Preface

This book has been written to provide an overview for the interdisciplinary and correlated fields of Tissue Engineering and Regenerative Medicine. The book describes different regenerative approaches to achieve tissue restoration, bringing together the fundamental and the innovative concepts in this innovative biomedical field.

This book covers several key themes. It presents different interdisciplinary strategies aiming toward the restoration of tissue homeostasis after trauma, as well as degenerative diseases or cancer. Through the chapters, the book describes several translational approaches in different fields such as tissue engineering, drug delivery, regenerative medicine, cell biology and nanoscience.

Each chapter has been written by authors who have established knowledge in their relative field and it is well supported by schematics and figures. The book is organized and edited in order to provide useful information to established scientists as well as to novices in the field. It could be a precious reference for those laboratories that will continue to develop biomedical approaches to tissue regeneration.

- Panseri Silvia, Ph.D.
- Taraballi Francesca, Ph.D.
- Cunha Carla, Ph.D.
About Editor

Silvia Panseri received her PhD in Biology at the University of Milan-Bicocca (Italy) working on regenerative medicine in traumatic injury of central nervous system (Vescovi’s Lab). She was awarded with SIBS award (16° ed.) Best PhD Thesis of 2009 in Biological Field (83° National Congress of Italian Society of Sperimental Biology).

She completed a Post Doctoral fellowship (2009-2012) with Dr. Marcacci at Rizzoli Orthopaedic Institute in Bologna (Italy). In 2011 she was a visiting fellow (Marco Polo Fellowship for young Italian Researchers to research periods abroad - University of Bologna) at Department of Biomedical Engineering, Cellular Engineering Laboratory - Columbia University (NYC, USA). Since 2013 she is a researcher in the Bioceramics and Bio-hybrid Composites Group at Institute of Science and Technology for Ceramics of National Research Council of Italy in Faenza (RA).

Since the 2006 she has been worked on different biomaterials applied to regenerative medicine. She has expertise in three dimensional stem cell culture with several scaffolds (self-assembling peptide, electrospun fibrous materials, ceramic, polymeric and bio-hybrid composite) and in vivo regenerative medicine.

Her interests are focused on novel approaches in tissue engineering and nanomedicine based on innovative biomaterials and 3D cell culture.

-Dr. Silvia Panseri
Francesca Taraballi earned her B.S. in Biological Sciences and her M.S. in Biochemistry at University of Milan - Bicocca, Italy and a Ph.D. in Nanostructures and Nanotechnologies from a joint program of the Materials Science Department of University of Milan - Bicocca with the Lawrence National Berkeley Laboratory (LBNL) in 2009. Her research focused on tissue engineering for different applications (spinal cord injury, cardiovascular, musculoskeletal). Dr. Taraballi worked in different laboratory such as the Department of Biomedical engineering of TAMU (College Station, TX), the Molecular Foundry at LBNL (Berkeley, CA) and the School of Materials Science and Engineering of NTU (Singapore). Dr. Taraballi developed in her career many tissue engineering platforms applied to different regenerative medicine approaches, from neural to bone and cartilage regeneration. She has authored multiple papers and book chapters in the field in nanomedicine and materials science, as well as multiple patent applications. She definitely moved to the United States in January 2013 as a senior research associate under the supervision of Dr. Ennio Tasciotti at Houston Methodist Research Institute, Department of Regenerative Medicine with a research focus on biomaterial functionalization for immune modulation.

-Dr. Francesca Taraballi
Carla Cunha completed her University Degree in Biology in 2002 in the Faculty of Sciences from the University of Porto. She was then selected as a fellow of the GABBA PhD programme. In 2007 she obtained her PhD degree in Biology granted by the University of Porto, with a PhD project in neurobiology of learning developed at the San Raffaele Institute in Milan. Carla Cunha has since then been working on the area of Biomedical Engineering in Italy, USA and Australia. Research interests are centred on the combined used of stem cells and biomaterials for tissue engineering strategies in different target tissues. In particular, research has focused on nerve reconstruction, including development of in vitro 3D scaffolds made of functionalized self-assembling peptides for culture of neural stem cells and in vivo nerve regeneration strategies. Later on, osteochondral tissue engineering strategies were pursued, with several novel biomaterials being tested in vitro and applied into osteochondral animal model defects. In 2012 she moved to Portugal to join INEB, where tissue engineering strategies focus the intervertebral disc.

-Dr. Carla Cunha
Acknowledgement

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Introduction

Tissue regeneration can be considered a process of renewal and growth of a tissue that exclusively starts after damage or disturbance to the tissue homeostasis. The process of tissue regeneration is characterized by a well-coordinated sequence of physiological events: hemostasis, inflammation, proliferation, and final tissue remodeling. However, regeneration and restoration cannot be confused with the process of healing. Tissue healing implies the repair process has not allowed for full restoration of the anatomical architecture. For many tissues, the healing process may lead to tissue fibrosis, which creates an obstacle to proper physiological function. Tissue engineering and regenerative medicine are growing fields of research that aim to improve the tissue restoration functions using different approaches. The first is investigating embryological development in order to understand the molecular mechanism at the base of specific-tissue regeneration. The second is studying adult tissue organization (e.g. extracellular matrix composition and structure) in order to build a similar structure that is essential as support to the tissue restoration (scaffold). The third is identifying the cell’s phenotype and source that may help, encourage, and enhance the process of tissue repair. The final approach is characterizing the molecular signal and its kinetics in order to give the correct information in a spatio-temporal fashion to the surrounding cells, leading to the recovery of tissue homeostasis.

Recently, biomimicry is an effective strategy that produced considerable results in regenerative biology. Biomimicry, the imitation of natural things or processes, generated cutting-edge designs in the engineering and technological fields. In regenerative medicine, novel scaffolds have been designed to recapitulate extracellular matrix-like structures, binding of ligands, controlled release of specific growth factor, and mechanical properties of the tissue to restore. Furthermore, different phenotypes of a cell have been isolated and characterized in order to be used to recreate the natural niche and direct the tissue response. The biomimetic approach can range from the mimicking of tissue form and function to the mimicking of biological processes and systems.

In this book, we present a selection of biomimetic approaches. This includes cell source characterization and selection through the use of drug delivery and scaffold design that function to merge together with the same aim: functional tissue restoration. We decided not to select a specific tissue, but rather present some general guidelines in regenerative biology research.

We believe that, because biomimicry is the driving force in different research fields, further research could move the biomedical field forward. Clinical trials with a focus in genomics. That includes but is not limited to clinical scientists, clinical biomarker developers, clinical statisticians and clinical program or protocol directors. Therefore, this eBook is tailored to address some issues we encountered that impact these people’s everyday life and to share with this targeted audience those lessons from which we personally benefited tremendously. Since a lot of early phase investigational compounds fail in early clinical trials, those lessons learned become extremely important in preparing and planning for the future clinical trials. With the advent of personalized medicine or precision medicine the success rate for drug development appears to be improving. However, the last thing we want to see is for a drug development program to be terminated not based on the drug efficacy but due to the sample testing failure resulting in the unexpected poor enrollment rate, or a biomarker does not live up to the expected performance based on the analytical validation results. Several
chapters in this eBook touch upon these issues and provide some solutions to address
them. Although most of the authors in this eBook are clinical biomarker developers, we
tried to make sure no significant bias toward how the clinical biomarker program evolves
and operates compared to the view points from a clinical monitor. The most frequently
encountered clinical genomic assays, including Next Generation Sequencing (NGS), gene
expression signature and Single Nucleotide Polymorphism (SNP) assays are discussed in this
eBook with focus not on the technologies themselves but instead on the current issues and
challenges, and in many cases proposed solutions. Since the recent model of outsourcing
clinical assays by pharmaceutical companies to contract research organizations appeared
to be more matured, we also dedicated a chapter to discuss the challenges, benefits and
lessons learned of this model using our experiences gained through the last 5-6 years
of practice. Finally, we are very excited to be able to dedicate a chapter to discuss the
challenges and dilemmas of developing Companion Diagnostics (CDx) including genomic
CDx.
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Abstract

Bone tissue regeneration is a complex well-orchestrated physiological process, involving a number of cell types and intracellular and extracellular molecular signalling pathways. With the increased life expectancy and the consequent aging of population, scientific efforts to speed up the body's natural bone healing process and to reduce and treat associated complications are highly requested. The research in this field has been firstly focused extensively on developing CaP-based ceramics endowed of osteo-conductive properties and after, on degradable polymeric scaffolds for the controlled localized delivery of bioactive agents. Recent advantages in the field of engineered scaffolds have been obtained from the investigation of composite scaffolds designed by the combination of bio ceramics and/or biodegradable polymers in order to obtain osteo-conductive structures that can mimic the natural properties of bone tissue. Recently, nano materials used in bone tissue regeneration for the delivery of tailored drugs have shown to offer new opportunities to provide more focused and fine-tuned treatment of diseases at a molecular level, thus enhancing the therapeutic efficacy of drugs and reducing side effects. In this chapter natural, synthetic, hybrid and nano materials will be discussed summarizing and analyzing their chemical and mechanical properties as well as the results of in vitro or in vivo release studies and their biocompatibility and ability to induce bone tissue regeneration.

Introduction

Ever growing interest is highlighted for bone tissue engineering and regeneration therapies, in parallel with the rise in trauma victims and musculoskeletal disorders often associated with the increase in life expectancy [1-3]. Bone regeneration is a complex, well organized physiological process, in which different cell types and their activated signaling pathways are involved. Mesenchymal Stem Cells (MSC) play a pivotal role, and their differentiation is regulated by specific signaling molecules such as growth factors, cytokines and hormones and their activated intracellular networks [4]. The engineering of bone tissue can provide bone grafts for clinical use or offer solutions to be implemented in the bone lost that occurs, for example, to degenerative, surgical or traumatic processes.
In addition, there is the need to accelerate the healing of bone fractures and to treat some problematic non-union fractures. The development of controlled release systems for the regeneration of bone, cartilage, and osteo chondral interface is one of the hot topics in the field of tissue engineering and regenerative medicine and a variety of therapeutic drugs have been evaluated in combination with tissue-engineering approaches for bone regeneration. Among them, Bisphosphonates (BP) are well-established drugs, used in the development of metabolic bone disorder-related therapies, such as osteoporosis and Paget’s disease, tumour-induced hyperkalaemia, and inflammation related bone loss [5-6]. Increasing evidences on the advantages of BP in combination with scaffolds in tissue-engineering strategies are emerging and the related literature addressed for the delivery and sustained release of these therapeutic drugs will be discussed in this chapter. Moreover, the controlled delivery of growth factors, nucleic acids, small molecules and cells within biomaterial carriers can enhance and accelerate functional bone formation aiding in recapitulating signals present in bone development and healing, regenerating interfaces of bone with other connective tissues and enhancing vascularization of tissue engineered bone [7]. These carrier systems can be designed with pre-programmed release kinetics to deliver bioactive molecules in a localized, spatiotemporal manner, most similar to the natural wound healing process and can also acts as extracellular matrix mimicking substrates for promoting osteo progenitor cellular infiltration and proliferation for integrative tissue repair [8]. The need for new drug delivery strategies for bone tissue regeneration is also due to the peculiar target, the bones, which are peripheral organ with limited blood supply. The current drugs used for the pathologies related to bone loss are administered orally or parenterally and are exposed to various physicochemical and biological factors which affect the bioavailability of the drugs. Thus, the current anti-osteoporotic drugs need to be administered at higher doses to account for pharmacological interactions, thus exposing the patients to adverse effects such as the cancer risks of postmenopausal women who took estrogen replacement therapy and patient compliance for the drugs that have to be administered for prolonged time [9]. Several potential drug delivery systems which are able to contain the anti-osteoporosis drugs and release them slowly to the targeted bone have been proposed. Among them CaP-based ceramics Hydroxyapatite (HA) and beta tricalcium phosphate ceramic materials, polymers, micro-sponges and other various carriers, have proved to increase drug efficacy and reduce adverse effects. These delivery systems allow the drugs to be administered locally at the targeted bone for longer time, thus reducing drug frequency and improving patient’s compliance [10-12]. The materials used for these purposes as well as bone substitution materials must possess biocompatibility and osteo conductivity, namely the ability to stimulate themselves the regeneration of the bone tissue. Moreover, an ideal material must be non-antigenic, resistant to infection, easily adaptable and of course, readily and sufficiently available to trigger osteogenesis. The development of new medical technologies and the achievements in material science, biochemistry, molecular biology and genetic engineering allowed the creation of new combined synthetic materials for bone grafting and bone DDS. Modification of the materials bulk structure, which brings their structure closer to natural bone tissue, including drugs, cytokines, growth factors and cells into their composition enables to provide synthetic materials with not only osteoconductive but also osteo inductive properties as well as the control the speed of biodegradation, bringing it closer to the kinetics of osteogenesis.

The aim of this chapter is to assess the materials that can be used as drug delivery systems in bone tissue regeneration, summarizing and analyzing the results of studies and developments in the field of natural and synthetic osteoplastic materials. In light of the immunological and disease transfer risks from allogeneic bone, the research in this field has been focused extensively on developing alloplastic bone substitutes predominantly based on ceramics, such as CaP-based ceramics Hydroxyapatite (HA) and beta tricalcium phosphate, calcium sulfates, and bioactive glasses with osteoconductive and bioactive properties [13]. Since within bone tissues, HA is present in the form of nanosized crystals, great interest
was also devoted in developing HA nanocrystals which are in a dynamic equilibrium with the biological environment in the resorption/mineralization cycle and are endowed of high level of mechanical properties [14,15]. However, despite the positive biological properties, the drawbacks of most CaP-based materials are their poor mechanical durability and slow resorption in the body tissues. Thus, new biodegradable polymeric scaffolds for the controlled localized delivery of bioactive agents have been investigated and will be here reported. Polymer composition, hydrophobicity, crystallinity and degradability can positively affect the rate of drug release as well as the rate of tissue ingrowth. Next-generation materials for bone tissue engineering should combine the tunable macro/microporosity and osteoinductive properties of ceramic material with the mechanical/physical properties of biodegradable polymers. In this context, a lot of research is now focused on hybrid ceramic/polymer scaffolds, as matrices for the sustained delivery of therapeutic drugs and/or biomolecular signals, such as growth factors. Composites made of synthetic HA in the forms of powders, granules and gels in combination with the polysaccharides chitosan, alginate, hyaluronic acid, collagen, peptides, embryonic stem cells, drugs and other preparations have been proposed, expanding the possibilities of reconstructing pathologically modified mineralized tissues [16-18]. Moreover, in recent years there has been growing interest in the use of nanoscale structures for biomedical application. Materials converted into nano size provide unique surface properties which critically may influence their interaction with the biological systems. Thus, nanocomposites have been investigated for the delivery of tailored drugs or other bioactive agents in bone tissue regeneration, these materials have shown to provide more focused and fine-tuned treatment of diseases at a molecular level, thus enhancing the therapeutic efficacy of drugs and reducing side effects.

Ceramic Materials

A primary objective in selection of a bulk biomaterial to be used in bone tissue engineering is to mimic native bone tissue. Calcium Phosphate (CaP) ceramics quite satisfied this requirement and have been widely used in the biomedical engineering and bone substitution/regeneration [1,19]. These materials were introduced more than 40 years ago and are considered bioactive as they bond to bone and enhance bone tissue formation as bone substitutes. This bioactivity has been attributed to their composition and structure which is similar with the mineral phase of bone [20]. The most common types of CaP materials investigated for tissue regeneration derive from natural origin, such as coralline hydroxyapatite or by synthesis, such as synthetic HA (Ca\(_5\)(PO\(_4\))\(_3\)OH), β-tricalcium phosphate (Ca\(_3\)PO\(_4\))\(_2\), β-TCP), Biphasic Calcium Phosphates, consisting of a mixture of Tricalcium Phosphate and Hydroxyapatite (BCP), and multiphasic bio-glasses (Figure 1) [21].

![Chemical Formulas of Hydroxyapatite (HA) and β-Tricalcium Phosphate (TCP).](image)

Figure 1: Chemical Formulas of Hydroxyapatite (HA) and β-Tricalcium Phosphate (TCP).

On the basis of their composition and stoichiometry, the physical properties such as degradation rate, modulus, and process ability of these materials can be significantly changed [22,23]. As example, some synthetic composition of HA degrade too slowly to allow native tissue integration, while TCP materials has revealed that the degradation rate is too rapid. In this context, the development of BCP and bio-glasses which have shown
better degradation rates [24]. A pivotal physical attribute in the development of these materials in bone tissue engineering and in particular, for synthetic bone scaffolds is the high degree of porosity which have to mimic the natural bones on the other hand, when synthetic CaP ceramics such as TCP, HA and BCP are formed into porous scaffolds, the macroscopic mechanical properties are often inadequate for load bearing surfaces, because of their inherent brittleness and low mechanical stability which seriously may limit their use in clinic [25]. Thus, rather than in 3D scaffold applications, these materials are most successful used as implant coatings.

Synthetic and natural CaP formulations has been shown to be biocompatible and to promote osteoblast adhesion and migration/infiltration \textit{in vitro} and several papers report the use of this material for bone tissue regeneration [3,26,27]. Fellah et al., have summarized a model for the biointeraction between CaP materials and bone cells; the authors demonstrated that CaP materials and in particular BCP have superior stability and \textit{in vivo} osteogenic properties with respect to autologous bone grafts in critical-sized bone defects [28]. Short and long-term \textit{in vitro} and \textit{in vivo} studies have reported the ability of CaP materials to induce osteogenic differentiation, to promote Mesenchymal Stem Cell (MSC) migration, and to allow bone tissue growth and integration [29,30].

CaP materials have been used as delivery carriers for antibiotics, anti-inflammatory agents, analgesics, anticancer drugs, growth factors, proteins and genes (Table 1) [31,32]. These Drug Delivery Systems (DDS) can be easily synthesized and the drugs can be incorporated via different routes, such as wet chemical processes, solid state reactions, hydrothermal and micelle-mediated processes [33,34]. These nano scaffolds can serve multiple functions, such as drug delivery, directing cell growth or tissue generation, and mechanical support. Another great major advantage of these DDS with respect to the other materials used for tissue regeneration is due to their degradation ions are Ca$^{2+}$ and PO$_4^{3-}$ ions, which already exist in the body in high concentrations [35]. Moreover, nanotechnology-derived CaP materials have also proved to maintain a sustained and steady drug release over time by controlling tailoring calcium phosphate nanoparticle grain size, the surface area and calcium/phosphorus ratios [36]. CaP-based DDS have been widely studied for the release of antibiotics after surgical interventions aimed at the implantation of prosthesis or for the prevention from bacterial infections. In fact, the use of these drugs either orally or intravenously, because of the very little accessibility of the site of infection, often prolongs the treatment of bone infections. Several materials have been proposed in the past for the release of antibiotics such as the implantation of Poly-Methyl Methacrylate Spheres (PMMA) loaded with gentamicin sulphate in the infection site, this method led to a non-resorbable material that must be removed after some months and replaced with new material [37]. The use of calcium sulphate dihydrate (Ca$_2$SO$_4$·2H$_2$O), proposed as alternative material, showed the drawback to have low mechanical strength and very high resorption rate [38].

<table>
<thead>
<tr>
<th>Delivery System</th>
<th>Type of drug</th>
<th>Drug</th>
<th>release study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTCP and DCPA</td>
<td>Antibiotic</td>
<td>flomoxef</td>
<td>\textit{in vitro}</td>
<td>39</td>
</tr>
<tr>
<td>Sr-TCP</td>
<td></td>
<td>doxycycline</td>
<td>\textit{in vitro}</td>
<td>41</td>
</tr>
<tr>
<td>HA</td>
<td></td>
<td>tetracycline</td>
<td>\textit{in vitro}</td>
<td>42</td>
</tr>
<tr>
<td>HA</td>
<td></td>
<td>cephalexin</td>
<td>\textit{in vitro}</td>
<td>44</td>
</tr>
<tr>
<td>TCP and MCP</td>
<td></td>
<td>gentamicin</td>
<td>\textit{in vitro}</td>
<td>44</td>
</tr>
<tr>
<td>HA</td>
<td></td>
<td>vancomycin</td>
<td>\textit{in vivo}</td>
<td>45</td>
</tr>
<tr>
<td>TTCP and DCP</td>
<td></td>
<td>gentamicin</td>
<td>\textit{in vivo}</td>
<td>46</td>
</tr>
<tr>
<td>TTCP/DCP/HADCP</td>
<td>Anti-inflammatory</td>
<td>acetylsalicylic acid</td>
<td>\textit{in vitro}</td>
<td>47</td>
</tr>
<tr>
<td>TTCP, DCP, HA</td>
<td></td>
<td>indomethacin</td>
<td>\textit{in vitro}</td>
<td>48-51</td>
</tr>
<tr>
<td>HA</td>
<td></td>
<td>indomethacin</td>
<td>\textit{in vivo}</td>
<td>49, 51</td>
</tr>
</tbody>
</table>
### Table 1: Examples of Investigated Drug Delivery Systems Ca-P ceramics-based.

<table>
<thead>
<tr>
<th>System</th>
<th>Drug Delivery System</th>
<th>Hormones</th>
<th>Bisphosphonates</th>
<th>Growth factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTCP and DCPD</td>
<td>Anti-cancer</td>
<td>mercaptopurine</td>
<td>pamidronate</td>
<td>VEGF</td>
</tr>
<tr>
<td>HA</td>
<td>in vitro</td>
<td>estradiol</td>
<td>in vivo</td>
<td>in vivo</td>
</tr>
<tr>
<td>Ha</td>
<td>in vitro</td>
<td>estradiol</td>
<td>in vivo</td>
<td>in vitro</td>
</tr>
<tr>
<td>CP</td>
<td>in vitro</td>
<td>Zoledronate</td>
<td>in vivo</td>
<td>rhBMP-2</td>
</tr>
<tr>
<td>HA</td>
<td>zoledronate</td>
<td>alendronate</td>
<td>in vitro</td>
<td>HGF</td>
</tr>
<tr>
<td>HA</td>
<td>Zoledronate and alendronate</td>
<td>in vitro</td>
<td>in vitro</td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>alendronate</td>
<td>ibandronate</td>
<td>in vitro</td>
<td>BMP-2</td>
</tr>
<tr>
<td>OCP</td>
<td>alendronate</td>
<td>clodronate</td>
<td>in vitro</td>
<td>EGF and FGF-2</td>
</tr>
<tr>
<td>HA</td>
<td>alendronate</td>
<td>in vivo</td>
<td>in vitro</td>
<td>in vivo</td>
</tr>
<tr>
<td>TCP and DCPA</td>
<td>VEGF</td>
<td>in vivo</td>
<td>in vivo</td>
<td>in vivo</td>
</tr>
<tr>
<td>HA</td>
<td>HGF</td>
<td>in vitro</td>
<td>81,82</td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>BMP-2</td>
<td>in vitro</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>EGF and FGF-2</td>
<td>in vitro</td>
<td>85</td>
<td></td>
</tr>
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</table>

The combination of antibiotics with CaP material showed enhanced properties which may overcome these drawbacks. Takechi et al., reported the effect of incorporating different concentrations of flomoxef in Tetracalcium Phosphate (TTCP) and Dicalcium Phosphate Anhydrous DCPA [39]. The authors observed a strong reduction of mechanical strength when the amount of antibiotic increased, this effect was attributed to increased porosity and to some inhibition of the setting reaction. The drug release followed the typical profile observed in a skeleton-type drug delivery system with a drug release of 55% and 60%, after 72 h. In other studies, the modification of the CaP ceramics properties can be due to some chemical interactions with the drug as example, the addition of tetracycline to an apatitic cement caused a strong reduction in mechanical properties which was attributed to the ability of this antibiotic to chelate Ca-atoms [40]. Alkhraisat et al., investigated new Sr-substituted calcium phosphate cement loaded with doxycycline hyclate in order to elucidate the effect of strontium substitution on antibiotic delivery. The cement was prepared using as reactants Sr-substituted β-tricalcium phosphate (Sr-β-TCP) and acidic monocalcium phosphate monohydrate. The results of this study revealed that the Sr-substituted cement efficiently adsorbs the antibiotic, probably for the enhanced accessibility to the drug-binding sites within the matrix, thus suggesting that Sr substitution in secondary calcium phosphate cements improves their efficiency for the drug adsorption and release [41]. Ratier et al., observed a decrease in compressive strength with an increase in tetracycline concentration together with morphological changes [42]. These effects were reported to be related to the strong affinity of tetracycline hydrochloride to CaP matrix, a maximum of 7% drug was incorporated without affecting the mechanical properties of the material. Hesaraki et al., investigated different types of Ca-P cements as potential matrices for incorporating different types of antibiotics. In this study, the release of cephalolin monohydrate from a macroporous HA was investigated over 0.5–300 h in simulated body fluid. Results showed that the release rate of the drug from porous CaP was higher than that of the nonporous CaP but same release patterns were experienced in both types of cements, observing a time-dependent controlled release of the incorporated drug from macroporous matrix [43]. An increase in setting time was also reported by Bohner et al., due to gentamicin sulphate incorporation into the CaP matrix [44]. Hamanishi et al., examined the incorporation of vancomycin to a CaP matrix formed by TTCP and DCPD for treating osteomyelitis caused by methiciline-cefem resistant Staphylococcus aureus (MRSA)-specific antibiotic [45]. The in vitro studies revealed an effective release of vancomycin
within 2 weeks, in the case of CaP containing 1% vancomycin, and within 9 weeks for CaP containing 5% of vancomycin in both cases, the released concentrations were higher than the effective concentration against different types of MRSA. The rate of drug release depended on the crystallinity of the cements, but for the two periods studied up to 95% of the antibiotic was liberated in both cases. The in vivo release study implanting HA loaded with 1%, 2% and 5% of vancomycin in tibial condyles of rabbits showed that, for the HA sample loaded 5% of vancomycin, after 3 weeks, the concentration of vancomycin in bone marrow was 20 times higher than the minimum value clinically required. In another in vivo study, Stallmann et al., investigated and compared the effect of adding gentamicine and the peptide hLF1-11 (a fragment of human lactoferrin) to commercial CaP matrix constituted by based in TTCP and DCP [46]. These materials implanted in the femoral channel of rabbits and vaccinated with Staphylococcus aureus revealed a reduction in the development of osteomyelitis for both drugs and a more effective reduction in the case of the gentamicine. Calcium phosphate ceramics have been also used for the delivery of anti-inflammatory, analgesic and anticancer drugs. Otsuka et al., investigated the in vitro drug-release rate of a drug-delivery system based on TTCP/DCP/HA containing acetylsalicylic acid (aspirin) as a model drug. The rate of drug liberation was found to increase with the higher porosity of the material, which can be easily controlled by liquid-to powder ratio [47]. Indomethacin, an anti-inflammatory, non-steroidal drug usually used in different pathologies of muscle skeletal system was incorporated in CaP ceramic materials such as TTCP, DCPD and HA [48-51]. Different concentrations of the drug loaded in these CaP materials were analyzed in vitro in Simulated Body Fluid (SBF) as well as in Phosphate Buffer Solution (PBS) and in both cases the amount of drug released increased with the amount of drug loaded in the material. In vivo studies performed by subcutaneous implantation of the Ca-P materials in rats showed that the initially indomethacin release, measured in plasma, was very fast, decreasing after 1 day and in total prolonged for 3 weeks [49]. The authors reported that the half-life of indomethacin in plasma was much higher when drug was introduced via implantation, rather than when it was injected subcutaneously; moreover, the drug release prolonged over one month. The in vitro release of mercaptopurine, a drug able to inhibit the proliferation of tumoral cells, showed a similar behaviour to that observed for indomethacin. Otsuka et al., studied the incorporation of mercaptopurin in TTCP and DCPD [52]. A clear dependence between release rate and ceramic porosity was observed also in this case, which can be easily controlled by modifying the liquid-to-powder ratio of the ceramic. The rate of release of estradiol, a feminine sexual hormone with estrogenic activity, which can be used in the treatment of mineral resorption and bone loss, was studied by the same research group, incorporating the drug in HA and investigating the release in SBF [53]. The authors also reported an in vivo study, by subcutaneous implantation in rats [54]. The in vivo release of estradiol was faster in rats which had lower concentrations of vitamin D and Ca, with respect to healthy rats, thus suggesting the autoregulatory mechanism of estradiol release.

Bisphosphonates (BP) are an important family of drugs, used in various bone- and calcium-related pathologies, such as cancer, hypercalcemia, Paget’s disease, or osteoporosis [55]. These drugs have great affinity with mineral hydroxyapatite: they are pyrophosphate (P–O–P) analogs in which the oxygen has been replaced by a carbon to give a P–C–P backbone. Substitutions on the central carbon give rise to a large family of compounds with different properties and potencies (Figure 2). Their biological activities in calcium-related disorders are due to the direct interaction with osteoclasts, causing their inhibition and increasing their apoptosis [56]. The major drawbacks of this class of drugs are related to their pharmacokinetics and poor oral absorption from the gastrointestinal tract, which is typically less than 1%. In addition, BPs has been associated with adverse gastrointestinal effects in humans [57]. Thus, in addition to oral administration, different strategies have been proposed over the last years, such as nasal delivery and subcutaneous or intramuscular injection [58-60].
Figure 2: Bisphosphonates.

<table>
<thead>
<tr>
<th>Drug</th>
<th>( R_1 )</th>
<th>( R_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etidronate</td>
<td>-OH</td>
<td>-CH(_3)</td>
</tr>
<tr>
<td>Clodronate</td>
<td>-Cl</td>
<td>-Cl</td>
</tr>
<tr>
<td>Tiludronate</td>
<td>-H</td>
<td>-S-Cl</td>
</tr>
<tr>
<td>Pamidronate</td>
<td>-OH</td>
<td>-CH(_2)-CH(_2)-NH(_2)</td>
</tr>
<tr>
<td>Neridronate</td>
<td>-OH</td>
<td>-(CH(_2))(_5)-NH(_2)</td>
</tr>
<tr>
<td>Olpadronate</td>
<td>-OH</td>
<td>-(CH(_2))(_2)N(CH(_3))(_2)</td>
</tr>
<tr>
<td>Alendronate</td>
<td>-OH</td>
<td>-(CH(_3))-NH(_2)</td>
</tr>
<tr>
<td>Ibandronate</td>
<td>-OH</td>
<td>-(CH(_2))(_2)N-(CH(_2))(_4)-CH(_3)</td>
</tr>
<tr>
<td>Risedronate</td>
<td>-OH</td>
<td></td>
</tr>
<tr>
<td>Zoledronate</td>
<td>-OH</td>
<td></td>
</tr>
</tbody>
</table>

The challenge for bisphosphonated based drug delivery systems is to achieve improved bioavailability and safety. Moreover, it has been proved that BP can be beneficial when administrated after bone replacement; a localized delivery of the drug could have a favorable effect to produce a more stable and integrated interface between the implant and the bone at the early stage of implantation, when a significant bone loss can occur. This effect was proved in several studies using different BP [61,62]. Yoshinari et al., evaluated the bone response to titanium implants treated with a thin Ca-P coating and pamidronate [61]. These surface-treated implants were inserted into edentulous areas in the mandibular molar region of five beagle dogs and after 4 and 12 weeks, the bone implant interface was evaluated histologically and histomorphometrically. After 4 weeks a higher percentage of bone contact was found around the coated implants with respect to the control group. The highest percentage of bone contact was found around the bisphosphonate loaded systems after 12 weeks of implantation. These data suggest that the bisphosphonate-immobilization can be effective in the promotion of osteogenesis on surfaces of implants. Peter et al., have grafted zoledronate to Hydroxyapatite (HA) coating of titanium implants which were inserted in rat condyles with various zoledronate concentrations [62]. A positive concentration-dependent effect was observed on the peri-implant bone density and on different histomorphometric parameters. Moreover the authors report that the mechanical fixation of the implants was increased by the local presence of zoledronate. Boanini et al., demonstrated that zoledronate containing Hydroxyapatite Nanocrystals (HA-ZOL) can be synthesized as a single crystalline phase up to a zoledronate content of about 7 wt% by direct synthesis in aqueous solution. From this study emerged the good structural fit between zoledronate and hydroxyapatite, which involves calcium ion coordination geometry and the possibility of hydrogen bonds. The synthesized HA-ZOL nanocrystals showed the ability to promote osteoblast proliferation and activity, to prevent osteoclast formation and to promote osteoclast apoptosis [63]. The authors also reported the results of an in vitro study aimed to investigate the effects of alendronate incorporation into hydroxyapatite on bone cells response in osteoblast-like MG63 cells and human osteoclasts which were cultured on nano crystals at different alendronate content. The results of these investigations highlighted an even greater influence off HA-ZOL than HA-AL on osteoclast apoptosis. Zhao et al., have
investigated CaP material as drug delivery system for the release of alendronate [64]. The authors reported an increased alendronate release rate with increase in drug concentration and a sustained release over 21 days. The system showed good biocompatibility in terms of proliferation of rat mesenchymal stem cells. Niu et al., investigated the mechanisms through which BP enhance bone-implant integration in a state of peri-implant high bone turnover [65]. In this study, the authors synthesized HA composite coatings loaded with alendronate and evaluated the efficacy of the composite coating into the proximal region of the medullary cavity of the left tibia in rabbits. The results indicated that the alendronate based HA composite coating reduces peri-implant high bone turnover, improves bone-implant integration, bone quality, and implant stability; and inhibits particle migration. In vitro results suggest that the system can afford long release duration. The results of this study indicated that the presence of alendronate enhances osteoblast activation and extracellular matrix mineralization processes, without any abnormal collagen degradation. Moreover, the osteoclast number on the composite nanocrystals decreased, thus indicating that the bisphosphonate exerts its inhibitory effect on osteoclast proliferation even when incorporated into hydroxyapatite. The Matrix Assisted Pulsed Laser Evaporation (MAPLE) was applied in order to synthesize alendronate-hydroxyapatite thin films on titanium substrates. Alendronate-hydroxyapatite composite nanocrystals with increasing bisphosphonate content were suspended in deionised water, frozen at liquid nitrogen temperature and used as targets for MAPLE experiments [66]. Biological assays performed on osteoblast-like MG63 cells and human osteoclasts cultured on the thin films up to 14 days, showed enhanced bioactivity; the systems were able to promote osteoblast differentiation and to inhibit osteoclast proliferation. The same research group also investigated the Octacalcium Phosphate (OCP) as suitable substrate for alendronate local action towards bone cells. The results of this study showed that soaking OCP into alendronate solutions led to the deposition of long crystalline rod-shaped formations, most likely a calcium/alendronate complex, onto the calcium phosphate. The amount of drug loaded into the CaP material increased as a function of the bisphosphonate concentration in solution [67]. The synthesized DDS displayed a beneficial effect on osteoblast activity and differentiation and inhibition of osteoclast proliferation and differentiation. The authors reported that also OCP matrix displayed a greater stimulating effect than HA on osteoblast differentiation and AL promotion of osteoblast differentiation and mineralization was enhanced in OCP-AL with respect to HA-AL. Zhang et al. have investigated the effects of ibandronate combined with HA on the morphology and resorptive activity of osteoclasts [68]. The resorptive activity of the system was evaluated on osteoclasts cultured with ibandronate-HA; the results of this study demonstrated osteoclasts morphological alterations and a significantly lower hydrogen ion concentration with respect to the control. Oliveira et al., have incorporated sodium clodronate, in a biomimetic Calcium Phosphate (CaP) coating, previously formed on the surface of a starch-based biomaterial by a sodium silicate methodology, as a strategy to develop a site-specific drug delivery system for bone tissue regeneration applications [55]. The authors reported a stimulatory effect on osteoblastic activity, thus suggesting that these coatings can be used for regulating the equilibrium on osteoblastic/osteoclastic activity, leading to a controlled regenerative effect at the interface between the biomaterial and bone. Luo et al., reported a system where clodronate is grafted to the surface of HA scaffold by chelation [69]. The cell culture test and gene expression test were performed and evaluated the effect of clodronate modifying the HA on co-culturing osteoblast with HA scaffold in vitro. The cell culture test indicated that the cells actively proliferated on the scaffolds. The results of RT-PCR showed there was no significant difference between two groups in expression of RANKL gene, while the expression of OPG gene showed higher expression in Clodronate-HA group.

Growth Hormones (GHs), Growth Factors (GFs), Bone Morphogenetic Proteins (BMPs) and/or Mesenchymal Stem Cells (MSCs) have been also investigated in order to improve the biological properties of porous scaffolds, to induce osteogenesis by osteoinduction or angiogenesis by vascularization [70,71]. Growth factors are soluble signaling proteins
secreted by cells to induce specific biological responses such as cell survival, migration, differentiation and proliferation [70]. These proteins act by binding to cell surface receptors and after this binding can affect gene expression, alternatively, the internalized growth factor-receptor complexes can phosphorylate intracellular signal transduction proteins and regulate gene activation. The growth factors tend to diffuse only for short distances through the ECM, and may act on cells near the site of their production. Moreover, they only act on cells expressing their receptors and are subject to proteolytic degradation [72]. Thus it is necessary to use available materials which can act as substrates or carriers for these biologically active factors and allow for the controlled administration of these factors at adequate therapeutic levels, and their vectoring towards local tissue targets and cells. Bone Morphogenetic Proteins (BMPs) have been most frequently used in bone tissue engineering but since current clinical therapies often require much greater quantities of growth factor to positively impact bone formation, recombinant DNA technology allowed the synthesis of human growth factors in hosts including bacteria and mammalian cell lines [73]. Moreover, synthetic peptides able to mimic growth factor activity have been produced [74-76]. These short peptide sequences can easily be modified with chemical groups to control their presentation and are able to mimic other growth factors important for bone formation, such as analogues for Fibroblast Growth Factor (FGF) [77] and Vascular Endothelial Growth Factor (VEGF) [78]. The direct printing of brushite and hydroxyapatite bioceramics at room temperature was used by Gbureck et al., to construct model implants onto which VEGF and copper sulfate were adsorbed [79]. In this study, one macroscale Y-shaped channel within each scaffold was printed and loaded with a VEGF solution. The growth factor bioactivity \textit{in vivo} was maintained as the vascular tissue infiltrated the channel during peritoneal implantation in mice. This low-temperature direct approach offers several practical advantages and may find application in bone grafting for the delivery of combinations of drugs or growth factors. The osteoinductive capability of recombinant human bone morphogenetic protein-2 (rhBMP-2), known for its osteoinductive potential in bone tissue engineering, was evaluated in porous Ca-P cement discs loaded with this growth factor, \textit{in vitro} and implanted subcutaneously in New Zealand white rabbits. The histological analysis of retrieved specimens revealed only in rhBMP-2 loaded porous Ca-P discs evident bone formation after 10 weeks of implantation [80]. Several papers demonstrated the ability of these growth factors carriers to act as bone formation scaffolds. Zambonin et al., reported that hydroxyapatite coated with Hepatocyte Growth Factor (HGF) stimulates human osteoblasts \textit{in vitro} and further increases the bioactivity of CaPs [81]. Hossain et al., showed an enhancement of osteoblast differentiation when cultured on HGF adsorbed HA-based CaP compared to CaP without any HGF [82]. The presence of microporosity in a scaffold is undoubtedly beneficial for new bone formation. Woodard et al., showed that the presence of microporosity (2–8 μm) in HA scaffold improved osteo conductivity as compared to scaffolds without any microporosity [83]. Polak et al., showed that the presence of microporosity accelerated the healing process twice, as compared to scaffolds without any microporosity [84]. The authors showed that the addition of BMP-2 in microporous HA accelerated the healing process four times fast with respect to scaffolds without any BMP-2. Martin et al., investigated the influence of a series of growth factors on the growth and bone formation capability from Bone Marrow Stromal Cells (BMSCs) which are considered as progenitors of skeletal tissue system [85]. The authors reported that Endothelial Growth Factor (EGF) and fibroblast growth factor (FGF-2) significantly influenced the BMSCs growth. In an \textit{in vivo} mice model, they also showed that BMSCs seeded on HA induced bone formation; no bone formation was observed in the control collagen sponge seeded with BMSC. Roldan et al., investigated the effect of highly porous Biphasic Calcium Phosphate (BCP) ceramic in presence of BMP-7 combined with VEGF and Mesenchymal Stem Cells (MSCs) in an ectopic mouse model [86]. The author reported that only the BMP-7 group showed the highest new bone formation, no statistically significant differences were observed in new bone formation in the presence of BMP-7/VEGF, BMP-7/MSCs compared to
BMP-7 alone in the investigated scaffolds. The osteoinduction in the control ceramic was induced by the presence of micro- and macropores.

Although the above reported great advantages showed by CaP ceramics, compared with other biodegradable polymeric systems, these materials have some major drawbacks which are mainly related to their brittleness and their degradation/dissolution rates that happen in vivo, because of osteoclastic activity which dramatically increase the extracellular concentrations of Ca and P. Thus, polymers and polymer-ceramic hybrid composites are the principle materials investigated for the development of synthetic bone scaffolds and as drug delivery systems.

**Biodegradable Polymers**

In order to develop materials more biocompatible attention was devoted not only to inorganic scaffolds but also to organic-polymer which can show characteristics of biostability, first and in recent years also biodegradation and biocompatibility. The main goal is facing the possibility of developing temporary scaffold that can carry bioactive molecules or drugs directly on the site concerned thereby exerting their biological function. The suitable modulation of the release kinetics of these systems is critical to proper control not only of the quantity of drug released but also the release kinetics [87-92]. The intensive research on biodegradable polymers has opened up new frontiers in modern medicine, biodegradable polymers are particularly indicated where there is the need to exploit a transient material such as sutures, tissue adhesives, hemostats, scaffolds for tissue regeneration because of the hydrolytically unstable linkages in their backbone and tunable biodegradation rate, biodegradable polymers offer great potential in controlled delivery of drugs and bioactive molecules that can be covalently bound to polymers or physically entrapped inside a polymer matrix [93,94] and released as the polymer degrades in the physiological environment. Biodegradable polymers degrade into products that are normal metabolites of the body or into products that can be completely eliminated from the body with or without further metabolic transformations. The fundamental properties of a biodegradable polymer to be used as biomaterial are that its degradation products should be nontoxic. As is evident, the advantages of biodegradable polymers compared to bio-stable polymers are that once implanted they obviate the need for a second surgical procedure as well as avoid tissue rejection phenomena. Synthetic biodegradable polymers, in addition, offer the advantages that, due to their synthetic flexibility, it is possible to tune a wide spectrum of properties with excellent reproducibility; furthermore, the properly design of their structure allows to a fine-controlling of the degradation rate of these polymers and hence the desired control of the release kinetics. Biodegradation of polymers proceed via hydrolytic bulk or surface degradation [95,96]. In bulk degradation, the rate of water penetration is higher than the degradation and/or solubilization rate of surface molecules resulting in a bulk material degradation and consequently the loss of macroscopic mechanical properties of the scaffold. Polymers which undergo bulk degradation have a high hydrophilicity and may therefore be unsuitable as delivery system for drugs that are unstable in an aqueous environment. In surface degradation, that occurs particularly on polymer with a small pore size or a high hydrophobicity [97,98] the surface molecules degrade and/or solubilize faster than the water penetration rate resulting in surface erosion while bulk material can maintain its structural integrity. These materials show a more hydrophobic character, may better preserve the activity of molecules within the polymer matrix [99,100] the release kinetic is easier to control because it is proportional to the surface area of the polymer and have typically longer residence times in vivo [101-104]. On the other hand, it is known that hydrophilic surfaces are usually preferable for cells meaning that the selection of the optimal biodegradable polymer for the drug-delivery function in bone engineering scaffolds should be aimed at balancing both hydrophilic and hydrophobic aspects. The hydrolytic degradation involves the cleavage of chemical bonds in the polymer network with a constant decrease in the molecular weight chain scission can occur random, in any part of the polymer chain, or chain-end, at the end
of the polymer chain [105,106]. When the molecular weight is reduced to a sufficiently low number (5 kDa or less) polymer fragments diffuse out of the bulk or surface polymer matrix and the degradation process is so complete, the ease of chain cleavage is proportional to the degree of crystallinity of the polymer, with amorphous region more reactive than crystalline [106-108]. In practice, polymer degradation depends also by several external factors such as the micro and nano scale structure of the scaffold, (2) the polymerization of multiple polymers/polymer types and (3) the presence of hydrolytic accelerators or suppressors. For example, the in vivo degradation rate of polyesters is significantly enhanced compared with in vitro degradation rates due to the presence with optimum concentration of ester degrading enzymes such as lipases in the human body [105,109-111]. Furthermore, the design and selection of a biodegradable polymer for synthetic bone scaffold have to taking into account other key design criteria such as:

1) Degradation rate must be comparable to tissue development/ingrowth rate.

2) Biodegradation products must be biocompatible, and

3) Properly polymer process ability and functionalization (i.e., ease of copolymerization, covalent attachment of drug or biological molecules).

The following discussion reports many biodegradable, polymeric drug delivery systems for bone tissue repair. Polymer composition, hydrophobicity, crystallinity and degradability, as well as the method of drug loading, represent the main parameters to be tailored for a properly tuning of the drug release effectiveness. Polyesters, polyether-esters, and polyester-amides belong generally to the bulk-degrading polymers, while poly-anhydrides and poly-orthoesters show mainly a surface degradation behavior [99,100,112,113]. However, it is very important to keep in mind that changes to the microarchitecture and surface properties of polymer based drug-release systems or the chemical and biological characteristics of the host body may greatly affect the polymer degradation with significant changes in the release of the drug [114,115]. Additionally, if the drug delivery scaffolds results from copolymerization of two or more polymers, hydrolysis often proceeds through both bulk and surface degradation.

**Polyesters**

**I) Poly (l-lactic) acid (PLLA):** The degradable aliphatic polyesters represent the first and most widely investigated polymeric drug carriers for use in many clinical applications, including surgical sutures, pins, clips and staples [99,114,116]. The polyester poly (lactic acid) was investigated as a coating for implants releasing gentamicin and proved its validity for sustained release of this antibiotic at the minimum inhibitory concentration toward the bacteria *Staphylococcus aureus* for approximately four weeks [117]. On this basis, researchers have extended the use of these polyesters for the design of more complicated systems for tissue regeneration. Table 2 lists the most investigated polymers as carriers of therapeutic molecules for bone repair. Poly (lactic acid) is formed by ring-opening polymerization of lactide, the dimerization product of lactic acid. Two optical isomers of lactic acid exist, corresponding to l-lactide or d-lactide. Poly(l-lactic acid) (PLLA), formed from the naturally occurring isomer is a semi-crystalline polymer with a relatively high melting point (178°C) and glass-transition temperature (65°C), PLLA scaffolds show high tensile strength and extended degradation times (3-5 years) to PLLA scaffolds. Since the degradation of PLLA yields the naturally occurring stereoisomer of lactic acid, a normal intermediate of carbohydrate metabolism, this form of poly(lactic acid) is generally preferred to the poly(D-lactide) (PDLA) or poly(D, L-lactide) (PDLLA) form with the last one showing faster degradation rate than the other forms due to its amorphous structure [118,119]. Systematic investigations was focused on the effect of PLLA molecular weight on degradation rate so that to fix the optimum range appropriate for bone implants [120]. Specifically, bone morphogenetic protein was incorporated in PLLA particulates of various molecular weights and the resulting delivery system implanted in the dorsal muscles.
of mice. No bone formation was visible after three weeks with PLLA particulates of high molecular weight (greater than 3300 Da), since the slow degradation rate of these polymers restricted tissue ingrowth while tissue necrosis was observed with particulates of extremely low molecular weight (160 Da), owing to the high acidity and rapid degradation of these formulations. An intermediate PLLA molecular weight (650 Da) gave the best results with observed bone formation [120]. Successful improvement in the optimization of PLLA based drug delivery systems were obtained with the development of more advanced scaffold fabrication techniques, such as solvent-casting, gas-foaming, and emulsion freeze-drying [121]. For instance, recombinant bone morphogenetic protein-2 (rhBMP-2) was reconstituted in a collagen solution and then adsorbed to the surface of pre-fabricated PLLA disks before implantation in critical-size rat calvarial defects. After four weeks, enhanced bone formation was observed in defects treated with these delivery systems with respect to PLLA disks seeded with osteo-progenitor cells and unloaded PLLA controls [122]. Furthermore, more precise control over protein-release kinetics, leading to a lower and still therapeutic drug loadings, were achieved by incorporating the desired protein directly inside the polymer network [123,124]. For example, using an air-drying phase-inversion process, PLLA porous membranes mixed with Platelet Derived Growth Factor (PDGF) demonstrated sustained PDGF release over the course of 28 days in in vitro release experiments. Release rates could be increased through dual loading of both PDGF and Bovine Serum Albumin (BSA) into these matrices. Furthermore, bone formation within critical-size rat calvarial defects was achieved using these delivery systems within approximately two weeks after implantation, demonstrating maintenance of protein activity with this scaffold fabrication technique [123].

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Chemical structure</th>
<th>Degradation type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(lactic acid)</td>
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<td>bulk</td>
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<tr>
<td>Poly(lactic-co-glycolic acid)</td>
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<td>bulk</td>
</tr>
<tr>
<td>Poly(ε-caprolactone)</td>
<td><img src="image" alt="Poly(ε-caprolactone) structure" /></td>
<td>bulk/surface</td>
</tr>
<tr>
<td>Polyglyconate</td>
<td><img src="image" alt="Polyglyconate structure" /></td>
<td>bulk</td>
</tr>
<tr>
<td>Poly(lactic acid)-poly(ethylene glycol)</td>
<td><img src="image" alt="Poly(lactic acid)-poly(ethylene glycol) structure" /></td>
<td>bulk/surface</td>
</tr>
<tr>
<td>Poly(ethylene glycol) diacyclate</td>
<td><img src="image" alt="Poly(ethylene glycol) diacyclate structure" /></td>
<td>bulk/surface</td>
</tr>
<tr>
<td>Oligo(poly(ethylene glycol) fumarate)</td>
<td><img src="image" alt="Oligo(poly(ethylene glycol) fumarate) structure" /></td>
<td>bulk/surface</td>
</tr>
<tr>
<td>Poly(carboxyphenoxy sebacic acid) propane-</td>
<td><img src="image" alt="Poly(carboxyphenoxy sebacic acid) structure" /></td>
<td>bulk/surface</td>
</tr>
<tr>
<td>Poly(propylene fumarate)</td>
<td><img src="image" alt="Poly(propylene fumarate) structure" /></td>
<td>bulk/surface</td>
</tr>
</tbody>
</table>

Table 2: Biodegradable polyester-based polymers investigated in bone drug delivery.
II) Polyglycolic acid (PGA): Polyglycolic Acid (PGA) is the simplest linear aliphatic polyester; it is highly crystalline (45–55%), has a high melting point (220°C) and a glass transition temperature of 35°C. Furthermore, PGA shows a high modulus (7 GPa), and completely degrades in vivo within 4-6 months [125]. Like PLA, PGA has also been investigated as biodegradable polymer for bone tissue engineering applications. However, most researchers copolymerize PLA and PGA to find a tunable control over degradation rates and mechanisms for a specific application. PGA shows higher acidic and hydrophilic properties than PLA. PGA can be processed by the common fabrication techniques such as extrusion, injection, and compression molding and can be fabricated into foam and porous scaffolds. The chemical, physical and mechanical properties and the degradation rate can be affected by the type of processing technique. Degradation of PGA proceeds in two degradation steps. In the first step, water diffuses easily into the amorphous regions of matrix and gives rise to hydrolytic chain scission of the ester group in the second step of degradation only crystalline areas of the polymer remains, which are more difficult to be hydrolyzed. Although the degradation product, glycolic acid, is resorbable at high concentration, it may cause the increase of the localized acid concentration which will cause the tissue damage. PGA was also investigated for development of bone fixation device (Biofix) [126,127].

III) Polycaprolactone (PCL): Poly (ε-caprolactone) is semi-crystalline polyester with a glass transition and melting temperature of approximately -60°C and 60°C, respectively. The degradation time for PCL is similar to PLA (2 years in vivo). The crystallinity of PCL increases with the decrease of molecular weight of the material. PCL is synthesized by the ring-opening polymerization of cyclic monomer ε-caprolactone in the presence of stannous octoate, serving as a catalyst. The degradation mechanism of PCL and its copolymers are similar to PLA with random hydrolysis ester cleavage and weight loss through the diffusion of oligomeric fragments from the bulk. Because of it relatively slow degradation rate, and high modulus compared with other FDA approved biodegradable polyesters, it is well suited for orthopedic and drug delivery applications [106,128]. Additionally, PCL degradation products are easily resorbed through metabolic pathways and, as opposed to polylactides and glycolides, do not produce local acidic environments. PCL scaffolds have demonstrated enhanced osteoblast functionality in vitro and bone formation in vivo as a result of controlled delivery of calcium phosphates and growth factors [129-132]. In vitro analysis with adipose-derived stem cells showed that PCL scaffolds functionalized with laminin-derived peptide sequences was suitable to enhance cellular adhesion and proliferation [133].

IV) Polypropylene Fumarate (PPF): Poly (propylene fumarate) is synthetic, unsaturated linear polyester; the synthesis of PPF occurs by reaction of diethyl fumarate reacts with an excess of propylene glycol to produce is hydroxypropylfumarate in the presence of zinc chloride as acid catalyst. PPF achieves high mechanical strength when it is properly cross linked. Due to this property it is highly recommended in bone replacement scaffold. Additionally, the porous PPF scaffold gives the osteoconductive surface for bone ingrowth [126,134].The mechanical properties of the PPF may change with different choice of composition of the PPF. PPF is a biocompatible polymer and the main products of biodegradation, propylene glycol and fumaric acid, are biocompatible and can be easily removed from the body. PPF shows bulk degradation and the degrading time depends on the structure as well as the other factors, such as molecular weight, cross-linking agent, crosslink density, pore size and volume of scaffold, PH value of surroundings, and also the other copolymer or constituent ratio in the PPF composites [135]. PPF has the characteristic to be also injectable into the body and makes it appropriate for the orthopedic implant in minimally persistent procedures; in fact, before cross linking it is in liquid form, which makes the polymer easy to handle. PPF scaffolds doped with rhTGF-β1 were effectiveness in bone formation for the repair of rabbit cranial defects [136]. Microparticles encapsulating the therapeutic peptide TP508 have successfully been integrated into both the pores and the polymer network of PPF scaffolds for the sustained release of this molecule [137] showing fast and slow release rate for pore and network sites, respectively [138]. Thus, scaffolds
based on this polymer not only impart improved mechanical properties, but also provide a means of controlled drug delivery by changing the porosity of the polymer.

**Polyorthoesters**

Poly(orthoesters) are amorphous, hydrophobic, biodegradable polymers. The main feature of poly (orthoesters) is that the rate of degradation is pH sensitive and can be used in the development of several drug delivery systems. Poly (orthesters) is hydrophobic and can be easily dissolved in organic solvents such as chloroform, methylene chloride and dioxane. These polymer materials show slow degradation in water while they can be while they can be degraded in the presence of anhydrides, glycolic acid or lactic acid by surface erosion. The mechanical properties of poly (orthoesters) also vary over a wide range depending the starting materials with different compositions and molecular weights. The viscous ointment like consistency of the polymeric material allows the incorporation of drugs into the carrier by simple mixing at room temperature, without the use of solvents.

**Polyphosphazene**

Polyphosphazenes are one of the most versatile and rapidly developing classes of biomedical polymers [139,140]. They are typically synthesized as linear polymers, composed of an inorganic backbone with nitrogen and phosphorous atoms. Biodegradable polyphosphazenes have been developed by reacting the highly reactive phosphorus-chlorine bonds of Poly Dichloro Phosphazene (PDPP) with alkoxide, primary (or secondary) amines, and organometallic reagents [139]. Because there are numerous substituents capable of being introduced into the backbone, a broad spectrum of polyphosphazenes can be synthesized by choosing the type and ratios of appropriate side groups. When exposed to an aqueous solution, these polymers are cleaved into nontoxic, low molecular weight products such as phosphates, ammonia and the corresponding side groups. They can degrade by both surface and bulk erosion, depending on the lability of the bond and hydrophobicity of the polymer [139]. It is feasible to control the hydrolysis of polyphosphazene over hours, days, months or years by precise controlling the species of side group's substituents [141]. A few examples, synthesized for biomedical applications, are polyphosphazene-bearing amino acid ester [142,143], imidazole [142,144], glucosyl amino [145] and glycolic acid ester and lactic acid ester side groups [146]. Polyphosphazene represents an excellent class of materials for drug delivery and tissue-engineering applications [147]. A matrix of amino acid ester polyphosphazene nano fibers, with or without hydroxyapatite, has been developed obtaining an open scaffold which favors the quick proliferation of osteoblasts and the accelerated augment of bone tissue. Polyphosphazene can be processed in electro spinning, solvent casting particulate leaching, and emulsion freeze drying for the bone scaffold [148]. Polyphosphazene is suitable to blend or copolymerize with the widely used poly (lactic glycolic acid) (PLGA) in order to exploit the synergistic properties of these two polymers. The presence of polyphosphazene is useful to neutralize the acidic hydrolysis of PLGA and the rate of degradation results also delayed; at the same time the PLGA acts as mechanical reinforcement towards the polyphosphazene [149].

**Polyanhydrides**

Another class of biodegradable polymers suitable for the delivery of hydrolytically unstable molecules is often based upon more hydrophobic polymers like polyanhydrides. These polymers are synthesized by melt polycondensation. Typically a diacid monomer is reacted with excess acetic anhydride, yielding an anhydride oligomer [100]. The polymer's degradation rate depends not only from the degree of crystallinity but also from the degree of monomer hydrophobicity [99,100]. Although polyanhydrides were best to the drug delivery application but because of their surface degradation/eroding properties, polyanhydrides have a low load bearing and mechanical properties. To get the good mechanical properties polyanhydride is copolymerized with polyimide such as poly-[trimellitylimidoglycinr-co-bis
(carboxyphenoxy) hexane] and polypyrromellitylimidoalanine-co-1, 6-bis (carboxyphenoxy) hexane]. Poly(anhydride-e-imides) show a considerable enhanced mechanical properties. Poly (anhydride-co-imides) is established with succinic acid trimellitylimidoglycine and trimellitylimidoalanine reaching compressive strength in range of 50-60 Mpa. The mechanical properties of poly (anhydride-co-imide) were also investigated as scaffold for bone tissue engineering application. The osteocompatibility of these polymer materials was examined via the rat tibial modal. It was shown that untreated imperfections cured in 12 days. In comparison, the imperfections treated with poly (anhydride-co-imides) created endosteal bone growth on the 3rd day and formed the bridges of cortical development bone around the implanted matrices on the 30th day representing the osteocompatibility of matrices [150].

Another widely investigated polyanhydride is a copolymer of sebacic acid and 1,3-bis(p-carboxyphenoxy)propane, known as poly(carboxyphenoxy propane-sebacic acid) (PCPP-SA) [99,113]. The sebacic acid monomer in the copolymer network represent the short-time degradable fragment owing to the relative hydrophilicity of this monomer and high sebacic acid content allows to achieve relatively short degradation times [151,152]. Drug release from PCPP-SA and other surface-erosing polymers has been shown to directly coincide with the rate of degradation [99,100,153,154]. Polyanhydride based systems were examined for the localized delivery of antibiotics or for the controlled delivery of a number of different bioactive molecules [155]. A model drug (insulin) was entrapped within PCPP-SA microparticles synthesized by a hot melt microencapsulation procedure [156]. Insulin-incorporated microspheres injected into diabetic rats resulted in normoglycemia for a period of approximately five days [151]. Polyanhydrided based scaffolds have also been fabricated for the controlled delivery of active proteins using compression molding and results showed a positive effect on osteogenesis intramuscularly in mice [89].

In order to enhance the mechanical properties of polyanhydride scaffolds, researchers are investigating the use of dimethacrylated anhydride monomers to favor polymer crosslinking. Dimethacrylated sebacic acid and 1,3-bis(p-carboxyphenoxy)hexane were photo-crosslinked into networks with respective compressive strengths of about 34 and 39 MPa obtaining values comparable to the compressive strength of trabecular bone (5-10MPa), but far away from the strength of cortical bone (130–220 MPa) [157].

Copolymers

To gain more control over the degradation rate, hydrophobicity, degree of crystallinity and subsequent biological functionality, researchers combine two or more polymers in a chemical process called copolymerization so that to synthesize a new composite material that bring together several properties arising from each component showing desirable behaviors.

1) Poly(lactic-co-glycolic acid) (PLGA): Additional efforts to achieve further control over protein release led to the copolymerization of lactide and glycolide to form Poly(Lactic-Co-Glycolic Acid) (PLGA). Copolymerization of lactide with glycolide disrupts the crystallinity of these monomers, leading to an amorphous network with a rate of degradation and protein release dictated by the monomer ratio [116]. PLGA with a lactide-to-glycolide ratio of 7:3 is used commercially to produce surgical staples marketed as Lactomer [99]. Solvent casting/porogen-leaching techniques have been utilized to fabricate porous PLGA foams which support osteoblast and mesenchymal stem cell attachment and proliferation [158-160].

To control the release of therapeutic agents from PLGA-based materials, a number of scaffold processing techniques have been employed. Emulsion freeze-drying methods have been used to fabricate and model release of active rhBMP-2 from scaffolds of various pore sizes [161,162]. Innovative methods for encapsulating proteins within PLGA microspheres have also been developed [163,164]. The most popular of these methods, a double-emulsion-solvent-extraction technique, has been shown successfully to entrap rhBMP-2, TGF-β1, and
IGF-1 within polymeric micro-particles without significant loss in protein activity [165-167]. Protein-release kinetics from PLGA micro-particles can be further controlled by altering the loading of additional components, including Poly (Ethylene Glycol) (PEG), BSA, and gelatin [167,168]. Utilizing these and other processing techniques for the fabrication of both PLGA micro-particles and scaffolds, researchers now have the ability to create composite materials with precise release profiles [137,169]. For instance, delivery of multiple growth factors at specific rates is now possible. Slow release of Platelet-Derived Growth Factor (PDGF) (approximately 0.1 pmol/day) and fast release of vascular endothelial growth factor (VEGF) (1.7 pmol/day) was achieved using a novel scaffold design. PDGF was first encapsulated within PLGA microparticles, while VEGF was mixed PLGA particulates. The microparticles and particulates were then combined and processed into porous foams using a gas-foaming technique. Four weeks after implantation in the hind limbs of mice, these dual-release systems demonstrated enhanced vasculature when compared with systems releasing only PDGF or VEGF [169].

II) Polylactic Acid-Polyethyleneglycol (PLA-PEG): PLA<sub>y</sub>-PEG<sub>x</sub> block copolymers have been investigated as biodegradable. These copolymers consist of alternating olygomeric sequences of PLA and PEG, whose respective molecular weights <i>y</i> and <i>x</i> are controlled by the polymerization reaction conditions. The hydrophilic nature of the repeating PEG unit helps not only to neutralize the acidity of low molecular weight PLA segments but also to modulate the degradation rate [170]. When loaded with recombinant human bone morphogenetic protein (rhBMP-2), block copolymers with a 7:3 PLA-to-PEG ratio demonstrate promising bone formation. rhBMP-2 was incorporated into PL<sub>6500</sub>PEG<sub>3000</sub> devices and then implanted into the back muscles of mice. After approximately three weeks, 79% of the copolymer was degraded and bone formation with osseous trabeculae was apparent. However, BMP-2 release from implants with a higher PLA-to-PEG ratio did not result in osteoinduction owing to the low value of degraded polymer (2-6%) observed after three weeks [171]. The optimal total block copolymer molecular weight for BMP-releasing implants with a PLA-to-PEG ratio of 7:3 was found to be close to 6400 Da, allowing for complete in vivo degradation by three weeks [172]. Additional research demonstrated that the degradation rate of these block copolymers could be further tailored by introducing a random linkage of other constituents or by using innovative preparation method and as the material properties of a delivery system are improved, the minimal effective dose of a particular drug or growth factor is often substantially reduced. N-succinimidyl tartrate monoamine PEG-PLA (ST-NH-PEG<sub>x</sub>-PLA<sub>y</sub>), an amine-reactive polymer was synthesized using innovative chemical techniques [173]. The amine group of this polymer was shown to facilitate covalent attachment of model proteins in both solution (insulin and somatostatin) and the solid phase (trypsin). Similar results were found with a novel thiolreactive polymer, synthesized by attaching N-succinimidyl 3-maleinimido propionate to monoamine PEG-PLA [173]. In vitro and in vivo studies with similar protein tethering methods suggest that these methods should not alter protein activity [174,175]. Taking this technology even further, an inventive means of fabricating scaffolds with interconnected pore networks was developed through incorporation of lipid micro-particles during ST-NH-PEG<sub>x</sub>-PLA<sub>y</sub> precipitation into n-hexane. This method avoids an aqueous environment, preserving the amine group from hydrolysis, and thus permitting the covalent attachment of proteins. Upon polymer precipitation into three-dimensional structures, lipid micro-particles are subsequently extracted by melting to yield porous scaffolds [176]. Biodegradable carrier based on poly-d, l-lactic acid-p-dioxanonepolyethylene glycol block copolymer (PLA-DX-PEG) [177-179] exhibited promising degradation characteristics for BMPs release systems and new bone formation effectively and showed also enough osteogenicity to repair large bone defects [180].

Other Copolymers

Hydrogel copolymers represent an attractive carrier matrix for controlled drug delivery applications. Hydrogels are hydrophilic, three dimensional networks capable of adsorb
large amounts of water or biological fluids [181]. The 3-D network in hydrogels can be created by physical or chemical crosslinking reactions. The physical cross-linking of polymers includes entanglement and crystallites, while chemical cross-linking occurs using difunctional cross-linking agents and/or high-energy radiation [182-184]. The hydrogel-based carrier systems show good biocompatibility, easy encapsulation of the drug and fine modulation of the rate of drug release by properly design the extent of cross-linking. In addition, hydrogel based carriers can be also designed to act as pulsed drug delivery system able to releases drug following an intermittent release kinetic. Pulsed release systems can be classified into two types: intelligent pulsed delivery where the drug release depends from the occurrence of an external or internal stimulus and programmable delivery where the matrix automatically releases drug at a predetermined time [185]. In fact, the swelling behavior and hence the rate of drug release from hydrogel-based drug delivery systems can be controlled by external stimuli such as pH, ionic strength, and temperature [186,187]. Ionic hydrogels formed from polymers having acidic or basic pendant group’s exhibit variable swelling patterns depending on the pH of the environment. Thus, polymers having carboxyl or sulfonic acid groups show a greater swelling property due to ionization of the pendant groups when the pH of the environment is greater than the pK\textsubscript{a} of the polymer [181] on the other hand, polymers having basic amino group’s show higher swelling when the pH of the environment is below the pK\textsubscript{a} of the polymer. Similarly, the extent of swelling can be significantly influenced by the ionic strength of the swelling agent [181,188]. Biodegradable temperature-sensitive hydrogels based on polyesters and polyphosphazenes were also developed for application in controlled drug delivery [189,190]. Other than pH, ionic strength, and temperature, several other external stimuli such as light [191], magnetic field [192], ultrasound [193], electrical current [194] and the presence of some chemical [195] or biological molecules [196] can stimulate pulsatile release of drug from appropriate matrices.

Programmable drug delivery systems have been developed using multilayered polymeric matrices having different hydrophobicity/hydrophilicity and consequently different degradation rate. Programmable drug delivery systems regard a core-shell cylindrical structure composed of a hydrophobic coating on a cylindrical core of alternating biodegradable polyanhydride and poly-phosphazene layers. A pulsed drug release pattern can be obtained from this system based on the pH-sensitive degradation of the polymers. Further, it has been demonstrated that the lag time or duration of drug release step can be modulated by varying the nature and amount of the different biodegradable polymers in the system [190]. Toward the goal of minimizing implantation surgeries, researchers have also been developing novel injectable drug delivery systems. Many of these carriers have a repeating PEG unit within their backbone, since the hydrophilicity of PEG often imparts water solubility. Upon thermal or photoinitiated reactions, these polymers are converted from their soluble state into crosslinked hydrogels. For instance, macromers of PEG with acrylated end groups have been photo-cross-linked into hydrogels and utilized for protein delivery [197]. Likewise, macromers of PLA-PEG-PLA with acrylated end groups have been examined for osteogenic protein or cell delivery [198-200]. Another PEG-based macromer, Oligo(PEG Fumarate) (OPF) has been extensively examined in vitro [201-203] and utilized in vivo for the repair of both bone and soft tissue defects by modification with bioactive peptides [204]. The ester linkage in the backbone of this macromer facilitates hydrolytic degradation, while the double bond facilitates crosslinking through thermal initiation. Thus, OPF can be cross-linked into degradable hydrogels at physiological temperatures [205]. Furthermore, transforming growth factor (TGF-\(\beta\)) release from OPF hydrogels can be tailored by altering the swelling ratio and mesh size of these networks [206]. However, since all of these PEG-based materials form water absorbent gels, their utility will be limited to soft tissue defects and as a carrier for drugs which are highly stable in an aqueous environment. Copolymers of \(\varepsilon\)-caprolactone (that degrades in about 2 years) and dl-lactide have been synthesized in order to reduce the time of polymer degradation and so of drug release. However, when implanted
in femoral defects in rats, this material still remained after one year and appeared to retard bone formation when compared to untreated defects [207] so that they can found application to protect bone grafts from displacement and rapid resorption [208,209]. Following a similar strategy to tailor both polymer strength and degradability to tissue engineering applications, additional research groups have synthesized PGA-based copolymers. In particular, polyglyconate, a copolymer of glycolide and trimethylene carbonate, is utilized in surgical sutures, tacks, and screws. Copolymers with a 2:1 glycolide-to-trimethylene carbonate ratio demonstrate increased flexibility and faster degradation times (seven months) than pure PGA [113]. Similar trends are reported when glycolide is polymerized with both trimethylene carbonate and/or \( p \)-dioxanone [113,210].

**Hybrid Ceramic/Polymer Composites**

Next-generation materials for bone tissue regeneration should exhibit the appropriate combination of mechanical support and morphological guidance for cell proliferation and attachment while at the same time serving as matrices for sustained delivery of therapeutic drugs and/or biomolecular signals, such as growth factors. Following this objective nowadays there is a lot of research ongoing on the so-called ceramic/polymer scaffolds which should combine the tunable macro/microporosity and osteo inductive properties of ceramic material with the mechanical/physical properties of biodegradable polymers [211]. Among these, the most used polymers belong to polylactic/polyglycolic acid derived polymers, protein and carbohydrates polymers. This approach can tune the release profiles of different drugs since different polymers show different degradation rates and mechanisms. Beyond the selection of the biomaterials (polymer and ceramic), the development of composite scaffolds involves the optimization of several parameters such as the volume fraction, size and shape of the inorganic phase, porosity and establishment of a suitable bonding at the polymer-bioceramic interface in order to allow a sustained release [212]. Drugs can be loaded in a scaffold by attachment to the surface or by entrapment within the scaffold. Mainly are mainly three methods for drug loading:

1) Pre-encapsulation of the agent (using as example, micro- or nanospheres), followed by loading of the encapsulating system into the scaffold.

2) Surface immobilization of the agent by nonspecific mechanisms such as hydrophobic, electrostatic or van der Waals interactions.

3) Specific interaction, which may be introduced through the incorporation of functional groups on the molecular agent or in the biomaterial to achieve a better control of the binding as well as the incorporation of the molecular agent within the carrier [213].

An overview of the various Drug Delivery Systems ceramic/polymer composites based polymers discussed in this section is given in Table 3.

**Hybrids With Polylactic/Polyglycolic Acid Derived Polymers**

Polylactic/polyglycolic acid derived polymers have been deeply investigated and many types of hybrid composites have been proposed. This group of polymers include Polylactic Acid (PLA), polyglycolic acid (PGA), Poly-(D/L-Lactic Co-Glycolic) Acid (PLGA) and Poly-E-Caprolactone (PCL). They are highly biocompatible and their degradation properties, due to hydrolysis reactions can be tuned changing different parameters such as the molecular weight. Thus most of ceramic/polymer composites. The most used ceramic particles added to the polymer to make it osteo conductive and/or to increase mechanical properties are porous or porous or dense of structure HA, ACP and BCP [214-220]. Soriano et al., used a calcium phosphate/PLA blend for the release of gentamycin. The drug, incorporated in the polymer showed a burst release followed by a slower sustained release, typical for PLA polymers [221].
Drug release from degradable PLLA/PLGA microspheres was studied extensively and has proven to be useful for the release of proteins/drugs [222-227]. Schnieders et al., reported the controlled release of gentamycin from calcium phosphate-Poly(Lactic Acid-Co-Glycolic Acid) (PLGA) composite bone cement [228]. In this study, scaffolds containing up to 20 wt% of microspheres were loaded with gentamycin (10-30 wt%) and showed a sustained in vitro release pattern, while the release pattern from separate microspheres exhibited a typical burst release. Liu et al., developed a biodegradable scaffold for bone regeneration or tissue engineering with the capacity of controlled drug delivery [229]. Ceftazidime as a model drug was encapsulated in Ethyl Cellulose (EC) microspheres, which were incorporated in a Hydroxyapatite/Polyurethane (HA/PU) composite scaffold to generate an antibiotic drug delivery system. The incorporation of microspheres into scaffolds significantly reduced the initial burst release, and the system exhibited a sustained release of the model drug for up to 60 days. A ciprofloxacin implant formulation composed of hydroxyapatite, tricalcium phosphate, poly (DL-lactide) and 40% ciprofloxacin was characterized in vivo for use in treatment of multi-bacterial bone infection [230]. The implant, inserted in the femur of rabbits showed approximately 90% of the total ciprofloxacin released within 8 weeks, maintaining therapeutic levels in the femur and tibia. Miyai et al., developed a composite of Poly-Epsilon-Caprolactone (PCL) loaded with gatifloxacin, and a β−TCP) by a solvent-free process in which no toxic solvent was used [231]. The composite released GFLX for 4 weeks in Hanks’ balanced solution, and had sustained bactericidal activity against Streptococcus milleri and Bacteroides fragilis for at least 1 week. The composite, implanted in an osteomyelitis lesion induced by S. milleri and B. fragilis in the rabbit mandible, showed to be effective in controlling infection at the bone defect formed by debridement, and supported bone tissue reconstruction at the bone defect. Hydroxyapatite (HA) porous scaffold was coated with HA and Polycaprolactone (PCL) composites, and antibiotic drug tetracycline hydrochloride was entrapped within the coating layer. Highly porous HA scaffolds, fabricated by a polyurethane foam reticulate method, were coated with hybrid coating solution, consisting of Poly (Epsilon-Caprolactone) (PCL), HA powders, and the antibiotic vancomycin [232]. The encapsulated drug within the coated scaffold was released in a highly sustained manner as compared to the rapid

<table>
<thead>
<tr>
<th>Delivery System</th>
<th>Type of Drug</th>
<th>Drug</th>
<th>release study</th>
<th>Reference</th>
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Table 3: Examples of Investigated Drug Delivery Systems ceramic/polymer composites based.
release of drugs directly adsorbed on the pure HA scaffold. Bone tissue engineering implants with sustained local drug delivery provide an opportunity for better postoperative care for bone tumor patients because these implants offer sustained drug release at the tumor site and reduce systemic side effects. Chen et al., developed a macroporous polycaprolactone scaffold embedded with a porous matrix composed of chitosan, nano clay and β-tricalcium phosphate by freeze-drying [233]. This composite scaffold was evaluated on its ability to deliver the anticancer agent doxorubicin and to promote formation of mineralized matrix \textit{in vitro}. The scaffold without nano clay released 95\% of the drug within 4 days and can fulfill the requirements for both bone tissue engineering and local sustained release of an anticancer drug \textit{in vitro}. Son et al., investigated the inductive effect in osteogenic culture of Dexamethasone (DEX)-loaded PLGA microspheres on hydroxyapatite scaffold surfaces [234]. Release profile of DEX over a 4 week immersion study indicated an initial burst release followed by a sustained release. \textit{In vivo} evaluation of the defects filled with DEX-loaded HA scaffolds indicated enhanced volume and quality of new bone formation when compared to defects that were either unfilled or filled with HA scaffolds alone. The release of bisphosphonates from ceramic/polymer composites was investigated by Puppi et al., which developed a composite bioactive scaffolds made of biodegradable three-arm branched-star PCL, HA and the clodronate, a bisphosphonate drug that has demonstrated efficacy in the treatment of various bone diseases and as an anti-inflammatory drug PCL fibrous mats [235,236]. Wang et al., investigated the situ release of alendronate (AL) and DEX from PLGA)/HA sintered microspherical scaffold. \textit{In vitro} osteogenesis was successfully achieved with synovium-derived Mesenchymal Stem Cells (SMSCs), which intrinsically have a strong chondrogenic tendency, as indicated by high yields of Alkaline Phosphatase (ALP) and bone calcification. PLGA microsphere/calcium phosphate cement composites have been investigated also as possible carrier of growth factors [237]. \textit{In vitro} release studies with rhBMP-2 showed a sustained, small release (5-10\%) after 4 weeks in medium; the \textit{in vivo} release characteristics with low and high molecular weight PLGA microspheres showed higher release efficiencies (25–50\%). The discrepancy between \textit{in vitro} and \textit{in vivo} results could be referred to a more dynamic flow \textit{in vivo} and to the presence of high amounts of salts and proteins which positively influenced the release properties.

\textbf{Protein-Based Polymers}

Composites of CaP-ceramics with protein based polymers have been extensively investigated because of the possibility to produce organic–inorganic composites with mechanical/physical and biological characteristics, similar to human bone. Among these, collagen and gelatin \textit{have} been proposed in tissue-engineered constructs. Collagen is usually retrieved by processing different animal tissues and a lot of collagen types and forms exist. Gelatin is a processed form of collagen that is usually derived from pig-skin with an acidic process or from bovine origin with a basic process [212]. Both collagen and gelatin materials are degradable biopolymers by the proteolysis reaction of collagenase or gelat inase, respectively. Several porous gelatin/collagen scaffolds with ceramic particles have been formulated especially so-called gelatin sponges that are prepared by freeze-drying a gelatin/calcium phosphate mixture [238-240]. A different ceramic/gelatin porous scaffold was prepared by Tampieri et al., [241]. In this study a macroporous HA ceramic was soaked in gelatin solution, and a 10\% gelatin composite was formed in which the gelatin penetrated in the bulk of the ceramic. Bigi et al., introduced 18\% of gelatin inside α-TCP and studied the biological response as well as the properties of the composite [242]. The authors found an increase in compression strength due to the high density of the gelatin and an excellent biological response with an improvement of osteoblast activity and differentiation. Several authors observed an improvement in cell differentiation and proliferation using collagen or gelatin ceramic composites [243-245]. Kim et al., loaded gentamycin to HA-particle containing gelatin sponge and investigated the \textit{in vitro} drug entrapment and release as a function of crosslinking density of the gelatin, amount of HA and drug loading [246]. The authors showed that increasing the crosslinking density leads to a higher drug entrapment,
a decreasing initial drug release and a more sustained release pattern. Zhou et al., reported the controlled release of vancomycin in gelatin/beta-TCP composite scaffolds [247]. In this study, biodegradable gelatin sponges containing different contents of β-tricalcium phosphate ceramic was prepared and the results showed that the composite scaffolds could achieve local therapeutic drug levels over an extended duration. Takahashi et al., investigated gelatin sponges containing β-TCP particles that were loaded with the growth factors BMP-2 [248]. The BMP-2 loading was performed by simply dropping a solution of the growth-factor to freeze-dried sponges prepared by chemical crosslinking of the gelatin in the presence of different amounts of β-TCP. The drug release was followed in vivo by labeling the BMP-2 with 125I. Bone formation was also observed with all types of composites. Itoh et al., investigated in vivo the release of rhBMP-2 using a collagen scaffold with HA nanoparticles. A positive influence of the added drug was observed in a loaded implant model in dogs [249].

The release properties of collagen/ceramic composites were investigated by Lode et al., for the release of VEGF from a collagen containing apatite cement [250]. The authors reported a cumulative release (25%) and burst release from the composite, which was higher than that observed from the ceramic alone, thus indicating a looser binding of the VEGF to the collagen. Other than gelatin and collagen also other protein structures have been used as composite material for ceramic/polymer composites. Fibrin, and especially fibrin glue was applied for the introduction of ceramic particles or mixed together with calcium phosphate cement [251-253] Studies have been performed using TCP particles in a casein and soybean protein scaffold and polypeptides into CaP cement, because of their biodegradability, biomimetic properties and mechanical reinforcement [254,255].

**Carbohydrate Based Polymers**

Another interesting group of biodegradable polymers are carbohydrate-based biopolymers such as chitin and chitosan, a derivative of chitin, alginate, hyaluronan and cellulose. Among these, chitosan is often applied together with CaP-ceramics because of its biodegradability by lytic enzymes and the improved mechanical/physical properties of ceramic/chitosan composites [256,257]. Moreover, chitosan can be added to a CaP-ceramics as adjuvant to make the cement more injectable without substantially modifying the setting reaction [258,259]. Takechi et al., investigated the in vitro flomoxef sodium release from TTCP-cement/chitosan composite which was measured from preet disks for 3 day [260]. Results of drug release profile showed the typical profile observed in skeleton type drug delivery which was characterized by an initial burst, followed by a more sustained release. The authors reported that the addition of chitosan in different amounts did not influence total release after 72h. Similar to other polymer/ceramic composites, the opposite construction with the insertion ceramic particles in a porous chitosan composite was also formulated [261,262]. The added ceramic particles gave extra osteogenic potential and the composite materials showed enhanced osteoblast proliferation and differentiation. Macro-porous chitosan scaffolds reinforced by beta-tricalcium phosphate and calcium phosphate invert glasses were fabricated using a thermally induced phase separation technique [263]. The controlled drug release of antibiotic gentamicin-sulfate loaded on these scaffolds and morphology of osteosarcoma MG63 cells cultured on the scaffolds were studied. The authors reported an initial burst release that was diminished by addition of the ceramic particles and a total release of 90% after 3weeks. The more sustained release observed from the particle-containing composite was suggested to be due to the higher extend of chitosan crosslinking. Teng et al., explored the potential of chitosan/hydroxyapatite composites to act as a controlled drug delivery system for tetracycline hydrochloride by developing functional scaffolds with a gradient of structure and drug concentration [264]. The authors reported for the composite, a sustained, drug-release pattern without any initial drug burst. Cabañas et al., reported the fabrication of beta-tricalcium phosphate/agarose scaffolds prepared by a shaping technique that allows to obtain pieces at a temperature low enough to simultaneously include active substances susceptible of heat degrading such as the antibiotic vancomycin [265]. The drug release was also controlled by the drying procedures employed to process the obtained
scaffold, which resulted in the generation of different pore architectures and certain chemical interactions that yielded different drug release patterns. Lee et al., investigated the release of Platelet-Derived Growth Factor (PDGF) to the chitosan-TCP scaffolds and measured the in vitro release by $^{125}$I-labelled PDGF and in vivo bone regeneration in calvarial defects in rats [266]. The release test showed an initial burst, followed by a slower maintained release. Moreover, the addition of PDGF in vivo, further enhanced bone formation. Hydroxy Propyl Methylcellulose (HPMC) a carbohydrate derived from cellulose was also applied in combination with ceramic particles or as an addition to calcium phosphate cement. These composites showed poor mechanical properties but a fast in vivo dissolution, followed by replacement with newly formed bone [267-269]. Ongpipattanakul et al., prepared injectable pastes comprised of amylopectin with β-TCP granules loaded with rhTGF-β by absorbing the growth factor on the granules [270]. The in vitro results showed a release of 80% after 24h incubation in serum. When implanted in a rabbit unilateral segmental defect model a positive bone response of the added TGF-β was observed after 56 days. Hyaluronan is another carbohydrate-based polymer for tissue engineering; it is present in the human body and is proteolytically degradable by hyaluronidases and was investigated bone grafts consisting of HA granules in a hyaluronan carrier [271]. The use of alginate in inorganic–organic scaffolds for bone tissue engineering is limited due to their poor biodegradation. However, recent studies reported the development of alginate scaffolds more biodegradable by shortening the chains and by chemical modifications [272]. Moreover alginate has been added as a cohesion promoter for calcium phosphate ceramics in biological medium. Also other hydrocarbonate biopolymers show the ability to improve cement cohesion. Khairoun et al., therefore investigated the cohesion properties of a commercial HA after the addition of a wide range of these polymers such as hydroxyethyl starch, starch, sodium dextran sulphate, α/β/λ-cyclodextrins, alginic acid, hyaluronic acid and chondroitine sulphate [273]. Results for all these composites show a reduced cohesion time and small changes in setting time and mechanical properties when compared to the original cement.

Nanosized Composites

In recent years there has been growing interest in the use of nano scale structures for biomedical application. Materials converted into nano size provide unique surface properties, which are different from those of the bulk materials or single molecules and this aspect critically may influence their interaction with the biological systems [274]. Nano size biomaterials have been developed as drug and protein delivery systems, micro patterned devices, systems for biological recognition and as scaffold materials in tissue engineering [275]. Nano composites used in bone tissue regeneration for the delivery of tailored drugs have shown to offer new opportunities to provide more focused and fine-tuned treatment of diseases at a molecular level, thus enhancing the therapeutic efficacy of drugs and reducing side effects. The proper designing of these nano systems can make them capable for being independent in the normal tissue environments and selective at the diseased pharmacological site [276]. Nanosized hydroxyapatite materials have received much attention in the context of their advanced biomedical applications, including tissue engineering and drug delivery systems. Hybridization of nanosized HA with organic or inorganic molecules is a promising approach to facilitate the preparation of HA nanomaterials [277]. A controlled drug delivery nanosystem CaP-ceramic and/or biopolymer based able to attract and deliver bio-agents in a controlled manner can be achieved by the use of Magnetic Nanoparticles (MNP). These nanoparticles functionalized with bio-agents such as drugs, growth factors and stem cells act as carriers able to transport the bio-agents towards and inside the magnetic scaffold under the effect of a magnetic field, thus improving the bone regeneration processes [278-285]. Moreover there are increasing evidence shows that magnetic fields alone and magnetic responsive scaffolds can play unique roles in promoting bone repair and regeneration. highlighting the synergistic effects of magnetic scaffolds in response to external magnetic fields on the bone regeneration in situ. [286]. Bock et al., developed a simple and inexpensive
method to transform standard commercial scaffolds made of hydroxyapatite and collagen, currently used for bone graft substitution, in magnetic scaffolds by dip-coating the scaffolds in aqueous ferro fluids containing iron oxide nanoparticles coated with various biopolymers [278]. The obtained magnetization values have shown to be suitable for generating magnetic gradients, enabling magnetic guiding in the vicinity and inside the scaffold. Moreover, biological studies indicated the ability of the magnetic scaffolds to support adhesion and proliferation of human bone marrow stem cells in vitro. The same research group developed new porous HA ceramic composites incorporating magnetite at three different ratios [279]. The authors have evaluated in vitro the ability of this magnetic hydroxyapatite scaffold to enhance tissue regeneration using human osteoblast-like cells cultures. Results indicate high biocompatibility, similar to a commercially available HA bone graft, with no negative effects arising from the presence of magnetite or by the use of a static magnetic field. The implantation of the nano-composite HA/Fe$_3$O$_4$ 90/10 in vivo in a critical size lesion of rabbit condyle shown good level of histocompatibility, thus opening new perspectives for the application of a magnetic field in a clinical setting of bone replacement, either for magnetic scaffold fixation or magnetic drug delivery. Tampieri et al., proposed a bioinspired mineralization process to develop biomimetic hybrid scaffolds made of (Fe$^{2+}$/Fe$^{3+}$)-doped hydroxyapatite nanocrystals nucleated on self-assembling collagen fibers and endowed with super-paramagnetic properties in order to minimize the formation of potentially cytotoxic magnetic phases such as magnetite or other iron oxide phases [280]. In vitro biological studies, performed by seeding human osteoblast-like cells on magnetic and nonmagnetic materials indicated the biocompatibility of the materials moreover, the magnetization of the super-paramagnetic scaffolds, induced applying an external static magnetic field have shown to improve cell proliferation in comparison to the nonmagnetic scaffold. Magnetite nanoparticles have been also used for the preparation of polymer based nanocomposites for biomedical applications [281]. Singh et al., reported magnetic nanofibrous scaffolds of poly(caprolactone) incorporating magnetic nanoparticles and their effects on physico-chemical, mechanical and biological properties for bone regeneration purpose [287]. The nanocomposites exhibited magnetic behaviors, which increased gradually with MNP content. The in vitro evaluation of apatite forming ability in simulated body fluid confirmed the substantial improvement gained by the addition of MNPs. The in vitro biological studies on osteoblastic cells reported an improved initial cell adhesion and subsequent penetration through the nanofibers, compared to pure PCL. The same nanocomposite, subcutaneously implanted in rats exhibited minimal adverse tissue reactions, while inducing substantial neoblood vessel formation, which was greatly limited in pure PCL. In a similar study, Meng et al., reported a nanofibrous composite scaffold composed of super-paramagnetic γ-Fe$_2$O$_3$ nanoparticles, hydroxyapatite nanoparticles and poly lactide acid, prepared using electrospinning technique [284]. The scaffolds, which well responded to extern static magnetic field, were implanted in white rabbit model of lumbar transverse defects. The authors reported that MNP incorporated in the nanofibers endows the scaffolds super-paramagnetic responsive under the applied static magnetic field, which accelerates new bone tissue formation and remodeling in the rabbit defect. In a recent study De Santis et al., reported 3D fully biodegradable and magnetic nanocomposite scaffolds for bone tissue engineering, consisting of a poly (ɛ-caprolactone) (PCL) matrix reinforced with iron-doped hydroxyapatite (FeHA) nanoparticles for bone regeneration; the scaffolds were designed and manufactured using a rapid prototyping technique [288]. The in vitro results showed that the cell growth in the magnetized scaffolds was 2.2-fold greater than that in non-magnetized ones. In vivo experiments further suggested that, after only 4 weeks, the PCL/FeHA scaffolds were completely filled with newly formed bone, proving a good level of histocompatibility. In the search of new biocompatible nano materials for tissue engineering applications, functionalized Carbon Nanotubes (CNT) have shown to be promising for casting bone scaffolds because of its high mechanical strength [289]. The idea to inject the CNT into a bone fracture for supporting the growth of new tissues and to heal the fracture has been examined by Haddon’s group [290,291]. This research group functionalized Single Walled
Carbon Nanotubes (SWCNT) with phosphonates and poly (aminobenzene sulfonic acid) in the solution phase and as films on substrates. Microscopy studies showed that hydroxyapatite nucleated and crystallized on the surface of the functionalized SWCNT, the negatively charged functional groups on SWCNT attracted the calcium cations and lead to self-assembly of HA. The thickness of the HA layers was found to be a function of the mineralization time [290]. In a successive work, the same research group evaluated the ability of SWCNT and MWCNT with different functional groups on their surface to control cell growth in osteosarcoma ROS 17/2.8 cells [291]. The authors found that CNT carrying neutral electric charge sustained the highest cell growth and production of plate-shaped crystals. The results of these studies demonstrated that the presence of CNT in the scaffold improves the cell adhesion, which is crucial for cell growth, proliferation, differentiation and migration within the scaffold. Liao et al., demonstrated another method of nucleation and growth of hydroxyapatite by using MWCNT [292].

These evidences prompted search for developing strategies and studying the mechanism of bio-mineralization with CNT in order to obtain advanced nano composites to be used for hard tissue repair and replacement. Jell et al., reported CNT-reinforced porous polyurethane nanocomposite scaffold for osteoblast growth and mineralization [293]. The synthesized nanocomposites did not cause any cytotoxicity on SaOS-2 osteoblast cells; moreover, an increased level of production of vascular endothelial growth facto angiogenic factor, in proportion to the CNT loading in the scaffold was registered. Marrs et al., have investigated the mechanical properties of the MWCNT (0-10 wt%) incorporated in polymethyl methacrylate, a common polymer material for bone cement and dental prostheses [294]. The authors found that the incorporation of MWCNT favorably alters the static and fatigue mechanical properties of the polymer offering thermal benefits and improving the longevity of the implants; this effect can be attributed to the high thermal conductivity of the carbon nanotubes. In vivo studies have also been performed to explore the effect of CNTs on bone formation and the bone-tissue compatibility. Usui et al., implanted MWCNT particles into mouse skull sub-periosteum and tibial bones and found that the MWCNT did not cause any major inflammatory reaction [295]. Moreover the incorporation of rhBMP-2 with MWCNT, implanted into the bone can accelerate the new bone formation. Wang et al., also demonstrated the biocompatibility of CNT for bone regeneration [296]. MWCNT-polycarbosilane composites instilled into rat femur leads to only an insignificant inflammation in 4 weeks, and the newly formed bone is remodeled around the nanotubes. In a similar study MWCNT-chitosan composite scaffolds, adsorbed with rhBMP-2 and implanted in a muscle tissue were investigated for their ability to induce ectopic formation of bone tissue [297].

In order to combine the ability of magnetic nanoparticles and carbon nanotubes to promote bone formation, Cunha et al., reported the synthesis of specifically tailored Fe$_3$O$_4$/carboxylated MWCNT and evaluated in vitro their biocompatibility and their potential for tissue engineering applications [283]. These magnetic hybrid composites were analysed in vitro by incubation with mesenchymal stem cells for 1, 3 and 7 days, either in the presence or absence of a static magnetic field. The results of this study demonstrated that the introduction of magnetite into the MWCNT structure increases biocompatibility of oxidized MWCNTs. In addition, the presence of a static magnetic field further increases Fe$_3$O$_4$/MWCNT influence on cell behaviour. In a successive work the same research group investigated magnetic hydroxyapatite-based nano materials as bone-specific systems for controlled drug delivery [282]. The synthesized hydroxyapatite, decorated with magnetite nanoparticles by a deposition method (HA/Fe$_3$O$_4$) and the nanocomposite system obtained using magnetic MWCNT as filler for HA (HA/MWCNT/Fe$_3$O$_4$), have been investigated for their biological behavior and also doped with clodronate in order to evaluate the drug releasing properties of the nanosystems. The preosteoclastic RAW264.7 cell proliferation by MTT assay confirmed the high biocompatibility of the three systems. The clodronate-doped systems were able to release the drug in vitro and the analysis of TRAP staining of RAW 264.7 conditioned with
sRAKL to induce osteoclastogenesis, showed an higher reduction of the osteoclast formation, compared to the parent ones without clodronate, the osteoclast inhibition activity for the magnetic hydroxyapatite clodronate-doped system has shown to be comparable with that exerted by clodronate alone. Recently, graphene-based materials have been explored for wound healing, stem cell engineering, regenerative medicine and tissue engineering [298]. This nanomaterial has excellent mechanical properties and various functionalities can be inserted on its flat surfaces. Thus, it can be potentially used as a reinforcement material in hydrogels, biodegradable films, electrospun fibers and other tissue engineering scaffolds leading to the development of scaffolds with properties fine-tuned for target organ/tissues. Graphene-reinforced chitosan films have shown enhanced mechanical properties and no toxicity when tested on murine fibrosarcoma L929 cell culture [299]. Also Graphene Oxide (GO) chitosan hydrogel scaffolds prepared by covalent conjugation of chitosan amino groups with the carboxylic groups of graphene showed a significant improvement in cell adhesion, differentiation, proliferation and calcium phosphate deposition by mouse pre-osteoblast MC3T3-E1 cells [300]. Lu et al., investigated graphene-based composite materials for wound healing by preparing chitosan–PVA nanofibrous scaffolds containing graphene [301]. In this study three materials have been investigated: chitosan–PVA–graphene, electrospun fibers and chitosan–PVA fibers without graphene and control in order to assess the wound healing potential of graphene-based composite in mice and rabbit. The samples containing graphene healed completely and at a faster rate as compared to others in both mice and rabbit. Besides wound healing, a number of studies have been performed exploring the use of graphene for stem cell engineering and musculoskeletal tissue engineering [302-304]. 3D composite scaffolds were also fabricated using gelatin methacrylate and GO [305]. The synthesized hydrogels showed enhanced mechanical and electrical properties with no adverse effect on encapsulated fibroblast cells. In addition to its mechanical and electrical properties, the graphene functionalization with protein/peptides is expected to be very useful for tissue engineering applications and several micro- and nano fabrication approaches can be employed to achieve spatial patterning of cells and/or proteins [306,307].

Conclusions

As discussed in this chapter, many materials have been developed for the delivery of bioactive agents for tissue regeneration. The research in this field has been firstly focused extensively on developing CaP-based ceramics such as hydroxyapatite, endowed of osteoconductive and bioactive properties. Despite their positive biological properties, their poor mechanical durability related to their brittleness and their degradation/dissolution rates in vivo, led the search for new biodegradable polymeric scaffolds for the controlled localized delivery of bioactive agents. Polymer composition, hydrophobicity, crystallinity, and degradability can positively affect the rate of drug release as well as the rate of tissue ingrowth. Next-generation materials for bone tissue engineering should combine the tunable macro/microporosity and osteoinductive properties of ceramic material with the mechanical/physical properties of biodegradable polymers. Following this objective a lot of research is now focused on hybrid ceramic/polymer scaffolds, as matrices for the sustained delivery of therapeutic drugs and/or biomolecular signals, such as growth factors. In recent years there has been growing interest in the use of nanoscale structures for biomedical application. Materials converted into nano size provide unique surface properties which critically may influence their interaction with the biological systems. Nano composites used in bone tissue regeneration for the delivery of tailored drugs or other bioactive agents have shown to offer new opportunities to provide more focused and fine-tuned treatment of diseases at a molecular level, thus enhancing the therapeutic efficacy of drugs and reducing side effects. By optimizing the interplay between the material used as drug delivery system and the bioactive components, the field of tissue engineering will undoubtedly revolutionize the treatment of bone degenerative disorders. With improved experimental animal models, able to mimic not only the human defect but the human response, controlled delivery
systems will certainly provide efficient methods to clinically regenerate bone in the future. Researchers now have the material and processing tools to build more effective materials for drug delivery; the close collaboration among chemists, engineers and biologists will allow the development of highly efficient release systems and to study how the kinetics of bioactive agents release from these systems can affect bone repair.

References


Introduction

The existence of stromal cells with progenitor characteristics in bone marrow was first hypothesized by Cohnheim in the 19th century. In the early 20th century, Maximow elucidated the importance of stromal cells for support of hematopoietic tissue in bone marrow [1]. The foundation for the research field of mesenchymal stromal cells or stem cells was then laid by Friedenstein and his group in the 1960’s and 1970’s [2,3]. Their early experiments regarding bone marrow transplantation and the expansion of bone marrow stromal cells on plastic, led to the discovery of a fibroblast-like cell population. These cells were highly proliferative, formed colonies and exhibited the potential to mature into different cell types of mesenchymal origin, which earned them the name of Mesenchymal Stem Cells (MSCs). With the field of mesenchymal stem cell research taking off in the late 1980’s and the early 1990’s, scientists made significant observations that highlighted the importance of adult mesenchymal stem cells for regenerative medicine, including the existence of mesenchymal stem and progenitor cells in most adult tissues [4-6]. Various projects arising from these initial observations refined isolation methods and characterization of these cells and ultimately lead to their translation into clinical therapies. In the 1990s, MSC’s were introduced into clinical trials for bone marrow transplantations and future treatments for immunological diseases [7-11]. The possibility to isolate autologous stem cells from tissues held the promise for personalized regenerative therapies without both ethical concerns associated with embryonic stem cells and concerns of histocompatibility associated with allogeneic stem cell transplantation. Within three decades, the literature on mesenchymal stromal cells or stem cells increased to over 15,000 original publications with the majority aiming to establish MSCs as a viable therapeutic strategy to overcome a wide range of diseases. Although scientists around the world have made immense strides in adult stem therapy research, the treatment has yet to make its way into everyday clinical practice. Initially hypothesized as a universal tool to regenerating damaged tissues, autologous adult stem cells have not yet been proven as effective in practice. Moreover, a large number of promising results in vitro and in animal models did not translate into a significant therapeutic result in clinical studies [12,13]. Nevertheless, certain characteristics of stromal cells, especially their damage response secretome, have marked them as a potentially powerful tool to enhance the body’s own regenerative capacity [13]. Recent clinical data from various medical institutions have
demonstrated that autologous, and in some cases allogeneic transplantation of adult stem cells into chronically inflamed sites, or chronic wounds enhanced regeneration not by differentiation into the target tissue, but by modulating the immune response and activating and recruiting tissue resident progenitors [14]. Due to the ease of access and availability, adult stem cells from adipose tissue play an increasingly dominant role in clinically applied stem cell therapy. This chapter will therefore focus on this source and will characterize the regenerative capacity of adult stem cells. Further, the chapter highlights the current state of clinical studies and provides an outlook to what we can expect from adult stem cell therapy in these upcoming years.

**Characteristics of Mesenchymal Stem Cells**

**Isolation and characterization**

As mentioned in the previous section, adult mesenchymal stem cells can be derived from various mesenchymal tissues such as the umbilical cord, bone marrow and adipose tissue [15-17]. Most histological analyses of these tissues have revealed a perivascular location of MSCs [18-23]. The most abundant and easily accessible tissue for the isolation of MSCs is adipose tissue. Regardless of tissue type, the primary isolate from these tissues are called stromal cells or Stromal Vascular Fraction (SVF). The primary cell isolate consists of a heterogeneous cell population that varies in its composition from tissue to tissue and donor to donor [4,14].

![Figure 1: Isolation method for MSCs from adipose tissue, umbilical cord and bone marrow.](image)

While MSCs from Bone Marrow (BM-MSCs) are purified by differential culture expansion of bone marrow aspirate, the MSCs of Umbilical Cord (U-MSCs) and Adipose Tissue (A-MSCs) are isolated by enzymatic and mechanical tissue processing with subsequent cell culture expansion of the plastic adherent cell population (Figure 1) [2,4,14,24,25]. The SVF of bone marrow aspirate, umbilical cord and adipose tissue vary significantly in their composition of cell types and percentages of MSCs present [15,17]. In bone marrow aspirate, the predominant cell type are hematopoietic progenitors as well as naïve and mature lymphocytes [2,3]. The SVF of adipose tissue and umbilical cord consists mainly
of mesenchymal stromal/stem cells (MSCs), hematopoietic and endothelial progenitors, as well as endothelial cells [15,16,23]. Even after purification of MSCs through cell culture, some phenotypical differences remain. Independent from the origin of the primary cell isolate, hematopoietic markers like CD4, CD8 and CD45 are lost during the early passages of cell culture expansion due to loss of the hematopoietic population [24,26-28]. The main difference between uncultured SVF derived from umbilical and adipose tissue and bone marrow is the lack in expression of CD34, a marker of hematopoietic and vasculature associated progenitor cells, in bone marrow derived cell preparations [29-31]. All MSCs have in common that, once cultured on plastic, they up regulate the expression of surface proteins such as CD44, CD90, CD105 and CD73 [32] (Table 1).

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Table 1: Important cell surface markers expressed in the cells of the SVF, the bone marrow nucleated cell (BM – NC, primary bone marrow aspirate), A-MSCs, BM-MSCs. ++>70%, +=30-70%, ±=2-30%, -=<2%. Also displayed are the colony forming units (CFU-F).

MSCs from umbilical cord and adipose tissue additionally loose the expression of CD34, which in part can be explained by their removal from the perivascular niche [33,34]. Cultured BM-MSCs may be differentiated from A-MSCs by the lack of expression of CD36 [25,35]. Since these changes in cell surface antigen expression proved to be consistent and reproducible, the use of flow cytometry has become an integral part in the characterization and identification of MSCs. Nevertheless, it is important to note that, despite the homogenous phenotype of MSCs based on cell surface proteins, the cells still maintain a heterogeneous functional phenotype that is highly dependent on cell culture technique [36]. In addition to characterizing MSCs by cell surface marker expression, functional assays are applied. The International Federation for Adipose Therapeutics and Science (IFATs) and the International Society for Cellular Therapy (ISCT) committees published a joint guideline in 2013 that summarizes current methods of characterizing A-MSCs and highlights the differences between the SVF, ASCs and Bone Marrow derived Mesenchymal Stem Cells (BM-MSCs) [26]. These minimal requirements need to be present to define cells as MSCs and entail the expression of previously mentioned cell surface molecules as well as the potential to differentiate into chondrocytes, osteocytes, adipocytes and the ability to form colonies in culture. The differentiation potential of MSCs beyond the aforementioned tri-lineage potential remains one of their most controversial characteristics and will be discussed in the next section. The guidelines that functionally characterize MSCs do not provide any prediction regarding their therapeutic potential. As discussed in the following sections, one of the most important therapeutic effects MSCs exhibit is their paracrine character. The secretion of cyto and chemokines is highly variable and mechanisms of release are not yet well understood, which is why to date prior to therapeutic application of MSCs clinicians and scientists still depend on the minimal requirements for characterization published by IFATs and ISCT.

**Differentiation Potential**

MSCs are progenitor cells that exhibit the potential to form a wide range of mature mesenchymal cell types. Their primary function in situ is the maintenance of tissue integrity and compensation of cell turnover, suggested by their abundant location in all tissue types
of the body [1,4,21,25]. When these cells are isolated from their natural perivascular niche and cultured ex vivo, they can be subjected to conditions that promote differentiation into specific cell lineages. While ontogenesis, adipogenesis and chondrogenesis are well accepted characteristics of BM-MSCs, A-MSCs and U-MSCs in vitro and in vivo, their capacity for giving rise to other mesenchymal tissues or showing plasticity for non-mesenchymal cell types beyond in vitro models is highly disputed (Figure 2) [14,26]. Most in vitro differentiation protocols incorporate a complex multi-stage sequence of growth factors, cytokines and small molecules that are difficult to reproduce and do not reflect the physiological environment of MSCs in their perivascular niche [18,21,23]. Therefore, it has to be assumed that the in vitro differentiation capacity of MSCs does not predict their in vivo differentiation capacity. One complicating factor when characterizing the differentiation capacity and plasticity of MSCs is the heterogeneity of the isolated cell population. Research of the past 20 years has aimed at identifying a small multi or pluripotent subpopulation within the various MSC isolates. To date, there is still conflicting data regarding the existence of such a pluripotent subpopulation [36-38]. Furthermore, for therapeutic translation of stem cell transplantation, it is important to keep in mind that the chances for success heavily depend on a minimally manipulative process for isolation, identification, maintenance and transplantation of MSCs. To assist in their in vivo differentiation capacity different matrices for MSCs are used to promote in vivo differentiation into target tissues, such as bone or cartilage [39-43]. Also, in aesthetic and reconstructive surgery the potential of MSCs, especially from adipose tissue, has been demonstrated. The supplementation of lipografts with SVF or A-MSCs supports the formation of architectural integer tissues, which can be linked to the engraftment and differentiation of MSCs into stromal tissue [44-46]. Most studies looking at the bioavailability of MSCs after transplantation show that only a small percentage of cells can be found within the first week of transplantation. When injected intravenously, most cells will get trapped in the first capillary bed, i.e. the liver and the lung without the formation of ectopic mesenchymal tissues in these organs [47-49]. The therapeutic effect seen after systemic administration of MSCs is more likely due to their paracrine potential [50].

Paracrine function of mesenchymal stem cells

When MSCs were first discovered in the bone marrow, they were believed to solely replenish the mature mesenchymal cells the bone marrow [1-3]. Studies investigating their interactions with hematopoietic progenitor (HPCs) and mature immune cells then uncovered their regulatory function in protecting the bone marrow from inflammatory insults and
supporting the differentiation process of HPCs [14,51]. Similarly, the paracrine potential of MSCs was observed in other tissues, like the adipose tissue [52]. Their perivascular location makes them ideal responders to inflammation and tissue damage which in turn helps to orchestrate wound healing processes. Early MSC transplantation studies only showed minimal engraftment and differentiation effects, while an increasing number of recent studies have begun exploring on the paracrine function of MSCs. In vitro and in vivo studies impressively demonstrated a plethora of mechanisms by which MSCs from bone marrow, adipose tissue, and umbilical cord modulated the response of mature immune cells to pro-inflammatory cues [53-58]. It became clear that MSCs are modulators of immune and injury response through secretion of cytokines and trophic factors [Figure 3] [14,59-61]. This highly differentiated response mechanism of MSCs makes them an ideal biological drug for treatment of complex inflammatory disease patterns.

![Figure 3: Mechanism of action for SVF and MSCs. Recent studies comparing SVF to BM-MSCs and A-ASCs have demonstrated the greater secretory capacity of SVF.](image)

The mechanisms that allow MSCs to modulate the phenotype of mature immune cells were mainly uncovered by trans-well culture experiments of MSCs with cells of the native and adaptive immune system, as well as hematopoietic progenitor cells [62]. The results of these studies demonstrated that MSCs mainly interact with immune cells by secreting cytokines such as IL-6, IDO, PGE2, GM-CSF and TGF-beta2 [62] (Figure 4). In an inflammatory environment, MSCs act as immune suppressors by interacting with naïve T-cells, monocytes, dendritic cells and natural killer cells. In macrophages, MSCs are capable of inhibiting the switch of Macrophages to the pro-inflammatory M1-phenotype and support the switch to the anti-inflammatory M2-phenotype [60,63-65].
Figure 4: Schematic of immune modulatory potential of MSCs. This diagram depicts the reported cytokines that are released by MSCs in a pro-inflammatory environment and whether they inhibit (red arrow) or activate (green) the response of the immune cell. The activated or differentiated forms of the immune cells are depicted as diamonds. The general response of the immune cell in a pro-inflammatory setting is listed next to the cell type. NO=nitric oxide, TNF=tumor necrosis factor, IL=interleukin, GM-CSF=granulocyte macrophage colony stimulating factor, TGF=transforming growth factor, PGE2=prostaglandin E2, DC=dendritic cell. INF=interferon. Modified and updated based on Glenn et al. and Hoogduijn [60, 65].

The so called polarization of macrophages towards the M2-phenotype via secretion of PGE2 reduces tissue inflammation and promotes wound healing. Additionally, MSCs have been shown to suppress the respiratory burst and apoptosis of neutrophils via secretion of IL-6 while leaving their phagocytosis, chemotaxis and matrix adhesion intact [66,67]. This differential modulation of neutrophil regulation further promotes tissue repair. MSCs also secrete Indoleamine-2,3-dioxygenase (IDO) and are capable of reducing the sensitivity of natural killer cells to MHC-molecules, depending on activation stage and duration of exposure to inflammatory cues [68]. While the mechanisms by which MSCs modulate the immune response of T-cells is still under current investigation, the published data consistently demonstrates the effect of MSCs on activation, differentiation and proliferation of T-cells [69-71]. The heterogeneity of proposed mechanisms might be due to the different sources of MSCs that were used and due to the difference in time points used for modulating the T-cell response. The presence and the lack of pro-inflammatory cues and damage signals also seems to have an effect on the interaction of MSCs with T-cells [65]. In vitro data consistently showed that MSCs effectively suppress the proliferation of T-cells by secretion of IDO in an inflammatory environment [65,69-71]. In non-inflamed tissues MSCs may also act as pro-inflammatory agents by secreting IL-7 and therefore promoting the differentiation and proliferation of T-cells [72,73]. A field of current research is the effect of MSCs on differentiation of naïve T-cells towards Th1, Th2, Th17 and regulatory T-cells (Treg) [74-76]. Current studies show that MSCs activate regulatory Treg-cells immune suppression [74,77-80]. MSCs themselves do not activate the adaptive immune system, since they do not express MHC-class II molecules [26]. This characteristic of MSCs makes them also ideal candidates for allogeneic stem cell transplantation.

In addition to their immune modulatory potential, MSCs are also capable of
secreting trophic factors that promote tissue regeneration [14,52]. The exact underlying mechanisms are less understood than the immune modulatory capacity. *In vivo* data suggests the secretion of angiogenic cytokines, like VEGF and a correlation between the magnitude of VEGF levels post transplantation and angiogenesis was observed [14,81]. The published *in vivo* data favors A-MSCs over BM-MSCs for their angiogenic potential, even though *in vitro* data is less conclusive in this regard. As mentioned above, the heterogeneity of the results can be contributed to cell culture artifacts. Particularly when using adipose derived SVF, the presence of endothelial progenitors does suggest implantation and differentiation into mature endothelial cells and capillaries in addition to the paracrine effect of MSCs [26]. This explains the superiority of SVF in regards to the angiogenic potential when compared to A-MSCs and BM-MSCs. In addition to cyto and chemokines, microRNAs and exosomes have been also hypothesized to play a role in the trophic potential of MSCs. The exact mechanisms are subject of current investigations [65].

**Clinical Use of Mesenchymal Stem Cells**

The following section will highlight the current use of MSCs in clinical trials and the mechanism by which MSCs may alleviate clinical symptoms or even cure certain conditions (Figure 5). As the field of MSC-based therapies is still expanding and relatively new, only representative clinical studies of the most important disciplines will be highlighted in this chapter. As of today, approximately 500 clinical studies are registered world-wide using MSC-based therapies. Particularly in China, the number of studies has significantly grown in the past 10 years to surpass the overall numbers of clinical trials being conducted in the U.S. or in Europe (Figure 6). For more information on current and completed trials please visit www.clinicaltrials.gov.

![Figure 5: Clinical applications of MSCs.](image)
Plastic and Reconstructive Surgery

The field of plastic and reconstructive surgery, especially for cosmetic interventions, has expanded significantly. Due to the revenue created by these interventions and the drive to find minimally invasive and natural techniques to aesthetically improve and rejuvenate the outer appearance of customers, MSCs are increasingly applied in a variety of procedures.

A clear benefit of MSC use in reconstructive surgery can be found when combined with fat grafting (Table 2) [44-46,65]. Plastic surgeons have applied this technique since the early 20th century to compensate for tissue loss due to aging, trauma or disease [46]. Generally, fat is harvested from subcutaneous locations in the abdominal or hip region and mechanically processed before injection. The most common fat grafting applications are breast augmentation or reconstruction, as well as correction of facial deformities (e.g. pectus excavatum, hemifacial microsomia and lipoatrophy) and age related facial rejuvenation [82,83]. The main challenge with fat grafts is their survival and engraftment over time, which is due to volume loss of the graft [45]. It is hypothesized that the harvesting technique of fat grafts (lipoaspiration) yields only insufficient numbers of A-MSCs that would promote the vascularization of the grafts and allow the formation of long lasting stromal tissue [34,84]. Based on this assumption and considering the advancements of clinically approved aseptic techniques for isolation of adipose derived SVF, plastic surgeons have started to enrich fat grafts with freshly isolated autologous SVF, called Cell-Assisted Lipotransfer (CAL) [85]. Multiple animal and pre-clinical human trials have shown that fat graft survival can be significantly increased using this technique which has not been shown to be associated with any major side effects [44-46,85]. Fat grafting is a useful approach for women that underwent mastectomy in course of their breast cancer treatment have benefited from this procedure for breast reconstruction, with reported high satisfaction rates after one year of follow-up [45]. Concerns yielding from animal experiments regarding the transfer of undetected micro-tumors and associated concerns related to recurrence of breast cancer are not supported by any clinical reports yet [86]. Nevertheless, due to the novelty of this approach, there are no long-term follow-up study results available to evaluate this possibility. Table 2
<table>
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Table 2: Important clinical studies. This is a selection of the most important clinical studies performed in the last 15 years. Only studies with published study results were selected. For active studies please visit [link to clinicaltrials.gov].

**Orthopedics**

In the upcoming years, regenerative medicine will take an increasingly important role in orthopedics. With an increasingly aging society, the incidence of trauma and/or degeneration of the skeletal system are rising and with it the need for alternatives to prosthetic replacements and minimal invasive interventions. While the functionality and durability of prosthetic replacements of joints and limbs have increased, they still pose a burden to patients. Especially in elderly patients, who suffer from degenerative osteoarthritis and cannot sustain major surgical interventions, regenerative cell based therapies are a viable alternative. Even in young patients, implantation of artificial joints poses the problem of potential revisions later in life, despite the improved durability of prosthetics. In both traumatic and degenerative injuries to the joints, the limited wound healing capacity of articular cartilages causes the biggest challenge to successfully restoring full functionality and alleviating painful scar formation [82]. The self-regenerative capacity of cartilage is limited due to the lack of vascularization, slow cellular turnover and low metabolic rate of chondrocytes [87]. Surgical interventions aimed at treating these defects through abrasion, micro fracturing or cell-based therapies, such as autologous culture-expanded chondrocytes had only limited success [87]. The chronic inflammation that is associated with damaged and degenerated cartilage is one of the therapeutic targets for MSC-based therapies. As described above, the paracrine factors secreted by MSCs may result in recruitment and activation of resident progenitors and modulate wound healing by limiting damaging
inflammation. Additionally, the sustained reduction in inflammation in both the damaged joint as well as in the osteoarthritic joint after MSC injection also leads to a significant reduction in pain and discomfort [88] (Table 2). In clinical studies, both BM-MSCs as well as A-MSCs have been shown to be effective in reducing symptomatic osteoarthritis in patients that are resistant to traditional therapy such as anti-inflammatory drugs and surgery [88-92]. Beyond the use in osteoarthritis and traumatic cartilage injuries, MSCs have been used for critical size bone defects and craniofacial reconstruction surgery [82,93,94]. As to whether A-MSCs or BM-MSCs are more beneficial in above mentioned applications, the published data has been inconsistent [87,95-98]. Most studies rely on in vitro and in vivo data, which may favor one over the other, depending on donor population, culture condition, and isolation method. Regarding safety considerations, the use of either cell preparation is equal, but with A-MSCs being more accessible and available in larger quantities than B-MSCs [14]. Future large cohort studies may elucidate efficacy of both A-MSCs and BM-MSCs in the field of orthopedics.

Diseases of the Immune System

Due to their immune modulatory capacity, the potential therapeutic role of MSCs has been investigated in various animal models and in small clinical trials. In vitro, in vivo and clinical results regarding the application of MSCs have been promising, specifically in Crohn’s Disease (CD), Multiple Sclerosis (MS) [99], Systemic Lupus Erythematosus (SLE) [100-102], Graft-Versus-Host Disease (GvHD) [56,62,103-105] and type I Diabetes Mellitus (DMI) [106] (Table 2). GvHD may occur as a complication after allogeneic hematopoietic stem cell transplantation and is associated with a high mortality and morbidity. While corticosteroid therapy remains the gold standard for first line therapy, a subset of patients does not respond to medical therapy. Due to their immune suppressive potential BM-MSCs and later on also A-MSCs were evaluated for their potential in reversing or at least alleviate the symptoms of GvHD [103,104,107,108]. The results of these studies demonstrated that both autologous and HLA-mismatched donor systemic administration of MSCs were effective to reverse the disease in some patients. It must be noted though that due to a small study population, the data might not be representative for all GvHD cases [56]. What is more, the failure to meet the study endpoint of an industry initiated phase III clinical study initiated the discussion whether this form of treatment is a viable alternative to the traditional regimen [109]. The failure of this study could be accounted to the lack of understanding for the pharmacokinetics of MSCs and it underscores the importance for further evaluating the timing for injection as well as further optimization of the treatment regimen. Larger clinical studies using BM-MSCS or A-MSCs are currently underway and may further elucidate the efficacy of MSCs in GvHD.

In the field of auto-immune disease, Crohn’s disease was among the first to be evaluated for MSCs therapies in human patients [110-114]. Crohn’s disease is a systemic disease that manifests within the gastrointestinal tract with recurring fistulas. The disease drastically affects the quality of life and may entail multiple surgical interventions. MSCs have been evaluated for both systemic and local administration clearly favoring local administration of MSCs for fistula repairs [112]. The mechanism of action in this treatment was linked to the increase in regulatory T-cells (T<sub>reg</sub> cells) by MSCs [111,112]. Clinical data suggests that the combination of surgical fistula repair with administration of MSCs at the resection site promises a lasting therapeutic effect, while improving patients’ quality of life for [113,114]. Similar to the mechanism of action in Crohn’s disease, there is some limited clinical data that suggests that injection of allogeneic MSCs in SLE may also cause remission by increasing the number of circulating T<sub>reg</sub>-cells [100-102].

Altogether, MSCs show promise in becoming a supplemental tool for managing complex autoimmune disease. However, optimal dosing, timing and involved mechanisms of action remain unclear. Larger clinical trials are needed to confirm existing preliminary results.
Cardiovascular Disease

Cardiovascular Disease is the leading cause of death in the United States and in the world [115]. Advancements in pharmaceutical, surgical and minimal invasive interventions make it possible to slow progression of cardiovascular disease. Additionally, preventive measurements have proven to be successful in shifting the onset of cardiovascular disease towards later decades of life. For regeneration of the damaged heart, MSCs seem to be the most promising treatment to successfully augmenting the limited regenerative capacity of the adult heart [116]. The pathophysiological changes in the cardiovascular system that occur after an ischemic insult of the heart tissue can be linked to the hemodynamic changes that occur due to decreased contractile function of the myocardium due to scar formation. Interestingly, when observing the regeneration of cardiac tissue in infants and newborns, the regenerative capacity of the myocardium appears to be still intact [117,118]. The loss in numbers of cardiac progenitors and the massive inflammatory response after myocardial ischemia in adult hearts have been made responsible for the formation of large fibrous scars [118,119]. MSC-based therapies are believed to express the capacity of promoting the formation of a more functional scar. Contrary to the initial hypothesis of injected MSCs, replenishing the cardiac progenitor pool, close to no lasting engraftment of MSCs was observed. Regardless, reduction in scar formation, revascularization, and improvement of contractility was observed in pre-clinical large animal studies as well as in first clinical studies [120-124] (Table 2). Hence, the therapeutic effect of MSCs in cardiac regeneration was linked to the paracrine secretion of pro-angiogenic and anti-inflammatory factors into the freshly infarcted myocardium [125]. In vivo animal data demonstrated a reduction of T-cell infiltration, decrease in border zone apoptosis of cardiomyocytes and increase de novo capillary formation explaining the cardio protective effect of MSCs [124,126]. Similarly, the injection in older scars (older than 4 weeks) was able to induce an immune reaction and recruitment of resident progenitors to restructure the already formed scar and to promote the differentiation of cardio-myocytes and capillarization of the scar [127]. Data from various clinical studies such as the APOLLO (NCT00442806) [128], ADVANCE (NCT01216995) [129], PRECISE (NCT00426868) [127], PROMETHEUS (NCT00587990) [121] and POSEIDON (NCT01087996) [130] trials has been less conclusive on the clinical outcomes after MSC-injection [116,121,127,131,132]. While safety investigations regarding intra-myocardial MSC injections have demonstrated no adverse effects, one of the most important efficacy parameters, the left ventricular ejection fraction, did not improve significantly. On the other hand, MRI-images did show a reduction in fibrous scar formation and other hemodynamic parameters that coincided with an improvement of the clinical presentation of the patients [133]. The heterogeneity of results and the lower-than-expected performance of MSCs in these trials might in part be explained by the different isolation and culture methods applied to the MSCs. Additionally, in case of autologous use of MSCs, the co-morbidities of the stem cell donor may have an impact on clinical outcome as well. In the cases of allogeneic transplantations of MSCs it still remains to be investigated whether histocompatibility does play a role in the performance of MSCs. It is not clear whether MSCs start expressing MHC molecules following engraftment and are then rejected by the host’s immune system.

Future Directions

The field of MSC therapeutics has expanded rapidly in these past ten years and the continued commercialization of MSC therapeutics has been one of the main drivers for translating the scientific discoveries into the clinic. The experience we gained from clinical trials underscores that MSC will play an important role in future regenerative therapeutics. The greatest challenges that may hinder the advancement of MSC therapeutics beyond the clinical trial phase is the heterogeneity of study outcomes. The greatest obstacles are gaps of knowledge regarding the biology of MSCs, their mechanism of action, and clinical application. Recent developments like the formulation of xeno-free defined culture media, have contributed to a reduction of the amount of cell culture artifacts introduced by the
use of fetal bovine serum [110]. The use of xeno-free media for MSC expansion has the potential to reduce donor to donor variation in response to MSC treatments. The second aspect of MSC therapeutics is their pharmacokinetics. The published data consolidates the role of MSCs as a biological agent with its own pharmacokinetic profile [50]. Future studies will have to further investigate the modes and kinetics of cytokine release by MSCs to improve standardization of dosage, frequency and timing of MSCs transplantation and injection. Future projects will also need to be directed towards a better understanding of the mechanism by which stem cell function is impaired in disease and age. In an aging society, the number of potential beneficiaries of autologous stem cell preparations with multiple co-morbidities and ages beyond the 6th decade will increase. In vitro studies have already shown that age may have an impact on the secretome, functionality and viability of MSCs [134,135]. Identifying clinical markers that could predict the potential therapeutic outcome may help to select patients who would actually benefit from MSC-based therapeutics. Furthermore, results from a recent study aiming at reversal of age related defect in adult stem cells could also improve the performance of autologous MSC therapies in elderly patients [136]. In this study, BM-MSCs were transfected with myosin D and telomerase and successfully rejuvenated with the result of improved therapeutic outcome in mice. Hence, understanding the mechanisms that lead to declining functionality of MSCs with age reversing this process could hold open the path to autologous stem cell therapy for all patient collectives. In recent years, MSCs derived from umbilical cord gained importance in the field of stem cell therapeutics. Their origin from fetal tissue, lack of extensive exposure to exogenous noxae and compared to A-MSCs and BM-MSCs increased proliferative capacity makes them interesting candidates for therapeutic applications [137-139]. Due to the novelty of this field we recommend to closely observe upcoming clinical studies that involve U-MSCs. The outcome of these studies will show whether the results from pre-clinical studies using U-MSCs can be confirmed by clinical studies.

To summarize, MSCs will be a powerful tool for various clinical applications and uncovering their therapeutic mechanisms will allow physicians to design personalized therapeutic regiments for their patients. However, to increase predictability of regenerative cell therapies future studies will have to focus further on the biology of MSCs in situ and their behavior ex vivo.

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Resident Stem Cells Stimulation: New Promise for Tissue Regeneration?

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Glossary

PSC: Pluripotent Stem Cells
ESC: Embryonic Stem Cells
MSC: Multipotent Stem Cells
iPSC: Induced Pluripotent Stem Cells
ECM: Extracellular Matrix
MSC-CM: Multipotent Stem Cell-Conditioned Medium
MSC-MVs: Multipotent Stem Cell-Derived Microvesicles
MV: Microvesicle
Oct4: Octamer-Binding Transcription Factor 4
Sox-2: SRY (Sex Determining Region Y)-Box 2
Klf4: Kruppel-Like Factor 4
c-Myc: avian myelocytomatosis virus oncogene cellular homolog
IFN-γ: Interferon Gamma
VPA: Pluripotency-Related, Valproic Acid
LIF: Leukemia Inhibitory Factor
PTN: Pleiotrophin
SR1: StemRegenin 1
FGF: Fibroblast Growth Factors
BMP: Bone Morphogenetic Protein
TGFβ: Transforming Growth Factor Beta

Abstract

Stem cells are a population of undifferentiated cells characterized by the ability to extensively proliferate and to differentiate into different cell types. As demonstrated by the great number of clinical trials developed, stem cells show anti-apoptotic, immune-
modulatory, angiogenic and chemo attractant properties, which are required to promote tissue repair. In the last decades the transplant of stem cells or their differentiated derivatives has represented the central strategy for regenerative medicine applications. Despite the significant advances in stem cell biology, several issues have limited their application in cell-based therapy, including the ethical controversies and the risk of tumor formation associated with embryonic stem cells, or the possible rejection following adult stem cell transplant. For these reasons, researchers are focusing on the development of stem cell-free therapeutic approaches to manipulate the tissue microenvironment and provide resident stem cells with the physical, chemical and biological cues needed to facilitate a functional tissue restoration. Current approaches count with the in situ administration of MSC derivatives (including conditioned medium or micro vesicles), the application of small molecules, and the development of biomimetic biomaterials that are less complex than cells. Such approaches offer the advantage to restore tissue functionality exploiting the patient’s own machinery, without the need for biopsy, ex vivo cell expansion and manipulation, or autologous/heterologous transplantation.

**Keywords:** Conditioned Medium; Extracellular Matrix; Regenerative Medicine; Stem Cells; Microvesicles; IPSC; Stem Cell Niche; Tissue Repair; Small Molecules;

**Stem cells**

Stem cells are defined as a population of immature, non-specialized cells able to self-renew and proliferate. When exposed to specific stimuli stem cells are capable of differentiating into several specialized cellular lineages [1,2] and represent the building blocks of tissue and organs. Stem cells have been identified and isolated from all tissues in the body and can be classified based on their ability to differentiate, which is tightly dependent on the tissue they are derived from [3]. They can be categorized into totipotent, pluripotent, and multipotent cells. Totipotent cells are so called because of their ability to generate an entire organism, including the placenta’s trophoblasts [4,5]. This type of cells is only represented by the zygote. Pluripotent Stem Cells (PSC), are able to generate the entire organism (endoderm, ectoderm, and mesoderm lineages) but not to produce placenta and the supporting tissues [6,7]. Pluripotent cells are derived from the inner cell mass of the blastocyst and are called embryonic stem cells (ESC, Figure 1A). Adult tissues include a population of multipotent stem cells (MSC, Figure 1B), which are able to produce a limited number of cell lines, mainly those associated to the mesoderm lineage (i.e. adipocytes, osteoblasts and chondrocytes) [8]. Ethical issues associated to the isolation of ESC, which involves the destruction of the embryo, as well as their tumorigenic potential, limit the use of these cells for clinical applications.

![Figure 1](image.png)

**Figure 1:** Cell morphology. Images represent mouse embryonic stem cells (ESC, A) and mouse bone marrow-derived mesenchymal stem cells (BM-MSC, B). ESC form 3D clusters, whereas BM-MSC display a fibroblastic-like morphology and grow in monolayer. 10x magnification, scale bars: 10 μm.
To overcome these limitations regarding the use of embryos in research and clinics, in 2006, cells with ESC-associated features were derived from terminally differentiated cells by using a combination of factors, including Oct4, Sox-2, Klf4, and c-Myc. Cells obtained through this approach were named induced Pluripotent Stem Cells (iPSC). Since then, a number of different reprogramming factors have been identified [9-16] and significant advancements in methods/techniques employed to deliver these factors have been developed (Table 1). Since their discovery, iPSC have been used in many research and clinical studies including disease modeling, drug discovery and cytotoxicity trials and regenerative medicine [3]. The relevance of iPSC is mainly due to their potential to mimic the human cellular microenvironment and metabolism, thus overcoming the use of animals as disease models, which is limited by the existing variability in their genetic make-up compared with human individuals [17]. Moreover, the opportunity to obtain a patient specific iPSC represents an enormous advantage in the development of specific therapeutic regimens.

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<tr>
<td>Non-integrating methods</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PiggyBAC</td>
<td>Oct-4, Sox2, Klf4, c-Myc</td>
<td>Fibroblast</td>
<td>0.01</td>
<td>[25]</td>
</tr>
<tr>
<td>Poly-arginine tagged polypeptide</td>
<td>Oct-4, Sox2, Klf4, c-Myc</td>
<td>Neonatal fibroblast</td>
<td>0.00</td>
<td>[26]</td>
</tr>
<tr>
<td>RNA modified synthetic mRNA</td>
<td>Oct-4, Sox2, Klf4, c-Myc</td>
<td>Human fibroblast</td>
<td>4.40</td>
<td>[27]</td>
</tr>
</tbody>
</table>

Table 1: Integrating and non-integrating methods developed to generate iPSC.

**Stem Cell Niche**

The dynamics of progenitor cells within a tissue is tissue-dependent. Indeed, the environment the cells are embedded in highly affects their fate [28]. The stem cells niche represents a specialized microenvironment, which regulates cell behavior, maintaining a balance between quiescence, self-renewal and differentiation. The proper interaction between stem cells and their niche is essential to prevent stem cell exhaustion [29] and to protect them from the accumulation of gene mutations that is thought to be the cause of their malignant transformation into cancer cells [30]. Since the original definition proposed by Schofield in 1976 for the hematopoietic microenvironment, the concept of the niche has become more and more complex [31]. Niches show a defined anatomical localization and are composed by undifferentiated as well as supportive stromal cells, tightly interacting each other with the surrounding extra cellular matrix (ECM) through cell surface receptors, gap junctions and soluble factors [32,33]. Also inflammatory and other circulating cells are often found within a niche as they are recruited into the niche in response to specific stimuli [34]. Secreted factors produced by cells normally activate signaling cascades and gradients and physical cues, including shear stress, oxygen tension and temperature, thus controlling or affecting stem cell behavior [35,36]. Figure 2 is a schematic representation of the dynamic interaction occurring within a tissue between cell components, secreted factors, the extracellular matrix, physical cues and metabolism-associated cues within the niche.
Stem Cells and Regenerative Medicine

The first attempt to use stem cells for regenerative medicine purposes in vivo was performed in 1950s, laying the foundations for the current bone marrow transplantation as a therapy to face blood disorders [37]. Time after time it became evident that bone marrow includes a subset of cells that are able to support tissue regeneration [38]. Since then, adult MSC have been identified, isolated and exploited as therapeutic tools to regenerate tissues in various disorders, including but not limited to Parkinson’s, Alzheimer’s, cardiovascular disease, and disorders affecting muscles, lung, liver and other organs [39-44]. Although with a more limited differentiation capability compared to their embryonic counterparts, adult stem cells show anti-apoptotic, immune-modulatory, angiogenic and chemo attractant properties required to promote tissue repair [45,46]. Moreover, MSC offer the advantage of being autologously transplanted, without raising issues of rejection or ethical controversies. As demonstrated by clinical trials (clinicaltrials.gov), MSC transplantation in humans is considered to be safe. No pro-tumorigenic effect of MSC and their derivatives has been yet observed in human clinical studies with a follow-up period ranging from 1 month to 6.8 years [47,48].

Toward The Achievement of Endogenous Tissue Repair

In the last decade, several clinical trials involving the use of multipotent stem cells have been developed for regenerative purposes (clinicaltrials.gov). However, although many mechanisms by which MSC contribute to tissue regeneration have been highlighted, much remains to be understood. The regenerative mechanisms ascribed to MSC can be classified into 3 categories and include:

**CELLULAR COMPONENTS**
- Tissue-specific cells
- Blood cells
- MSC
- Immune cells
- Fibroblasts
- Integrins
- Fibronectin/Collagen
- Basal membrane

**SECRETED FACTORS**
- Hormones
- Chemokines
- Chemokines receptors
- Exosomes
- Cytokines
- Growth factors receptors
- Calcium receptor
- Oxygen
- Lipids

**EXTRACELLULAR MATRIX**
- Ca2+

**HYPOXIA AND METABOLISM**

**Figure 2:** Schematic representation showing the bidirectional dynamic interactions between resident stem cells and their niche. Stem cell niche is a complex environment where structural, physical, chemical and cellular components communicate, through specific factors acting as mediators.
i) Differentiating toward reparative or replacement cell types

ii) Enhancing the nutrient supply and

iii) Improving the survival and function of the endogenous cells via paracrine actions. Although engraftment of the transplanted MSC has been documented in some cases [49-51] because of the inhospiteable microenvironment of the injured or degenerating tissues, a large proportion of the implanted MSC may die or undergo apoptosis in a short period post-transplantation [52,53].

Only a small percentage of the injected MSC engrafts successfully in various models of myocardial infarction and acute kidney injury [54,55], suggesting that paracrine-mediated signals must be implicated in endogenous cells activation [56] and tissue regeneration [56-58].

**Paracrine-Mediated Mechanisms**

Paracrine mechanisms have been proposed to be responsible for mediated communication between transplanted MSC and tissue-resident cells, where one MSC release bio-chemical mediators to its immediate environment, resulting in a change in the behavior of endogenous cells in their adjacent environment. Understanding how paracrine signaling is involved in the repair of damaged tissue could be important to the development of new regenerative therapies. Numerous suggestions as to how the paracrine effects observed in many different model tissue repair systems are mediated have been proposed. It has been proposed that paracrine effects contribute to the improvement of a tissue function following tissue insult and injury by modifying factors such as inflammation, fibrosis, apoptosis, neovascularization, and repair [59]. Researchers have demonstrated such effects to be mediated by molecules secreted by MSC, thus suggesting the MSC-Conditioned Medium (MSC-CM) as a critical player rather than MSC themselves [59-61]. Recently, we demonstrated that MSC-CM have the potential to improve endometrial cell replenishment when low proliferation is associated to pregnancy failure in vitro [62] and to be sufficient to stimulate the structural and functional regeneration of cardiac [54,63], renal [61,64,65], tendon [66], spinal cord [67] tissues in vivo. These evidences denote that the beneficial effects exerted by MSC in facilitating regeneration can rather be attributed to the activation of resident cells [68]. These mechanisms may be accounted for bioactive soluble factors (lipids, gases, growth factors and cytokines) known to inhibit apoptosis and fibrosis, enhance angiogenesis, stimulate mitosis and/or differentiation of tissue-resident progenitor cells and modulate the immune response [69]. Specifically, transplanted MSC have been reported to induce:

The up-regulation of anti-apoptotic and anti-inflammatory cytokines and growth factors (i.e. hepatocyte growth factor, vascular endothelial growth factor, insulin-like growth factor-1, interleukin-10,basic fibroblast growth factor,tumor growth factor alpha and B-cell lymphoma-2 and

The down-regulation of pro-inflammatory mediators (i.e.interleukin-1β, tumor necrosis factor-α, interferon-γ and inducible nitric oxide synthase.

**iPSC as Potential Alternative to MSC-Based Therapy**

In regenerative medicine, the use of iPSC has been proposed as an alternative to MSC-based therapy to repair injured or degenerated tissues and, as mentioned above, several attempts to treat a number of disorders have been performed. So far, conditions treated include hematopoietic disorders, musculoskeletal injury, spinal cord injury, liver damage, cardiovascular diseases, retinal pigmentosa and age-related macular degeneration. The successful application of iPSC in regenerative medicine relies in their ability to integrate with existing cells in the tissue and guarantee the appropriate balance in function between the number of engrafted and resident cells and tissue-specific geometrical arrangement. In the
case of iPSC-based treatments, however, unaddressed concerns over safety still remain, not least because of iPSC potential to generate teratomas. For this reason, to be transplanted in vivo, patient-specific iPSC must be differentiated in vitro [22] but the immature nature of cells derived in this way has been generally recognized, thus representing a limitation in their clinical applicability. This is the case of degenerative diseases, such as Parkinson’s and Alzheimer’s disease that require the transplanted cells to be sufficiently mature to replace the lost cells of similar type to ensure proper function. Similarly, cell maturity is also critical for the treatment of diseases where the transplanted cells need to correct lost secretory function, such as pancreatic islet β cells, hepatocytes or hematopoietic cells. Other limitations associated with iPSC exist, including the risk for the viral systems used in generating such cells to get incorporated with the host genome, causing genetic aberration.

**Alternative Approaches**

Based on the recent findings, however, many groups are working at the development of stem cell-free therapeutic approaches. Targeting the stem cell microenvironment, modulating individual or multiple components of the niche, represents an alternative strategy to induce endogenous repair within the injured tissue. This goal can be achieved through systems able to recapitulate the niche and/or to provide resident cells with the physical, chemical and biological cues needed to induce a functional restoration. The main advantage of this strategy is to allow the tissue to be regenerated exploiting the patient’s own cells, without the need for biopsy, ex vivo cell expansion, manipulation and transplantation [33]. Such approaches include:

- The administration of MSC-derived microvesicles (MSC-MVs),
- The application of small molecules and
- The development of biomimetic materials (Figure 3).

![Figure 3: Alternative approaches to stimulate the stem cell niche. Administration of MSC- microvesicles, small molecules or biomimetic materials to exploit the own patient’s machinery toward a functional regeneration of tissue, by inducing resident stem cell recruitment, proliferation, and differentiation.](image-url)
Stem Cells Derived Microvesicles

Stem cell-released vesicles are becoming a valid alternative therapeutic approach to cell-based therapies in regenerative medicine. Clinical studies have been already initiated to treat graft-versus-host disease with MSC-derived extracellular vesicles, as they are easily and safely administered for the treatment of autoimmune and inflammatory diseases and possibly for tissue regeneration [70]. The term vesicles include exosomes and Microvesicles (MVs) that can be differentiated by their size and content [71]. Exosomes are defined as extracellular vesicles ranging from 40 to 100 nm in size, whereas MVs are those with a larger size (100-1000nm). Although exosomes and microvesicles have been reported to be structurally and morphologically distinct, they are both composed by a phospholipid bilayer and retain the surface properties of the cells they are released by, including the presence of phosphatidylserine and expression of a specific antigenic profile. MVs cargo include specific proteins, mRNAs and microRNAs of their parent cells. It is widely recognized MVs are responsible for cell-to-cell communication, by transferring such cargo to target neighboring and distant cells [68,72]. The therapeutic effects of MSC-MVs have been actively investigated in animal models of various diseases [48,73-76] demonstrating both, the biological effect to repair tissue damage and immune-modulatory properties. A schematic representation of the MV-mediated exchange of information between injured cells, resident stem cells and tissue-specific cells favoring tissue regeneration is shown in Figure 4. One example is represented by the induction of peripheral tolerance through the arrest of T cell differentiation exerted by adipose-tissue derived MVs and the consequent reduction of IFN-y levels. Other biological functions include the stimulation of angiogenesis and resident cell proliferation and differentiation, the resolution of inflammation and the induction of coagulation.

![Figure 4](Figure4.png)

Figure 4: Schematic representation of the cross-talk between tissue-injured cells and resident stem cells through Microvesicles (MVs). Following an injury, cells secrete factors able to stimulate stem cells recruited from the circulation or resident stem cells to release MVs containing the information (miRNA and mRNA) needed to acquire tissue-specific features, or to produce soluble paracrine mediators, thus favoring tissue regeneration.

Clinical trials using MSC-MV have been already registered, primarily in the field of cancer and diagnostics and therapeutics (clinicaltrials.gov). For regenerative medicine applications, however, several factors still need to be considered, such as the type of effector cargo molecule (proteins, mRNA and miRNA) and the ability of that specific molecule to localize into the vesicles. Moreover, the precise mechanisms regarding the interaction of MSC-MVs and the injured tissues, the homing and biodistribution of MVs need further investigation.
Furthermore, being MVs cargo and function determined by the cell of origin, differences in the MSC-source of election may provide a novel approach for stem cell selection in the regeneration of specific tissues.

Administration of Small Molecules

Although their use is still in an early stage, regenerative medicine research is also focusing on small molecules as novel cell therapy approaches for augmentation of endogenous cells for tissue regeneration, facilitating the generation of target cells for cell therapy, improving the interactions between cells and the ECM and enhancing endogenous stem cell function for tissue regeneration [77]. Specifically, interest in their use is increasing due to a better understanding of signaling pathways that control stem cell fate, both in vitro and in vivo, and the development of technologies for high-throughput screening [78,79].

The advantages in the use of small molecules over other approaches rely on their potential to:

i) Be more convenient to use

ii) Provide a higher degree of temporal (rapid and reversible effects) and spatial (effects confined to different cell or tissue compartments) control over protein function and

iii) Be tuned by varying their concentrations and combinations.

Screenings of small molecules to modulate specific targets or stem cell phenotypes have led to the generation/validation of efficient compounds for enhancing cell-based therapy, facilitating the development of therapeutic drugs to spatially and temporally target endogenous progenitor cells to treat degenerative diseases and injuries [80,81]. Small molecules also represent a cell replacement-based therapy to expand [82] and differentiate embryonic and adult stem cells into a desired cell type before their transplantation in vivo, as opposite to their direct injection, which can lead to the limitations reported above.

Small molecules have been used to produce sufficient quantities of functionally normal cells, to make them immunologically tolerable to the recipient, remaining free of contaminants that could harm the individual. For example, specific small molecules derived from stem cells, including metabolites of cellular essential nutrients, have been applied to control, enhance, inhibit specific signaling, or molecular mechanisms, through their ability to modify proteins or act as receptor ligands or cofactors [83]. Table 2 summarizes some of the most used small molecules to facilitate stem cell maintenance and modulate their key developmental pathways in vitro.

<table>
<thead>
<tr>
<th>Small Molecules</th>
<th>Function</th>
<th>Species</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pluripotin</td>
<td>Long-term self-renewal</td>
<td>Mouse</td>
<td>[84]</td>
</tr>
<tr>
<td>PD0325901</td>
<td>MEK pathway inhibition</td>
<td>Mouse</td>
<td>[85]</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>GSK3 pathway inhibition</td>
<td>Mouse</td>
<td>[85]</td>
</tr>
<tr>
<td>PD0325901/CHIR99021 and LIF</td>
<td>Long-term self-renewal</td>
<td>Mouse, Rat</td>
<td>[12,86,87]</td>
</tr>
<tr>
<td>Y27632</td>
<td>ROCK pathway inhibition, dissociated cells survival</td>
<td>Human</td>
<td>[88]</td>
</tr>
<tr>
<td>Tzv, Ptn</td>
<td>Adherent and dissociated cell survival</td>
<td>Human</td>
<td>[89]</td>
</tr>
<tr>
<td>StemRegenin1 (SR1)</td>
<td>Long-term self-renewal</td>
<td>Human</td>
<td>[82]</td>
</tr>
<tr>
<td>SB421542</td>
<td>TGF-β type 1 receptors (ALK4, ALK5 and ALK7) pathway inhibition</td>
<td>Human</td>
<td>[90]</td>
</tr>
</tbody>
</table>
### Table 2: List of small molecules used to support stem cell renewal or to induce differentiation.

<table>
<thead>
<tr>
<th>Small Molecules</th>
<th>Function</th>
<th>Species</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt, FGF, Hedgehog, Notch, and BMP/TGFβ</td>
<td>Neural induction</td>
<td>Human</td>
<td>[80,91,92]</td>
</tr>
<tr>
<td>Noggin, SB431542, LDN193189, CHIR99021, SUS402, DAPT, LDN193189, SB431542</td>
<td>Endoderm differentiation</td>
<td>Human</td>
<td>[93]</td>
</tr>
<tr>
<td>IDE</td>
<td>Cardiac specification</td>
<td>Human</td>
<td>[94,95]</td>
</tr>
<tr>
<td>Wnt, KY02111 and CHIR99021, CHIR99021 and IWR-1endo, CHIR99021 and IWP</td>
<td>Neurogenic differentiation maintenance</td>
<td>Human</td>
<td>[90]</td>
</tr>
<tr>
<td>SB421542</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Development of Biomimetic Materials

The development of biomimetic materials for tissue healing is widely described in this book. It is clear that the ideal biomaterial for tissue engineering approaches should be able to support tissue regeneration exploiting the self-healing capabilities of the patient. The most current relevant approach to achieve these goals is biomimicry, which aims at designing materials able to closely resemble the target tissue. Although this approach is very promising, biomaterials for tissue-engineering applications have to reproduce the complex mechanisms of living systems. As mentioned above, inside a tissue, cells of different phenotypes are interconnected by a complex network of macromolecules comprising of proteins and polysaccharides secreted by the cells themselves. This natural environment refers to the ECM and has the role to structurally and functionally organize the overall tissue. Within the ECM gradients of growth factors play key role in both tissue regeneration and developmental biology, as they provide a mechanism by which cells can obtain spatial and directional cues. Thus, another crucial role of the ECM is regulating and integrating different and consecutive key processes during the wound healing: hemostasis, inflammation, proliferation and remodeling of the injured tissue. Several biomaterials mimicking the chemical, physical and topographical cues of the target tissue have been developed so far and have been lately engineered with nanostructured delivery systems to precisely reproduce the biochemical gradients occurring in tissues and control over the platform for endogenous tissue-specific and progenitor cells growth. To this end and with the goal of achieving a successful and functional restoration of the tissue, a biomaterial should not only provide the main building blocks for the formation of new tissue and be totally resorbable, but also activate and recruit body’s own progenitor cells, promoting their proliferation and differentiation. Moreover, “smart” biomaterials actively direct cell behavior and activity surrounding the implant, thereby encouraging more desirable interactions soon after the surgery. Since biomaterial implantation is usually accompanied by the injury provoked by the surgical procedure, the immune-modulatory ability of the implant to correctly regulate the immune system is crucial in determining the successful outcome.

### References


Abstract

Magnetic scaffolds have recently attracted significant attention in tissue engineering, due to the prospect of improving bone tissue formation by conveying soluble factors such as growth factors, hormones and polypeptides directly to the site of implantation, as well as to the possibility of improving implant fixation and stability. In particular, increasing evidence shows that the synergistic effect of magnetic fields and magnetic responsive scaffolds can play unique roles in promoting bone repair and regeneration. In this chapter we review the recent breakthrough innovations in the field of bone tissue engineering which exploit the possibility to enhance bone cells adhesion and proliferation as well as affect bone tissue growth by means of magnetic biomaterials combined with static magnetic fields.

Introduction

The repair of critical size bone and osteochondral defects caused by degenerative diseases, prosthetic implant revisions, tumor excisions and traumas still pones serious challenges in the orthopedic field [1]. During normal healing process, undifferentiated Mesenchymal Stem Cells (MSCs) in combination with growth factors and regulatory cytokines are recruited to the defect site and eventually differentiate into bone cells such as osteoblasts and osteoclasts leading to new bone matrix deposition, endochondral ossification up to woven bone and finally lamellar bone formation [2]. However, when the size of the defect exceeds a critical value, which depends on a variety of factors including bone type, segment and animal species, defects fail to heal and a surgery intervention is required [3].

Current orthopaedic practice for repairing of critical-sized defects is the implantation of autologous bone grafts or allografts, used in approximately 2.2million orthopedic procedures annually [4]. Although autografts still represent the gold standard for bone defect repair, their usage is significantly thwarted by donor-site morbidity and limited supply [5]. Besides, limitations of using allografts include immunogenic response by the host to the foreign tissue of the graft and the potential for disease transmission [6]. A common cause for graft failure is the lack of rapid and stable vascularization [7].

Due to the above-mentioned issues, several alternative solutions have been proposed in the latest decades, spanning from ceramic or polymeric scaffolds, seeded cells to the...
use of soluble factors such as Vascular Endothelial Growth Factor (VEGF) and Bone Morphogenetic Protein (BMP) [8]. Exploiting the fact that bone is a mechano-sensitive material, mechanical force stimuli have been successfully used to promote bone regeneration [9]. Besides mechanical stimuli, magnetic stimulation originated from Static Magnetic Field (SMF) has been also explored for many years [10]. Inspired by the magnetic stimulation effects, magnetic biomaterials are attracting increasing research interests for bone tissue engineering applications. In particular, Superparamagnetic Nanoparticles (MNPs) with size below 20 nm, demonstrated high potential for \textit{in vivo} applications thanks to the possibility of not retaining any residual magnetization upon removal of the magnetic field and thus to avoid MNPs aggregation after withdrawing the magnetic field, according to an on/off mechanism [11]. Whereas the possibility to guide MNPs and drug-carriers by using external magnets has been attracting significant attention in the medical field thanks to the wide range of suitable applications, from the anti-tumoral hyperthermia effect to contrast agent for MRI and magnetic drug delivery [12,13], the attractive application of MNPs in tissue regeneration by incorporating them into scaffolds has been only recently investigated. These superparamagnetic scaffolds may be activated through the application of an external magnetic field indeed the application of a SMF to the system induces a high magnetic gradient field that causes displacement of the particles along the gradient vector with the production of compression and tensile forces on the cell membrane resulting in cytoskeleton deformation and cell dragging [14]. Mechanical forces transmitted to the cytoskeleton by membrane receptors such as integrins are envisaged to activate a number of intracellular signaling pathways including changes in intracellular calcium levels and Mitogen-Activated Protein Kinases (MAPK) activity that replicate the effect of mechanical loading and regulate osteocyte and osteoblast function eventually leading to the development and normal function of bone tissue [15]. Figure 1

![Figure 1: Sketch of the most investigated magnetic scaffolds for bone tissue engineering.](image)

**Bio-Resorbable Magnetic Scaffolds as Biological Stations**

A major issue related to conventional scaffolds used in bone graft substitution is the impossibility of re-loading them with bio-agents necessary to support and induce tissue regeneration after implantation. Bioactive magnetic scaffolds could be imagined as fixed stations that offer a long-living assistance to neighboring tissues: these stations will be reloaded repeatedly after implantation, and in this sense they will mimic the production and consequent delivery of human body growth factors in order to direct tissue regeneration and
angiogenesis processes. Magnetic scaffolds are envisaged to take up, close to the area to regenerate, functionalized MNPs that bind growth factors, stem cells and many bio-agents under the effect of an external magnetic field. Once close to the scaffolds, the magnetic carriers release the bio-agents and successively they can be reloaded. According to this vision, Bock and coworkers suggested an innovative, reproducible and non-damaging technique to easily magnetize biocompatible porous scaffolds, by infiltration of Hydroxyapatite (HA)/collagen (70:30 w/w) and pure collagen scaffolds with different Ferro fluid solution, i.e. an aqueous dispersion of magnetite nanoparticles [16]. Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES) analysis indicated that MNPs were retained by all the scaffolds, whereas magnetic characterization showed a higher loading potential of the composite HA/collagen with respect to the collagen scaffold. The biocompatibility of novel magnetic scaffolds was preliminary tested \textit{in vitro} by evaluating the adhesion and proliferation of human bone marrow stem cells (hBMSC) an increase in the number of viable cells was observed from day 5 to day 15 for both scaffolds, indicating a suitable biocompatibility of the newly-developed magnetic scaffolds.

In 2010, Tampieri’s group succeeded in synthesizing magnetic bio-hybrid porous scaffolds, prepared following a biologically-inspired mineralization procedure, according to which apatite nanocrystals \textit{in situ} nucleated on self-assembling collagen, in presence of magnetite NPs [17]. Energy Dispersive X-ray Spectroscopy (EDS) analysis revealed an uniform distribution of the magnetite phase within the scaffold framework, whereas X-Ray Diffraction (XRD) analysis showed the main peak of magnetite besides the low-crystalline HA ones, typical of biologically inspired HA/collagen materials. High-Resolution Transmission Electron Microscope (HRTEM) micrographs showed that calcium phosphate NPs covered most of the surface of the collagen fibers and exhibited a hollow spherical/ellipsoidal morphology with an average diameter ranging from 10 to 50 nm, whereas MNPs were made of magnetite nanocrystals 20-40 nm sized, aggregated on the collagen fibers. MTT and live/dead tests revealed that, other than not being cytotoxic, magnetic HA/collagen scaffold promoted hBMSCs cell adhesion, growth and proliferation, as indicated by a 10-fold increment in cell growth after 10 days of cell culturing.

Ferrofluid-infiltrated and \textit{in situ} bio-hybrid scaffolds were tested for the first time \textit{in vivo} by Panseri and co.\[18\] magnetic and not-magnetic scaffolds were implanted in rabbit distal femoral epiphysis and tibial mid-diaphysis, in order to evaluate biocompatibility, osseointegration and bone healing progression in both trabecular and cortical bone. At 2 weeks from surgery, new bone formation, significant scaffold resorption and low residual iron were detected, independently from the magnetic scaffold type. Interestingly, at 4 weeks the bio-hybrid scaffold resulted more reabsorbed and replaced by more woven trabecular bone when compared to the infiltrated scaffold. The different \textit{in vivo} behavior of the two magnetic scaffolds was ascribed by the authors to the different magnetic phase distribution within the two groups: while the magnetic phase within the bio-hybrid scaffold was completely integrated and finely distributed into the fibrous matrix, in the infiltrated scaffold, the MNPs were just physically ensnared on the surface of the collagen fibrils.

**Synergetic Effect of Static Magnetic Fields and Magnetic Scaffolds to Improve Implant Fixation**

In view of the application of magnetic scaffolds as fixed stations for conveying magnetized soluble factors on the site of implant, an external or internal SMF is required. In particular, an internal magnet close to the magnetic scaffold may also improve the settling and fixing of the magnetic scaffold itself by decreasing micro-motions at the scaffold/tissue interface. The SMF above mentioned can be generated in the bone microenvironment by rare-earth magnets such as Neodymium-Iron-Boron Magnets (NdFeB), which attracted great attention in the latest years thanks to the potential of stimulating bone formation and inhibiting bone density decrease, caused for instance by surgical infiltrations. Ferrofluid-infiltrated
and bio-hybrid magnetic scaffolds were implanted in the lateral condyle of rabbit distal femoral epiphysis, in contact with titanium-coated cylindrical NdFeB (1.2 T of permanent magnetization), non-magnetic HA/collagen scaffolds were employed as control scaffold [19]. Histological analyses at 4 weeks from surgery indicated no inflammatory reactions due to the presence of the permanent magnet. Both magnetic scaffolds appeared well integrated with the surrounding bone, whereas control scaffold was completely reabsorbed. Newly-formed bone tissue was characterized by thin immature trabeculae within the scaffold and more mature trabeculae at its periphery. Interestingly, magnetized collagen fibers aligned in the same direction of the magnetic field lines generated by the permanent magnet.

At 12 weeks from the implantation, a cooperative effect between the magnetic forces generated by the permanent magnet and the magnetized collagen fibers of the re-absorbable scaffold was observed. This was associated with accelerated bone formation and promoted the development of a dense and ordered pattern of trabeculae, orthogonally oriented with respect to the magnetic field lines (unpublished data). In contrast, enhancement of bone formation was limited when a non-magnetic scaffold or only the permanent magnet were implanted. The authors concluded that the implantation of magnetic biomaterials together with a permanent magnet generated a force gradient that was more efficient in determining bone structure in respect to the physiologic repair process.

Besides highly-resorbable scaffolds, slowly-resorbable magnetic scaffolds were designed for application as bone substitute of large defects, wherever high stress resistance is required. Microporous HA composite ceramic construct/insert embedding different amounts of magnetite NPs (wt.% 95/5, 90/10, 50/50 and 100/0 w/w) were fabricated via a foaming technique and sintered at high temperature in a controlled atmosphere [20]. XRD results revealed only HA and magnetite phases, as expected; but for higher magnetite content higher than 10%, hematite was detected as secondary phase. In vitro tests using human osteoblast-like cells were carried out with or without an applied magnetic field (320mT). After 7 days from seeding, cells completely coated the whole external surface of the scaffold, filling a good part of the inner micro-pores after 14 days. Cell proliferation was higher for the 90/10 compound with respect to the other formulations, both at 7 and 14 days from seeding, irrespectively of the presence of the external magnetic field. As a consequence of its better in vitro behavior, the 90/10 scaffold was selected to be tested in vivo in a critical-sized lesion of a rabbit femoral condyle. After 4 weeks, novel mineralized bone tissue was found within the interconnected porous structure of the scaffold, both in the magnetic and control scaffold, suggesting good histocompatibility of the newly-developed magnetic scaffold.

Zeng and co. investigated the behavior of rat osteoblast and mice pre-osteoblast cells on a series of HA scaffolds magnetized with different MNP contents (from 0 to 2 wt%) [21]. The results demonstrated that the magnetic scaffolds enhanced cell adhesion, proliferation and differentiation with respect to non-magnetic scaffolds. Interestingly, a significant improvement of cell proliferation was detected when combining the magnetic scaffolds with an external SMF, suggesting a synergistic effect between the magnetic scaffolds and the static magnetic field. Finally, a positive correlation between MNPs content and cell proliferation was observed.

Gu's group fabricated magnetic HA and HA/Tricalcium Phosphate (TCP) composite scaffolds containing superparamagnetic nanoparticles [22]. The two scaffolds were cultured with rat Ros17/2.8 and human MG63 osteosarcoma cells in order to evaluate their ability to sustain the cell proliferation and differentiation. Results indicate that the composites have good biocompatibility with the bone cells. It is also demonstrated that the super-paramagnetic nanoparticles integrated in the composites do not affect the function of the BMP binding to the composites. In the rat-subcutaneous implantation model, the composite composed of HA-TCP, MNPs and BMP-2 accelerated new bone-like tissue formation. Polymeric materials allow more processing options for the scaffold fabrication when used as substrates.
Intrinsically superparamagnetic Fe\(^{2+}/\)Fe\(^{3+}\) scaffolds

As the long-term toxicity effects in the human body of iron oxide-based phases such as magnetite and maghemite have not been fully estimated, a novel synthesis procedure to obtain a superparamagnetic (Fe\(^{2+}/\)Fe\(^{3+}\)) lattice substituted hydroxyapatite (Fe-HA) was recently proposed [23]. The author's idea was to introduce both ferrous (Fe\(^{2+}\)) and ferric (Fe\(^{3+}\)) ions into the HA lattice at different Ca\(^{2+}\) sites, thus to create two iron sub-lattices, whose interaction gives rise to the superparamagnetic behavior, reducing at the same time the formation of magnetite as secondary phase into the scaffolds. Three different modified-classical neutralization synthesis procedures were undertaken: a reductive method, using a source of Fe\(^{3+}\) ions to replace the Ca\(^{2+}\) ions during the nucleation of HA, with subsequent reduction of a certain fraction of Fe\(^{3+}\) into Fe\(^{2+}\), an oxidation method, exploiting the spontaneous oxidation of Fe\(^{2+}\) species in the environment reaction to synthesize Fe-HA powders; a simultaneous method, where sources of Fe\(^{2+}\) and Fe\(^{3+}\) were simultaneously added during the neutralization reaction. The Rietveld analysis indicated that in the Fe-HA powders synthesized according to the reductive method, only Fe\(^{3+}\) was present in the lattice; further, after the thermal treatment no magnetic phase was detected, likely due to the fact that the reduction process of Fe\(^{3+}\) trapped into the HA lattice was not homogeneous in the whole bulk material. Similarly, unwanted magnetite peaks where found in the powders synthesized according to the oxidation method. The simultaneous method provided Fe-HA powders with the higher magnetic potential with no traces of magnetite phase. Magnetization curves revealed a superparamagnetic-like behavior of single-domain MNPs. Hyperthermia measurements, i.e. the evolution of heat as function of time of exposure to a magnetic field, showed a temperature increase of about 40°C in 60 s, faster than the one provided by HA-magnetite control powders. The biocompatibility of the superparamagnetic Fe-HA MNPs was assessed in vitro by evaluating Saos-2 human osteoblastic-like cell proliferation either in the absence or presence of an externally applied SMF and compared to the behavior of commercial HA nanoparticles [24]. Four different MNPs concentrations (2000, 1000, 500 and 200 μg/ml) were investigated. Live/dead assay results demonstrated the Fe-HA MNPs enhanced cell proliferation compared to the control group. The application of a 320 mT SMF on cell cultures induced a significant increase in cell proliferation from day 1 to day 7 compared to groups without SMF in particular, the 200 μg/ml concentration promoted the highest cell proliferation. Biocompatibility of Fe-HA granulate (400-600 μm) was also evaluated in vivo on a rabbit critical bone defect model bone tissue was well visible around the biomaterials in both the magnetic and control group, indicating good integration of the scaffold into the surrounding bone tissue.

The same group managed to directly nucleate superparamagnetic Fe-HA crystals on self-assembling collagen fibers (telopeptides-free type I, from horse tendon) using a biologically inspired mineralization process [25]. Different synthesis temperatures were evaluated, i.e. 25°C (Fe-HA/coll25), 40°C (Fe-HA/coll40) and 50°C (Fe-HA/coll50). Non-magnetic scaffolds made of pure collagen and HA/collagens (60/40 w/w) were prepared as reference materials. XRD pattern of the hybrid composites synthesized at 25°C displayed the typical shape ascribable to a CaP phase without long-range periodic regularity close to an amorphous one, while those of the scaffolds prepared at higher temperatures exhibited the characteristic broad diffraction peaks of Nano crystalline apatite with very low coherent length. Interestingly, Fe-HA nuclei grew in tight contact with the fibers with their c-axis preferentially oriented parallel to the direction of collagen fibers orientation. FT-IR spectra of hybrid scaffolds confirmed the presence of the CaP phase nucleated on collagen fibers, as expected, and the resolution of the distinctive bands increased as a function of temperature due to the different CaP phase crystallized at different temperature, i.e. amorphous CaP at 25°C and apatite at 40 and 50°C respectively. Scanning Electron Microscope (SEM) analysis revealed that in Fe-HA/coll25, the small apatite Nano crystals closely bound to the collagen fiber, gave rise to a micro-porous framework, whereas higher synthesis temperature yielded
an external layer of large apatite crystals exhibiting a plate-like morphology, leading to an increase of the collagen fiber thickness and reduction of the total porosity. Magnetic measurements showed that higher magnetization values were registered for the scaffolds prepared at higher synthesis temperatures, suggesting that higher temperatures promoted the substitution of the Fe ions in the lattice, as also confirmed by quantitative chemical analyses. Finally, viability of osteoblast-like human cells cultured on the magnetic hybrid composites was evaluated either in the absence or in the presence of an applied external magnetic field of 320 mT intensity. Fe-HA/coll25 provided the best results both in terms of cell adhesion, viability and proliferation, independently of the presence of the static magnetic field.

**Polymer-based magnetic scaffolds**

In 2011, De Santis and co-prepared by rapid prototyping poly (ε-caprolactone) (PCL) magnetic scaffolds embedding magnetite (Fe$_3$O$_4$) MNPs [26]. The 3D fiber-deposition technique allowed manufacturing highly controlled, well-defined and customized scaffold with a fully-interconnected porous structure. 3D Nano composite fibers were obtained by extruding and alternatively depositing the straight fibers along the 0° direction and the 90° direction between two successive layers, thus obtaining a 0°/90° pattern. PCL/Fe$_3$O$_4$ Nano composite scaffolds were fabricated starting from PCL/Fe$_3$O$_4$ pellets (90:10 wt%), starting from PCL pellets and Poly Vinyl Pyrrolidone (PVP)-coated Fe$_3$O$_4$ NPs exhibiting the same weight ratio. Results of tensile tests carried out on PCL and PCL/Fe$_3$O$_4$ Nano composite fibers (340-360 µm and 380 µm in diameter, respectively) revealed a ductile behavior for both groups. The presence of the magnetic phase mechanically reinforced the PCL matrix, the elastic modulus and the maximum stress increased about 10% and 30%, respectively, however, the maximum strain decreased of about 50%, suggesting also increased brittleness. Magnetic measurements performed at T=310 K indicated that the magnetic PCL scaffolds showed a super paramagnetic behavior. Finally, biological in vitro tests revealed a marked increase of adhesion and spreading of hBMSCs on magnetic PCL scaffolds compared to pristine PCL scaffolds, indicating improved biocompatibility of the PCL matrix by addition of the magnetic phase.

In order to obtain magnetic scaffolds with minimum content of magnetite, the same group recently developed a fully biodegradable and magnetic Nano composite for bone tissue engineering consisting of a PCL matrix embedding bio-resorbable iron-doped Hydroxyapatite (Fe-HA) MNPs [27]. Fe-HA MNPs were integrated into a PCL matrix with three different polymer-to-particle weight ratios (90/10, 80/20 and 70/30 w/w). XRD analysis revealed that fabrication process did not alter the structure and crystallinity of the Fe-HA component, while SEM-EDS mapping indicated a homogeneous distribution of MNPs within the matrix. Small punch tests indicated that PCL/Fe-HA (90/10) scaffolds were the strongest and toughest of the analyzed scaffolds. Hyperthermia test curves collected on the PCL/Fe-HA (90/10) scaffold under an alternating field of 27 mT with a frequency of 260 kHz, indicated a magnetically induced thermal response suitable for in vivo applications, thanks to the super paramagnetic nature of MNPs embedded in the HA nano-powders, proportional to the amount of magnetic phase. Even in this case, the biocompatibility of the newly-developed magnetic scaffolds was tested in vitro by using hMSCs. Interestingly, hMSCs exhibited higher proliferation on the PCL/FeHA nanocomposites when compared to PCL, both at 7, 14 and 21 days. In particular, PCL/Fe-HA 70/30 and 80/20 w/w specimens yielded the highest cell proliferation values.

Magnetic biodegradable Fe$_3$O$_4$/chitosan (CS)/poly (vinyl alcohol) (PVA) nanofibrous membranes have been recently fabricated by Wei and co. [28]. Membranes were fabricated by electrospinning with average fiber diameters ranging from 230 to 380 nm. By adding a Fe$_3$O$_4$ NPs concentration lower than 5 wt%, a homogeneous and smooth Nano fibrous composite could be obtained without altering the crystalline structure of Fe$_3$O$_4$, CS and PVA.
and with the MNPs evenly distributed in the fibers. Tensile test showed that the stiffness of the composite improved as MNPs concentration increased. Finally, the biocompatibility of the novel constructs was assessed by *in vitro* evaluation of the behavior of MG63 human osteoblast-like cells. MTT assay and SEM observations clearly indicated good suitable cell adhesion and proliferation, suggesting that these magnetic nano-fibrous membranes can be promising biomaterials for promoting osteogenesis.

Magnetic nanofibrous films based on Polylactic Acid (PLA) were obtained by Meng and co. [29]. MNPs of γ-Fe₂O₃ with an average diameter of 14 nm, HA nanoparticles with an average diameter of 50 nm and PLA with a molecular weight of 10 kD were mixed together and then processed into fibers by electro spinning. The MNP mainly located inside the fibers, while HA nanoparticles distributed near the surface of the fibers. Interestingly, the films could be folded and fixed to pellets thus to be suitable as bone substitutes. In their study, pre-osteoblast MC3T3-E1 cell line was cultured on the samples with or without the application of an external magnetic field of 0.9–1.0 mT. The presence of the SMF significantly improved cell proliferation rate, which clearly indicates super-paramagnetic nanoparticles integrated in the scaffold played a synergy role in cell proliferation with the applied magnetic field. Interestingly, when exposed to the magnetic field, cells growing on MNP/HA/PLA films expressed significantly more ALP than those growing on the HA/PLA films over the experimental period of 17 days.

The same group, encouraged by previously reported *in vitro* results, validated *in vivo* the osteogenic potential of the magnetic nanofibrous scaffolds coupled with an external SMF [30]. The scaffolds were implanted in the lumbar transverse defect of New Zealand white rabbits. In order to have the rabbits exposed to an external magnetic field, animals were housed in cages with fixed permanent magnets in the two opposite sides, while animals housed in standard cages were taken as a control. The final evaluation of defect healing was performed 110 days after the surgery. Histological observations indicated that after 10 days from the implantation, the scaffolds had recruited host-derived cells migrating to the defects area, including macrophages and fibroblasts. At longer times, the implanted scaffolds started to break into small pieces, osteoblast cells appeared and new bone tissue formed around the scaffold pieces as well as blood vessels. Higher bone apposition rate, Osteocalcin (OC) and collagen deposition levels were detected when using the external magnets. Computed Tomography (CT) images interestingly showed also that animal’s undergone external magnetic field presented a more homogenous and close-to-natural bone morphology when compared to animals not exposed to the magnetic field. Finally, scaffolds degraded faster under the effect of the external magnet; the authors speculated that the recruited macrophages were more active when exposed to the external magnetic field, contributing to fast scaffold resorption.

**Magnetic Bioactive Glasses**

Recently, the possibility to fabricate magnetic coatings by the incorporation of iron oxide into the structure of bioactive glasses has gained interest. Magnetic bioactive glass coatings (CaO–SiO₂–P₂O₅–Fe₃O₄, MBGCs) were recently used by Chen and co. as sacrificial templates in order to fabricate Magnetic HA Coatings (MHACs) with oriented nano rods arrays [31]. MBGCs were converted to MHACs in a simulated body fluid (SBF) via a dissolution–precipitation reaction. HA nanorods with a preferential (002) crystal plane orientation, orthogonal to the coating surfaces were obtained. The authors demonstrated that magnetite nanoparticles present in the coating improved the nucleation rate of HA; indeed, when no MNPs were incorporated into the bioglass coatings the HA nanorods turn into blocky HA particles. Further, the magnetic coating exhibited much higher hydrophilicity (contact angle of 10.8°) than the non-magnetic coatings ascribable to the presence of magnetite nanoparticles. The biocompatibility of the HA nanorods was tested by using human bone marrow stromal cells (hBMSCs) as cell models. The hBMSCs showed improved cell adhesion,
spreading and proliferation on the MHACs when compared to the BGCs or MBGCs thanks to the presence of the HA phase, higher hydrophilicity and oriented nanorods arrays, suggesting the great potential for bone implants.

Wu and co. [32] developed a multifunctional mesoporous bioactive glass scaffold system for hyperthermia and drug delivery applications. To this end, iron scaffolds with a hierarchical macroporous structure (300–500μm) and mesoporous (4.5nm) were prepared by incorporating 5% or 10% Fe into bioglass using co-templates of the non-ionic block polymer P123 (poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide)-based triblock copolymer (EO)_{20}(PO)_{70}(EO)_{20}) and polyurethane sponges, the former responsible for the formation of the mesoporosity and the latter of the macroporosity. The incorporation of Fe into mesoporous MBG glass scaffolds enhanced the mitochondrial activity and the expression of bone-related genes (ALP and OC) in BMSCs attached to the scaffolds. The Fe-MBG scaffolds obtained also possessed high specific surface areas and demonstrated sustained drug delivery.

Conclusions

Magnetic scaffolds have unambiguously demonstrated to have a significant influence on cellular aspects such as adhesion, proliferation and differentiation. In particular, MNP-embedded and in situ hybrid HA, HA/collagen, polymeric and bio glass scaffolds demonstrated an enhanced effect on cell behavior, likely due to the remote activation of the mechano-transduction pathway which in turn triggers the biochemical one. Finally, magnetic scaffolds present an excellent alternative and improvement in bioreactor and scaffold design, as they can provide mechanical cues that can be enhanced upon the application of a remotely generated external magnetic field.

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References


Abstract

In recent years, the importance of the surrounding microenvironment as modulator of cell activity has been greatly recognized and its maintenance is crucial to preserve tissue homeostasis. The Extracellular Matrix (ECM) is a particularly important component of tissue microenvironment, providing cells a mechanical support and a plethora of biochemical stimuli. Within this context, Tissue Engineering (TE) aim to develop scaffolds that mimic one or multiple features of the natural ECM or specific of developmental or wound healing programs.

In this chapter we revisit the main biomaterials used in TE to mimic ECM. Some of them are derived from naturally occurring bio-macromolecules, others are constituted by synthetic polymeric compounds and others combine both materials to obtain highly complex scaffolds. A great effort has been made to prepare scaffolds based solely on natural polymers. However, much work is still needed to obtain clinically successful materials. Thus, new strategies are being developed to combine natural compounds with easier modifiable synthetic materials, resulting in highly adjustable hybrid scaffolds. Nevertheless, concerns on material performance in vivo still remain.

Ultimately, the goal of TE is to obtain a biocompatible, biodegradable, bio-resorbable, non-toxic, non-immunogenic, cell-instructive 3D structure capable of being modulated by cell activity, adapting and evolving along time. In the future, the impact of biomaterials in regenerative medicine will greatly rely on the ability to integrate multiple signals and deliver...
a combination of bioactive factors in a spatio-temporally controlled manner, just like the tissue microenvironment does.

**Introduction**

Cells within a tissue continuously sense and integrate a multitude of stimuli from their surrounding microenvironment that modulate cell function and activity by providing chemical, topographical, mechanical and biological cues. In turn, such signals are transduced into a cellular effect, often capable of shaping the microenvironment itself [1]. These stimuli are provided by diverse elements in the microenvironment, including the Extracellular Matrix (ECM), which mainly has a structural role. At the molecular level, the ECM is a tri-dimensional network of proteins, such as collagens, elastin, glycoproteins and proteoglycans, among others. It also functions as a reservoir of growth factors and proteolytic enzymes [2].

In recent years, the importance of the ECM as a modulator of cell activity has been greatly recognized. Thus, the maintenance of the native microenvironment of a cell is crucial to maintain its normal function. As so, Tissue Engineering (TE) aims to develop artificial scaffolds that mimic one or multiple features of the natural ECM, or of developmental or wound healing programs, in order to provide a temporary Three-Dimensional (3D) template that maintains tissue function and homeostasis by modulating cell recruitment, seeding, adhesion, proliferation, differentiation, diffusion of nutrients and metabolic waste products, as well as *de novo* tissue formation [3].

However, because the process of *neo* tissue synthesis is not exactly the same as in a developmental or wound healing setting, a complete replica of the native mature matrix or its derivatives may not always be the ideal scaffold for tissue regeneration. In a TE approach, an accelerated regeneration process (comparing to the natural developmental program) is intended. As so, particular features of artificially designed scaffolds (like porosity, pore shape and size, interconnectivity, orientation, etc.) must be engineered to enhance tissue regeneration [3]. Several scaffold materials have been developed to be used as 3D porous scaffolds or as hydrogel matrices for distinct medical applications. As discussed in this chapter, some are derived from naturally occurring bio-macromolecules, others are constituted by synthetic polymeric materials, and others combine both materials, being used as cellular or cell-seeded biomaterials (Figure 1). Nevertheless, independently of the strategy followed for the construction of the scaffold, the development of an appropriate regenerative matrix must always take into account:

(i) Its biomechanical and biochemical properties.

(ii) The incorporation of cell adhesion ligands.

(iii) The presence of degradation sites that enable scaffold remodelling and replacement with new tissue and

The addition of signalling mediators (e.g., cytokines, growth factors, chemokines, hormones, exosomes, microvesicles) to recruit competent cells, induce cell proliferation and differentiation, and control the inflammatory response [4]. Ultimately, TE attempts to create a biocompatible, biodegradable, bio-resorbable, non-toxic, non-immunogenic, cell-instructive 3D structure that is capable of being modulated by cell activity, adapting and evolving along time, in order to avoid an additional surgery to remove it.
Biomimetic Scaffolds with Basic Configurations

**Scaffolds Composed of Natural Materials**

Over the past decade, natural-derived biomaterials have been increasingly used for...
tissue regeneration. Such natural based polymers are one of the most attractive options in the TE field, mainly due to their similarities with the ECM, chemical versatility, as well as good biological performance [10]. Natural scaffold materials are usually used in a gel-like phase either without alterations, in combination with synthetic polymers or after chemical modifications. The latter have been used to enable modulation of cell release or grafting of bioactive molecules (which can be easily incorporated during gel formulation) [3,11,12]. Being analogous to biological macromolecules, they can be recognized as part of the microenvironment, avoiding stimulation of chronic inflammation, toxicity and immunogenicity, often detected with synthetic polymers [10].

Collagen and Gelatin: Collagen type I is the major protein of most ECMs and, among the natural derived materials, is the most often used for biomedical applications [4]. An advantage of using these matrices is their ability to support cell adhesion and spreading, while being rapidly degradable, allowing in vivo matrix remodelling [13]. Collagen type I can be purified from skin, cartilage, tendon and placenta, among other human tissues [3]. Its main limitations are the potential for disease transmission and its possible immunogenicity [14]. To avoid these risks, recombinant collagen expression has been explored in multiple eukaryotic systems [15]. In vivo, its application is also limited by reduced mechanical strength. Still, several procedures like chemical glycation, enzymatic crosslinking, heat treatment or hydrogel compression have been developed to overcome this issue [16-19]. These methods also enable retention of additional signalling molecules such as growth factors, which enhance collagen bioactivity, promote extended release of distinct molecules and increase therapeutic effect [10,20,21].

Gelatin, which is denatured collagen type I, is less antigenic and more cost-effective than collagen itself [14,22]. It can be produced with different net charges, driving interactions with numerous signalling molecules [4]. Like collagen, gelatin hydrogels present weak mechanical properties that can be improved by physical or chemical cross-linking [23-25].

Hyaluronic Acid: Hyaluronic Acid (HA), also called hyaluronan or hyaluronate, is an anionic glycosaminoglycan originally extracted from rooster combs, shark skin, bovine eyes, or human umbilical cords [26]. It is also an important constituent of connective tissue and synovial fluid [10]. HA synthesis in large scale through bacterial fermentation results in a highly reproducible and controllable molecular weight product that circumvents the risk of animal-derived pathogens [10,26]. Owing to its ionic character, this material absorbs a large amount of water, thus providing compressive strength and forming viscous solutions that facilitate the filling of irregularly shaped defects [10]. For that reason, HA constitutes an attractive material for clinical applications [4]. However, its mechanical properties and biological functions must be improved. Interestingly, photo-polymerization can control HA mechanical properties and slow degradation rates, being an advantage for minimally invasive procedures, which minimize patient discomfort, infection risk, scar formation, cost and treatment time [27]. Still, HA is mainly used as a protein carrier rather than a scaffolding material [4,10]. Although HA naturally interacts with several cell surface receptors, it is unable to bind important adhesion receptors, like integrin’s. Several chemical groups and polymers have been used to try to overcome this issue [4].

Fibrin: Although Fibrin is not a regular component of the ECM, this provisional matrix is one of the most widely used hydrogels in the clinic. It is derived from polymerization of circulating fibrinogen, which occurs in the presence of thrombin, during blood coagulation. For that, Fibrin can be isolated from patient blood plasma, reducing the potential for disease transmission and immunogenic reactions [4,10]. This protein has been used as a cell delivery matrix, since it can bind several receptors such as integrins, promoting cell adhesion [28]. Cell migration in these matrices is almost exclusively dependent on proteolytic activity, in contrast to collagen matrices, in which migration occurs through both dependent and independent proteolytic degradation mechanisms. One of the major advantages of using fibrin matrices is that they can naturally bind to several proteins.
Therefore natural enzymatic crosslinking \[29\] can be easily exploited to functionalize these scaffolds with multiple cell-signalling molecules \[30,31\].

**Alginate:** Alginate is the monovalent form of alginic acid. This linear polymer is an anionic polysaccharide easily extracted from brown algae cell walls and cytoskeleton \[10\]. For this reason, it is cost-effective and able to be gelled under mild conditions \[4\]. Alginate is safe to use in multiple clinical applications \[32-34\], but it remains a challenge to match its physical properties with particular applications \[4\]. One of its major limitations is its slow *in vivo* degradation \[35\]. To improve cellular interactions, chemical functionalization through covalent attachment has been implemented to incorporate cell signalling molecules \[36\]. Chemo-enzymatic methods have also been used for similar purposes \[37\].

**Chitosan:** Chitosan is a natural polymer mostly obtained from crustaceans and fungal mycelia \[38\]. It is soluble in diluted acids and further neutralized with glycerol phosphate \[39\]. Chitosan degree of acetylation influences physico-chemical properties, such as solubility, reactivity, biodegradability and cell response \[40\]. An important limitation of this material is the fact that the higher the degree of acetylation, the stronger the inflammatory response \[10\]. However, water-soluble chitin derivatives with unique features like biocompatibility, biodegradability, hydrophilicity and adsorption capability, can also be obtained, extending the domain of applications, particularly in the biomedical field. Such versatile polymers can be processed as fibres, sponges, membranes, beads and hydrogels \[10\].

In addition to the examples mentioned above, many other molecules, like Fibro-nectin or silk proteins, have also shown a great potential to be used as scaffolds for TE applications, and are reviewed elsewhere \[10,41\].

**Scaffolds Composed of Synthetic Materials**

Synthetic polymers can be used as an alternative to minimize some of the possible risks associated with the use of tissue derived materials, as immune rejection, blood coagulation or tissue hypertrophy. Furthermore, their nature enables their application in the reconstitution of tissues with very distinct mechanical characteristics.

For bone or cartilage regeneration, for instance, a scaffold must provide an appropriate mechanical strength, stiffness or elasticity to replace the damaged tissue \[3\]. Metals are an excellent choice for bone-replacing medical implants given their superior mechanical properties, but they lack degradability in a biological environment \[42,43\]. On the other hand, inorganic/ceramic materials, such as calcium phosphates, have good osteoconductivity and are being studied for mineralized TE, but are fragile and difficult to mould into highly porous structures. In contrast, synthetic polymers have great design flexibility because their composition and structure can be tailored to meet specific needs. Therefore, they have been extensively studied in various biomedical applications \[42\].

Synthetic polymers have been used in the form of foams, sponges, gels and hydrogels, both as scaffold or release materials, delivering biological active agents to induce tissue growth.

Such scaffolds include bio-resorbable polymers like linear polyesters (e.g. Polylactic Acid (PLA) and Polyglycolic Acid (PGA) and their derivatives), polyethylene, polypropylene, polycaprolactones and various co-polymers, as Poly (Ethylene Oxide) there phthalates (PEOT). \[44\] In addition to pure homo- and co-polymers, a broad variety of corresponding polymer-ceramic composites are being developed, circumventing limitations of both types of materials alone.

The major challenge with synthetic materials is biocompatibility, since degradation products may accumulate in the body and reach cytotoxic levels. Simultaneously, there is an associated acidification at the implant site due to pH lowering as a result of acid monomers release. This may cause inflammation, giant cell reaction and cell death. However, porosity
variation might reduce this effect. Furthermore, synthetic polymers do not possess natural adhesion sites, which is a limitation for cell attachment [3].

**Biomimetic Scaffolds with Elaborate Configurations**

**Self-Assembling and Protein-Engineered Scaffolds**

Semi-natural mimetic matrices for TE applications can be synthesized by self-assembling of peptide chains. This process is driven by hydrophobic and electrostatic interactions established between peptides in defined media conditions. Such peptides can be produced by chemical synthesis or recombinant protein engineering and their primary amino acids sequence may be created *de novo* or be derived from proteins naturally composing the ECM [45]. Depending on the amino acids incorporated into the peptide building blocks, the physical, mechanical and chemical properties of the scaffolds can be controlled to resemble the ECM of different tissues and to create tunable materials, responsive for instance to changes in pH and ionic strength of the media. In addition, the incorporation of different functional peptides in the polymer allows the construction of a multi-modular biomaterial with each domain putatively having different bioactivity.

In general, Self-Assembling Peptides (SAPs) originate gel-based biomaterials that can be injected for clinical applications, being particularly explored for soft tissue regeneration. Most of the SAPs currently used in TE form mainly β-sheet structures. One of the best examples are RAD (arginine-alanine-aspartate) based peptides [46]. They have been shown to be biocompatible *in vitro* with a variety of cell types and their supportive role for soft tissue regeneration was already demonstrated *in vivo*, promoting axonal regeneration [47], neovascularisation [48], and skin repair [49]. Additional examples of SAPs include peptide sequences of ECM molecules, as is the case of the IKVAV domain of laminin α-1 chain, being used either alone for scaffold formation or as a functional domain of more complex SAPs [50]. Other peptide sequences fold into α-helices that supercoil with each other, forming coiled coils. Usually those sequences contain a heptad repeat with hydrophobic aminoacids at defined positions responsible for the strong interaction of the different α-helices [51]. The most common peptides forming coiled coils are leucine-zippers. Their biocompatibility was demonstrated both *in vitro* [51] and *in vivo*, recruiting cells into the scaffold and escaping foreign body reaction [52].

Other commonly used peptides are amphiphiles SAPs, composed by a hydrophobic alkyl tail and a hydrophilic peptide head [53]. In aqueous solutions, the amphiphiles self-assemble into fibres typically with a diameter of 6-7nm, with tails aggregating inwards and the peptide head exposed towards the solvent. Hydrogels of amphiphile peptides were shown to be biocompatible and biodegradable *in vitro* [54]. Furthermore, their functionalization with IKVAV resulted in the regeneration of motor and sensory fibres with simultaneous inhibition of glial scar formation in a murine model of spinal cord injury [55].

In order to create biomaterials that better resemble the ECM, several domains other than IKVAV are being added to the basic peptide backbone of SAPs. Some domains mainly intend to promote cell adhesion to the matrix, as RGD (present in fibro nectin), YIGSR (present in laminin), functional sequences from the different types of collagen and fibrin, etc. [56,57]. Others intend to modulate cell functions like differentiation and migration, as is the case of osteogenic peptides, myelo-regulatory peptides and homing peptides [56,58]. Cytokines, chemokines, growth factors, and extracellular vesicles can also be incorporated into SAP matrices [1,10,59,60]. Scaffolds can even be modulated to be more responsive to cell activity, for instance by the incorporation of peptides recognized by cell proteases, facilitating matrix remodelling along with cell infiltration [61].

The major drawback in the construction of SAPs matrices is the possible loss of self-assembling capacity upon incorporation of bioactive domains, and loss of peptide bioactivity due to conformational changes upon molecule interaction.
Biphasic Composite Scaffolds

The addition of a mineralized phase within a scaffold, for bone or osteochondral applications, is a process that further challenges biomaterials development. Bone tissue is composed of hydroxyapatite (HA) nano-crystals grown on collagen fibers. As such, numerous studies have reported the preparation of bio-hybrid scaffolds that combine naturally derived polymers with inorganic materials like calcium phosphate ceramics (reviewed in [62-64]).

Chitosan with β-1,3-glucan [65], electro-spun chitosan [66], chitosan with polygalacturonic [67], poly-etheretherketone [68] and bio-glasses, among others, have all been explored (reviewed in [69]) in an attempt to create the biohybrid composite that best mimics natural bone composition.

Even more audacious is the capability to control the mineralization process itself. As referred in Sect. 1, a biomimetic ECM must not be merely an inert support but an active element guiding cell behaviour. The concept of *in situ* bio-mineralization has been proposed by Kokubo in 1990 and is a complex process that involves controlled nucleation and growth of carbonated HAp (a bone-like mineral) onto polymer scaffolds. [70] This process mimics to some extent natural bone mineralization, as the carbonated HAp that is generated was found to be more similar to bone apatite than to its synthetic (highly pure and crystalline) form. This bone-like mineral presents characteristics such as low crystallinity and a nano-scale size, that are important for bone reabsorption and remodelling [70-72]. The control of the mineralization process itself has also been achieved through functionalized biomaterials, by using peptides capable of controlling the synthesis of specific inorganic phases. Chung et al., identified a 12-aminoacid peptide resembling type I collagen that can bind to single crystals of hydroxyapatite, the main component of bone’s inorganic phase. This peptide is able to initiate the nucleation and growth of crystalline hydroxyapatite [73].

The design of a scaffold intended for osteo-chondral applications, which incorporates both an osseous phase and a cartilaginous phase, is even more challenging. Such scaffolds must reproduce the complex physico-chemical characteristics of the articular cartilage and of the underlying subchondral bone, from the macro- to the nanoscale. A poly-vinyl alcohol/gelatin-nano-HA/polyamide6 (PVA-n-HA/PA6) bilayered scaffold has been developed by ensuring an alternating porous and non-porous microarchitecture and was successfully used for articular cartilage and subchondral bone regeneration in a murine model [74]. In a different approach, a 3D biomimetic scaffold (MaioRegen®) was obtained by nucleating collagen type I fibrils with nanostructured HA nanoparticles, in tri-layers with different proportions of collagen and HA, to reproduce the osteochondral anatomy. The mineral phase was directly nucleated onto collagen fibers during their self-assembly, by HA nanocrystal precipitation and growth along the fibril axes [75]. Such biphasic composite scaffold has been widely used in the clinic for large osteochondral lesions of the knee [76] and of the tibial plateau [77].

Decellularized Matrices

Decellularized tissue is mainly composed of ECM and cell residues, such as DNA, RNA, cell membrane and debris and can be obtained by different approaches. Independently of the method used, the process of cell removal results in a template composed of ECM proteins that still possess a native-like geometry and architecture, maintaining its biomechanical and hemodynamic properties [78]. Conservation of the complex biochemical and structural matrix composition offers a physical support to the cells, enables cell-matrix interactions and provides a natural reservoir of growth factors and cytokines, presenting a great potential for authologous therapies [79].

Numerous methods have been developed to remove an organ or tissue’s cellular components. These include physical, chemical and enzymatic treatments. Physical treatments use agitation, sonication, mechanical massage, pressure or freezing and thawing
to disrupt the cell and release its contents. Chemical treatments use alkalis, acids and detergents, organic solvents, chelating agents, hypotonic solutions or hypertonic solutions to break down cell membranes and the bonds responsible for intercellular and extracellular connections. Enzymatic treatments use proteases or nuclease to cleave the peptide or nucleotide bonds [80].

All in all, the de-cellularization process is crucial to minimize residual immunologic potential of biological matrices. By eliminating cellular antigens, it can prevent disease transmission, inflammation, as well as post implantation rejection [79]. Given that ECM proteins are quite conserved among species, decellularization is enough for explants to be well tolerated [81].

The advantages of using decellularized matrices are evident. However, several problems and challenges might arise during tissue processing. Generally, different approaches are combined to maximize decellularization while minimizing adverse effects on the composition, biological activity, integrity and biomechanical properties of the remaining ECM. Appropriate methods should be selected and refined for specific tissues and organs [80]. Another major drawback is the fact that ECM composition can vary depending on the gender, age, ethnicity, lifestyle habits and pathological conditions of the donor [79]. Such alterations can influence resident and/or transplanted cell behaviour, ultimately resulting in mis-repopulation of the implanted decellularized matrix [82].

In contrast, cell-formed decellularized matrices have the advantages of unlimited availability and adaptability for different developmental stages and should thus be considered. Still, their use also presents some drawbacks like the difficulty to mimic the native ECM-like intricate architecture and the differences in the ECM secretion pattern of cells cultured in vitro when compared to that of an in vivo setting.

Years ago, decellularization was focused on simple tissues such as small intestinal submucosa [83], amniotic membrane [84], skin [85], heart valves [86] and blood vessels [87]. In recent years, with the development of new and advanced decellularization methods, the decellularization of complicated organs, such as the heart [88], liver [9], trachea [89] and lung [90], has become realistic.

Several decellularized materials have already been FDA-approved and are now being commercialized for therapeutic applications. Among them, the most representative ones include dermis tissue (AlloDerm®; LifeCell), porcine heart valves (Synergraft®; Cryolife) and porcine urinary bladder (Urinary bladder matrix; ACell) [91].

Still, to facilitate clinical applications, regulatory issues critical for the development and commercialization of decellularized scaffolds should be taken into account. These include production regulation, tissue and organ donor management, effectiveness assessment, safety evaluation, quality control, application protocols and guidelines, as well as standardization and ethical concerns [80].

**Conclusions**

With the help of emerging technologies like nanoscience and nanotechnology, tissue engineering experienced an exponential growth during the last decade [42].

Despite extensive research to engineer hydrogels for regenerative purposes, only few have been successfully translated into the clinic. This was due to the difficulty of reproducing the complexity of biological microenvironments that regulate tissue repair, and of combining multiple signals to fully control different regenerative stages. Past research mostly focused on the development of materials that integrated few biological cues, without considering the way they participate together in the tissue microenvironment. Nowadays, it has become clear that the mechanical, biological, and chemical properties of biomaterials need to be integrated and fine-tuned according to each application [4].
In the field of TE, a great deal of effort has been put in to prepare different formulations based on natural polymers. However, much work is still needed to obtain clinically successful materials [10]. Because the mechanical properties of synthetic hydrogels are easier to modulate than those of natural scaffolds, new strategies are being developed to combine them, resulting in highly adjustable hybrid materials. However, some concerns still remain, concerning bio-affinity, material degradation and biocompatibility of degradation side products. In addition, the therapeutic application of growth factors can be accompanied by undesirable side effects due to the difficulty to control their release in a dose-dependent manner [3]. Such issues should be addressed in future studies.

In conclusion, the future impact of biomaterials in regenerative medicine will depend on the capacity of integrating multiple signals and delivering a combination of bioactive factors in a spatio-temporally controlled manner, while exploiting synergistic interactions [3,92,93] and mimicking the natural ability of the ECM to do so during tissue morphogenesis. This will greatly enhance the safety, cost effectiveness, and efficacy of biomaterials for tissue repair and regeneration [4].

Acknowledgments

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References


Abstract

Organs transplantation or implantation of synthetic devices is the currently available and most used methods to treat loss of tissues and organs in humans. However, there is a continue demanding of new solutions and approaches for tissues failure since the definitive solution is far to be achieved. For this reason, regenerative medicine and tissue engineering are becoming of great interest as the alternative strategy to repair or regenerate damaged tissue. Among different regenerative strategies, the development of synthetic scaffolds able to induce tissue restoration has been widely explored. The main goal of this approach is to develop functionalized platforms releasing growth factors or bioactive molecules that can lead desired cell response in order to enhance and accelerated tissue healing. A plethora of materials natural, synthetic and hybrid have been exploited to develop an adequate system for the sustained and controlled release of regenerative signals for the specific application. Recently, available delivery systems have some drawbacks such as growth factors loss of bioactivity, limited control over administrated dose and non-targeted delivery. Thus, the field of drug delivery systems for tissue engineering is still investigating new approaches and with the support of materials science new smart solutions to overcome these limitations. This chapter discusses carrier-based growth factor delivery systems with a particular focus on materials engineering for bone and cartilage tissue regeneration.

Introduction

Regenerative medicine and tissue engineering aim to develop functional substitutes for defective tissues by mimicking natural ones: the self-healing capacity of a patient is, therefore, altered and enhanced to cause the regeneration of tissues and organs that would not heal otherwise [1].

An encouraging strategy to restore tissue function and composition is to mimic the biological niche and establish ad hoc combinations of Growth Factors (GFs), cells, and biomaterials able to replace and recreate crucial regenerative pathways taking place during the tissue healing process such as proliferation, migration and differentiation [2-4]. The ideal materials are biodegradable, biocompatible, and able to serve as a supporting artificial
Extracellular Matrix (ECM) until the natural tissue is produced by the neighboring cells as the biomaterials gradually degrade. The use of GFs has become greatly attractive to achieve the above mentioned goals, because their activity affect and regulate many cellular processes involved during tissue healing [5]. The controlled delivery of GFs can boost the self-healing capacity of patients while accelerating multiple processes involved in tissue regeneration. GFs act as signaling molecules between cells, transferring information between cell populations and their micro-environment during the healing process resulting in accelerated functional reparation of the damaged tissues [6-8]. A limited control over the release kinetics, fast degradation in vivo, payload bioactivity loss and toxicity are some of the drawbacks that require the presence of a delivery system platform to protect the payload. Biocompatible and biodegradable delivery systems are crucial to obtain a successful usage of GFs in clinically approved regenerative medicine therapies [9]. The presence of a carrier, for the most part, enables the embedded GFs to release at an advantageous dosage and rate, and persist within the trauma area for a sufficient period of time to recruit progenitor cells and speed up tissue healing processes. Temporo-spatial control of GFs bioactivity after introduction into the body is imperative to accomplish a tangible therapeutic effect, in fact, uncontrollable pharmacokinetics have shown adverse effects [10]. The design of the appropriate GFs delivery platform is influenced by multiple factors.

Qualitative and quantitative information about GFs distribution within the microenvironment of the target tissue, the biocompatibility and biodegradability of the material, and the efficiency and cost of platform production all affect the final design. Moreover, the selection of shape, structure, and formulation of the carrier system can affect the GFs local and systemic administration and enhance cellular infiltration and growth. The characteristics of an ideal GFs carrier for regenerative medicine should fulfill the following parameters:

a. Biodegradability that enables tissue remodeling and reconstruction
b. Macro-porosity to enhance cell ingrowth within the composite
c. Mechanical stability
d. Biocompatibility to avoid or reduce foreign body reactions

This chapter aims to strictly highlight new developments and limitations of the materials design and processing methods suitable to incorporate and release GFs in a controlled and defined manner. Up to date status of biomaterial substrates and sophisticated delivery systems as hydrogels, microcomposite particulate and scaffolds will be examined in details. Finally, issues and possible future research paths for GFs applications in regenerative medicine will be discussed.

**Growth Factor’s roles in Tissue Regeneration**

A plethora of bioactive molecules plays a crucial role in the process of tissue regeneration [11]. Among those, GFs are proteins that enable and lead different cellular processes involved in tissue healing; such as infiltration, growth, differentiation, migration, cell metabolism and apoptosis. The use and controlled delivery of GFs can reinforce tissue restoration by speeding up and regulate the aforementioned processes [12]. They modulate cellular activity by acting as signaling molecules between cells, transferring information between cell populations and their micro-environment resulting in quickened functional reestablishment of the damaged tissues [6-8,13]. There are various ways by which GFs can reach the target cell such as: paracrine, endocrine, intracrine and autocrine mechanism. The paracrine method employs cells releasing GFs that subsequently reach the neighboring cells by molecular diffusion. Through the endocrine process, the bloodstream works as a carrier for the GFs secreted by the endocrine gland [13]. The intracrine method consists in the internalization of the GFs/receptor system [14]. The autocrine mechanism involves
secretion of GFs by cell to stimulate themselves; it is very common in tumor cells and frequently cause the excessive generation and secretion of GFs [4].

These different ways of action must be taken into consideration when designing the delivery platform in order to tune the proper release kinetics of the GFs depending on the mechanism that you want to elicit.

Moreover, the structure and the properties of the GFs must be maintained and preserved during its release by the delivery system. It is crucial that the fabrication of the material does not involve any loss of payload features. Strong solvents, non-physiologic temperatures and harsh pH conditions can lead to a partial loss of GFs bioactivity and bioavailability while undergoing material preparation.

Thus a deep understanding of GFs biochemical features is mandatory.

A variety of GFs has been identified and they have been summarized in table 1 with their principal biologic activity and source. Transforming growth factor-β (TGF-β) family play a pivotal role in embryonic development, tissue morphogenesis, cell proliferation and cell differentiation[15]. TGF-β, Growth Differentiation Factors (GDF) and Bone Morphogenetic Proteins (BMPs) have a homologous structure that confers them analogous biological properties. Several TGF-β family members have been related to the biological processes of bone induction, including mesenchyme cell recruitment, proliferation, and ECM production[16]. BMPs, particularly BMP-2 and BMP-7 are the most extensively studied proteins to induce bone formation even in critical size defects [17-19]. Recombinant human BMP-2 (rhBMP-2) has been successfully used with FDA approval to treat Grade III open tibia fractures [20]. Some other GFs have been reported to induce bone healing including insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF) and platelet-derived growth factors (PDGFs) [21]. BMP-2, BMP-7 and PDGFs have been approved by the Food and Drug Administration (FDA) for orthopedic application. Nevertheless FGF-2 has been extensively used also for periodontal regeneration[22] and cervical spinal cord injury [23], and PDGF and VEGF for diabetic foot ulcers [24]. A decisive step, during tissue restoration, is the formation of a mature vascular system, and GFs fulfill a lead role in angiogenesis [25]. Vessels transport oxygen and nutrients fundamental for the growth and differentiation of circulating cells essential for the formation and homeostasis of bone [34,35]. VEGF delivery has been proved to enhance blood vessel density and stimulate bone regeneration in rabbit [26] and rat [27] critical size bone defects.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Source</th>
<th>Principal role</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>Primary liver</td>
<td>Promotes proliferation and inhibition of many cell types</td>
<td>[28,29]</td>
</tr>
<tr>
<td>IGF-II</td>
<td>Variety of cells</td>
<td></td>
<td>[28]</td>
</tr>
<tr>
<td>FGF</td>
<td>Variety of cells</td>
<td>Promotes migration, proliferation and survival of endothelial cells. Inhibits differentiation of embryonic stem cells. Promotes periodontal regeneration and is used to treat cervical spinal cord injury</td>
<td>[22,23]</td>
</tr>
<tr>
<td>KGF</td>
<td>Epithelial cells</td>
<td>Promotes wound healing during epithelialization-phase</td>
<td>[30,31]</td>
</tr>
<tr>
<td>VEGF</td>
<td>Endothelial cells, platelets, fibroblasts</td>
<td>Regulates blood vessel formation, sustains the proliferation and differentiation of different cell types, regulates endothelial cell proliferation and angiogenesis</td>
<td>[24]</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelets</td>
<td>Regulates embryonic development, proliferation, migration, growth of endothelial cells, promotes angiogenesis</td>
<td>[24]</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Activated T helper (TH1) cells, Natural killer (NK) cells</td>
<td>Anti-inflammatory factor, promotes wound healing and inhibits macrophage and lymphocyte proliferation</td>
<td>[32]</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Musculoskeletal tissues</td>
<td>Stimulates chondrogenic differentiation of mesenchymal stem cells (MSC)</td>
<td>[33]</td>
</tr>
<tr>
<td>BMP-7</td>
<td></td>
<td>Stimulates cartilage maturation and renal development</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: GFs commonly used in tissue regeneration along with their source and principal role
The direct administration of GFs resulted unsuitable for regenerative medicine application due to the reduced bioavailability of the proteins, the potential toxicity after delivery and their short half-life in the biological environment [34]. GFs stimulation is required for a short window of time, direct administration remains a popular method due to its immediate effect at the site of action, its safety and reproducibility of the results. However the necessity of having a sustained and controlled delivery of the payload led to linking the payload onto polymeric surface so as to diminish the loss of bioactivity during diffusion [35]. GF can be immobilized to the carrier by either non-covalent or covalent binding.

Whenever the GFs are required only in a localized area, the use of a delivery system is preferred. During tissue regenerative processes, GFs might, in fact, be encapsulated and delivered in situ through a carrier. In situ release of GFs is responsible for immediate effect at the site of action and helps avoid toxic effects on the surrounding healthy tissues. Within the most popular vehicles, there are hydrogels, particulate systems and scaffolds.

This chapter will discuss the role of various GFs and their combinations with different delivery platforms involved in bone healing augmentation and osteo-chondral tissue regeneration. The chapter will focus on how the selection of materials, configurations and processing affects GFs delivery and regenerative efficacy.

**Importance of Biomaterials for growth factors delivery system**

As mentioned above a strategy for the regeneration of tissue is using biomaterial platforms to deliver a signaling molecule that can induce cell migration, growth, and differentiation. The short-ranged signaling in combination with short half-lives limit the extent of impact GFs may have over a regenerative process [6]. When blended with a biomaterial into a delivery system, bioactive proteins overcome the obstacle of limited diffusion and can be controlled to maintain a sustained release over an extended period of time [14]. Generally a GF delivery system needs to meet four requirements:

1. Identify the key GF to achieve the desired effect.
2. The mode of delivery must target the specific cell population and minimize the transduction in non-targeted cells.
3. The platform must stabilize the GF to ensure longevity of the bioactivity, and finally
4. The release from the system should be controlled [36].

Another factor limiting the ability of a GF to deliver a particular message is based completely outside of the identity of the GF. The population of the targeted cell, the type of receptor on the cell, and the mechanism for signal transduction all affect the extent of influence a GF may have. To increase the efficiency of a GF, Lee et al., and Babensee et al., reported that GFs can be bound to a macromolecule on the platform, chemically immobilized into or onto the platform biomaterial during fabrication, or physically encapsulated in a delivery system [6,14] (Figure 1). Binding GF to a biomaterial can be done by utilizing biological molecules to indirectly attach GFs to the biomaterial. Fibronectin [37], laminin [38], fibrin [39], collagen [40], and heparin sulfate [41] are all examples of macromolecules that have been employed as ECM-mimicking materials to immobilize GFs for delivery. Heparin as a method of functionalization has shown to deliver a maintained release of various heparin binding GFs to regulate cell growth [42,43]. The major benefit of utilizing this method is the improved storage, release and preservation of the GF [41]. The second method, chemical attachment to the biomaterial, tends to prolong the release of growth factors when compared to the physical binding [6]. Functional groups are incorporated into the material through copolymerization or chemical or physical treatment [44]. The last commonly used method to incorporate GFs for delivery is encapsulating the desired GF into the delivery system. Encapsulation can occur through the use of synthetic polymer
capsules [45], liposome-based particles [46] and natural biomaterial microspheres [47]. Whether the GF is incorporated through an indirect link, direct immobilization, or physical encapsulation, GFs can either be released from the degradation of the platform or chemically bound to the material system so that they are readily available for the cells that infiltrate the material [6]. When GFs are added to biomaterials, these materials have been able to induce various forms of regenerative processes depending on the application including but not limited to the formation of neo-cartilage [48], osteo-blastic differentiation [49], stem cell differentiation [50], neo-vascular formation [51], skin formation [52], neurodegenerative disease treatment [14], and bone regeneration [53]. Biomaterials are designed to interact with a patient’s body, aim to direct new tissue formation, and then degrade over time [54]. The biomaterial mimics the specific microenvironments that cells need for proper tissue regeneration. Normally, the environments include specific cell to cell communication, cell-matrix interactions, appropriate biological signals, and mechanical stresses [55]. By creating a 3-D environment and using GFs to simulate the proper cell-niche interactions, biomaterial based platforms have proven to be an important tool in tissue engineering [50]. In many cases, the specific reactions of a patient’s body can be used as a trigger for the release of the GFs. A change in temperature [56], or a change in the pH of a system can be used to trigger the release of GFs from a system. Protease action can also be used to trigger the release. The most commonly used system is based on the catalytic matrix metalloproteinase (MMP) [57]. The different platforms used for GFs delivery can be split into three categories: hydrogels, particulate systems, and scaffolds. Each have their advantages and disadvantaged and will be described in further detail in the next section. The specific biomaterials themselves can be grouped into either natural materials or synthetic materials.

Natural Materials

Natural biomaterials can be derived from components found in the ECM, plants, insects or animal sources. The major advantages of natural materials are that they are bioactive, biocompatible, water soluble and have similar mechanical properties as the native tissue. They can naturally present receptor-binding ligands and are susceptible to cellular proteolytic degradation and remodeling [72]. Being of a natural source allows these
materials to work on a molecular level while reducing chronic inflammation at the site [6]. At the same time, their solubility allows for mild fabrication conditions that do not affect the bioactivity of the embedded GFs. Unfortunately, there is limited control over their physical-chemical properties, it is difficult to modify their degradation rates, and there are difficulties in sterilizing and purifying the materials [50]. Typically, chemical modifications of the natural material are done to assist in the control of degradation rates or to modify their bioactivity [73-81].

<table>
<thead>
<tr>
<th>Natural Material</th>
<th>Source</th>
<th>Type</th>
<th>Possible Applications</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>ECM component</td>
<td>Protein</td>
<td>MSC Seeding and Proliferation, Cell Attachment, Osteogenesis</td>
<td>[50]</td>
</tr>
<tr>
<td>Matrigel</td>
<td>ECM component</td>
<td>Protein</td>
<td>Cell Culture applications</td>
<td>[60]</td>
</tr>
<tr>
<td>Fibrin</td>
<td>ECM component</td>
<td>Protein</td>
<td>Neural differentiation, Cell culture, Chondrogenesis</td>
<td>[61]</td>
</tr>
<tr>
<td>Hyaluronic Acid</td>
<td>ECM component</td>
<td>Polysaccharide</td>
<td>Maintenance of pluripotency</td>
<td>[64]</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>ECM component</td>
<td>Mineral</td>
<td>Osteogenesis</td>
<td>[65]</td>
</tr>
<tr>
<td>Silk Fibroin</td>
<td>Silkworm Cocoons</td>
<td>Protein</td>
<td>Osteogenesis, Chondrogenesis</td>
<td>[66]</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Crustacean Shells</td>
<td>Polysaccharide</td>
<td>Osteogenesis</td>
<td>[68]</td>
</tr>
<tr>
<td>Coralline</td>
<td>Exoskeletons of Marine species or coral</td>
<td>Calcite mineral</td>
<td>Osteogenesis</td>
<td>[69]</td>
</tr>
<tr>
<td>Alginate</td>
<td>Algae Cell Walls</td>
<td>Polysaccharide</td>
<td>Angiogenesis, Cell encapsulation and differentiation</td>
<td>[70]</td>
</tr>
</tbody>
</table>

Table 2: Commonly used Natural Materials in Tissue Engineering

The disadvantages have not prevented the advancement of several materials into becoming commercially available. Chitosan, hyaluronic acid and silk have controlled reproducible properties and are being well characterized [82-84]. Each specific source of material can be used with different applications and different chemical modifications. A list of natural biomaterials is listed in Table 2, including their source, type, and possible applications. Natural materials found in the ECM include collagen, fibrinogen/fibrin, hyaluronic acid and hydroxyapatite (HA) [50]. Collagen is a readily available ECM component that allows for cellular infiltration and remodeling [36,85]. For the most part, collagen is used as either a scaffold or sponge for GFs delivery. When used to deliver BMP, it has been shown to regenerate long, spinal, and craniofacial bone, due to its properties of being a major non-mineral component of bone [36]. Collagen has also proven to be a 3D system capable of maintaining chondrogenesis in vivo [85]. Collagen is also part of a commercially available product called Matrigel™. Matrigel is composed of other ECM components including laminin and heparin sulfate proteoglycans [86]. It is typically used in normal cell culture but has shown an angiogenic effect in an ischemic mouse model [87]. Fibrinogen and fibrin are another natural material that can be used for GF delivery through scaffolds [88]. Fibrin has shown to be an effective platform for neural differentiation and nerve regeneration by delivering nerve growth factor (NGF) in a biologically active form in vitro and in vivo [89].

Hyaluronic acid has been modified and is commercially available as Hyaff® [90, 91]. Depending on the chemical configuration, the rate of degradation can change. For example, Brun et al. demonstrated that Hyaff® 7 is degraded within 30 days in vitro and 60 days in vivo, while Hyaff® 11 is degraded 60 days in vitro and 110 days in vivo. These differences are due to the type of ester with the hyaluronic acid. Hyaluronic acid takes part in cell behavior and cell signaling and can be easily modified into fibers, membranes or microspheres. Hyaluronic acid has also been modified into a hydrogel and has demonstrated the maintenance of pluripotency in embryonic stem cells [64]. Hydroxyapatite (HA) on the other hand is known for its osteogenic potential and can encourage bone in-growth [92,93]. HA is a natural inorganic component of bone mineral and is considered a calcium phosphate.
This natural mineral can also be synthesized and used as a synthetic material [94]. It has excellent biological performance but on its own has a low mechanical strength limit and thus restricts HA’s application in new bone generation at load bearing sites [54]. To compensate for the mechanical weakness, HA can be blended with a polymer, e.g., in one study, PLGA and HA were mixed at a 1:1 ratio and then loaded with BMP-2 producing cells. The results were a continuous production of BMP and thus acted as a long-term, sustained osteoinductive stimuli [95]. Silk fibroin is isolated from silkworm cocoons and has been developed into porous scaffolds and can also be used for surgical sutures [96]. The silkworm silk contains fibroin, an insoluble protein that creates a unique mechanical property to slow the rate of biodegradation. These silk fibroin scaffolds have been shown as a successful method of GFs delivery through the induction of chondrogenesis by releasing IGF-I [67]. Chitosan is a commercially available polysaccharide isolated and processed from crustacean shells. It is hydrophilic, biodegradable, biocompatible and has similar properties to mammalian glycosaminoglycans [97]. Chitosan can be used in a sponge platform and has been used to deliver cross-linked PDGF to obtain bone regenerative effects. The chitosan sponge degraded to be replaced by newly formed bone and the controlled release of the GFs significantly enhanced bone healing and regeneration [98,99]. Chitosan can also be blended with coralline, a calcite mineral isolated from the exoskeletons of marine species or coral [100]. The last major natural material comes from the cell walls of algae. Alginate is a natural polysaccharide that has been shown to enhance angiogenesis. Silva et al., demonstrates that the alginate led to a 2.5 fold increase in bioactivity of the released VEGF [101]. The numerous sources of natural materials offer varying benefits and weaknesses. Their biocompatibility, bioactivity, and resemblance to native tissue set the standards for the fabrication of synthetic materials to strive for.

**Synthetic Materials**

Synthetic biomaterials have a distinct advantage over natural: the features of the material can be altered and controlled. These materials create 3-D microenvironments where mechanical properties, degradation rates and porosity can be manipulated [50]. Synthetic materials can have comparable biocompatibility, but do not have the same bioactivity as a natural material and can produce acidic by-products upon degradation [102,103]. To overcome these challenges, it is critical to modify synthetic materials to mimic biological interactions and achieve an appropriate cellular response. By imitating characteristics of natural materials, synthetic biomaterials can be designed to have different structures, as in a hydrogel, and release GFs at a constant rate with a specific concentration [72]. Another aspect that synthetic materials allow for is the combination of multiple GFs and the investigation of their combinations. Specifically for growth factor delivery, we focused our attention on polymers.

**Polymers**

The most utilized biodegradable polymers in tissue engineering are saturated poly-α-esters (including Polylactic Acid (PLA), Polyglycolic Acid (PGA) and copolymer Polylactide-Co-Glycolide (PLGA) [104]. Synthetic polymers can be categorized by the type of degradation that they undergo either bulk degradable polymers or surface erodative polymers. The poly-α-esters fall under the category of bulk degradable polymers. Bulk erosion is the degradation of all parts of the scaffold, the surface and the interior simultaneously. The degradation process occurs through hydrolytic degradation of the ester bonds and once degraded the monomeric byproducts are cleared away through the tricarboxylic acid cycle and other natural pathways [50,105]. Due to this bulk degradation, PLA and PGA tend to mechanically fail prematurely. To avoid failure, PLA and PGA have been blended into the copolymer PLGA. Other factors besides the copolymerization ratio can affect the degradation kinetics of polymers. The molecular weight, the morphology, the chemical makeup, crystallinity, environmental conditions, mechanical stress, and the distribution of chemically reactive
compounds all affect the rate at which the polymer degrades [54]. Some of these physical properties, such as varying pore sizes and polymer composition, have shown to significantly influence embryonic stem cell differentiation and have been used for the evaluation of cell behavior [50]. During the fabrication of the polymers, the polymer molecular weight, copolymerization ratio, and polydispersity can also be manipulated to suit the particular need [50,54]. The ability to tune the composition and other physical properties of this polymer is what makes them suitable as delivery system for GFs. By using a polymer with a low molecular weight, GFs, drugs, and antibiotics can be delivered to the desired tissues [106]. A few GFs and their successful polymeric delivery system are listed in Table 3. The copolymer PLGA can be selected to degrade quickly to facilitate cellular ingrowth [107]. To control the degradation rate, the ratio of PGA to PLA can be increased taking advantage of the faster rate of erosion of the PGA [54]. PLGA may also be combined with other polymers for better mechanical properties. In fact, when 50:50 PLGA has been combined with PLLA, it provided an enhanced mechanical stiffness to support 3D scaffolds. By including TGF-β, this polymer has induced the formation of tissue that produced ECM similar to cartilage [107]. Another polymer that is part of the poly-α-ester family and widely used in tissue engineering is Poly (ε-caprolactone) (PCL). PCL has been involved in many different studies of adipogenesis [108], chondrogenesis [109,110], and osteogenesis [111]. It has also proven to have good biocompatibility, has the capacity of supporting various cell types [112], and has a slower degradation rate [113,114]. Due to its slower degradation rate in comparison to the other members of the poly-α-ester family, PCL can be a more suitable polymer in tissue engineering applications [109,115]. It has successfully been used to entrap antibiotics and has been used in the treatment of bone defects [54].

<table>
<thead>
<tr>
<th>Polymetric Delivery System</th>
<th>Growth Factor</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polylactide-co-glycolide (PLGA)</td>
<td>VEGF, TGF-β</td>
<td>[6, 36, 107]</td>
</tr>
<tr>
<td>D,L-Polylactic acid (PDLLA)</td>
<td>TGF-β</td>
<td>[36]</td>
</tr>
<tr>
<td>Polylactic acid (PLA)</td>
<td>PDGF</td>
<td>[36]</td>
</tr>
<tr>
<td>Polyethylene glycol (PEG)</td>
<td>IGF-1, TGF-β</td>
<td>[116, 50, 116]</td>
</tr>
</tbody>
</table>

Table 3: Polymers that have successfully incorporated and delivered Growth Factors

Finally, another large category of polymeric materials are the acrylated polymers that can be used to form hydrogels. Polyethylene Glycol (PEG) and its derivatives have already been used for mesenchymal and embryonic stem cell studies [50]. PEG has unique characteristics that make it an optimal selection for hydrogel preparation: such as solubility in water and organic solvents, nontoxicity, low protein adhesion, and non-immunogenic [117]. PEG polymers have played a role in skin regeneration [118], nerve regeneration [119], angiogenesis [120] and bone regeneration [121]. The surface modification of PEG can further lower protein binding and enhance the surfaces biocompatibility rendering the material a stealth like attribute [122]. This feature of PEG can be applied to nanoparticles enclosing GFs to enhance their delivery to the desired site [8]. They have been used for the delivery of IGF-1 and TGF-β1 to injured cartilage tissue [116] and have been used to encapsulate embryo bodies to improve chondrogenesis [55].

In summation, the many distinct characteristics of the various materials allow for the selection of an optimal material for a targeted purpose. By selecting a particular material, specific physical characteristics can be designated to hydrogels and scaffolds for GF delivery and optimize the system.

**Drug Delivery Systems in Tissue Engineering**

A grand majority of the approaches in tissue engineering have been concentrated toward the use of biomaterials and the particulate system [156]. Particle implementation
has been investigated as a complement to biomaterials. These particles can be utilized as
an injectable or moldable platform in which cells can adhere and/or proliferate in a solid
foundation. Moreover, the use of this approach has other utilizations. The encapsulation
of bioactive molecules or prior sheathing of cells may be distributed to the area of interest
using particles as transporters [157]. Previous authors have characterized particles by their
size; Microparticles (MP) usually consist of sizes ranging from 1-1000 micrometers, versus
Nanoparticles (NP) which range are smaller than a micrometer. These have been developed
into various forms which alter their physical properties to tailor them for specific applications.
Microcapsules are identified as cystic cavities enveloped within a polymeric membrane,
versus microspheres, which are packed with a specific matrix [158]. As mentioned before,
MPs have been modified for various functions: they may be tailored to be integrated into
hydrogels or porous scaffolds. Advancements have been made for the formation of an
intricate delivery system for bioactive molecules, such as a dual phase release system. Their
implementation for bone regeneration has been studied extensively [92]. There are many
advantages to using MP for GF delivery they possess a minimally invasive nature, which
permits the possibility of administration by injection to a desired area. MP assembled utilizing
biodegradable polymers avoid the need for surgical interventions that were previously
required for application of materials that were non-absorbable. This also imparts protection
of the payload, from a phenomenon known as the burst effect, were the contained bioactive
molecules are released at an accelerated rate, decreasing the biological effects desired. For
this reason, the utilization of polymers grants a homeostatic release of GFs, which mimic the
similar response the body has for auto-regeneration. Particulate/hydrogel combination offer
synergistic effects for the GF payload. Additionally, the degradation of these biomaterials
decreases the risks for foreign body reactions. Many factors play an important role in the
physiology and performance of MP: particle size, distribution, porosity, pore structure and
surface area [160]. To this extent, there exist various studies that have attempted to identify
the ideal properties for different therapeutic areas of interest. Nanoparticles (NP) can be
categorized into lipid-based, polymer-based or a miscellaneous component, which includes
dendrites and micelles. The former, include liposomes, Solid Lipid Particles (SLN), and
lipid nanocapsules, which are ideally variations of the outer shell, with unique cores [161].
Regarding polymer based materials, nanocapsules and nanospheres can be manufactured
through identical methods as MP and are morphologically comparable [162]. NP systems
have demonstrated numerous advantages. They possess a large total surface area due to
the inverse proportion to the third power of the diameter [163]. NPs offer the capability of
penetrating finer capillaries and epithelial linings, which permit more effective biological
responses [164]. Nano-scale characteristics impart greater properties in contrast to MP,
such as solubility, increased biodistribution, diffusivity, release profile, and immune-
regulation [96]. In addition, GF delivered through NP systems are primordially affected by
the physiochemical attributes rather than that of the protein [8]. Advancement in the NP
field led to the possibility of delivering dual payloads, which are loaded into a particle. In
an in vitro study, Zhang and colleagues studied the effects of Nerve Growth Factor (NGF)
and basic Fibroblast Growth Factor (bFGF) for the regeneration of nerve tissue after injury.
Growth factors were encapsulated in a heparin and e-Poly-l-Lysine (PL) NP polymer. In vitro
release of the GF demonstrated positive outcomes, including a sustained release and the
absence of an initial burst effect. Regarding the bFGF, ~42.5% of the payload was released
within 20 days; in contrast to NGF, ~59.4% of the loaded bioactive agent was discharged
within the same duration. The authors associate this phenomenon due to the lower binding
affinity NGF has to heparin [165]. Many methods have been described for preparation of
MPs and NPs for GF delivery [157,166,167]. In fact, there is an established framework
for optimizing the controlled release profile of particles and can be adjusted for specific
applications and needs. It is important to have a precise control over particle size, shape,
surface characteristics and porosity to obtain an optimized delivery system. Emulsification
has been identified as the most commonly utilized approach for manufacturing MP [157].
Growth factors can be added through a modified process of emulsification; bioactive molecules
are added via emulsion or dispersion in the system which permits GF encapsulation, this method is referred to as the double emulsion technique. The disadvantage of this technique is the production of varying particle sizes [168]. A recent paper, published by Nakaguchi et al., looked at neuron brain cell regeneration through application of GF within gelatin hydrogel MP [169]. They administered the MPs directly to the subventricular zone where neural stem cells reside eleven days after middle cerebral artery occlusion to stimulate cerebral neuron injury. The two GF focused in the study were insulin-like growth factor (IGF-1) and Hepatocyte Growth Factor (HGF). Administration of solely IGF-1 in Phosphate Buffered Saline (PBS) did not show significant change in neuronal population. However, administration with a gelatin-hydrogel suspension in PBS showed a relative increase in the number of new neurons, quantified through anti-doublecortin antibodies after seven days [169]. The combination of hydrogels and particulate systems shows great promise for the delivery of GFs.

Hydrogels have been a key branch of biomaterial applications for more than three decades. Scientists and engineers have worked together to improve this platform finding it useful especially in cartilage regenerative applications [123-126]. Hydrogels are considered three-dimensional, hydrophilic, polymeric networks that present a thermodynamic compatibility with aqueous media allowing them to swell in water and biological fluids. Some environmental factors can also affect the swelling behavior of physiologically-responsive hydrogels, such as pH, temperature, electromagnetic radiation and ionic strength. Hydrogels are composed of polymers, and are insoluble due to the presence of chemical crosslinks (e.g. tie-points, junctions), or physical crosslinks (e.g. entanglements or crystallites). The structure and physical integrity of the network is provided by the physical crosslinks or electrostatic/hydrophobic interactions [124,127-129]. The classification of hydrogels can be based on two aspects of the materials. The nature of their components is one of the main features, classifying them into two categories: synthetic or biological. In addition to the just described classification between synthetic and natural, it is possible to characterized the hydrogels by their specific features such as the nature of the side groups, the preparation method, the mechanical and structural characteristics, and their physical structure provide a further degree of classification (Table 4) [122,123,127,130].

<table>
<thead>
<tr>
<th>Hydrogel Components Nature</th>
<th>Synthetics</th>
<th>Biological</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nature of side Group</td>
<td>Neutral</td>
<td>Ionic</td>
</tr>
<tr>
<td>Preparation Method</td>
<td>Homopolymer</td>
<td>Copolymer</td>
</tr>
<tr>
<td>Mechanical / Structural</td>
<td>Affine Network</td>
<td>Phantom Network</td>
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<tr>
<td>Physical Structure</td>
<td>Amorphous</td>
<td>Semicrystalline</td>
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<td>Hydrogen-bonded</td>
<td>Supramolecular Structures</td>
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<td></td>
<td>Hydrocolloidal Aggregates</td>
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Table 4: Hydrogel Classification Criteria [122,123,127,130].

All synthetic hydrogels can be specifically modified to obtain a desired crosslink density, a specific mechanical strength and biodegradation ratio, and the previously mentioned response to the surrounding environment. There are several characteristics that make these covalently cross-linked PEG polymers the most commonly used type of synthetic hydrogel. The non-immunogenic and the non-toxic properties of PEG are two of the reasons why it has been used in several clinical studies and treatments approved by the FDA. The second most used and studied hydrogel systems are those synthesized from PHEMA. The transparent properties of the material and the high stability in water make this a good and safe material for biomedical uses as in the case of contact lenses and drug delivery system (e.g. insulin
carriers[122, 130, 131]. As the rest of hydrogels, cross-linkage plays an important role on the physical and structural characteristics of the material. PHEMA hydrogels are not degradable in physiological conditions. For this reason they have been synthesized with dextran modification to allow enzymes to degrade them.[132, 133] PEG has been approved by the FDA for clinical use due to its biocompatibility, non-immunogenic qualities and low rates of toxicity. With a low capacity to degrade naturally, the peptide sequence of Ala-Pro-Gly-Leu was added to improve the degradation ratio of the hydrogel. UV photo-polymerization is the most common method for the synthesis of PEG [132, 134]. PVA hydrogels have been utilized in tissue engineering for regeneration of artificial articular cartilage and bone-like apatite formation. There are three processes that can be used to obtain PVA from poly-(vinyl acetate). These processes are alcoholysis, hydrolysis, and aminolysis. The use of repeated freezing/thawing technique helps to avoid toxicity from the materials, to enhance structural stability at room temperature, and to develop a higher elasticity ratio. These materials are used as permanent/long term scaffolds since PVA is not degradable in most physiological situations [132, 135]. On the other hand, a plethora of biological macromolecules can be used as natural hydrogels. Among those, alginate and chitosan are commonly used materials that can be found in some types of fungi, bacteria and seaweed on the contrary, fibrin, collagen, and Hyaluronic Acid (HA) are some examples of materials from animal sources. Although these type of materials offer more effective platforms for cellular growth, their mechanical weakness when not chemically modified is one of their drawbacks. As previously mentioned, the strength of the hydrogel depends on the crosslinking of the polymer. To enhance the mechanical properties and subsequently the strength of these hydrogels, crosslinking with UV light or temperature, and mixing with polymeric agents or using chemicals, are techniques that have been employed [122, 130, 131, 136]. Chitosan is increasing in interest as a promising vehicle for the particulate system. The polymer forms a colloidal particle capable of encapsulating GFs, which is accomplished through chemical and ionic cross-linking, as well as ionic complexation. This biomaterial demonstrates antibacterial, anti-inflammatory, and regenerative capabilities [137]. It is easily soluble in the presence of acidic conditions and insoluble in neutral conditions as well as in most organic solvents. [132, 138]. Skop and colleagues investigated central nervous system regeneration through delivery of neuronal stem cells and FBF-2 through the development of a biocompatible chitosan-based films and micro-particles [139]. They were successful in sustaining survival and growth of neural stem cells compared to the control group. Another example is the alginates, usually prepared through gelation. Different strategies have been used to increase mechanical features of alginate such as the blending with either ceramics or other polymers [140]. It possesses many favorable properties such as biocompatibility and low toxicity, but is unable to interact with mammalian cells due to its hydrophilic character, which discourages protein adsorption. To enhance the ligand-specific binding properties, alginate was modified with lectin, specific for protein binding, and in this way provided adhesion, and proliferation capabilities to the modified alginate [132,138,141] Collagen is the most commonly used biological polymer. It represents the most abundant protein of the human body, which exemplifies its superior biocompatibility, biodegradability and weak antigenicity [142]. [143] Chemical crosslinking can improve its physical properties, such as the incorporation of chondroitin sulfate or fibronectin or enhancing the level of crosslinking. [144] Fibrin gels avoid inflammatory reactions and are non-toxic. Additionally the gel itself is capable of being formed in the presence of thrombin and at room temperature by the enzymatic polymerization of fibrinogen. Although widely used in wound healing, due to its capabilities to promote cell migration and proliferation through the incorporation of platelet-derived growth factors, their used have been reduced due to the limitations on its mechanical strength [132, 145].

Hydrogels are used as three-dimensional platforms to engineer new tissues due to their resemblance to the ECM. These materials permit the delivery of cells to a specific site and improve diffusion of GFs and nutrients. The hydrogel can promote a better cell
incorporation, and regulate the function and structure of the new tissue. Cartilage, ligaments, bone, skin and tendons are some of the tissues that have been able to be engineered using this technique [122,130,132]. Kim et al., demonstrated that matrix metalloproteinase sensitive HA hydrogels with BMP-2 delivery could improve bone regeneration in a calvarial defect model [146]. The material maintains the capability to induce calcification through the enhancement of the proliferation of calcium phosphate crystals. This ability is due to the addition of a ceramic phase to the basic composition of the material, which most cases is collagen type I. The most commonly used inorganic phases used are calcium phosphates (e.g. b-TCP, amorphous calcium phosphate, and hydroxyapatite) and bio glasses.

**Scaffolds**

The recent advances in tissue engineering and regenerative medicine have made it possible to incorporate innovative therapeutic approaches in orthopedic field. The capability to deliver GFs and chemo/cytokines, through bio-conductive scaffolds has become a robust approach for surgical procedures, to decrease post-surgical recovery time, increase tissue repair, regeneration and wound healing [147]. Although the idea of delivering bioactive molecules to tissues of interest is not a novel concept, an ideal carrier, able to mimic the autologous steps of the tissue regeneration process, still needs to be designed. For this reason, bolus application of GFs is ineffective for tissue healing; in addition, there are adverse side effects that have been identified (e.g. respiratory compression and dysphagia [10]. A suitable solution to diminish and circumvent this problem is using a functionalized scaffold able to control the GFs release both spatially and temporally [54, 72]. Understanding how GFs can be loaded and released from a scaffold (e.g. loading efficiency) is crucial. Scaffolds enriched with GFs, in fact are proved to reduce complications involved with scaffolds’ implantation [148] and to provide support for the growth and differentiation of different cell types [149]. To date, it is possible to design and functionalize ad hoc materials in order to fulfill the specific needs of the target application and to enhance their bioactivity. Many cellular processes depend on the spatial distribution of the GF. Spatial gradient of GFs can guide the cells away from their source to reach the new forming tissue. As proved by Park et al., scaffolds enriched with PDGF improved bone cell migration and speeded up the regeneration process in bone regeneration [98]. Preliminary in vitro tests are necessary to examine the physical properties of the scaffold in order to ensure long-term success of the implant [149]. Furthermore, scaffold are particularly advantageous as it is possible to tailor their porosity, charge and mechanical strength by introducing various functional groups [150]. The commonly used methods to fabricate GF-loaded scaffolds can be categorized into two main groups:

1. GF attachment to the scaffold
2. GFs embedding within the scaffold.

The first method consists of the adsorption of the GF onto the scaffold or chemically cross-linking the GF to the polymeric matrix. The second one consists in the encapsulation of the GF within the scaffold matrix. Growth factors can also be integrated in the scaffold mesh by layer deposition, by electro spinning or self-assembly techniques [151]. These techniques augment the materials capabilities for controlled release of the payload, avoiding the burst release effect as well as retaining the GFs efficacy over the degradation period. Lee and al. developed a bilayered scaffold for blood vessel restoration using the electro spinning technique [152]. Electro spinning techniques allow incorporating bioactive molecules within the bulk of the nanosized material fibers. The thickness and porosity of the electrospun material can be easily tuned to control the release kinetics of GFs [153]. The electrospun material can be shaped ad hoc depending on which application it will be utilized for. Another advantage to this fabrication method is the large surface area it
retains, which allows for more tissue to come in contact with the bioactive material. Electrospinning has been utilized in various approaches such as for wound healing and nerve regeneration through anastomosis of severed ends with guiding conduits. It has also been used for the augmentation of vascularity in areas of interest with GFs including VEGF and PDGF in vitro. GFs functionalized electrospun scaffolds have been successfully employed also in the treatment of challenging fractures and large osseous defects [154]. Kolambkar et al., proposed using a hybrid system composed by electrospun polymeric tubes filled with an injectable peptide-modified alginate hydrogel. The group successfully tested the ability of this system to deliver recombinant bone morphogenetic protein-2 (rhBMP-2) to repair critically-sized segmental bone defects in a rat model [155]. As previously discussed, in order to enhance wound healing and diminish the adverse effects of the GFs, the release of multiple molecules at the same time and in appropriate doses may be required during treatment. Recently, several efforts have been done to develop methods to spatially control the immobilization of different GFs in distinct volumes in 3D biomimetic scaffolds [156]. Kim et al. showed that two or more drugs could be incorporated within a PLGA-based electrospun mat through electro-spinning [157]. Saif et al. produced a PLGA based scaffolds releasing a combinations of VEGF, hepatocyte growth factor, and angiopeptin-1 in a murine hind limb ischemia model. They obtained an increased cell migration and the incorporation of vasculogenic progenitors, as well as a more efficient vessel stabilization and enhanced capillary density [158]. On the contrary the release of two drugs with distinct behaviors could not be controlled due to the severe burst release of each drug. As a solution, scaffolds with nanostructured delivery systems embedded in the structure represent a promising strategy to obtain better controlled release kinetics of GFs [159]. Kim et al. introduced an interesting microparticle-based scaffold fabrication technique, as a method to create 3D scaffolds with spatial control over multiple bioactive molecules using uniform PLGA microspheres [161]. They demonstrated that embedding the PLGA MPs into the scaffolds led to more sustained release of the payload. Recently, Minardi et al., presented a novel approach for the creation of a multiscale biomimetic scaffold, capable of controlling both spatially and temporally the protein kinetics [162]. This result was achieved due to the natural ability of the collagen matrix to interact with PLGA-porous silicon microspheres (PLGA-pSi), effectively constructing a simple and tunable system for various tissue engineering applications. As above mentioned, the most commonly used natural polymers for tissue engineering applications are collagen/gelatin, chitosan, silk, alginate, and hyaluronic acid [163,164]. Some synthetic polymers are also available but presented some disadvantages. The main drawback with the use of synthetic polymers is their inadequacy during cell attachment. Chemical modification of the scaffold surface can improve the biocompatibility of materials and help cell growth [165]. Cheng et al. functionalized electrospun poly(L-lactide) scaffolds with Ar/NH3 plasma to introduce amine groups in order to ameliorate cell attachment, growth, and infiltration [166]. Ravichandran et al., used a polymer solution of PLLA, poly-(α,β)-DL-aspartic acid (PAA), type I and type III collagen to obtain Nano fibrous scaffolds by electrospinning and then functionalized them to augment the hydrophilicity in order to allow cell attachment [167]. Different BMPs were loaded into diverse delivery systems and then incorporated into scaffolds. This delivery approach ameliorated cell differentiation and provided a better control of the release kinetics. Simson et al., in order to regenerate cartilage, developed a chondroitin sulfate-bone marrow hydrogel mixed with BMP-2 [168]. A major drawback in the use of scaffolds for tissue engineering application is the limited diffusion of oxygen within the bulk structure [169]. When engineers are designing the microarchitecture of the scaffolds they must ensure that cells can penetrate the scaffolds structure but more importantly, that the cells can have access to nutrients until neo-vascularization occurs [170]. It has been proved that an irregular distribution of cells is associated with an uneven oxygen percentage in the scaffolds. Figure 2
Conclusions

Important advancements have been made to understand GF biology, their interaction within the tissue microenvironment and to refine polymeric platform to control the delivery of them in order to enhance tissue regeneration. On the other hand, GFs delivery platforms require improvements related to release control and dosing to guarantee better safety and efficacy. The ability to tune and control the response would be crucial for both understanding and manipulating tissue processes.

Moreover, an ideal delivery system should simulate the natural healing process of the damaged tissue which involves the puzzling concurrence of multiple GFs having each a distinct function within the tissue and performing their functions in a specific sequence and at specific concentrations. For this reason, controlled delivery of a combination of GFs would be a promising approach paying attention to modulate the sequence of the multiple GFs in order to enable the right order of events in the process of tissue regeneration. Developing and refining effective sustained GFs carrier in the following year will have a huge influence on various therapeutic approaches. The material and the form in which it is used could influence and determine important properties of the GFs release; therefore, it will not be unexpected if researching the ad hoc materials lead the way in the future years. Creating and ameliorating GFs delivery platforms, able to provide appropriate chemical and mechanical cues in a timely and spatially controlled fashion, will likely be helpful and of a crucial importance to accurately tune regenerative processes. The use of hydrogels in the medical field is still new, but the possible uses for these platforms are countless and the benefits that they could bring to the world of tissue engineering and drug delivery are immeasurable. There is still work to do on this field but the future looks promising. A large variety of GFs based products have already entered the market, and many others that are currently in clinical trials such as IL-21 and IL-29 (by Zymogenetics) are expected to bear on the market in the near future [171,172].

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Abstract

Due to the increasing annual incidence of neurotraumas, advanced tissue engineering approaches could represent the resolute remedies for patients with strongly lesioned central nervous system and have a great repercussion in the public healthcare cost. Unfortunately the current clinical treatments in traumatic brain injury and spinal cord injury show several limitations. While the clinical management of neurotraumas has been greatly improved in the last years with the development of standardized approaches to patient’s care, numerous clinical trials have been unable to find a specific and effective medical treatment targeting neural regeneration promotion and in reducing mortality or limiting disability following trauma.

Over the last decades, several therapies, coming from tissue engineering and nanotechnology, have demonstrated an unquestionable potential to regenerate damaged tissues and organs by using biomaterials capable of creating favourable microenvironments for tissue ingrowth. Tissue engineering approaches have great prospective also in virgin field like central nervous system regeneration, enhancing the restoration of functional neural circuitries and leading to recovery of lost sensory motor and cognitive capabilities and emotional deficit.

Keywords: Biomaterials; Nanotechnology; Spinal Cord Injury; Tissue Engineering; Traumatic Brain Injury;

Introduction

The nervous system is considered the most complex structure in the body: it works as control center for all the activities (e.g conscious and unconscious), responding to any changes by transferring electrochemical impulses to the involved organs.

The nervous system is classified into the Central Nervous System (CNS) and the Peripheral Nervous System (PNS). CNS is the part of the nervous system enclosed in the meninges and surrounded by cerebrospinal fluid. It consists of the brain, protected by the skull, and the spinal cord protected by the vertebral column. The PNS consists of the cranial nerves...
arising from the brain, the spinal nerves arising from the spinal cord and sensory nerve cell bodies (dorsal root ganglia) and their processes. Peripheral nerves innervate muscle tissue, transmitting sensory and excitatory input to and from the spinal column.

The brain has a fundamental role in the management of all the functions of the body (e.g. consciousness, walk, thoughts, language and memory. Some involuntary movement resulting from a sensory stimulus, known as reflex movements, can happen without the participation of brain structures via spinal cord pathways. Cerebrospinal fluid surrounds the brain and the spinal cord and also circulates within the cavities (called ventricles) of the central nervous system. The lepto meninges surround the brain and the spinal cord. The cerebrospinal fluid circulates between 2 meningeal layers called the Pia matter and the arachnoid (or pia-arachnoid membranes). The outer, thicker layer serves the role of a protective shield and is called the Dura matter.

**Neurotraumas**

Damages to the CNS are destructive due to the scarce capability of the CNS itself to reconnected injured nervous system and restore the functions. Moreover, after an injury, a complex cascade of events causes a secondary damage with significant tissue degeneration.

The ability of the adult neurons of CNS to regenerate after an injury, although restricted respect to the peripheral nerves, could be observed if a permissive environment is provided to the neurons. Even if this was already known at the beginning of the Twentieth Century [1], only 30yrs ago new experiments were performed to confirmed this regenerative theory of the CNS [2]. These new findings demonstrated the importance of the environment in supporting central nervous system.

**Traumatic Brain Injury**

Traumatic Brain Injury (TBI) represents the leading cause of morbidity and mortality in individuals under the age of 45yrs in the world and it presents a major worldwide health, social and economic problem. One-third of severe TBI patients with brain contusion develop intracranial post traumatic poro-encephalic cysts surrounded by a glial scar. TBI cases are becoming an urgent issue in the scientific community due to the ever increasing number of cases and the severity and mortality level of the trauma. In fact, one of the TBI main causes is related to a social need in western countries such as motor transport. The annual incidence of TBI is estimated to be up to 500 per 100,000 inhabitants/year in Europe with an average of 235 cases per 100,000, a mortality of 15 per 100.000 inhabitants [3]. The World Health Organization (WHO) predicts that deaths from road traffic incidents (primarily due to TBI) will double before 2020 and TBI will rise to the third leading cause of global mortality and disability by 2020 (WHO, 2009). The resulting direct and indirect costs to society are extremely high.

The initial inflammatory response after TBI results in neuronal injury and disruption of the blood-brain barrier [4-6]. Microglial cells become activated within minutes and resemble peripheral macrophages by acting as Antigen Presenting Cells (APCs) releasing pro-inflammatory cytokines and chemokine’s [7,8]. Activated microglia also produce other neurotoxic products after injury such as Nitric Oxide (NO) and superoxide free radicals that generate Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS).

A phenomenon that adds to the complexity of regenerative failure is the process of glial scarring or reactive gliosis that has been considered to be one of the major impediments to axonal regeneration [9,10]. Although the functional role of glial scarring is not completely understood, it has been suggested that it presents a mechanism by which CNS restores homeostasis through isolation of the damaged region [11,12]. Astrocytes are thought to play crucial roles in response to injury. While astrocytic hypertrophy, hyperplasia, and glial scar formation have negative effects on regeneration, more recent evidence favors a positive
role for astrocytes in brain injury. Astrocytes produce essential neurotrophic factors (e.g. nerve growth factor, transforming growth factor beta, basic fibroblast growth factor) which are more growth-supporting in the young as compared to the adult brain [13,14]. Insights into the cellular process underlying reactive astrogliosis will provide target for therapeutic intervention designed to minimize astrocytes proliferation.

TBI patients may develop impaired cognition, seizures and PNS injury that could result in severe paralysis. In addition, it seems that TBI patients could have a higher incidence in developing progressive degenerative diseases (e.g. Parkinson’s disease, Alzheimer’s disease).

Current clinical approaches for treating TBI: While the clinical management of TBI has been greatly improved with the development of standardized approaches to patient’s care, in particular for the treatment of intracranial hypertension [15], unfortunately numerous clinical trials have been unable to find a specific and effective medical treatment targeting neural regeneration promotion and in reducing mortality or limiting disability following TBI [16]. Currently, the mainstay of therapy for TBI is the removal of hematomas and the repair of significant skull fractures, along with supportive therapies aimed at maintaining perfusion and oxygenation to tissues. For patients who do not have operable lesions, the control of intra cranial pressure (ICP, <20 mmHg), cerebral perfusion pressure (CPP, >60 mmHg) and systemic, and perhaps local, oxygenation are the cornerstones of intensive care unit management. Other indispensable medical interventions are traditionally employed, for example early enteral feeding, maintenance and control of cerebrospinal fluid volume, and prevention and treatment of the most common complications (hyperthermia seizures, stress-related mucosal bleeding, hypernatremia, pneumonia and venous thromboembolism).

The primary injury in TBI is considered irredeemable, for this reason, the major opportunity for interventions is in reversing or preventing secondary injuries with the aim to prevent further neurological decline. After decades of research, several experimental TBI models have been developed to understand the mechanisms that occur after trauma, and many potential surgical and pharmacological therapeutic treatments have been developed. Over the last three decades, thanks to preclinical studies at least 30 compounds and therapeutic interventions being the subject of more than 50 clinical trials. Despite the encouraging results, non-considerable improvements in the clinical outcome have been reported [17]. Moreover, most of the promising therapeutic strategies obtained after experimental studies in animal models have failed in clinical trial setting of TBI. Glucocorticoids is considered a mainstay of TBI treatment but, as known, it has shown important systemic side effects [18,19]. The trial of magnesium sulfate was stopped when a significantly higher mortality rate was found in magnesium sulfate treatment group than in the control group [20]. More recently, citicoline, a neuro protective endogenous substance showing properties that facilitate neurorepair, seems not to improve the clinical function and cognitive status of patients after injury [21].

Traumatic Spinal Cord Injury

With the term Spinal Cord Injury (SCI) is referred the damage to the spinal cord resulting after trauma (e.g. a road accident), disease or tissue degeneration (e.g. cancer). Even if no reliable estimate of global occurrence exists and annual global incidence estimation is 40 to 80 cases/million inhabitants. Up to 90% of these cases are due to traumatic causes [22-24].

The injury can radically disrupt the normal human spinal cord architecture. SCI is heterogeneous in cause and outcome and it results from contusion, maceration of the spinal cord, compression or penetration [25]. The effects of SCI consist of death of neurons, oligodendrocytes, astrocytes and precursor cells, moreover, the cavities and cysts resulting from SCI may interrupt descending and ascending axonal tracts, though the circumferential white matter is often spared. After the initial insult to the spinal cord, active secondary processes can lead to loss of additional structure, as induced oligodendrocytes apoptosis
and loss of myelin [26]. After human SCI is possible to observe demyelinated axons up to a decade, and several efforts are nowadays invested to understand the survival of unmyelinated axons or the possible process of regeneration of myelin [27,28]. Inflammatory cells (e.g. neutrophils, microglia, macrophages and T cells) have a series of destructive and reparative roles [29]. SCI culminates in glial scarring, a multifactorial process that involves reactive astrocytes, glial progenitors, microglia and macrophages, fibroblasts [30-32]. The scar is often oriented perpendicular to the neuraxis and appears impenetrable. The scar also contains secreted and trans-membrane molecular inhibitors of axon growth [10,33]. Cortical sensory motor zones can functionally reorganize and, at the subcortical level, the rubro-spinal system can reorganize and compensate for much of the function lost after cortico-spinal injury [34]. Therefore, although there is some spontaneous repair after CNS injury, it is incomplete. Additional recovery of function will require a combination of affective and safe therapeutic interventions.

**Current clinical approaches for treating SCI:** For SCI the current clinical treatments are insufficient. If bone fragments exist near the site of injury, then surgery may be performed to reduce any risk of secondary injury.

In the 1990s, the discovery that the steroid drug, methylprednisolone, could reduce damage to nervous cells if given early enough after injury (between 3 and 8 hours after injury) gave an encouraging treatment option [35,36]. This anti-inflammatory drug is often also administered to reduce swelling and secondary injury. However it cannot be said at this point that methylprednisolone has no beneficial effects in the treatment of acute spinal cord injury, but it seems clear that if any benefit exists, it is probably small and has not been demonstrated by the National Spinal Cord Injury Study (NASCIS) studies [37].

Unfortunately, there is currently no treatment available to restore nervous function. After swelling from the injury subsides, patients begin a long period of rehabilitation during which time they train remaining nerves to compensate for the loss due to injury. Improved motor function is often seen in mammals with incomplete and even complete SCI following exercise or rehabilitation [38]. Loco-motor exercise enhances the ability of many spinally transected animals to walk on a treadmill when body-weight support is provided [39,40]. This improvement is related to the maintained activity of spinal circuitries below the injury site that could respond to peripheral inputs and to the considerable plasticity of nervous system [41].

**Tissue Engineering Approaches**

Nervous regeneration and functional recovery subsequent to a traumatic injury continues to be a clinical challenge in regenerative medicine. Over the last decades, several approaches, coming from tissue engineering and nanotechnology have demonstrated an unquestionable potential to regenerate damaged tissues and organs by using biomaterials capable of creating favourable microenvironments for nervous ingrowth.

A gold standard biomaterial has to be biocompatible without inducing inflammatory, immunogenic and cytotoxic responses. Moreover, the scaffold porosity has to mimic the Extra Cellular Matrix (ECM) of native tissue allowing the diffusion of the nutrients and permitting the cell adhesion and migration.

These characteristics have been recently introduced by innovative nanotechnology approaches, which allow the design and modification of nano-scale suitable biomaterials for the scope of Neural Stem/Progenitor (NPCs) migration, proliferation and differentiation on the injured site and subsequent CNS regeneration.

In fact stem cell transplantation presents a viable strategy for the repair of CNS injury. However, following transplantation cell death is prevalent and limits the efficacy of this technique. Two of the factors that contribute to poor cell survival are anoikis (cell
apoptotic response to the absence of cell–matrix interaction) and growth factor withdrawal. Biomaterials can be modified with cell adhesion proteins or motifs to improve cell attachment and minimize cell death caused by anoikis. Furthermore, survival factors, such as growth factors, can be encapsulated into the biomaterial to enhance cell survival.

**Neural Stem/Progenitor Cells**

The mature nervous tissue has been for long time considered incapable of cell renewal and structural remodelling, especially in mammals. The adult brain of both rodents and primates has been shown to embody undifferentiated, mitotically active precursor cells that are multi-potential in nature and can contribute new, differentiated neurons and glia to specific regions of the mature brain such as the olfactory bulb, the hyppo-campus, the cortex and the striatum [42-46]. NPCs displaying stem cell characteristics have been isolated from the adult human brain [47]. NPCs are multi-potential, self-renewing cells that have been known to play a critical role in regeneration of different types of brain damage from neurodegenerative disorders to traumatic brain/spinal cord injuries [48]. The neural stem cells can be found in the Sub-Ventricular Zone (SVZ) and the Sub-Granular Zone (SGZ) [49]. Stimulation of endogenous NPCs, injection of exogenous NPCs or a mixture of both treatments could be efficient in enhancing recovery in neuronal diseases [50].

The NPCs are able to react to several stimuli by proliferation and migration towards the injured sites [51]. Moreover NPCs can also differentiate into differentiated neural cells [52,53]. Severe injuries limit the capability of CNS to regenerate the nervous tissue (e.g. regenerate lost cells, replace damaged myelin sheets and reestablish functional neural connections [54]. This limitation is due to the insufficient recruitment of NPCs, migration difficulties and/or the lack of cell differentiation [54,55].

Moreover the source of somatic neural stem cells for clinical applications has been a major issue for many years in the field of CNS regeneration. Recently the development of reprogramming techniques has allowed reprogramming of adult fibroblasts into embryonic-like stem cells (called induced pluripotent stem cells, iPSCs), from which NPCs can be derived and used for clinical applications. However, the administration of NPCs derived from iPSCs possesses some risks associated with the capacity of non-converted pluripotent cells to generate teratomas in vivo. In the last couple of years technologies have been developed to directly reprogram fibroblasts into NPCs, such cells are called induced neural stem cells (iNSCs) [56]. These cells have been shown to be genetically identical to somatic NPCs and they overcome the ethical issue.

**Biomaterials for Nano-scaffolds**

The principle aim of tissue engineering is to design a well-defined biomimetic environment that surrounds the cells and promotes specific cell interactions in order to control and direct cell behaviour to reassemble into structures that resemble the original tissue.

Biomaterials must be able to match to the sizes of the injury and maintain a proper shape after implantation. Extra features to be considered are the immune response, the biomaterials sterilization, the degradation rate and the capability of the biomaterial itself to be used as a controlled drug/growth factors delivery system.

The biomaterials used for enhancement of nervous regeneration can be divided in natural, synthetic and semi-synthetic materials.

Natural materials are attractive for neural regeneration due to several properties. The most important is that they exhibit similar features to the native tissue as cell adhesion domains.

Collagen, fibronectin and hyaluronic acid are the main structural proteins of the ECM and continue to be the common natural materials employed in the field of tissue engineering.
The use of natural biomaterials has been reported in several studies in peripheral nerve regeneration in spinal cord healing and scar formation [57-60]. Notwithstanding their advantages natural materials may cause immuno response, if they are not well purified. Moreover natural materials are less flexible to modification compared to synthetic materials, which are cheaper and more easily characterized.

Synthetic materials have several advantages for their use as tissue engineering scaffolds. They are cheaper and more easily characterized. Moreover they can be custom-made by reacting together diverse polymers to combine the features that are unique to each. For example it is possible to obtain biomaterials with different mechanical properties changing the ratio of two or more polymers. Clearly, scaffolds fabricated from biodegradable polymers are preferable to non-biodegradable polymers because of the advantage of avoiding a second surgery to remove the scaffold.

Several tubular nerve guides are made by poly (α-hydroxy esters). With these synthetic polymers is possible to easily obtain bioresorbable tree-dimensional scaffolds that degrade via hydrolysis in CO₂ and H₂O.

Several studies, mainly focused on the PNS regeneration, have synthetized tubular nerve guides, that have shown minimal inflammatory response, made of Poly(Glycolic Acid) (PGA), Poly(Lactide Acid) (PLA), Poly(Lactide-Co-Glycolide Acid) (PLGA), Poly(L-Lactic Acid) (PLLA), Poly(L-Lactic Acid)-Caprolactone (PLLA-PCL) and Poly(DL-Lactide-Co-Glycolide) and Poly(ε-Caprolactone) (PCL/PLGA) [61-63].

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Several products are already in clinical applications for nerve regeneration (e.g. Stryker NeuroMatrix, Integra NeuraGen, Axogen Avance Nerve Graft, Polyganics Neurolac), unfortunately for spinal cord lesion none of the above mentioned solutions have yet reached the clinic applications.

The greatest disadvantage of synthetic materials, however, is the lack of cell-recognition signals and presenting therefore few cellular interactions. Toward this end, efforts are being made to incorporate cell-adhesion peptide motifs into synthetic biomaterials.

The combination of synthetic materials with cell-recognition sites of naturally derived materials is very attractive.

Several studies focused on the design of these semisynthetic biomaterials that due to their characteristics hold clinical capacity to promote tissue regeneration. Once transplanted to the injury site, these biomaterials could also actively and temporarily contribute in the tissue regeneration process by providing a scaffold on which cell-triggered remodelling could occur [64,65].

Hydrogels are very promising biomaterials for nervous system regeneration. Hydrogels characteristics as tissue-like mechanical properties, high water content or the possibility to inject them in the injured site, enhance cell attachment and growth in both TBI and SCI conditions. Self-assembling process is an essential event that often happens in nature and allows the formation of material patterns or structures without external influence but only thank to electrostatic forces, hydrophobic interactions and hydrogen bonding. It is a key concept in “bottom up” synthesis and individual molecular units represent the main actors in simple or complex hierarchal cascade of events including lipid bilayers (micelles), DNA, proteins and viral capsids formation [66]. The key in self-assembled structures is design of base units that have two distinguishing components: a segment for directing the aggregation process and a biologically active moiety that encodes cell-specific instruction [66]. Self-assembling peptides consist in a well-defined amino acid sequence that self-assemble under physiological conditions forming a nano scaffold with very thin fibers (~10 nm in diameter).

In the last few decades, they have been studied as biomaterials not only useful for specific 3-Dimensional (3D) tissue cell cultures but also for tissue repair and regenerative therapies.
These peptide scaffolds can be commercially and custom-tailor synthesized, modified readily at the single amino acid level at will inexpensively and quickly. Furthermore, these designer self-assembling peptide scaffolds have recently become powerful tools for regenerative medicine to repair infarctuated myocardia, to stop bleeding in seconds and to repair nervous tissue, as well as being useful medical devices for slow drug release [67-70].

**Innovative Nanodesign**

The future of regenerative medicine technology depends on optimization of the scaffold geometry, mechanical properties and cross-sectional area, in order to increase the number of cell survival/differentiation and to restore tissue functionality.

The development of nanofibers has greatly enhanced the scope of fabricating scaffolds that can potentially meet this challenge. Tissue engineering scaffolds should be analogous to native ECM in terms of both chemical composition and physical structure. For the achievement of this goal, functionalized nanofibers are continuously developed in order to induce the best tissue response and to enhance regeneration. In native tissues cells are often regularly oriented and this cell orientation is of crucial importance for tissue function. This is particularly true for neural tissue, where neuronal/axonal growth have a precise directionality. The scope of the application of aligned polymer nanofibers in tissue engineering is exactly to control cell orientation. Aligned electro-spun PLLA nano/micro fibrous materials investigated the suitability of the scaffold for NSC culture in terms of their fiber alignment and dimension. The results suggest that the aligned nanofibers help to improve neurite outgrowth when they are in a highly oriented status, whereas the fiber diameter do not show any significant effect on cell orientation. On the contrary, the NSC differentiation rate is higher for the nanofibers scaffold than that of microfibers, but it was independent of the fiber alignment [71].

For tissue engineering scaffold applications, different fiber diameters are required depending on the tissue. The typical diameter of proteins in native ECM range from several tens to 300 nm, therefore nanofibers are usually desired. In electro-spinning, self-assembling and phase separation techniques the diameter of the nanofibers should be controlled by adjusting parameters including polymer concentration, amino acid sequence, flow rate of the polymer solution, solvent conductivity and temperature [72].

Surface modification of biomaterials, with the intent to improve not only biocompatibility but also target cell/tissue response has been extensively studied in order to recreate the native nervous structure and to do it in the shortest time possible. An increasingly employed approach for emulating the ECM involves identifying bioactive motifs present in these molecules and grafting synthetic analogues of these signals onto a material. For example, cells engage with ECM ligands via receptors such as integrins. They are known to bind to several common polypeptide motifs like Arginine-Glycine-Aspartic Acid (RGD). The possibility to include these motifs in the biomaterials structure can promote cell interactions and enhance nervous regeneration.

An important feature for promising biomaterials is its porosity. Pores positively influence the size of Tissue Bridge by allowing inward diffusion of growth factors and ECM proteins, and outward diffusion of waste products. Hydrogel porosity is essential to stabilize the post-injury environment by permitting nutrient flow into and out of the scaffold. Porosity also affects cell infiltration, cell distribution, as well as cell growth, proliferation, vascularization and local angiogenesis [73]. Hydrogel interaction with host cells is also largely a result of the hydrogels’ chemical and mechanical compatibility. Mechanically, injectable hydrogels with storage moduli similar to surrounding tissue have been the most successful because they are able to more closely mimic the native environment [73,74].

An additional consideration when using biomaterials in vivo is the shape of the biomaterial. In vivo injuries result in cavities of varying size and shape and subsequently biomaterials
that can adapt to these complex shapes are desirable. Moreover, injectable biomaterial setting and resulting structures can easily be tuned by controlling the crosslinking density in gel matrix [75].

Furthermore, injectable biomaterials confer an additional advantage as they offer an easy method of delivery [76-78] since they may dictate not only where a drug is delivered, but also when and with which interval it is released [77]. The stimuli that induce various responses of the hydrogel systems include physical (temperature, electric fields, light, pressure, sound and magnetic fields), chemical (pH, ions) or biological/biochemical (biomolecules) ones [77]. Temperature-sensitive hydrogels are probably the most commonly studied class of environment-sensitive polymer systems in drug delivery research. These hydrogels are able to swell or shrink as a result of changing in the temperature of the surrounding fluid (e.g. physiological temperature).

References


