CHAPTER 5

Bioactivated Polymer Scaffolds for Tissue Engineering

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Summary

A critical step of all tissue engineering techniques is the use of a tridimensional structure which, mimicking the extracellular matrix (ECM), serves as scaffold which is able to promote and guide actively the tissue regeneration process. The ability of the scaffold in releasing signalling molecules, such as growth factors (GFs), in a controlled fashion is critical to achieve a successful tissue development and repair. In fact, direct injection of GFs into the regeneration site or their simple dispersion into the porous scaffold could be ineffective because of the intrinsic instability of GFs toward chemical and physical inactivation, and the relatively long time contact with tissues requested to obtain the desired effect. Furthermore, a spatial localization of the signalling molecule may enable to control not only the extent, but also the pattern of tissue formation. A promising approach to overcome these issues and tune spatially and temporally the concentration of GFs consists in siting biodegradable microspheres that release the protein for long time-frames within the scaffold. This chapter gives a brief description of ECM-mimicking scaffolds and strategies for their bioactivation, with particular regard to the use of GF-loaded biodegradable microspheres.

We highlight the relevant aspects of microsphere fabrication and characterization, including the issues related to the evaluation of GF release kinetics, which is essential for the design of microsphere-integrated polymer scaffolds. In the last part of the chapter, a technique to follow protein release from biodegradable microspheres directly within the polymer scaffold is presented.

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Extracellular Matrix (ECM)-mimicking scaffolds

ECM composition and functions

ECM is the natural medium in which cells proliferate, differentiate and migrate, and therefore is the gold standard for tissue regeneration (1, 2). Cell-ECM interaction is specific and biunivocal. Cells synthesize, assembly and degrade ECM components responding to specific signals and, on the other hand, ECM controls and guides specific cell functions. This continuous cross-talk between cells and ECM is essential for tissue and organ development and repair.

In physiological conditions, ECM composition derives from homeostasis, a fine dynamic balance of regeneration, differentiation and programmed cellular death (apoptosis), which continuously remodels ECM through protein breakdown and synthesis (1). Natural ECM is a condensed matrix mainly composed of locally secreted proteins and polysaccharides, arranged as a molecular network formed by an intricate agglomerate of weaves, struts and gels interconnecting cells with matrix proteins. Amounts and organizations of these molecules are variable with tissue site and type, and during tissue development, in terms of resistance to tensile and compressive forces and transport properties. Dynamic properties of ECM are controlled by proteoglycans, and a number of signalling molecules, such as growth factors (GFs), which mediate cell-ECM and cell-cell interactions. All these molecules are embedded in an amorphous, fundamental substance represented by glycosaminoglycan chains, which form the highly hydrated gel structure imbibing the matrix. The ECM components and their role in ECM dynamics are summarized in Table 1.

Component	Function	Location	Ref.
Collagens	Tissue scaffolding, tensile strength Cell-ECM interactions Cell-cell interactions Fibroblast activation	Ubiquitous	3-4
Proteoglycans	Collagen embedding Tissue resistance to compressive forces Transport of nutrients Fibroblast and chondrocyte proliferation Endothelial and epithelial cell differentiation	Ubiquitous	5-6
Hyaluronic acid	Transport of metabolites and nutrients Tissue resistance to compressive forces Cell migration Cell proliferation	Ubiquitous	7-9
Laminins	Intracellular signalling Cell differentiation Cell shape/movement	Basement membranes	10
Fibronectin	Cell attachment to ECM Cell migration Cell proliferation	Ubiquitous	11
Growth factors	Cellular signalling	Ubiquitous	12-14

Table 1. ECM components and their role

ECM and GFs: a complex interplay controlling tissue growth and repair

For the formation of complex tissues from single cells, and for tissue maintenance, large amounts of information are needed and must be transported from cell to cell and from cells to ECM. GFs are protein molecules specific for intercellular and cell-ECM signalling involved in ECM dynamic properties through specific surface receptors, driving GFs regulatory activity (12-14).

GFs are released by many cell types for immediate signalling and activate specific pathways controlling cell migration, differentiation and proliferation. During tissue morphogenesis the presence of soluble GFs guides cellular behaviours, thus governing neo-tissue formation and organization. GFs are normally synthesized as membrane-bound or high molecular weight precursors that must be modified to release the active form. GFs are often bound to ECM molecules, such as glycosaminoglycans (e.g. heparins). The interaction with these molecules alters GF action, by retaining the active/latent forms near cells and modifying GF transport properties.

The sequestration of GFs within ECM in inert form is necessary for rapid signal transduction, allowing extracellular signal processing to take place in time frames similar to those occurring inside cells. In addition, GF storage in ECM is crucial to maintain homeostasis through continuous GF activation upon ECM degradation. ECM, therefore, serves as a sustained release reservoir for GFs, this aspect being crucial for molecules that are released over a short period but stimulate processes involved in tissue regeneration that take extended periods to be carried out (e.g. angiogenesis).

Tissue engineering 3D-scaffolds

Tissue engineering aims to create biological substitutes that might restore, maintain or improve tissue functions. For a successful tissue engineering approach, an appropriate cell source must be identified, isolated and amplified on 3D-scaffolds. The correct synthetic or natural material to be used as a substrate should be chosen and

manufactured in the desired shape and dimension (15, 16). A tissue engineering scaffold serves as a temporary ECM, which should support cells and enhance the subsequent 3D tissue regeneration. Thus, a critical step in this process is the mimicking of some ECM characteristics to provide cells with an adequate mechanical stability and biological environment for tissue growth and integration (15).

A wide variety of materials, both synthetic and natural, can be manufactured into scaffolds, taking into account the need of avoiding any adverse foreign host response (16). In the case of *in vivo* application, the properties of ECM-mimicking scaffold must be tailored according to the body site because the template must temporarily bear mechanical stresses after implantation, avoiding potential stress discontinuities at the tissue-implant interface until the surrounding tissue is fully regenerated.

A suitable scaffold for tissue engineering should provide: (i) mechanical integrity to tissues by acting as a support for neo-tissue growth; (ii) guidance for biological response through the promotion of the dynamic interaction with surrounding tissues; (iii) a space for host cell survival, enhancing the transport of nutrients and metabolites through maximization of biological and/or pharmaceutical response; (iv) adequate biocompatibility/biodegradability, with degradation kinetics suitable to match neo-tissue formation, thus minimizing toxicity in terms of both tissue and systemic response; (v) manufacturing feasibility.

The selection of the material for a scaffold still remains a key factor in the design and development of tissue engineering constructs, especially if it is considered that the biomaterial employed must produce controlled and predictable interactions with cells. Several options are available (Table 2), although it should be taken into account that each application implies a unique environment for cell-cell and cell-material interaction (17).

Material		Ref.
Natural organic materials	Collagens	18
	Hyaluronic acid and derivatives	19-20
Synthetic organic materials	Poly(glycolic acid) (PGA)	21-23
	Poly(lactic acid) (PLA)	
	Poly(lactic-co-glycolic acid) (PLGA)	
	Poly(ε-caprolactone) (PCL)	26-29
	PEG and copolymers	30
Inorganic materials	Hydroxyapatite (HAP)	31-32
	β-tricalcium phosphate (TCP)	

Table 2. Materials for tissue engineering scaffolds

Naturally-derived molecules, such as proteins and polysaccharides, find wide application in tissue engineering. Collagen has been used especially for regeneration of soft tissues either alone or in combination with other agents (18). Naturally derived polymers in general, and collagen in particular, though having poor mechanical properties, are interesting because they do not induce a host response, and may enhance the biological recognition in the growing neo-tissue, encouraging the normal cellular functions (17). For the replacement of soft tissues, there are many strategies employing HA as a scaffold material. Through chemical modifications of HA it is possible to obtain HA derivatives, which exhibit better mechanical features but are not well recognized by cells, thus impairing biological activity (19). On the other hand, semi-interpenetrating (semi-IPN) gels made of collagen and HA, have been demonstrated suitable to realize scaffolds for tissue engineering applications because the structures of collagen-HA gels strongly resemble the organization of ECM in soft tissues (20).

Among synthetic materials, biodegradable polyesters approved by the Food and Drug Administration (FDA) for human use are rapidly gaining recognition in the field of

tissue engineering as they can be easily processed into porous scaffolds. Poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and their copolymer poly(lactic-co-glycolic acid) (PLGA) have been widely applied in bone and cartilage repair (21-23). Poly(ε-caprolactone) (PCL), which degrades at significantly lower rates than PLA, PGA, PLGA, is less attractive for tissue engineering applications. However, PCL-based scaffolds have been studied for skin replacement and other tissue engineering applications (24-26). Furthermore, novel degradable PCL networks, PLGA/PCL/PLGA tri-block copolymers and PCL-chitosan matrices are more hydrophilic, degrade faster and possess desirable mechanical properties as compared to PCL (27-29).

Also hydrogels are very attractive candidates in some tissue engineering applications because they can fill irregularly shaped defects, and incorporate cells and other bioactive materials (30). Concerns about the toxicity of the cross-linking agents remain and limit the feasibility of *in vivo* applications. Among synthetic hydrogels, poly(ethylene glycol) is frequently employed. Attempts have been made to impart biodegradability to PEG by copolymerization with PGA and/or PLA, and by introducing degradable linkages into the PEG backbone.

Inorganic compounds are sometimes used for the regeneration of bones and other mineralized tissues. Among these materials, β-tricalcium phosphate (TCP), hydroxyapatite (HAP), its derivatives, and their combinations, are the most frequently used (31, 32).

Controlled delivery of growth factors for tissue engineering

During tissue morphogenesis, the presence of GFs in soluble form drives cellular behaviours and governs neo-tissue formation and organization (12-14). Thus, specific GFs released from a delivery device or from co-transplanted cells would aid the engineering of a new tissue *in vitro* and/or *in vivo* (15). Indeed, GF use in tissue engineering strategies is becoming more and more promising in many applications, including neo-vascularization, bone regeneration, and wound healing (33, 34).

The ideal transformation of a neo-tissue into a functional substitute is a stepwise process, which should recapitulate the natural sequence of biological signals, related to the existence of chemotactic gradients. Such gradients should be created and maintained throughout the period of tissue repair. A spatial coordination is also necessary to keep a pseudo-stationary dynamic balance between the rate of biological signal diffusion down a concentration gradient, and their removal by metabolism and degradation. The maintenance of stable gradients finally leads to the formation of neo-tissues (34) .

The control over the cascade of events leading to neo-tissue formation may be carried out through strategies for GF delivery, with the aim of mimicking natural biological patterning and tissue architecture. To date, the way to make GFs effectively available at the site of action (i.e. in the bioactive form, at the appropriate concentration level, for the necessary time interval) remains a matter of primary importance (35).

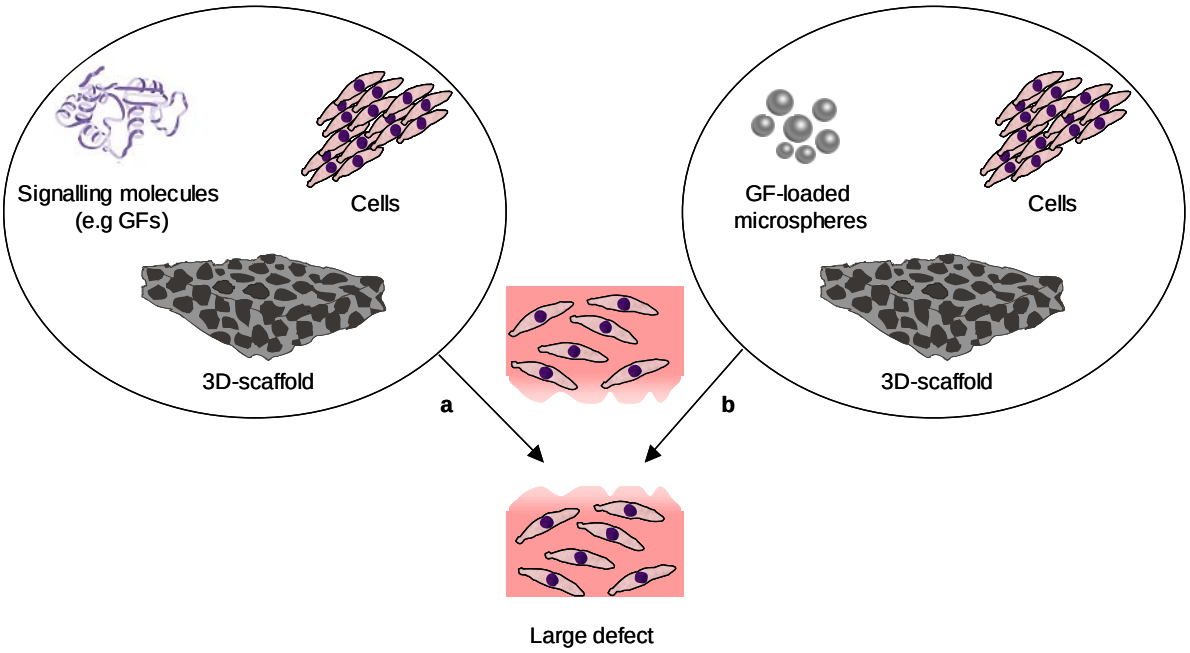

Fig. 1: Basic GF delivery strategies: a) direct incorporation of GFs and cells within a 3D-scaffold; b) 3D-scaffolds embedded with GF-loaded microspheres and cells (e.g. chondrocytes for cartilage; keratinocytes for epidermis, *etc.*). After a given time-frame *in vitro*, necessary for cell attachment and growth, the scaffold is implanted *in vivo*. A new tissue will be formed within the biodegradable scaffold by cells infiltrating from and to the surrounding healthy tissue.

The most widely employed strategy to face this issue consists in the local delivery of GFs by their direct incorporation within the scaffold and cell seeding (Fig. 1a) (35, 36). The first significant limitation of this approach relies upon GF enzymatic degradation, which rapidly occurs when the macromolecule is exposed to the *in vivo* environment. Furthermore, another important limit of this strategy is the failing in controlling protein local concentration over time inside the scaffold. In fact, as already underlined, the engineering of different tissue structures presumably requires the adjustment of the local concentration of signalling molecules and eventually its changing over time (i.e. spatial and/or temporal control). Actually, poor or no control over protein release can be accomplished by its incorporation due to its rapid leaching out of the porous polymeric materials commonly used for cell attachment and growth.

To enable GFs to exert efficiently their biological effect at the site of action, the use of drug delivery systems (DDS), which can protect and deliver signalling molecules exactly within the scaffold, has been suggested (35, 36). As temporally controlled systems, DDS can release the protein at a specific time (i.e. multiple factors released at different time intervals) and for long time frames during tissue development. Above all, a DDS intended to release GFs or other signalling molecules into tissue engineering scaffolds requires long-term maintenance of protein biologic activity. Afterwards, GF release profiles from the system should be temporally and spatially controlled to be suitable for a specific tissue injury and disease. To meet these design criteria, the use of controlled release biodegradable microspheres incorporating signalling molecules has been proposed (Fig. 2b). The entrapment of polymeric microspheres within the scaffold may prevent both GF rapid diffusion out of the site of action and its enzymatic degradation. Novel porous alginate scaffolds incorporating microspheres have been demonstrated to effectively control the release of basic fibroblast growth factor (bFGF) in its biologically active form (37). Transforming growth factor-β1 (TGF-β1)-loaded microspheres incorporated in 3D chitosan scaffolds have shown an enhancing effect on cartilage formation (38, 39). Furthermore, multiple growth factors may work in concert to promote tissue regeneration by embedding controlled release microspheres in the

scaffold. For the first time, the combined release of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) was effectively achieved by dispersing free VEGF and PDGF-loaded PLGA microspheres within tissue engineering scaffolds (40). Insulin-like growth factor (IGF-I) and TGF-β1-loaded microspheres were contemporarily photoencapsulated with bovine articular chondrocytes in PEO-based hydrogels for cartilage tissue engineering (41).

In perspective, a highly regulated network could be achieved by micropositioning the reservoirs within the scaffold, generating spatial and temporal gradients of the bioactive agent to control not only the extent, but also the pattern of tissue formation.

Biodegradable microspheres for the controlled release of growth factors

Biodegradable microspheres as protein delivery systems

Microspheres are particulate delivery systems which can incorporate small drugs or macromolecules (42-45). Polymers with different physico-chemical characteristics (e.g. chemical nature, composition, molecular weight, hydrophilicity, degradability) can be used to produce microspheres with different final properties. Specific polymers commonly employed for protein microencapsulation are reported in Table 3.

Type	Class	Polymer	Protein encapsulated	Ref.
Natural polymers	**Polysaccharides**	Alginates	Bovine serum albumin Polysaccharide-protein conjugate	49-51
		Chitosan	Human growth hormone Transforming growth factor-β1 Meningococcal conjugate vaccine Interleukin-2 Ovalbumin	52 38 53 54 55
		Dextrans	Immunoglobulin G Interleuchin-2	56, 57 58
	Polypeptides and proteins	Albumin	Endothelial cell growth factor Glycoprotein fragment	59 60
		Collagen	Bone morphogenic protein-4	61
		Gelatin	Lysozyme Fibroblast growth factor Bovine serum albumin	62 63, 64 65
Synthetic polymers	**Polyanhydrides**	Poly(SA) Poly(FAD-SA) Poly(SA-co-CHP)	Albumin, immunoglobulin, lysozyme, ovalbumin, trypsin	66-68
	Polyesters	Poly(ε-caprolactone)	Bovine serum albumin, muramyl dipeptide, oral vaccine delivery	69
		Poly(lactide)	Growth factors Vaccines Other proteins	70, 71 72-74 75, 76
		Poly(lactide-co-glycolide)	Growth factors Vaccines Other proteins	37, 40, 41, 77-90 48, 91 47
	Polyorthoesters		Bovine serum albumin	92
	Polyphosphazenes		Insulin	93
	Polyphosphoesters	Poly(DAGP-EOP)	Nerve growth factor	94

Table 3. Biodegradable polymers employed in protein microencapsulation

As well-known, biodegradable polymers have attracted increasing attention in microsphere development because, differently from non degradable systems, they do not require further manipulation after introduction within the body. Among synthetic biodegradable polymers, a wide range of biomaterials with specific mechanical, processing and release properties can be found, e.g. PCL, PLA and its derivatives. In particular, PLGA copolymers have gained strong success because their release properties can be easily tailored by varying composition (lactide/glycolide ratio), molecular weight and chemical structure (i.e. capped and uncapped end-groups) (45). PLGAs characterized by very different *in vivo* life-times, ranging from 3 weeks to over a year, are available and approved for human use. Protein microencapsulation within PLGA copolymers is regarded as a powerful mean to protect effectively the macromolecule from *in vivo* degradation, occurring at the administration site, and to achieve its sustained release for long time-frames (47, 48).

Fabrication of protein-loaded PLGA microspheres

A large number of techniques have been developed to encapsulate drugs within PLGA biodegradable microparticles (95, 96). Among them, solvent evaporation/extraction methods are the most widely employed in the case of therapeutic peptides and proteins (96, 97). The polymer is dissolved in an appropriate organic solvent immiscible with water (e.g. methylene chloride, ethyl acetate) and the solution dispersed in an aqueous continuous phase containing a stabilizer (e.g. polyvinylalchool). Hydrophilic drugs may be either incorporated as solid micronized particles (e.g. freeze-dried proteins) suspended in the organic phase – that is the solid in oil in water (s/o/w) encapsulation technique - or emulsified in the organic phase as a water solution - that is the double or multiple emulsion (w/o/w) technique (Fig. 2).

In both solvent evaporation and extraction techniques, the organic solvent has a certain solubility in the external aqueous phase so that partitioning into the continuous phase can occur leading to phase separation of the matrix material. In solvent evaporation, the

capacity of the continuous phase is insufficient to dissolve the entire volume of the disperse organic solvent, which must evaporate to yield hardened microspheres. In solvent extraction, the amount and composition of the continuous phase are chosen so that the entire volume of the disperse phase solvent can be dissolved. For the microencapsulation of highly hydrophilic compounds, the replacement of the aqueous continuous phase with an oily phase (e.g. paraffin oil) – that is the solid in oil in oil (s/o/o) technique - prevents undesirable drug escape from the internal to the external aqueous phase and allows the achievement of high encapsulation efficiencies (98).

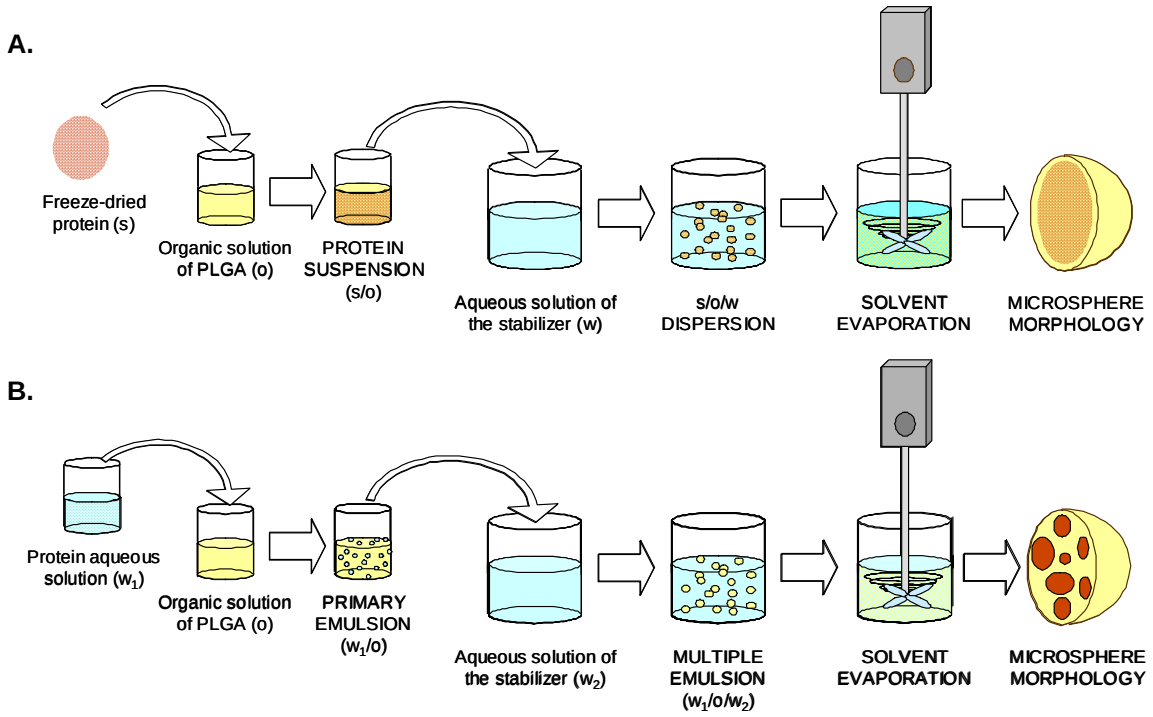

Fig. 2: Solvent evaporation methods of microencapsulation: A) solid in oil in water (s/o/w) – solvent evaporation technique; B) water in oil in water (w_1/o/w_2) – solvent evaporation technique. As shown by microsphere cross section, the internal morphology of the particles depends on the protein physical state (e.g. solid form or aqueous solution) in the polymer organic solution. In case A, a protein solid dispersion within the polymeric matrix is obtained. In case B, protein is located within cavities (*macropores*) originated during microsphere production. In solvent extraction technique, the amount and composition of the continuous phase are chosen so that the entire volume of the dispersed organic phase can be removed by dissolution in the external phase.

The solvent evaporation/extraction techniques are extremely useful for the incorporation of high molecular weight hydrophilic drugs, such as proteins (47, 96). Nevertheless, a crucial point to consider for the successful microencapsulation of proteins is the maintenance of their biological activity. On this matter, the ProLease® technology (Alkermes Inc., USA), which is a cryogenic and nonaqueous microencapsulation method, is particularly suitable to maintain protein integrity during encapsulation and achieve high protein encapsulation efficiencies (99). The process consists of the following steps: 1) spray freeze-drying of a protein solution containing stabilizing excipients, by fine atomization into liquid nitrogen and subsequent product lyophilization; 2) preparation of a protein/polymer suspension in an organic solvent followed by sonication; 3) production of frozen drug/polymer microspheres, using an ultrasonic nozzle, which atomizes the suspension in liquid nitrogen; 4) extraction of the polymer solvent with ethanol, a miscible non-solvent; 5) removal of ethanol and extracted polymer solvent by filtration. The final powder product is achieved after vacuum-drying and sieving to remove residual solvent and large particles, respectively (Fig. 3).

During microencapsulation, protein denaturation can be associated with dispersion methods (i.e. magnetic stirring, homogenization, sonication) due to the generation of shear and cavitation forces. Moreover, interface formation in emulsion-based microencapsulation techniques can maximize protein mobility and results in its aggregation, further increased by polymer/protein interactions (100). In theory, a protein suspended in pure organic solvent (i.e. s/o/w technique; ProLease® technology) displays less conformational flexibility than in a w/o emulsion, thus leading to a more preserved 3D structure and impaired denaturation (98). However, in order to obtain an anhydrous powder, the protein must be freeze-dried or spray-freeze dried, i.e. processes which can cause structural perturbation due to freezing, pH-shifts and loss of hydrating water shell (100).

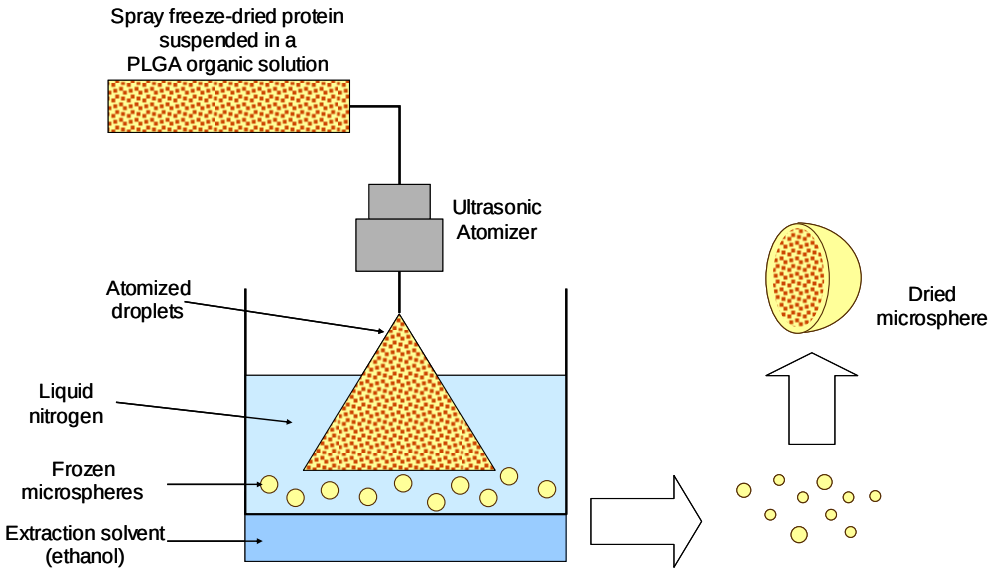

Fig. 3:The ProLease® microencapsulation technique. The spray freeze-dried protein is suspended in a polymer organic solution (generally a methylene chloride solution of PLGA), atomized through an ultrasonic nozzle and frozen. After the liquid nitrogen evaporates, ethanol melts and extracts the organic solvent from the microspheres which are then filtered and dried.

In this case, lyoprotectants, such as mannitol or threalose, can be added to improve protein stability (77). Even if emulsion-based microsphere processing may induce protein inactivation and aggregation at w/o interface, it should be noted that great progresses have been made in formulation optimization and, to date, straightforward strategies for protein stabilization have been developed (98, 100, 101). Among them, a widely employed approach involves the co-encapsulation of a surface-active protein, such as BSA, which displaces the therapeutic protein from w/o interface (i.e. *sacrificial lamb approach*).

Protein release from PLGA microspheres

Upon immersing microspheres in an aqueous medium, water penetrates toward the centre of the particle (hydration phase) and activates drug diffusion through the innate micropores of PLGA (angstrom- or nanometer-dimension) and the macroporous structure (macropores) of the particle formed during processing (i.e. double emulsion microencapsulation method). In the case of macromolecules, the diffusion in the porous network is highly limited due to the cramped space available and therefore extremely slow until pores grow in size and/or coalesce because of polymer erosion (Fig. 4). Thus, drug release rate from biodegradable PLGA microspheres is mainly controlled by polymer erosion, structure of the porous microenvironment and drug diffusion (102, 103).

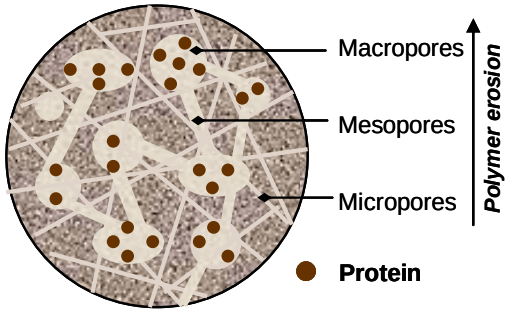

Fig. 4: Porous microenvironment of a PLGA microsphere produced by the double emulsion technique during the release phase: intrinsic *microporous* structure (i.e. angstrom- or nanometer dimension) of the copolymer; *macropores* possibly formed during the preparation process; *mesopores* formed during PLGA erosion phase. The protein is initially located primarily within *macropores*. Adapted from Baticky *et al*. (102).

These properties can be generally regulated by selecting adequate formulation conditions, such as polymer type and preparation method (104). Actually, the use of PLGAs differing for molecular weight and copolymer composition can change initial hydration and erosion rate of the matrix (105, 106). Much more difficult is to modify the release features of microspheres once a polymer type and a preparation technique have

been selected. In this case, control over the release rate could be exerted by either modifying the internal morphology of the system (i.e. internal porosity) or adding a third component that alters drug effective diffusivity in the polymeric matrix (107).

Degradation of PLGA microspheres was regarded as homogeneous (bulk erosion) and related to the hydrolytic cleavage of ester bonds (46). PLGA degradation generates acidic products, and the resulting acidic microclimate within the particle is regarded as the most detrimental factor affecting protein integrity during release incubation (109). An incomplete protein release may be also ascribed to moisture-induced aggregation or adsorption to polymer surface.

Growth factor incorporation in PLGA microspheres: the state of the art

Several examples of successful GF delivery through PLGA microspheres are reported in the literature. PLGA-based microspheres appear an attractive system for the localized rate-controlled delivery of VEGF to improve local angiogenesis (77-80). Different PLGA-microsphere formulations for the sustained release of bioactive nerve growth factor (NGF) in the treatment of neuronal diseases (81-84) and IGF-I in bone healing have been developed (41, 85-87). EGF released from PLGA microspheres was found to improve transplanted hepatocytes survival (88). TGF-β1 was effectively incorporated into biodegradable microparticles of PLGA and poly(ethylene glycol) (PEG) blends and allowed a modulated cellular response during bone healing at a skeletal defect site (89, 90). Finally, the controlled delivery of bFGF from PLGA microspheres integrated within alginate scaffolds has been demonstrated to effectively enhance matrix vascularization after implantation for tissue engineering (37).

In most cases, the microencapsulation of GFs within PLGA microspheres is carried out by solvent evaporation/extraction techniques. GF incorporation as solid micronized particles (e.g. freeze-dried/spray freeze-dried proteins) by the s/o/w microencapsulation technique has been regarded as a way to prevent protein

degradation during the emulsification step (78, 79). Nevertheless, the control over GF encapsulation efficiency and release profile is much more difficult to attain and microspheres display very high burst, reasonably due to the preferential location of the protein close to microsphere surface (79). To overcome this issue, straightforward strategies for protein stabilization during microencapsulation by classical multiple emulsion techniques make use of additives in the internal aqueous phase. The use of bovine/human serum albumin, in high weight ratios to GFs, has been demonstrated as an effective strategy to encapsulate bioactive GFs (37, 41, 78, 80, 82, 83, 86). Another approach, applied to stabilize IGF-I during microencapsulation, is the addition of succinylated gelatine hydrolysate (Physiogel®) (86, 87). Physiogel® addition within the PLGA formulation results in an effective preservation of IGF-I bioactivity, which was confirmed by the enhancement of new bone formation achieved after IGF I-loaded microparticles administration at the site of bone injury.

The proprietary ProLease® technology has been especially used by Cleland and colleagues and diversely applied for the microencapsulation of NGF (81, 84), IGF-I (85) and, more recently, VEGF (77). Different excipients added to PLGA formulations protect GFs from degradation during spray freeze-drying (e.g. trehalose, poloxamer 188 and PEG) although they did not prevent protein aggregation during *in vitro* release stage. Only the formation of an insoluble NGF-zinc complex by addition of zinc acetate stabilized the protein during both microencapsulation and release (84). VEGF, released from PLGA microspheres formulated with threalose, aggregated and hydrolyzed over time and lost heparin but not receptor affinity (77). In the case of heparin-binding GFs (e.g. bFGF, VEGF), the co-encapsulation of heparin has been regarded as a powerful mean to stabilize protein conformation also during the release phase (37). It is worth of note that the co-encapsulation of GF stabilizing excipients affects the release rate from microspheres. Therefore, this aspect should be carefully taken into account during microsphere design and engineering in view of their specific therapeutic application.

PLGA microspheres embedded in collagen-based scaffolds: evaluation of release kinetics

The design of tissue engineering constructs based on the combined use of microspheres and scaffolds requires the exact knowledge of the release profiles of signalling molecules directly within microsphere-integrated scaffolds and the mechanism controlling the release. As for release in buffer solution, minor variations in formulation and process parameters can significantly modify protein release from PLGA microspheres. Nevertheless, in the case of microsphere-integrated scaffolds, the chemico-physical properties of the scaffold (e.g. hydrophilicity, composition) may be an additional feature affecting protein release kinetics and must be properly taken into account.

Evaluation of release kinetics in solution

In vitro studies of protein release from microparticulate systems are commonly accomplished by sampling-separation methods (104). A known amount of microspheres is suspended in the release medium, which often corresponds to a buffered solution at pH 7.4 (e.g. phosphate buffered saline), and kept at 37°C in a thermostated bath, thus mimicking *in vivo* conditions. Samples of the dispersed system are collected at predetermined time intervals, the particles separated from the release medium, and the medium assayed for the amount of protein released (Fig. 5). The separation can be accomplished in few minutes by centrifugation, but its duration dramatically increases to achieve a complete sedimentaton of submicron particles. Adequate analytical techniques are selected to evaluate protein concentration within the release medium, such as High-Performance Liquid Chromatography (HPLC), UV-vis spectrophotometry, Enzyme Linked Immunosorbent Assay (ELISA), radioactivity assay or, if encapsulated proteins are fluorescent or marked with a fluorescent probe, by spectrofluorimetry.

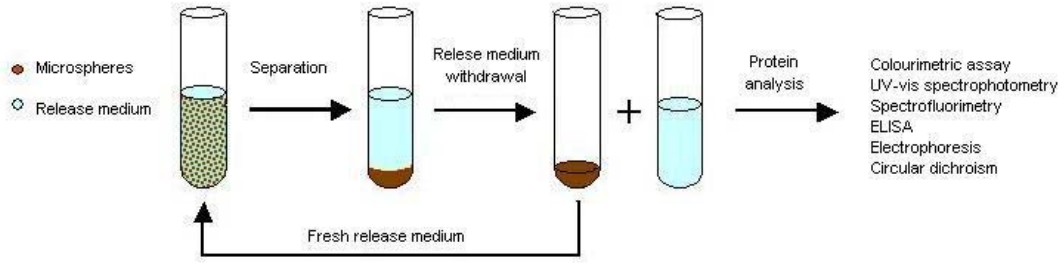

Fig. 5: *Sampling-separation methods* commonly used to follow protein release from PLGA microspheres in buffer solutions. A known amount of microspheres is suspended in the release medium and kept at 37°C in a thermostated bath. Samples of the dispersed system are collected at predetermined time intervals after separation of the particles from the release medium (i.e. centrifugation). The withdrawn medium is assayed by adequate analytical techniques for the amount of protein released.

Various experimental factors may affect *in vitro* protein release kinetics, such as buffer capacity of incubation medium, amount of incubated microspheres, volume of buffer solution, sampling method (104). Even if in most cases the experimental release rate is only indicative of the *in vivo* release behaviour, these factors must be settled to better reflect what happens in the human body (i.e. perfect sink experiment) (105).

Sampling-separation methods can be carried out in both static and dynamic conditions. The static experimental protocol applies very well if microspheres are injected in a tissue and form a depot system. In fact, if not adequately stirred, particles tend to settle at the bottom of the test tube forming sediment from which the protein is continuously released in solution. However, during static experiments, only a fraction of the device surface area is available for protein transport towards the supernatant. To overcome this issue, dynamic tests may be carried out by imposing continuous stirring. In this case, the convective contribution added to protein transport and, above all, the mechanical stresses imposed to the macromolecule, must be properly taken into account.

GF release kinetics from PLGA microspheres intended for tissue egineering 3D-scaffold integration have been commonly assessed by sampling-separation methods (37-41). The cumulative release of IGF-I and TGF-β from PLGA microspheres was followed by static incubation of the particles in in phosphate buffer at 37°C. The concentration of active protein within the release medium at each time interval was determined by an ELISA (41). However, microsphere depots formed in static experiments do not reflect what happens in a solid or gel-like scaffold, where the whole surface area of the device is exposed to the template. Thus, very different release profiles may be realised *in vivo*, after microsphere-embedding and scaffold implantation. More realistic information may be obtained following protein release from the whole template (i.e. microsphere-integrated scaffold). GF release studies from alginate composite scaffolds containing bFGF-loaded PLGA-microspheres were carried out by dynamic incubation of the integrated scaffolds in buffer solution at pH 7.2 and 37°C (37). The adopted experimental protocol indicated the ability of PLGA microspheres to sustain bFGF release within the aqueous environment sorrounding the scaffold. However, no indication about protein release profiles and subsequent information on GF concentration gradients achievement within the scaffold could be obtained.

Evaluation of release kinetics within 3D-scaffolds: the potential of Confocal Laser Scanning Microscopy technique

The embedding of microspheres in solid or gel-like scaffolds requires different approaches for release studies, because the device exposes the whole surface area to the release environment, and because microspheres cannot be separated from the template in a non-destructive way.

An alternative strategy to evaluate release kinetics from microspheres dispersed in a 3D scaffold is the use of Confocal Laser Scanning Microscopy (CLSM), which is a non-invasive technique allowing the visualization of fluorescent molecules or molecules covalently bound to a fluorescent probe (fluorophore). In a confocal microscope a laser

light is concentrated in a very thin focal plane (1 to 10 μm), therefore a high resolution of the sample, which can be optically dissected in any desired number of co-planar cross sections, may be obtained.

The suitability of the technique was assessed following the release of rhodamine-labelled BSA (BSA-Rhod), a model protein, from PLGA microspheres embedded in collagen-based scaffolds (submitted for publication). Microspheres loaded with BSA-Rhod were produced by the multiple emulsion-solvent evaporation technique and embedded in a ECM-mimicking scaffold, made of collagen and collagen/HA, at different HA concentrations. For release experiments, microspheres were dispersed in collagen-based solutions and incubated at 37°C to promote collagen fibrillation and the subsequent release period up to CLSM observations.

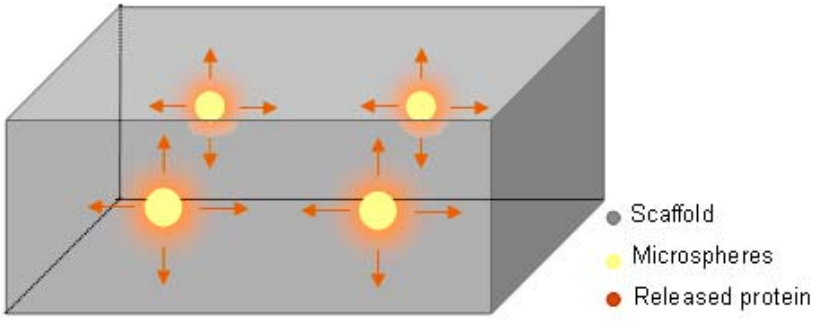

Fig. 6: *In vitro* protein release within collagen-based scaffold followed by CLSM. Microspheres are first dispersed in a collagen solution and, then, kept at 37°C for fibril formation and release. Microspheres are isolated each other so that the whole surface area is exposed to the release medium.

Release kinetics were evaluated directly in the scaffold, following the fluorescence decrease inside the microspheres with time (Fig. 7). The relationship between BSA-Rhod concentration in the microsphere and the CLSM signal was defined by evaluating the fluorescence of aqueous solutions of BSA at known concentrations, at predetermined confocal parameter settings, in a concentration range suitable to follow BSA-Rhod depletion. A linear dependence of overall fluorescence ratio on BSA-Rhod adimensional concentration was found out. The calibration equation is:

$$\frac{\bar{\phi}}{\bar{\phi}_0} = h + k\gamma$$

where:

$\bar{\phi}$ = integral average fluorescence of microspheres at time t, as given by CLSM software

$\bar{\phi}_0$ = integral average fluorescence at time zero.

$\gamma = \frac{C_{BSA}}{C^0_{BSA}}$ = adimensional BSA-Rhod concentration

C^0_{BSA} = initial BSA-Rhod concentration

h and k were -1.00 10^{-2}, and 1.007, respectively, and R^2 was 0.9978. Starting from fluorescence values, adimensional BSA-Rhod concentration is immediately derived. The same relation, in terms of adimensional amounts, was obtained in the range of concentrations and detector gains used for all observations, after fixing CLSM parameters (i.e. laser power, pinhole aperture and amplifier offset). Release percentage is easily calculated since:

$$R_{\%} = 100[1 - \gamma(t)]$$

The bleaching effect was evaluated by a prolonged exposure of microspheres to the laser light set at maximum power, and found to be negligible. CLSM parameters were set at time zero and kept constant throughout the release phase. Thus, the fluorescence depletion could be exclusively ascribed to BSA-Rhod diffusion out of the microparticle.

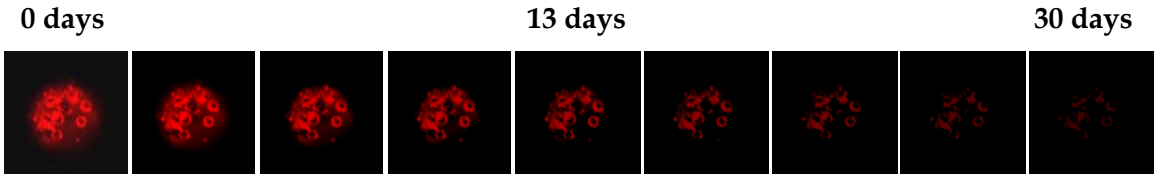

Fig. 7: Fluorescence decrease with time within a BSA-Rhod loaded PLGA microsphere embedded in a collagen-based scaffold as detected by CLSM. Microsphere fluorescence decrease with time is related to BSA-Rhod diffusion out of the device.

For comparison, release kinetics of BSA-Rhod from PLGA microspheres were evaluated also in solution by the sampling-separation method in both static and dynamic conditions. On the same PLGA microsphere formulation, *in vitro* experimental conditions strongly affects BSA-Rhod release profile from the particles (Fig. 8).

The hypothesis of a faster protein release within the collagen scaffold due to the increased surface area exposed to the template is confirmed by CLSM release data. To better reproduce what happens within the scaffold, dynamic conditions should preferentially be employed to evaluate release kinetics in solution (Fig. 8). However, one of the drawbacks in this case is related to the instability of released protein, which is continuously exposed to mechanical stresses. Furthermore, the use of classical sampling-separation methods could not account for the effect of scaffold composition on BSA-Rhod release kinetics. Actually, by comparing release profiles obtained by CLSM analysis of microspheres embedded in collagen and collagen/HA gels, it was demonstrated that the addition of HA significantly decreased BSA-Rhod diffusion out of the device, this effect being more evident during the first release stage (Fig. 9).

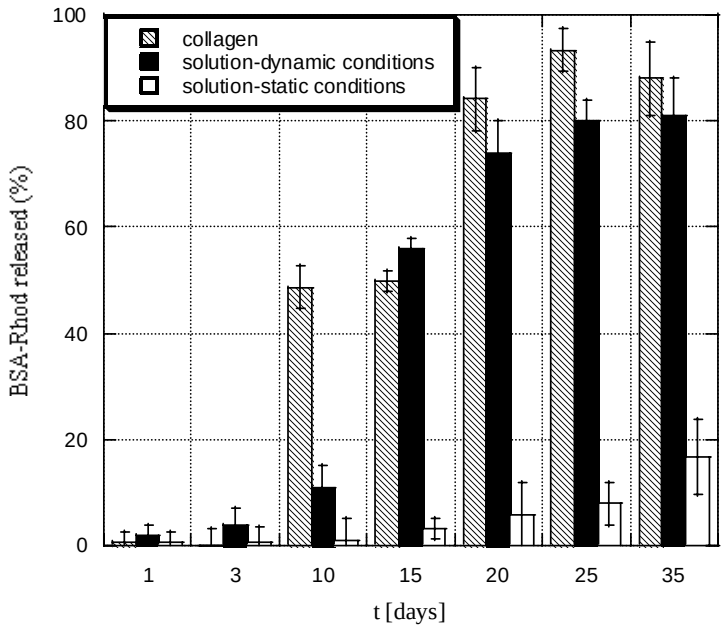

Fig. 8: Effect of the experimental protocol used for the evaulation *in vitro* BSA-Rhod release kinetics from a PLGA-based microsphere formulation as followed by CLSM. The slowest release is obtained in solution in static conditions, as a result of the reduced surface area exposed to the medium. When the suspension medium is continuously stirred (i.e. dynamic conditions), the release rate is much higher, as a result of the convective contribution. Though taking place in static conditions, BSA-Rhod release in collagen scaffolds is faster, because isolated microspheres expose the whole surface area to the release medium.

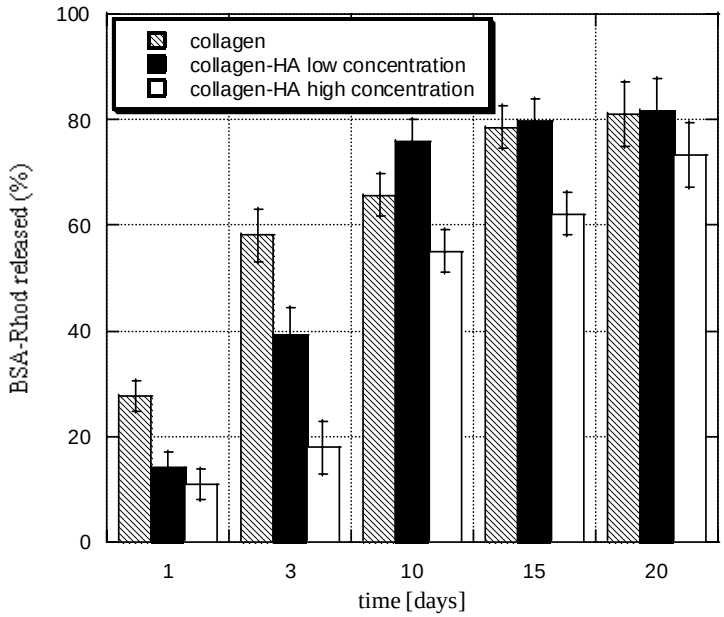

Fig. 9: Effect of scaffold composition on the release kinetics of BSA-Rhod loaded from a PLGA-based microsphere formulation as followed by CLSM. Release in a collagen, HA-free scaffold is the fastest one. HA addition to collagen scaffold induced a slower release rate, this effect being more evident adding even higher HA concentration.

Taken all together, release data highlight that, in case of GF-loaded microsphere-integrated scaffolds, the intrinsic limits of sampling-separation methods can be overcome by the use of CLSM. The technique allows a non-invasive description of protein release from isolated microspheres inside the polymeric scaffold, taking into account the effect of scaffold nature and composition on water diffusion in and protein release out of the particle. Thus, the analysis of a number of single microspheres throughout the release phase would permit to define the intrinsic release kinetics of the designed system, which is the main project parameter in bioactivated scaffold engineering.

Conclusion

The combined use of polymeric scaffolds and microspheres for GF-controlled release is a promising strategy to improve cell and tissue guidance for tissue regeneration. Through the fine tuning of microsphere formulation and scaffold structure/composition, it is possible to realize platforms able to control the microenvironmental conditions, in terms of time and space evolution of bioactive signalling molecules. Definition and evaluation of release mechanisms controlling the delivery of bioactive molecules from microsphere-integrated scaffolds is crucial for the design of novel bioactivated polymer scaffolds. Further study along this direction should be devoted to develop novel strategies and approaches to engineer templates by placing microspheres, releasing GFs at established rates, in a predetermined and optimized spatial distribution.

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