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Review

3D bioprinting of structural proteins

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ABSTRACT

3D bioprinting is a booming method to obtain scaffolds of different materials with predesigned and customized morphologies and geometries. In this review we focus on the experimental strategies and recent achievements in the bioprinting of major structural proteins (collagen, silk, fibrin), as a particularly interesting technology to reconstruct the biochemical and biophysical composition and hierarchical morphology of natural scaffolds. The flexibility in molecular design offered by structural proteins, combined with the flexibility in mixing, deposition, and mechanical processing inherent to bioprinting technologies, enables the fabrication of highly functional scaffolds and tissue mimics with a degree of complexity and organization which has only just started to be explored. Here we describe the printing parameters and physical (mechanical) properties of bioinks based on structural proteins, including the biological function of the printed scaffolds. We describe applied printing techniques and cross-linking methods, highlighting the modifications implemented to improve scaffold properties. The used cell types, cell viability, and possible construct applications are also reported. We envision that the application of printing technologies to structural proteins will enable unprecedented control over their supramolecular organization, conferring printed scaffolds biological properties and functions close to natural systems.

1. Introduction

The extracellular matrix (ECM) constitutes the material part of the natural cell microenvironment [1–4]. From a materials perspective, the ECM can be considered as a multicomponent hydrogel composed of structural proteins, functional proteins, glycoproteins and proteoglycans [1,3]. The main structural proteins in the matrix are collagens, elastin, and fibrin. These proteins form a variety of supramolecular structures and constitute the mechanical scaffold and natural support of embedded cells [1]. The main functional proteins are fibronectin, vitronectin and laminins [1,3]. They provide binding sites for cell membrane receptors and mechanically connect the living and non-living components of the cellular microenvironment. All abovementioned compounds are arranged in unique, tissue-specific 3D patterns [5]. The structural organization of the ECM, including fibrillar networks of different porosities and fiber diameters, is a consequence of the self-assembly and cross-linking of the constituent proteins [6] and has a crucial influence on tissue mechanics and function. For example, distribution of collagen fibers in a tendon or muscle (i.e. highly aligned) is different than the architecture of small intestinal submucosa (i.e. spiral arrangement, not aligned with long axis of the intestine), determining the mechanical behavior of the tissue [5]. The architecture of the tissue can impose cell fate (stemness *vs* differentiation) [7], cell behavior (e.g. migration *vs* adhesion) [5,6], cell shape, migration mode and directions, by providing spatial determinants, such as specific protein alignment, network density, and porosity [6,8]. Arrangements of those parameters influence dispositions of cell adhesion receptors and cytoskeletal organization, directly impacting cellular response [8]. The patterned deposition of bioactive ligands (particular ligand type, density, spacing) is as well of significant influence [9]. Resembling the properties of the ECM and obtaining credible ECM mimics remain key objectives in biomaterials design [4,10–12]. Yet, obtaining complete, synthetic ECM is not possible since the material compounds and their dynamic assembly and interactions to generate patterned and functional morphologies remain unknown to a major extent [5].

3D bioprinting has evolved as a promising method to produce scaffolds with appropriate spatial distribution of different

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components. Bioprinting is a layer-by-layer deposition of protein solutions, enabling fabrication of complex, multicomponent systems with precise control over material allocation, currently down to a micrometer range [10,13]. Fabrication of scaffolds with defined porosity and interconnectivity is straightforward. Multiple materials and different cell types can be deposited simultaneously or sequentially during the same printing process. This allows production of hierarchical patterned structures with defined spatial distribution of different materials, cell types, or bioactive domains to build up complex co-culture systems, tissue, or organ models. Bioprinting opens unprecedented opportunities to support functional vasculature development in the printed scaffold, as it can deliver different cells on preselected positions and particular environments. This is a particularly relevant and unsolved issue in tissue engineering, crucial for *in vivo* tissue regeneration [14]. Fully individualized scaffolds in complex shapes can be relatively easily obtained at reasonable cost, without forming predesigned molds.

The field of additive manufacturing in biomaterial science has experienced a boom in the last 3 years. More flexible, faster and higher resolution printing techniques have been developed. In contrast, ink development is still in its infancy [15]. Multiple general reviews in bioprinting have become available recently [13,16–38], though critical ink-specific reports comparing the material parameters and experimental approaches are rare. Herein, we review the state of the art in bioprinting focused on printing structural proteins typically applied as scaffold biomaterials (collagen, silk, fibrin, dECM and Matrigel). We describe the printing parameters and physical (mechanical) properties of the bioinks. We present applied printing techniques and specialized cross-linking methods for different bioinks, highlighting the modification implemented to improve scaffold properties. The used cell types, cell viability, the biological function and possible applications of the printed scaffolds are also reported.

1.1. A short description of bioprinting methods

3D bioprinting evolved from the 3D printing used in other fields of material science. Specifically for *bio*printing, deposited material, so called *bioink*, is constituted by biomaterials, biochemical molecules, living cells or any mixtures of them [13,39,40]. If the bioink is constituted only by cells in suspension, the pre-prepared hydrogel scaffold on which the bioink is printed is called *biopaper* [41–43]. The material development and fabrication based on bioprinting usually involve consecutive steps: ink design and preparation, computer aided design of the scaffold structure, script reading by the software, deposition of the material into final shape by the hardware, material testing, and culturing in the bioreactor [24,37] (Fig. 1).

There are three most commonly applied strategies of bioprinting: inkjet, robotic dispensing and laser-based printing. These techniques have been described in multiple recent reviews [13,16–30,40,44–46]. Inkjet printing (Fig. 2A), also called drop-on-demand printing, is a non-contact strategy based on the deposition of bioink drops in a predesigned manner to form a final multilayer pattern. Drops of a defined volume, in the picoliter range, are generated by pressure pulses induced by thermal or piezoelectric changes. Commercial thermal printers use a system in which the heating element in contact with ink is heated for few microsecond to ~300 °C in order to cause vapor bubble formation and ink droplet ejection [47]. To enable printing in the z-direction, the substrate is placed on a micro-positioning stage. Inkjet printing ensures fast scaffold production at low costs, though the printing resolution is relatively low. Materials with low viscosity and low cell concentrations are required in order to prevent clogging of the nozzle.

In robotic dispensing strategy two approaches can be

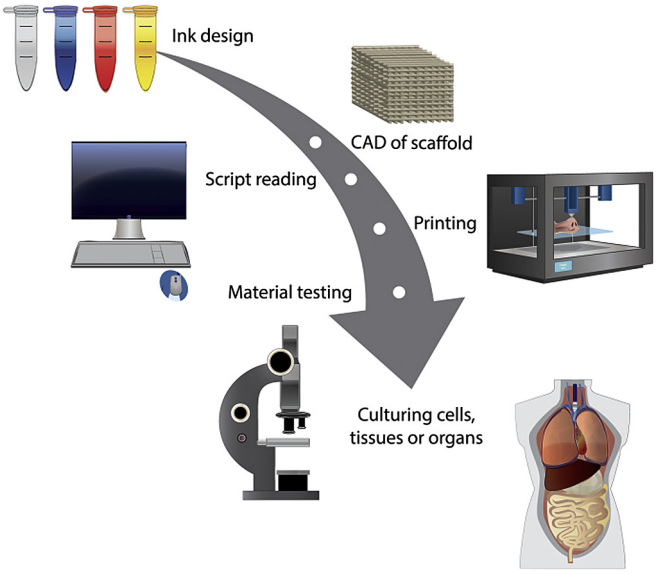

Fig. 1. Schematic of the consecutive steps involved in the process of (new) material printing.

distinguished: continuous extrusion (Fig. 2B) (1) and microvalve-based droplet ejection (Fig. 2C) (2). In extrusion bioprinting, also called direct writing, the ink is dispensed by a pneumatic (high pressure) or mechanical force (piston or screw), usually continuously as a strand. The dispenser is placed on the robotic stage, which ensures motion of the printing head in 3 directions. This printing method is suitable for deposition of higher viscosity inks, and higher cell densities. The risk of clogging is smaller than in inkjet printing. The limiting factor is typically the compromised cell viability under the shear stress-induced deformation during ink deposition. Induced shear rate is an important parameter since it determines shear stress applied to printed cells, influencing their viability. The droplet ejection strategy can be seen as a technique in between the inkjet and standard extrusion techniques. Although pressure is applied for the ink deposition and the robotic stage is often used, the ink is dispensed in the form of droplets. The merging of droplets allows for struts and scaffold's mesh formation. The droplets are formed by gating the ink flow through the nozzle, by means of the plunger, where opening time is controlled by a magnetic field generated in the valve coil (solenoid). The droplet size is controlled by changes in the deposition pressure and the opening/closing time of the valve and is typically in the order of nanoliters – one order of magnitude bigger than the droplets in the inkjet printing. Due to the small diameter of the microvalve, the viscosity of the printed solution can be lower than in extrusion.

Finally, laser-based printing (Fig. 2D) is based on the transfer of the bioink (cells or biomaterial) from a donor substrate to a receiving substrate, which is placed directly below. The transfer is controlled by laser beam pulses that target precisely defined positions on the energy absorbing layer (i.e. titanium or gold) deposited on top of the donor substrate. Based on the absorbed energy, defined in size droplets of bioink are formed. This method allows for printing high viscosity materials and high cell densities at very good resolution, however is limited by the high costs and lack of suitability to print large constructs.

1.2. Natural polymers in bioprinting

Water soluble polymers forming hydrogels are the major material group used as bioinks for bioprinting, due to their chemical