REVIEW OF CURRENT RESEARCH ON CHITOSAN AS A RAW MATERIAL IN THREE-DIMENSIONAL PRINTING TECHNOLOGY IN BIOMEDICAL APPLICATIONS

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Abstract

Three-dimensional (3D) biomaterial manufacturing strategies show an extraordinary driving force for the development of innovative solutions in the biomedical sector, including drug delivery systems, disease modelling and tissue and organ engineering. Due to its remarkable and promising biological and structural properties, chitosan has been widely studied for decades in several potential applications in the biomedical field. However, tools in the form of 3D printers have created new possibilities for the production of chitosan models, implants and scaffolds for cell cultures that are much more precise than existing ones. The article presents current achievements related to the possibility of using chitosan to create new materials for 3D printing in the form of chitosan bioinks, filaments, resins and powders dedicated for bioprinting, fused deposition modelling, stereolithography/digital light processing and selective laser sintering methods, respectively.

1. Introduction

Three-dimensional printing (3DP) is one of the ways of producing objects through controlled deposition of material layers until its final structure is obtained [1]. Due to subsequent addition of layers of the material during printing, 3DP is also called additive manufacturing (AM) [2]. This is the basic difference that distinguishes 3DP from available manufacturing technologies that involve subtractive processing of a larger amount of material in order to shape it properly, like drilling, milling, broaching, etc. [3]. Moreover, AM is efficient: it produces less waste and a large amount of energy. Hence, it uses less energy-demanding machinery [4]. 3DP technology and its unused potential is a gateway to the new era of printing self-organizing objects after leaving the printer as a result of exposure to the fourth dimension – time. The stimuli acting in an additional dimension may be a change in the water content, pH or pressure in the vicinity of the object, energy supply, e.g. in the form of heat, or chemical reactions occurring in the environment in which the printed object is located [5]. While the manufacturing and printing advancements are state-of-the-art technologies with a great scope in various fields such as automobiles, medical implants, electronics, aerospace and robotics, the biomedical sector is the one in which the use of AM technology is extremely hopeful [6, 7]. Many researchers are involved in using 3D printers for various applications such as drug delivery, disease modelling and tissue and organ engineering [8, 9]. Until recently, 3D printers were mostly used for prototyping devices for design and manufacture. More recently, 3D bioprinting has been expanded for applications such as cell and tissue printing with great hope for a quick transition from research and testing models to surgical planning, device manufacturing and tissue or organ replacement [10].

In line with the current focus on the sustainable economy, the exploration of natural-derived and renewable biopolymers, instead of fossil-fuel-based plastics, for various products' fabrication has received tremendous attention. Biomass from marine, woody and agricultural residuals, the most abundant renewable feedstocks on earth, has shown a promising potential as alternatives to fossil resources [11–13]. Development of biomass-based materials instead of fossil oil-based plastics for different 3DP technologies provides an opportunity to realize a truly sustainable and recycling economy [14]. In general, the material property requirements for 3DP applications in medicine and pharmacy include, but are not limited to, printability, biocompatibility, degradability (safe degradation by-products and good degradation kinetics), tissue biomimicry and appropriate mechanical properties [15, 16].

From a technical point of view, materials should have adequate processability due to the utilized 3DP technique, e.g. the ability for thermoplastic processing in the fused deposition modelling technique (FDM) or shear thinning behaviour and zero shear viscosity, enabling accurate and easy 3DP of high-resolution structures, high shape accuracy and structural stability in the stereolithography technique (SLA) [17, 18]. From the point of view of the biomedical application, biocompatibility should be assessed in terms of cell and tissue compatibility of the ink components, the printed structure, any washable products or degradation by-products from the printed structure or material [19]. Normal biocompatibility tests include *in vitro* tests for DNA damage, cytotoxicity, cell proliferation and quantification of specific proteins, as well as necessary *in vivo* tests according to the categorization of the medical device according to tissue contact and duration of contact [20]. For scaffolds that are not intended for permanent implants, printed structures should break down into biocompatible by-products in a controlled manner, enabling cells to produce their own extracellular matrix or allowing embedded components to achieve desired release profiles [21]. Tissue biomimetics place demands on printed constructs to imitate the natural shape of organs and tissues and represent their complex, heterogeneous nature, including the desired biological functionality, sufficient strength and rigidity to maintain structural integrity and macro- and micro-porous

architecture. The attempt to present these characteristics is becoming a key challenge for biopolymers for 3DP for biomedical applications, especially in tissue engineering [22]. More specific requirements for 3DP materials or printed structures should be considered for the specific end use and its printing approach. Today's most promising 3DP techniques in terms of creating three-dimensional objects for biomedical issues and involving the use of natural polymers are described below. The principle of operation of these techniques is shown in Fig. 1.
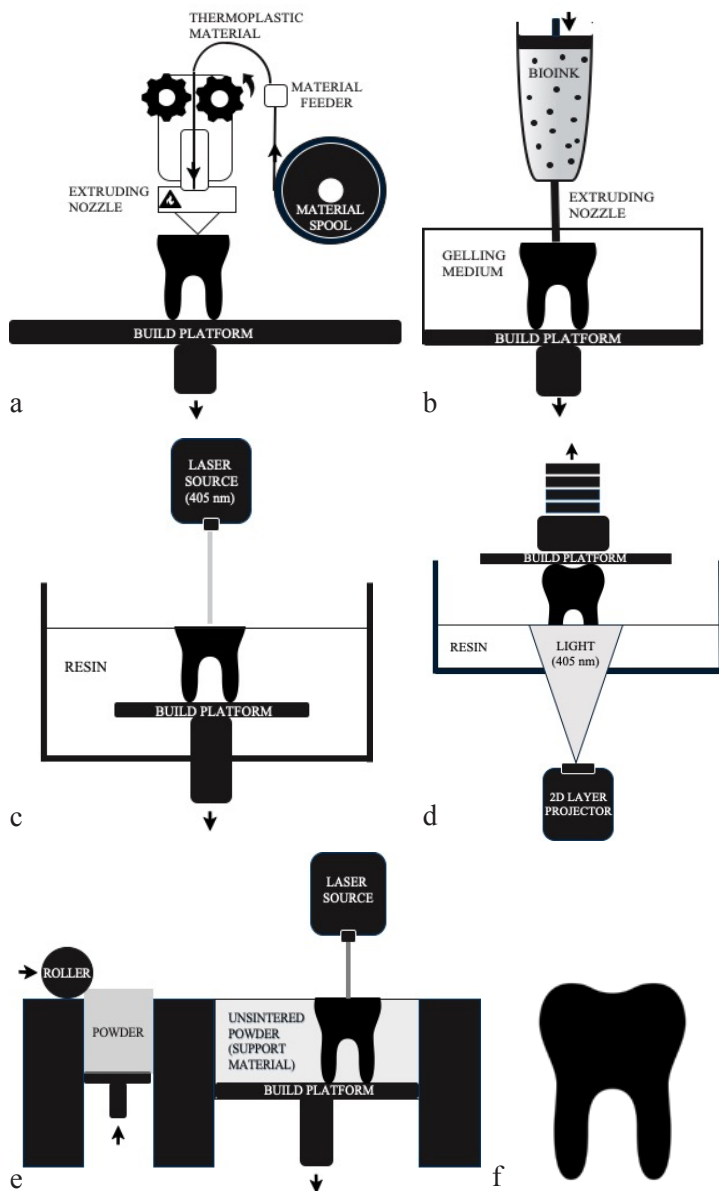
Figure 1. The principle of operation of the major commercial three-dimensional printing methods in which the usage of natural polymers and their derivatives is currently being investigated: (a) fused deposition modelling, (b) bioprinting, (c) stereolithography, (d) digital light processing and (e) selective laser sintering; (f) the printed model.

- ***Fused deposition modelling (FDM)*** is currently the most common method in which thermoplastics are used in printing. Objects are created by applying successive layers of semi-fluid material extruded from a heated nozzle. This material has the form of a line with a constant diameter of 1.75 or 2.85 mm wound on a spool. FDM uses various types of thermoplastic materials that differ in strength and melting point: Acrylonitrile-Butadiene-Styrene copolymer (ABS), polycarbonates, polyphenyl sulphides and waxes (Fig. 1A) [23].

- ***Bioprinting (BP)*** is the utilization of 3DP-like techniques to combine cells, growth factors, and biomaterials to fabricate biomedical parts that maximally imitate natural tissue characteristics. BP generally utilizes the layer-by-layer method to deposit material known as bioink to create tissue-like structures that are later used in medical and tissue engineering fields (Fig. 1B) [24].

- ***SLA and digital light processing (DLP)*** are photopolymerization techniques, which in general refers to the curing of photo-reactive polymers by using a laser, visible or ultraviolet (UV) light [25]. The example of 3DP technologies that employ photopolymerization are SLA (Fig. 1C) and DLP (Fig. 1D). DLP is similar to SLA in that they both work with photopolymers, But the light source is different. DLP uses a more conventional light source, such as an arc lamp with a liquid crystal display panel. It can act on the whole surface of the work area (build platform) while a single layer photopolymerization of resin. DLP is usually faster than SLA, in which reaction takes place only at the one point of irradiation of the laser beam [26].

- ***Selective laser sintering (SLS)*** uses either an electron beam or laser to melt or fuse the material powder together. The example of the materials used in this process are metals, ceramics, polymers or their composites or hybrids (Fig. 1E) [27].

Liu and co-workers [28] listed the most important biopolymers that have so far been successfully adopted in the above-mentioned 3DP methods (Fig. 1). These include cellulose, sodium alginate, starch, poly(lactic acid) (PLA), agar and their modified derivatives and composites. Chitosan is another natural polymer in which one can see the application potential. Chitosan is the N-deacetylated derivative of chitin, a linear and semi-crystalline polysaccharide composed of glucosamine and N-acetyl glucosamine units linked by β-(1→4) glycosidic bonds [29]. Chitosan is a natural, biodegradable, nontoxic and biocompatible hydrophilic polymer with antimicrobial properties, which due to its excellent properties can prove itself in 3DP technology dedicated for biomedical applications. The commercial exploitation of chitosan faces significant barriers because there are difficulties in preparing homogeneously reproducible chitosan in large quantities from various marine organisms around the world. Derivatization of chitosan further increases the overall price and possible differences in character uniformity. These limitations can be overcome thanks to research and technological progress that will give impetus to the growing applications and demand of chitosan and its derivatives. Although a number of examples of chitosan derivatives have been used in biomedical areas, only a few, including carboxymethylated chitosan, trimethylated chitosan and polyethylene glycol (PEG) ylated chitosan, have achieved a well-established and potentially characterized application profile [30]. Therefore, much research still needs to be done to fully exploit the benefits of chitosan and its derivatives in biomedical applications via 3D printers. This review presents the latest achievements related to the use of chitosan and their derivatives in the most commonly used 3DP techniques.

2. Chitosan Filaments for FDM

The main raw materials used in the production of filaments dedicated for FDM printing are biodegradable aliphatic polyesters, such as polyhydroxybutyrate and its copolymer polyhydroxybutyrate-co-valerate, PLA, poly(glycolic acid), poly-caprolactone and their copolymers. The ester bonds in these synthetic polymers hydrolyse to nontoxic natural metabolites [31]. Chitosan has proven to be the right raw material for creating bioconstructions due to their properties, so the idea of adding it to thermoplastics dedicated to FDM printers is fully justified. However, the major issue was combining two raw materials with such extremely different properties while maintaining thermoplasticity.

The easiest way seemed to be physically mixing polymers and subjecting them to co-extrusion. We conducted such preliminary tests by extrusion PLA pellets with chitosan powder to assess the possibility of obtaining filaments with antimicrobial activity. The results have shown that chitosan increased porosity and decreased the density of the obtained PLA/chitosan filaments. PLA and chitosan formed only a physical mixture after extrusion. Chitosan caused deterioration of the mechanical properties of filaments, especially elongation at break and Young's modulus, but improved their ability to crystallize. It provided at most the antibacterial properties against *Escherichia coli* and *Staphylococcus aureus* at a 3% mass addition in the filament. The 10% share of chitosan in the filament completely reduced its printability [32]. Therefore, to create homogeneous and thermoprocessable (printable) filaments with chitosan, it is crucial to ensure adequate raw grinding and the use of auxiliary compounds capable of reacting with functional groups of both extruded polymers.

Wu et al. [33] used PLA and chitosan to create a PLA/chitosan composite. Chitosan was first made into powder by grinding; it was then mixed into PLA and finally grafted by maleic anhydride (MA) into PLA-g-MA/chitosan to increase interfacial adhesion of the blend and enhance the mechanical properties of the PLA/CS composite in reference to the neat PLA (Fig. 2). This process produced morphologically consistent composites with a chemical structure different from the structure of raw materials and
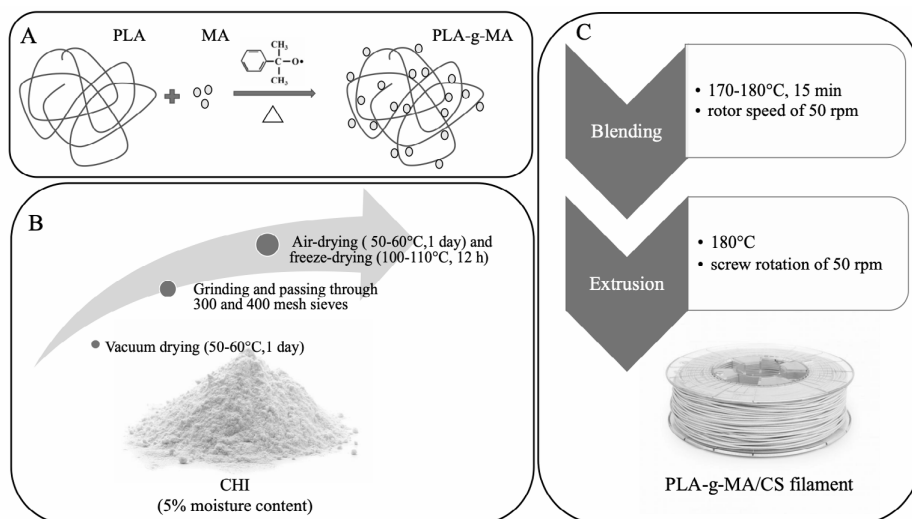
Figure 2. Fabrication of (C) three-dimensional printable filament strips from (A) maleic anhydride (MA)-grafted polylactide (PLA-g-MA) and chitosan (CS) composite [33].

increased tensile strength and water resistance. The 10% share of chitosan in PLA-g-MA/chitosan membranes provided them antibacterial activity.

Another way to increase the compatibility of chitosan with aliphatic polyesters is to convert it into a so-called thermoplastic form; this endeavour involves combining chitosan powder with the addition of acid and polyol such as sorbitol, glycerol, etc. [34]. Grande et al. [35] described a method for effectively producing thermoplastic poly(vinyl alcohol)/chitosan blends that can be melted and dispersed by extrusion without the negative effects of acetic acid residues into a polyester matrix such as PLA. PLA/ chitosan blends were obtained by solution processing, which involved conventional oven drying and using new approaches such as freeze- and spray drying. However, browning of chitosan-containing materials has not been completely eliminated [35].

Georgios et al. [36] used the findings from the above-mentioned studies for the production of film based on PVA (film-forming activity), xylitol (plasticizer) and chitosan (improvement of mucoadhesiveness) for buccal delivery of a model hydrophilic drug (diclofenac sodium) [36]. The manufactured products exhibited acceptable structural features and dose uniformity. The solid-state characterization indicated effective plasticization of the polymer, complete blending of the integrated components and amorphization of the drug. The presence of chitosan affected the *ex vivo* performance of formulated films, demonstrating enhanced mucoadhesion and permeation properties.

Research from the last few years has focused mainly on exploring aliphatic polyester/ hydroxyapatite/chitosan systems, in which the content of chitosan in printable filaments does not exceed 5%, with 10% hydroxyapatite [37–39]. Filaments containing up to 20% chitosan were developed by Thuaksuban and colleagues [40]. Their main ingredient is polycaprolactone (PCL). Chitosan and PCL are milled separately using a freezer-mill machine, then sieved through a 75 µm sieve, mixed together in an 80:20 ratio of PCL:chitosan by weight and melted in an extruding machine to obtain filament, from which scaffolds were printed using the melt stretching and multilayer deposition technique. In this study, the ability of obtained scaffolds for bone regeneration was evaluated within a rabbit's calvarial defects. The results confirmed that a chitosan scaffold can provide small amounts of new bone regeneration that is insufficient for reconstructing larger bone defects in terms of inducing some specific inflammatory cells. However, the concept of melt stretching and multilayer deposition (MSMD) scaffold is still valuable and proves that the barrier of combining hydrophilic chitosan with thermoplastic materials has been overcome.

3. Chitosan Inks for BP

Bioink can be defined as a mixture of cells, biomaterials and bioactive molecules, which are applied layer by layer using additive manufacturing to create biocompatible 3D objects. Therefore, the dispersion medium in which cells, growth factors or drugs are suspended are most often natural polymer solutions, including chitosan solutions. This polymer possess the ability to form a gel itself by neutralizing the amino groups. It can also form an ionic crosslinked hydrogel in the presence of anionic components under relatively mild gelation conditions. Covalently crosslinked chitosan hydrogels can be prepared by treating chitosan with various chemical reagents, such as glyoxal, glutaraldehyde and genipin [41]. Chitosan is widely used in wound care, cartilage repair and drug delivery. When present in dressings, it facilitates the haemostatic process by interacting with red blood cells with a negatively charged membrane due to its cationic nature [42]. Chitosan-based hydrogels are often used in bio-applications, but there has been little research on printing chitosan using BP methods. Gelation and fabrication time are the two essential parameters leading to suitable hydrogel cell retaining ability, and they rely on hydrogel rheological behaviour. Pisani and co-authors proposed bioink meeting most of the requirements for biomedical applications using chitosan/poly
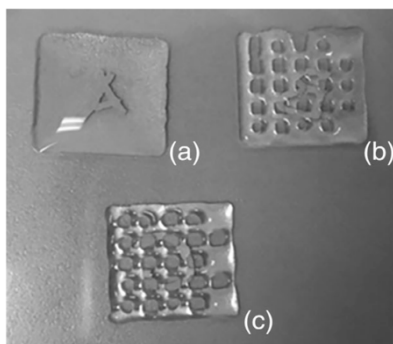
Figure 3. Pictures of three-dimensional-bioprinted layers using chitosan (CS) bioink:
(a) 6% CS 2% poly(gamma-glutamic acid) (γ-PGA) solutions, 90% infill; (b) 4.5%
CS/2% γ-PGA solutions, 70% infill; and (c) 6% CS/2% γ-PGA solutions, 70% infill [43].

(gamma-glutamic acid) (γ-PGA) [43]. The principle of the method is based on the use
of a commercial INKREDIBLE + 3D bioprinter equipped with two dosing heads, and
the electrostatic interaction between amino groups of chitosan and carboxylic groups
of γ-PGA.

The number of reactive functional groups from both polymers determines the quality
of the printed polyelectrolyte hydrogel (IPECs). The aim of the study was to find the
optimal composition of both polymer solutions in terms of the quality of printouts and
evaluate the survival of human fibroblast cells that were suspended in chitosan solution
during printing. The results showed that IPECs formed between 4.5% or 6% chitosan
and 2% γ-PGA 2% were stable in a grid shape up to about 37 Pa (370 dyne/cm^2) shear
stress (Fig. 3), much higher pressure than those present in most human arteries. Cell
survival in the bioink and after 3D BP was excellent.

To create another bioink, Lee and colleagues proposed the use of catechol-conjugated
chitosan (Chi-C), which in the first stage is produced by chemical coupling of both
substrates using 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDC) [44]. To 2%
Chi-C derivative solution in culture medium, vanadium oxide sulfate hydrate is added. The
vanadyl ion accelerates gelation by quickly generating a catechol radical and forming
oxygen complexes with hydroxyl groups present in the catechol aromatic ring (Fig. 4).

Chi-C can interact with blood components to form self-sealing membranes. The
phenomenon was observed in blood, so a similar type of rapid complexation with foetal
bovine serum was tested. The measured viability of L929 (mouse fibroblast) cells
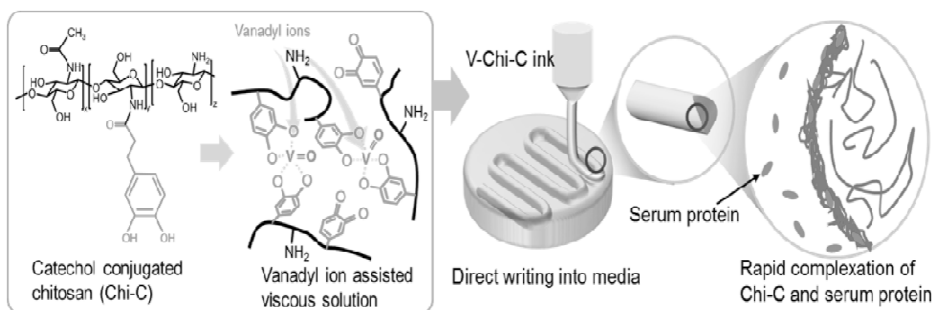
Figure 4. Schematic illustration for preparation of the vanadyl ion-catechol-conjugated
chitosan (V-Chi-C) bioink [44].

encapsulated in the printed scaffolds was almost $90 \pm 5\%$. These results show the unexplored potential of vanadyl ion-Chi-CS for *in situ* printability in serum containing media by rapid complexation between Chi-C and serum proteins without any extrinsic physical factors such as UV light. The small amount of vanadyl ion (1 mM) showed nearly no cytotoxicity. Moreover, the cell viability was 89 ± 8 % even after a 5-day incubation.

4. Chitosan Resins for SLA/DLP

Many studies have described the possibility of using chitosan derivatives in the field of biomaterials, such as soft tissue reconstruction [45], cartilage tissue engineering [46], cartilage regeneration [47] and drug release [48]. However, the procedure for these methods has so far been complex, the cost was high and during the process a toxic segment was introduced that changes the properties of the chitosan derivative. Research works from the last decade have clearly indicated the superiority of the Michael reaction in constructing chitosan photocured resins. The Michael reaction is a well-known process of adding various amines to α, β-unsaturated carbonyl compounds. Many studies have reported chemical modification of chitosan with a double bond monomer in the Michael reaction under mild reaction conditions [49, 50].

Ma and colleagues [51] presented a method of producing photopolymerizable chitosan derivative prepared by the Michael reaction of chitosan and poly(ethylene glycol) diacrylate (PEGDA). They reacted a 1% solution of chitosan in 1% acetic acid with PEGDA in a 1:14 mass ratio of polymers at 40°C for 24 h. Then, the reaction mixture was concentrated to a yellow viscous liquid. By using a 2% addition of Irgacure 2959 photoinitiator, they confirmed the ability to photopolymerize the solution after 15 min of UV irradiation (30 mV/cm²), but printability was not checked using a 3D printer. The obtained N-alkylated photopolymerizable chitosan derivative (PEGDA-CS) exhibited good solubility in water, an amorphous structure, lower thermal stability than chitosan and antimicrobial properties against *E. coli*, but lower than the chitosan used for modification.

Cheng and Chen [52] presented a way to implement chitosan in PCL-DA/PEG-DA resin in order to harness its many biological benefits and evaluate the effects of chitosan on structure wettability, cell adhesion and cell proliferation. The first stage consisted of PCL diol end-capping reaction with acrylate groups to form a crosslinkable and polymerizable PCL-DA macromer. In the second step, PEGDA was added to pre-dissolved 6% chitosan solution in 1% acetic acid heated to 40°C and finally mixed with PCL-DA/TPO (photoinitiator)/acetone solution for 10 h. Samples containing 5%, 10% and 15% chitosan in resin were used in the tests with a DLP 3D printer. All obtained 3D constructs had a melting temperature well above body temperature of 37°C and thus can be considered thermally stable for implantation. The increase in chitosan content increased the hydrophilicity of resulting resin in a possible acceleration of the bioabsorption rate and provided a surface favourable to L929 mouse fibroblast adhesion. At the same time, the crosslinking structure of printed scaffolds, especially above 10% chitosan addition, was weakened. Studies have also confirmed that the addition of chitosan reduces the crystallinity of the resin and its shrinkage after photocuring, what is an advantage from a technical point of view.

Cebe et al. [53] presented another example of using chitosan scaffolds created by photocuring to increase cell growth. Their work demonstrated a method of obtaining methacrylic derivatives of chitosan (MAC) and gelatin (MAG) via the Michael reaction. Dialysis-purified derivatives are lyophilized and then dissolved at a concentration of 10% in a culture medium dedicated to the cell line with 0.8% addition of Irgacure 2959 photoinitiator and 4% addition of sucrose and laponite nanosilicate. Scaffolds were
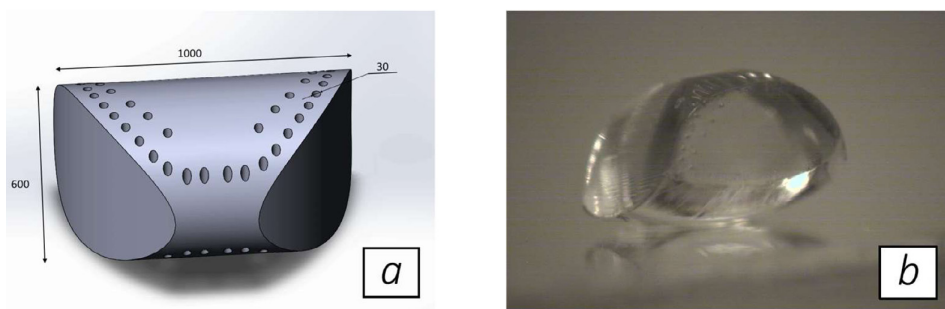
Figure 5. (a) Three-dimensional model and (b) scaffold fabricated by two-photon-induced micro-stereolithography [55].

printed using UV light (10–40 mW/cm^2). The results confirmed that the use of chitosan and laponite nanosilicate in scaffolds obtained by 3DP can enhance the formation of biomineral in osteoprogenitor cells during osteogenic differentiation due to the increased affinity of chitosan to enhance MC3T3 cell growth, elevation phosphate to amide formation, and forming the Ca–P biomineral nodules on the surface of the scaffold in a relatively short time frame as compared with gelatin-based scaffolds. However, this method is multi-stage and complicated, which rather excludes it in industrial use. Shen and colleagues [54] very recently proposed an easier way to use the same raw materials.

Bardakowa and co-workers [55] proposed a less complicated way for implementation of chitosan in production of biodegradable scaffolds for spinal cord regeneration by 3DP. They first obtained the chitosan-g-oligo (L, L-lactide) copolymer by reactive blending of chitosan powder and oligo (L, L-lactide) at 55°C using the twin-screw extruder. An amount of the obtained copolymer (4.9%) was dissolved in a 3% acetic acid solution. After separation of the insoluble fraction, the solution was mixed with PEGDA and a biocompatible Irgacure 2959 initiator in the mass ratio of components 5:5:1 in the final resin, respectively. This method developed a 3D model for treating spinal cord injuries (Fig. 5). It is a truncated cylinder that is 1000 μm long and 600 μm high. There is an array of holes 30 μm in diameter on the convex side of the scaffold. The device provided a high survival rate of cortical neurons and the formation of neural networks and thus could be considered biocompatible and suitable for neuroregeneration [55].

The Liu team investigated the problem of SLA printout shrinkage. One way to prevent this phenomenon is using inert fillers as an additive in resins [56]. They decided to use calcium sulphate whiskers (CSW), due to the many advantages like thermal stability, chemical resistance, high strength and whole surfaces. CSW were coated with chitosan by immersion in its solution and formed polyelectrolyte complexes (CS@CSW). The derivative obtained in this way was dispersed in N,N-dimethyl- acetamide (DMAC) with an excess amount of acryloyl chloride and triethylamine related to chitosan polymer and the mixture was stirred at room temperature for 24 h. The obtained m-CS@CSW was mixed with oligomer epoxy acrylate and tripropylene glycol diacrylate (TPGDA) and TPO photoinitiator and photocured. Fourier transform infrared spectroscopy, water contact angle (WCA) and thermogravimetric analysis showed that the modification was succesful and the printed models showed high accuracy and resolution. Tests of viscosity and volumetric shrinkage showed that CSW modified with chitosan has little effect on viscosity and leads to a significant reduction in volumetric shrinkage of the printout. The use of CSW modification in a 5% chitosan solution increases tensile strength and impact strength of cured samples by 19.4% and

6.6% compared to cured pure resin, respectively. It is simultaneously one of the alternative method of introducing chitosan to the final resin.

5. Chitosan powder for SLS

The idea of using chitosan in SLS seems to be interesting because of the unique, functional properties of chitosan, which determine the possibility of using this polymer for biomedical applications. The second reason is the ability to use the raw material in the form of a powder, so the use of classic solvents to obtain a polymer solution is unnecessary. The printing process, however, requires large amounts of energy that allow point bonding of the powder layer by layer in accordance with the information contained in the file of the printed object.

Brysch and others [57] showed that chitosan degradation starts above 220°C but can also occur at lower temperatures (180°C) at longer sintering times (12 h). Shorter sintering times seem to favour the strength of the chitosan. Those conditions resulted in accelerated polymer breakdown, as evidenced by the characteristic exothermic reaction or by the appearance of ash [57]. The intra- and intermolecular hydrogen bonds, which generate a stable and rigid semi-crystalline structure of chitosan, also make it degradable before melting because of the high melt viscosity, which is typical for polysaccharides with extensive hydrogen bonding. Hence, the use of chitosan in the printing method by sintering its powder is a difficult task. There is information in the literature on the creation of, for example, tissue engineering scaffolds using SLS simultaneously containing chitosan. However, these are mainly at least two-stage methods, in which a porous scaffold is first made, e.g. from polycaprolactone powder or a titanium composite, which is then subjected to postprocessing by immersion in a solution of chitosan or chitosan with hydroxyapatite to improve the biocompatibility and cell proliferation activity [58, 59].

The latest work in which chitosan had been directly subjected to sintering in SLS is from Sun and colleagues [60]. They presented a method of using chitosan for the production of composite membrane for adsorption and catalysis, with the base thermoplastic polyurethane (TPU). Membranes were obtained by simple physical mixing both powders in a 1:1 mass ratio and treated by a carbon dioxide (CO_2) laser. The results showed that sintering temperature caused by laser power and scanning speed was an important factor affecting the formation of chitosan/TPU composite membrane during sintering. Under suitable laser power and scanning speed, the TPU melts and wets the CS to promote its formation of a membrane structure. Due to the existence of chitosan, the membrane has super-hydrophilic properties and can effectively adsorb copper and lead metal ions in water. In addition, a palladium-laden chitosan/TPU membrane can be used as a catalyst for p-nitrophenol reduction with sodium borohydride (conversion rate could reach 96% in 20 min), and has the advantages of excellent stability, repeatability and ease of separation from the reaction system.

6. Conclusions

Research in recent years has shown significant progress in the use of 3DP technology in biomedical applications. Medical materials used in 3DP consist of metals, polymers and ceramics, with many materials usually integrated to achieve complex functions in printed components. Further methods of combining raw materials, including chitosan, to improve the mechanical behaviour of personalized scaffolds, to ensure their appropriate porosity, as well as biodegradation time are currently being described. Much remains to be done before printed bioactive tissues and organs are routinely used in the clinic, and these tasks include searching for high-performance materials compatible with various commercial 3D printers, mastering precise 3D production using them, creating uniform standards for printed objects and conducting clinical tests.

7. Acknowledgements

8. References

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