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Teaser Incorporation of nano- and micro-objects in composite inks and bioinks enables improved bioprinting for regenerative medicine applications.

Micro- and nano-formulations for bioprinting and additive manufacturing

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Recent developments in bioprinting have enabled an optimized formulation of bioinks by incorporating pharmaceuticals into cell-containing gel matrices. The proof-of-printability of a variety of forms has been provided, such as particles and fibers in the nanometric or micrometric range like dendrimers or micelles, although this is still lacking for some (liposomes for example). Resulting composite bioinks have the advantage of (i) improving cell growth and differentiation, (ii) delivering active molecules or (iii) improving mechanical properties of bioinks, printed scaffolds or the printing process. Improvement of these properties brings bioprinting one step forward toward clinical applications. Applications are reviewed for each field of improvements.

Introduction

Bioprinting has recently been redefined as the 'use of computer-aided transfer processes for patterning and assembly of living and non-living materials with a prescribed 2D or 3D organization to produce bioengineered structures serving in regenerative medicine, pharmacokinetics and basic cell biology studies' [1]. This strategy consists of printing living single cells or cell aggregates, biomaterials and bioactive molecules [2]. To do so, a printing material, an ink (generic term), is needed. The bioink is currently defined as a 'formulation of material(s) and biological molecules or cells processed using bioprinting technologies' [1]. Consequently, when the formulation does not contain the cells, the term ink is used strictly.

Additive manufacturing (i.e., 3D scaffold construction followed by cell seeding) is considered as bioprinting when inducing cell development into tissues [1,2]. Although being a particular case of 'bioprinting', according to the definitions here, additive manufacturing uses inks (and not bioinks) – cells being seeded afterwards. Alternatively to bioprinting, bioassembly (i.e., fabrication of hierarchical organized constructs from previously constructed building blocks) is the second major strategy for biofabrication of living and functional structures used in tissue engineering and regenerative medicine [1,2]. In line with the subject of this review, bioprinting, we focus here on bioprinted building blocks only. Recent developments have allowed regenera-

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tive medicine to address the highly challenging issue of designing living and complex functional tissues and organs replacing previous inert prostheses [3].

Bioprinting is currently considered as a highly promising technique in a variety of applications of regenerative medicine, including bone and cartilage regeneration [4–8], skin substitutes [9], cardiac valve [10] and cardiovascular system repair [11], craniofacial reconstruction [12], management of severe spinal conditions [13], musculoskeletal applications [14], hand surgery [15], reconstructive surgery [16], otorhinolaryngology [17], dental applications [18] and imaging [19], but also in the domain of biosensors [20]. Different technologies of bioprinting (i.e., extrusion, particle-fusion-based methods, light-assisted methods and inkjet) were recently reviewed and it was highlighted that the design of bioink needs to be adjusted to each technology chosen [21].

The matrices serving to formulate cell-containing bioinks include polymers, hydrogels and composite gels, combining several technologies including micro- and nano-forms. Among them, hydrogels are of particular interest, providing a microenvironment that mimics the extracellular matrix (ECM) with the versatility of its mechanical properties, degradation and functionalization which could be printed along with cells. The shear-thinning properties allow injection, which is of translational interest [21]. Molecular design, synthesis, physicochemical properties and fine-tuning of supramolecular assemblies in relation to stem-cell behavior have recently been described [22,23]. The characteristics of a suitable bioink for scaffold printing should involve: sufficient mechanical strength and material degradation rate; modulation of a 3D cellular microenvironment; supporting cellular adhesion, proliferation and tissue formation; and enabling adequate nutrient and waste exchange for cells contained in the bioink [24]. Matrigel®, a protein hydrogel commonly used in non-printed regenerative medicine, presents poor mechanical properties and, unless modified, has not been considered as a good bioink candidate for tissue constructs [25]. To meet the requirements of a suitable bioink, nanoengineering has recently been introduced in the bioink design. Using nanoforms to functionalize the surface, deliver growth factors or drugs or increase the viability and differentiation of cells have been described for many fields of regenerative medicine [20,26,27]. Chimene *et al*. [28] recently reviewed new perspectives and strategies for 3D printing ink development such as multimaterial inks (to overcome the limitations of single-component hydrogels), interpenetrating networks (hydrogels in which each polymer network has limited interactions with the other) and nanocomposite bioinks (i.e., bioinks containing nano-objects). The major part of research work reviewed by Chimene *et al*. was not printed, however their interest for the applications in regenerative medicine was demonstrated.

The functionality and effective differentiation of stem cells depend not only on chemical signs and growth factors but also on biophysical signals. For this reason, the nanotopography has recently gained interest in regenerative medicine [29]. Approaches like electrospinning to produce nanofibers, soft lithography for specific outcomes like nanodots, nanoridges and grooves and nanotechnology providing nanoparticles, nanotubes, nanostructured hydrogels and other nanoforms have been shown to be effective for tissue engineering applications. Several mechano-transduction pathways, coupled with several growth factors that mediated signaling pathways to regulate stem cell development, were proposed to explain the observed effects [29]. Additionally, pharmaceutical forms can be used in regenerative medicine for their common pharmaceutical purposes like delivery of active molecules, or for tissue engineering specific applications, related to tissue regeneration and functionality.

The progress in bioprinting and nanotechnology could render viable the approach of composite bioinks for the *in vivo* regeneration of tissues immediately after injury or during surgery [30]. These outlines raise the question regarding what the considered contributions and added values of composite bioinks containing pharmaceutical forms are that we address in this review. Here, we focus mainly on microforms and nanoforms included in inks or bioinks and printed. First, a general overview of major printed pharmaceutical forms for regenerative medicine applications is presented. Based on these data we propose to classify the applications according to the role that each pharmaceutical form has in a specific bioink for a specific application. Then, applications for each role are developed to highlight how the current challenges of bioprinting in regenerative medicine could be addressed by using pharmaceutical forms included in composite (bio)inks.

Pharmaceutical forms used in composite inks

Nanomaterials commonly used in regenerative medicine include nanoparticles (magnetic, gold, silver, ceramic, polymeric), liposomes, carbon nanotubes, micelles, dendrimers and quantum dots [30]. Microparticles and microfibers can also be used [31]. However, a persisting challenge is to include these pharmaceutical forms into composite inks and successfully print them. Significant changes in mechanical properties including stiffness, shear-thinning and different kinetics of degradation could be observed for nanoengineered hydrogels compared with single-component inks. An important parameter to check is therefore the impact of the nano- or micro-formulation inclusion on printability and on printing parameters of the composite ink [28] – but this is not systematically the case in the literature.

An overview of successfully printed pharmaceutical forms is given in Table 1. A distinction is made when additive manufacturing, as a particular case of bioprinting, was used. Among the most common printed forms, various nanoparticles and microparticles can be cited with a major application in bone or osteochondral tissue engineering. Additionally, fibers of various sizes (micrometer on nanometer range) but also micelles, dendrimers and nanotubes have performed their proof-of-printability. Brief definitions, details of applications and references of these forms are given in Table 1. However, in the literature related to the regenerative medicine and tissue engineering, liposomes [32–35], niosomes [36] and magnetoliposomes [37] have been shown to be of great potential. To the best of our knowledge, these forms have not been printed yet but would be of interest for bioprinting providing their proof-of-printability. A graphical illustration of printed and potentially printable forms, matrices and supports is presented in Fig. 1.

When comparing the contribution (i.e., the added value that inclusion of nanoforms and microforms brings to the ink used) some common features can be driven out, summarizing the main roles of composite inks compared with simple inks and the challenges that could be addressed using composite inks. Here, we

TABLE 1

Summary of major pharmaceuticals added to composite inks and bioinks for additive manufacturing and bioprinting applications

Definition of pharmaceutical forms and subtypes		Application scope	Details on the form	Contribution	Biofabrication technique	Refs
Additive manufacturing						
Nanoparticles Nanometric range objects made from various organic or inorganic, natural or synthetic materials; e.g., bioceramics, magnetic particles, nanotubes, polymeric nanoparticles	**Bioceramics** Biocompatible ceramic nanoparticles (commonly nanospheres) with inert or resorbable material, similar to biological hydroxyapatite, commonly used in bone regeneration	Bone tissue engineering	Biphasic calcium phosphate containing hydroxyapatite or tricalcium phosphate	Cell proliferation and viability	3D printing	[39]
			Polylactic-co-glycolic acid solution post treatment of the ceramic scaffold	Enhanced mechanical properties		
			TiO2 and bioactive glass nanoparticles	Induction of a lag time in cell adhesion, proliferation and differentiation	3D printing	[41]
			Mesoporous bioactive glass and mesoporous silica nanoparticles	Local drug delivery	Extrusion printing	[79]
			Nanoclays incorporated into N-acryloyl glycinamide hydrogel	Improved mechanical properties of printed scaffolds and further increased adhesion, proliferation and cell differentiation	Extrusion printing	[44]
			Mesoporous silica nanoparticles incorporated in a polyglycidol and hyaluronic acid hydrogel	Drug release	Extrusion printing	[81]
			Collagen-nanosilicate-based hydrogels	Improved hydrogel shear-thinning mechanical properties. Promoted osteoblast differentiation and induction of production of mineralized extracellular matrix	Fused deposition modelling	[45]
Nanofibers Nanometer range fibers made of natural or synthetic polymer chains, produced by various methods including electrospinning		Cartilage and bone tissue engineering	Polycaprolactone nanofibers	Improved cell viability	Electrospinning and inkjet	[8]
		Bone tissue engineering	Poly(lactic-co-glycolic acid) (PLGA) nanofibers	Improved cell adhesion and mechanical stability of the scaffold	Electrospinning and fused deposition modelling	[48]
		Musculoskeletal tissue engineering	Polystyrene submicron fibres coated with fibroblast growth factor	Growth factor local delivery	Inkjet printing	[31]
Bioprinting						

TABLE 1 (*Continued*)

Definition of pharmaceutical forms and subtypes		Application scope	Details on the form	Contribution	Biofabrication technique	Refs
Nanoparticles Nanometric range objects made from various organic or inorganic, natural or synthetic materials; e.g., bioceramics, magnetic particles, nanotubes, polymeric nanoparticles	**Bioceramics** Biocompatible ceramic nanoparticles (commonly nanospheres) with inert or resorbable material, similar to biological hydroxyapatite, commonly used in bone regeneration	Bone tissue engineering	Bioactive glass and hydroxyapatite nanoparticles	Increased strength, making extracellular matrix deposition easier and upregulating bone-related gene expression	Ink jet printing	[46]
			Hydroxyapatite nanoparticles and β-tricalcium phosphate microparticles	Induction of osteoclast activation	Extrusion printing	[40]
			Magnesium synthetic nanosilicate clays (Laponite) incorporated to an alginate and methylcellulose bioink	Drug delivery and improvement of mechanical properties (colloidal ligand)	Extrusion printing	[43]
			Core-shell PLGA nanospheres incorporating transforming growth factor beta 1 (TGF-β1)	Local drug delivery	Stereolithography and electrospraying	[72]
	Magnetic nanoparticles Particles made of material expressing magnetic properties (e.g., Fe2O3)	Shape construction in uterine and vascular tissue engineering	Magnetized cells with magnetic nanoparticle assembly (i.e., NanoShuttle)	Scaffold architecture and cell placement to form a required shape	Extrusion printing	[57,58]
	Nanotubes Mono- or multi-layer nanometric hollow tubes formed by carbon atom assembly	Cardiac surgery medical device engineering (patch)	Carboxyl functionalized carbon nanotubes (CNTs) incorporated in an alginate and methacrylated collagen hydrogel	Reinforce the 3D printed structure and improve it electrical conductivity	Extrusion printing	[51]
Microfibers Micrometer range fibers made of natural or synthetic polymer chains connected via covalent bonds produced by various methods including electrospinning		Bone tissue engineering	Bone morphogenetic protein 2 bound on collagen microfibers	Growth factor delivery	Extrusion printing	[75]
Microparticles Micrometric range object from various form and wide variety of materials (e.g., polymeric microcapsules, microcarriers, microspheres)	**Polymeric microcapsules** Microparticles with a thin polymeric shell commonly containing a liquid core	Proof of concept (no specific application defined)	Poly-L-lysine and chitosan microcapsules	Enhanced cell–cell contact, cellular proliferation and aggregation. 3D controlled constructs bead-by-bead	Laser-assisted bioprinting	[63]
	Polymeric microspheres Matrix microparticles composed by a polymeric network	Bone tissue engineering	Gelatine microspheres loaded with bone morphogenetic protein 2	Growth factor release	Extrusion printing	[69]
		Vessel engineering	Vascular endothelial growth factor loaded gelatine microspheres	Prolonged release of growth factor in vitro and in vivo	Extrusion printing	[70]
		Osteochondral tissue engineering	Mesenchymal-stromal-cell-laden polylactic acid microcarriers, encapsulated in a gelatin methacrylamide-gellan gum bioink	Improved compressive modulus of the bioink and hydrogel constructs, facilitated cell adhesion, support osteogenic differentiation and bone matrix deposition by MSC	Extrusion printing	[62]
		Scaffold engineering	DNA-coated carboxylate microspheres	Gel shape maintenance, improved scaffold sturdiness, viability and cell growth	Extrusion printing	[101]

classify these issues into three main fields of added value for the contribution of pharmaceutical forms to printed constructs: (i) cell functions and cell fate, (ii) delivery of bioactive molecules and (iii) improvement of mechanical ink properties, printing process or printed scaffolds. Inclusion of micro-objects or nano-objects into composite inks for the printing process allows one to address several challenges (Box 1). Each contribution is further illustrated by representative applications in the following sections.

BOX 1

Contribution of printed pharmaceutical forms in regenerative medicine

Cell functions and fate
- Cell viability enhancement, growth and differentiation promotion
- Cell encapsulation

Delivery of bioactive molecules
- Growth factors
- Drugs
- Genetic material

Improvement of mechanical properties of
- (Bio)inks for printing process optimization
- Scaffolds for optimal scaffold design

Improving cell functions and fate

The contribution of nanoforms to cell function and fate includes the cell survival and growth, but also their differentiation to fully assume all the functions necessary to replace the injured tissue. The aim of regenerative medicine is to be a functional and long-term viable replacement and not a mechanical prosthesis. In addition, encapsulation of cells allows them to get better contact between them on the one hand and to use cell-containing capsules as building blocks for tissue construction on the other hand. The first point joins the idea of cell viability, growth and differentiation enhancement, whereas the second point allows the structural precision of scaffolds.

Cell viability, growth and differentiation enhancement

To fulfil the renewal of injured tissues and provide a viable and functional tissue substitute, cells need not only to survive but also to differentiate properly. Micro- and nano-engineered composite inks and bioinks have been developed to enhance cell viability, differentiation and, hence, the functionality of the new tissue. Some illustrations of these applications are given in Fig. 2. Several studies concerned engineered scaffolds for bone regeneration and the input of nanoparticles and microparticles, in particular bioceramics, on cell growth and differentiation. Bioceramic scaffolds are commonly printed without cells, which are added by seeding afterwards using an additive manufacturing strategy. Hence, they are not considered as bioinks but still comply with the definition of bioprinting [1,2,38]. The chemical structure of bioceramic nanoparticles is naturally of importance, but nanotopography can also have a major impact on cell fate.

Porous bioceramic printed scaffolds have been shown to be biocompatible with good cell proliferation and cell ingrowth [4]. In particular, calcium phosphate bioceramics are widely used in bone tissue engineering owing to their excellent bioactivity, osteoconductivity and similarities in composition to bone [5]. Comparing different calcium phosphate derivatives, the bone remodeling potential of 3D printed biphasic calcium phosphate (BCP) microparticles showed an enhanced cytocompatibility, cell adherence, cell viability and cell proliferation seeded on these scaffolds. BCPs were found to be more effective in inducing elaborate bone formation at the ectopic location compared with calcium triphosphate or hydroxyapatite scaffolds [39,40].

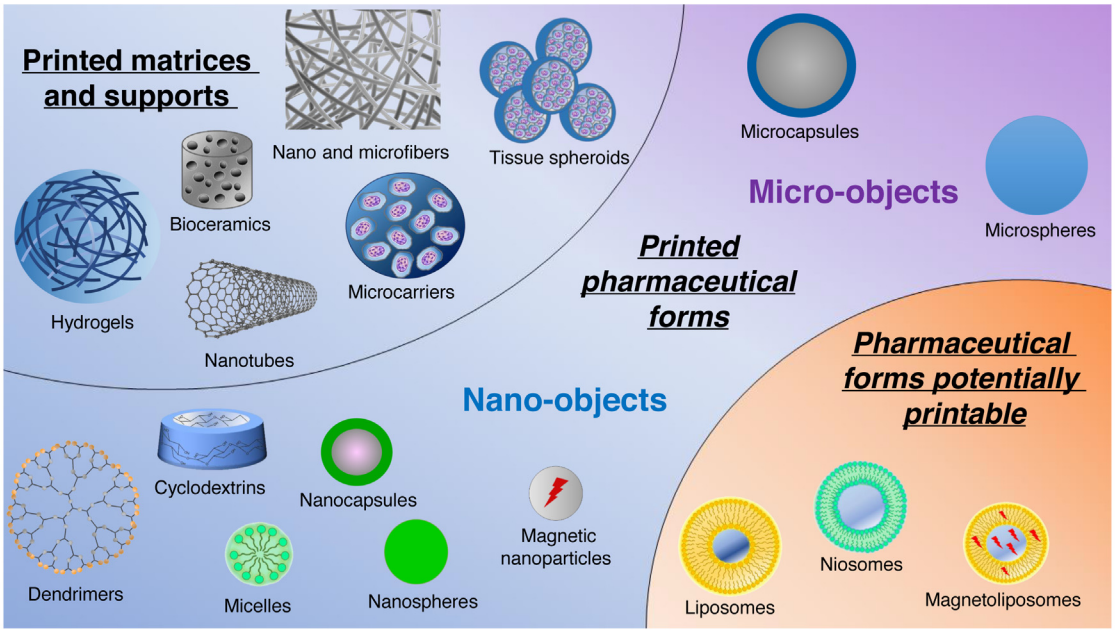

FIGURE 1

Printed and potentially printable pharmaceutical forms, matrices and supports in regenerative medicine.

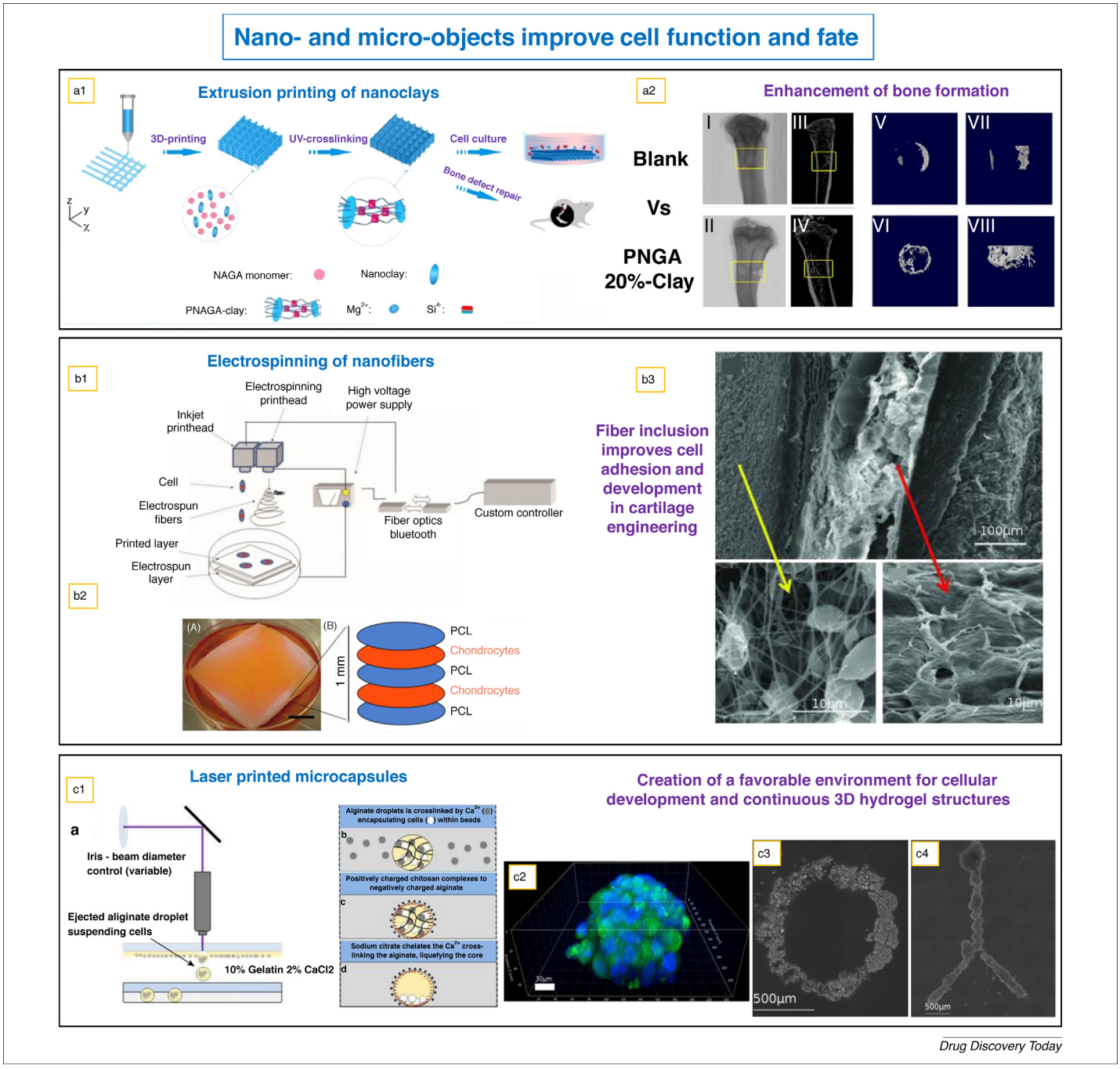

FIGURE 2

Contribution of nano-objects incorporated in inks and bioinks for cell function and fate improvement in printed constructs. **(a1)** Procedure of 3D-printing poly (*N*-acryloyl glycinamide (PNAGA-Clay) scaffold incorporating nanoclays. **(a2)** Nanoclays of enhanced new bone formation implanted in rat tibia (yellow area): **(a2 I–IV)** micro-computed tomography (sagittal cut), and **(a2 V–VIII)** reconstructed 3D images (V and VI: top view; VII and VIII: side view) [44]. **(b1)** Schematic representation of the hybrid printing system: the electrospinning printhead (generating a polymeric fiber-based scaffold) is coupled to an inkjet printhead. **(b2)** Successively, a layer of polycaprolactone (PCL) fibers is electrospun and followed by the printing of a chondrocytes plus fibrinogen and collagen hydrogel solution. **(b3)** Microstructural analysis of the printed hybrid constructs using scanning electron microscopy: PCL porous microstructures promote cellular infiltration and integration within the 3D-printed structure; above (cross-section of the 3D-printed constructs: PCL layer (yellow arrow, beneath left) is followed by a chondrocyte plus collagen layer (red arrow, beneath right) [8]. **(c1)** Schematic representation of the 3D-laser-printed microbead formation. Microbeads fabricated by a single laser pulse that volatilizes a sacrificial layer (thin film of 10% gelatin plus 2% alginate), ejecting a droplet of transfer material (cells suspended in 2% alginate) to a receiving substrate; formation of capsules by alginate crosslinking, addition of chitosan, sodium citrate core liquefaction. **(c2)** Representation of cell-loaded microcapsule after 7 days of development (confocal imaging), illustrating the formation of a 3D cellular aggregate within the chitosan shell. The structures formed [**(c3)** circular and **(c4)** bifurcated strand] present continuous cell growth and differentiation within the structure after 10 days in culture [63].

Beside chemical derivatives of bioceramics, the available surface and its nanotopography is another important issue for cell fate. The uniform cell growth in porous scaffolds was shown to be curvature-dependent for nonsoluble ceramic particles and material-dependent for soluble fillers like bioactive glass nanoparticles [41]. When the surface of the 3D-printed polycaprolactone scaffolds was decorated with TiO_2 and bioactive glass nanoparticles to mimic ECM nanoarchitecture, a lag time in cell differentiation was induced [41]. Scaffolds doped with SrO and MgO induced faster tissue regeneration, leading to a fully mineralized bone after 12 weeks, in contrast to non-doped scaffolds [42].

Combining nanoparticles with synthetic or natural hydrogels has been further considered. Alginate- and methylcellulose-bioink-containing synthetic layered silicates (Laponite®) allowed >70% cell viability 21 days after extrusion [43]. Recently, it was shown that incorporating nanoclays (i.e., nanoparticles of layered mineral silicates) into gels from acrylate derivatives (*N*-acryloyl glycinamide hydrogel) significantly improved cell adhesion, proliferation and differentiation of primary rat osteoblasts when used for bone regeneration (Fig. 2a) [44], induced production of mineralized ECM [45] and upregulated bone-related gene expression [46].

Scaffolds containing nanofibers and microfibers based on natural polymers (e.g., collagen, gelatin, chitosan, fibrin and hyaluronic acid) or synthetic polymers [e.g., poly(ε-caprolactone) (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(urethane) (PU)] as the raw material could be designed [47]. The nanofiber-like physiological structure of bone and cartilage tissue makes these nanostructures of particular interest in bone and cartilage tissue engineering and they have been shown to efficiently enhance mesenchymal stem cell and chondrocyte proliferation, differentiation and infiltration in nanofibrous scaffolds, as recently reviewed [26]. The interest of fiber inclusion into viable 3D-printed constructs was further shown for cartilage regeneration of rabbit elastic chondrocytes (Fig. 2b) in a fibrin–collagen hydrogel *in vitro* and *in vivo* [8]. When bioengineered PCL scaffolds were further covered with PLGA electrospun fibers, mesenchymal stem cells seeded using an additive manufacturing approach were able to differentiate into osteogenic and chondrogenic lineages and showed greater cell adhesion compared with noncoated scaffolds [48]. Thus, several micro- and nano-forms (e.g., particles, fibers, nanotubes) have shown their uses for cell growth and differentiation by offering an appropriate nanosurface.

Current biomedical applications of carbon nanotubes include their role as a support for cell adherence, differentiation and proliferation in bone, cartilage and neural tissue engineering. Enhanced neuronal impulse conduction, neural network formation and neurite outgrowth on carbon nanotube and graphene substrates and enhanced osteogenesis or chondrogenic differentiation have recently been shown [29,49,50]. In addition, titania nanotubes used as a surface modification strategy in dental nonprinted applications have gained in interest because they can promote bone cell functions, mainly in relation with their increased surface roughness or energy, incorporation of fluoride ions from the anodization electrolyte and ability to mechanically stimulate cells [18]. Further loading of nanotubes could add a local therapeutic action or enhanced modulation of various cellular functions and, hence, faster integration of dental implants in the bone and soft tissues. Combining titania nanotubes with 3D-printing technology could achieve the desired dual micro- and nano-structured surface of future implants for early osseointegration and soft tissue sealing [18]. Recently, carboxyl-functionalized carbon nanotubes were successfully printed within hybrid cardiac patches for myocardial tissue engineering [51]. Indeed, incorporated to an alginate framework and cell-laden methacrylate hydrogel, these nanotubes improved the bioink stiffness, reinforced 3D-printed constructs and, moreover, facilitated electrical conductivity of the implant.

Besides bioceramic nanoparticles, fibers or nanotubes, bone regeneration was also addressed using magnetic nanoparticles. Magnetic bioprinting, despite potential toxicity of magnetic particles for cell behavior, has been considered to enhance cell differentiation [52]. Fe_3O_4 particles and exposure to electromagnetic fields were used to induce human bone marrow stem cell differentiation [53]. Furthermore, superparamagnetic particles were shown to promote the proliferation of human mesenchymal stem cells by quenching H_2O_2 [54]. In addition, inclusion of iron oxide nanoparticles covalently conjugated to glial-derived neurotrophic factor (GDNF) enhanced the stability and bioactivity of the hydrogel, as it significantly increased neurite outgrowth from dorsal root ganglia [55]. However, in these examples of applications, nanoparticles were not bioprinted.

However, printed magnetic nanoparticles have also been used for spatial patterning of bioprinted scaffolds. Spatial patterning enables formation of cell assemblies for tissue development (i.e., blood vessels, skeletal muscle or bone tissue) by using magnetic properties of nanoparticles incorporated in the cells to induce a levitation under the action of a magnetic field and design desired patterns. It has been shown to play an important part in stem cell fate through influencing juxtacrine and paracrine cellular signaling, and directing early differentiation by modifying the cell microenvironment and cell communication [56]. For example, cells could be magnetized with nanoshuttle particles (nanoparticle assembly consisting of gold, iron oxide and poly-L-lysine) subjected to levitation, which induced ECM production of cell aggregates. A design of uterin and vascular smooth muscle segments, made of cells bioprinted in 3D hollow rings to study their contractility properties, was achieved using magnetic bioprinting [57,58]. Magnetic nanoparticles also permitted the ring-shaped homogeneous distribution of cells on a polysaccharide porous scaffold for biomimetic vascular graft design [59] and further allowed post-transplantation tracking of cells [60].

To limit the adverse effects and cytotoxicity related to magnetic nanoparticle degradation, magnetoliposomes (i.e., liposomes containing magnetic particles) have been employed [37]. Beside the conventional properties granted to liposomes (e.g., configuration facilitating incorporation in cellular membranes, the possibility to graft PEG to increase encapsulation efficiency, their biocompatibility and biodegradable properties) magnetoliposomes highlight some shield properties by limiting magnetic particle degradation by lysosomes (i.e., phospholipases) and reducing aggregation [37]. Not yet printed, magnetoliposomes could easily find their place among inks and bioinks for applications in regenerative medicine to limit adverse effects of magnetic particles.

An alternative way to differentiate promoting composite inks has been described using cyclodextrins. Singh *et al.* [61] worked on

hydrogel formed by α-cyclodextrin (α-CD) nanobeads threaded on PEG chains. They demonstrated that α-CD PEG hydrogel led to chondrogenic differentiation and increased cartilage matrix production of mesenchymal stroma cells (MSCs). According to chemical substitution of α-CD hydroxyl function, different cell types (i.e., adipogenic or osteogenic) were observed, thus demonstrating that the chemical environment modulation influenced the tissue development [61]. In view of all these results, most of them concern inclusion of nanoparticles, mainly bioceramics, into scaffolds for bone regeneration. Beyond this application, fibers and tubes offer an appropriate support for cell growth and differentiation. The applications of magnetic particles cited are rather specific and difficult to generalize for cell fate improvement.

Cell encapsulation as tissue engineering building blocks

An alternative strategy to bioprinting is bioassembly, which consists of using building blocks (microcarriers or tissue spheroids) to address the challenges in fabricating functional grafts of clinically relevant size [1]. Microcarriers (MCs) are 40–600 μm polymeric microspheres that permit adhesion and growth of cells by offering an appropriate surface for cell development and facilitating cell–cell contact for optimal differentiation. They could be used as such by a bioassembly strategy, or combined for bioprinting [62]. For instance, MCs included in gelatin methacrylamide-gellan gum bioinks and bioprinted enabled high MSC concentrations and high viability by facilitating cell adhesion and supporting osteogenic differentiation of cells [62]. Alternatively, bioprinted microbeads can be further coated to form microcapsules. Kingsley *et al.* [63] encapsulated cells containing beads using a laser-direct writing process, offering the interior shell surface of the capsule for optimal cell development (Fig. 2c). Encapsulating several cell types such as fibroblasts, endothelial and smooth muscle cells could enable the creation of blood vessel structures with functional lumens [63].

The exact mechanism-of-interaction of cells with carriers is still not completely elucidated; MCs induce cell shape change and consequent pathways via RhoA modulation for mesenchymal stem cells or E-cadherin modulation for pluripotent stem cells [64]. Furthermore, MCs can play an important part in stem cell differentiation while being loaded with bioactive molecules and releasable factors [i.e., collagen-coated MCs; polylactic acid MCs; fibronectin-coated PLGA MCs releasing transforming growth factor (TGF)-β3] [65].

To further scale-up the size of constructs, tissue spheroids have been considered and reviewed as an alternative to bioprinting of sheets or rods and layer-by-layer assembly for tissue reconstruction [66]. They could be produced by a diversity of methods including hanging-drop culture or microwell culture microfluidic techniques (e.g., rotary culture, spinner culture, nonadhesive surfaces) [66] or printing cell-laden droplets into a crosslinking solution [56]. The key idea is to use hydrogel microbeads as volume pixels, or 'voxels', for cell capsulation and further structuring them into mode complex constructs like microstrands and bifurcations [56]. These spheroidal microcapsules ensured cell–cell interaction, differentiation and 3D cell growth [56], such as that described for microcarriers.

Taken together, using building blocks has been shown to be efficient for cell fate improvement even if all mechanistic details are not yet completely elucidated. Microcarriers and tissue spheroids can be used alternatively to improve cell interaction and outcome. In the cited applications, these building blocks were bioprinted. Independently from the production method, spheroids can further be used as a bioink for tissue bioprinting, providing flowability, nozzle-compatible size and sufficient physical integrity of spheroids. The tissue fusion process would then be crucial for 3D tissue construction and viability [66].

Delivery of bioactive molecules

As commonly used in the pharmaceutical field, micro- and nanoforms could deliver active compounds, like drugs. Genes or nucleic acids could be another type of delivered active biomolecule. In the field of regenerative medicine, growth factors need to be delivered as well and the advantage of controlled or site-specific delivery is interesting for optimal tissue regeneration. Spatially and temporarily controlled delivery is of key importance for efficient cell differentiation and function. Some examples of composite inks delivering active molecules are illustrated in Fig. 3.

Growth factor delivery

Controlled and prolonged release of growth factors is one of the key challenges for optimal cell growth and differentiation. The delivery systems need to comply with general requirements like biocompatibility, predictable biodegradability, low immunogenicity and antigenicity, affinity with growth factor and tissue, maintenance or enhancement of growth factor activity, ease of manufacture, safety, stability, sterility, availability and cost-effectiveness, as well as approval by regulatory agencies [67]. Commonly, collagen, alginate, chitosan, hyaluronic acid, hydroxyapatite and some synthetic biodegradable polymers or composite materials for bone grafts can be used as delivery systems by direct inclusion of factors into matrices [67], but this approach does not provide the optimal control and prolonged efficient local concentrations of growth factors.

To further optimize controlled and prolonged release, pharmaceutical drug release systems have been tested for growth factor delivery. Growth-factor-loaded nanoparticles have been considered for stem cell engineering, delivering growth and differentiation factors for bone and cartilage regeneration, adhesion molecules, extracellular matrices, tight junction proteins and signaling molecules for vascular tissue engineering [26,68]. Alternatively, tissue growth agents were also incorporated in the polymeric nanofibers for wound dressing [26,68]. These applications have not been bioprinted but, from these developments, the application of microparticles and nanoparticles loaded with growth factors was extended to bioprinted matrices and applied to various tissues. For example, bone morphogenetic protein (BMP)-2 loaded in gelatin microparticles and formulated in alginate bioink containing goat multipotent stromal cells allowed continuous 3-week BMP-2 release, enabling significant osteogenic differentiation and bone formation in rats and mice [69]. Site-specific bioprinting of vascular endothelial growth factor (VEGF)-loaded microspheres allowed regional differences in vasculogenesis of scaffolds dependent on VEGF release *in vivo* [70].

Bioprinting was further used for multi-cell-type printed constructs to specifically control growth factor release, which was

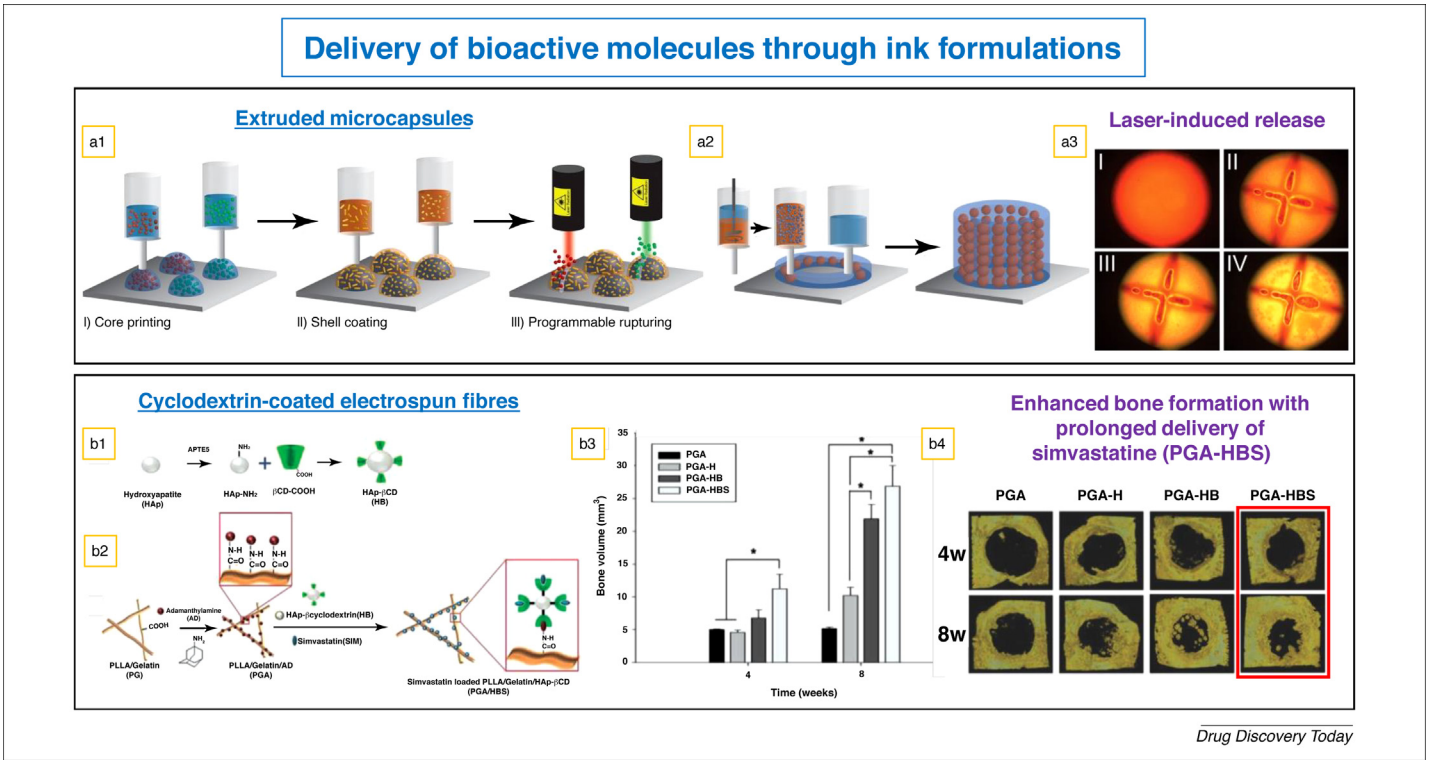

FIGURE 3

Delivery of bioactive molecules and drugs through formulations of composite inks and bioinks. **(a1)** Schematic representation of programmable printing and rupturing of capsules: (I) aqueous printing core containing payloads; (II) coating with PLGA solution containing AuNRs (nanorods); (III) capsule-selective rupturing adapted to the absorption wavelength of the nanorods. **(a2)** Schematic illustration of layer by layer 3D construct of complex capsule arrays, using emulsion ink in hydrogel. **(a3)** Rupture and kinetic release (from 15 min (II) to 2 h (IV)) of fluorescein dye from an emulsion capsule [82]. **(b1)** Synthesis of β-cyclodextrin-grafted hydroxyapatite. **(b2)** Preparation of simvastatin-coated poly(L-lactic acid) (PLLA) and gelatin fibrous scaffold. Adamantylamine plays the part of linker between PLLA plus gelatin fibers and simvastatin-loaded hydroxyapatite. **(b3)** Recovered rabbit calvarial bone area implanted with PGA, PGA-H, PGA-HB and PGA-HBS in the defect site. **(b4)** Microcomputerized tomography scan images of rabbit calvarial after scaffold 4 and 8 weeks of implantation [90]. Abbreviations: PGA, PLLA plus gelatin plus adamantylamine; PGA-H, hydroxyapatite-coated PGA; PGA-HB, β-cyclodextrin-coated PGA-H; PGA-HBS, simvastatin-coated PGA-HB.

adjusted locally to each cell type. A biomimetic 3D nanocomposite scaffold for osteochondral regeneration containing growth factors for chondrogenic and osteogenic differentiation (TGF-β1 and BMP-2, respectively) was designed. The bone layer and cartilage layer were functionalized with nanospheres for efficient growth factor encapsulation and sustained delivery. Significantly improved stem cell adhesion and differentiation in bone and cartilage layers was obtained simultaneously [71]. A similar approach was adopted using the sustained release properties of core–shell nanospheres to deliver TGF-β1 to human bone marrow MSCs within a bioprinted cartilage construct [72]. Nanospheres properly distributed in the cartilage construct permitted a TGF-β1-progressive delivery along the 21 days of the study, which improved chondrogenic differentiation of encapsulated MSCs.

Local delivery of biomolecules, including growth factors, has also been shown to be feasible on printed ceramic matrices [73,74] or using magnetic particles bound to growth factors that could be of interest for delivering growth factors at a precise time (controlled release) and at a specific place using external magnetic stimulation [52]. Beside particles, fibers have also been shown to be effective in controlled growth factor release. Bioprinted BMP-2-bound collagen microfibers enabled bone mesenchymal stem cell differentiation into osteocytes more efficiently compared with microfibers without growth factor [75]. Additionally, a spatially controlled specific cell differentiation has been achieved by functionalizing oriented submicron fibers with different printed growth factors [76], leading to myocyte, tenocyte and osteoblast differentiation where no growth factor, fibroblast growth factor (FGF)-2 or BMP-2 were locally printed, respectively [76].

Niosomes could also be used for efficient and sustained growth factor delivery but, to the best of our knowledge, have not been bioprinted yet. Indeed, in a recent study, Moghassemi *et al.* [36] reported the formulation and evaluation of basic fibroblast growth factor (bFGF)- and BSA-loaded nano-niosome-hydrogel allowing sustained growth factor release to modulate human umbilical vein endothelial cell (HUVEC) behavior. Although not printed in this application, this strategy could be used in printed and bioprinted scaffolds to locally release bioactive agents over time. In view of these results, several formulation approaches, including particles, fibers and vesicles, have been shown to be efficient for growth factor delivery. However, attention should be paid because some data tend to demonstrate that bioprinting could affect biological activity of loaded growth factors. For example, when printed in bioceramic matrices using ink jet technology, biological activity of recombinant BMP-2 was decreased by 10% compared with the non-printed control solution [77].

Drug and gene delivery

Using antibiotics and anti-inflammatory substances in regenerative medicine is a common clinical approach to reduce infections and inflammatory reactions. Including these drugs in scaffolds allows spatially and temporally controlled drug release by incorporating drugs into nanoparticles or fibers. Nanostructured materials including polymeric micelles, polymersomes, nanogels, nanoparticles, nanocapsules and dendrimers were reviewed [26] for their biomedical (non-printed) applications and, more specifically, for tissue engineering and regenerative medicine. In general, the solid, hollow or porous nanoparticles are suitable for bone and cartilage applications. Several synthetic and natural polymers, including PLA, PCL, PLGA, polyurethane, polyvinyl alcohol, dextran, chitin, chitosan, cellulose acetate, gelatin and collagen, were considered as candidates for dressing materials [26]. Recently, an additional step forward was done by incorporating some drug-loaded particles and fibers into composite bioinks and printing them along with biomaterials and cells.

Antibiotic-loaded ceramic particles were considered for the treatment of several infectious bone diseases including osteoarticular tuberculosis [78,79] and osteomyelitis [7]. Mesoporous bioactive glass was used as a drug delivery system and co-printed with isoniazid and rifampicin for bone tuberculosis treatment after surgery. Local drug release and good osteogenic potential in a rabbit bone defect model demonstrated the interest of this approach for treating osteoarticular tuberculosis [78]. The same approach was found successful using mesoporous silica nanoparticles as a composite scaffold loaded with isoniazid and rifampicin [79]. Analogously, calcium phosphate scaffolds were loaded by adding powder antibiotics to bioceramics before printing, or by infusing an antibiotic solution into the scaffolds via a second set of inkjets – vancomycin- and rifampicin-laden and co-printed for osteomyelitis treatment after surgery [7]. Further studies of vancomycin, ofloxacin and tetracycline sorption and release, loaded onto 3D-powder-printed microporous bioceramics (hydroxyapatite, brushite and monetite), showed delayed antibiotic-dependent drug release. Additional polymer impregnation of the drug-loaded matrix with polylactide-polyglycolide (50:50) polymer solutions further sustained the antibiotic drug release [80]. However, this approach was unsuccessful for poly(methyl methacrylate), which could not be co-printed with vancomycin or rifampicin because of interference with polymerization [7]. Moreover, as demonstrated for mesoporous silica nanoparticles incorporated into polyglycidol and hyaluronic acid 3D-printed hydrogel, surface particle charges should be taken into account for appropriate drug release within 3D-printed scaffolds and integration by cells [81].

Beside particles, fibers could be loaded with drugs for local and controlled delivery in printed scaffolds. A recent review [47] summarized fabrication methods of drug-loaded fiber-based composite scaffolds and their application in tissue regeneration. In a general manner, microfibers could achieve a more sustained drug release compared with nanofibers [47]. Stimuli-responsive microcapsules were embedded in a hydrogel matrix to release biomolecules from a PLGA shell when excited by a laser, as shown in Fig. 3a. Dyes, biomolecules or enzymes were printed on a hydrophobic solid substrate and recovered by a PLGA shell containing gold nanorods, printed over the hydrogel matrices. This functionalization of capsules allowed their selective rupturing when irradiated with a laser and, hence, temporal and spatial 2D and 3D control of release from grafted microcapsules [82]. However, analogously to growth factors, (bio)printing could affect the biological activity of loaded drugs. The biological activity reduction of heparin and vancomycin following spraying through the ink jet nozzles was between 1% and 18% [77]. For vancomycin, a further loss of 11% of biological activity was found following incorporation into a cement and subsequent *in vitro* release [77]. Printing parameters like printing temperature, pressure and time between two layers need to be adapted for composite inks and bioinks, and taken into account to avoid a negative effect on drug delivery after printing [47].

Several additional formulation strategies have been shown to be of interest in regenerative medicine, including liposomes, micelles, dendrimers and cyclodextrin solubilization but, to the best of our knowledge, have not been printed yet for drug delivery applications in regenerative medicine. Liposomes are commonly used as carriers in drug delivery, using their ability in protecting and delivering over time a wide range of molecules. Recently, they were formulated as carriers for the sustained and local delivery of drugs but also bioactive agents as growth factors or genetic materials (i.e., DNA, RNA or derivative RNA) within a scaffold used as a support for cell development [32–34]. They could be incorporated in a desired place within the scaffold, with a programmed kinetic profile (i.e., based on lipid composition, particle size, morphology, surface charge, a drug's loaded charge), possibly triggered with a stimuli-responsive ability (i.e., release with temperature, pH, UV influence) [34,35,83]. Additionally, several liposome-loaded biohydrogels (e.g., chitosan, gelatin, dextran) were developed [35] to protect drugs and minimize their burst-release in biohydrogels. Their biocompatibility and their physical and rheological properties underlined their therapeutic potential in regenerative medicine [83] but, surprisingly, to the best of our knowledge, liposomes have not yet been used in bioprinting.

Tissue engineering applications of dendrimers include drug delivery or controlled delivery of nutrients and growth factors as nanocarriers [84]. This specific application has not been printed, but the proof-of-printability of dendrimers has been done for other applications [85] – the reason why we considered dendrimers as a form having shown proof-of-printability in Fig. 1. In addition to non-printed applications of liposomes and dendrimers, solubilization approaches using micelles or cyclodextrins have been reported and could be of interest for future bioprinting developments. Rey-Rico *et al.* [86] recently published supramolecular polypseudorotaxane gels as scaffolds that can durably deliver recombinant adeno-associated viral vectors encapsulated in polymeric micelles in human mesenchymal stem cells for applications in cartilage regeneration. Poloxamer PF68 and poloxamine T908 polymers combined with alpha-cyclodextrin, hyaluronic acid or chondroitin sulfate were of particular interest to encapsulate viral vectors. Compared with free vectors, the gels provided higher cellular concentrations of vectors and sustained levels of transgene expression over time. The same approach had been shown to be efficient in human osteoarthritic chondrocytes using linear or X-shaped copolymers [87]. These formulations were not bioprinted in this specific application, but the proof-of-printability of micelles is being carried out for other applications [88], considering bioprinting genetic material containing micelles would be of interest for the next step of development in gene delivery.

Additional studies have been performed on cyclodextrin drug delivery applied to bioprinting and regenerative medicine. For example, γ-cyclodextrins were immobilized on a hydrogel to release osteogenic agents (e.g., dexamethasone) to human-adipose-derived stem cells (hASCs), allowing efficient bone regeneration [89]. Furthermore, Lee *et al.* [90] designed poly(L-lactic acid) (PLLA) and gelatin fibrous scaffolds (illustrated in Fig. 3b) on which hydroxyapatite particles were coated with simvastatin (osteogenic agent) loaded β-cyclodextrin and adamantylamine (linker to hydroxyapatite) loaded β-cyclodextrin. This construction prolonged simvastatin release by a factor of two-to-four, enhancing *in vivo* and *in vitro* bone regeneration [90]. This application was not bioprinted but the proof-of-printability of cyclodextrins was done for other applications [91]. In conclusion regarding the delivery of bioactive molecules, unsurprisingly common pharmaceutical forms like nanoparticles and microparticles are found. Applications using loaded fibers allow the combined delivery of growth factors or active substances with the appropriate nanotopography for optimal cell adhesion. Among unprinted forms, liposomes, commonly used in the field of pharmacy, appear promising, providing their proof-of-printability.

Improvement of mechanical properties of inks and scaffolds

Beside the cell fate and the delivery of biomolecules, the inclusion of forms into bioinks could be used to reach optimal properties of the bioink for the printing process (Fig. 4). This could be achieved by modulating the nanotopography, bioink stiffness and porosity, while maintaining shear-thinning properties. Optimal mechanical properties of the printed scaffold as a final object, such as the scaffold's hardness and porosity, its rate of degradation and its architecture, are also essential for efficient tissue regeneration.

Improvement of mechanical properties of inks

The appropriate modulation of mechanical properties of the designed bioink is one of the major challenges for successful bioprinting, because these properties impact the printing process and the fate of the printed scaffold. Several approaches have been tested to modulate mechanical properties of bioinks. Inclusion of particles to improve mechanical properties of hydrogels was the chosen strategy. Initially added to facilitate cell adhesion and differentiation, microcarriers significantly influence the bioink stiffness in a size-dependent manner [65,92]. For instance, polylactic acid microcarriers provided an increased compressive modulus of the gelatin methacrylamide-gellan gum bioink for bone and osteochondral constructs allowing a successful printing of bilayered osteochondral graft models of clinically relevant sizes [62]. The preparation of these microcarriers had previously been shown as biocompatible and the potential of drug release was demonstrated using fluorescent probes [93]. Furthermore, the excipients used for microcarriers should be nontoxic, and the rate of degradation should correspond to the desired properties of the optimal scaffold [94]. However, the technical challenge of the technique is the successful delivery of the microcarriers during the printing stage and assembly in a 3D structure, because the microcarriers could be at the origin of nozzle obstruction [94].

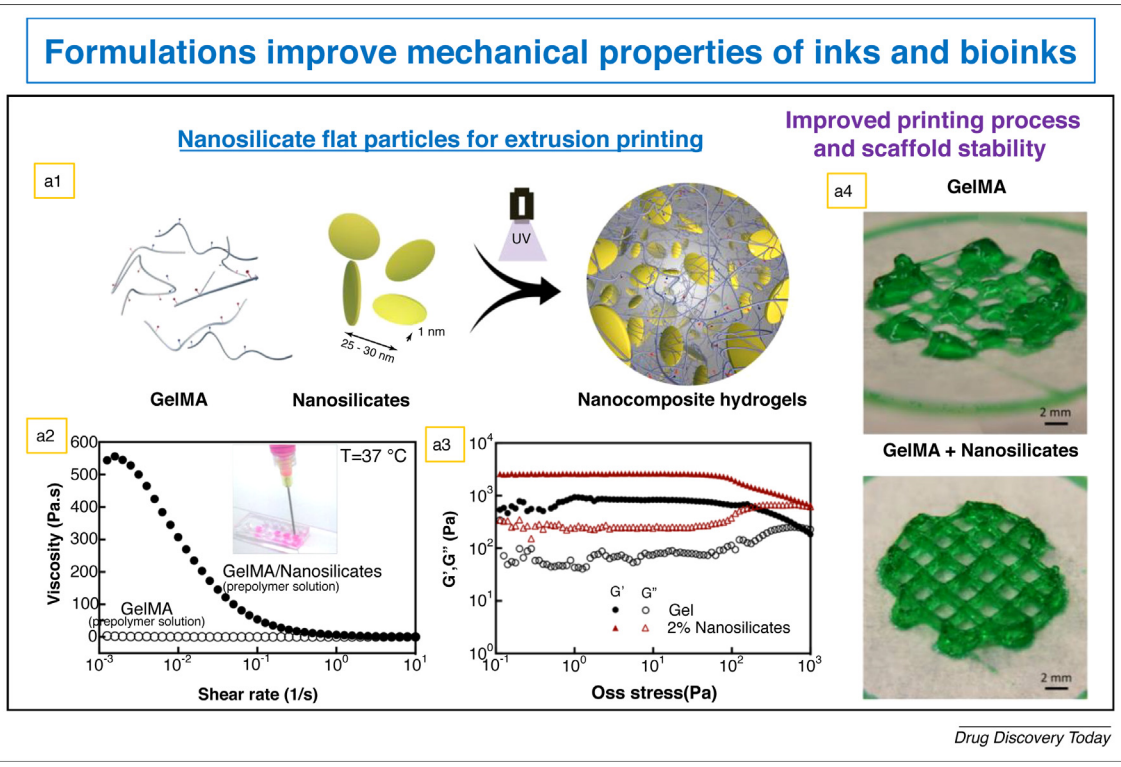

FIGURE 4

Contribution of formulations to improve mechanical properties of printing process. **(a1)** Schematic representation of fabrication of nanocomposite hydrogels from gelatin metacrylate (GelMA) and nanosilicates by covalent crosslinking under UV radiation. **(a2)** Viscosity shear rate and **(a3)** rheological analysis highlight shear-thinning characteristic of the prepolymer solution GelMA loaded with nanosilicates. **(a4)** Illustration of printed hydrogels showing the contribution of nanoparticles to increase printing precision and strengthen mechanical properties of the scaffold [45].

To avoid obstruction-related issues during printing, nanoparticles could be used. For instance, nanoclays incorporated into *N*-acryloyl glycinamide hydrogel significantly improved the mechanical properties of 3D-extrusion-printed scaffolds for bone regeneration [44]. Furthermore, the size was not the only important parameter while incorporating objects into hydrogels. According to the shape of incorporated objects, the hydrogel could benefit from shear-thinning properties, allowing liquefaction of a gel under physical constraint for an easy administration, and regelling *in situ* afterwards. For instance, shear-thinning nanocomposite hydrogels composed of synthetic silicate nanoplatelets (disks) and gelatin were developed as injectable hemostatic agents that promoted *in vitro* and *in vivo* coagulation [95]. Inclusion of coin-like 2D synthetic silicate clay nanoparticles (Fig. 4a) to gelatin methacrylate composite ink using an additive manufacturing approach resulted in strong, reversible, noncovalent interactions leading to shear-thinning mechanical properties [45]. Another possibility for tuning mechanical properties of bioinks is to use Pluronic® F127 diacrylate micelles, which have reportedly [88] provided a suitable rheological behavior and shear-thinning properties for 3D printing of living responsive materials and devices with programmed bacterial cells as responsive components. Additionally, fibers could be used for optimizing mechanical properties. PLGA fibers were integrated into the 3D-printed polycaprolactone matrices. The tensile moduli were comparable for nude scaffolds, scaffolds coated with nanofibers and cell-seeded scaffolds. Higher mechanical stability of scaffolds and greater cell adhesion were obtained by coating scaffolds by PLGA nanofibers [48]. The fiber size was shown to be of key importance not only for conferring a physiologically relevant environment for bone and cartilage regeneration but also for the properties of the printed scaffold. Although nanofibers could better reproduce ECM layout, larger pores obtained with microfibers provide better cell migration inside the scaffold and better mechanical support. Microfiber diameter influences porosity of the scaffold, the migration and development of cells [47]. Further reinforcement of fibers was studied recently by adding short fibers to a matrix of cellulose-modified gypsum powder, which allowed ten-times more work of fracture values compared with nonreinforced printed samples [96].

Alternatively to stiffening hydrogels with incorporation of individual objects to optimize bioink properties, glue-like molecules could be used to structure the scaffolds and optimize mechanical properties of printed constructs. For this purpose, dendrimers have recently been used in tissue engineering and regenerative medicine as an inter-particle or inter-cell glue for building larger constructs. For example, cyclodextrin-functionalized polystyrene nanoparticles were assembled in monolayers using guest-functionalized complementary adamantyl-terminated poly(propylene imine) dendrimers as a noncovalent supramolecular glue [97]. Furthermore, as an intercellular glue, 32-arm dendrimers were considered optimal for cell linking while preserving viability and cell functions and proliferation (however modification of cells using sodium periodate to introduce aldehyde groups onto the cell surface was necessary) [98]. Dendrimers have the potential to cross cell membranes and can deliver the entrapped bioactive inside the cell *in vivo*. Their functionalization greatly improved the toxicity profile of the parent dendrimer, reducing their cytotoxicity significantly after functionalization [99], which increases their potential for future applications in bioprinting. As cited above, dendrimer bioprinting has not yet been described for regenerative medicine but printed dendrimers were used as a model for the studies of biomineralization of cell walls by nanoprecipitation of silica nanoparticles [85].

An alternative approach [100] to improve printed scaffolds described the use of cyclodextrins as a crosslinker to mechanically reinforce networks and viscoelastic features of conventional hydrogels (e.g., hyaluronic acid and collagen). The supramolecular interactions thereby generated between cyclodextrin units, mostly engaged by photopolymerization, permit use of these syringeable hydrogels in bioprinting. A bioink for extrusion bioprinting was developed [91] where a hyaluronic acid hydrogel was functionalized with adamantanes and β-cyclodextrins, in a guest–host interaction that reinforced the original hydrogel network formation. Moreover, the cyclodextrin intermolecular connection was also reported in electrospinning to enhance the tensile strength of polymeric nanofibers or facilitate the electrospinning of nonprocessable polymers [100]. Allen *et al*. [101] used DNA-coated carboxylate microparticles that could assemble by DNA–DNA interactions to form the 3D shape of the scaffold. Microparticle-laden polystyrene gel was extruded with a 3D printer and the 3D assembly solely held together by DNA interactions. They also coextruded cells with microparticle-laden hydrogel and demonstrated that cells had better viability and growth when coextruded with hydrogel compared with polystyrene gel alone [101].

Printing process and improving mechanical properties of scaffolds

Scaffold hardness and porosity are of high importance for efficient tissue regeneration and, as described here above, can be successfully tuned by appropriate bioink formulation, taking into account bioink mechanical properties. In addition, the printing process can also significantly affect the properties of scaffolds. The kinetics of printed scaffold degradation, of key importance for efficient tissue regeneration, is dependent upon various factors, including process conditions [28,102]. Mechanical properties of scaffolds are particularly important for bone defect regeneration. They are mainly related to the porosity, which is physiologically high (>50%) in the inner part of the bone but low (<10%) at the external parts [5]. Scaffold porosity has been shown to be essential for bone reconstruction. Pores were dependent on 3D-printing technology and post-processing that can harden the scaffolds [5].

Common printing technology of bone scaffolds is available in printing ceramic particles but, alternatively, bioceramic bone scaffolds could be obtained by 'powder-based 3D printing' [39], technology renamed in recent consensus terminology only as '3D printing' [1]. The technique consists of printing a binder solution over a powder layer of calcium phosphate, alpha-tricalcium phosphate or hydroxyapatite, doped or not by adding SrO and MgO [4,5,42]. The quality of scaffolds obtained by 3D printing used commonly for bone regeneration depends on parameters such as powder packing density, powder flowability, powder layer thickness, binder drop volume, binder saturation and powder wettability. The obtained porosity and mechanical properties are of great importance for bone scaffolds [5]. The 3D printing is followed by de-powdering and post-processing treatments, commonly by dip-

ping them in a binder solution or by sintering, to enhance their mechanical properties [4].

To better understand required key powder parameters and printing accuracy which conditions the final outcome of printed scaffolds, a 3D printability study of calcium phosphate powders was performed. Results showed that particle size, powder flowability and compaction rate were of key importance for the final outcome of the printed object [6,103]. However, it has been shown that the particle size also impacts the kinetics of bone formation, not only mechanical properties. In a recent study, different particle sizes of calcium polyphosphate, crushed to obtain submicron and 1–3 μm particulates were considered as a filler in a rabbit model of bone defects. Higher new bone formation was obtained *in vivo* with low degradation kinetics (i.e., higher size particulates) [5,104].

Mechanical properties can also be tuned after printing, using a post-printing treatment of scaffolds to harden them. Post-treatment of the biphasic calcium phosphate and hydroxyapatite scaffolds with a polylactic-co-glycolic acid solution enhanced the mechanical properties by a factor of 8. Scaffolds showed bone remodeling potential for 3D-printed individual and complex bone substitutes [39]. To conclude, on improvement of mechanical properties of inks, each inclusion of micro- or nano-objects modifies mechanical and rheological properties of the matrices by stiffening the gel used. According to geometric properties of objects, additional shear-thinning properties can be achieved. Alternatively, glue-like approaches through dendrimers or cyclodextrins were shown to be efficient. The improvement of printed scaffolds mainly depends on the printing process or post-printing treatment. The geometry of printed scaffolds has also been shown to impact biological performances but the impact is not fully understood yet [105].

Remaining challenges and future outcomes

Most of the remaining challenges and future outcomes of composite-formulation-based bioprinted constructs are related to the field of bioprinting in general, micro- and nano-objects being already at the commercial level. Ongoing research for bioprinting of functional tissues and organs concerns several applications, including skeletal, muscular, integumentary, nervous, endocrine, reproductive, respiratory, digestive, urinary, lymphatic and circulatory systems [106,107]. Recent estimations indicate that we are about two decades from bioprinting a fully functional complex organ like the heart [108]. The analysis of manufacturing readiness level (i.e., manufacturing maturity, risk and gap identification for technology to manufacturing transition) shows that bioprinting is starting the translational phase [109]. Although the number of encouraging results of research applications is growing daily, printing of viable tissues and organs for clinical applications of regenerative medicine remains a challenge. Several issues still have to be addressed for a successful clinical translation of bioprinting technologies for the pre-bioprinting phase (e.g., biopsy, cell expansion), bioprinting (e.g., bioink issues, technology, resolution, biocompatibility) and post-bioprinting (e.g., conditioning, affordability) [110]. Regarding commonly limited availability of primary cells, the availability of sufficient quantities of biological material for bioprinting a clinically relevant size of tissues is a hurdle. Recent developments on upscaling stem cell culture [111] are addressing this barrier. The technology issues are also currently addressed to be able to print sterile hard and soft tissues containing matrix and viable cells of clinically relevant size [107]. In addition, the very first *in situ* bioprinting proof-of-concept results need further development [108]. The technology readiness level is considered as level 3 out of 10, meaning that experimental proof-of-concept is done, but full rate production is still far from being reached [109].

Tissue growth or tissue fusion post-printing is another challenge that can be addressed with computational studies [106], as well as 4D bioprinting development (i.e., considering the time as the fourth dimension for printed tissue evolution upon response to intrinsic or external stimuli) [112,113]. The fate of implanted printed objects needs to be taken into account for efficient long-term replacement of tissues by integrating unavoidable, mechanical and biological changes in the development. *In situ* innervation and vascularization is of key importance for tissue survival. Fine-tuning of supramolecular assemblies of low-molecular-weight gelators has been shown to control stem cell behavior [22]. Formulation of new inks or bioinks with tunable mechanical and rheological properties, as well as stimuli-responsive hydrogels and further mechanobiological studies on cell–ink interaction, can allow an optimal outcome of printed objects [106]. Including microparticles or nano-objects in inks or bioinks can address some challenges, as discussed in this review. These objects are already at a commercialized scale; however, the impact of the addition of such objects to inks needs to be assessed on the mechanical and biological levels for each specific application. Furthermore, addressing the ethical, social and regulatory issues concerning bioprinting still needs to be completed before implementing bioprinting in everyday clinical practice [106,114]. For efficient patient management, bioprinting needs to become commercially viable and safe [115]. Through 5-year anticipated advances, overall medical costs could be reduced using printing technology [108].

Concluding remarks

Recent advances in bioprinting and additive manufacturing have led to a significant improvement of regenerative medicine, allowing the development of viable and functional tissues that advantageously replace prostheses in numerous applications. However, this development has raised several challenges related to the mechanical and biological issues related to viable constructs. Some of these challenges can be efficiently addressed by incorporating pharmaceutical forms into bioinks for bioprinting. In this review, we have classified the challenges into cell fate (viability, growth and differentiation) related issues, biomolecule delivery issues and mechanical improvement of either inks or printed constructs. The added values of pharmaceutical forms included in nanoengineered inks and bioinks are reviewed. The results show that composite inks and bioinks bring the regenerative medicine one step forward from the bench to the bedside, providing that inherent rheological modifications of the ink or the consequences of printing process on the forms are considered. Inclusion of pharmaceutical forms like nanoparticles, microparticles and fibers allowed improvement of cell growth and differentiation, encapsulating biomolecules or improving the printing process and scaffold outcome.

Printing stimuli-responsive materials, currently studied for magnetic particles, is one possible strategy, but a variety of other stimuli (temperature, pH, light, etc.) could also be explored. Bioprinting could benefit from recent developments of smart delivery platforms [116]. Future perspectives will possibly further combine the main therapeutic advances and bioprinting technology. For instance, recent findings on cell-mediated delivery of nanoparticles showed that, by chemical modification, nano- and micro-particles could be bound to the cell surface in a noncovalent (e.g., chitosan- or hyaluronic-acid-mediated adhesion, antibody-targeted adhesion, biotin-avidin adhesion) and covalent (e.g., coupling to cell surface amine groups, maleimide-thiol coupling) way [117]. Future developments of bioprinting could print cells along with encapsulated particles containing everything cells need for proper differentiation and optimal tissue replacement.

Currently, little is known about how to clean cell-elimination products. Accumulation of metabolites and cell waste could be harmful in immature constructs, where vascularization is not yet optimal, and could limit the maximal size of printed constructs where elimination is diffusion based. An *in vitro* detoxification system using polydiacetylene nanoparticles in a liver-inspired hydrogel structure was efficiently used to detoxify toxins [118]. Analogous biocompatible applications could be considered for printed tissue constructs in regenerative medicine to assure optimal conditions for the lag time of tissue development and maturation. Combining bioprinting with recent advances in nanotechnology, printing technology and also precise and fine understanding of physiological processes of tissue development and differentiation, allows regenerative medicine to get closer to the clinics and to personalized medicine.

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