The Impact of Fabrication Parameters and Substrate Stiffness in Direct Writing of Living Constructs

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Biomolecules and living cells can be printed in high-resolution patterns to fabricate living constructs for tissue engineering. To evaluate the impact of processing cells with rapid prototyping (RP) methods, we modeled the printing phase of two RP systems that use biomaterial inks containing living cells: a high-resolution inkjet system (BioJet) and a lower-resolution nozzle-based contact printing system (PAM^2). In the first fabrication method, we reasoned that cell damage occurs principally during drop collision on the printing surface, in the second we hypothesize that shear stresses act on cells during extrusion (within the printing nozzle). The two cases were modeled changing the printing conditions: biomaterial substrate stiffness and volumetric flow rate, respectively, in BioJet and PAM^2. Results show that during inkjet printing impact energies of about 10^{-8} J are transmitted to cells, whereas extrusion energies of the order of 10^{-11} J are exerted in direct printing. Viability tests of printed cells can be related to those numerical simulations, suggesting a threshold energy of 10^{-9} J to avoid permanent cell damage. To obtain well-defined living constructs, a combination of these methods is proposed for the fabrication of scaffolds with controlled 3D architecture and spatial distribution of biomolecules and cells. © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.,* 28: 1315–1320, 2012
Keywords: biofabrication, living systems, scaffold, biomaterials, cell culture

Introduction

In recent years, tissue engineering (TE), a multidisciplinary subject that comprises biology, medicine, and material science,[1] has evolved to include other disciplines such as computer science, chemical and material engineering, and robotics to better understand biological system function, and thus to recreate its overall structural complexity. Indeed, biological tissue structure is intricately linked with its function. Natural tissue architecture is in fact an intricate mesh, characterized by the presence of multiple cell types, biomolecules, ligands, fibrous proteins, and many other factors. All of them are precisely organized in three dimensions (3D), and can be grouped in: (i) cellular, (ii) biochemical, and (iii) architectural/mechanical components. The aim of TE is to reproduce this complex system through the design and fabrication of scaled-constructs, restoring the function of native tissue and providing the basic 3D topological and mechanical framework for cells. In this perspective, the function of scaffolding biomaterials, as analog of the natural extracellular matrix (ECM), is to provide mechanical support to cells and regulate cell behavior. In other words, generating biological in vitro constructs involves the use of engineering and material science methods to fabricate ECM substitutes with specific properties across multiple length scales. A number of microfabrication techniques aimed at controlling cell–material interaction have been developed. In particular, rapid prototyping (RP) systems are commonly used to fabricate 3D structures with a high degree of control over the geometrical features. RP direct-write technologies based on computer-aided designs (CADs) include many additive techniques capable of depositing, dispensing, or processing different types of materials over various surfaces. A typical direct-write approach prints patterns or layered structures using CAD with a fine control over the spatial resolution (ranging from 1–10 to 0.1–1 mm). Cell printing through direct-writing of living material evolved about a decade ago thanks to significant advances in microfabrication technology. This approach enables the production of layer-by-layer heterogeneous 3D constructs embedding living cells in a scaffolding material. As it solves many of the problems associated with cell seeding on 3D scaffolds, such as cell loss due to inefficient seeding and inadequate penetration, printing cell suspension is actually useful for TE applications. In addition, the fabrication of 3D living constructs using robotic dispensing systems enables fine control over cell positioning as well as material architecture, both of which influence cell function and organization. Inkjet printing as a RP method to deposit cells was first demonstrated by Mironov.[2] Since then, many researchers have adapted commercial printers to deliver cells in high-resolution patterns.[3–5] In most reports, it is generally accepted that the cells are subjected to high stresses in the cartridge of the printer (i.e., heat and mechanical forces), and during the impact of the drop on the substrate, however, few reports on cell printing actually quantify

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cell viability or damage after inkjet deposition.[6] Other direct-writing systems, such as the PAM^2 (piston and pressure assisted microsyringe) system, make it possible to push the boundary from the 2D printing to 3D miniaturized constructs. Custom and user-defined microarchitectures have been used to provide guidance for the formation of multicellular living microstructures.[7–12]

In this study, we demonstrate the possibility of printing living biological materials using two RP systems: the Olivetti BioJet thermal inkjet printer and the PAM^2 system. As both of these systems have already been characterized in printing of spatially controlled patterns, attention was dedicated to the feasibility of processing viable "living inks" (i.e., cell suspensions). In particular, as processed cells are subject to high forces and stresses, finite element methods (FEMs) were used to calculate the average energy exerted on cells: models were then experimentally validated by assessing cell viability after both the printing processes. Specifically, attention was focused on the mechanical impact of ink drops with the printing substrate in the BioJet printer and on the fluid dynamics in a narrow nozzle in the PAM^2 system. Different printing substrates and volumetric flow rates (parameters that can be easily changed and controlled) were considered both in the numerical simulation and in the experimental tests. In this article, we demonstrate that printing energies acting on printed cells can be modeled to predict cell survival during and after the printing phase and then used for the fabrication of living constructs. A new design approach for the fabrication of 3D architectured microsystems including living and viable cells, as well as biomolecules, with controlled mechanical properties is thus proposed.

Methods and Materials

BioJet

BioJet (Olivetti I-Jet, Arnad, Italy) is a patented thermal inkjet technology, able to precisely and accurately dispense small amount of liquid (10–100 pL) in high-precision 2D patterns (10 μm lateral resolution). The main limitation of inkjet systems resides in ink composition: viscosity and surface tension ranges of such inks should be well matched with the ejection conditions of thermal inkjets, and typical values are similar to those of water ($\sim$1 mPa s viscosity and 64 mN/m surface tension, although maximum values depend on nozzle properties). For this reason, only water-based inks can be used, and in general, it is difficult to deposit scaffolding material together with the cell suspension. To use cell suspensions as ink, the print cartridges were modified and sterilized before use.

PAM^2

The PAM^2 system represents a new concept of RP system, in which several biomaterials (e.g., water-like, viscous, and thermo- or light-sensitive inks) having different properties can be processed on the same platform to fabricate multimaterial and complex-shaped microstructures in a biologically friendly environment. It consists of a 3D axis micropositioner with modular add-on processing heads.[13] System add-ons can be used to extrude liquid-like inks (0.7–20 mPa s) through a pressure actuated nozzle and viscous inks (0.05–1 Pa s) through a piston actuated nozzle, or control ink temperature (25–80°C). Using purpose designed software, it is possible to have a fine control of the preprocessing, processing, and postprocessing of complex and multimaterial microsystems, with high fidelity layered patterns (deposited material with lines of 10–100 μm width according to deposition head, ink viscosity, and characteristics of the nozzle).

Cell cultures

LivingInk. 3T3 fibroblasts (mouse embryonic fibroblast cell line, ATCC-LGC, UK) were used to prepare water-like cell suspensions (henceforth referred to as "LivingInk"). LivingInk was used for all inkjet experiments described in this work. 3T3 cells were cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10% v/v fetal bovine serum, 4 mM L-glutamine, 100 U of penicillin, and 100 μg of streptomycin. A cell suspension with a concentration of 5×10^6 cells/mL in medium was prepared, injected into sterile print heads, and ready to be used in printing tests. During the printing phase, LivingInk was deposited directly on different surfaces controlling the number of printed cells (indirectly derived via ejected volume of LivingInk). The surfaces used in this study were: (i) polystyrene culture plates, (ii) cross-linked collagen, and (iii) a 1% w/v solution of gelatin in medium. Surface properties (i.e., stiffness and contact angle) were experimentally measured using compressive mechanical tests and a contact angle goniometer, respectively. Cell viability was monitored using the Cell-Titer Blue assay at 6, 24, and 48 h after printing. Viability was expressed as a percentage relative to unprocessed controls with the same initial cell density.

ScaffoldingInk. HepG2 cells were used to prepare cell-incorporated scaffolds. The viscous ink used with PAM^2 (termed "ScaffoldingInk") was prepared gently suspending cells to a final concentration of 4×10^6 cells/mL in 6% w/v alginate solution in phosphate buffered saline (PBS). A sterile and commercial syringe (10 mL, 165 μm inner diameter stainless steel needle) was then filled with ScaffoldingInk and mounted on the PAM^2 system using the piston actuated printing head. During printing, scaffolds volume was controlled to maintain a constant cell number per sample (about 200,000 cells were included in each scaffold). Scaffolds with different volumetric flow rates (i.e., 4 and 9 μL/s) were fabricated and immediately physically cross-linked with 0.5 M calcium chloride solution as described by Tirella et al.[14] After fabrication, HepG2 cell viability in the scaffolds was assessed using Cell-Titer Blue for up to 48 h, using the same volume of pipetted cell-laden alginate as controls.

FEM Modeling

The fabrication of living scaffolds using direct writing systems is a critical process, which can cause cell damage. FEM analyses can be used to predict the stresses imposed on cells during the printing phase. In this work, FEM analyses of both droplet generation and impact on a substrate and extrusion phase of viscous ink through a syringe needle were performed with Comsol Multiphyiscs Software (COMSOL, Stockholm, Sweden) coupling Navier–Stokes equations with other physic modules. In particular, the phase field method[15] was used to track the interface between two fluids. Printing processes were modeled in two different ways, focusing the attention on tracking drop shape and on evaluating cell shape and motion, respectively, in BioJet and PAM^2.

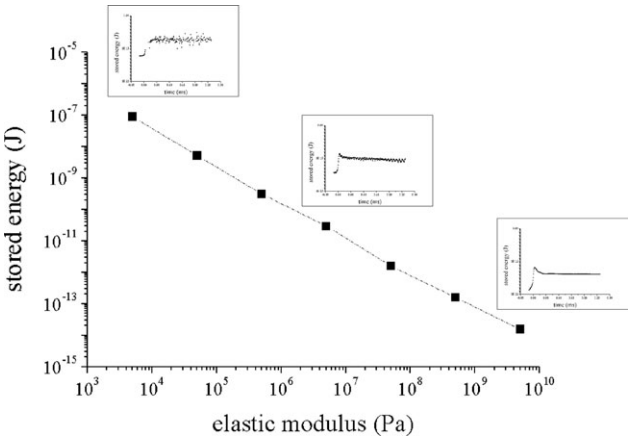

Figure 1. Impact energy exerted in LivingInk during drop landing as a function of substrate stiffness evaluated using FEM models.

The insets represent impact energy in time on different substrates.

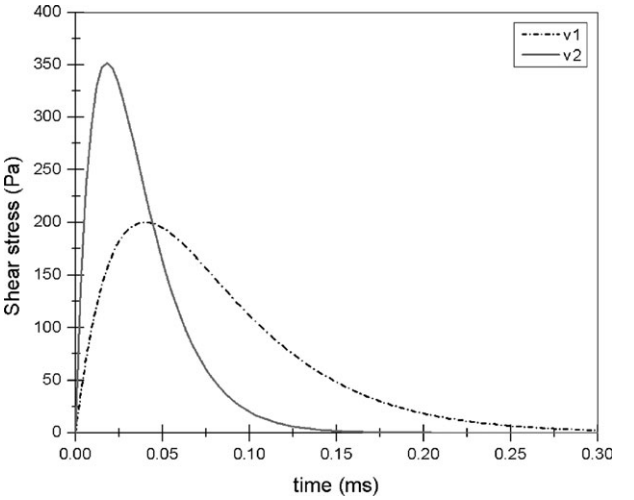

Figure 2. Time dependence of extrusion forces (shear stresses) acting on a cell membrane.

Numerical integration of the curves gives the total force, which could be used as a measure of the cumulative damage to cells during extrusion. The lower outflow (4 μL/s) delivers a higher impulse per unit area on the cells than the higher (9 μL/s).

Starting from its formation at the nozzle of the ink print head drop shape was tracked at various phases after inkjet ejection: (i) as it flies toward the substrate, (ii) on its impact with a specific planar substrate, and finally, (iii) its remodeling after landing.

In the PAM^2 printing model, cell motion within a viscous ink was evaluated as it passed through the narrowest zone of the nozzle: stresses acting on a single cell immersed in a viscous domain can be analyzed and monitored, as a force is applied.

Briefly, the models were represented as incompressible fluids with constant density using Navier–Stokes equations (Eq. 1), which describe mass and momentum transport. A specific term was included in the model to consider the surface tension force (expressed as $G\nabla\varphi$). Details of the models are described in the sections later.

$$\rho\left(\frac{\partial \mathbf{u}}{\partial t} + \mathbf{u}\cdot\nabla\mathbf{u}\right) - \nabla\cdot\left(\mu\left(\nabla\mathbf{u} + (\nabla\mathbf{u})^T\right)\right) + \nabla p = G\nabla\varphi$$
$$\nabla\mathbf{u} = 0 \qquad (1)$$

Here, ρ is fluid density, μ is the dynamic viscosity, $\mathbf{u}$ represents the velocity field, p denotes the pressure, G is the chemical potential, and φ is the phase field variable. The phase field variable is a globally defined function (a step function that equals zero in one domain or phase and one in the other), which describes the interface separating the two fluid phases. This function should be initialized with a time step proportional to the mesh size before the FEM analysis starts. Then, plotting the globally defined function allows interface tracking within the domain. Fluid dynamic variables were coupled with the mechanical module for the evaluation of stresses in both models.

BioJet and substrate stiffness

Hypothesizing that the impact of a drop on a substrate is the phase with the highest stress and that the impact is the principal determinant of cell viability, a detailed FEM analysis was performed in this direction. The landing phase of a LivingInk drop on substrates with specified mechanical properties was analyzed, evaluating the role of the substrate in determining impact energies. The phase field variable was used to describe the diffuse interface separating air, LivingInk, and substrate. To evaluate forces developed within droplet and substrate, the fluid dynamics variables were coupled with the mechanical module (i.e., imposing the loading force to be equal to the one exerted by fluid and the droplet boundary velocity equal to the displacement velocity of the substrate). The coupled fluid-dynamic and structural mechanics analysis was then used to quantify impact energies acting on the substrate. Substrates used in the model were (i) hard substrate (empty polystyrene well), elastic modulus 5 GPa; (ii) stiff substrate (3 mg/mL rat-tail collagen, prepared using the method described by Elsdale and Bard,[16] cross-linked with 10× culture medium) elastic modulus 5 MPa; and (iii) viscous substrate (1% w/v of gelatin dissolved in medium) elastic modulus 5 kPa. It should be noted that while substrate stiffness was used because it is directly related to impact energy, the contact angle between droplet and surface was only used here to model droplet penetration into the surface during the impact (data not shown).

PAM^2 and shear stresses

Shear stress acting on cell membrane is a well-known problem affecting cell viability during high-velocity extrusion using viscous solutions. Therefore, to quantify shear forces acting on processed cells, attention was dedicated to model the extrusion phase of ScaffoldingInk through a syringe needle. In particular, the model considers two immiscible fluid phases: a spherical liquid domain (a single suspended cell, shaped with a circle of 25 μm diameter) and a viscous phase (i.e., alginate solution used in the experimental phase). A single cell model was chosen, because at the concentration used in our experiments two cells at the most can pass through the narrowest part of the nozzle; given that the nozzle is 165 μm in diameter, it is unlikely that cells can interact. The cell domain was defined imposing a density[17] of 1,280 kg/m^3 and a viscosity[18] of 1.2×10^{-3} Pa s, according to the literature values. Alginate domain

properties (i.e., density and viscosity) were set to experimentally measured values (1,160 kg/m^3 and 4.23 Pa s, respectively). The interface separating the cell membrane/alginate was tracked during the extrusion process and used to evaluate shear stresses.

Results

FEM Modeling

Drop Collision on Substrate: Impact Energy Evaluation. The evaluation of impact energy acting on LivingInk during printing was indirectly derived considering results of substrate strain energy values. This assumption was based on the hypothesis that the energy stored in the droplet during the landing phase is elastically transmitted to the substrate. In this evaluation, low values of dissipation energy mean high forces still acting on the droplet, whereas high dissipation energy values correspond to low forces exerted on the droplet. To appreciate the effect of substrate properties, the impact energy stored in the substrate as a function of substrate stiffness is represented in Figure 1.

In the same figure, the time dependence of the stored energy for different elastic moduli shows how soft substrate energy dissipation is distributed over time.

As expected the simulations underline the influence of substrate stiffness in impact forces and energies. Soft or liquid-like materials absorb higher energy and store it in time, reaching a constant value once the drop spread over the substrate. On the contrary, solid-like materials (i.e., stiff and hard substrates) absorb less energy and in a shorter time, resulting in higher energy storage within the droplet (Figure 1).

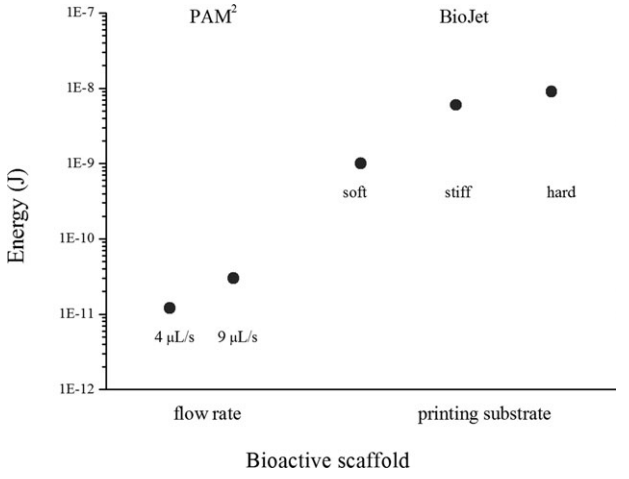

Figure 3. Energy values obtained from FEM models acting on printed cells.

Cell damage due to the mechanical impact during cell inkjet printing has been observed, and it is a possible hurdle for broad applications of fragile cell direct writing.

Extrusion of Cell Suspension: Shear Stress Evaluation. Modeling the motion of a fluid spherical body in a viscous domain can reveal cell membrane deformation due to viscous forces exerted by fluid motion. Numerical models return shear values of about 200–350 Pa acting during the first 10 μs on the fluid–fluid interface, this happens as the cell starts to move in the needle. Results show that shear stresses are higher than the ones which cells experience in physiological conditions (it is reported that hepatocytes can tolerate shear stress value of 0.05 Pa[19]). However, these stresses act only in a short time period, allowing a rapid recovery. Analyzing FEM results underline that the maximum shear stress imposed during the printing with the PAM^2 system is lower than the one exerted and tolerated by cells during inkjet printing,[20,21] however, it acts for a longer time (Figures 1 and 2, respectively, for BioJet and PAM^2). To better compare the two systems, the extrusion energy in PAM^2 system was evaluated considering the time dependence of the shear forces developed during material expulsion (Figure 2): considering shear stress, cell surface area and the velocity of the suspension in the nozzle, it was possible to calculate the energy transmitted to cells over the extrusion time (Figure 3).

In both volumetric flow rates used to print and fabricate living scaffolds, once the extrusion begins (time 0), there is an immediate increase in shear stress; the shear stress quickly drops to zero after less than half a second once the cell exits the needle (Figure 2). Remarkably, the maximum shear force is greater with the lower volumetric flow rate (4 μL/s) than at higher volumetric flow rates (9 μL/s), however, cells remain in the syringe for a longer time prolonging the action of forces on cell membrane. Figure 3 shows that the stored energies obtained do not vary significantly, suggesting that extrusion conditions do not influence printed cells as much as substrate stiffness.

Living constructs fabrication and FEM validation

Direct printing of living constructs was performed with both the systems to validate FEM results.

Inkjet Printing on Specific Substrates. The viability assay performed on fibroblasts shows a considerable difference between soft and solid substrates particularly at 6 h after printing (Table 1). The capability of liquid-like substrates to absorb impact forces is reflected on cell viability: a linear relationship between viability and elastic properties was apparent even after few hours from printing. These results correlate very well with the FEM results (Figure 1), which shows an inversely proportional relationship between adsorbed strain energy and substrate elastic modulus. A very small portion of cells survives the printing process on the

Table 1. Viability Assay Results of Fabricated Living Scaffolds

	BioJet				PAM^2 System		
	Printing Substrates				Volumetric Flow Rates		
Time (h)	Control*	Polystyrene	Collagen	Gelatin	Control**	4 μL/s	9 μL/s
6	100	27.1 ± 11.2	59.7 ± 8.6	115.4 ± 5.5	100	86.2 ± 11.3	106.4 ±4.1
24	150.9 ± 7.8	20.2 ± 17.2	178.3 ± 19.0	154.5 ± 40.1	100	95.8 ± 9.9	96.6 ± 5.5
48	452.6 ± 37.8	123.4 ± 44.5	409.7 ± 25.3	386.9 ± 51.9	100	79.3 ± 14.7	94.1 ± 12.8

Reported values are expressed as % respect to controls. The results underline the printing parameters that cause cell damage or death.

*The control refers to a 2D cell culture at time 0.

**The control refers to a cell suspension in alginate at each time point.

stiffest substrate, whereas for softer substrates no significant differences were measured with respect to the control 48 h after printing.

Deposition Using Controlled Volumetric Flow Rate. To assess the effect of the extrusion process on cells, in this study, we evaluated cell proliferation and metabolic profiles of HepG2 cells encapsulated in 6% v/w alginate scaffolds using different volumetric flow rates. Cell viability over 48 h was analyzed (Table 1). As shown, scaffolds fabricated with higher volumetric flow rates (9 μL/s) exhibit viability similar to controls, whereas viability is slightly but significantly decreased using lower volumetric flow rates (4 μL/s). At 9 μL/s, no significant changes occur immediately after the printing phase (6 h) or a few days after, and this demonstrates that the shear stresses exerted using this system, thus the fabricating parameters used, do not damage cells.

Discussion

Cells sense, interact, and dynamically respond to their microenvironment, which has an important role in controlling cellular spatial organization and function. TE uses different approaches to evaluate cell–material interactions. Patterned 2D topological cues and bioactive motifs use surface modification techniques to investigate how biocompatible surfaces can control cellular interactions on the micrometer and submicrometer scales[22] or transmit extrinsic signals through focal adhesions and integrins using microcontact printing techniques.[23] Other approaches use multigrooved fibers[24] or electrospun nanosized mesh networks[25] to increase cell attachment and control cellular alignment. Given the dramatic difference between culturing cells in 2D or 3D cell systems (cell shape, gene expression, growth, morphogenesis, etc.),[26] there is an increasing interest in the study of 3D environments. Inkjet and RP systems have been used to recreate the structural aspects of ECM, controlling size, shape, and mechanical properties of the fabricated constructs at the microscale.[4,11,27–31]

Beyond the need to mimic the architectural aspects of ECM, it is also interesting to deposit cells with highly controlled spatial cues to confer spatially specific bioactivity to the scaffold.[32] However, during the fabrication of bioactive scaffolds, cells can experience high stresses due to impact and shear forces. In general, using a trial-and-error approach, suitable printing conditions are empirically derived to obtain scaffolds containing living and viable cells. To improve these fabrication methods, we evaluated stresses occurring during the printing of living materials (using two printing techniques) via FEM analyses.

In particular, attention was first dedicated to modeling the printing steps that may affect cell viability. In case of inkjet printing, we considered the impact of a drop on substrates with different elastic moduli, assuming that the impact energy exerted on the drop is elastically transmitted to the cells. The elastic energy transfer during droplet landing and the total energy in the system during impact is then shared between the strain energy of the substrate and the stored energy in the droplet. We therefore reason that the stored energy is transferred to living cells dispersed within the droplet and thus may permanently damage cells if it exceeds a threshold value.

In case of the nozzle-based direct-writing system PAM^2, the key parameter controlling spatial resolution of hydrogel scaffolds is the volumetric flow rate.[14] Therefore, the analysis, varying flow rate value, was concentrated on the narrowest part of the printing system: the nozzle. FEM results show that stored energy (evaluated from shear stress values acting on a cell membrane) does not differ significantly as a function of the imposed volumetric flow rate; these data were also confirmed by cell viability tests.

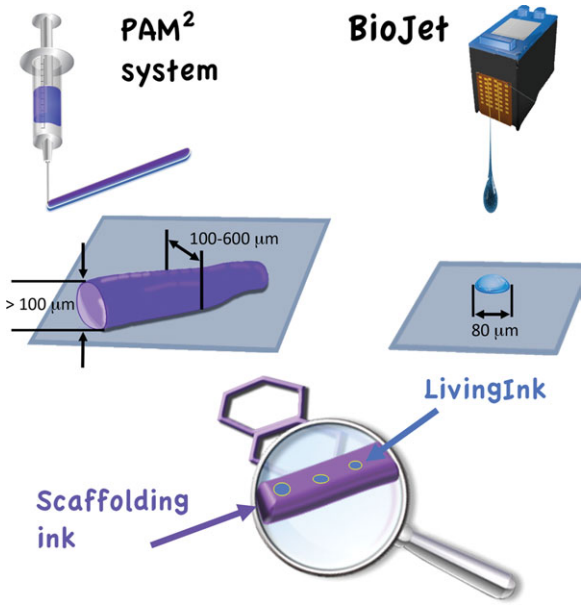

Figure 4. PAM^2 and BioJet printing principles and obtained constructs.

The two systems can be integrated to print 3D constructs containing cells. As summarized here the PAM^2 system can be used to direct-write soft living constructs with ≈300 μm resolution without inducing cell damage. Successively the BioJet can print cells and biomolecules in controlled locations with 80 μm resolution onto the surface of the soft constructs, thus minimizing cell damage due to substrate impact.

Comparing FEM results, it is possible to correlate energies transmitted to LivingInk and ScaffoldingInk to predict the most suitable printing conditions to preserve cell viability and, at the same time, obtain a well-defined shaped structure. In Figure 3, it is evident that drop impact is more critical than shear stress during printing of cell suspensions. In particular, cell viability experiments have shown that impact energies higher than 10^{-9} J cause irreversible damage. Using the PAM^2 system, this threshold is unlikely to be reached. In contrast, using BioJet, and inkjet systems in general, attention should be dedicated to the nature of the printing substrate to determine impact energy values.

In the perspective of fabricating more complex scaffolds with different features (e.g., materials, biomolecules, and cell types) and at different length scales (from nanometer to millimeter), we propose the integration of the two printing systems to design new scaffolds. Together the two methods can be used to direct-write soft living constructs as summarized in Figure 4. In detail, we propose the use of the PAM^2 system to fabricate 3D living scaffolds: the fabrication parameters can be dimensioned to avoid damage on cells due to shear stresses. Then by controlling the viscosity and gelling properties of the ScaffoldingInk composition, it is possible not only to fix the microarchitecture but also to finely tune the mechanical properties of the construct as a substrate for subsequent nanoscale printing with the BioJet. In this case, on one hand, the construct should be stiff and tough, so that printed droplets do not spread or diffuse through the matrix, and spatial resolution can be preserved; on the other

hand, it should be viscous or soft, absorbing all the forces developed during droplet landing phase and impact.

The integration of these two methods combined with a proper choice of soft material will allow fine control over: (i) the spatial resolution, (ii) the amount of material used, (iii) the cell types and their location, and (iv) the dispensing of biomolecules. All these parameters will determine the final 3D architecture of a printed microsystem (Figure 4). Precisely, these living microsystems (containing one or more cell types in cell laden layout) can be selectively functionalized using other cell types, nanoparticles (as sensors or for drug release) or biomolecules (as selective ligands). Thus, complex and spatially controlled microsystems can be used to monitor and control the fate of 3D living constructs.

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