products in stimulation of growth and especially mineralization of foetal long bones, as reported by Maroothynaden and Hench (Polak & Hench 2005).

An implication of the above studies is that it is now feasible to design the dissolution rates and architecture of bioactive, resorbable inorganic scaffolds, including second phases, to achieve specific biological effects *in vivo* that

synchronize with the progenitor cell population present *in situ*. This offers the potential to design biomaterials for specific patients and their individual clinical needs.

8. TWENTY-FIRST CENTURY BIOCERAMICS CHALLENGE NO. 4: CONTROL OF INFECTION

Bacterial adhesion to biomaterials that causes biomaterial-centred infection and poor tissue integration is a problem that limits the lifetime of some medical devices. An increasing problem throughout the world is the expanding population of bacteria that are resistant to common antibiotics. An increasing incidence of chronic wounds of the lower limbs and feet that often lead to amputation offers another major challenge. Localized control of bacteria and inflammation that lead to cellular de-differentiation is needed. The *in vitro* bioactivity and antibacterial action of a novel sol–gel-derived glass, AgBG, in the system SiO_2–CaO–P_2O_5–Ag_2O offer a new twenty-first century biomaterials approach towards solving these problems (Bellantone & Hench 2001; Bellantone *et al.* 2001, 2002; Blaker *et al.* 2004; Saravanapavan *et al.* 2004; Lohbauer *et al.* 2005*a*,*b*; Jones *et al.* 2006*c*). The incorporation of 3 wt% Ag_2O in the bioactive gel glass system confers antimicrobial properties to the glass without compromising its bioactivity.

The bacteriostatic and bactericidal properties of this new material and of two other bioactive glass compositions, 45S5 Bioglass and BG without Ag, have been compared using *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* as test microorganisms (Bellantone & Hench 2001; Bellantone *et al.* 2001, 2002). Concentrations of AgBG in the range of 0.05–0.20 mg of AgBG per millilitre of culture medium inhibit the growth of these bacteria. Not only was AgBG bacteriostatic, but it also elicited a rapid bactericidal action. A complete bactericidal effect was elicited within the first hours of incubation at AgBG concentrations of 10 mg ml^{-1}. 45S5 Bioglass and BG had no effect on bacterial growth or viability. The antibacterial action of AgBG is attributed exclusively to the leaching of Ag^+ ions from the glass matrix. Analytical measurements rule out any contribution to AgBG-mediated bacterial killing by changes in pH or ionic strength or the dissolution of other ionic species from the biomaterials. Observations of the dissolution profiles of Ag^+ from AgBG in the presence and absence of bacteria are consistent with silver accumulation by the bacteria. On the basis of XRD patterns, FTIR spectra and ICP data, the bioactive behaviour of the Ag-doped gel glass is equivalent to bioactive gel glasses that have FDA approval for use in bone repair.

Inclusion of silver into bioactive sutures (Blaker *et al.* 2004) and bioactive gel glass foam scaffolds (Lohbauer *et al.* 2005*a*,*b*; Jones *et al.* 2006*c*) for TE and regenerative medicine applications has also been achieved. The amount of silver released from Ag-doped S70C30 bioactive gel-glass foams is well above the minimum bactericidal concentration (0.1 ppm), but below the cytotoxic concentration (1.6 ppm) for human cells. Primary human osteoblasts proliferate on the silver-doped gel glasses. The *in vitro* bioactivity tests of two bioactive gel-glass compositions (58S and S70C30) and their silver-containing equivalents lead to the following conclusions.

— The rate of formation of HCA on the silver-doped powders is slower than their un-doped counterparts.
— The presence of silver does not compromise the *in vitro* bioactivity of the gel glasses.

Particle size can be used as a means to control the release rates of active ions that are bactericidal (silver) and those that stimulate cellular response (silica and calcia). Cell culture experiments of the new binary Ag-doped CaO–SiO_2 gel glasses using human fibroblasts, osteoblasts and keratinocytes established the safe limits of controlled silver release when exposed to human cells involved in soft and hard tissue repair (Saravanapavan *et al.* 2004; Lohbauer *et al.* 2005*a*,*b*; Jones *et al.* 2006*c*).

9. TWENTY-FIRST CENTURY BIOCERAMICS CHALLENGE NO. 5: PREDICTIVE *IN VITRO* TESTS OF TOXICITY AND BIOCOMPATIBILITY OF BIOMATERIALS AND NANOPARTICLES

Present day dependence on *in vivo* animal testing to establish safety of new biomaterials, TE constructs and nanoparticles is a societal concern for both ethical and economic reasons. Developing predictive *in vitro* tests based upon human cells that are sufficiently reliable and cost effective is an important twenty-first century challenge. There are several concerns regarding *in vitro* tests that must be addressed in order to ensure relevance to eventual *in vivo* applications. First, a mature cell phenotype must be present in culture that is characteristic of the same type of human cell *in vivo*. At present, many cell culture tests are conducted using immortal cell lines that are not capable of expressing the complex arrays of proteins characteristic of mature phenotypes. Second, the status of cell cycles in culture needs to be monitored and correlated with response to the material being tested. Third, mature cell phenotype needs to be maintained in the cell culture during testing. This requires monitoring of cell phenotype, preferably *in situ*, because the material being tested might not be sufficiently toxic to kill the cells, but could induce de-differentiation and alter the healing response of the tissues. Fourth, the *in vitro* tests should provide information regarding the molecular biological changes taking place in the cells during exposure to the material. Fifth, the *in vitro* tests should be capable of statistical analyses in order to discriminate between small changes in the cell population. Sixth, cost and ease of use are equally important criteria.

New approaches that satisfy the above requirements are needed with some urgency. Two innovative technologies that offer hope of meeting this challenge are reviewed in this section. They are: (i) use of bio-Raman spectroscopy for *in situ* cell-based testing and (ii) use of a human autologous modular immune *in*

vitro construct (MIMIC) to interrogate innate and adaptive immune responses.

9.1. Bio-Raman test methods

Our studies show that it is possible to meet most of the requirements listed above for a predictive *in vitro* test system using bio-Raman spectroscopy of cells living in a special environmental chamber fitted with a microscope and a gas support system. The bio-Raman method uses specific wavelengths of lasers, new spectrometers and detectors to obtain Raman signals from living cells. Raman spectral signatures can be obtained with a high signal-to-noise ratio on living cells with 2 min exposure to 115 mW of 785 nm laser light without damage to the cells (Notingher *et al.* 2002, 2003*a*). A series of investigations showed that the bio-Raman method makes it possible to monitor the viability, cell cycle, metabolism, mitosis, differentiation, de-differentiation, mineralization and onset of death of single cells and cell assemblages (organoids) in real time without damage to the cells (Notingher *et al.* 2002, 2003*a*,*b*, 2004*a*–*e*, 2005; Owen *et al.* 2004, 2006*a*,*b*; Verrier *et al.* 2004*b*; Jones *et al.* 2005; Chan *et al.* 2006; Notingher & Hench 2006; Swain & Stevens 2007; Jell *et al.* 2008; Pyrgiotakis *et al.* 2008; Swain *et al.* 2008*a*,*b*; Gentleman *et al.* 2009). Single cells or three-dimensional assemblages of cells living in a specially designed microscope chamber can survive for long periods of time (several days) while being interrogated as to their state of health without alteration or damage to the cells (Notingher *et al.* 2002, 2003*a*). When the cells are exposed to test agents introduced into the cell chambers, the shifts in spectroscopic signatures can be observed and quantified in seconds to minutes, depending upon the level of resolution required. The time course of exposure can be followed by monitoring the spectroscopic signatures on a preselected number of cells in the chamber. The changes in spectroscopic signatures of the cells are related to alterations of DNA, RNA, proteins, lipids and carbohydrates within the cells owing to exposure to the agent being tested.

The many advantages of bio-Raman microspectroscopy for testing of living cell–biomaterial or cell–nanoparticle interactions are listed in table 4. Bio-Raman analyses applied to numerous human and animal cell types in the authors' laboratory are shown in table 5. Use of this analytical method by various investigators for other types of cells and tissues are reviewed in Notingher & Hench (2006).

A summary of several findings follows. Differences between the biological actions of cytotoxic agents (Triton X-100) versus genotoxic agents (Etoposide, VP16) can be detected by use of bio-Raman spectroscopy. This is achieved by determining the mechanism of cell death by either disruption of the cell membrane (cytotoxin) or breaking of DNA (genotoxin; Notingher *et al.* 2003*a*, 2004*c*,*e*, 2005; Owen *et al.* 2004, 2006*a*,*b*; Verrier *et al.* 2004*b*; Notingher & Hench 2006; Swain & Stevens 2007). This bio-Raman methodology was used to achieve an important milestone in our DARPA-funded research programme; i.e.

Table 4. Advantages of bio-Raman micro-spectroscopy.

non-invasive, no labels required
no damage to cells
measurements on cells maintained in physiological conditions
rapid data collection (1–2 min per spectrum, less than 10 min per cell)
high biomolecular chemical specificity
in situ test dosages are easy to control and monitor
simultaneously monitor changes in cell morphology
statistical sampling is possible at pre-selected levels of confidence
can monitor state of differentiation and phenotype of cells being tested
individual cells, cell assemblages, co-cultures or organoids can be tested
low cost
does not require training in advanced level of molecular biology to conduct analysis

Table 5. Cell types analysed with bio-Raman micro-spectroscopy.

primary human osteoblasts
human osteoblast cell line (MG63)
gene-transfected human osteoblasts
human monocyte cell line (U937)
human lung-like cell line (A549)
primary human chondrocytes
mouse lung epithelial cells (MLE-12)
mouse embryonic stem (ES) cells
mouse fibroblasts (3T3)
mouse neurons
neonatal calvarial osteoblasts
adult bone-marrow-derived mesenchymal stem cells (MSC)

detection and discrimination between two important chemical agents used in terrorist attacks, ricin and sulphur mustard (Notingher *et al.* 2004*e*). The collaborative paper with scientists at the Porton Down Chemical and Biological Warfare Laboratory in England was published in 2004 (Notingher *et al.* 2004*e*). The results showed that it was possible to rapidly discriminate between these two toxic agents owing to their differing modes of attack of A549 human lung-like cells.

One of the essential requirements for *in vitro* test methods for assessing biomaterial–cell interactions listed above is confirming the presence of a mature cell phenotype in the culture that is characteristic of the same type of cell *in vivo*. The bio-Raman system offers the potential of monitoring and maintaining cell phenotype by *in situ* spectroscopic analysis of the cells (Notingher *et al.* 2003*b*, 2004*b*; Jones *et al.* 2005; Jell *et al.* 2008; Swain *et al.* 2008*a*; Gentleman *et al.* 2009). Notingher *et al.* show that two statistical-based spectroscopic data management programs, called principal component analysis and linear discriminant analysis (LDA), are powerful tools for distinguishing cell phenotypes in real time. Bio-Raman spectroscopic analyses were made of MG63 immortal human osteosarcoma-derived osteoblasts and compared with primary

human osteoblasts, obtained from surgically excised femoral heads (Jell *et al.* 2008).

An LDA model prepared from the data showed high cross-validation sensitivity (100%) and high specificity (95%) for discriminating the MG63 cells from the primary cells, with 96 per cent of the cells being correctly classified either as tumour-derived or non-tumour-derived cells. A similar analytical method has been used to monitor cell cycle dynamics (Swain *et al.* 2008*b*) and to analyse the molecular changes of stem cells undergoing differentiation towards a specific cell phenotype (Notingher *et al.* 2004*a*,*b*,*d*; Jones *et al.* 2005; Chan *et al.* 2006; Jell *et al.* 2008; Swain *et al.* 2008*a*).

An important issue addressed in challenge no. 3 discussed in §7 is achieving a quality of tissue that is equivalent to natural tissues. One of the difficulties is quantitative assessment of tissues grown *in vitro*. Bio-Raman spectroscopy has been used to attack this problem for growth of bone *in vitro*. The results are seminal for the field. The paper of Gentleman *et al.*, from Professor Molly Stevens' group at Imperial College London, used bio-Raman spectroscopy to reveal cell source-dependent differences in interactions between multiple bone-like mineral environments (Gentleman *et al.* 2009). They showed that although osteoblasts and adult stem cells exhibited bone-specific biological activities and created a material with many of the characteristics of native bone, the mineralized bone nodules formed from ES cells were an order of magnitude less stiff and lacked the distinctive architecture and complex biomolecular and mineral composition present in native bone. This is an example of the power and potential of obtaining *in situ* analyses of complex tissue constructs by use of bio-Raman spectroscopy.

An example of the use of bio-Raman spectroscopy to study cellular response to micro- and nanoparticles is a recent study of a traditional Indian medicine used to treat ailments such as diabetes and eye diseases (Chan *et al.* 2006). This treatment involves the use of specially prepared particles of zinc oxide, known as *Jasada Bhasma*, that have a wide particle size distribution from 15 nm to 3 μm diameter. Fractioned particles were spherically shaped with a narrow size distribution of a mean size of 30 nm (Thian *et al.* 2006). The effect of the particles was examined using human lung adenocarcinoma (A549) cells with MTT assays performed to determine the mitochondrial activity after treatment with different doses ranging from 37.5 to 2400 ppm of the particles and after different exposure times of 24 to 72 h. Quartz particles at 600 ppm were used as a positive control. Raman spectroscopy was performed on cells seeded on MgF_2 plates and incubated for 24 h at 37°C and 5 per cent CO_2. Spectra from 30 different cells were collected for each sample group. The study showed by use of Raman spectroscopy combined with MTT assay that cellular damage of nucleic acids, proteins and lipids associated with ageing and caused by oxygen free radicals is mitigated by treatment with zinc oxide particulate systems. The treated cells are able to maintain higher intracellular protein and carbohydrate concentrations compared with the controls.

9.2. *New* in vitro *test system* (*MIMIC*) *for toxicology testing*

Establishing the environmental health and safety limits for new materials, especially nanoscale particles, is an important challenge of the twenty-first century. One of the difficulties posed by testing of nanoparticles is the need to study their interaction with cells of the human immune system. A new *in vitro* test system has been established that provides a predictive assay model focused on the human inflammatory response (Schanen *et al.* 2009).

This system, termed a modular immune *in vitro* construct (MIMIC), comprises several components that together permit interrogation of the short-term inflammatory response as well as the long-term memory response in either separate or longitudinal studies. The peripheral tissue equivalent of the MIMIC system is principally composed of blood vein endothelial cells. These cells participate in inflammatory reactions by secreting soluble factors and regulate the flow of immune cells from the vasculature. The second component is monocyte-derived dendritic cells. These are a critical antigen-presenting cell population that bridges both short-term (innate) and long-term (adaptive) responses and stimulates T-cell responses. It is the synergistic effect of the cell types that enables evaluation of the early immune responses associated with exposure to a foreign body. The MIMIC system has been used to assess the immunogenicity of titanium dioxide nanoparticles: anatase (7–10 nm), rutile (15–20 nm) and TiO_2 nanotubes (10–15 nm diameter and 70–150 nm length). The nanoparticles led to elevated levels of pro-inflammatory cytokines and increased maturation and expression of co-stimulatory molecules on dendritic cells. The *in vitro* assays indicated a response to the nanoparticles characteristic of an *in vivo* inflammatory response. The success of this type of human cellular-based immunological assay combined with the bio-Raman spectroscopic analyses described above offers a powerful combination of technologies that could well circumvent the need for many of the animal tests normally required for environmental health and safety testing of new materials and chemicals and toxic agents.

10. IMPLICATIONS FOR THE FUTURE

A genetic basis for development of a third generation of biomaterials provides the scientific foundation for molecular design of scaffolds for TE and for *in situ* tissue regeneration and repair, preferably using minimally invasive surgery. There are significant economic advantages to each of these new approaches that may aid in solving the problems of care for an ageing population. It should be feasible to design a new generation of gene-activating biomaterials tailored for specific patients and disease states. New, predictive analytical methods are becoming available that can aid in developing such innovative approaches to affordable healthcare. Perhaps of even more importance is the possibility that bioactive stimuli can be used to activate genes in a preventative treatment to maintain the

health of tissues as they age. Only a few years ago this concept would have seemed impossible. We need to remember that only 40 years ago the concept of a material that would not be rejected by living tissues also seemed impossible. This is now a clinical reality that has benefited millions of people and should stimulate new concepts in the years ahead.

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