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Multiscale Patterning of a Biomimetic Scaffold Integrated with Composite Microspheres

Silvia Minardi,

Department of Bioceramics and Bio-hybrid materials, National Research Council of Italy – ISTEC,
Via Granarolo 64, 48018, Faenza RA, Italy

Department of Nanomedicine, Houston Methodist Research Institute, 6670 Bertner Ave. Houston,
TX 77030 (USA)

Monica Sandri,

Department of Bioceramics and Bio-hybrid materials, National Research Council of Italy – ISTEC,
Via Granarolo 64, 48018, Faenza RA, Italy

Jonathan O. Martinez,

Department of Nanomedicine, Houston Methodist Research Institute, 6670 Bertner Ave. Houston,
TX 77030 (USA)

Graduate School of Biomedical Sciences, University of Texas Health Science Center at Houston,
6667 Bertner Ave; Houston, TX 77030 (USA), Houston, TX USA

Iman K. Yazdi,

Department of Nanomedicine, Houston Methodist Research Institute, 6670 Bertner Ave. Houston,
TX 77030 (USA)

Department of Biomedical Engineering, University of Houston, Houston, TX USA

Xeuwu Liu,

Department of Nanomedicine, Houston Methodist Research Institute, 6670 Bertner Ave. Houston,
TX 77030 (USA)

Mauro Ferrari,

Department of Nanomedicine, Houston Methodist Research Institute, 6670 Bertner Ave. Houston,
TX 77030 (USA)

Bradley K. Weiner,

Department of Nanomedicine, Houston Methodist Research Institute, 6670 Bertner Ave. Houston,
TX 77030 (USA)

Department of Orthopedic Surgery Weill Cornell Medical College, The Methodist Hospital, 6550
Fannin St. 77030, Houston TX, USA

Anna Tampieri, and

Corresponding authors: Dr. E. Tasciotti, Department of Nanomedicine, Houston Methodist Research Institute, 6670 Bertner Ave. MS
R7-1414 Houston, TX 77030 (USA), etasciotti@HoustonMethodist.org.

Supporting Information

Supporting Information is available online from the Wiley Online Library or from the author.

Department of Bioceramics and Bio-hybrid materials, National Research Council of Italy – ISTEC,
Via Granarolo 64, 48018, Faenza RA, Italy

Ennio Tasciotti*

Department of Nanomedicine, Houston Methodist Research Institute, 6670 Bertner Ave. Houston,
TX 77030 (USA)

Abstract

The ideal scaffold for regenerative medicine should concurrently mimic the structure of the original tissue from the nano- up to the macro-scale and recapitulate the biochemical composition of the extracellular matrix (ECM) in space and time. In this study, a multiscale approach is followed to selectively integrate different types of nanostructured composite microspheres loaded with reporter proteins, in a multi-compartment collagen scaffold. Through the preservation of the structural cues of the functionalized collagen scaffold at the nano- and micro-scale, its macroscopic features (pore size, porosity and swelling) are not altered. Additionally, the spatial confinement of the microspheres allows the release of the reporter proteins in each of the layers of the scaffold. Finally, the staged and zero-order release kinetics enables the temporal biochemical patterning of the scaffold. The versatile manufacturing of each component of the scaffold results in the ability to customize it to better mimic the architecture and composition of the tissues and biological systems.

Keywords

biomimetics; silicon; PLGA; controlled release; tissue engineering

1. Introduction

The fate of a cell is determined by a complex set of biomolecules, which create the tissue-specific biochemical *milieu*, and by the nano- and micro-scale physical features that ultimately define tissues macroscopically.^[1] The elucidation of the stimuli necessary to achieve proper tissue regeneration has focused scaffolds' design toward the mimicry of the chemical and structural determinants of the tissue of choice.^[2, 3] A wide range of signaling molecules has been utilized to enhance the recruitment, proliferation and differentiation of autologous cells in the scaffolds. As an example, Bone Morphogenetic Protein-2 (BMP-2)^[4] and Transforming Growth Factor Beta (TGF- β),^[5] are extensively used for bone and cartilage regeneration.

A plethora of biomaterials based on polysaccharides (e.g. hyaluronic acid and alginate), proteins (e.g. fibrin, gelatin, and collagens) or synthetic polymers (e.g. poly(lactic acid) (PLA), poly(lactic acid)-co-(glycolic acid) (PLGA)) have been tested for tissue engineering applications.^[5-7] Collagen has attracted material scientists because it is one of the main components of human tissues. Its fibers spontaneously organize in supra-molecular networks, whose nano-topography is critical for conserving both biochemical (protein adsorption and growth factor retention)^[8] and biological (cell adhesion, migration, and differentiation)^[1] mechanisms.

The initial strategy was to add the growth factors by directly soaking the scaffold in a solution containing the factors of choice.^[9] Unfortunately, a recent controversy spurred from the adverse effects of excessive and uncontrolled release of recombinant human – BMP-2 in patients treated with collagen implants for spine fusion, brought the attention on the need to control the spatial and temporal distribution of the growth factor in the scaffold.^[10, 11]

To recapitulate how these growth factors are naturally presented, it is necessary to engineer scaffolds able to mimic the sequential release of multiple molecules from various intra-scaffold compartments.^[12, 13] The control of the material's structure at the nano-, micro- and macro-scale is of paramount importance to ensure the optimal three dimensional (3D) release and distribution of bioactive factors.^[14, 15] Nanostructured porous silicon^[16] revealed a valuable material in tissue engineering for its osteoconductivity,^[17, 18] and to develop porous particles able to accommodate high amount of proteins.^[19] Thus, pSi has been efficiently incorporated in a wide range of synthetic polymers for composite scaffolds' fabrication,^[20, 21] or for the development of composite delivery systems able to load, store and release multiple proteins from days to weeks and months.^[22, 23] These composite delivery platforms have the advantage to be easily tunable to match their release rates to those of native tissue, and to be more stable than the scaffold in which the delivery systems were added through surface adsorption.^[24–26] The lack of a tight confinement and protection of the delivery systems implies that once the scaffolds are implanted, they are exposed to the fluctuation of the microenvironment (e.g. pH, enzymatic activity, abnormal accumulation of fluid, etc) and to phagocytic cells of the immune systems which alter the composition of the scaffold and the stability of its individual components.^[27] However the dispersion of particles in a microfibrinous polymer such as natural type I collagen is challenging as well as the homogenous and effective retain of the particles in the final 3D scaffold. Type I collagen has the advantage to closely resemble ECM, but its hierarchically organized fibrous structure does not allow the use of the common methods of fabrication (e.g. electrospinning, sonication), without affecting its structure, which is key for its biological functions.^[28]

Here we propose a systematic approach for the synthesis of multiscale fibrous type I collagen scaffolds patterned with PLGA–pSi composite microspheres for the temporal and spatial release of proteins, addressing all the current limitations. This proof of concept study shows that the integration of the delivery systems within the two layers of a collagen scaffold, allows the triple-controlled release of two reporter proteins, the spatial confinement of the microspheres and the preservation of the nano- micro- and macro-scale features of the scaffold.

2. Results and Discussion

2.1. Nano- and Micro-Patterning of the Scaffold

2.1.1. Nanostructured pSi Particles—Nanostructured porous silicon particles (pSi) have been chosen as the core of the delivery systems for their biocompatibility,^[29] controllable biodegradation,^[22] high porosity (51%) and thus surface area,^[30] that allow for the storage and preservation of high amounts of different payloads (Figure 1A, B).^[16, 31] The ability to tailor the physicochemical properties of porous silicon at the nano- and micro-scale confers versatility to this material.^[30] Also, their preparation method allows for the

fabrication of highly reproducible, monodisperse particles, favoring their loading and encapsulation.^[23, 31, 32] pSi shells were fabricated with an average diameter of 3.2 μm , 600 nm shell thickness. To improve loading efficiency, pSi surface was modified with (3-Aminopropyl)triethoxysilane (APTES) that increases its hydrophilicity by introducing amine groups on the surface of the particles.^[32]

2.1.2. pSi Encapsulation in PLGA Microspheres—Although silicon revealed a valuable materials to develop delivery systems able to load, store and release multiple proteins, their degradation in physiologic conditions resulted very fast (in the order of hours),^[22] and thus not compatible with tissue healing, which take place from several weeks, up to months. The creation of composites, based on pSi and polymers, contributes to enhance material stability and thus prolong release kinetics.

PLGA can be produced in different molecular weights and copolymer ratios, yielding materials with tunable degradation rates.^[33, 34] To obtain the stable and efficient blending with pSi in the organic solvent and the following integration with collagen in an aqueous solution, we used the 50:50 PLGA co-polymer that resulted in the optimal surface charge and polarity. pSi were encapsulated in the PLGA shell through a modified double emulsion method.^[23] The use of monodisperse pSi was key to optimize PLGA-pSi size distribution, as confirmed by scanning electron microscopy (SEM) (Figure 1C), where PLGA-pSi displayed an average size of $11 \pm 3 \mu\text{m}$ in diameter (Figure 1D, blue data set), with sizes ranging between 3 μm to 25 μm , with a reduction of the size range of the 50% compared to previous reports.^[35]

Optical microscopy demonstrated that median number of $3 (\pm 0.2307)$ pSi (Figure 1D, yellow data set) were encapsulated in the PLGA microsphere (Figure 1E, F). The overall improvement of PLGA-pSi fabrication and pSi dispersion through the microspheres was crucial to ensure a more homogenous release of payload.

2.1.3. Collagen Functionalization with PLGA-pSi—Collagen type I was chosen because it is the most abundant structural element in connective tissues,^[36, 37] where its hierarchically organized structure plays a prominent role in maintaining the biologic and structural integrity of the ECM.^[38]

Exploiting collagen's ability to dock and blend with PLGA, we optimized a procedure to stably integrate PLGA-pSi within the scaffold's matrix. To demonstrate the spatial confinement of the microspheres in the scaffold we compared three groups: (i) blank scaffolds (CTRL); (ii) scaffolds with adsorbed PLGA-pSi microspheres (ADS), included as current standard; (iii) scaffolds with PLGA-pSi integrated in the collagen matrix (INT). Figure 2 illustrates the morphology and microstructure of the CTRL (Figure 2A, B, C), ADS (Figure 2D, E, F) and INT (Figure 2G, H, I) scaffolds. The PLGA-pSi fully integrated within the collagen matrix of INT scaffolds, while in the ADS scaffolds the microspheres were simply adsorbed on the pore walls. In fact, the collagen matrix formed a consistent coating surrounding the PLGA-pSi, as illustrated in Figure 2J, where a high voltage electron beam during SEM imaging was used to damage the collagen coating and expose the PLGA-pSi. The overall morphology of the scaffolds and their pore shape and size were preserved in

both ADS and INT scaffolds. Similarly, PLGA-pSi did not affect the structure of the collagen fibrils which maintained the characteristic D-band of approximately 67 nm (Figure 2K, L and Figure S1 in Supporting Information).^[39, 40] Thus, herein we developed a method of scaffold functionalization with PLGA-pSi which preserves such asset, and which is also exploited as a further level of control over the temporal and spatial control over the reporter proteins.

2.1.4 Characterization of PLGA-pSi Integration in Collagen Scaffolds—To

confirm the complete embedding of the PLGA-pSi within the collagen matrix, confocal laser microscopy was used to acquire Z-stacks, and reconstruct the 3D volume around the integrated microsphere (Figure 3A). As exposed in the orthogonal views in Figure 3B, the fibers of the collagen (blue) completely enclosed the PLGA-pSi (red and green, respectively). Pore wall's thickness was measured from the Z-stack acquisitions, and appeared to be in the range of 10–15 μm in the control scaffold. By embedding PLGA-pSi of $\sim 11 \mu\text{m}$, in the collagen matrix we provided a $\sim 2 \mu\text{m}$ thick collagen coating of PLGA-pSi. Reaching a more homogenous distribution of PLGA-pSi along the pore walls, as well as the consistent thickness of the collagen coating on all microspheres, was crucial for a homogenous release from the bulk scaffold.

Microspheres' blending with the collagen matrix was analyzed through Fourier transformed infrared spectroscopy (FTIR). CTRL, INT scaffold and ADS scaffold have been all freeze dried following the same procedure, previous FTIR analysis. FTIR spectra of PLGA-pSi microspheres resembled a hybrid spectra, resulting by the addition of the peaks of pSi and PLGA, confirming the formation of a pSi-PLGA composite (Figure 3C). In the INT scaffolds (Figure 3D), a pattern with broader FTIR peaks, especially in the regions that correspond to amine groups ($3500\text{--}3300 \text{ cm}^{-1}$, stretching),^[41] and carbonyl groups ($1750\text{--}1735 \text{ cm}^{-1}$, stretching) can be observed,^[42] suggesting the interaction of the carbonyl groups on the surface of the PLGA-pSi microspheres with the amine groups of the collagen. A moderate contribution in the broadening of the Amide I peaks of INT scaffold by physisorbed water was excluded for the absence of any broadening in the correspondent peak in CTRL. On the contrary, the Amide I peak intensity and area in the range $3000\text{--}3600 \text{ cm}^{-1}$ (OH- stretching) are comparable in both samples, and do not appear to be affected by the presence of physisorbed water. On the contrary, the FTIR spectra of ADS scaffolds displayed either the spectrum of pure type I collagen or of PLGA-pSi, depending on the spot analyzed (10 random spots were evaluated) (Figure S2).

Analysis of the mean pore size and surface area confirmed the anisotropic structure of all scaffolds (Figure 4A and B).^[43, 44] CTRL presented a porosity of 91%, with a mean pore area of $20 \cdot 10^3 \mu\text{m}^2$, and with a diameter in the range of $250\text{--}350 \mu\text{m}$. The changes in the average porosity and pore area of ADS and INT, respect to CTRL, are shown in Figure 4C. While the integration of PLGA-pSi did not result in a significant decrease of the porosity in the INT scaffolds, the pore size and porosity of ADS scaffolds were significantly different from CTRL. This discrepancy was attributed to the obstruction of the pores by the adsorbed microspheres. The swelling of both ADS and INT scaffolds was slightly lower than the control scaffold, though not significantly altered, and demonstrated that the integration of PLGA-pSi in the collagen matrix did not affect its swelling, a key feature for both the

mechanical and biological properties of the scaffold (Figure 4D). Pore size and porosity of the scaffold were tuned during the freeze drying process through the adjustment of the freezing temperature, heating ramp, and the content of water in the collagen slurry.^[45] None of these features were affected by the incorporation of the PLGA-pSi microspheres.

Confocal imaging of the scaffolds allowed the identification and characterization (e.g., diameter, distribution, and relative distance) of individual microspheres within scaffolds (NIS-Elements software, Nikon). The density of microspheres in the ADS and INT scaffolds was $2 \cdot 10^3 (\pm 24)$ per mm^3 . The type of integration did not change the size of PLGA-pSi confirming the stability of the microspheres throughout the integration process. The mean distance between PLGA-pSi was calculated automatically in ten random positions on the Z plane. For INT scaffolds, the mean distance between neighboring PLGA-pSi was 46 μm . The moderate standard deviation supports the homogenous distribution of microspheres within the INT scaffolds. On the other hand ADS scaffolds, possibly due to the uncontrolled distribution and agglomeration of PLGA-pSi, exhibited mean distances of 70 μm , correlating with a much higher standard deviation (Figure S3). All these data are summarized in Table 1. Through the tuning of the design parameters (PLGA-pSi size, density, number of PLGA-pSi per layer, mean distance between microspheres, etc.), it is possible to reverse engineer scaffolds with pre-defined parameters, to allow for the customization of scaffolds with defined geometries, release patterns and kinetics.

The stability of the integration of PLGA-pSi in the scaffold was tested comparing the retention of microspheres from ADS and INT scaffolds. After two soaking steps, ADS scaffolds lost three times more PLGA-pSi than INT (15% and 5% respectively). At two weeks 94% of PLGA-pSi was lost from the ADS scaffolds, compared to only 17 % lost from INT, exhibiting an almost 6 fold increase in retention (Figure S4, Supporting Information). The role of the collagen in retaining scaffold's nano- and micro- components and features (e.g. functional groups, hierarchical architecture, pore size, swelling), ultimately preserving the entire macro-structure, enabled the stable and homogeneous confinement of PLGA-pSi. All these results confirmed that PLGA-pSi became part of the scaffold, when integrated to the collagen at the slurry state, during the last steps of fibers self-assembling. All these data together demonstrated that this approach of functionalization represents a more efficient alternative to particle adsorption in collagen sponges, which lack of any control over particles' distribution and retention.

2.2. Macro-patterning of the Scaffold with PLGA-pSi

2.2.1. Spatial Patterning of the Reporter Protein—A bi-layered scaffold for the staged release of 488-BSA and 680-BSA from each individual layer was fabricated according to Scheme 1. 488-BSA was encapsulated in a PLGA-pSi with a low-density polymer (LD) coating to achieve a faster release rate; while 680-BSA was incorporated in high-density polymers (HD) for a slower release rate. Composites were characterized also by Thermo-Gravimetric Analysis to confirm the different content of polymer and silicon (Figure S5 in Supporting Information). Quantification of BSA loading revealed that 12 ± 3 μg of 488- or 680-BSA was accommodated into 10^6 pSi exhibiting no appreciable loss of BSA during encapsulation with PLGA (Figure S6). The high loading capacity of the pSi core

facilitates the prolonged release of proteins and is amenable to the concurrent loading of multiple bioactive factors. This is particularly relevant as most clinical applications require hundreds of nanograms of bioactive factors per day to achieve an effective dose.^[46, 47]

The structure of the bi-layered scaffold and of each layer is depicted in Figure 5. PLGA-pSi microspheres were completely enclosed within the collagen layer forming the pore walls. The interface between the two layers exhibited no delamination and near-perfect integration (see dotted line in Figure 5A). The optimized freeze-drying process led to a monolithic scaffold showing structural continuity between different layers (Figure 5B and C), and devoid of signs of delamination under physiological conditions (Figure S7). When the two layers are stacked at their slurry state the exchange of fibers between the unstructured collagen molecules allow the interweaving of the two layers. The controlled freezing and heating ramp, triggered the directional growth of water crystals from the bottom to the top of the slurry, resulting in further integration and stability of the two layers. This fabrication technique allowed for the creation of several intra-scaffold compartments in which the confinement of the PLGA-pSi occurred in all dimensions (Figure S8). The ability to accomplish the 3D patterning of the scaffold is particularly relevant for applications in interface tissue engineering, where there is the need to simultaneously mimic the architecture and biochemical environments found at the interface of different but contiguous tissues.^[48]

2.2.2. Temporal Patterning of the Reporter Proteins: In Vitro Release—To

investigate the contribution of the collagen scaffold to the release kinetics of BSA from LD and HD PLGA-pSi, we investigated four experimental groups: (1) LD and HD; (2) 488- and 680-BSA soaked in a bilayered collagen scaffold (SKD); (3) LD and HD adsorbed in a bi-layered (LD/HD ADS); (4) LD and HD integrated in a bi-layered scaffold (LD/HD INT). The release kinetics of each experimental group are reported in Figure 6A. For clarity, a single release profile was reported for SKD, as the release kinetics for 488- and 680-BSA were almost identical. As expected, the SKD group showed a burst release, up to 80% lost within the first 12 hours. In addition, LD exhibited a considerably faster release rate compared to HD with complete release plateauing at day 18 compared to day 50 for HD samples.

Similar release kinetics were found for both LD and HD and the corresponding LD/HD ADS (Figure 6B). The results suggested a negligible contribution of the collagen scaffold to the release kinetics when the microspheres were adsorbed on its surface. Considering the loss of PLGA-pSi from ADS, presented in the supporting information session, it is reasonable to believe that the similar release kinetics of free LD/HD and LD/HD ADS could be attributed to the massive loss of PLGA-pSi (82.5%) from the ADS scaffolds within the first two weeks. The release from LD/HD INT was significantly delayed compared to all other groups (Figure 6C), confirming the contribution of the collagen matrix in modulating the release kinetics by creating an additional coating layer over the microspheres. As expected, the release of BSA from HD INT scaffold exhibited a slower release rate compared to the corresponding free HD (Figure 6D).

The quantification of the total mass of BSA released at the individual time points revealed that significant amounts of BSA were discharged by LD SKD and HD INT as early as 48 hours (5× and 7× respectively) (Figure 6E). After seven days, free and LD ADS demonstrated similar cumulative release (~78%), while INT released 50% and 35% of BSA from LD and HD respectively (Figure 6F). LD and HD INT scaffolds showed substantially different kinetics in the two layers (HD INT release rate being 50 percent slower than LD INT). While these results confirm the creation of temporal gradients of proteins, the observation that the BSA released from each layer, remained entrapped within its own layer, proved the spatial confinement of these protein regardless of their release during time. All together, these studies demonstrated that the integration of microparticles within the collagen scaffold matrix played an important role in controlling the staged release and spatial retention of two reporter proteins within the two separate compartments of the scaffold.

3. Conclusions

With this study we present a novel approach for the creation of multiscale biomimetic scaffold, capable of generating spatial and temporal protein patterns. The result was achieved by leveraging the natural ability of the collagen matrix to interact with PLGA-pSi microspheres, effectively constructing a simple and tunable system for various tissue engineering applications. Optimizing the synthesis of monolithic multilayered collagen scaffolds, we stably integrated PLGA-pSi within the collagen matrix without altering collagen's nano- and micro- structure. The results demonstrate that this modular approach to fabricate multi-compartment scaffolds allow for tissues mimicry at a multiscale level. At the nanoscale, the type I collagen conserved its fibrillar structure and typical D-bands appearance, while the pSi nanostructure allowed the loading and release of reporter proteins. At the microscale, the PLGA coating of pSi created a composite delivery platform for the tunable release of the reporter proteins, while the collagen coating on PLGA-pSi enabled for their spatial confinement in the scaffold. Finally, at the macroscale, all these elements combined, without altering the feature of the material, such as pore size, porosity and swelling upon PLGA-pSi integration. pSi, PLGA and collagen boundaries contributed to accomplishing the temporal patterning of the proteins in the multi-layered scaffold, through a triple controlled release, enabling for the zero-order release kinetics of reporter protein up to 50 days. The release of controlled amounts of drugs over long periods of time would favor on-scaffold regeneration while avoiding adverse effects due to the diffusion of high doses of therapeutic molecules in the tissues surrounding the scaffold. By grafting distinct sets of PLGA-pSi composites, one can envision the creation of multiple unique biochemical niches within a 3D biomimetic scaffold, while protecting the payload and the delivery system from the cellular and enzymatic attack of cells of the immune system (mainly macrophages) and from the surgical procedures (washing, debridement, bleeding, fluid aspiration) applied during the implantation of the scaffold which challenge the integrity of the material.

Experimental Section

Silicon Particles Fabrication and Surface Modification

Hemispherical porous silicon particles of 3.2 μm in diameter and 600 nm thick were fabricated by photolithography and electrochemical porosification of patterned silicon wafers, allowing for precise control over particle size and shape, as previously reported.^[23, 30] Silicon particles were oxidized as extensively described elsewhere.^[23, 32] The surface of the pSi particles was modified with APTES (Sigma–Aldrich) as previously described.^[23, 30]

PLGA-pSi Optimization and Characterization

2×10^8 APTES modified pSi were dispersed, by sonication, in a solution of PLGA (50:50) (LACTEL) dissolved in dichloromethane (DCM) (375 μL , 50 mg/ml) (Sigma-Aldrich). The organic phase containing the pSi was mixed with poly (vinyl alcohol) (PVA) (Fisher Scientific) (1,5 mL, 25 mg/ml) by homogenization. The emulsion was dropped into an aqueous solution (50 mL) containing PVA (5 mg/ml). The resulting suspension was stirred for 6 h to allow the evaporation of the dichloromethane, then washed three times with DI water. Empty PLGA microspheres were eliminated by differential centrifugation, as we previously implemented.^[23] PLGA-pSi's morphology of the microspheres was characterized by SEM (FEI Nova NanoSEM 230). They were sputtered coated by a Plasma Sciences CrC-150 Sputtering System (Cressington 208HR), and examined under a voltage of 7 KV. Also, they were imaged through optical microscopy (Nikon Eclipse TS 100), and PLGA-pSi size distribution was measured through an automated measurement tool of the software NIS-Element (Nikon).

Preparation of the collagen slurry

1 g of type I collagen (Sigma-Aldrich) were dissolved in an acetate buffer (pH 3.5) to reach the desired concentration (20 mg/ml).^[44] The collagen suspension was precipitated by the addition of sodium hydroxide (0.1 M) solution at pH 5.5. The collagen was washed three times with DI water. The collagen was cross-linked through dispersion for 48 h in a 1,4-butanediol diglycidyl ether (BDDGE) (Sigma-Aldrich) aqueous solution (2.5 mM), setting up the 1, 4-butanediol diglycidyl ether (BDDGE)/collagen ratio (1 wt%).^[44] The collagen was washed 3 times in DI water.

Fabrication of CTRL, ADS and INT scaffolds

the cross-linked collagen (20 mg dry weight) was suspended in DI water (5 mL), and three groups of collagen scaffolds were fabricated: (i) CTRL; (ii) ADS; (iii) INT. To fabricate the ADS, PLGA-pSi were suspended in water (2 mg/mL) and the scaffolds were swollen in the dispersion. The same amount of particles was instead integrated during fabrication of INT, at their slurry state. The materials were freeze-dried with a controlled freezing ramp from 25 $^{\circ}\text{C}$ to -25°C and a heating ramp from -25°C to 25 $^{\circ}\text{C}$ in 50 min under vacuum conditions ($P = 0.20$ mbar), and collagen scaffolds (20 mm³) were produced. Blank collagen scaffolds were used as CTRL. The evaluation of scaffolds morphology was performed through SEM. A set of fluorescently labeled PLGA-pSi were prepared for confocal laser microscopy

imaging (A1 Nikon Confocal Microscope). PLGA was labeled with Rhodamine B (Sigma-Aldrich), while pSi was conjugated with Dylight488 (Invitrogen).^[31] The blending of PLGA-pSi and collagen was also validated through FTIR (Nicolet 6700 FT-IR Spectrometer).

Pore Size, Porosity and Swelling

1 mm thick sections of the scaffolds were imaged with fluorescence microscopy (Nikon Eclipse Ti equipped with a Hamamatsu Orca Flash 2.8 digital camera), and the pore are determined via an automated measurement tool of the NIS-Element software (Nikon). The volumes of the scaffolds (V_s) were measured from the scaffold geometry (cylinders of 5 mm in diameter and $\times 1$ mm height). The volume of the pores was calculated by an ethanol infiltration method.^[49] The volume of the pores was defined as in Equation (1):

$$V_p = (W_e - W_0) / \rho_e \quad (1)$$

where W is scaffold's weight before (W_0) and after incubation in ethanol (W_e), and ρ_e (0.789 mg mL^{-1}) represents the ethanol density at room temperature. The porosity of the scaffolds was calculated according to Equation (2).

$$P = V_p / V_s \cdot 100\% \quad (2)$$

To determine the PBS uptake property, the completely dried scaffolds were weighted and afterwards incubated in PBS at 37°C . The hydrated scaffolds were taken out at the desired time intervals, wiped superficially with a filter paper to remove the surface water, and weighed (W_w). The uptake ratio was defined as swelling %, as in Equation (3). The value is expressed as means $\pm$ SD ($n = 5$).

$$Sw\% = (W_w - W_d) / W_d \cdot 100 \quad (3)$$

PLGA-pSi Integration Assessment

PLGA-pSi confinement stability over time was investigated incubating the CTRL, ADS and INT scaffolds (5 per group) in PSB at 37°C in mild mixing (100 rpm). Particle loss was quantified counting the fluorescently labeled microspheres (Rhodamine B) through the automated counting tool of the NIS-Element Software (Nikon) of the fluorescence microscope, collecting the PBS and substituting it with fresh one at defined time points, up to 2 weeks. Also, other 5 scaffolds, with the same formulation were used to assess PLGA-pSi distribution in ADS and INT.

Loading of 488-BSA and 680-BSA into APTES modified pSi particles

488-BSA and 680-BSA (FITC-BSA, Sigma-Aldrich; Dylight680-BSA, Invitrogen) solutions (10 mg/mL) were prepared by dissolving the BSA powder in DI water. 2×10^8 APTES modified particles were immersed into the 488-BSA (Sigma-Aldrich) and 680-BSA (Invitrogen) solution ($500 \mu\text{L}$) respectively. The suspensions were mixed at 37°C for 2 h, to allow proteins loading in the pores of the pSi. The particles were recovered by centrifugation

at 4000 rpm and washed three times with PBS to remove the 488-BSA or 680-BSA adsorbed on the surface. The particles were then lyophilized overnight. The amount of protein loaded was measured by the difference between the protein concentrations of the stock solution and of the supernatant using SpectraMax M2 spectrophotometer (Molecular Devices) at 493/520 nm and 680/715 nm to quantify 488-BSA and 680-BSA, respectively.

Loaded pSi encapsulation in PLGA-pSi

BSA-loaded pSi particles were coated with PLGA by a modified S/O/W emulsion method, as described above. Briefly, 2×10^8 488-BSA and 680-BSA loaded pSi were dispersed, by sonication, in PLGA (50:50) (LACTEL) dissolved in DCM (375 μ L) (Sigma-Aldrich) (50 mg/ml and 200 mg/mL). Thus, two sets of PLGA-pSi with different PLGA coating were obtained as follow: 5 wt% (LD) and 20 wt% PLGA in DCM (HD). A quantitative characterization to assess the PLGA and pSi content in the two formulations (LD and HD) was performed via thermogravimetric analysis (TA Instruments Model Q600), with a heating ramp of 5°C/minute, from 25°C to 500°C.

Bi-layered BSA-loaded scaffold fabrication

PLGA-pSi loaded with 488- or 680- BSA were dispersed in 1 mL of DI water and added to separate crosslinked collagen slurries (as described above), mixed and finally centrifuged at 4000 rpm in a Legend X1R Centrifuge (Thermo Scientific) for 5 minutes. Through layer-by-layer assembly the two collagen slurries were piled on to each other, in a metallic mold. Finally, a monolithic scaffold was generated through freeze-drying process as described above. Scaffold's morphology was characterized by SEM, fluorescence and confocal laser microscopy.

Evaluation of 488- and 680- BSA in vitro Release

as a control, LD and HD were dispersed in phosphate buffer saline (PBS) (20 mL) at 37 °C. Also bylayered scaffolds ADS and INT were immersed in PBS in glass bottles as well as CTRL, at 37°C, and mixed at 100 rpm. At predetermined time intervals, the suspension was centrifuged (4500 rpm; 5 min), and 10% of the total volume of supernatant (2 mL) was collected, and replaced with fresh PBS, up to 50 days. The release was quantified by SpectraMax M2 spectrophotometer (Molecular Devices) at a wavelength of 493/520 nm and 680/715 nm to quantify 488-BSA and 680-BSA respectively. Three replicates for each experimental group were utilized.

Statistical analysis

The number of samples used in each experiment is noted in the text of the experimental section. Dependent variables are expressed as means $\pm$ SD. The differences in the means were tested using ANOVA and Student T-test to check for statistical significance ($P < 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization of PLGA-pSi microspheres. (A) SEM image of pSi illustrating their typical quasi-hemispherical shape; (B) Maximum, mean, and minimum pSi size; (C) SEM image and (D) size distribution of PLGA-pSi microspheres and number of pSi per PLGA microsphere. (E) Optical microscope image of PLGA-pSi composite microspheres (PLGA: transparent sphere; pSi: brown hemispheres); (F) sliced view of a PLGA-pSi microsphere showing full integration of pSi.

Figure 2. Scaffold integrated with PLGA-pSi microspheres. (A, B, C) Scanning electron microscopy images at different magnifications of control collagen scaffold (CTRL); (D, E, F) scaffold with PLGA-pSi adsorbed (ADS) on the surface and (G, H, I) with PLGA-pSi integrated (INT). (J) Type I collagen created a coating on the microspheres, indicated by the arrows, (K, L) while preserving collagen ultrastructure.

Figure 3.

Qualitative analysis of PLGA-pSi blending with collagen matrix. (A) 3D reconstruction of confocal microscopy images illustrating PLGA-pSi integrated in the collagen scaffold and completely wrapped by collagen (collagen: blue; PLGA: red; pSi: green); (B) sagittal view showing pSi encapsulated within PLGA and coated by the collagen matrix. (C) FTIR patterns of the multiscale components of PLGA-pSi and (D) of the functionalized scaffold.

Figure 4. Characterization of blended scaffolds. (A) Fluorescent image of the entire collagen scaffold at low and (B) high magnification used for pore area calculation. (C) Porosity and pore area of scaffolds adsorbed or integrated with PLGA-pSi compared to a control scaffold. (D) Swelling characteristics comparing control, adsorbed (ADS), and integrated (INT) collagen scaffolds over time.

Figure 5.

PLGA-pSi functionalized bi-layered scaffold. (A) SEM, (B) fluorescent and (C) bright field images, merged with fluorescence, of the interface between the two layers of the collagen scaffold functionalized with LD loaded with 488-BSA (green layer) or HD loaded with 680-BSA (red layer). (D, E) Maximum intensity projections and 3D reconstruction of CTRL, (F, G) LD INT layer and (H, I) HD INT layer, respectively.

Figure 6. Release kinetics from bi-layered functionalized scaffolds. (A) Overall results of all the groups investigated. (B) Release of BSA from SKD, LD PLGA-pSi and LD PLGA-pSi ADS. (C) Comparison of BSA release from LD PLGA-pSi and INT. (D) Comparison of BSA release from HD PLGA-pSi and INT. The statistical significance between the amounts of BSA release over time per each group is marked with asterisks ($p < 0.05$) for the time points at (E) 48h and (F,G) at 7 days.

Scheme 1.

Schematic showing: (A) the setting of LD PLGA-pSi (PLGA: blue; pSi: green) and HD PLGA-pSi (pSi: red), in the collagen fibers (yellow), (B) in the bi-layered collagen scaffold.

Table 1

Comparison of the characteristics of ADS and INT respect to their controls.

	CTRL	ADS	INT
Pore Area [μm^2]	$20.3 \cdot 10^3$	$14.3 \cdot 10^3$	$18.4 \cdot 10^3$
Porosity [%]	91	83	88
PLGA-pSi Diameter [μm]	11 ± 3	10 ± 4	10 ± 3
Mean Distance of PLGA-pSi [μm]	/	70 ± 21	46 ± 7