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Sphyga: a multiparameter open source tool for fabricating smart and tunable hydrogel microbeads

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Abstract

Hydrogel microbeads are used in many biological applications, particularly for cell, protein or drug encapsulation. Although there are several methods for fabricating microbeads with controlled shapes and dimensions, many are limited to a small range of materials or sizes. We describe a compact open source tool—the spherical hydrogel generator (Sphyga)—for the fabrication of highly reproducible hydrogel based microbeads with predictable shapes and diameters ranging from 100 to 2000 μm. The unique feature of the system is the ability to modulate multiple parameters independently, so as to create a wide range of working conditions for fabricating tailored microbeads. Hence, by combining the different fabrication parameters, hydrogel beads with chosen shapes, sizes and materials can be generated with Sphyga. A multiparameter working-window was obtained by fixing the concentration of the base material, alginate, and varying the viscosity of the solution along with Sphyga's fabrication parameters (needle size, external air pressure, and material outflow). To validate the multiparameter working window, components such as proteins, cells, dyes and nanoparticles were also used to fabricate composite microbeads. The results show that the architecture of hydrogel microbeads can be engineered by considering the viscosity of the initial solution, which depends principally on the pH and composition of alginate solution. Coupled with Sphyga's multiple working parameters, material viscosity can then be used to tune hydrogel domains and thereby generate complex biologically relevant microenvironments for many biomedical applications.

Keywords: biofabrication, hydrogel, microbeads, *in vitro* models, 3-way PCA

(Some figures may appear in colour only in the online journal)

1. Introduction

There is great interest in the development of smart and tunable biomaterials as three-dimensional (3D) scaffolds for engineering tissues, drug delivery, and for designing controlled *in vitro* microenvironments for the study of disease models or toxicity. Microencapsulation is an emerging method that addresses a number of the problems associated with traditional scaffolds (e.g. non-biocompatibility of processing methods and nutrient limitations in 3D constructs). In microbeads, cells are enclosed in a hydrogel biomaterial which provides a 3D microenvironment with characteristic dimensions small enough to ensure an adequate supply of oxygen and other nutrients in the media as well waste removal.

Hydrogels have received much attention as scaffolding biomaterials due to their hydrophilicity and structural similarity to the extracellular matrix (ECM). Gelation processes can be controlled to be compatible with cell viability,

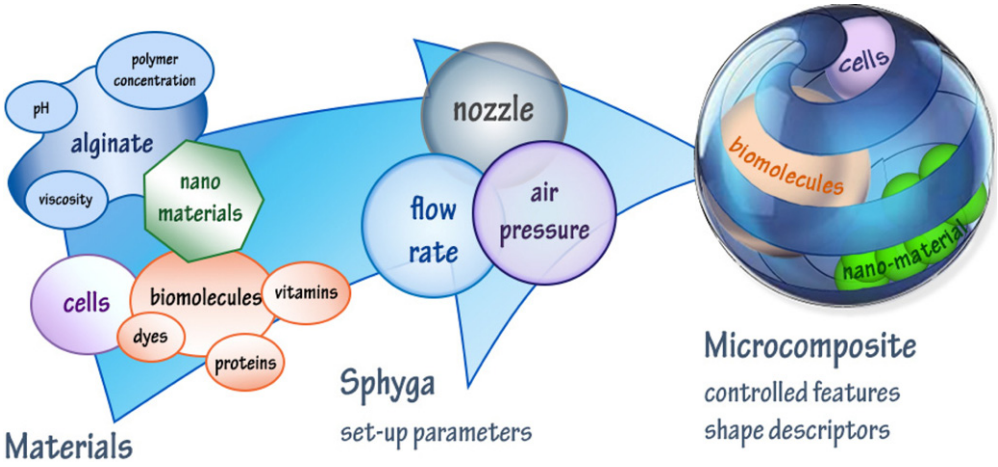

Figure 1. Fabrication scheme: experimental conditions and working parameters which influence the shape and dimensions of engineered microbeads.

thus hydrogels could enable effective solute transport in and out of the scaffold as well as biocompatibility and biodegradability [1–3]. To date, various fabrication methods have been developed to generate 3D spherical hydrogel beads encapsulating many types of cells. In particular, micro-sized hydrogel beads are fabricated by three well-known methods: coacervation, emulsification, and liquid droplet formation.

Phase separation or coacervation is one of the oldest and commonly used method for preparing microbeads [4]. Two immiscible solutions are generally used (one forming the membrane, the other is the encapsulated core) to fabricate nanosized spheres for drug delivery purposes [5, 6]. In recent years this method was modified for the production of microbeads containing enzymes [7], yeast or bacteria [8].

Emulsification generally uses an oil-in-water technique to generate gel microbeads [9, 10]. The size of the microbeads formed by this technique is highly-dependent on the stirring speed and the rate of the addition of the crosslinking solution. However this method is not suitable for fabricating *living* or cell containing microbeads, since it involves the use of harsh chemical reagents such as ethyl ether to remove the oil at the end of the process.

Liquid droplet formation or the spraying method is nowadays one of the most commonly used techniques. It uses an extrusion device with a small orifice: a solution may be ejected using a variety of methods—spraying, ejection in a stirred crosslinking bath [11], falling drops through a fine mist of hardening solution [12], air atomization [13], droplet-based microfluidic devices [14, 15]. The microbead membrane is instantaneously formed when the liquid contacts the hardening or gelling media. Several materials can be used with this method, but alginate, chitosan and their blends are commonly employed.

A general overview of liquid droplet microbead preparation uses a solution loaded into a reservoir mounted on a syringe pump. The solution is then ejected through a small orifice with a defined diameter ($<$1000 μm) at the tip (note that smaller diameter orifices can be used, but may run the risk of orifice clogging/plugging by the high viscosity of the solution employed). The sizes of these beads can be controlled by: (i) the flow-rate of the syringe pump, (ii) the viscosity of the solution used, (iii) the spraying principle or (iv) the distance between the orifice and the surface of the crosslinking solution (in which microbeads are crosslinked and collected). Almost all of these techniques are based on the physical gelation of sodium alginate in the presence of calcium ions. Sodium alginate solution can be dispensed with standard syringe at a constant flow rate and made to fall dropwise into a crosslinking solution with divalent cations [16, 17]. However, most of these droplet devices are expensive, and due to their large size, do not work under sterile conditions. Moreover they are generally difficult to use in terms of setting the working parameters, requiring a long trial-and-error approach to obtain microbeads with regular shape and size.

It is known that the diameter of the beads depends on a large number of parameters. Of these, the most important are the size of the needle used and the viscosity of the alginate solution: a larger needle diameter and lower viscosity solutions will produce larger beads (diameter $>$1000 μm). Moreover, the viscosity of sodium alginate, which depends on its concentration and pH [18], also influences the shape of the microbeads produced.

In this paper the spherical hydrogel generator (Sphyga), a system able to fabricate multicomponent spherical hydrogel microbeads with controlled and predictable shape and size is presented. Sphyga combines an easy and straightforward fabrication process, tuning multiple working parameters (flow rate, external air pressure and needle diameter) to obtain uniform hydrogel microbeads with controlled radii and composition (figure 1). The device is compact so can be placed under a hood to guarantee sterile working conditions and operated remotely by a PC. In this study, we present the fabrication of alginate microbeads with different sizes obtained by changing the working parameters of Sphyga. The geometrical features of microbeads were analyzed as a function of Sphyga's parameters using 3-way PCA principal component analysis (PCA) [19] to identify the most important contributions to shape and size. Using these data, we generated a multiparameter working-window, which enables setting up the system prior to fabrication to obtain microbeads

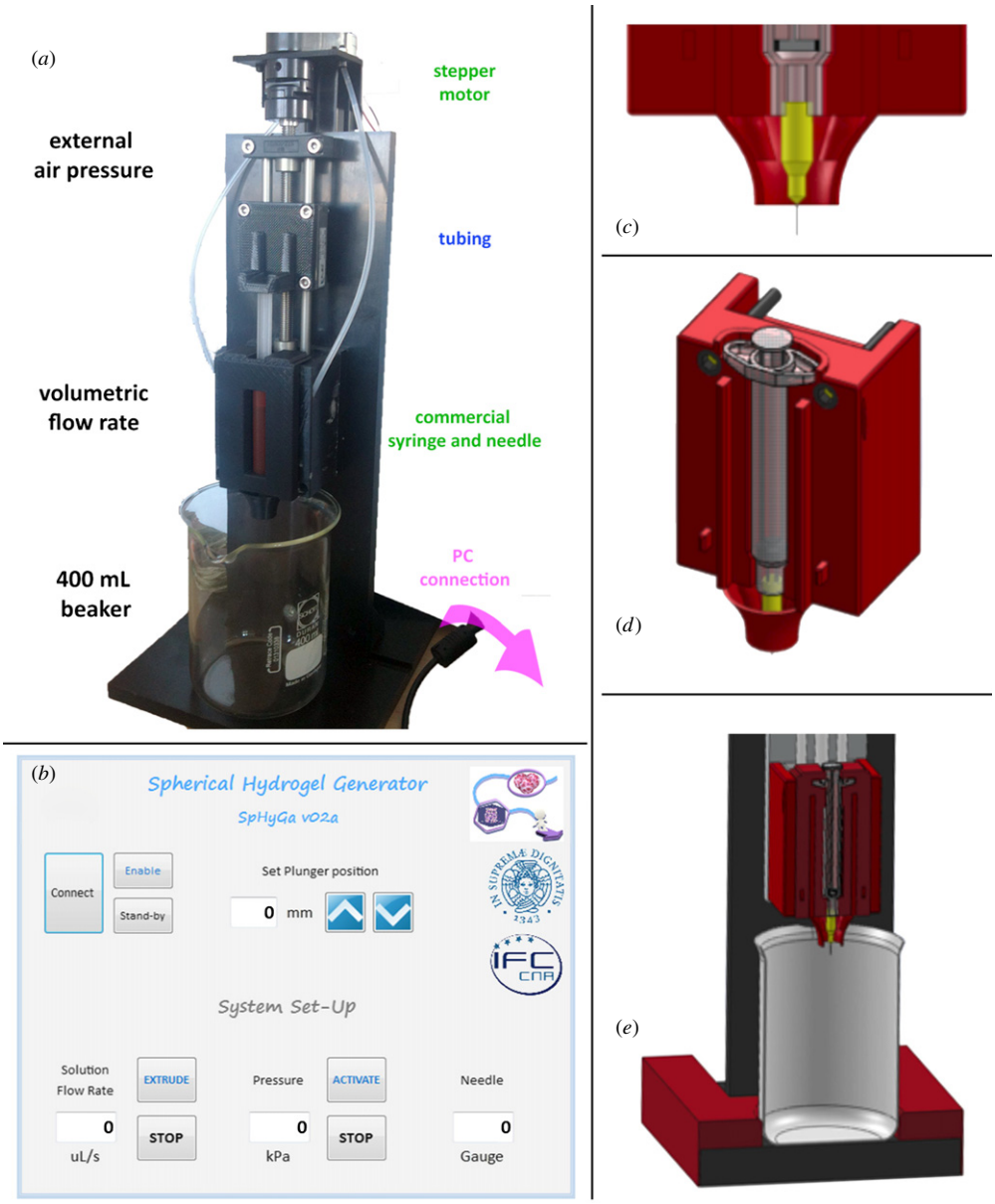

Figure 2. Sphyga: (*a*) the hardware, (*b*) the GUI, (*c*) design of tubing directing the external airflow on the nozzle tip, (*d*) the mechanical framework for the syringe and (*e*) coaxial design to direct droplets into the beaker.

with known shape and dimensions. Finally, we show how the working-window can be used to engineer composite microconstructs including cells, ECM and nanoparticles (NP) to generate controlled microenvironments for a wide range of biomedical applications.

2. Methods

2.1. Sphyga overview and working principle

The spherical hydrogel generator (Sphyga, shown in figure 2(*a*)) is composed of an extrusion module (i.e. commercial syringe and linear actuation stage) combined with an external air supply; a dedicated software allows the control of the system parameters (figure 2(*b*)). As the syringe is actuated, the solution flows through a nozzle with a given internal diameter and a drop is generated on nozzle tip. An external sterile airflow, co-axial with syringe needle, is used to guide the formation and pinch-off of the droplet. The liquid droplet lands in a beaker filled with a crosslinking solution (Sphyga design detail are shown in figures 2(*c*)–(*e*)), whereupon the droplet forms a hydrogel bead.

Identifying stable operating conditions under which controlled and reproducible beads are generated is challenging, particularly in biological applications. First, surface tension, nozzle diameter and solution viscosity influence the shape of droplets as they form on the nozzle tip. Then the external airflow determines the volume of the detached droplet, which immediately forms a gel when it falls into a beaker filled with a crosslinking solution. The landing phase [20], the solution and the gelation phase determine the final shape of fabricated beads. In Sphyga, highly reproducible and precisely controlled spherical micro-sized hydrogels (diameter <1000 μm) can

be fabricated by appropriately tuning of four independent parameters (i.e. nozzle diameter, solution viscosity, solution flow rate and external air pressure).

2.1.1. Hardware. A syringe holder and a linear actuator compose the core of Sphyga. A stable and compact framework is designed to align the syringe holder, the linear stage (DryLin® SHTP mini—small, Igus, Germany), a stepper motor (50:1 reduction, P542-M481U-G17L82, resolution 0.1 mm McLennan Servo Supplies, UK) and a 400 mL beaker. The syringe holder, stepper motor cover and beaker holder were designed using CAD software (computer aided design, Dassault Systèmes SolidWorks Corporation, US) and then printed using a 3D printer (Dimension Elite, Stratasys Ltd, US). The device is simple, compact and light and can easily be positioned under a sterile hood. Moreover, the syringe holder is designed to allow coaxial airflow on the needle's tip and to house a 3 mL commercial syringe (7030 series, Techcon System, Italy). An external air supply controlled through a pressure regulator (ITV0011–2BL-Q, SMC Corporation, Japan) is connected to the system and directs the airflow at the nozzle tip (TE series Techcon System, Italy). The PhidgetStepper Bipolar 1-Motor controller (Phidgets Inc, US) and the Arduino Uno (Arduino.cc, Italy) are used to control the stepper motor and the pressure regulator, respectively. Using the dedicated software, solution flow rate and air pressure can be set and controlled in the range of $(5 \div 20 \pm 1)$ μL s^{-1} and $(1 \div 100 \pm 0.4)$ kPa.

2.1.2. Software and graphical user interface. The Sphyga control software was developed in Microsoft Visual Studio 2010. The libraries to control all the hardware components were compiled with F#. NET, while the graphical user interface (GUI) was designed using windows forms application. In case of the air pressure regulator, the microcontroller was programmed in the Arduino IDE environment. To enable easy installation, a single executable file was compiled including all the .NET libraries. The GUI (figure 2(*b*)) helps the user to control microbead fabrication by: (i) enabling the stepper motor and pressure regulator, (ii) setting the system in stand-by mode, (iii) moving the plunger actuator to a target position and controlling the extrusion of the solution through the nozzle, or (iv) setting a target external air pressure.

2.1.3. Open access repository. The 3D CAD assembly designed using SolidWorks, as well as software source library and installation files can be downloaded at www.centropiaggio.unipi.it/research/sphyga-system.html.

2.2. Alginate microbead fabrication

In order to obtain stable, highly hydrated and soft hydrogels with rapid gelation, low viscosity alginate-based solutions (Alginic acid sodium salt from brown algae, A0682, Sigma-Aldrich, Italy) were physically crosslinked using calcium ions (0.1 M $CaCl_2$ in deionized water from anhydrous calcium chloride powder, C5670 Sigma-Aldrich). For microbead characterization, alginate solutions were buffered at different pH values, thus changing their dynamic viscosity [18]. Moreover, different needles were used (with internal diameters (μm) of 110, 160, 210 and 260 corresponding to gauges (G) of 32, 30, 27, and 25) and the external air pressure was regulated to different target values (i.e. 60, 75 and 100 kPa). To reduce the number of variables, the alginate solution concentration and flow rate were kept constant at 2% w/v and 12 μL s^{-1}, respectively. This value was chosen in accordance with a previous study, in which we observed that shear stress induced damage on cell viability was minimal at this flow rate [21]. Microbeads were produced simply by: filling the syringe with the alginate solution, setting the parameters through the GUI and finally collecting the beads in the beaker containing 250 mL of $CaCl_2$ solution (figure 2(*e*)). Newly formed alginate microbeads were kept immersed in the $CaCl_2$ solution for 5–10 min to allow homogeneous crosslink formation. They were then collected, rinsed with deionized water and characterized as described in section 2.3.

2.3. Microbead characterization

Microbead features were characterized as function of fabrication parameters acquiring bright-field images immediately after fabrication and at different time points (up to 2 weeks) using an inverted microscope (Olympus IX81, Italy). Images were analyzed using the *Analyze Particles* tool (ImageJ [22]), retrieving dimensions (e.g. area, perimeter) and shape descriptors (e.g. roundness, aspect ratio defined according to Russ [23]). In order to construct the *Sphyga multiparameter working-window* for obtaining microbeads with known dimensions, the data on shape descriptors as a function of working parameters were evaluated using the *3-way PCA* plug-in for ImageJ [24]. 3-way PCA is a powerful statistical method for analyzing complex datasets to establish trends and determinants [25]. Here, needle size was set as the objects, airflow as the variables and alginate solution as conditions. The dataset used is summarized in table 1.

3. Results

In biomedical applications such as controlled release or *in vitro* tissue models, it is important that microbeads possess predictable or stable shape descriptors over time. For this reason, a batch of sterile microbeads was maintained in cell culture media in an incubator and monitored over time: the beads did not change significantly in size (less than 5% variations, data not shown) and circularity values were constant (about 0.9 for all beads independent of their composition).

3.1. Sphyga multiparameter working-window

Average diameters were measured for alginate microbeads as function of: alginate solution, external air pressure and needle size (table 1). The *working-window* was generated integrating the information obtained from these datasets. The 3-way PCA analysis shows that solution viscosity is the main parameter that influences microbead features (i.e. radius and circularity,

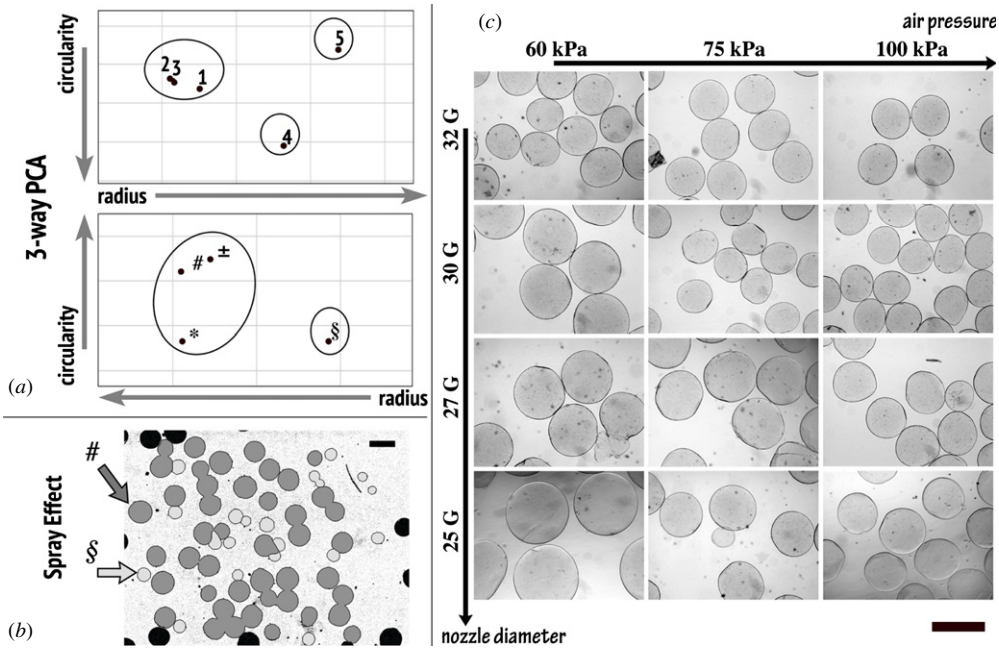

Figure 3. Analysis of size (radius) and shape (circularity) of 2% w/v alginate microbeads fabricated with Sphyga (at a fixed flow rate of 12 μL s^{-1}): (*a*) 3-way PCA results of conditions (pH 5(1), pH 6 (2), pH 6.5 (3), pH 7.2(4), pH 9 (5)) and objects (needles, 25G (§), 27G (*), 30G (#), 32G ($\pm$)). (*b*) Representation of the 'spray effect' which generates two different populations of microbeads with average diameters of 160 μm (#) and 100 μm (§); (*c*) Montage of alginate microbeads obtained varying needle size and external air pressure, using a 2% w/v alginate solution buffered at a pH value of 6.5. Circularity >0.875 for all beads. Scale bar 200 μm.

Table 1. Summary of Sphyga working parameters and average values of fabricated sphere radius ($n > 30$) used for the 3-way PCA analysis. Micro-spheres were fabricated using a constant flow rate of 12 μL s^{-1}.

Needle inner diameter (G, μm)	Buffered pH value of 2% w/v alginate solution (note that solution viscosity is inversely proportional to pH value)					Pressure (kPa)
	5	6	6.5	7.2	9	
(32 110)	389.0 ± 17.3	241.8 ± 38.5	265.7 ± 5.0	663.6 ± 11.9	794.1 ± 13.8	60
	343.7 ± 37.8	227.1 ± 26.0	122.6 ± 61.4	502.1 ± 8.3	607.9 ± 26.8	75
	255.4 ± 21.0	205.0 ± 5.1	147.4 ± 37.3	392.0 ± 42.6	230.2 ± 6.4	100
(30 160)	317.6 ± 8.3	302.9 ± 35.2	264.6 ± 11.1	590.8 ± 19.8	691.8 ± 5.8	60
	271.7 ± 10.2	240.3 ± 16.9	230.8 ± 12.7	418.2 ± 8.4	542.4 ± 11.2	75
	248.8 ± 35.0	231.9 ± 13.2	224.9 ± 4.7	238.0 ± 62.2	520.6 ± 8.3	100
(27 210)	459.3 ± 21.1	358.2 ± 1.4	323.4 ± 26.4	820.3 ± 15.1	854.1 ± 42.9	60
	290.6 ± 11.6	353.6 ± 22.1	326.9 ± 26.8	389.4 ± 11.7	647.8 ± 33.0	75
	314.6 ± 22.4	262.4 ± 19.5	285.9 ± 10.2	262.2 ± 18.7	563.3 ± 14.4	100
(25 260)	401.7 ± 25.6	343.5 ± 43.7	409.6 ± 35.7	319.2 ± 56.8	381.5 ± 70.5	60
	539.0 ± 73.1	322.5 ± 13.0	298.3 ± 29.5	257.3 ± 43.8	244.3 ± 62.7	75
	208.1 ± 98.1	213.9 ± 31.7	272.0 ± 34.1	275.8 ± 91.0	199.5 ± 57.2	100

as shown in figure 3(*a*)). The minimum bead diameter in these conditions is just over 100 μm. Interestingly, this analysis also clusters higher nozzle diameter as a working parameters which gives rise to homogenous circular beads (figure 3(*a*)). The role of external air pressure is underlined in figures 3(*b*) and (*c*). Figure 3(*b*) illustrates the so-called 'spray effect': high pressure values ($\geqslant$100 kPa), particularly if coupled with larger nozzles, may cause the generation of two different sized microbead populations. The 3-way PCA clusters the 25G needle in the region of smaller beads due to this effect, which can also be recognized in a larger distribution of bead dimensions (table 1). Keeping all other parameters constant, an increase in air pressure results in a decrease of bead size (figure 3(*c*)). These effects are also highlighted in the *working-window* represented in figure 4.

3.2. Composite microbeads and validation of the working-window

Focusing on models of hepatic metabolism, sensing and nanoparticle delivery, further experiments were conducted to show how Sphyga, together with the *working-window*, can be used for fabricating purposely designed microbeads. A brief

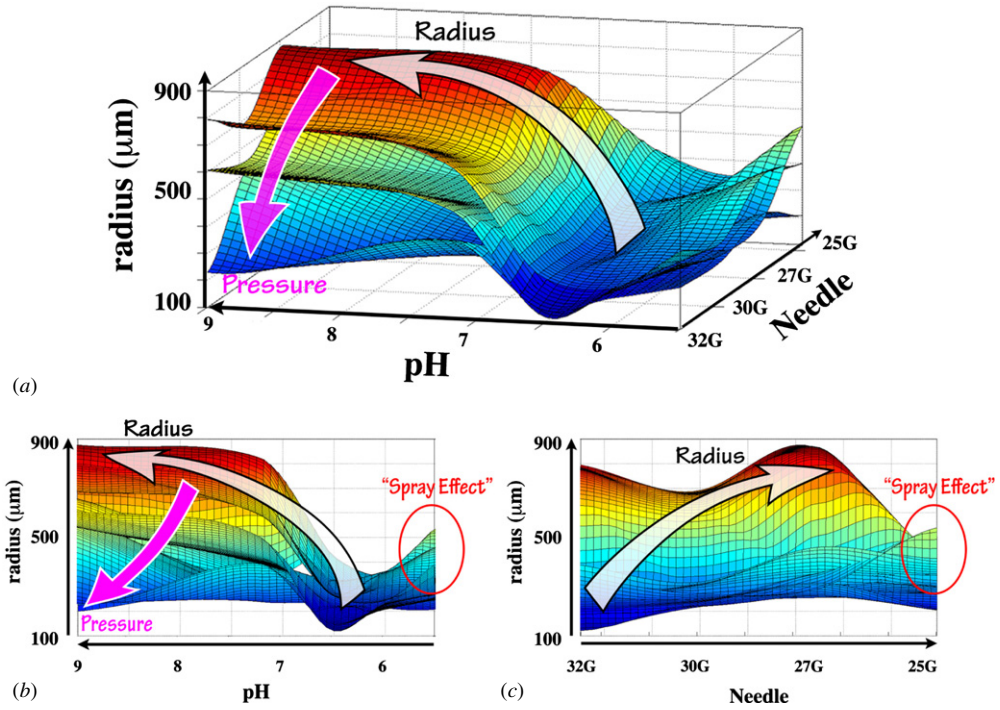

Figure 4. Sphyga working-window: analysis of microbeads fabricated with a constant flow rate and varying the pH of alginate, the needle internal diameter and the applied external airflow. Surface plots (*a*) indicate microsphere radius as a function of the external air pressure (red highest radius, blue lowest). The effect of solution pH and applied external pressure is highlighted in (*b*), while in (*c*) the 'spray effect' due to higher pressure coupled with larger needle size is shown. The white arrows highlight the increase in microsphere size, while the purple arrows indicate the increase of the applied air pressure.

Table 2. Summary of composite microbeads fabricated with Sphyga: design criteria, fabrication parameters and features of microbeads obtained.

Alginate-based solution			Sphyga parameters			
Material	Measured pH value	Design requirements: radius size (μm), application	Needle size (G)	Volumetric flow rate (μL s^{-1})	Air pressure (kPa)	Measured radius/ circularity
Collagen 1 mg mL^{-1}	5.8	~200 μm, ECM mimic	30	15	90	(219 $\pm$ 16) μm 0.86
ddECM 1 mg mL^{-1}	7.6	~200 μm, ECM mimic	30	15	90	(238 $\pm$ 40) μm 0.66
DCF (0.001–100 mM)	~7.0	<200 μm, free radical sensing	32	12	100	(162 $\pm$ 8) μm 0.75
FITC-NP 5 mg mL^{-1}	6.8	400 μm, nanoparticle delivery	25	15	60	(418 $\pm$ 10) μm 0.80
HepG2 5 $\times$ 10^6 cell mL^{-1}	6.2	150–200 μm, hepatic tissue mimic	27	12	100	(224 $\pm$ 20) μm 0.71

overview of microbead composition and design requirements is given in following sections and summarized in table 2.

3.2.1. ECM mimetic microbeads (alginate/protein). Microbeads with a composition mimicking the hepatic ECM were fabricated using a mixture of alginate, to represent the polysaccharide component, and either collagen or decellularized porcine liver extra cellular matrix (ddECM) as the protein component [26]. Alginate/protein solutions, with 2% w/v of sodium alginate and 1 mg mL^{-1} of collagen or ddECM were prepared in cell culture medium and stirred at room temperature for 2 h allowing complete alginate dissolution. Since collagen is prepared in acid solution while ddECM is slightly basic, the viscosity of the two alginate/protein solutions is different [18]. As shown in figures 5(*a*) and (*b*) and reported in table 2, alginate/collagen microbeads are more spherical and smaller than the somewhat pendant shaped alginate/ddECM beads.

3.2.2. Sensing fluorescent microbeads. Alginate microbeads can be used as fluorescent sensors and for nanomaterial confinement and delivery. Microbeads sensitive to reactive oxygen species were fabricated using the fluorescent probe DCFH/DCF [27]. The beads were prepared by dissolving alginate and 2',7'-dichlorofluorescein (DCF, Sigma-Aldrich) in deionized water to give solutions with different molar concentrations (ranging from 0.001 and 100 mM) of DCF in 2% w/v alginate and stirring in the dark for 2 h. The addition of DCF did not visibly alter the viscosity of the solution, as verified by the match between predicted and measured circularity and radius (table 2). It should be noted that due to diffusive phenomena, the concentration of this dye

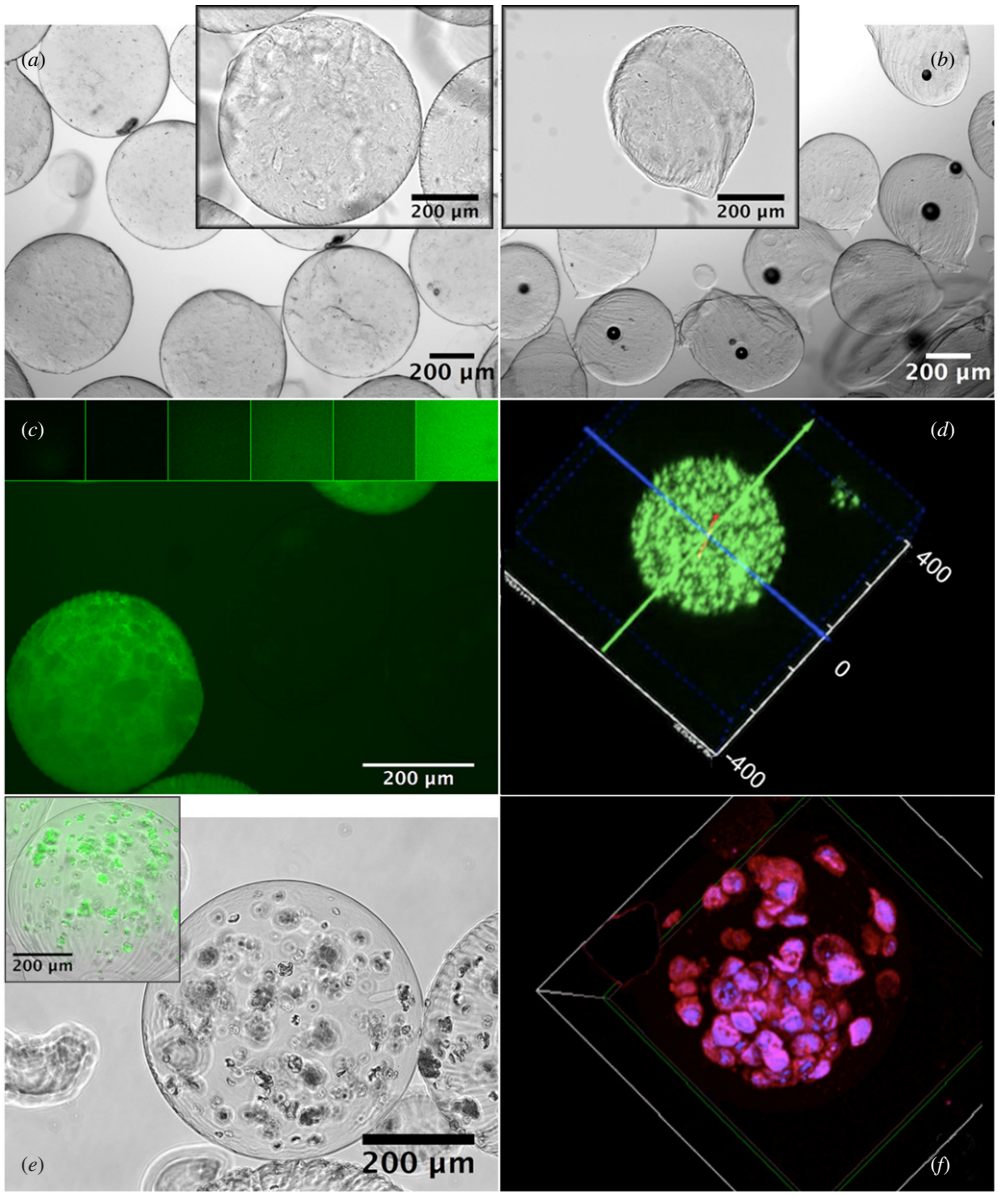

Figure 5. Composite alginate-based microbeads fabricated with Sphyga: (*a*) alginate/collagen, (*b*) alginate/ddECM, (*c*) DCF/alginate - the inset shows a calibration of portions of microbeads having different DCF concentrations ([0.001:100] mM), (*d*) 3D rendering of alginate/FITC-NP, (*e*) live/dead stain of alginate/HepG2 microbeads and (*f*) 3D rendering of DAPI/Phalloidin stained hepatocytes in micro-spheres. Brightfield and phase contrast images were obtained using an inverted microscope (Olympus IX81), fluorescent detection was performed using a confocal microscope (Nikon A1).

might change in time. However DCFH/DCF oxidation is instantaneous, thus for the purpose of this application, loss of the dye to the aqueous environment can be neglected.

Microbeads as models of NP delivery were prepared by adding FITC (fluorescein isothiocynatae)-labeled polystyrene NP (Fluoresbrite® 50 nm Polysciences Inc, Germany) to obtain 2% w/v alginate solution with 5 mg mL^{-1} NP. The suspension was stirred in the dark till the alginate dissolved and sonicated to suspend NP homogeneously prior to fabrication. NPs were then embedded in fabricated microbeads, and may be released in specific locations in the presence of chelating moieties that can interact with calcium ion (e.g. sodium carbonate).

Fluorescent alginate/DCF and alginate/NP micro-spheres are shown in figures 5(*c*) and (*d*).

3.2.3. Living microbeads (alginate/protein/cells). HepG2 cells, as *in vitro* models of hepatocytes, were cultured as described by Vinci *et al* [28]. Aliquots of 5×10^6 cells were prepared, centrifuged and then gently re-suspended in 1 mL of a 2% w/v alginate/1 mg mL^{-1} collagen solution in EMEM, obtaining an *Alginate/collagen/hepatocyte* suspension. The viscosity of the alginate-based solution did not significantly change with the addition of cells. In fact, the size and shape of living microbeads were as predicted by the *working-window* (figure 5). The cells were viable with more than 80% live cells (live/dead staining after 7 days of culture, shown in figure 5(*e*), and the 3D spherical shape of living microbeads

can be appreciated in the DAPI/phalloidin stained constructs (figure 5(*f*)).

4. Discussion

Currently there are many methods for fabricating hydrogel spheres, but only a few of them are able to precisely control their size and roundness. To address this, an open source tool is presented for the fabrication of multi-component hydrogel microbeads. A statistical analysis performed on Sphyga's working parameters (i.e. 3-way PCA) enabled the identification of the most relevant ones for the fabrication of micro-sized alginate hydrogel spheres. As shown in figures 3(*c*) and 4(*a*) and (*b*), external air pressure plays a significant role in determining the final dimensions, while solution viscosity and needle size are more important in the definition of the resultant shape. We demonstrate that varying solution viscosity, nozzle diameter and external air pressure it is possible to fabricate hydrogel microspheres with different sizes using Sphyga. In this work, the smallest reproducible bead dimension is about 100 μm, and is limited by solution viscosity and needle internal diameter. Having identified a suitable range of fabrication parameters (flow rate of 10–15 μL s^{-1}, external air pressure of 60–100 kPa, alginate based solution with pH values of 5–7.2 and needle size of 150–250 μm), composite and sensitive microspheres including living cells, proteins and NP were then generated with Sphyga to validate the *working-window.*

The beads have well-defined spatial features (size and roundness) and are highly controlled both in composition and in architecture (uniform distribution of components), providing a tailored biomimetic microenvironment that allows an effective exchange of nutrients and other molecules whilst providing an adhesive 3D framework.

5. Conclusion

Sphyga is an open source, low cost and compact tool that helps the user to generate highly controlled microbeads with an easy GUI and a fabrication working-window.

Examples of microbeads with different compositions were illustrated, showing how Sphyga parameters can be chosen to match the required design criteria for the fabrication of microbeads including biomolecules, proteins, cells and nanoparticles in an alginate-based solution. The simultaneous inclusion of sensitive domains, living organisms (e.g. cells, bacteria) and biologically relevant biomaterials are just an example of the constructs that can be fabricated with Sphyga to generate engineered microenvironments for biomedical applications such as drug delivery, toxicology, *in vitro* models and regenerative medicine.

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