TITLE OF INVENTION: DEVICE AND METHOD FOR MICROFLUIDICS-BASED 3D BIOPRINTING

TECHNICAL FIELD

The present invention relates to a device and a method for building a 3D object. The present invention further relates to a method of obtaining a hydrogel.

BACKGROUND

3D printing technologies can be applied to build tissue-like structures, e.g. in the field of medicine and tissue engineering. Generally, these methods are referred to as 3D bioprinting. Typically, printing inks are used that are synthetic, e.g. polymers, or natural. Also materials from plants such as alginate can be used. In particular with natural materials there can be significant batch-to-batch variations, which has an impact on reproducibility and sustainability of the bio-printed 3D structures.

In 3D bioprinting usually a pre-polymer viscous solution is used to print in 3D, and after printing, either an initiator or (UV or visible) light is used for the polymerization of the 3D construct. Several factors are important for bioinks to be suitable for 3D bioprinting. These include biocompatibility, biomimetic structure, biodegradability, porosity and mechanical strength.

Despite recent advances in 3D bioprinting, there is still a need for improved devices and methods for printing of 3D objects.

SUMMARY OF THE INVENTION

The objective of the present invention is to provide a device and a method for building a 3D object, wherein the device for building a 3D object and the method for building a 3D object overcome one or more of the problems of the prior art.

A first aspect of the invention provides a device for building a 3D object from a representation of the object stored in a memory, the device comprising:

- a first inlet configured to take in a bioink solution, in particular a peptide solution,
- a second inlet configured to take in a buffer solution capable of inducing gelation of the bioink solution, preferably instantaneous gelation of the bioink solution,
- a third inlet configured to take in a dispersion,
- a fluid duct for mixing the bioink solution, the buffer solution and the dispersion to obtain a hydrogel, in particular a peptide hydrogel, and
- a nozzle configured to eject a hydrogel, in particular a peptide hydrogel to build the 3D object.

The device can comprise a 3D arm which guides the nozzle to build the 3D object with the desired three-dimensional structure. The memory storing the representation of the object can be part of the device. In other embodiments, the memory is external to the device.

Preferably, the device comprises two or more arms which can move in three dimensions and comprise at least two or three axes. The incorporation of multiple robotic arms allows for simultaneously printing different materials and thus can reduce overall print time.

In embodiments, the first inlet can be connected to a tank with the bioink solution and/or the second inlet can be connected to a tank with the buffer solution. The buffer solution can be in particular a phosphate buffer solution.

Bioink refers to a material that mimics an extracellular matrix environment. The bioink can be suitable to support the adhesion, proliferation, and differentiation of mammalian cells. The bioink can have the ability to be deposited as filament during an additive manufacturing process, e.g. 3D printing.

The bioink solution can be a peptide solution wherein the peptides are made from synthetic, but natural amino acid sequences. Such bioinks can closely resemble body like materials, thus avoiding immunological problems and potential inflammatory processes.

The fluid duct can be a microfluidic channel. In particular, the fluid duct can have a diameter of less than 1000 µm, preferably less than 200 µm.

The device of the first aspect can be used with peptide-based bioinks and offers a unique and novel combination for 3D bioprinting. The advantage of peptide-based bioinks is that these are synthetic amino acid sequences and closely resemble body-like materials. In particular, a synthetic, but naturally occurring and cost-effective peptide bioink can be provided with superior physical and chemical properties enabling instantaneous gelation.

The representation of the object can comprise information about a mixture level between the bioink solution and the buffer solution. For example, the representation can comprise information that in a first region of the object, a 2:1 ratio between bioink solution and buffer solution should be used and in a second region a 1:1 ratio between bioink solution and buffer solution should be used.

The representation can also comprise information about which pressure or flow rate should be used to eject the peptide hydrogel.

The device of the first aspect can be used to fabricate 3D tissue scaffolds or structures at the physiological milieu of choice, with suitable pH, ionic composition, and molarity, thus protecting the native cell function, which gives an advantage over existing printing devices that use chemical initiators or photo-polymerization (UV or visible light treatment).

The peptide bioinks also allow the printing of different materials (e.g. peptide micro or nanoparticles, gold or silver nanoparticles, nanowires, graphene, carbon nanotubes and quantum dots, for example) in 3D structures that can perform various functions. For example, the built structures can be used for imaging, sensing, catalysis and tuning of the mechanical properties of the peptide hydrogel. The versatility of printing the nanomaterials is demonstrated by printing the quantum dots and by the *in-situ* synthesis of silver nanoparticles in the 3D printed structures.

The device of the first aspect can have applications in materials science and in the field of biomedical engineering such as regenerative medicine, tissue engineering, wound healing (printing at the wound site), tissues and organs printing, prosthetics, medical implants, *in vitro* models and 3D tissue models for drug testing and biosensing.

The device of the first aspect can also be used directly in the surgical room during surgery to provide patients in need immediately with the printed tissue construct. A platform based on the device of the first aspect can also include a 3D image reconstruction system which enables the printing devices to take the image of an object and print it according to the user specifications.

Preferably, the nozzle comprises the fluid duct. Preferably, the fluid duct is arranged within the nozzle. For example, the fluid duct can be arranged along an axis of the nozzle. The fluid duct can be arranged such that it ends inside of the nozzle.

In a first implementation of the device for printing a 3D object according to the first aspect, the fluid duct comprises a first region for mixing the bioink solution and the dispersion to obtain a bioink-dispersion mixture and a second region for mixing the bioink-dispersion mixture with the buffer solution.

In a second implementation of the device for printing a 3D object according to the first aspect, the dispersion comprises a cell culture media. The cell culture media can for example comprise primary human skin fibroblast cells.

Preferably, the cell culture media comprises a plurality of cell types. In other embodiments, the cell culture media comprises only one type of cell types.

In a third implementation of the device for printing a 3D object according to the first aspect, the dispersion comprises micro and/or nanoparticles, preferably peptide microparticles, peptide nanoparticles, silver nanoparticles, gold nanoparticles, nanowires, quantum dots and/or carbon nanotubes.

The peptide micro or nanoparticles loaded with different drugs can be used for controlled drug delivery in the 3D printed structure. The advantage of using other types of nanoparticle such as silver nanoparticles, gold nanoparticles, nanowires, quantum dots and carbon nanotubes is that these nanoparticles can add imaging, sensing and catalysis abilities to the 3D printed peptide hydrogel structure.

In a fourth implementation of the device for printing a 3D object according to the first aspect, a plurality of cell types are encapsulated in the peptide microparticles and/or immobilized on the peptide microparticles, in particular on the peptide microparticle surface.

The advantage of encapsulating the plurality of cells in the peptide microbeads and subsequent 3D printing with the peptide hydrogel allow the precise positioning of different cell types in the 3D construct. This will allow an increase in vascularization of the 3D printed construct which is a requirement for the 3D bioprinted constructs.

In a further implementation of the device for printing a 3D object according to the first aspect, the device further comprises a heating module configured to heat the microfluidic fluid duct.

The heating module can comprise a coil with windings that are wrapped around the nozzle or a heating jacket. The heating module can be configured to heat the fluid duct to more than 60°C, preferably more than 80°C.

In a further implementation of the device according to the first aspect, the device further comprises a micromixer. Said micromixer can be used to enhance mixing of the peptide/bioink solution and cells to get a more homogeneous distribution of the cells across the 3D bioprinted construct. Said micromixer can be located in the fluid duct, in particular in said first region for mixing the bioink solution and the cell/dispersion to obtain a bioink-dispersion mixture.

The device can also comprise a heating module and a micromixer.

In a further implementation of the device according to the first aspect, the device further comprises one or more light emitters configured to irradiate the microfluidic fluid duct.

The light emitters can be configured to lead to a heating of the peptide hydrogel without heating other components or can be used to initiate a chemical process for faster gelation. In particular, the fluid duct can be surrounded by transparent material, such that the light from the light emitters can pass through to the fluid duct or the light can be focused at the end of the nozzle outlet.

In a further implementation of the device for printing a 3D object according to the first aspect, the light emitters comprise a first LED with a first wavelength and a second LED with a second wavelength different from the first wavelength.

The device can be configured to separately switch the first and second LED. Thus, different components in the fluid duct that have different light absorption spectra can be heated or activated separately.

In a further implementation of the device for printing a 3D object according to the first aspect, the bioink solution comprises one or several peptides, said peptides preferably having a general formula selected from:

a) Z_o-X_nBX_mW-Z'$_p$, and

b) Z_o-WX_mBX_n-Z'$_p$,

wherein Z is an N-terminal protecting group and Z' is a C-terminal protecting group, with o and p being independently selected from 0 and 1;

wherein **X** is, independently at each occurrence, an aliphatic amino acid selected from isoleucine, norleucine, leucine, valine, alanine, glycine, homoallylglycine and homopropargylglycine with n and m being integers being independently selected from 0, 1 and 2, with the proviso that $m+n \leq 2$,

wherein B is an aromatic amino acid selected from phenylalanine and tryptophan, or is an aliphatic counterpart of said aromatic amino acid, said aliphatic counterpart being selected from cyclohexylalanine, 4-hydroxy-cyclohexylalanine, 3,4-dihydroxycyclohexylalanine.

Wherein W is a polar amino acid selected from aspartic acid, glutamic acid, asparagine, glutamine, lysine, 5-N-ethyl-glutamine (theanine), citrulline, thio-citrulline, cysteine, homocysteine, methionine, ethionine, selenomethionine, telluromethionine, threonine, allothreonine, serine, homoserine, tyrosine, histidine, arginine, homoarginine, ornithine, lysine, N(6)-carboxymethyllysine, histidine, 2,4-diaminobutyric acid (Dab), 2,3-diaminopropionic acid (Dap), and N(6)-carboxymethyllysine,

wherein said polar amino acid is preferably selected from the group consisting of aspartic acid, asparagine, glutamic acid, glutamine, serine, threonine, methionine, arginine, histidine, lysine, ornithine (Orn), 2,4-diaminobutyric acid (Dab), and 2,3-diaminopropionic acid (Dap).

Examples for such peptides are IVZK, IVFK, IVYK, FIVK and others. Alginates may also be used.

Experiments have shown that these components are particularly suitable for 3D printing.

A second aspect of the invention refers to a method for printing a 3D object, preferably an *in situ* method for printing a 3D object. The method comprises:

- mixing a bioink solution, in particular a peptide solution, a cell culture medium and a buffer solution capable of inducing gelation of the bioink solution to obtain a hydrogel, in particular a peptide hydrogel, and
- ejecting the hydrogel, in particular the peptide hydrogel, out of a nozzle to build the 3D object.

The methods according to the second aspect of the invention can be performed by the device for printing a 3D object according to the first aspect of the invention. Further features or implementations of the method according to the second aspect of the invention can perform the functionality of the device for printing a 3D object according to the first aspect of the invention and its different implementation forms.

In a first implementation of the method for printing a 3D object of the second aspect, the mixing is performed in fluid duct, in particular a microfluidic channel, wherein preferably the nozzle comprises the fluid duct.

The fluid duct can comprise elements within the fluid duct which lead to an improved mixing between the components. For example, the elements can be configured to create turbulences within the fluid duct, which may lead to an improved mixing.

In a second implementation of the method for printing a 3D object of the second aspect as such or according to the first implementation of the second aspect, the nozzle comprises the microfluidic fluid duct.

In a third implementation of the method for printing a 3D object of the second aspect as such or according to any of the preceding implementations of the second aspect, air pressure and/or microfluidic pumps are used to eject the peptide hydrogel.

In a fourth implementation of the method for printing a 3D object of the second aspect as such or according to any of the preceding implementations of the second aspect, the method further comprises an initial step of scanning a 3D source object.

By obtaining a 3D scan of the 3D source object, the method can create a target object that closely resembles the source object, thus acting as 3D copying method.

In a fifth implementation of the method for printing a 3D object of the second aspect as such or according to any of the preceding implementations of the second aspect, the cell culture media comprises a plurality of cell types.

In a further implementation of the method for printing a 3D object of the second aspect, the dispersion comprises micro and/or nanoparticles, preferably peptide microparticles, peptide nanoparticles, silver nanoparticles, gold nanoparticles, nanowires, quantum dots and/or carbon nanotubes.

In a preferred embodiment, the method of the present invention is carried out under physiological conditions.

The buffer solution capable of inducing instantaneous gelation used in the method of the invention can be phosphate buffer saline or buffer(s) containing salt(s), such as sodium chloride or calcium chloride.

In one implementation of the *in situ* method for printing a 3D object, the bioink solution is a peptide solution as defined herein above.

A further aspect of the invention refers to a method of obtaining a hydrogel, the method comprising:

- providing a bioink solution, in particular a peptide solution, through a first inlet (410) into a first airbrush (401),
- providing a buffer solution capable of inducing instantaneous gelation of the bioink solution to a hydrogel, in particular a peptide hydrogel, through a second inlet (420) into a second airbrush (402), and
- air-spraying the bioink solution and the buffer solution simultaneously through the nozzles (411, 421) of said first and said second airbrush (401, 402), onto the same site on a surface, thereby creating the hydrogel, in particular the peptide hydrogel.

In a first implementation of the method of obtaining a hydrogel, the bioink solution is a peptide solution as defined herein above.

In a further implementation of the method of obtaining a hydrogel, the buffer solution capable of inducing instantaneous gelation is phosphate buffer saline or buffer(s) containing salt(s), such as sodium chloride or calcium chloride.

In a further implementation of the method of obtaining a hydrogel, the airbrushes are used with a device as defined herein above as first aspect of the invention.

The airbrushes can be used with a device according to the present invention as defined herein. The airbrushes can also be used as a hand held device.
The bioink solution and the buffer solution are preferably directly sprayed onto a surface, such as a wound site, where the hydrogel forms.

In particular, the airbrush can be used with a device such as a robotic arm as shown in Fig.4A, 4B and 4C for direct spray of a peptide hydrogel onto a surface such as a wound site. The air brush assembly can also be used as a hand held device to directly spray the peptide hydrogels onto a surface such as a wound site.

BRIEF DESCRIPTION OF THE DRAWINGS

To illustrate the technical features of embodiments of the present invention more clearly, the accompanying drawings provided for describing the embodiments are introduced briefly in the following. The accompanying drawings in the following description are merely some em-

bodiments of the present invention, modifications on these embodiments are possible without departing from the scope of the present invention as defined in the claims.

FIG. 1A is a schematic of a peptide hydrogel formation using a 3D bioprinter,

FIG. 1B are two pictures of the microfluidic printing nozzle containing three different inlets that can be connected to the microfluidic pumps,

FIG. 1C shows the microfluidic printing device in a specially designed holder fitted on a robotic arm,

FIG. 1D shows a printed structure containing IVZK peptide bioink and human skin fibroblast cells using the 3D bioprinter.

FIG. 1E is a schematic of a device for building a 3D object.

FIG. 2 shows two devices for building a 3D object, wherein the first device comprises a heating unit and the second device comprises a light treatment module.

FIG. 3A is a schematic representation of the device/nozzle developed for 3D bioprinting of peptide bioinks, as also shown in Figure 1A.

FIG. 3B is a schematic representation of the device/nozzle including a micromixer (240) that can be used to enhance mixing of the peptide and cells to get a more homogeneous distribution of the cells across the 3D bioprinted construct.

FIG. 3 C-H shows a breakdown of the different regions of the device/nozzle, namely:

(C) with the micromixer (240) and the main nozzle,

(D) with the micromixer (240), the buffer nozzle and the main nozzle,

(E) with the buffer nozzle and the main nozzle,

(F) with mixing of the cells, peptides and the buffer to form the hydrogel,

(G) and (H) with the microheater (220) on the device/nozzle (A) and (B), to control the temperature of the peptide gelation process, if necessary.

FIG. 4 A is a schematic illustration of a 3D bioprinter platform containing multiple robotic arms equipped with multiple nozzles for printing of different peptides or cell types.

FIG. 4 B Robotic arm 3D bioprinting set-up. The figure shows a schematic picture of robotic 3D bioprinter inside the biosafety cabinet showing microfluidic pumps, robotic arm and printing nozzle. All the bioprinting experiments were performed inside the biosafety cabinet.

FIG. 4C Scheme of the bioprinting nozzle installed on a commercially available dual arm robotic system and integrated with microfluidic pumps to create 3D structures. The 3D scanner scans the structure and sends the information to a computer where a software reconstructs the image of the 3D structure. The robotic arms then move the nozzles according to the design of the 3D structure to create the final 3D shape. The stage is temperature controlled and flexible to move into different directions and can also rotate in different directions.

FIG. 5 illustrates the printability of peptide-based bioinks (IVFK and IVZK) into different shapes such as circle, square, grid and letters/text. The scale bar in all figures is 10 mm.

FIG. 6A and 6B are fluorescence confocal microscopy 2D and 3D images of human skin fibroblast cells bioprinted using peptide-based bioink (IVZK).

FIG. 7 is a cell viability assay as measured from ATP levels in 3D bioprinted human skin fibroblast cells using IVFK, IVZK peptides and alginate as bioinks.

FIG. 8 *In-situ* synthesis and inclusion of nanomaterials in 3D bioprinted peptide hydrogels

FIG. 8A shows *in-situ* generated silver nanoparticles inside the 3D bioprinted IVZK peptide bioink. 1mM silver nitrate solution was mixed with IVZK bioink and after bioprinting UV exposure was done for 10min at 254nm. Yellowish colour of the bioprinted text is due to the formation of silver nanoparticles.

FIG. 8B, C shows the transmission electron microscopy (TEM) images of silver nanoparticles. The average diameter calculated from the TEM images using imageJ was 4.4 nm.

Fig. 8D, E 3D bioprinted IVZK peptide bioink after inclusion of green colour quantum dots in the peptide solution without (D) and with (E) UV excitation at 365nm.

FIG. 8F confocal microscopy image showing the emission from green color quantum dots which adsorb on the peptide fibers inside the scaffold and clearly showing the fibrous network of 3D bioprinted peptide bioink (IVZK).

FIG. 9 shows representative fluorescence confocal microscopy images of human skin fibroblast cells.

FIG. 10 shows scanning electron microscopy studies of 3D printed peptide hydrogel constructs.

FIG. 11A Fluorescence confocal microscopy images of 3D bioprinted HDFn cells using IVFK, IVZK, and alginate-gelatin bioinks at different days of cell culturing (nucleus is shown in blue, F-actin is shown in red and vinculin is shown in green).

FIG. 11B Picture of hydrogels printed on day 1 and on day 21 after cells had been cultured in the media for 21 days.

FIG. 11C 3D cell viability assay of HDFn cells 3D bioprinted in IVFK, IVZK, and alginate-gelatin bioinks at various time points.

FIG. 12A Fluorescence confocal microscopy images of 3D bioprinted human bone marrow-derived mesenchymal stem cells (BM-MSCs) cells using IVZK and alginate-gelatin bioinks at different days of cell culturing (nucleus is shown in blue, F-actin is shown in red and vinculin is shown in green).

FIG. 12B 3D cell viability assay of BM-MSCs cells 3D bioprinted in IVZK and alginate-gelatin bioinks at various time points.

FIG. 13A Schematic presentation of the mode of action to spray both peptide and phosphate buffer using the air brush. The nozzles of the air brushes are aligned at an angle such that both the peptide solution and phosphate buffered saline (PBS) streams, coming out from the nozzles, combine at a certain point to create the peptide hydrogel.

FIG. 13B Pictures of two air spray brush nozzles that were assembled in a casing made from Perspex sheet at an angle such that the stream from both the nozzles meet at one point. One nozzle contained the peptide solution (5mg/ml IVZK) and the second nozzle contained the phosphate buffer solution (10X).

FIG. 13C Scanning electron micrographs (SEM) of peptide hydrogel sprayed on a silicon surface. The SEM images confirmed the formation of the peptide fiber network.

DETAILED DESCRIPTION OF EMBODIMENTS

FIG. 1A is a schematic illustration of a peptide hydrogel formation using a 3D bioprinter. A bioink solution and cell culture media containing different cell types mix together in a first region. The mixture is then mixed with a buffer solution in a second region, which is a microfluidic fluid duct, to induce gelation and form the peptide hydrogel. The peptide hydrogel is then ejected out through outlet nozzle. The buffer solution can be a phosphate buffer solution, e.g. a phosphate buffer saline (PBS).

Benefit of using peptide bioinks for biological applications include their resemblance to natural, body-like features compared to non body-like materials (e.g. polymers) or natural, but not from human background, and undefined material (alginate from crustaceae, plants, etc.) with significant batch-to-batch variations which will has an impact on the sustainability of the bioprinted 3D structures. Also, biofunctionalization can be more smoothly fitted and added to a peptide compound than to a polymer structure. Peptide bioinks also allow addition of different

materials (peptide micro or nanoparticles, gold or silver nanoparticles, nanowires, graphene, carbon nanotubes and quantum dots, for example) and subsequent printing in 3D structures that can perform various functions. For example, the built structures can be used for imaging, sensing, drug delivery, catalysis and tuning of the mechanical properties of the peptide hydrogel.

FIG. 1B is a picture of the microfluidic printing nozzle containing three different inlets that can be connected to the microfluidic pumps. The cells were pre-loaded in the tubing loop with inner diameter of 250 μm connected with the nozzle in order to get a uniform distribution of the cells throughout the 3D construct.

FIG. 1C shows the microfluidic printing device in a specially designed holder fitted on a commercially available robotic arm that can print 3D structures. The nozzle needle close to the petri dish is used to print the peptide hydrogel bioink into a 3D circle.

FIG. 1D shows a printed structure containing IVZK peptide bioink and human skin fibroblast cells using the 3D bioprinting device.

FIG. 1E shows in detail a device 100 for building a 3D object. The device comprises three inlets 112, 114, 116 for taking in a bioink solution, a phosphate buffer saline and a dispersion, e.g. a cell culture medium. The three inlets 112, 114, 116 are connected to a fluid duct 110, which comprises a first part 110A and a second part 110B. The bioink solution from the first inlet 112 and the dispersion from the third inlet 116 are brought together in the first part 110A. The mixture of these two components is then pushed through the second part 110B and meets the phosphate buffer solution from the second inlet 114. The second part 110B forms a microfluidic fluid duct towards an outlet nozzle 122 where the mixture is ejected from the device 100. The term fluid duct herein refers to any tube, canal, pipe, or conduit by which the bioink and the other components can be conducted or conveyed and mixed with the other component or components.

FIG. 2 shows two devices 200A, 200B for building a 3D object. Both devices comprise a first inlet 212 for taking in a bioink solution, a second inlet 214 for taking in a phosphate buffer saline and a third inlet 216 for taking in different cell types dissolved in a cell culture media. The devices 200A, 200B also comprise a fluid duct 210 which is arranged within the nozzle

222. The fluid duct 210 receives the bioink solution, the phosphate buffer saline and the cell culture media. The three components are mixed within the fluid duct to obtain a peptide hydrogel. The peptide hydrogel is then ejected from the outlet of the nozzle 222.

The first device 200A comprises a heating unit 220, which comprises a heating wire which is wrapped around the nozzle 222 and thus around the fluid duct 210.

The second device 200B comprises a light emitter 230 which is configured to irradiate the fluid duct 210 within the nozzle. The nozzle 222 is completely made of transparent material. In other embodiments, only a part of the nozzle 222 is transparent so that the light emitter can irradiate material within the fluid duct.

FIG. 3 shows different designs of the devices/nozzles. Two types of devices/nozzles can be used to perform 3D bioprinting of peptides. The device/nozzle 1 (Fig. 3A, also shown in FIG. 1A) contains two regions of mixing. In the first region, the peptide solution and cells in cell culture media were mixed, and in the second region, PBS was introduced to induce instant gelation of the peptide which results in the encapsulation of the cells into the peptide hydrogel (Fig. 3A). In principle, any cell type can be used for 3D bioprinting using this method. The device/nozzle 2 (Fig. 3B) contains a micromixer (240) that can be used to enhance the mixing between peptide and cells to get a more homogeneous distribution of the cells inside the 3D construct. Fig.3C-F show more details of different regions of the device/nozzle. Fig. 3G and Fig. 3H show the assembly of the microheater (220) on the devices/nozzles shown in Fig.3A and Fig. 3B to control the temperature of the devices/nozzles.

FIG. 4A is a schematic illustration of a 3D bioprinting platform 300 comprising two robotic arms 342A, 342 that are equipped with printing devices 344A, 344B for printing of different peptides or cell types. The printing devices 344A, 344B shown in FIG. 4A can be implemented as shown in FIG. 2.

The 3D bioprinting platform 300 comprises a 3D scanner 320 that is configured to scan an object 310 to be printed. After scanning the object 310, the 3D scanner transmits a representation 322 of the object to a control module 330 for controlling the robotic arms 342A, 342B, microfluidic pumps 340 and printing devices 344A, 344B. The printing devices 344A, 344B are arranged to print the target object 352 on an automated stage 350 with heating and cool-

ing. The automated stage 350 can comprise multiple holders to print e.g. in a petri dish or multiple well plates. It should be noted that the 3D printing and the device and method in accordance with the present invention is not limited to any particular or specific 3D pattern or 3D object. It can be used for any 3D object.

The printing devices 344A, 344B are connected to the microfluidic pumps through flexible tubes 346. There is one tube for each of the three components, such that the components can be mixed in the printing devices 344A, 344B.

In other embodiments, a single robotic arm can be equipped with multiple nozzles. Only two robotic arms are shown in FIG. 4A. Additional robotic arms can be included to print multiple cell types.

The printing platform 300 can be used in combination with peptide-based bioinks and allow a continuous printing of different cell types or other biological compounds into 3D structures with excellent biocompatibility.

The printing process can be based on using microfluidic pumps or air pressure to move the bioink solution, phosphate buffer saline (PBS) and different cell types (human skin fibroblast cells, for example) in cell culture media into individual fluid ducts, and after mixing, the peptide-based hydrogel containing the cells extruded from the nozzle or outlet for 3D bioprinting.

The robotic arms 342A, 342B move according to the 3D design in X, Y and Z direction to print the object information sent by the 3D scanner using peptide bioink extruded from the nozzle. A combination of two to three or more robotic arms can be used to print multiple peptide bioinks in parallel or one by one to print multiple cell types at the same time or different time intervals. Moreover, on one robotic arm, e.g. two or three nozzles can be attached to print 3D structures. This way, multiple cellular functionalities can be introduced in a single 3D bioprinted structure which is a requirement for a fully functional organ/tissue construct.

The robotic arms 342A, 342B each comprise four hinges 345A, 345B, thus allowing a high degree of flexibility when positioning the printing devices 344A, 344B.

Currently available 3D bioprinters are mainly based on linear systems, such as inkjet bioprinter systems, which are limited to 3-dimensional xyz movement. Thus, they offer 3 degree of freedoms (DOF) or an 3-axis system. Robotic systems have the advantage over linear systems to increase flexibility by increasing the number of DOFs. Robotic systems can start from 3-axis robots, to 4-axis robots and so forth, up to 7-axis robots. The system of FIG. 4A has two robotic arms thus giving additional degrees of freedom and allowing for supplementary angular movements when compared to a linear system or a one robotic arm system. In a variation of the system of FIG. 4A, where the base of the robotic arm can also rotate around itself, an increase in flexibility to a 13- or 14-axis printing system is achieved.

Since the robotic arms incorporate swift rotational movements, the system allows for faster and smoother printing. The quality of the printed biomaterial is enhanced due to the additional degrees of freedom. Multiple robotic arms can be equipped with multiple nozzles for printing of different types of bioinks or cell types. Robotic arms are also more compact in size than existing 3D bioprinters and can be more easily transported. This gives transport advantages of the printer when the operating procedures will take place at different surgical rooms. The system can be used for example for application toward plastic, reconstructive and aesthetic surgery.

FIG. 4B shows another integration of the device/nozzle on a commercially available robotic arm and using pumps. Human skin fibroblast cells together with IVZK and IVFK peptide solutions were 3D bioprinted using this set-up as a further example.

In more detail, a robotic arm 3D bioprinter was used to perform 3D bioprinting of normal human skin fibroblast cells (HDFn). The bioprinter was developed by integrating microfluidic pumps, a printing nozzle, and a robotic arm (FIG. 4B). A commercially available robotic arm 3D printer (Dobot Magician, Dobot) was converted to a bioprinter by exchanging the polymer extruder of the robotic arm with the bioprinting nozzle. The microfluidic printing nozzle was prepared by using two different syringe needles with different internal diameters (FIG. 4B). The main needle (18 Gauge) has the internal ID=840μm, and the top needle has an internal diameter of 80μm (34 Gauge) and the outer diameter of 190μm. Two holes were created at the side of the main needle, and PTFE tubing (PTFE # 30 AWG thin wall tubing, Cole Parmer) with an internal ID of 304μm was inserted from one side of the main nozzle by creating a hole of the same size as of the PTFE tubing's outer diameter for the flow of the cells. On the other side of the needle, PTFE tubing (PTFE#24 AWG thin wall tubing, Cole

Parmer) with an internal ID of 550μm was inserted and used for the flow of peptide solution. A transparent epoxy was used to seal the tubing and the top nozzle. The printing nozzle has three inlets and one outlet. A combination of different diameter needles can also be used to prepare different printing nozzles with different diameters. The tubing for the peptide solution and cell culture media (containing different cell types) inlets were inserted into the side wall of the main needle so that both the fluids can mix in this region. The second needle (34G) was inserted inside the main needle (18 Gauge) from the top such that the solution from this needle (10 x PBS) mixes with other two solutions to make the peptide hydrogel close to the end of the main needle. The length of the main needle was 2.5 cm and the mixing region for the buffer with the cells and peptide was approximately 2.0 cm. The mixing region for the buffer with the cells and peptide can be adjusted to make the peptide hydrogel. The length 2.0 cm can be varied depending on the formation of the peptide hydrogel inside the nozzle.

A further set-up using a commercially available dual arm robotic system that is integrated with microfluidic pumps to create 3D structures is shown in FIG. 4C. The 3D scanner scans the structure and sends the information to a computer where a software reconstructs the image of the 3D structure. The robotic arms then move the nozzles according to the design of the 3D structure to create the final 3D shape. The stage is temperature controlled and flexible to move into different directions and can also rotate in different directions.

In addition, the peptides such as IVZK and IVFK can also be sprayed directly onto different surfaces using a combination of air spray nozzles. FIG. 13A shows the schematic of the system in which two airbrush nozzles were used to spray at an angle so that both the solutions (peptide and phosphate buffer saline) meet on a certain point to form peptide hydrogel. FIG. 13B shows examples of such air spray nozzles. FIG. 13C shows the scanning electron microscopy (SEM) images of the IVZK peptide sprayed on a silicon wafer. The SEM images clearly showed the formation of the peptide fiber networks using the air spray nozzles. The spray nozzles can be incorporated on a robotic arm system, such as the ones shown in FIG. 4A to C, to directly coat different peptides either alone or in combination with different types of cells on a wound surface in an automated way. The spray nozzles can also be used as a handheld device to directly spray on a wound.

Experiments have been performed to show the technical examples using the 3D/4D bioprinting method.

Preparation of Peptide and Phosphate Buffer Solutions

All the peptide (Bachem, Germany) solutions were freshly prepared by dissolving different amounts of the peptides (IVFK and IVZK) in 1 ml of Milli-Q water and mixed for 30 seconds by vortexing to obtain a homogenous solution. In this example, 15 mg of each peptide was dissolved in Milli-Q water. The phosphate buffered saline (PBS, 10X concentration) was obtained from Sigma and used as received in the printing process.

The relative molecular mass of the IVFK and IVZK peptides was 546.71 and 552.76 with a purity of 97.1% and 97.2% (determined by HPLC method), respectively.

Cell Culture

Human dermal fibroblasts, neonatal (HDFn, C0045C) were purchased from Thermo Fisher Scientific, USA. These cells were first cultured using medium 106 (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. T175 or T75 cell culture flasks (Corning, USA) were used to maintain the cells in a humidified incubator with 95% air and 5% CO_2 at 37°C. The cells were subcultured by trypsinization at approximately 80% confluence. The cell culture media was replaced every 48 hours.

Fabrication of the Microfluidic Printing Nozzle

The microfluidic printing nozzle was prepared by using four different syringe needles with different internal diameters, selected from 100 µm, 150 µm, 230 µm, 300 µm, 450 µm, 500 µm, 600 µm, 700 µm, 800 µm, 1 mm and 1.2 mm diameters. The printing nozzle has three inlets and one outlet. A combination of different diameter needles can be used to prepare different printing nozzles with different diameters, depending on the individual solutions (viscosity) or depending on the writing structures giving rise to thinner or broader printed structures. Two holes were created on the two sides of the middle needle containing the outlet. The needles for the peptide solution and cell culture media (containing different cell types) inlets were inserted into the main needle so that both the fluids can mix together in this region. The third needle with a particular length was inserted from the top such that the solution from this needle (10 x PBS) mixes with other two solutions to make the peptide hydrogel.

Printing Procedure

The printing involves three different components such as peptide solution, phosphate buffer saline (PBS) and different cell types. As for peptides, solutions of different concentrations of IVZK or IVFK peptides were prepared by weighing different amounts of the peptide (15mg, for example) and dissolved in 1 ml Milli-Q water. The second solution contained 10 x PBS. The third solution contained primary human skin fibroblast cells in cell culture media (four million cells in 500 µl of the DMEM cell culture media, for example). Primary human skin fibroblast cells were used to demonstrate cell printing. Other cell types can be incorporated in the printing process similarly as described in the case of primary human skin fibroblasts. All three solutions were pumped to the nozzle using the syringe pumps at different flow rates. The flow rates were adjusted so that three solutions meet at the interface which causes gelation of the bioink and peptide hydrogel come out from the outlet of the printing nozzle. In a particular experiment, flow rates used were 20 µl/min for cell culture media containing skin fibroblast cells, 25 µl/min for the peptide solution and 20 µl/min for the 10x PBS. A simple ring structure was printed by the deposition of peptide hydrogel in a layer-by-layer fashion with a diameter of about 10 mm and thickness about 2 mm. Different structures, for example, circle and square and grid were printed instantaneously by using the movement of a 3D printer or a robotic arm 3D printer.

FIG. 5 shows the printability of peptide-based bioinks (IVFK and IVZK) into different shapes such as circle and square, grid or letters/text.

The constructs were printed onto 35 mm tissue culture petri dish. After bioprinting, the constructs were placed in biosafety cabinet for 3 min to further facilitate self-assembly of peptide bioinks. Then, the constructs were gently washed 2 or 3 times with culture medium. To each dish, 3 mL of culture medium was added and cultured in a humidified incubator at 37 °C and 5% CO_2. At pre-determined time points, the constructs were taken out to perform 3D assay and cytoskeletal staining of cells for fluorescence confocal microscopy studies.

Scanning Electron Microscopy (SEM) Analysis

For SEM analysis, the peptide hydrogel ring was directly 3D printed on a small piece of silicon wafer (20 x 20 mm). The sample was first fixed with 3.7% paraformaldehyde solution for 30 minutes. The peptide hydrogel ring was then washed with different concentrations of ethanol (10, 20, 30, 40, 50, 60, 70, 80, 90, 99.8 %) sequentially to remove water from the hydrogel. The sample was dried in a critical point dryer to evaporate ethanol and to preserve the

structure of the hydrogel. After this it was coated with 4 nm of Pt/Pd metals using a sputter coater. Finally, it was examined using Quanta 3D FEG SEM/FIB microscope and Magellan™ XHR SEM using an accelerating voltage of 10 kV and 2 kV, respectively.

Cytoskeletal Staining

Cytoskeletal staining of the 3D circular constructs containing human dermal fibroblast cells was performed at different time intervals after 1, 3, 7 and 14 days of culture. In brief, the 3D construct was treated with 3.7% paraformaldehyde solution for 30 minutes to fix the cells. After that it was incubated in a cold cytoskeleton buffer (3 mM MgCl2, 300 mM sucrose and 0.5% Triton X-100 in PBS solution) for 10 minutes to permeabilise the cell membranes. The permeabilised cells were incubated in blocking buffer solution (5% FBS, 0.1% Tween-20, and 0.02% sodium azide in PBS) for 30 minutes at 37°C, followed by incubation in FITC-phalloidin (1:200) for 1 hour at 37°C. Further, the construct was incubated in DAPI for 1 hour at 37°C to counterstain the nuclei of the cells. Fluorescence confocal microscopy (Zeiss LSM 710 Inverted Confocal Microscope, Germany) was used to image the labelled human skin fibroblasts in the 3D construct (see FIG. 6).

FIG. 6A shows a fluorescence confocal microscopy image of human skin fibroblast cells bioprinted using peptide-based bioink (IVZK). FIG. 6B shows a fluorescence confocal microscopy 3D image of human skin fibroblast cells bioprinted using peptide-based bioink (IVZK). The progress over 14 days is illustrated in FIG. 9. In particular, FIG. 9 shows representative fluorescence confocal microscopy images of human skin fibroblast cells bioprinted using peptide-based bioink (IVZK) (left panel) and fluorescence confocal microscopy 3D images of human skin fibroblast cells bioprinted using peptide-based bioink (IVZK) (right panel). Cells were stained for F-actin (green) and nuclei (blue) for fluorescence microscopy studies. The printed structures using IVZK were cultured for 1, 3, 5, 7 and 14 days. Both 2D and 3D confocal microscopy images were taken at different locations on the same sample.

In further experiments, see FIG. 11A and FIG. 12A, immunostaining was performed after 1, 3, 7, 14 and 21 days of culture. Briefly, the cells were fixed with 3.7% paraformaldehyde solution for 30 minutes and incubated in a cold cytoskeleton buffer (3 mM $MgCl_2$, 300 mM sucrose and 0.5% Triton X-100 in PBS solution) for 10 minutes to permeabilize the cell membranes. The permeabilised cells were incubated in a blocking buffer for 30 minutes at 37°C. The blocking buffer solution contains 5% FBS, 0.1% Tween-20, and 0.02% sodium

azide in PBS. In the next step, cells were incubated in an anti-vinculin (1:100) solution for one hour at 37°C and further incubated in anti-mouse IgG (whole molecule)-FITC and rhodamine-phalloidin (1:200) for 1 hour at 37°C. Finally, the cells were incubated in DAPI for 10 minutes at 37°C to counterstain the nucleus. The fluorescent dye-treated cells were imaged using a laser scanning confocal microscope (Zeiss LSM 710 Inverted Confocal Microscope, Germany). (Sundaramurthi *et al.,* RSC Adv., 2015, 5, 69205–69214).

3D Cell Proliferation Assay

FIG. 7 shows the cell viability assay as measured from ATP levels in 3D bioprinted human skin fibroblast cells using IVFK, IVZK peptides and alginate as bioinks.

The CellTiter-Glo® luminescent 3D cell viability assay was performed on 3D printed constructs containing human dermal fibroblast cells after different days (1, 3, 7 and 14) of culture. The quantification of the ATP using this method provides information about the metabolically active cells present in the construct. After each time point, the 3D constructs were washed twice with Dulbecco's phosphate-buffered saline (DPBS). Equal amounts of CellTiter-Glo® luminescent reagent and fresh cell culture medium were added to the samples. The contents were mixed for 2 minutes to digest the 3D hydrogel constructs and further incubated for 10 minutes. Finally, the luminescence was recorded using a plate reader (PHERAstar FS, Germany), see FIG. 7.

The CellTiter-Glo® luminescent 3D cell viability assay was also performed to determine the number of viable human bone marrow-derived mesenchymal stem cells (hBMSCs) in 3D hydrogels. After each time point, the hydrogels cultured with hBMSCs were washed twice with DPBS. Fresh medium was added to each well, and an equal amount of CellTiter-Glo® luminescent reagent was also added to the gels. The contents were mixed for 5 minutes to digest the hydrogels and then incubated for 30 minutes. After incubation, the luminescence was recorded using a plate reader (PHERAstar FS, Germany), see FIG. 12B.

Incorporation and In Situ Synthesis of Nanomaterials in the 3D Constructs

We show the versatility of the 3D bioprinting method using tetramer peptides by printing nanomaterials within the peptide bioinks. FIG. 8A shows the *in-situ* synthesis of silver nanoparticles inside the 3D bioprinted construct. The peptide bioinks provided sufficient nucleation sites for silver ions, and after UV irradiation, silver nanoparticles were formed inside the 3D

construct. The formation of silver nanoparticles appeared as yellowish color in the 3D bioprinted text (FIG. 8A) as compared to the transparent bioprinted text (FIG.5, lower panel).

FIG. 8B and 8C show transmission electron micrographs of silver nanoparticles prepared by *in-situ* synthesis of silver nanoparticles in the 3D printed structure of IVFK peptide bioinks. The 3D structure was printed using the IVFK peptide and silver nitrate (1 mM) mixture and subsequent illumination with UV light was used to produce silver nanoparticles.

FIG. 8F shows a fluorescence confocal microscopy image of green color quantum dots 3D printed with the peptide bioink (IVZK) showing that different nanomaterials can be incorporated into the printed structures, see also FIG. 8D and 8E. This shows that peptide based bioinks can be used to perform *in-situ* synthesis of nanomaterials in 3D printed structures.

Different nanomaterials such as quantum dots and nanoparticles can be incorporated in the 3D peptide hydrogel using this printing method. As an example, streptavidin modified CdSe/ZnS quantum dots (QD 525, Invitrogen) were mixed with the peptide solution and 3D printed using the same procedure as described earlier. Fluorescence confocal microscopy (Zeiss LSM 710 Inverted Confocal Microscope, Germany) was used to confirm the presence of the quantum dots in the construct (FIG. 8F). The excitation wavelength for the quantum dots was 405nm and emission wavelength maximum was 525 nm (green color). In another example, silver nanoparticles were generated *in situ* in the 3D peptide hydrogel construct by using the UV treatment (254nm). The solution of silver nitrate ($AgNO_3$, 1 mM) was mixed with the peptide solution and after printing, UV light (254 nm for 10 min) was used to generate silver nanoparticles in the construct (FIG. 8B and 8C). In this case, 100 mM Tris buffer (pH 8.5) was used instead of phosphate buffer saline (PBS).

Transmission Electron Microscopy (TEM) Studies

The TEM studies were carried out using FEI Titan G2 80-300 CT with a 300 kV emission gun. The TEM samples of 3D constructs containing in situ generated silver nanoparticles were prepared by transferring a small portion of the 3D construct on carbon coated copper grid using clean cotton swab. The grids were dried in normal air overnight before imaging (FIG. 8B and 8C).

FIG. 10 shows scanning electron microscopy studies of 3D printed peptide hydrogel constructs. For the low magnification images to show the circular ring (top), Quanta 3D FEG SEM/FIB microscope was used (10 kV). For high resolution images Magellan ^{TM} XHR SEM was used (2 kV).

In addition, human skin fibroblasts cells (HDFn) were 3D bioprinted into a simple ring structure in a layer-by-layer fashion with a diameter of about 8mm and thickness of 1.2 mm. The 3D bioprinted constructs were cultured up to twenty-one days to test the biocompatibility of the peptide bioinks. As control, HDFn were 3D bioprinted in the same 3D circular shape using alginate-gelatin as bioink with a commercially available 3D bioprinter. Fluorescence confocal microscopy images showed the cytoskeletal staining of 3D bioprinted human skin fibroblasts (Fig. 11A). It can be seen that HDFn cells bioprinted with IVFK and IVZK attained excellent spreading after 21 days of culture as compared to alginate-gelatin as evident from the actin network (Fig. 11A). After 14 and 21 days of culture, the F-actin were well spread, stretched and distinct in IVZK while on IVFK they were dense but not well pronounced. After 14 and 21 days of culture, the F-actin expression was relatively low and less distinct in alginate-gelatin when compared to IVFK and IVZK.

Shape fidelity of the 3D bioprinted construct after cell culture is another critical parameter to access the quality of 3D bioprinted constructs. The ring structures printed with peptide bioinks maintained their shapes after 21 days of culture (Fig. 11B). Moreover, 3D cell viability assay (Fig. 11C) was performed to quantitatively evaluate the potential of bioprinted constructs to support the proliferation of HDFn cells in 3D. These results showed a time-dependent increase in cell proliferation in all the bioprinted constructs. The cell numbers were comparable between the IVZK, IVFK, and alginate-gelatin until seven days in culture (Fig. 11C). After 14 days and 21 days of culture, the cell proliferation in IVFK and IVZK were comparable but higher than that of alginate-gelatin. This shows that IVFK and IVZK provide native cues and offer more surface area to the cells to divide and grow due to their nanofibrous structure resemblance with the native ECM as compared to alginate-gelatin bioink. Also, these peptide hydrogels have sufficient porosity to accommodate more cells and maintain viability. On the contrary, alginate-gelatin does not possess nanofibrous architecture and also degrades faster *in vitro*.

To show the versatile nature of our 3D bioprinting method and its potential to be used for other types of cells as well, we 3D bioprinted human bone marrow-derived mesenchymal stem cells (hBMSCs) using IVZK bioink and compared this with the alginate-gelatin bioink. Fluorescence confocal microscopy and 3D viability assay showed that peptide bioink allow better cell proliferation as compared to alginate-gelatin bioink (Fig. 12).

Peptide Spray

Two air spray brush nozzles were assembled in a casing made from Perspex sheet at an angle such that the stream from both the nozzles meet at one point (Fig. 13A and B). One nozzle contained the peptide solution (5mg/ml IVZK) and the second nozzle contained the phosphate buffer solution (10X). The peptide hydrogel was formed when both the streams intercepted each other on a silicon wafer surface. The sprayed peptide scaffolds were dried and finally imaged using scanning electron microscopy to study the fibrous network of the peptide hydrogel (Fig. 13C).

The airbrushes can be used with a device such as a robotic arm as shown in Fig.4A, 4B and 4C for direct spray of a peptide hydrogel onto a surface such as a wound site. The air brush assembly can also be used as a hand held device to directly spray the peptide hydrogels onto a surface such as a wound site.

The foregoing descriptions are only implementation manners of the present invention, the scope of the present invention is not limited to this. Any variations or replacements can be easily made through person skilled in the art. Therefore, the protection scope of the present invention should be subject to the protection scope of the attached claims.

CLAIMS

1. Device (100, 200A, 200B, 344A, 344B) for printing a 3D object (352) from a representation stored in a memory, the device comprising:
   - a first inlet (112, 212) configured to take in a bioink solution, in particular a peptide solution,
   - a second inlet (114, 214) configured to take in a buffer solution capable of inducing gelation of the bioink solution, preferably instantaneous gelation of the bioink solution,
   - a third inlet (116, 216) configured to take in a dispersion,
   - a fluid duct (110A, 110B, 210) for mixing the bioink solution, the buffer solution and the dispersion to obtain a hydrogel, in particular a peptide hydrogel, and
   - a nozzle (122, 222) configured to eject a hydrogel, in particular a peptide hydrogel to build the 3D object (352).

2. The device of claim 1, wherein the fluid duct (110, 210) comprises a first region for mixing the bioink solution and the dispersion to obtain a bioink-dispersion mixture and a second region for mixing the bioink-dispersion mixture with the buffer solution.

3. The device of claim 1 or 2, wherein the dispersion comprises a cell culture medium, wherein preferably the cell culture medium comprises a plurality of cell types.

4. The device of any one of the previous claims, wherein the dispersion comprises micro and/or nanoparticles, preferably peptide microparticles, peptide nanoparticles, silver nanoparticles, gold nanoparticles, nanowires, quantum dots and/or carbon nanotubes.

5. The device of claim 4, wherein a plurality of cell types are encapsulated in the peptide microparticles and/or immobilized on the peptide microparticle surface.

6. The device of any one of the previous claims, further comprising a heating module (220) configured to heat the fluid duct (110A, 110B, 210),

and/or further comprising a micromixer (240), preferably in the fluid duct (110A, 110B, 210).

7. The device of any one of the previous claims, further comprising one or more light emitters (230) configured to irradiate the fluid duct (210).

8. The device of claim 7, wherein the light emitters (230) comprise a first LED with a first wavelength and a second LED with a second wavelength different from the first wavelength.

9. The device of any one of the previous claims, wherein the bioink solution comprises one or several peptides, said peptides having a general formula selected from:

a) Z_o-X_nBX_mW-Z'$_p$, and

b) Z_o-WX_mBX_n-Z'$_p$,

wherein Z is an N-terminal protecting group and Z' is a C-terminal protecting group, with o and p being independently selected from 0 and 1;

wherein **X** is, independently at each occurrence, an aliphatic amino acid selected from isoleucine, norleucine, leucine, valine, alanine, glycine, homoallylglycine and homopropargylglycine with n and m being integers being independently selected from 0, 1 and 2, with the proviso that $m+n \leq 2$,

wherein B is an aromatic amino acid selected from phenylalanine and tryptophan, or is an aliphatic counterpart of said aromatic amino acid, said aliphatic counterpart being selected from cyclohexylalanine, 4-hydroxy-cyclohexylalanine, 3,4-dihydroxycyclohexylalanine.

Wherein W is a polar amino acid selected from aspartic acid, glutamic acid, asparagine, glutamine, lysine, 5-N-ethyl-glutamine (theanine), citrulline, thio-citrulline, cysteine, homocysteine, methionine, ethionine, selenomethionine, telluromethionine, threonine, allothreonine, serine, homoserine, tyrosine, histidine, arginine, homoarginine, ornithine, lysine, N(6)-carboxymethyllysine, histidine, 2,4-diaminobutyric acid (Dab), 2,3-diaminopropionic acid (Dap), and N(6)-carboxymethyllysine,

wherein said polar amino acid is preferably selected from the group consisting of aspartic acid, asparagine, glutamic acid, glutamine, serine, threonine, methionine, arginine, histidine, lysine, ornithine (Orn), 2,4-diaminobutyric acid (Dab), and 2,3-diaminopropionic acid (Dap).

10. *In situ* method for printing a 3D object (352), the method comprising:
- mixing a bioink solution, in particular a peptide solution, a dispersion and a buffer solution capable of inducing instantaneous gelation of the bioink solution to obtain a hydrogel, in particular a peptide hydrogel, and
- ejecting the hydrogel, in particular the peptide hydrogel, out of a nozzle (122, 222) to build the 3D object (352).

11. The method of claim 9, wherein the mixing is performed in a microfluidic channel (110B, 210), wherein preferably the nozzle (122, 222) comprises the microfluidic channel (110B, 210).

12. The method of any one of claims 10 and 11, wherein air pressure and/or microfluidic pumps (340) are used to eject the peptide hydrogel.

13. The method of any one of claims 10 to 12, further comprising an initial step of scanning a 3D source object (310).

14. The method of any one of claims 10 to 13, wherein the cell culture media comprises a plurality of cell types.

15. The method of any one of claims 10 to 14, wherein the dispersion comprises micro and/or nanoparticles, preferably peptide microparticles, peptide nanoparticles, silver nanoparticles, gold nanoparticles, nanowires, quantum dots and/or carbon nanotubes.

16. The method of any one of claims 10 to 15, wherein the buffer solution capable of inducing instantaneous gelation is phosphate buffer saline or buffer(s) containing salt(s), such as sodium chloride or calcium chloride.

17. The method of any one of claims 10 to 16, wherein the method is carried out under physiological conditions.

18. The method of any one of claims 10 to 17, wherein the bioink solution is a peptide solution as defined in claim 9.

19. Method of obtaining a hydrogel, the method comprising:
- providing a bioink solution, in particular a peptide solution, through a first inlet (410) into a first airbrush (401),
- providing a buffer solution capable of inducing instantaneous gelation of the bioink solution to a hydrogel, in particular a peptide hydrogel, through a second inlet (420) into a second airbrush (402), and
- air-spraying the bioink solution and the buffer solution simultaneously through the nozzles (411, 421) of said first and said second airbrush (401, 402), onto the same site on a surface, thereby creating the hydrogel, in particular the peptide hydrogel.

20. The method of claim 19, wherein the bioink solution is a peptide solution as defined in claim 9.

21. The method of claim 19 or 20, wherein the buffer solution capable of inducing instantaneous gelation is phosphate buffer saline or buffer(s) containing salt(s), such as sodium chloride or calcium chloride.

22. The method of any one of claims 19 to 21, wherein the airbrushes are used with a device as defined in any one of claims 1 to 8.

23. The method of any one of claims 19 to 21, wherein the airbrushes are used as a hand held device.

24. The method of claim 22 or 23, wherein the the bioink solution and the buffer solution are directly sprayed onto a surface, such as a wound site where the hydrogel forms.