3-D glass-derived scaffolds for bone tissue engineering: current trends and forecasts for the future

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Abstract

Biomaterials used in regenerative medicine are often designed to act as 3-D porous templates (scaffolds) able to support and promote the growth and repair of natural tissues. Some types of glasses have a great potential as scaffold materials, as they can bond to host bone, stimulate bone cells towards osteogenesis and resorb at the same time as the bone is repaired. This review article highlights the evolution of glass-based scaffolds for bone tissue engineering; specifically, the features, limitations and advantages of the different types of glass-derived scaffolds proposed in the literature (macroporous glass-ceramic, sol-gel glass, composite, graded, hybrid and hierarchical implants) are critically examined, discussed and compared. Future directions for the research are also suggested, highlighting the promise of multifunctional systems combining bone regeneration and drug release abilities, the increasing role of microtomographical analysis for scaffolds investigation and the potential of stem cells incorporation into scaffolds.

Keywords: Porosity; Bioactivity; Hybrid; Hierarchical systems; Bone regeneration.

INTRODUCTION

Bone is a connective tissue exhibiting excellent properties of mechanical resistance especially due to its unique structure, in which cells are encased in a composite matrix essentially formed by collagen fibres and apatitic mineral phase. Bone is usually in need of regeneration or substitution due to tumours removal, trauma or age-related pathologies, such as osteoarthritis and osteoporosis. Two alternatives are possible for bone replacement: (i) transplantation or (ii) implantation.[1] Transplants can be made by using living or non-living tissues; at present, the commonly recognized "gold standard" in reconstructive bone surgery consists in the use of autografts, that involve harvesting the patient's own tissue from a donor site and transplanting it to the damaged region. Autologous bone cause no immunological problems but can be collected in limited amount and its harvesting can induce death of healthy tissue at the donor site; in addition, problems related to second site morbidity, mismatching in mechanical properties with respect to host bone and tendency towards resorption may occur. A partial solution to these drawbacks is the use of allografts, *i.e.* the transplant of bone tissue from another living patient or from cadavers. However, allografts can cause disease transmission and carry the need of immunosuppressant drugs administration; furthermore, ethical and religious issues limit their use.

Implantation involves the substitution of damaged tissues by using, in most cases, man-made biocompatible materials that are designed to act as scaffolds, *i.e.* porous templates with proper 3-D architecture able to promote tissue regeneration and/or remodelling. The general requirements featuring an ideal scaffold for bone tissue engineering have been outlined by several authors[2-4]; briefly, the scaffold is required to (i) act as a 3-D template for bone in-growth, (ii) produce non-toxic degradation products, (iii) promote osteogenesis by inducing cells adhesion and proliferation, (iv) bond to the host bone creating a stable interface without the formation of scar/fibrous tissue, (v) possess mechanical properties matching those of natural bone, (vi) be tailored to match the shape and size of bone defects and (vii) be fabricated and sterilized according to international standards

for commercial production and clinical use. In addition, if the scaffold is temporary, it must resorb at the same time as the bone is repaired.

Bioactive glasses, due to their versatile properties which can be properly designed depending on their composition, are very attractive materials for producing scaffolds devoted to bone regeneration.[5] The first bioactive glass, belonging to the SiO_2-Na_2O-CaO-P_2O_5 system (Bioglass®), was synthesized by Hench and co-workers in the early 1970s[6]; since then, many other silicate, borate and phosphate glasses have been proposed by materials and medical researchers for bone tissue engineering applications. However, most of such glasses have been produced and tested in form of powders, particles, granulates or dense bulk of various shape and size, and only a limited group of them has been chosen to produce porous scaffolds.

In this work, glass-based scaffolds will be classified in three main groups: (i) glass/glass-ceramic scaffolds with porosity at the macro-scale (the macropores size is over 50 nm, according to IUPAC classification), (ii) glass/polymer porous composites and (iii) glass/glass-ceramic scaffolds with hierarchical porosity in the macro- (> 50 nm) and meso-range (2-50 nm). The main features of the scaffolds belonging to these three classes and available in the literature will be concisely examined, discussed and critically compared also in the light of recent advances and findings. This review is especially dedicated to 3-D bone tissue engineering scaffolds; the use of glasses in soft-tissue engineering – *e.g.* for the fabrication of fibre-based constructs as nerve guides – will be mentioned only, when necessary, for purpose of completeness. To avoid any confusion on pore classification depending on their size, the authors strictly refers to IUPAC terminology.

GLASSES FOR TISSUE ENGINEERING APPLICATIONS: SHORT OVERVIEW

In the past, the research for biomaterials to be implanted for bone replacement especially focused on as inert as possible materials that did not interact with biological environment. However, since the last 1960s the attention moved towards materials exhibiting chemical and crystallographic

similarity to natural bone mineral, such as hydroxyapatite (HA), fluoroapatite and other calcium phosphates. Finally, in the last decades bioactive materials able to stimulate bone regeneration have mainly attracted researchers' interest. A comprehensive picture about the ceramics used in medicine has been recently provided by Chevalier *et al*.[7] As regards the biomaterials devoted to hard-tissue substitution, bioactivity has to be intended as the ability of the material to bond to bone creating a stable interface with patient's host bone and, eventually, promoting natural bone regeneration.[6] Essentially, this ability is possible thanks to the formation on the biomaterial surface of a HA or apatite-like layer, mimicking the chemical and crystallographic features of bone mineral.[8]

From a compositional viewpoint, bioactive glasses can be basically divided into three groups, depending on the main oxide present in the composition that generates the network (former oxide): (i) SiO_2-based (silicate), (ii) B_2O_3-based (borate) and (iii) P_2O_5-based (phosphate) glasses. Specifically, the first group includes those glasses having a silica content below 60% mol., that was found to be the condition necessary, in melt-derived silicate glasses, for exerting bioactive responses.[8] Borate glasses are, at present, less known and investigated with respect to the other two groups, in spite of their very interesting bioactive properties, even superior to those of silicate glasses.[9,10] As far as the third group is concerned, it should be underlined that phosphate-based glasses may be both bioactive and bioresorbable[11]; however, most of studies has been focused especially on the resorption ability of phosphate glasses, and the literature about their bioactive behaviour is relatively scarce – also because their bioactivity has been found quite lower than that of silicate/borate glasses.[11]

Above SiO_2, B_2O_3 and P_2O_5, various amounts of other oxides can be added to impart peculiar properties to glass; for instance, CaO, K_2O, Na_2O and MgO are useful to adjust the rate of bioactivity process; ZnO, CuO, AgO and TiO_2 allow the release of proper ions that can impart anti-bacterial properties to the material; Al_2O_3 is helpful to strengthen the mechanical properties of glasses. It should be underlined that even a small variation in glass composition can deeply modify

the features of the material: in fact, different percentages of the same oxides will turn the glass properties towards bioinert, bioresorbable or bioactive.

In addition, some ions can be incorporated in glass composition especially to give the biomaterial useful and appropriate properties in the context of bone tissue regeneration. For instance, zinc[12,13] and magnesium[14] are known to exert a stimulatory effect on osteoblasts proliferation, differentiation and bone mineralization. Some research works carried out in the last two years have also demonstrated the great potential of strontium incorporation in the formulation of bioactive glasses as Sr^{2+} ions, when released from the glass, can reduce bone resorption, stimulate osteogenesis and accelerate bone healing processes.[15-17] In such a context, some excellent works highlighting the great potential of the "genetic design" of bioactive glasses have been recently published.[18,19]

Bioactive glasses are commonly produced by traditional melting-quenching routes or via sol-gel technique. Melt-derived glasses can be poured into moulds to produce rods, bars or as-cast components of various size and shape. The melt can also be quenched in cold water to obtain a "frit", *i.e.* granules and pieces of different size that can be easily powdered. Finally, the glasses – especially the phosphate ones – can be also spun to fabricate glass fibres, that in the last decade have attracted increasing interest for application in soft-tissue engineering as guides for muscle or nerve repair.

As above mentioned, the silica content should be less than 60% mol. to allow the glass to bond with bone, as shown by Hench.[8] However, HA layer formation and bone bonding can be also achieved with glasses with up to 90% mol. silica if the glass is obtained by a sol-gel process.[20] In general, sol-gel glasses were found to form the HA surface layer more rapidly than melt-derived glasses.[21]

Glass can be converted by heating into a glass-crystals composite containing various kinds of crystalline phases with controlled sizes and content depending on the thermal treatment parameters. Generally, the resulting glass-ceramic exhibits superior mechanical properties with respect to the parent glass, specifically higher elastic modulus, hardness, failure strength and wear resistance.

FIRST-GENERATION GLASS-DERIVED SCAFFOLDS: GLASS/GLASS-CERAMIC MACROPOROUS STRUCTURES

Various glasses of biomedical interest have been used as starting materials for fabricating porous scaffolds mimicking the 3-D trabecular architecture of natural spongy bone. Table I, compiled on the basis of the data available in the literature, reports an overview of the glasses specifically used for fabricating scaffolds; the features of the scaffolds produced from such materials, listed in Table II, will be described and critically examined in the following sections. It should be underlined that all known methods for producing inorganic glass-derived scaffolds (polymer/glass composite scaffolds are an apart category) require sintering treatments to ensure structural integrity; in most cases, the thermal treatment is carried out above the crystallization onset temperature and, therefore, the so-obtained sintered scaffolds are actually glass-ceramic due to crystals nucleation and growth.

Bioglass®: a pillar of bone tissue engineering

The invention of Bioglass®, synthesized for the first time about 40 years ago, led to a deep revolution in conceiving the role of biomaterials in human body: the perspective progressively moved from the seek for inert materials to be implanted towards the development of bioactive materials able to effectively promote natural tissue regeneration. Since the early 1970s, hundreds and hundreds of papers have been published about Bioglass® properties and applications. Just to cite a few examples, Peitl *et al.*[22] and Clupper *et al.*[23] examined in detail the effects of crystallization on the bioactivity of thermally treated glass-ceramic Bioglass® (GC-Bioglass®); Lefebvre *et al.*[24] and Huang *et al.*[25] investigated and modelled the sintering behaviour of Bioglass®; Xynos *et al.*[26,27] as well as Hench[28,29] studied the influence of the ions released by the glass on cells cycle and demonstrated that genetic design of bioactive glasses will be not only a dream in the next future. Recently, Hench published a very comprehensive review describing in detail the stages

involved in Bioglass® synthesis, biochemical/structural/biological testing and eventual commercialization.[30] Bioglass® is in clinical use since 1985 in form of fine particulate for dental application (Perioglas, NovaBone, US); in addition, since the mid 1990s to present Bioglass® has been also commercialized worldwide in form of dense blocks and granulates of various size for bone defect filling and orthopaedic applications.

The development of effective highly-porous Bioglass® scaffolds for clinical use is currently in progress: in fact, although Bioglass® was demonstrated to be an excellent bioactive material for promoting bone tissue regeneration, all porous bodies produced from it exhibited relevant brittleness and poor mechanical strength, as will be discussed later.

Glass scaffold beginning: porous sol-gel glasses

The first attempt to produce bioactive glass-derived scaffolds was carried out in 2002 by Sepulveda *et al.*[31] by using the sol-gel foaming process. It should be noticed that bioactive glass foams based on 58S sol-gel glass, and not on the so-called Bioglass®, have been manufactured. Specifically, the synthesis method involved the sol foaming with the aid of a surfactant; on gelation, the spherical bubbles formed after sol shaking become permanent in the gel and led to a spongy structure similar to that of trabecular bone. Sol-gel glasses, as well as their derivative scaffolds, exhibit an excellent bioactive behaviour showing HA-forming kinetics faster that traditional melt-derived glasses.[32] This is due to the high surface area available for ion-exchange phenomena with biological fluids: in fact, sol-gel foaming lead to scaffolds exhibiting a bimodal porous structure, constituted by large pores up to 500 μm connected by pores windows (10-100 μm) (Figure 1a) and a random-like mesoporous texture (10-50 nm). A very detailed and interesting study about the kinetics of apatite crystals formation on SiO_2-CaO sol-gel glass scaffolds during immersion in SBF was recently carried out by Fitzgerald *et al.*[33] These scaffolds can be considered the precursors of the third-generation scaffolds, in which, however, mesopores are arranged according to an ordered symmetry. We chose to present

sol-gel glass scaffolds here due to chronological reasons, as they were the first bioactive glass scaffolds to be fabricated.

Many variables of sol-gel processing can be used to control the scaffold pores network structure, *e.g.* surfactant agent and glass composition.[34,35] Jones *et al.*[36] showed that by properly adjusting the sintering temperature of 70S30C glass foams the compressive strength of final scaffolds (Table II) can be increased from 0.3 up to 2.2 MPa; however, this range of values is dramatically lower than the strength of spongy bone (2-12 MPa) as well as the strength exhibited by other bone substitutes, such as clinically used HA (~6 MPa).[8,37] The 3-D porous morphology of sol-gel foamed scaffolds has been recently investigated in detail by means of non-destructive X-ray computed microtomography (μCT)[38] and was found very similar to the trabecular 3-D architecture of cancellous bone. However, it can not be ignored that these scaffolds are quite brittle and, therefore, are not suitable for bone replacement in dynamic high-load environments, such joint regions.

Recently, Rainer *et al.*[39] fabricated bioactive glass-ceramic scaffolds via *in situ* foaming starting from sol-gel 70S26C glass powders. This technique involved the dispersion of sol-gel glass powders in an appropriate liquid monomers batch; after complete polymerization, glass-loaded polyurethane foams were obtained, and a final thermal treatment allowed the polymer burning-out and the sintering of glass particles. The glass-loaded foams underwent severe shrinkage (>75%) during sintering and, therefore, were characterized by relatively low porosity (48 %vol.) in comparison with spongy bone. The scaffolds were bioactive and the proposed fabrication method is very interesting and versatile for producing patient-tailored grafts, but no indications about scaffold strength were presented.

Bioglass®-derived scaffolds

In 2006, Chen *et al.*[40] prepared for the first time Bioglass®-derived scaffolds via sponge replication method (Figure 1b). A remarkable advantage of this method is the easiness to tailor the final

scaffold in terms of size and shape: in fact, the polymer template can be properly pre-formed and the scaffold geometry can be designed by taking in account the sample volumetric shrinkage due to sintering. The ability of such scaffolds to match bone defect dimensions is an important advantage for surgeons in the course of graft implantation procedures. However, the mechanical strength of the foam-like GC-Bioglass® scaffolds proposed by Chen *et al.*[40] was one order of magnitude lower than that of cancellous bone (Table II); this can be attributed to Bioglass® sintering behaviour that led to hollow trabeculae in GC-Bioglass® final scaffolds (Figure 1c).[25] It should be underlined that a so poor strength is a tremendous drawback that prejudices the clinical use of scaffolds, as difficulties related to samples manipulations by surgeons during surgical procedures, as well as problems of scaffold integrity in the early days after implantation, will occur. Some authors showed that the compressive strength of a scaffold can significantly increase *in vivo* due to tissue in-growth: in fact, cells adherent on scaffolds, newly formed tissue and the scaffold itself create a biocomposite *in situ*, thereby increasing the time-dependent scaffold strength.[41]

More recently, Wu *et al.*[42] prepared GC-Bioglass® scaffolds by using rice husk as pore former additive (Table II). The mechanical strength of such scaffolds is comparable to that of spongy bone and allows to avoid problems of scaffold integrity during handling and implantation, but their porosity content is below the lower threshold required for porous bone grafts (> 50%vol.).

Therefore, although Bioglass® has demonstrated to be an excellent bioactive and biocompatible material and it has been in clinical use since 25 years, a lot of work is to be done to optimize the mechanical competency of porous scaffolds derived from it in such a way to make them actually suitable grafts for clinical applications.

Scaffolds based on recently developed melt-derived silicate glasses

The compositions of new silicate glasses proposed in the literature since the early 2000s and specifically used for producing bone tissue engineering scaffolds are listed Table I. It should be

underlined that glass compositions must be carefully selected in order to impart biocompatible and bioactive properties to final materials. Almost all methods used for scaffolding require a sintering treatment and, therefore, both the crystalline phases nucleated and grown during the thermal treatment and the residual amorphous phase should not induce toxic effect on cells or negatively affect the kinetics of bioactive process.

The fabrication method deeply influences the structural properties of final scaffold, especially as far as pores features and mechanical strength are concerned (Table II). As already mentioned, the sponge replication, being easy, quick, versatile and inexpensive, is a very effective processing technique for making scaffolds. The morphology of foam-derived scaffolds[43-45] is quite similar – in terms of pores content, size and 3-D architecture – to that of natural cancellous bone (Figure 1d); however, sponge replication usually led to scaffolds with low mechanical strength[43-45] in comparison to cancellous bone (2-12 MPa)[8,37] (Table II). Vitale-Brovarone *et al.*[44] fabricated foam-like GC-CEL2 scaffolds exhibiting a compressive strength up to 1 MPa and an excellent biocompatibility with osteoblasts; very recently, the same research group successfully optimized the process parameters to obtain scaffolds with even higher strength (5-6 MPa[46]) actually within the range assessed for spongy bone. This achievement can be mainly ascribable to the soundness of GC-CEL2 scaffolds trabeculae, that, differently from sponge-replicated GC-Bioglass® scaffolds[40], did not present inward cavities (hollow struts).

It is interesting to compare the results – in terms of scaffold structural and mechanical features – obtained by using sponge replication method or organic additive burning-out techniques to the same starting glass. Specifically, by having a look at the features of GC-CEL2 and GC-FaGC scaffolds reported in Table II, it is evident that scaffolds produced by using polyethylene (PE) burning-out method[47,48] show mechanical strength higher than that the corresponding ones obtained via sponge replication[43-46]; however, it must be taken in account that this is possible at the expense of graft's architectural similarity with natural bone. In fact, in such scaffolds the pores are separated by dense regions without the presence of the trabecular structure observed in the samples manufactured via

replication method[43-46]: therefore, the higher mechanical strength is negatively balanced by a morphology quite different than the one featuring cancellous bone and by a lower degree of pores interconnection. The absence of an actual trabecular morphology, mimicking that of cancellous bone, also characterized other two kinds of scaffolds derived from experimental glasses, *i.e.* GC-SNCM[49,50] and GC-SCK[51] scaffolds. Scaffolds obtained by porogen additive burning-out, although having a low degree of pores interconnection, can be suitable for substituting load-bearing bone portions thanks to their high mechanical strength and good bioactivity.

For purpose of completeness, a few remarks about the peculiar bioactive behaviour of SCK are presented. At present, SCK is the only Na_2O-devoid silica-based bioactive glass used for making scaffolds, apart from sol-gel glasses (Table I). It is interesting to notice that the first stage of SCK bioactivity mechanism involves H^+/K^+ exchange phenomena, differently from Na_2O-containing bioactive glasses. K^+ has a quite large ionic radius (1.33 Å) in comparison with Na^+ (0.95 Å); therefore, K^+ release has a high disrupting effect on glass network enhancing SCK/GC-SCK specific surface area and reactivity. [51] In many glasses, both Na_2O and K_2O are present, as shown in Table II; in the case of GC-SCK scaffolds, the material reactivity is so high that cytotoxic effects on cells were detected due to an excessive pH increase (over 9).[51]

As previously mentioned, the currently used processing techniques comprise a thermal treatment for glass particles sintering and, therefore, lead to glass-ceramic scaffolds. An interesting exception is represented by 3-D foam-like scaffolds based on the so-called 13-93 glass, that have been recently proposed by Fu *et al.*[52]: in fact, X-ray diffraction investigations demonstrated that the final scaffold remained amorphous. This can occur thanks to the peculiar sintering behaviour of 13-93 glass: in comparison with Bioglass®, for instance, 13-93 glass has more facile viscous flow behaviour, less tendency to crystallize and, therefore, a longer "sinterability window".[53] As reported in Table II, the porosity content and mechanical strength of 13-93 glass scaffolds are actually comparable to those of spongy bone; in addition, 13-93 glass has been approved for *in vivo* use in Europe. Therefore, 13-

93 glass can be an effective resource for fabricating bioactive and mechanically competent scaffolds suitable for clinical applications.

An open and challenging field of research concerns the chemo/physical treatment of glass/glass-ceramic scaffolds for imparting them peculiar properties: for instance, Vitale-Brovarone *et al.*[43] recently proposed Ag-doped GC-FaGC scaffolds able to exert local antibacterial activity.

Borate glass-derived scaffolds

Bioactive glasses are, traditionally, silicate glasses in which silicon dioxide (SiO_2) acts as glass network former. In the last 1990s, Brink *et al.*[9] proposed for the first time boro-silicate glasses for biomedical applications, in which variable amounts of B_2O_3 were added to glasses composition to adjust their bioactive properties.

Because of their lower chemical durability, borate glasses react faster and convert more completely to HA than Bioglass®, according to a mechanism recently proposed by Huang *et al.*[10]: in fact, partial or full replacement of SiO_2 with B_2O_3 in glass composition results in a marked increase in the conversion of the glass to HA in aqueous phosphate solutions. The bioactivity of borate and boro-silicate glasses, as measured by its conversion rate to HA, can be varied over a wide range – from hours to months – depending on the glass composition. Thanks to their rapid and controllable conversion to HA, B_2O_3-containing bioactive glasses are attractive candidates for making scaffolds; in addition, from processing viewpoint, some borate/borosilicate glasses have been observed to undergo viscous flow sintering more readily than Bioglass®, thereby providing a more easily sinterable bioactive glass system for producing scaffolds.[54]

At present, a relatively low number of studies on B_2O_3-containing glasses for biomedical applications has been carried out in the literature, essentially by three independent research groups affiliated to Missouri University of Science and Technology, East China University of Science and Technology and Tongji University of Shanghai. Scaffolds derived from borate glasses have been

proposed for the first time in 2005 and produced by soft pressing of glass powders (Table I) followed by sintering treatment.[55,56] More recently, the interest of many researchers has been focused on 13-93B2 glass (Table I) as promising material for making foam-like scaffolds. Fu *et al.*[57] used polymer foam replication to successfully produce 13-93B2 glass scaffolds with microstructure nearly identical to human trabecular bone. However, biocompatibility tests showed that borate ions, leached out of the glass, inhibited the proliferation of bone marrow stromal cells if the boron concentration was above a certain threshold value (0.65 mM).[57] The toxic effect on cells could be alleviated by partial conversion of the borate-based glass to HA prior to cell culture, or by adopting dynamic cells culture conditions or else by carrying out proper dilutions of the phosphate solutions.[58] The strength of 13-93B2 glass scaffolds was increased up to 10 MPa (pores content ~70%vol.) by carefully optimizing the processing schedule adopted for scaffolds fabrication.[59] The mechanism of conversion of 13-93B2 scaffolds in HA after soaking in dilute phosphate solutions has been recently investigated in detail by Liu *et al.*[60] The peculiar bioactive properties of sponge-derived borate glass scaffolds[60], as well as their mechanical competence[59] and structural similarity to trabecular bone, make them very promising candidates for clinical applications as bone grafts (Table II). However, as demonstrated by Liu *et al.*[61], the progressive material degradation carries a significant drop of 13-93B2 scaffold strength (from 6.2 to 2.8 MPa after soaking for 15 days in phosphate solution).

Phosphate glass-derived scaffolds

In the last years, one of the major challenges of tissue engineering has concerned the design and development of materials able to safely dissolve once they have performed their function, thereby leaving the body to remodel the tissue to its natural form. For this purpose, since the last 1990s a novel group of glasses in which P_2O_5 acts as network former oxide has been proposed; the asymmetry of the [PO_4] tetrahedron unit, which represents the structural unit of phosphate glasses,

is believed to be the origin of their low durability, together with the ease of P–O–P bonds hydration.[62,63] Phosphate glasses can really have a great potential in regenerative medicine because their solubility is strongly dependent on their composition; therefore, their dissolution rate can be foreseen and tailored by adding metal oxides, such as TiO_2[64-66], CuO[67] and Fe_2O_3[68,69] to the glass composition. The two interdependent steps that take place during glass dissolution are hydration reaction, with a Na–H ion exchange, and phosphate network breakage in the hydrated layer due to the cleavage of P–O–P bonds.[63] Due to their versatility, phosphate glasses have been widely studied as controlled release vehicles of antibacterial ions, such as silver, copper, zinc, gallium[64-70]; in addition, they were proposed as smart materials for soft-tissue engineering. In fact, phosphate glass nerve guides, like tubes or meshes, have been developed and tested *in vivo*[71,72] with good results and 3-D constructs for the repair of the muscular tissue have been also studied.[73] In the field of hard-tissue engineering, they have been also proposed in the context of bone tissue regenerative materials especially in form of bulk or powders, alone or with polymers in composite materials.[11,64,65,74] It is interesting to mention that Abou Neel *et al.* very recently reported a detailed physical and structural characterization of a phosphate glass belonging to the Na_2O-CaO-SrO-P_2O_5 system and suggested its use as a bone regenerative material[75]: strontium-containing glasses can be promising materials in bone tissue engineering as strontium is known to reduce bone resorption and accelerate bone healing processes.

At present, very few phosphate glasses (Table I) have been specifically used for fabricating 3-D glass-derived scaffolds for bone grafting. In 2004, Navarro *et al.*[76] successfully fabricated 3-D trabecular scaffolds from phosphate glass by H_2O_2 foaming. By varying the amount of incorporated H_2O_2 and the thermal treatment conditions, the total pores content and size, as well as the percentage of crystallinity could be modulated. More recently, Vitale-Brovarone *et al.* manufactured phosphate glass-ceramic scaffolds both via PE burning-out[77] and sponge replication[78,79] by using ICEL2 powders as glassy inorganic phase. ICEL2 composition (Table I) was designed by modifying that of silicate CEL2 glass: specifically, the molar amounts of SiO_2 and

P_2O_5 in ICEL2 composition are inverted in comparison with CEL2 one. GC-ICEL2 scaffolds were found to be resorbable as, after soaking in different media (water, Tris-HCl, SBF), they underwent a process of continuous dissolution whose rate was both medium-dependent and time-dependent. In addition, GC-ICEL2 scaffolds were also bioactive, as a HA layer formed on their trabeculae after soaking in SBF. Bone marrow stromal cells cultured on the scaffolds maintained their metabolic activity, proliferation ability and seemed to be stimulated towards differentiation.[79] Very recently, Cai *et al.*[80] proposed the phosphate glass PG1 (Table I) as reinforcing phase in β-TCP-based scaffolds (percentage weight ratio: β-TCP : PG1 = 80 : 20). β-TCP/PG1 composite scaffolds exhibited enhanced mechanical properties (up to 6 MPa) with respect to pure β-TCP scaffolds (up to 2.3 MPa) as glass acted as viscous binder during sintering, thereby strengthening the final scaffold structure.

SECOND-GENERATION SCAFFOLDS: GLASS/POLYMER POROUS COMPOSITES

Conventional composite scaffolds

Since the last 1990s, porous ceramic (glass)/polymer composites have been widely investigated with the purpose of imparting to the scaffolds peculiar properties, *e.g.* more finely controlled tissue interactions and drug release ability. The first attempts of fabricating porous composites involved the use of HA or amorphous calcium phosphate as inorganic phase and PLLA or PLGA as organic one.[81-85] In the last decade, the researchers' interest has progressively moved towards bioactive glass/polymer composites; the most attractive reason driving the development of these for bone tissue engineering composite scaffolds was the need for conferring bioactive behaviour to the polymer matrix, which can be achieved by glass inclusions or coatings. In fact, the degree of bioactivity can be foreseen and properly designed by adjusting the volume fraction, size, shape and arrangement of bioactive glass in the composite. However, the presence of bioactive glass in the

porous composite can alter the polymer degradation behaviour, thereby affecting the bioresorption kinetics of the scaffold.[86,87]

Bioglass® has been the most commonly used glass phase used for making porous composite[86-97], but also other silicate[88], borate[89] and phosphate glasses[100-102] have been recently tested in combination with bioresorbable polymers. The porosity and strength of different glass/polymer composite scaffolds are compared in Table III, compiled from the data available in the literature. It is also worthy of mention here the work recently reported by Gentile *et al.*[103] who prepared HA/CEL2/gelatin composite films; as the material exhibited good bioactive properties and promising mechanical features, the authors suggested its use for making porous composite scaffolds, and the related work is currently in progress.

Many techniques have been developed to produce 3-D glass/polymer composite scaffolds with high pores interconnection; the most effective ones, *i.e.* thermally-induced phase separation[86,87,94,95], microsphere sintering[83,85,90] and coating methods[88,89,98], have been extensively reviewed by Rezwan *et al.*[104] and Mohamad Yunos *et al.*[105] Solid free-form fabrication, although being a powerful tool to produce wholly polymeric scaffolds, at present has been only used for fabricating calcium phosphate/polymer porous composites.[106,107] Very recently, Misra *et al.*[97] used a unique combination of solvent casting/particulate leaching by employing sugar cubes as porogen additive for fabricating poly(3-hydroxybutyrate) (P3HB)/Bioglass® composite scaffolds.

In general, the polymer makes the scaffold resorbable over time with degradation kinetics depending on the specific polymer used as matrix, whereas the glass inclusions or coating impart bioactive properties to the structure and contribute to mechanically reinforce the polymer matrix. In 2007, Bretcanu *et al.*[96] proposed a different approach and fabricated porous composites by using a GC-Bioglass® scaffold manufactured via sponge replication as porous inorganic matrix[40] and by coating it with P3HB. The polymer was specifically introduced to strengthen the GC-Bioglass® scaffold structure: in fact, P3HB layer acted as a glue thereby holding the inorganic particles

together when the scaffold struts start to fail. The compressive strength of such composite scaffolds (up to 1.5 MPa)[96] was twice than that of bare GC-Bioglass® scaffolds (up to 0.4 MPa).[40]

Glass/polymer composites have been mainly proposed for bone tissue engineering applications and, therefore, *in vitro* tested with some types of bone-related cells, *e.g.* bone marrow stromal cells[108], human osteosarcoma cells (SAOS-2)[90] and osteoblast-like cells line (MG-63).[91,109] However, Verrier *et al.* also suggested the use of Bioglass®/PDLLA scaffolds for lung tissue engineering describing the results of an *in vitro* culture with human lung carcinoma cells (A549).[93]

It can not be ignored that, as specifically remarked in some studies[98,104], the mechanical properties (strength, stiffness) of today's available polymer/glass composite scaffolds are inadequate if compared to the tissues they should temporarily replace. This drawback is particularly evident for implants designed for bone substitution, because polymer/glass porous composites are at least one order of magnitude weaker than natural cancellous bone, as reported in Table III. By comparing the mechanical properties of porous composites with those of purely polymeric scaffolds, a slight increase of strength and stiffness can be noticed, but a careful optimization of processing has to be done to reach values comparable to those exhibited by natural bone or its wholly glass-ceramic substitutes (Table II).

Nanocomposite and hybrid scaffolds

The poor or lacking bonding strength at the glass/polymer interface is, at least partially, responsible of the low increase of mechanical properties. In fact, it should be considered that glass phases are generally hydrophilic whereas polymers are hydrophobic; two possible solutions proposed by Rezwan *et al.*[104] to overcome this problem could be the use of surfactants chemisorbed on glass particles surface prior to composite processing and/or the use of nano-sized glass particles to enhance the interfacial area and, therefore, the polymer/glass bonding strength. The latter approach has been followed very recently by Misra *et al.*[109], who studied for the first time the effect of adding

bioactive glass nanoparticles (mean size ~29 nm) on the bioactivity, degradation and *in vitro* cytocompatibility of P3HB-based composites (Table III).

In recent years, some researchers have gone beyond these even good suggestions and have made important attempts for developing nanoscale composite scaffolds. The challenge is very attractive, as the aim of creating nanocomposites is to have a nanoscale interaction between the bioactive inorganic phase and the organic one, so that the scaffold could degrade as one material rather than having mismatched degradation rates of a glass and polymer phase. As recently underlined by Jones[110], this intimate interaction should allow cells to come into contact with both phases at one time, and the scaffold should degrade at a single rate. To the best of the authors' knowledge, the nanoscale interaction of composite constituents has been always performed through the use of sol-gel synthesis methods. The first approach, introduced by Pereira *et al.* in 2005[111,112], involved the introduction of the polymer directly into the sol; specifically, poly(vinyl alcohol) (PVA) was chosen due to its biocompatibility and solubility in water. The so-obtained PVA/bioactive glass nanocomposite scaffolds exhibited unsatisfactory mechanical strength and degraded quite rapidly in SBF since PVA was not covalently bonded to the glass phase. The latter problem was overcome by functionalizing the chosen polymers, so that they can form covalent bond with the silica network, thereby creating a "hybrid" nanocomposite material characterized by an intimate interaction between glass and polymer at the atomic level. These hybrid materials are often termed "ormosils" (organically modified silicates), and have been recently reviewed by Arcos and Vallet-Regi.[113] Relatively few biocompatible polymers have been tested to obtain hybrids of interest in the biomedical field. Turning their attention to synthetic polymers, some researchers synthesized silica/poly(ε-caprolactone) (PCL) hybrid nanocomposites.[114-118] The hydroxyl groups at both ends of the poly(ε-caprolactone diol) polymer chain were reacted with 3-isocyanatopropyl triethoxysilane, in order to obtain polymer chains bonded to triethoxysilyl groups. Eventually, this functionalized polymer was introduced into a sol to yield an interconnected PCL-silica network, thereby creating an intimate interaction between the two phases. Up to now, such hybrids have

been tested only as bulk materials that showed good bioactivity and promising mechanical properties; in the next future, studies on the nanocomposite processed as a scaffold would be very important. Some researchers tested also natural polymers, such as chitosan and gelatine, to synthesize hybrid nanocomposites. In a work reported by Zhu *et al.*[119], chitosan was reacted with methanesulphonic acid to form butyrylchitosan, able to react with acryloxypropyl trimethoxysilane to form a silanated butyrylchitosan, that was eventually introduced into a sol of hydrolysed TEOS to produce hybrid thin films. Ren *et al.*[120-122] functionalized gelatin molecules with 3-glycidoxypropyltrimethoxysilane before incorporation into the sol; hybrid scaffolds were then produced by soaking the gels in ammonia and freeze drying them. These hybrid scaffolds exhibited osteogenic properties with MC3T3-C cells, but no data about the mechanical properties have been reported yet.

A final mention should be devoted to the so-called "star gels", that are a particular type of ormosils having an organic core surrounded by flexible arms which are terminated in alkoxysilane groups able to form a silica-like network during the sol-gel process.[123] At present, only the star gel developed by Manzano *et al.* in form of monolith showed bioactive properties, as it induced the formation of an apatite-like phase after 7 days in SBF.[124] The fracture toughness of star gels are, in general, higher than that of sol-gel glasses and comparable with natural bone; therefore, as suggested by some researchers, they are expected to exhibit good long-term fatigue behaviour.[113,123]

THIRD-GENERATION-SCAFFOLDS: HIERARCHICALLY POROUS SYSTEMS

Many systems and structures in nature are characterized by a complex gradient of organization at multi-scale levels. If we consider bone tissue, for instance, its architecture exhibits non-uniform porosity distribution. The hierarchical porous organization of bone is particularly evident at the macro-scale in the longitudinal cross-section of long bones, in which the bone at the ends (epiphyses) has the appearance of a sponge (cancellous or trabecular bone) whereas the bone in the

central part (diaphysis) is rather dense with low pores content (cortical bone). In addition, at a finer scale, other hierarchically structured pores systems, *e.g.* Haversian and Volkmann canals (diameter ranging within 100-250 μm), bone lacunae (5-10 μm) and canaliculi (1-5 μm), can be found.[125]

As extensively reviewed by Miao *et al.*[126], in recent years many researchers have tried to mimic nature by developing bioceramic bone tissue engineering scaffolds which exhibited gradients of porosity: in fact, heterogeneity of pores features can result in optimized structural, mechanical and biological properties in comparison to monomodal porous biomaterials. Very recently, scaffolds with multi-scale porosity from the meso- to the macro-range have been developed to impart to biomaterials advanced properties over the traditional, such as drug uptake/release abilities.[127-130] It can not ignored that pore-graded and hierarchical multi-scale porous biomaterials are, in general, more difficult to fabricate than homogeneously porous materials. A short overview of such systems will be presented in the following sections.

Scaffolds with gradient of porosity at the macro-scale

As described in detail by Simske *et al.*[131], four levels of pores size would characterize an ideal porous implant devoted to bone grafting. The first level (1-100 μm) is essential to impart biomimetic features to the biomaterial, as the surface roughness provided by small pores can enhance cells adhesion on the substrate. The second level (100-500 μm) can promote bone in-growth, whereas larger pores (500-1000 μm), although contributing to decrease the mechanical strength, are useful to decrease the Young's modulus of the implant in order to reduce stress-shielding phenomena. Finally, pores over 1000 μm are useful for wires suture and fixation to patient's host bone during surgical procedures.

As recently reviewed by Miao *et al.*[126], HA, β-TCP and other calcium-phosphate ceramics, as well as some of their composites with biocompatible polymers, have been widely adopted for making

graded scaffolds able to mimic the pore-graded structure of natural bone. Among bioactive glasses, however, at present only Bioglass®[132] and CEL2[133] have been proposed for the same purpose.

Bretcanu *et al.*[132] described quick and inexpensive methods for manufacturing 3-D highly porous (> 80%vol.) foam-like GC-Bioglass® scaffolds (Table IV). The followed approach involved the use of pre-formed polyurethane sponges with tailored gradient of porosity as sacrificial templates for the replication technique. The porous polymer was pre-formed by compressing it in metal mould at low temperature (200 °C/1 h); by varying mould shape and size, PU templates with different porous features/gradients were successfully obtained. The pore-graded architecture is expected to reduce the dramatic brittleness of GC-Bioglass® structures with monomodal macroporosity[40], but no evidences were reported yet in the literature.

Vitale-Brovarone *et al.*[133] used CEL2 particles to fabricate graded glass-ceramic scaffolds by means of different processing methods, *i.e.* sponge replication, PE burning-out and enamelling, as well as various combinations of such techniques (Table IV). GC-CEL2 scaffolds able to mimic the porosity gradient of cancellous bone or to reproduce the trabecular/cortical bone system were obtained. In fact, as shown in Table IV it was possible to design the final scaffold – in terms of structural similarity to bone, pores features and mechanical strength – by varying the fabrication method, in order to fulfil specific criteria depending on the end use.

Glass-derived scaffolds with hierarchical porosity at the macro- and meso-scale

Mesoporous materials: short overview

Mesostructured materials belong to the class of nanomaterials, whose properties can be tuned at the nanometrical scale. Specifically, according to IUPAC nomenclature, mesoporous materials are characterized by pores ranging within 2-50 nm. These materials are generally obtained by coupling a sol-gel method, that is very effective to prepare glasses and ceramics at room temperature, with a

supermolecular self-assembling process. This particular approach is possible by taking advantage of hydrophobic/hydrophilic features of some molecules, *i.e.* surfactants, to prepare supermolecular aggregates (micellae). The first successful synthesis of pure-silica mesostructured materials was performed in the early 1990s, when surfactants as structure-directing agents were used by Mobil Oil researchers.[134] Since then, many classes of mesoporous materials with different pores features have been synthesized.

As regards the biomedical field, mesoporous materials, being characterized by an ordered texture of nano-sized pores, can easily host drug molecules and, therefore, are good candidates for designing and producing systems for controlled drug delivery. In addition, the silanol groups located on the walls of silica mesoporous materials may be not only useful to functionalize the walls for enhancing the drug adsorption ability of materials, but can also react with biological fluids to produce HA or apatite-like nano-crystals.[135] *In vitro* bioactivity studies, carried out by soaking SBA-15, MCM-41 and MCM-48 in a simulated body fluid (SBF), revealed that an apatite-like layer was formed on the surface of SBA-15 and MCM-48 materials after 30 and 60 days of immersion, respectively.[136] This behaviour is quite surprising as these mesophases, being constituted by pure silica, should not exhibit bioactive properties. In fact, according to Hench's definition of bioactivity[6,8], bioactive mechanisms can occur only if particular ion-exchange phenomena take place between material and surrounding fluids. On the other hand, it is obvious that mesoporous materials are non-traditional materials and, therefore, their mesoporous texture can impart them unexpected and fascinating properties. MCM-41 also exhibited a bioactive behaviour only if its walls were doped with phosphorus[137] or by adding small quantities of bioactive glasses.[138]

Mesoporous glasses

In order to overcome the problems related to the uncertain bioactivity of pure-silica mesophases, several researchers have recently synthesized mesoporous bioactive glasses (MBGs)[139-142], that

contain, above SiO_2 as former oxide, also variable amounts of CaO and P_2O_5. The role played by the textural and structural properties of MBGs on their bioactive behaviour is extremely important and, therefore, it is necessary to shortly speculate about this point. Since the early 1970s, bioactive glasses are known to be able to chemically bond to living bone without formation of fibrous tissue around the implant due to the growth of a bone-like apatite layer on its surface.[6] It was demonstrated that the HA formation on sol-gel glasses surface is related both to the structure and to the composition of the material, whereas melting-derived bioactive glasses show a direct dependence only from the composition.[143] An increase of the pores volume and specific area (up to 200 $m^2 \cdot g^{-1}$) in sol-gel glasses highly accelerates the deposition of HA, thus enhancing the bonding of the material to bone tissue.[21,144] Ordered mesoporous silicas possess a very high surface area and an ordered system of generally open mesopores, but are not properly suitable as filling materials for bone repair because of their almost complete lack of bioactivity, as shown by Horcajada *et al.*[138] and very recently underlined by Mortera *et al.*[144,145] Some authors reported a weak bioactive behaviour of SBA-15 and MCM-48, but only after relevant times of contact with biological fluids (> 30 days).[136] On the contrary, MBGs belonging to the SiO_2-CaO-P_2O_5 ternary system were found to exhibit a faster and higher bioactivity also in comparison with sol-gel glasses, thanks to their textural and structural properties (specific surface area up to 500 $m^2 \cdot g^{-1}$).[139] Therefore, considering their superior bioactivity, MBGs may be a very promising material for bone tissue regeneration.

Macro-/meso-structured scaffolds

Sol-gel glass scaffolds[31,34,39] can be considered the precursors of the hierarchically structured macro-/mesoporous glass scaffolds. However, although sol-gel glass scaffolds are characterized by both macro- and nano-pores, their mesoporous texture is not arranged in a well-defined symmetry, as it is intrinsically due to the sol-gel processing in itself.

In the last couple of years, some attempts for fabricating multi-scale glass-based scaffolds have been carried out by using properly mesostructrured material, in which nanopores size and arrangement can be carefully controlled and designed. The purpose of such scaffolds is twofold, as it combines the properties of traditional glass-derived scaffold, *i.e.* mechanical support in the defect zone, bioactivity, favoured osteointegration and bone tissue regeneration, with the unique features supplied by mesoporous materials, such as enhanced bioactivity and controlled drug adsorption/release ability for drug therapy *in situ*. Table V resumes the correlation between pores size and pores function in hierarchical porous scaffolds.

The first prototype of such a system was developed by Cauda *et al.*[127] and it involved the incorporation of SBA-15 mesoporous silicas inside a bioactive GC-SCK scaffold produced through PE burning-out method[51], in order to obtain a composite scaffold able to promote the successful integration of the graft and the local drug (ibuprofen) delivery in the implant surroundings. This composite system showed a drawback related to the SBA-15 synthesis conditions (strongly acidic) which led to the GC-SCK scaffold degradation during the incorporation of the mesophase by dipping route. Afterwards, the study was extended to MCM-41 silica spheres, which posses narrower pores size in comparison with SBA-15, and the synthesis conditions were optimized in order to avoid the scaffold damage (mild pH ~9).[128] Finally, a highly bioactive GC-FaGC scaffold was proposed as carrier for MCM-41 mesoporous spheres, that were found to play a key role in enhancing the drug adsorption ability of the whole composite system (Figures 2a and 2b).[129] Mortera *et al.* also showed that the size of MCM-41 spheres incorporated inside the scaffold may be carefully designed depending on synthesis condition to obtain mesophase spheres with narrow diameter distribution, without altering the drug uptake/release ability of the material.[130]

Although the obtained results were promising and encouraging, however some remarks have to be taken in account: (i) the proposed systems (bioactive glass-ceramic scaffold + silica mesophase) are composite, *i.e.* constituted by two different materials that can present problems of interfacial bonding; (ii) the synthesis process of whole system is easy, inexpensive but requires long time; (iii)

although Izquierdo-Barba *et al.*[136] reported the bioactivity of some pure-silica mesophases, this point is currently under debate in the literature and, specifically, several evidences seem to demonstrate the lacking of MCM-41 bioactivity.[145,146]

An attractive solution to overcome the last problem (lacking of bioactivity in pure-silica mesophases) is, for instance, the manufacturing of monomaterial (non-composite) bioactive scaffolds with multiscale porosity by using MBGs. Recently, Yun *et al.*[147] synthesized hierarchically porous 3-D MBG scaffolds with good *in vitro* bioactivity by using a combination of sol-gel, double polymers templating and rapid prototyping techniques. Li *et al.*[148] reported the synthesis of multiscale porous MBG scaffolds by using the block copolymer $EO_{20}PO_{70}EO_{20}$ (P123) and a PU macro-porous sponge as co-templates and demonstrated that a HA layer can form on scaffold surface after soaking in SBF for 4 h. Zhu *et al.*[149] successfully prepared 3-D porous MBG scaffolds by a combination of PU sponge and P123 surfactant as co-templates and evaporation-induced self-assembly (EISA) process (Figures 2c and 2d). Studies of biological compatibility showed that human bone-derived cells cultured on the scaffolds for 1, 3 and 7 days presented a good degree of attachment and spreading. In addition, hierarchical porous MBG scaffold exhibited a greatly enhanced bone-forming bioactivity as compared to traditional bioactive glass (BG) scaffold of same composition due to its high surface area and pore volume. Drug release studies by using gentamicin have been also performed.[150] The drug uptake ability of MBG scaffolds was over twofold higher than that of BG scaffold; in addition, as far as drug delivery is concerned, during the whole release period in SBF gentamicin was delivered from the MBG scaffold at a much lower release rate as compared to that from BG scaffolds.

At present, MBG scaffolds with multiscale porosity are at a preliminary stage of investigation, and a lot of research work has to be done in the future. For example, almost no indications about the mechanical strength of MBG scaffolds have been reported in the literature. In fact, only Wu *et al.*[151] very recently speculated about this point: firstly the authors tested as-such MBG scaffolds in compression (60 kPa), and afterwards they followed an approach similar to that proposed by

Bretcanu *et al.*[96] to reinforce GC-Bioglass® scaffolds by using a polymeric coating. Specifically, Wu *et al.*[151] soaked MBG scaffolds in silk solution and found that silk-induced modification improved the uniformity and continuity of scaffold pore network, maintained high porosity (~94 %vol.) as well as large pore size (200-400 μm) and increased the mechanical strength up to 250 kPa. It can not be ignored that the mechanical resistance of MBG-based scaffolds is over one order of magnitude lower than that of cancellous bone: this is a crucial drawback dramatically affecting any actual clinical applications, as the intrinsic mesoporous texture which features MBGs imparts high brittleness to scaffold structure, thereby causing problems related to sample manipulation and its safe implantation in patient's bone.

SUMMARY AND FUTURE CHALLENGES

The classification of glass-based scaffolds in three generations adopted in the present work does not involve that the scaffolds belonging to a next generation are destined to replace those of a previous one. In fact, a lot of work is to be done in all three groups: the scaffolds belonging to different generations can exhibit peculiar properties which allow to overcome some problems or to emphasize specific abilities (*e.g.* bioactivity, drug incorporation), but it can not ignored that all of them present some limitations.

By looking at the list of features that an ideal scaffold for bone tissue engineering should possess[2-4], we have to admit that, at present, a porous structure able to fulfil all these criteria does not exist.

Firstly, this occurs as it is very difficult to obtain glass-derived scaffolds exhibiting a satisfactory compromise between pores content and mechanical strength. For instance, although Bioglass® was invented four decades ago in the early 1970s[6] and, at present, it is the best-known and commercialized bioactive glass worldwide [30], nonetheless no scaffolds effectively mimicking both the trabecular structure and the mechanical strength of spongy bone have been successfully synthesized from it up to now.[40,42] A few consideration about this point, which has a crucial

importance, should be presented. Scaffold's architectural design is still a great challenge because, from a structural viewpoint, two competing requirements are to be basically fulfilled: on one hand, the scaffold should exhibit a sufficient mechanical competence, *i.e.* strength and stiffness comparable to those of natural bone, but, on the other hand, once the scaffold is implanted in human body it should allow new bone in-growth within its own structure. This typically requires a pores content above 50 %vol. to allow blood vessels supply, cells migration and new tissue in-growth, as well as the presence of pores over 100 μm. These features compete with mechanical requirements, that are further discriminated if the scaffold is resorbable, as its integrity progressively decrease over time. This specific drawback seems to be particularly dramatic for glass/polymer composite scaffolds[89-102], in which the structural and mechanical integrity is strongly affected by the progressive degradation of polymeric phase during the contact with biological fluids. On the other hand, porous composites carry unique properties for the incorporation and *in situ* release of biomolecules or organic moieties such as growing factors and antibiotics that can enhance, respectively, the new bone in-growth and the wound healing rate. However, it should be underlined that the assessment of long-term performances of such composite systems, with particular regard to their degradation over time, still remain a crucial work topic for foreseeing the actual scaffold behaviour *in vivo*; for this purpose, the effect of the incorporation of inorganic bioactive phases on scaffolds degradation and ion release kinetics should be still carefully studied, also by using properly developed predictive analytical models.

Scaffold properties should be carefully designed on the basis of the final clinical use (*e.g.* load-bearing needs, *in situ* drug release) and, in this sense, the choice of a proper method of fabrication plays a key role. There is a great variety of methods for scaffolds processing that lead to porous body with different structure, architecture, pores size and interconnection, and, at present, a "gold standard" method for scaffolding has not been defined yet. Sponge replication can be a good candidate in this sense due to its easiness, effectiveness, versatility and low cost; it was demonstrated that, by carefully setting the processing parameters[46] and/or by producing graded

structure[133], high-strength trabecular scaffolds can be successfully obtained. Solid freeform fabrication (SFF) methods could also be very suitable for manufacturing customized scaffolds. This class of techniques has been widely adopted to manufacture calcium phosphate[152] and polymer implants[2] with effective results, but up to now it has been very rarely used to process glass-containing or glass-derived devices. To the best of the authors' knowledge, this particular use of SFF has been reported only in two very recent works: Misra *et al.*[97] used solvent casting/particulate leaching to produce P3HB/Bioglass® composite scaffolds, and Bergmann *et al.*[153] adopted 3-D printing to manufacture β-TCP/Bioglass® composite implants. The major disadvantage of SFF techniques is, in general, the high production cost and, therefore, other less expensive processing methods have attracted the researchers' interest.

Finally, although there are procedures, guidelines and well-recognized standards for drugs and permanent implants (*e.g.* prostheses), there is not yet a regulatory system for devices, such as scaffolds, that will stimulate tissue growth and potentially resorb over time. Many issues still need to be defined more clearly, so that the researchers can have specific goals to aim for; hence, international standards for the production and clinical use of scaffolds are required to be developed. This point implies a deep and exhaustive knowledge of all the biological effects that can be potentially induced by scaffold materials in the human body. In fact, it was demonstrated in many research works that the ions released by bioactive glasses can exert a gene control regulation: for instance, silicon[26-29], zinc[12,13] and Mg[14] ions were found to promote osteoblastic cells proliferation, differentiation and thus bone mineralization, and strontium ions are known to reduce bone resorption and to accelerate bone healing processes.[15-19] In addition, it is possible to properly "dope" the scaffold in such a way that it can exert specific properties, such as antibacterial activity via ions release.[43] Therefore, the genetic design of bioactive glasses and their derivative scaffolds is a fascinating and attractive field of research able to open new perspectives towards a finely guided tissue regeneration.

Materials with hierarchical pores size distribution in the meso- (2-50 nm) and macro- (size > 50 nm) range can offer interesting properties in comparison with the ones exhibiting only monomodal porosity. In general, a hierarchically structured porous system should comprise a macroporous structure serving as a support inside which a material having smaller pores can be incorporated.[127-130] Ideally, the macroporous network aims to ensure the mechanical stability of the system, as well as good mass transport properties thanks to the high pores interconnection, whereas the mesophase can provide the functionality for a given applications, *e.g.* the encapsulation of biological or pharmaceutical agents. Glass-ceramic scaffolds are optimal candidates as substrates for the mesophases, as their relatively large geometric surface area (of the order of a few $m^2 \cdot g^{-1}$) and their highly interconnected 3-D pores network can allow the access of the mesophase also in the inner region of the structure.[127-130] The purpose of such a system is twofold, as it combines the properties of the glass-derived scaffold, *i.e.* mechanical support in the defect zone, bioactivity, favoured osteointegration and bone tissue regeneration, with the unique features supplied by mesoporous materials, *i.e.* controlled drug adsorption and release allowing the possibility of drug therapy *in situ*. Mesoporous materials carry a great potential for bone tissue engineering, due not only to their unique textural properties, but also thanks to the remarkable versatility of their pores structure, symmetry and arrangement. The ability to introduce different organic species in mesoporous matrices could open new applications of these materials in tissue engineering. For example, mesoporous materials could act as scaffolds with embedded proteins, peptides or growth factors that would be released in a controlled way in the physiological fluids to promote cells proliferation and differentiation. The use of pure-silica mesoporous materials[127-130] and MBGs[147-151] in tissue engineering scaffolds manufacturing is still at a preliminary stage of research but the results achieved up to now are promising and challenging, too. In the authors' opinion, efforts for fabricating MBG scaffolds with adequate mechanical strength, at least comparable to that of spongy bone, should be mandatory.

Controlled drug delivery from biomaterials and implants is a one of the most challenging issues not only of bone tissue engineering but also of the whole modern biomedical research. The release of therapeutic agents from scaffolds has been partially treated in the present work, especially as regards hierarchically porous glass systems; a comprehensive review about this topic was recently published by Mourino and Boccaccini.[154]

As a final comment, it is necessary to underline that a great help for improving scaffold design and tailoring, as well as a more detailed assessment of scaffold features, can be provided by recent advanced techniques of non-destructive investigation. For example, the use of X-ray μCT can be a reliable tool for quantifying in detail the features of porous structures (struts thickness as well as pores size, shape, distribution and interconnectivity) (Figure 3a). A comprehensive picture about the great potential carried by non-destructive imaging techniques for characterizing porous structures was recently provided by Jones *et al.*[155], who also underlined the need for appropriate algorithms for quantifying the μCT-derived parameters of interest. In addition, μCT data can be helpful to predict scaffold mechanical properties[156] and permeability as a function of specific pores networks, that can be imparted to the scaffolds by means of different fabrication processes. However, μCT analysis can be applied only to structures with pores in the macro-range (size of few hundreds of nanometers or above), as at present resolutions below 500 nm are not yet possible.

μCT can be also successfully used for the validation of mathematical models describing scaffold micromechanics[156,157], that can really act as powerful tools for scaffold design towards an ideal synthetic bone graft.

μCT, being a non-destructive technique of investigation, can also enable to analyze the scaffolds after *in vitro* and *in vivo* tests without damaging the samples. For instance, Renghini *et al.*[158] used μCT to have quantitative data about scaffold *in vitro* bioactivity, by monitoring the kinetics of HA formation on scaffold pores walls (Figures 3b and 3c). The potential of μCT in characterizing tissue-engineered scaffolds and bone was recently highlighted by Belicchi *et al.*[159], who showed that μCT can provide crux information about both mineral and organic phases (Figure 3d). In the

context of tissue engineering, μCT may be successfully applicable to monitor stem cells homing, after appropriate cells labelling with metal nanoparticles[159], thereby providing a powerful tool of investigation with superior performances to other non-destructive techniques such as magnetic resonance imaging.

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Figure legends

FIGURE 1. Some examples of first-generation scaffolds: (a) typical sol-gel glass scaffold (adapted from [36] with permission); (b) GC-Bioglass® scaffold (adapted from [40] with permission); (c) hollow centre of a single strut in a GC-Bioglass® scaffold (adapted from [40] with permission); (d) GC-CEL2 scaffold (the SEM micrograph was acquired in such a way that the 3-D architecture and pores network interconnection are emphasized).

FIGURE 2. Some examples of scaffolds with multi-scale porosity: (a) GC-FaGC/MCM-41 composite scaffolds (adapted from [129] with permission); (b) MCM-41 spheres anchored on scaffold walls (adapted from [129] with permission); (c) SEM micrograph of a MBG scaffold (adapted from [149] with permission); (d) TEM image showing the nanotexture (mesoporous channels) of MBG scaffolds (adapted from [149] with permission).

FIGURE 3. The potential of μCT in the characterization of bone tissue engineering scaffolds: (a) μCT image of a typical sol-gel glass scaffold (adapted from [110] with permission); (b) GC-CEL2 scaffold after soaking for 28 days in SBF (green = scaffold material, blue = newly formed HA) (adapted from [158] with permission); (c) isolation of HA phase grown on GC-CEL2 scaffold walls after *in vitro* tests (adapted from [158] with permission); (d) scaffold after implant for 16 weeks in mice (yellow = scaffold material, green = newly formed bone, blue = soft tissues) (adapted from [159] with permission).

Tables

TABLE I. Overview of the glasses adopted for making bone tissue engineering scaffolds.

Composition family [a]	Glass denotation	Scaffolds generation [d]	Synthesis [e]	Composition (%mol.)
Silicate, silica-phosphate	Bioglass® [b]	I, II	M	46.1SiO_2-26.9CaO-24.4Na_2O-2.6P_2O_5
	58S [b]	I, III	sg	60SiO_2-36CaO-4P_2O_5
	70S30C [b]	I, III	sg	70SiO_2-30CaO
	70S26C [c]	I, III	sg	70SiO_2-26CaO-4P_2O_5
	SNCM [b]	I	M	50SiO_2-16CaO-25Na_2O-9MgO
	SCK [b]	I, III	M	50SiO_2-44CaO-6K_2O
	FaGC [b]	I, III	M	50SiO_2-18CaO-7Na_2O-6P_2O_5-7K_2O-3MgO-9CaF_2
	CEL2 [b]	I, II	M	45SiO_2-26CaO-15Na_2O-3P_2O_5-4K_2O-7MgO
	13-93 [b]	I	M	53SiO_2-6Na_2O-12K_2O-5MgO-20CaO-4P_2O_5 (%wt.)
	80S15C5P	III	ms	80SiO_2-15CaO-5P_2O_5
Borate, boro-silicate, boro-silica-phosphate	Bor-0 [c]	I	M	20Na_2O-20CaO,-60 B_2O_3
	13-93B2 [b]	I	M	6Na_2O-8K_2O-8MgO-22CaO-36B_2O_3-18SiO_2-2P_2O_5
	0106 [b]	II	M	50SiO_2-22.6CaO-5.9Na_2O-4P_2O_5-12K_2O-5.3MgO-0.2B_2O_3
Phosphate, phospho-silicate	TiGlass [c]	I, II	M	44.5P_2O_5-44.5CaO-6Na_2O-5TiO_2
	ICEL2 [b]	I	M	45P_2O_5-26CaO-15Na_2O-3SiO_2-4K_2O-7MgO
	PG1 [c]	I	M	45P_2O_5-22CaO-25Na_2O-8MgO

[a] Depending on the glass network former oxides.

[b] Glass name found in the corresponding reference(s).

[c] Glass name assigned in the present article if not present in the corresponding reference(s).

[d] Glass use for making scaffolds of first (I), second (II) or third (III) generation, according to the classification followed in the present work.

[e] M = melt-derived, sg = sol-gel, ms = meso-structured.

TABLE II. Features of first-generation glass/glass-ceramic scaffolds.

Scaffold material [a]	Fabrication method	Mean porosity (%vol.)	Mean strength (MPa)	References [c]
GC-Bioglass®	Sponge replication	90.0	0.4	[25,40]
	Rice husk burning-out	43.5-47.2 [b]	5.4-7.2 [b]	[42]
58S	Sol-gel foaming	-	-	[31,34,35]
70S30C	Sol-gel foaming	82-88 [b]	0.3-2.2 [b]	[36,38]
GC-70S26C	In situ foaming	48	-	[39]
GC-SCNM	Starch (from corn, potato, rice) consolidation	40.0	6.0	[49,50]
GC-SCK	PE burning-out	60-62 [b]	1.5-6.0 [b]	[51]
GC-FaGC	PE burning-out	23.5-50.0 [b]	20.0-55.0 [b]	[47]
	Sponge replication	75	2	[43]
GC-CEL2	PE burning-out	48	7	[48]
	Sponge replication	53.5-72.8 [b]	1.0-5.4 [b]	[44-46]
13-93	Polymer foam replication	85	11	[52]
Bor-0	Soft pressing	40	-	[55,56]
13-93B2	Polymer foam replication	67.7-86.7 [d]	0.8-9.7 [d]	[57-61]
GC-TiGlass	H_2O_2 foaming	40-55 [d]	-	[76]
GC-ICEL2	PE burning-out	90	-	[77]
	Sponge replication	85	0.4	[78,79]
B-TCP/GC-PG1	Sponge replication	60-85 [b]	3.5-6 [b]	[80]

[a] If present, the notation "GC-" followed by the name of the glass (Table 1) means that the material is a glass-ceramic derived from the parent glass by means of a thermal treatment above the crystallization temperature.

[b] Different scaffolds batches were produced by varying the processing parameters in a controlled way (see the references for details).

[c] Refer to the text for reference numbering.

TABLE III. 3-D glass/polymer conventional composite scaffolds.

Materials		Mean porosity (%vol.)	Mean strength (MPa)	References [c]
Glass	Polymer			
Bioglass®	PLGA	43	0.42 [a]	89,90,95
	PLLA	77-80	1.5-3.9 [b]	92
	PDLLA	94	0.075 [a]	86,91,93,94
	P3HB	79-85	0.5-1.5 [a]	96,97,109
CEL2	PU	90	0.1 [b]	98
0106	PDLLA	68	0.6 [a]	99
TiGlass	PLA	95	0.020 [a]	100-102

[a] Compressive strength.

[b] Tensile strength.

[c] Refer to the text for reference numbering.

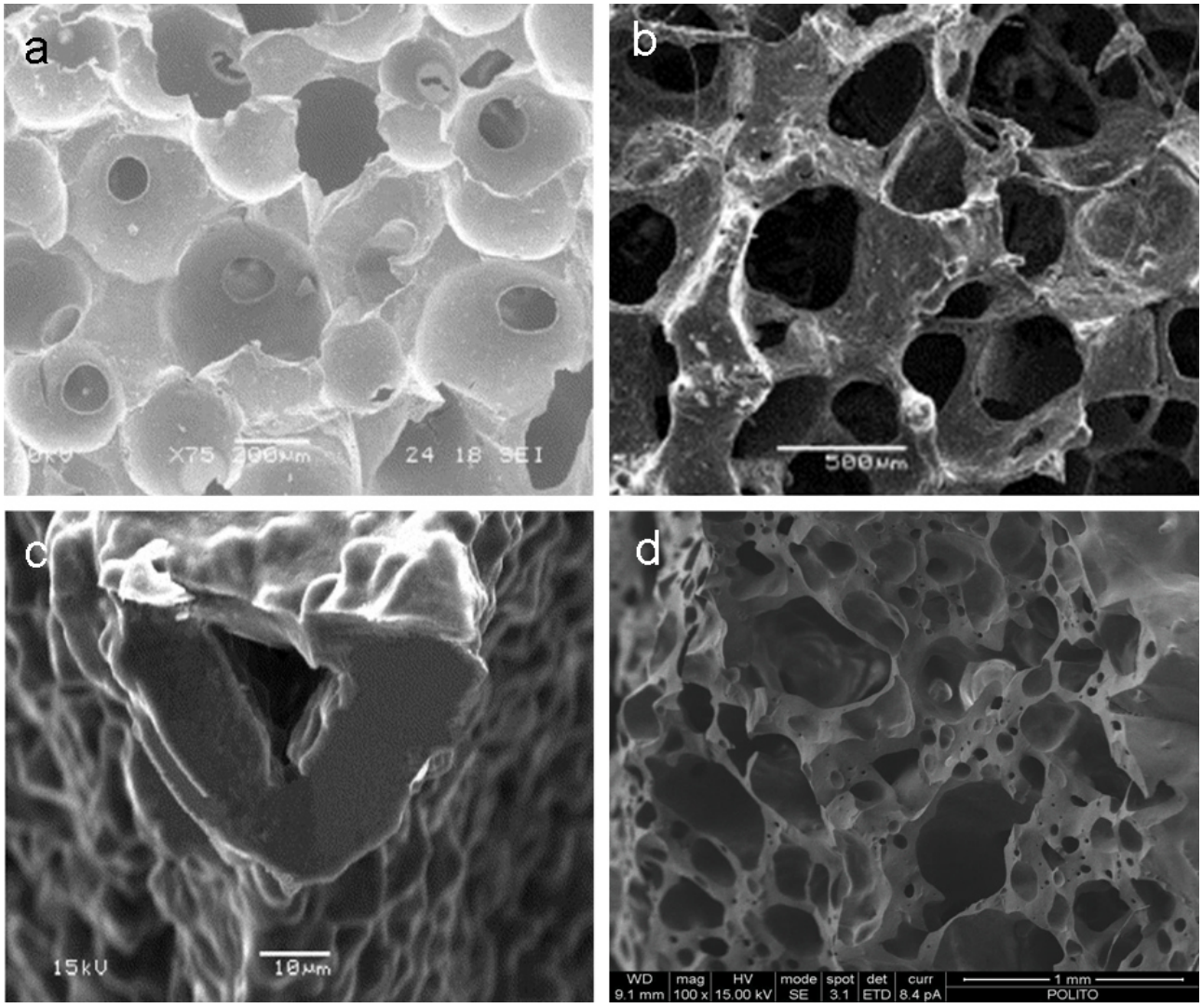
TABLE IV. Graded scaffolds fabricated by using glasses.

Scaffold material	Fabrication method	Correspondence with natural bone structure	Mean compressive strength (MPa)	References [a]
GC-Bioglass®	Graded sponge replication	Porosity gradient of natural cancellous bone	-	132
GC-CEL2	Differential PE burning-out	Porosity gradient of natural cancellous bone	11.5	133
	Differential sponge replication		1.9	
	PE burning-out + sponge replication		6.3	
	PE burning-out + enamelling	Cancellous/cortical bone system	18.0	
	Sponge replication + enamelling		9.7	

[a] Refer to the text for reference numbering.

TABLE V. Relationship between pores size and function in hierarchically structured porous bone scaffolds for bone tissue engineering.

IUPAC classification	Sub-level	Pores size	Function
Meso-scale	Lower meso-scale	2-10 nm	Drug uptake/release, bioactivity improvement
	Upper meso-scale	10-50 nm	Bioactivity improvement
Macro-scale	Lower macro-scale	50 nm-100 μm	Biomimetic properties, improved cells attachment
	Mid macro-scale	100-500 μm	Bone in-growth, blood vessels access, cells colonization
	Upper macro-scale	> 500 μm	Bone in-growth, facilities for surgical fixation by surgical wires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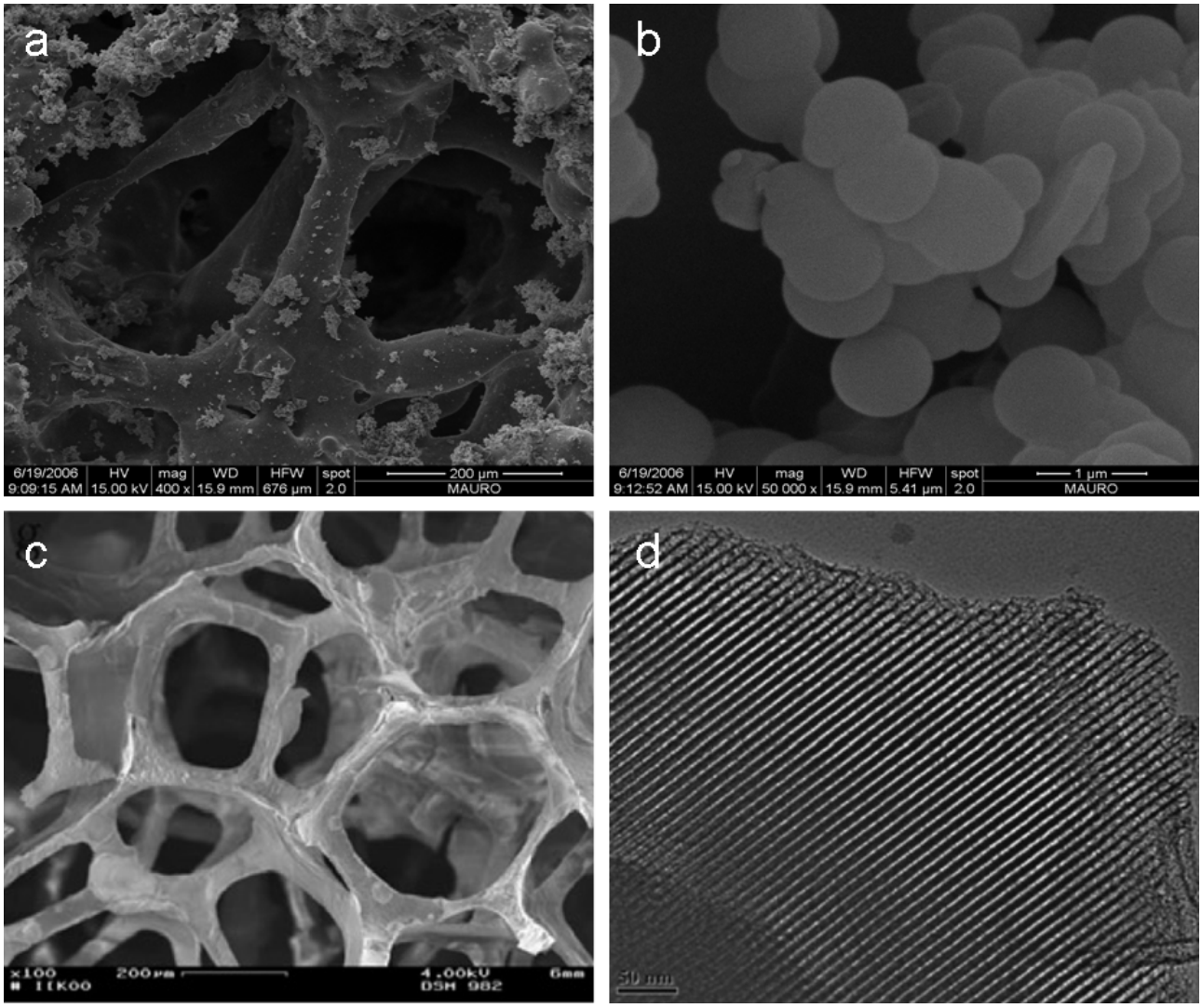
X75 200µm
24 18 SEI
b
500µm
c
15kV
10µm
d
WD 9.1 mm
mag 100 x
HV 15.00 kV
mode SE
spot 3.1
det ETD
curr 8.4 pA
1 mm
POLITO

Fig. 1


a
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HV 15.00 kV
mag 400 x
WD 15.9 mm
HFW 676 µm
spot 2.0
200 µm
MAURO
b
6/19/2006 9:12:52 AM
HV 15.00 kV
mag 50 000 x
WD 15.9 mm
HFW 5.41 µm
spot 2.0
1 µm
MAURO
c
x100
200µm
4.00kU
DSM 982
6mm
d
50 nm

Fig. 2

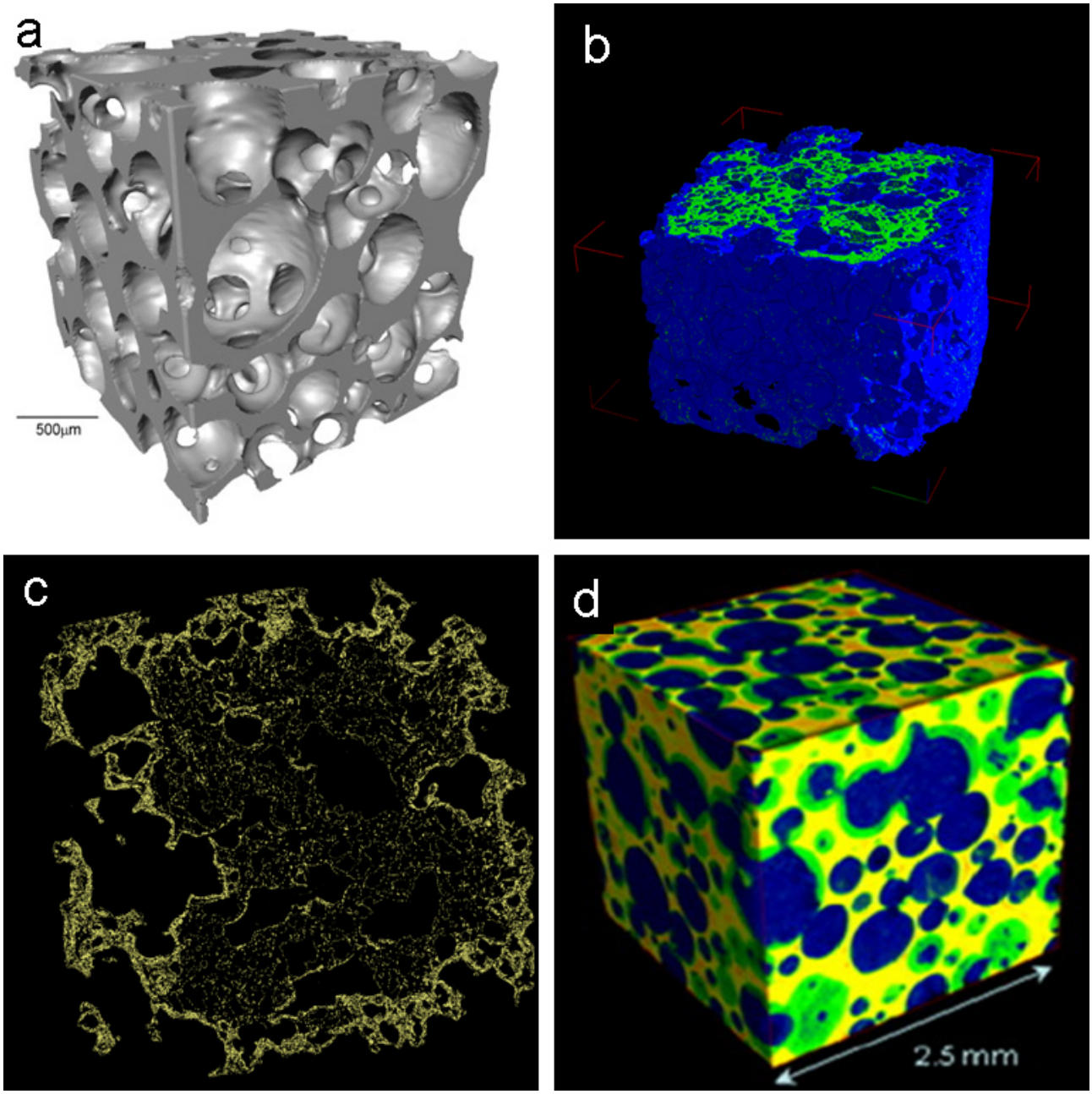

Fig. 3