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affiliée à l'Université de Montréal

**Three-Dimensional Printing of Protein/polysaccharide Mixed Gel Systems
for Food Applications**

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Thèse présentée en vue de l'obtention du diplôme de *Philosophiæ Doctor*

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Cette thèse intitulée :

Three-Dimensional Printing of Protein/polysaccharide Mixed Gel Systems for Food Applications

présentée par **Chi ZHANG**

en vue de l'obtention du diplôme de *Philosophiæ Doctor*

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DEDICATION

Life is unfair, kill yourself or get over it.

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RÉSUMÉ

Avec l'amélioration du niveau de qualité de la vie, il y a une demande croissante de formulations d'ingrédients alimentaires personnalisés et de conception d'aliments. La situation du vieillissement de la population a également accru les besoins en matière de composition des repas et de nutrition adaptées. De plus, avec la double augmentation attendue du nombre de personnes âgées de 65 ans ou plus au cours des trois prochaines décennies, le régime alimentaire en cas de dysphagie a attiré beaucoup d'attention. Cependant, l'apparence peu attrayante de ce régime (généralement à l'état écrasé ou en purée) entraîne une perte d'appétit, qui est directement liée à la malnutrition et à la perte de poids du patient souffrant de troubles de la déglutition. La fabrication additive (FA), également connue sous le nom d'impression 3D, est devenue une technologie émergente dans les applications alimentaires ces dernières années. Elle permet la fabrication d'aliments avec relativement moins de perte de matière, une forme plus complexe et un contrôle précis des ingrédients, montrant un grand potentiel pour compléter la cuisine familiale et en clinique.

Les protéines et les polysaccharides sont deux principaux types de macromolécules dans le système alimentaire. Elles jouent un rôle vital dans des fonctions nutritionnelles, structurales et texturales complémentaires, faisant le système à base de protéines et de polysaccharides un bon candidat modèle pour l'impression 3D alimentaire. Cette thèse vise à développer des formulations d'encres à base de gels mixtes protéine/polysaccharide pour la fabrication additive en vue d'applications alimentaires.

Tout d'abord, une encre alimentaire aqueuse à base de protéine/polysaccharide utilisant de la gélatine B (GB) et de la gomme de xanthane (XG) a été développée pour l'impression à écriture directe d'encre « direct ink writing » (DIW). GB et XG ont été choisis comme matériau d'encre, car ce sont des ingrédients alimentaires peu coûteux et couramment consommés. L'encre en gel de GB/XG présente des propriétés appropriées pendant et après le processus d'impression 3D, ce qui confère à un objet imprimé en 3D à 40 couches un motif clair à température ambiante. L'ajout d'ions calcium (Ca^{2+}), un micronutriment nécessaire au quotidien, améliore les propriétés rhéologiques de l'encre, donc une plus grande fidélité de forme. De plus, il a également été constaté que les propriétés texturales peuvent être ajustées par le pourcentage de remplissage des échantillons imprimés.

Ensuite, la performance en impression à écriture directe des encres aqueuses à base de protéine/polysaccharide a été étudiée plus en détail. Outre la paire GB/XG discutée précédemment, une autre protéine végétale fréquemment utilisée, l'hydrolysate acide de protéine de soja (SP), a également été étudiée en combinaison avec XG. Une carte de traitement a été établie montrant les effets du diamètre de la buse et de la teneur en XG sur divers types de géométries imprimées, y compris les filaments unidimensionnels (1D), les réseaux bidimensionnels (2D) et les structures 3D pour les systèmes GB/XG et SP/XG. La caractérisation rhéologique a montré que non seulement le comportement en écoulement peut être affecté par les concentrations de XG, mais aussi la rétention de la forme après impression. D'ailleurs, une cartographie de fidélité de forme a été générée pour l'évaluation post-dépôt des structures 3D imprimées sur la base des propriétés viscoélastiques des formulations d'encre et d'un indice de fidélité de forme des échafaudages imprimés. De plus, il a été prouvé que le type de protéine et les concentrations de XG sont critiques aux propriétés texturales des échantillons imprimés. Enfin, sur la base de l'établissement de cartographies du procédé et de fidélité de forme, une structure complexe de dauphin 3D à 18 couches composée de deux gels mélangés différents (c'est-à-dire 3 wt% GB/11 wt% XG et 8 wt% SP/15 wt% XG), a été imprimée avec succès par une technique d'impression DIW multi-matériaux.

Dans la dernière partie de la thèse, je me suis consacré à l'étude comparative d'encres alimentaires aqueuses de protéine/polysaccharide enrichies de nanocristaux de cellulose (CNC). Les encres candidates étaient le mélange GB/XG et l'isolat de protéines de lactosérum (WPI) avec XG pour l'impression 3D d'aliments pour la dysphagie. L'ajout de CNC améliore la stabilité thermique du système GB/XG avec une amélioration jusqu'à 15 % de la rétention de la zone latérale après chauffage à 50°C. Mis à part la concentration en CNC, la température post-dépôt a également eu un effet important sur les propriétés texturales, en particulier pour le système GB/XG. Enfin, une découverte originale a été observée sur un échantillon imprimé avec GB/XG/1.5CNC avec sa transition d'un régime dysphagique non approuvé vers un régime dysphagique haché et humide de niveau 5 en maintenant la forme imprimée à 4°C.

ABSTRACT

With the improvement of living standards, there is a growing demand for personalized ingredients and design in food. The serious aging situation has further expanded peoples' requirements for tailored meal compositions and nutrition. Moreover, dysphagia diet has attracted more attention due to the expected double increase in the number of elderly people above 65 and older in the next three decades. However, the unattractive appearance of common dysphagia-oriented diet (usually in mashed or pureed state) causes a loss of appetite, which is directly related to malnutrition and weight loss for the swallowing-impaired patient. Additive manufacturing (AM), also known as 3D printing, is becoming an emerging technology in food application in recent years. It enables food fabrication with less material loss, more flexible shapes and precise content control, showing great potential to enter the home kitchen and clinics.

Proteins and polysaccharides are two main types of macromolecules in food systems. They play a vital role in complementary nutritional, structural and textural functions, making the system based on protein and polysaccharide a good model candidate for 3D food printing. This thesis aims to develop ink formulations based on protein/polysaccharide mixed gels for additive manufacturing towards food application.

Firstly, an aqueous protein/polysaccharide food ink using gelatin B (GB) and xanthan gum (XG) was developed for direct-ink writing (DIW) printing. GB and XG were chosen as ink materials because they are low cost and commonly consumed food ingredients. The GB/XG mixed gel ink shows suitable properties during and after the 3D printing process, which endows a 40-layer 3D printed object with clear pattern at room temperature. The addition of calcium ions (Ca^{2+}), a daily required micronutrient, improves the rheological properties of the ink, giving rise to a higher shape fidelity. Furthermore, it was also found that the texture profile can be tuned by the infill percentage of the printed samples.

Secondly, the DIW performance of protein/polysaccharide inks was further investigated. Besides the GB/XG pair discussed previously, another frequently used plant-based protein, soy protein acid hydrolysate (SP), was also studied in combination with XG. A processing map was established showing the effects of nozzle diameter and XG concentration on various type of printed geometries including one-dimensional (1D) filaments, two-dimensional (2D) arrays and 3D structures for both GB/XG and SP/XG systems. Rheological results showed that not only the

flow behavior, but also the shape retention after printing could be affected by XG concentrations. In addition, a shape fidelity map was generated for the post-deposition assessment of printed 3D structures based on the viscoelastic properties of ink formulations and a defined shape fidelity index of printed scaffolds. Furthermore, it has been proved that both protein type and XG concentrations are critical to textural properties of printed samples. Finally, based on the established processing and shape fidelity maps, an 18-layer 3D complex dolphin shape composed of two different mixed gels (i.e. 3GB/11XG and 8SP/15XG) was successfully printed by a multi-material DIW printing technique.

Finally, a comparative study on cellulose nanocrystal (CNC)-enhanced protein/polysaccharide aqueous food inks was undertaken with GB/XG and whey protein isolate (WPI)/XG for 3D printing towards dysphagia diet. CNC addition improved the rheological properties, which resulted in a high shape fidelity right after printing and a ~90% retention of its original shape after 96 h for both GB/XG and WPI/XG systems. Additionally, CNC addition also affected the thermal stability of GB/XG system with a 15% enhancement on side area retention after heating at 50°C. Besides the CNC concentration, the post-deposition temperature also had an important effect on the textural properties, especially for the GB/XG system. Finally, an original finding was observed on the transition from non-approved dysphagia diet into level 5-minced and moist dysphagia diet by holding at 4°C on a GB/XG/1.5CNC printed sample.

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LISTE OF SYMBOLS AND ABBREVIATIONS

1D	one-dimensional
2D	two-dimensional
3D	three-dimensional
<i>A</i>	Pore area
AM	Additive manufacturing
<i>C</i>	Circularity
Ca ²⁺	Calcium ions
CFA	Capillary flow analysis
CNCs	Cellulose nanocrystals
COOH	Carboxyl groups
<i>D</i>	Diameter
DIW	Direct-ink writing
<i>G'</i>	Dynamic shear storage modulus
<i>G''</i>	Dynamic shear loss modulus
GB	Gelatin type B
<i>K</i>	Consistency index
<i>L</i>	Length
<i>l</i>	Perimeter of a square interstitial zone
LVE	Linear viscoelastic regime
<i>n</i>	Flow index
OH	Hydroxyl groups
pI	Isoelectric point
<i>Pr</i>	Printability

RR	Rotational rheometer
SAOS	Small amplitude oscillatory shear
SFI	Shape fidelity index
SP	Soy protein acid hydrolysate
$\tan \delta$	Loss tangent
TMF	Texture-modified food
WPI	Whey protein isolate
XG	Xanthan gum
ω	Angular frequency

CHAPTER 1 INTRODUCTION

1.1 Background and problem identification

With economic growth and the improvement of public living standards, there is a growing interest on food quality such as composition, design and properties (e.g., texture, flavor, mouthfeel) [1]. This desire for high quality food drives customized diets based on individual requirement as an emerging trend [2]. Additive manufacturing (AM), also known as three-dimensional (3D) printing, is a rising digitalized technology which is able to create computer-generated 3D objects directly by depositing the material through a layer-by-layer process [3]. Due to advantages such as less material loss, freedom design and simple processing [4, 5], the 3D printing technique is nowadays used in polymer processing, civil engineering and biomedical applications [3]. The food area is also stimulated by this technology which has great potential in the fabrication of food with customized design, personalized nutrition, and properties [5, 6]. The “Fab@Home model 1” is the first 3D food printer developed by the researchers from Cornell University in 2007 [1]. Later on, Massachusetts Institute of Technology (MIT) introduced a digital gastronomy concept into food printer design in 2011 [7]. In recent years, the 3D food printing technique also brought an increasing interest for some specific groups, such space and military staff and specific populations (e.g., elderlies, women and athletes) [2, 8].

Dysphagia is a neuropathic condition describing swallowing difficulties [9]. Up to 60% elderly people leaving in nursing homes have suffered from this condition in recent years [10]. Also, dysphagia will become more serious with the aging population. The traditional dietary therapy is to consume texture-modified food with unappealing puree/mashed forms, leading to a loss of appetite and nutritional deficiencies [11]. 3D food printing might address these issues, since it enables the fabrication of attractive food designs with the appropriate ingredients, nutrition and texture characteristics.

Recent advances in 3D food printing have used various ingredients such as milk-based products [12], surimi [13], cultured meat [14], banana paste [15] and fungi [16]. However, most studies are mainly concerned with food prototyping or ready-made food, while there is still a lack of fundamental investigation on how essential constituents of food perform during and after the 3D printing process [17].

Proteins and polysaccharides are two essential biopolymers in food systems, which perform complementary functional properties such as nutrition, structure and texture [18, 19]. They often coexist in actual food systems, such as the protein and polysaccharide mixtures in mayonnaise and ice cream [18]. Among various kinds of food composition, the interactions between proteins and polysaccharides play an important role in their thickening ability, surface stability and other features in the food system [17, 20-22]. 3D printing of protein and polysaccharide mixed systems imposes several challenges, since they may not have sufficient viscosity during the printing process and collapse during or after deposition [23-25]. Some complicated fabrication processes (e.g. using a supporting fluid bath) or/and chemical crosslinker are usually involved as seen in the previous literature, which is not suitable for food production. Hence, we seek to develop novel protein/polysaccharide mixed gel inks with a food adapted preparation strategy, which can be printed using a simple manufacturing process and keep a good post-printing stability for food applications.

1.2 Organization of the thesis

This thesis is based on three peer-reviewed articles published or submitted to scientific journals and is divided into 7 chapters. Chapter 2 provides a literature review considering the related issues. Chapter 3 defines the problem to be solved and brings out three major objectives of the research. Chapters 4, 5 and 6 present respectively the three main contributions of this work in the form of three peer-reviewed articles. A general discussion about the main results of the articles is reported in Chapter 7. Finally, Chapter 8 summarizes the conclusions of the whole project and several recommendations for future work are also proposed.

CHAPTER 2 LITERATURE REVIEW

2.1 Additive Manufacturing (AM) in food application

Additive manufacturing (AM), or three-dimensional (3D) printing, involves a computer controlled translation stage for fabricating a wide range of structures from a digital design into 3D objects [26, 27]. Compared to conventional fabrication technique, which needs molds to fabricate 3D structures and may cause large waste of materials, 3D printing can allow fabricating complex 3D structures via rapid prototyping [28, 29]. This fast developing technique entered the market in recent years and has great potential in food, aerospace and biomedical applications [30, 31]. Additive manufacturing of food is an emerging and potential new direction for industry [32]. In the field of food and health, several incentives drove its development: (1) increased requirement on customized dietary patterns according to gender, age and occupation [33]; (2) high-level life style [34]; (3) progress in dietary therapy owing to the breakthroughs in medicine [35]. As the first reported food 3D printer, the “Fab@Home Model 1” was released in 2007 at Cornell University [36]. Later on, Massachusetts Institute of Technology (MIT) introduced a digital gastronomy concept into food printer design in 2011 [7]. Figure 2.1 shows three representative commercial extrusion-based 3D food printers. Currently, the most popular and compatible 3D printing techniques for food applications are divided into several categories such as extrusion-based, jetting-based and power bed fusion printing [33, 37]. A brief comparison of these various techniques is presented in Table 2.1.



Figure 2.1 Three examples of existing commercial extrusion-based 3D food printers [38]: a) Choc Creator (Choc Edge, 2018) [39], b) Foodini (Natural Machines, 2020) [40] and c) Fab@Home (Creative Machines, 2020 [41]).

Table 2.1 Common AM techniques used in food applications [2, 33, 37]

Category	Principle	Advantages	Disadvantages
Extrusion-based printing	Extrude the ink material through a needle/nozzle using pneumatic air pressure	• Wider selection of ink materials • Possibility of more than one printing head	• Structure defects from bad layer deposition • Longer printing time

Jetting-based printing	Sprays droplets of ink material	• More simple operation process • Higher resolution	• Limit on ink materials selection • Droplet formation nonuniform
Powder Bed Fusion	Melt or fuse powdered materials by laser or electron beam	• Reduced material waste by recycling the powder • More flexible to build complex food design	• High porosity when the material powder is fused • Limit on ink materials selection

2.1.1 Extrusion-based 3D food printing

Extrusion-based printing is one of the most widely used 3D printing method, as it offers an easy and inexpensive way to fabricate 3D food [42]. During this process, the ink material is extruded through a nozzle upon the application of a pressure gradient ΔP along the effective length of the micro-extruder to build objects in a layer-by-layer fashion [43]. Figure 2.2 shows a typical extrusion-based 3D printing setup and the schematics of the printing process with a milk-based ink.

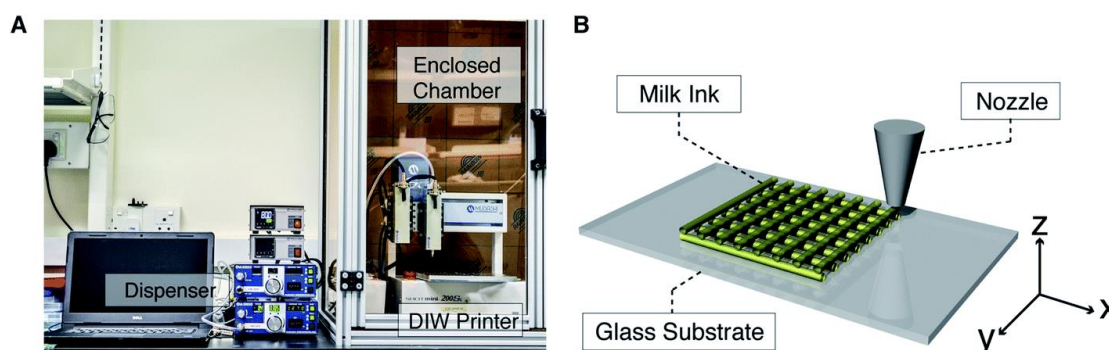


Figure 2.2 a) A typical extrusion-based 3D printing setup including a computer-controlled dispenser, an enclosed chamber and a printer. b) 3D extrusion-based printing process using a milk-based food ink by a layer-by-layer deposition [12].

Figure 2.3 shows the schematic representation of a complete 3D food printing process. Firstly, the 3D model is designed in some software such as computer-aided design (CAD). Then the

slicing software generates the 3D model into individual cross-section images and translates it into the printing code adapted by the printer. The printing process starts after choosing the food ingredient. Based on the food patterns generated from the 3D model, the food ink layer is formed by the relative movement of the stage and the printer head. In some cases, a post-treatment process such as baking may also be performed [44].

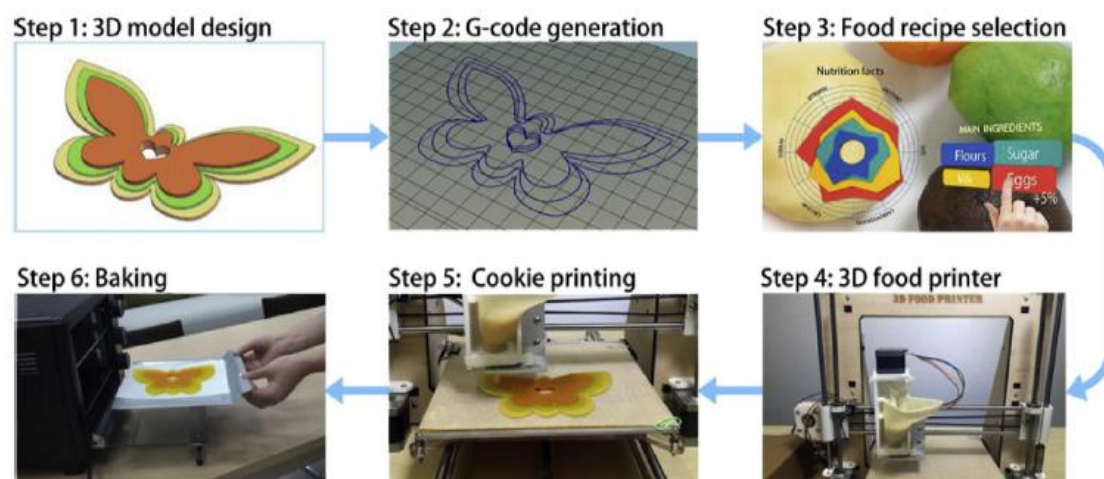


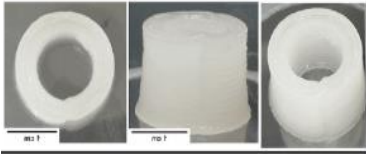
Figure 2.3 Schema of a complete 3D extrusion-based food printing step from the 3D model design to the final printed object [44]

Depending on the ink-material used and final object type requirement, the extrusion-based printing for food applications can be mainly divided into hot-melt extrusion, also called fused deposition modeling (FDM) and direct-ink writing (DIW), further described below [45].

2.1.2 Direct-ink writing (DIW)

The DIW printing is a technique based on the extrusion of native printable, self-supporting materials [46]. The ink material should have a semi-solid like behavior in order to guarantee adequate extrusion process and formation of 3D objects [47]. Hence, the rheological properties (viscosity, storage and loss modulus, yield stress etc.) are the most important parameters for qualifying its printability [37]. In recent years, the DIW technique has been applied for printing several essential food ingredients from animal and plant sources. Table 2.2 shows a brief summary of the latest applications of DIW food printing.

Table 2.2 Overview of latest applications of DIW food printing (abbreviations defined below)

Raw materials	Major findings	Presentative images	Reference
β -CD/ CS stabilized HIPEs	0.32 wt% CS addition in HIPE stabilized by β -CD at pH 5 showed the best printability		X. Li et al.[48] (2023)
<i>Spirulina platensis</i> (type of microalgae) residues	<i>Spirulina platensis</i> residues ink with middle moisture content showed shear thinning behavior, high viscosity and rapid recovery, promoting the high-value utilization of microalgae		M. Wang et al. [49] (2023)
DSF	Rheological properties of ink can be affected by particle sizes of DSF. Fine DSF can improve the textural properties of printed objects.		D. Nei et al. [50] (2023)
Potato starch and pectin	Adding 2 % pectin in potato starch ink showed excellent shape retention of printed sample after 14 days at 4°C.		N.E.Wedamulla et al.[51] (2023)

TP/ XG gel-like PE	XG addition enhanced the water holding capacity, gel strength, storage moduli and yield stress of ink. Best conditions were found: 70 ml oil/30 ml deionized water, 0.9g TP and 0.45g XG.		Z. An et al.[52] (2023)
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β -CD: β -cyclodextrin, CS : chitosan, HIPEs: high internal phase emulsions, DSF: Defatted soybeans, TP: Tea residue protein, XG: xanthan gum, PE: Pickering emulsion

2.1.3 Process-related parameters in extrusion-based printing

The printing process parameters have a significant effect on printability. In order to assess the printability, several key criteria including geometrical accuracy, shape stability and mechanical properties should be considered for extrusion-based printing. Hence, the processing factors such as nozzle diameter, extrusion flow rate and relative velocity need to be examined and improved during extrusion [45].

M.S. Huang *et al.* reported that the nozzle diameter had a significant effect on printing accuracy of brown rice [53]. Gravity played a greater influence on deposited filament when using a larger nozzle diameter. In addition, a small nozzle diameter decreased the deviation of the printed objects. L. Liu *et al.* and L. Zhang *et al.* also revealed that the dimensional resolution and surface quality of final 3D printed objects based on egg white protein and cereal-based dough improved by decreasing the nozzle diameter size from [54, 55]. Consistently, A. M'Barki *et al.* discussed the force on the printed sample after deposition, indicating that the gravitational slumping was more important when nozzle size increased from 250 μm to 840 μm [56]. Figure 2.4 shows the representative images of printed samples fabricated with different nozzle diameters.

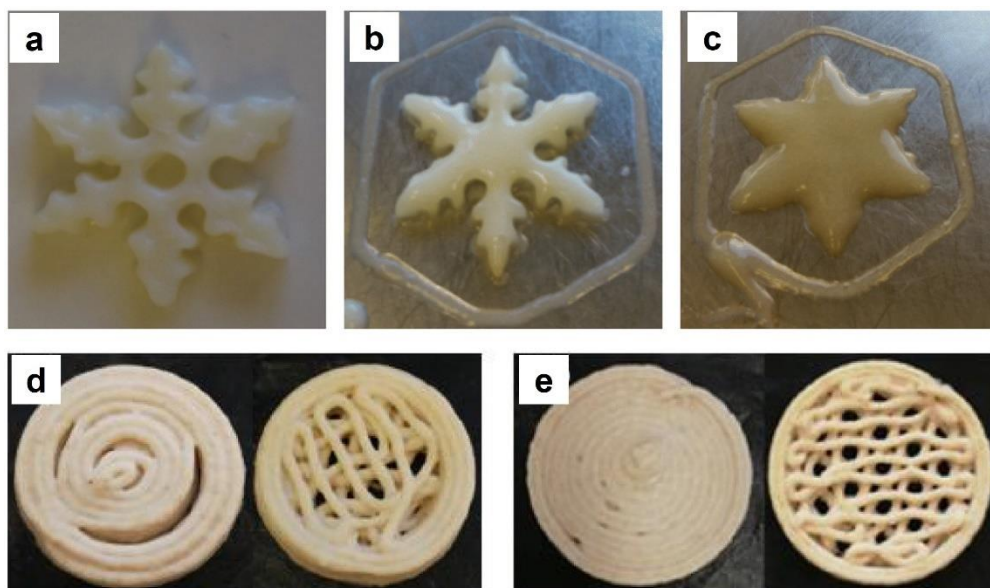


Figure 2.4 Geometry shape of printed samples using 5.0 % egg white protein with different nozzle diameter: a) 1.0, b) 1.5 and c) 2.5 mm [54]. Representative 3D printed shapes using cereal-based dough with d) 1.6 and e) 1.2 mm nozzle size [55].

Shape accuracy is also highly affected by the nozzle moving speed. A. Derossi *et al.* found that high printing speed at 200 mm/s caused a dragging effect, leading to the rupture of the printed filaments and a bad structure using a wheat dough ink. In addition, the corner parts of the 3D model was difficult to print at high nozzle speed [57]. Besides, L. Liu *et al.* and L. Zhang *et al.* reported that slow printing speed may lead to material overflow with a poor resolution of the printed object of egg white protein and egg yolk with rice flour blends [54, 58]. Figure 2.5 shows the representative images of printed samples fabricated with different nozzle movement speed.

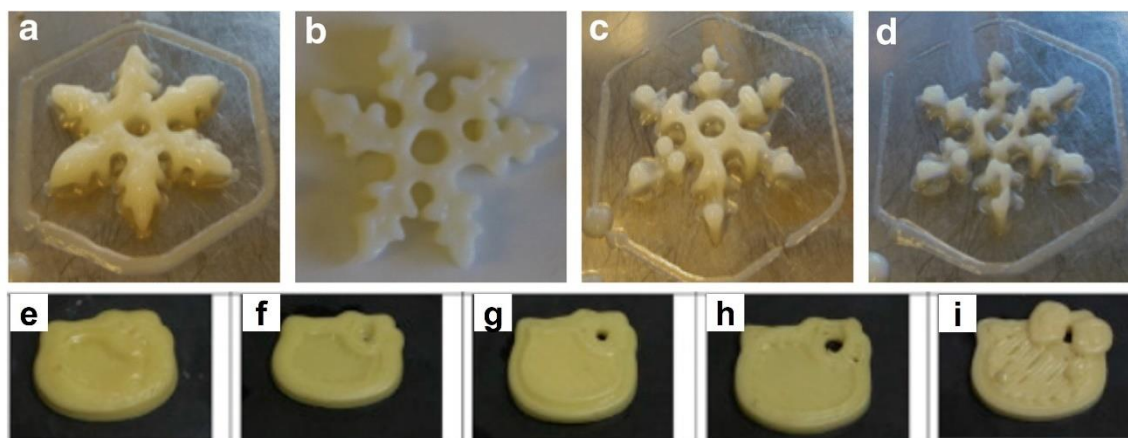


Figure 2.5 Geometry shape of printed samples using 5.0 % egg white protein with different nozzle moving speed: a) 60, b) 70, c) 80 and d) 90 mm/s [54]. Representative 3D printed shapes using cereal-based dough with e) 400, f) 600, g) 800, h) 1000 and i) 1200 mm/min [58].

Nozzle height is another important parameter influencing the stability of deposited filaments and the resolution of the printed structures in the extrusion-based printing process. K.Keerthana *et al.* [59] reported that the thicker filaments were extruded when the nozzle height was 25% of the nozzle diameter, resulting in a bad resolution of printed constructs with mushroom paste. However, parts of the filaments could not be well deposited on the substrate when turning a corner with a high nozzle height (equal to the nozzle diameter), then leading to a structure defect.

2.1.4 Application of 3D printing in dysphagia diet

Dysphagia is the medical term of a neuropathic disease, which refers to a swallowing difficulty due to a weak tongue or cheek muscles [60]. It is commonly caused by a stroke, Alzheimer's disease and Parkinson's disease etc., particularly among the geriatric population [61, 62]. Due to this swallowing disorder, parts of the food cannot be moved from the mouth to the stomach [63]. It directly leads to malnutrition and weight loss and may cause other serious medical complications [64], such as aspiration pneumonia and choking [64, 65]. Currently, besides the medical treatment, taking texture-modified food (TMF) is the most common intervention for dysphagic patients [66]. The traditional method for TMF fabrication is to soften the food by boiling or mashing. However, such a food generally presents a visually unappealing puree or mashed state, even accompanied by nutrition and flavor loss [67]. At the present, 3D food printing shows a great potential in dysphagia diet fabrication. It can improve the TMF by

personalizing nutrition, adjusting internal structures and creating attractive meals [64], which largely enhances the nutrients intake for dysphagic patients. Figure 2.6 explains schematically the advantage of 3D food printing in dysphagia diet.

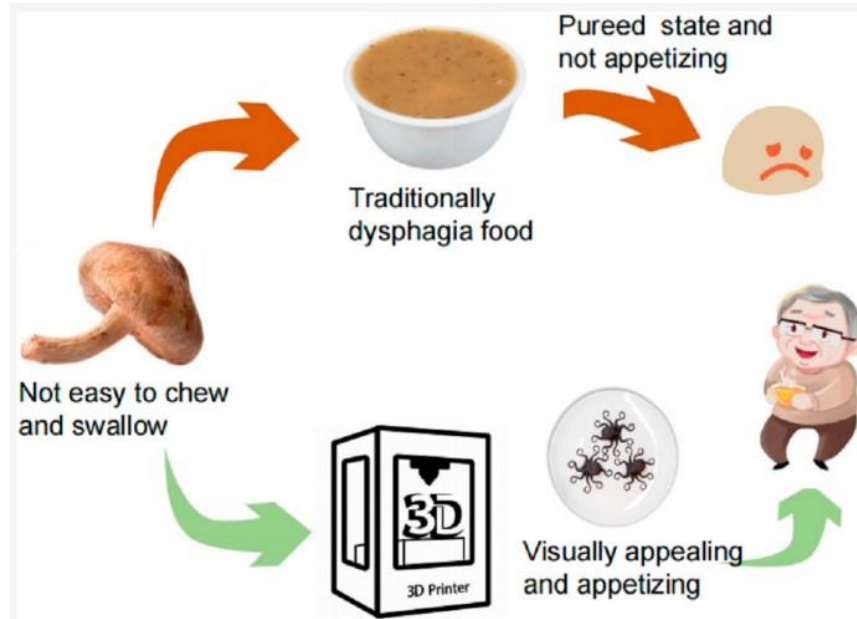


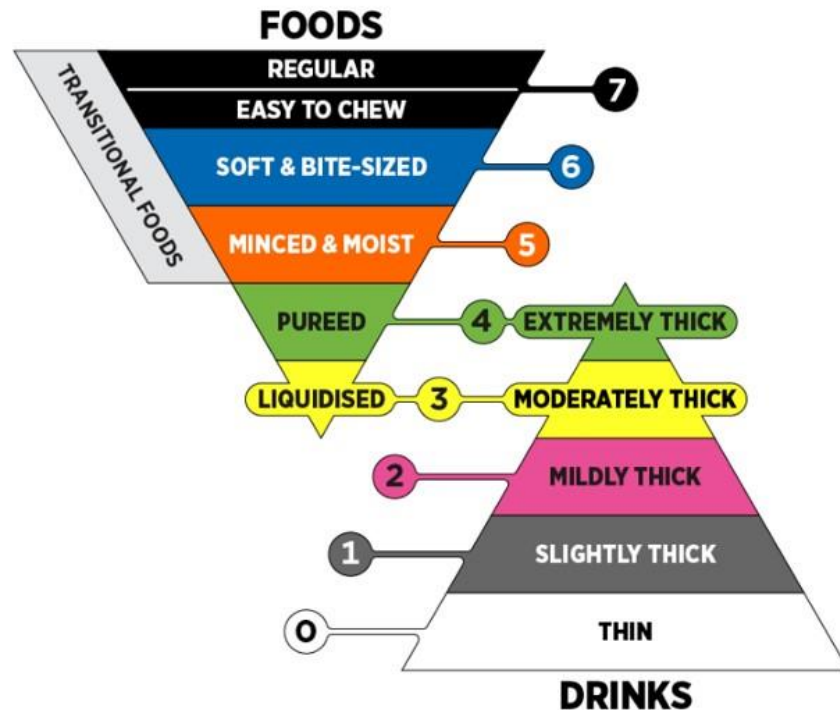
Figure 2.6 Schematic of how 3D food printing can improve the dysphagia diet [68].

The International Dysphagia Diet Standardization Initiative (IDDSI) framework has been released in 2016 [69]. This global initiative aims to develop a standardized food texture terminology worldwide. Figure 2.7 shows the IDDSI framework including different levels of drink and food adequate for dysphagic patients. According to the IDDSI framework, textural modified dysphagia diet can be classified into 8 levels (0-4 for thickened liquids and 4-7 for texture-modified foods).

Since the application of 3D food printing in dysphagia diet is an emerging field in recent years, the specialized studies are still not common. Table 2.3 presents a list of studies on 3D food printing in the dysphagia diet application.

The IDDSI Framework

Providing a common terminology for describing food textures and drink thicknesses to improve safety for individuals with swallowing difficulties.

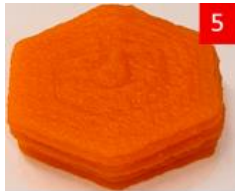


© The International Dysphagia Diet Standardisation Initiative 2019 @ <https://iddsi.org/framework/>
 Licensed under the Creative Commons Attribution Sharealike 4.0 License <https://creativecommons.org/licenses/by-sa/4.0/legalcode>.
 Derivative works extending beyond language translation are NOT PERMITTED.

Figure 2.7 IDDSI classification framework [70].

Table 2.3 Latest researches of 3D food printing in dysphagia diet (abbreviations defined below)

Raw materials	Advantages for dysphagia diet	IDDSI level	Representative printed images	Reference
Pork, XG and GG	Modified texture by controlling the contents of hydrocolloids	Level 6-soft&bite-sized or level 7-easy to chew		A.Dick <i>et al.</i> [71] (2020)

Beef, XG, GG, KC and LBG	Modified texture by controlling the contents of hydrocolloids	Level 5-minced&moist and level 6-soft&bite-sized		A.Dick <i>et al.</i> [72] (2021)
Freeze dried apple powder and rose powder, BSG and XG	Modified texture by controlling the contents of hydrocolloids and appealing meals design	Level 5-minced&moist		L.Qiu <i>et al.</i> [73] (2023)
Black fungus powder, AG, XG and KG	Modified texture by controlling the contents of hydrocolloids and appealing meals design	Level 5-minced&moist		X.Xing <i>et al.</i> [67] (2022)
Garden pea, carrot, bok choy, XG, KC and LBG	Modified texture by controlling the contents of hydrocolloids	Transitional food		A.Pant <i>et al.</i> [74] (2021)

XG: xanthan gum, GG: guar gum, KC: κ -carrageenan, LBG: locust bean gum, BSG: basil seed gum, AG: Arabic gum.

2.2 Proteins and polysaccharides

Proteins and polysaccharides are two natural biopolymers which have already been widely used as functional ingredients in several applications, including food and pharmaceutical engineering [75]. Hydrocolloids based on proteins and polysaccharides are interesting model systems, which have great potential to be applied in DIW 3D food printing. In this section, a basic understanding of the main food ingredients used in this project is reviewed. Afterwards, as a key factor in 3D food printing, the rheology of protein/polysaccharide mixed gels is discussed.

2.2.1 Proteins

Proteins are essential biological polymers containing great nutritional value and play a key role in bio-chemical processes in all living things. Several industrial scale proteins have already been applied in areas such as food, pharmaceutical, cosmetic, medicine, packaging, coating, etc. [76]. Proteins can have different structures, such as globular structure and linear proteins [77]. Globular proteins are formed by folding the polypeptide chains by entropy into a compact spheroid conformation or an ellipsoidal entities, such as whey protein and ovalbumin [78]. Linear proteins are formed by organizing the polypeptide chains in parallel along a single axis, such as the connective tissue of animals (e.g. collagen, gelatin) [78]. Several environmental conditions such as pH value, temperature, ionic strength and nature of solvent, could influence the structure of proteins. Isoelectric point (pI), where the net charge is zero, is an important character of protein. The charge of the protein is positive when the pH is below its pI. Otherwise the protein is negatively-charged [79].

2.2.1.1 Gelatin

Gelatin is a type of widely used proteins on the market in various applications, such as medicine, cosmetic products and food [80]. Figure 2.8 shows the situation including the expansion and prediction of the gelatin market from 2016 to 2027. As the most consumed hydrocolloid, the global gelatin market is estimated to reach 5.0 billion USD in 2025. According to the report of Grand View Research, the market size will even increase to 6.7 billion USD by the end of 2027 [81, 82].

There is commonly two type of gelatin according to the different denatured treatments from collagen: type A and B. Gelatin A results from the acid hydrolysis of collagen found in pig skin, which has an isoelectric point (pI) in a range from 8-9. Gelatin B is derived from alkaline treatment from collagen found in bovine hides. It has a lower pI in the range of 4.8-5.5 [81, 82].

Figure 2.9 shows a general denaturation process from collagen to gelatin and a typical sol-gel transition of gelatin by heat-cool process. Gelatin is a thermal-reversible protein with a helix-to-coil transition temperature around 20-30°C depending on gelatin sources [83]. In solution above the transition temperature, gelatin molecules are in the form of random coils, but on cooling it tends to order and form collagen-like triple helices [81, 82].

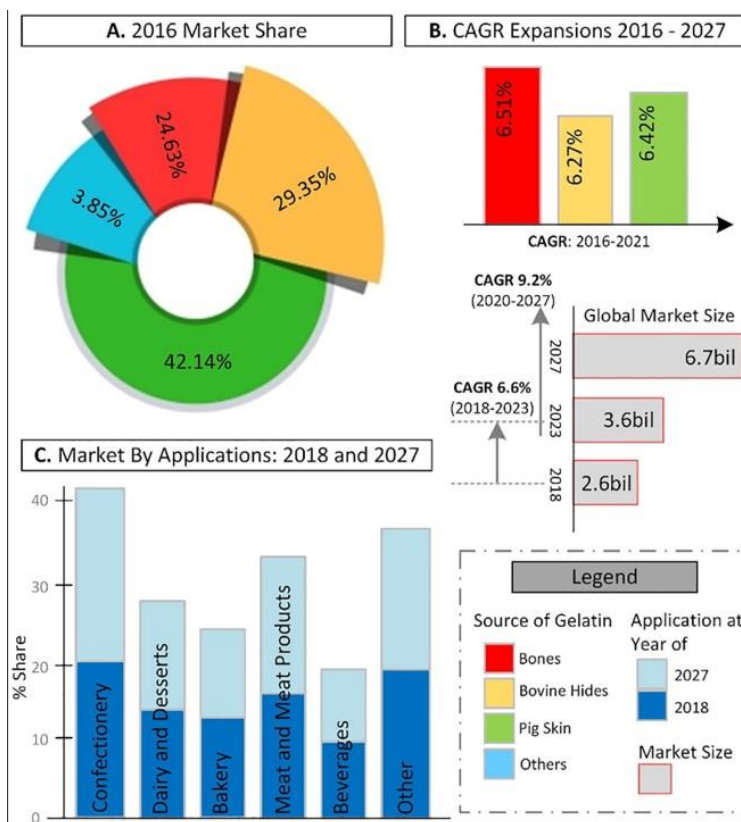


Figure 2.8 The market development and prediction of gelatin sales from 2016-2027 [82].

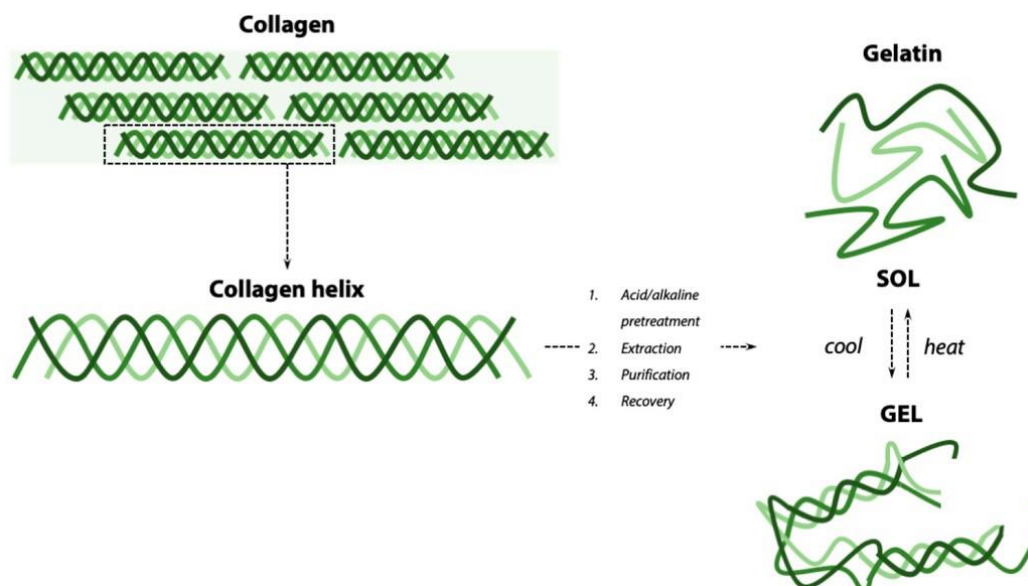


Figure 2.9 Schematic denaturation and sol-gel transition process of gelatin [81].

2.2.1.2 Soy protein

As an alternative to animal protein, soy protein has been extensively used in the food industry [84]. Soy protein isolate is the most refined form of soy protein with a high protein content (>90%) [85]. It has attracted high attention due to its abundant nutritional content and excellent functional properties [86]. Soy protein isolate is prepared from an alkaline extraction of the soybean white flakes flour, then followed by an acid precipitation at pH 4.5. After centrifugation, the final product is obtained by neutralization with an aqueous basic solution at pH 6.5-7.0 and then spray-dried [87].

Soy protein hydrolysate, another form of soy protein, can be produced by acid, alkaline or enzyme hydrolysis [88]. Due to its high solubility and protein digestibility with shortened amino acid chain, soy protein hydrolysate is becoming more popular than soy protein isolate especially among the individuals who have protein digestion disorders [89]. Clinical trials have shown that consumption of higher amount of soy protein may decrease the risk of cardiovascular disease and certain cancers [85, 90]. Its plant origin also provides more options for people who are allergic to dairy products or vegan [91].

2.2.1.3 Whey protein

Nowadays, whey protein has moved to the forefront of functional foods, owing to its low-cost, high protein content and fast absorption [92]. Figure 2.10 shows the constituents, different form and applications of whey protein. Normally, it is supplied in the form of whey protein concentrate (~80% protein) and whey protein isolate (~90% protein) [93]. Whey protein isolate (WPI) is the purest processed product by removing lactose and fat [93]. The most common methods to WPI manufacture is multiple membrane technology and ion exchange chromatography [94]. WPI is a globular protein consisting of β -lactoglobulin (60–70%), α -lactalbumin (12–25%) and some minor ingredients such as bovine serum albumin (5%), which contribute to various functions and applications as described below [92, 95]:

- 1) Muscles build up: contain certain types of amino acids which are beneficial muscles development;
- 2) Nutritional supplement: fill the protein gap fast if the daily protein consumption isn't enough;
- 3) Healing promotion: promote skin and organ recovery;

4) Immunomodulation: enhance immunity due to the immunoglobulins in the lactoglobulin fractions.

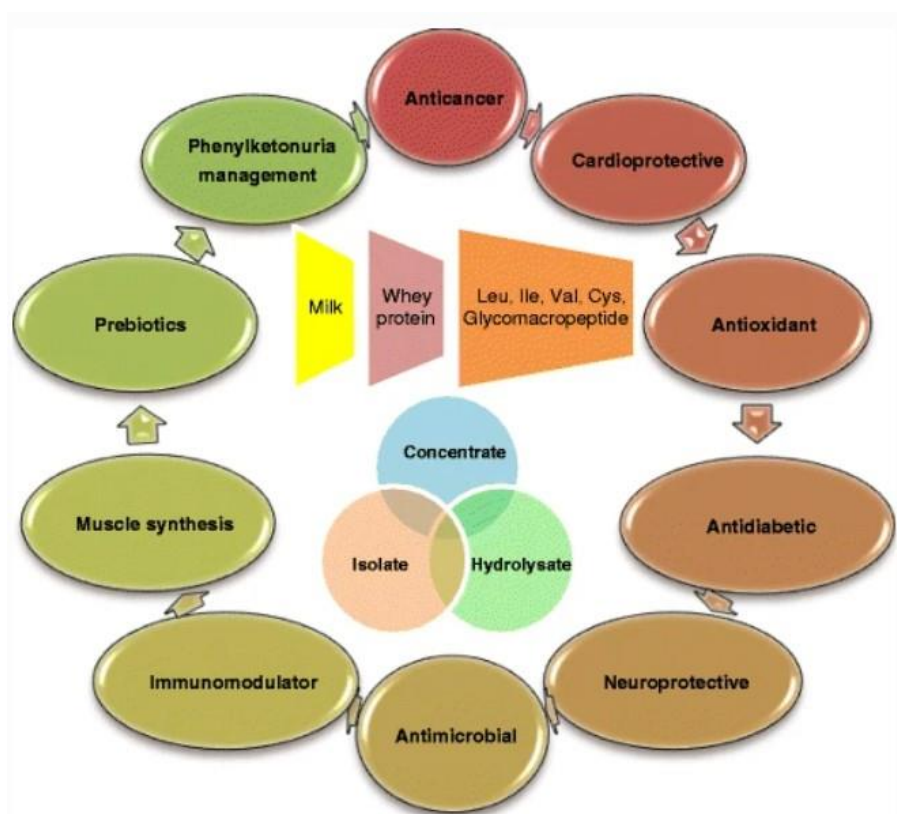


Figure 2.10 Composition, functions and applications of whey protein [95].

2.2.2 Polysaccharides

Besides proteins, polysaccharides are another family of biopolymers which are increasingly important in different applications such as food, cosmetics, pharmaceutical, medicine, packaging, and so on [96]. They are composed of at least 20 repeating monosaccharide units conjugated by glycosidic bonds [97]. The degree of polymerization (DP) represents the number of monosaccharide units in the polysaccharide. According to the component units, polysaccharides can be classified as homo-polysaccharides or hetero-polysaccharides [98].

The functional properties can be effectively affected by several molecular properties of the polysaccharides such as molecular weight, DP, charge, flexibility [99], to name a few. It is worth noting that the charge property of polysaccharides plays a very important role and it can be adjusted by controlling the pH value [100]. Generally, there are neutral (cellulose, starch, etc.),

negatively charged (xanthan gum, carrageenan, etc.) and positively charged (chitosan) polysaccharides, depending on the functional groups along the chain background and environmental conditions [100]. Anionic polysaccharides show neutral property at pH values below their pK_a but negative above, while cationic polysaccharides will be positively-charged below pK_a and neutral above their pK_a [101]. Different from proteins, the monosaccharide arrangement in polysaccharides is more uniform, which gives particular functional properties, such as gelation, and solubility [102].

2.2.2.1 Xanthan gum (XG)

Xanthan gum (XG) is an anionic food-grade polysaccharide produced by *Xanthomonas campestris* during aerobic fermentation [103]. Figure 2.11a shows the primary structure of a xanthan gum molecule. It is a heteropolysaccharide composed of long chain repeating pentose pentasaccharide units. XG consists of 1,4-linked β -D-glucose cellulosic backbone with charged trisaccharide side chains [104]. One D-glucuronic acid unit is positioned between two D-mannose on the trisaccharide side chains. A pyruvate group is linked to the terminal D-mannose at C4- and C6- positions, while another non-terminal D-mannose unit is usually linked with an acetyl group at C6 [105]. Due to the carboxyl groups on these acetate and pyruvate groups, XG demonstrates an anionic nature [103].

In aqueous solution, XG exhibits a fivefold ordered helix structure [106]. This stable form can protect XG from depolymerization, thus making its viscosity stable over a wide range of temperature and pH [107]. Besides, XG also has exceptional rheological properties. Due to its high molecular weight (10^6 - 10^7 Da) and rigid helical structure, it shows non-Newtonian and a strong shear-thinning behavior, which also benefit the flavor release and mouthfeel in food products [106].

Figure 2.11b demonstrates various applications of XG. Owing to its outstanding characteristics of non-toxic, thermal stability and biocompatibility, it has already been widely used in various applications such as food, agriculture, medicine, and so on [103, 104].

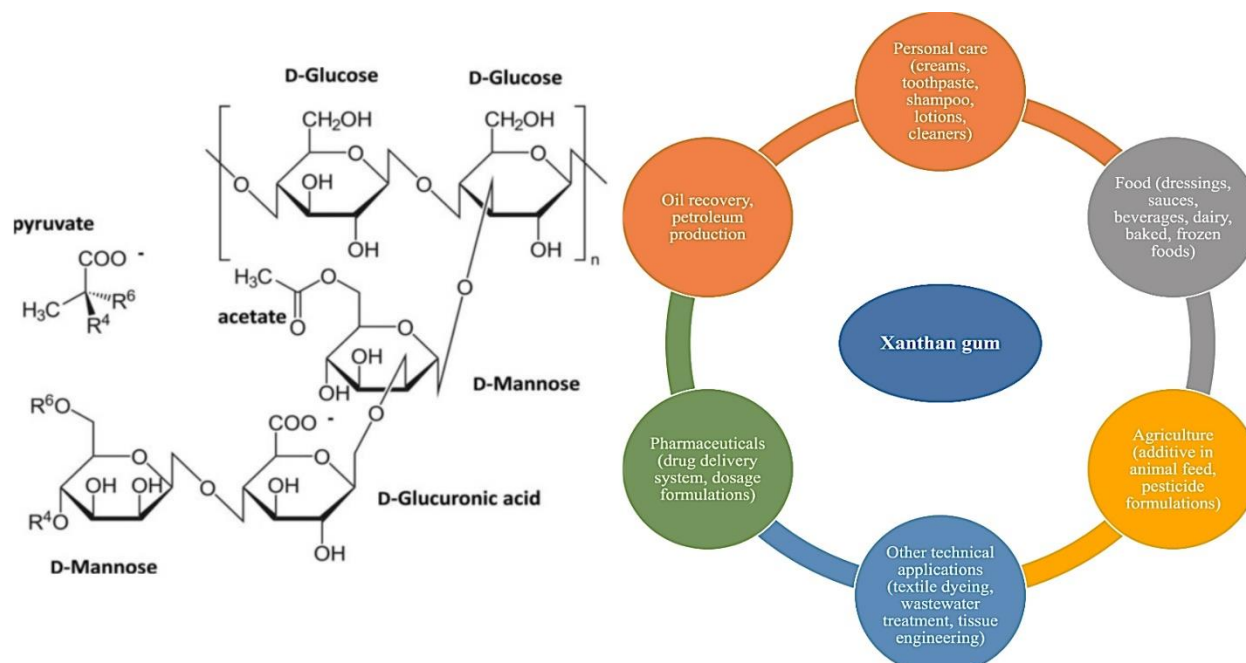


Figure 2.11 a) Molecular structure of xanthan gum molecule [103]; b) various applications of XG [103].

2.2.2.2 Cellulose nanocrystals (CNCs)

Cellulose is the most abundant macromolecular polysaccharide in nature, consisting of repeat anhydroglucose units ($C_6H_{10}O_5$) [108, 109]. Nanocellulose can be divided into cellulose nanocrystals (CNCs), cellulose nanofibers (CNFs) and bacterial nanocellulose (BNC), according to the dimensions, extraction method and nature[109].

The traditional method of CNC extraction is sulfuric acid hydrolysis [110]. Figure 2.12 shows schematically the CNC extraction process from the original natural source to the final CNC product. The original raw materials can be from agricultural products, office waste (paper, pencils) and microorganisms. Firstly, cellular walls containing cellulose fibrils are purified from the original source. Then the microfibrillar and the crystalline parts can be obtained through mechanical treatment. Acid hydrolysis is commonly performed to extract the CNC by destructuring the amorphous region within the pretreatment cellulose, leading to rigid rod-like CNCs [108-110].

Due to the advantages of non-toxicity, high crystallinity and large specific surface area, CNCs have already been used in many applications, such as food packaging and Pickering emulsion

stabilizer [109]. Specifically, as it can be extracted from some edible products, such as walnut shell [111], CNC can be a great candidate of ingredient in the food industry. Figure 2.13 presents the examples of application of CNC in the food industry.

However, as a nano scale material, CNCs still have some unknown risk to be applied directly in food products. So far, although CNCs have not been approved by the FDA (Food and Drug Administration) yet, it has been regulated as “Generally Recognized As being Safe” (GRAS). Moreover, the CNC from CelluForce company received regulatory clearance in Canada’s Domestic substances list (DSL) and Toxic Substances Control Act (TSCA) regulations in the US [108, 110]. In recent years, several studies have also focused on CNC safety. For example, 0.75 % w/w ingested CNCs showed almost no cytotoxicity in a cellular model of the intestinal epithelium [110]. Further studies focused on the nutritional properties and regulation for food application of CNC should be explored in the future [108].

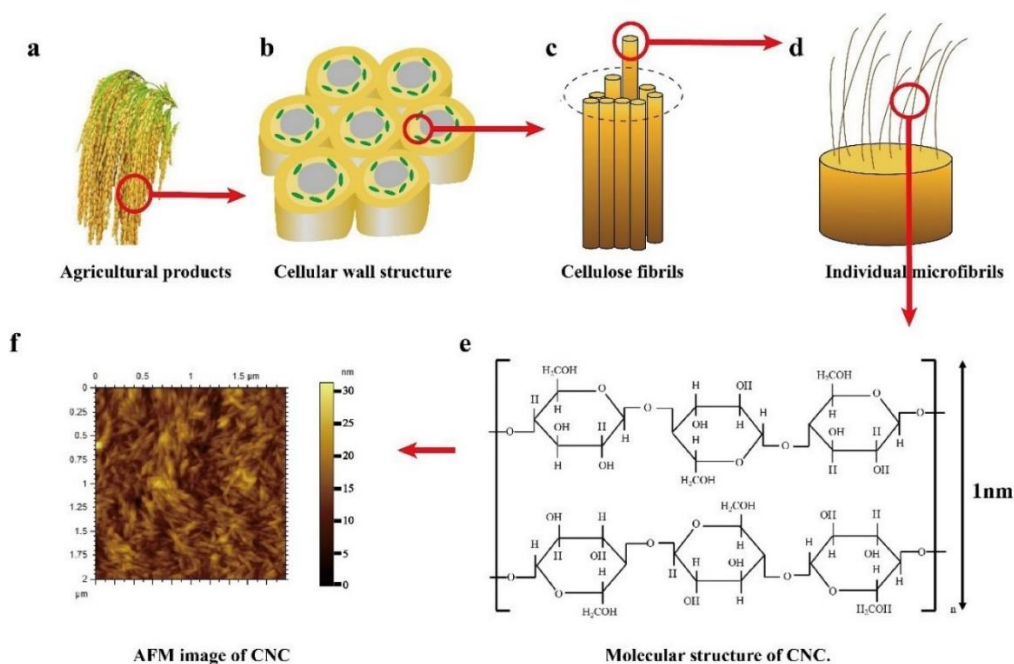


Figure 2.12 CNC extraction process from natural source [108].

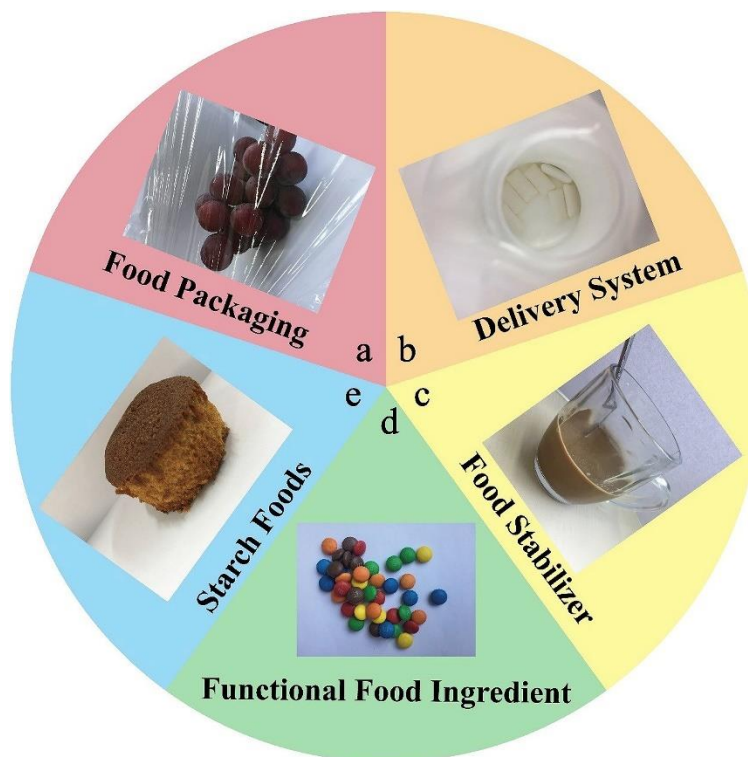


Figure 2.13 Applications of CNC in food engineering [108].

2.2.3 Proteins/polysaccharides gels

Generally speaking, protein and polysaccharide molecules can link to each other by both covalent bonds and physical interactions (non-covalent interactions like electrostatic and hydrophobic interactions, hydrogen bonding, steric exclusion, etc.) [112]. The amino groups from proteins and carboxylic groups from polysaccharides interact and then form the amide covalent bond in the presence of a chemical crosslinker. It is usually strong and permanent, which will not change by pH value and ionic strength [113]. On the other hand, the physical interaction is caused by ionic or non-ionic interactions [114].

Two different types of interactions can occur through the mixing of proteins and polysaccharides, namely associative phase separation (thermodynamic compatibility) and segregative phase separation (thermodynamic incompatibility), depending on the different biopolymers used and the environmental conditions such as pH, ionic strength and temperature, etc. [115]. Three different types of protein/polysaccharide mixed gel structures can be formed: interpenetrating, coupled and phase separated networks (Figure 2.14) [115].

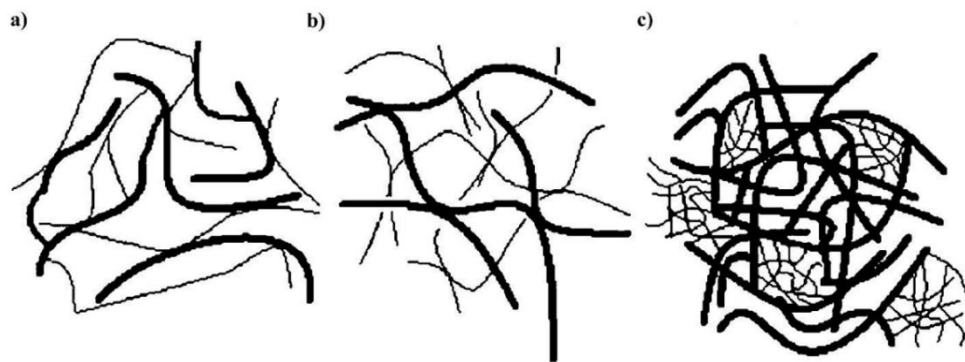


Figure 2.14 Network structures: (a) coupled network, (b) interpenetrating network, (c) phase-separated network [116].

2.2.3.1 Interpenetrating networks

Interpenetrating networks are the result of two polymers (proteins and polysaccharides) which contain two continuous independent networks, mixed with minimal bonding to each other [117]. Bovine serum albumin (BSA)/LM-pectin mixtures in the presence of calcium ions is a typical pair of interpenetrating networks [118]. The weak protein network formed by heating and the LM-pectin network formed by the presence of calcium ions can interpenetrate each other upon cooling [119].

2.2.3.2 Phase-separated networks

Phase-separated networks refer to the demixing between protein and polysaccharide phase under segregative conditions due to repulsion or/and hydrogen bond [120]. Phase-separated is the most common outcome among different mixed gel structures. In this case, the local polymer concentration will be increased, leading to gelation which halts the phase separation process [121]. The final structure could be controlled by the relative rates of phase separation and gelation process [122]. As its balance is mainly related to the electrical charges of the biopolymers, it can be adjusted by several factors including ionic strength, pH, heating/cooling kinetics [123]. Owing to the longer time to denature proteins, low heating rates could enhance phase separation compared to faster heating rates [124]. Protein phase will be distributed in large spherical zones with lower heating rates, which is better than being dispersed in small spherical zones with a faster heating rate [125]. Besides, with a pH value which is far away from the pI, it will extend the phase separation time with the increased electrostatic repulsion between proteins

and polysaccharides [126]. During the phase separation process, the water availability is reduced, which results in the components concentrating. Hence, it allows the biopolymers to form a gel at lower concentration than normal [127, 128]. Such protein/polysaccharide systems with phase separated gels have already been studied in the last three decades and been used in food engineering, such as whey protein (WP)/xanthan gum [129] and WP/kappa-carrageenan [130], to name a few.

2.2.3.3 Coupled gel networks

When two biopolymers are linked together through junction zones, coupled gel networks are formed through electrostatic associative conditions under several specific conditions such as slow acidification and quiescent conditions [131]. Even though the path of the gelling process is similar with complexes and coacervates, the soluble complexes connect with each other by interactions in order to form a network structure instead of coarsening and phase separation [132]. Most of polysaccharide-protein mixtures will give rise to form phase-separated gels. Thus, only few studies have focused on the formation of coupled gels in protein-polysaccharide mixtures. However, there are still some reports which have been well documented that certain proteins and polysaccharides will form coupled gel networks [133]. β -Lactoglobulin and xanthan gum was the first pair which has been investigated [134]. A gelation mechanism for gelatin/polysaccharide (i.e. XG or chitosan) was proposed by Wang et al. [115]. Some other protein-polysaccharide systems such as bovine serum albumin/xanthan gum [135], WPI/carrageenan and caseinate/gellan gum [136], have also been investigated.

2.2.4 Rheology of protein/polysaccharide gel inks for extrusion-based 3D printing

Regarding food applications, rheological properties are extremely important, since they are closely related to the printing process and post-deposition behavior. Several parameters should be considered for a successful printing:

- 1) Inks should have an appropriate viscosity to be extruded through the nozzle;
- 2) Inks should have sufficient self-supporting properties to prevent the printed stacked layers from sagging and collapsing;

3) Printed filaments should be stable enough to keep a good resolution of the printed structures.

Additionally, rheological properties also play an important role in the chewing/swallowing process, which is critical for dysphagia diet design. Hence, better understanding of the rheological properties of protein/polysaccharide gels gives numerous support in developing the mixed systems.

2.2.4.1 Viscosity

In the extrusion-based DIW process, the viscosity of ink should be low enough to be extruded through the nozzle during the printing process and high enough to retain its shape after deposition, which corresponds to shear-thinning materials that recover quickly. Among various shear-thinning models, the most common one of a viscoelastic material is the power-law model [137]:

$$\eta = K\dot{\gamma}^{(n-1)} \quad (2.1)$$

where η is the viscosity (Pa.s), $\dot{\gamma}$ is the shear rate (s^{-1}), K is the consistency index ($Pa.s^n$) and n (dimensionless) ($0 < n < 1$ for shear-thinning fluids) is the flow index.

The steady shear viscosity is obtained as a function of shear rate and usually measured by a rotational rheometer. However, in a higher shear rate region, the materials might be separate from the measuring plates and lead to unstable data. A method using capillary rheology to evaluate the process-related apparent viscosity in 3D printing, and proposed by Bruneaux et al. [138], can be applied in this situation.

The process-related shear stress can be calculated from:

$$\tau_w = \Delta P \cdot \frac{R}{2L} \quad (2.2)$$

where ΔP is the pressure gradient, R is the nozzle radius and L is the length of the nozzle. In order to calculate the process-related wall shear rate $\dot{\gamma}_w$, the Newtonian shear rate should be calculated first from:

$$\dot{\gamma}_{Newt} = \frac{4Q}{\pi R^3} \quad (2.3)$$

where Q is the volumetric flow rate. In case of non-Newtonian fluids, the Weissenberg-

Rabinowitsch correction should be applied in order to take into consideration a non-parabolic flow velocity profile for the wall shear rate $\dot{\gamma}_w$:

$$\dot{\gamma}_w = \frac{\dot{\gamma}_{Newt}(3n + 1)}{4n} \quad (2.4)$$

where the parameter n could be obtained by:

$$n = d\log(\tau_w)/d\log(\dot{\gamma}_w)$$

Hence, the process-related apparent viscosity can be calculated from the process-related shear stress and process-related shear rate as described below:

$$\eta_{app} = \frac{\tau_w}{\dot{\gamma}_w} \quad (2.5)$$

In some cases, the end effects should also be considered through the Bagley correction. It has been reported that the pressure entrance loss can be ignored for L/D larger than 50 [139].

2.2.4.2 Oscillatory rheology

Viscoelastic materials should have sufficient self-supporting properties to maintain their shape after deposition. Small amplitude oscillatory rheology is a common method to investigate the viscoelastic response of inks to dynamic shear stress. Storage modulus (G') represents the elastic component which reflects the energy stored in the network during deformation, while loss modulus (G'') represents the viscous component reflecting energy dissipated [140]. Another important viscoelastic parameter, loss tangent ($\tan \delta$), which is calculated by the ratio of G'' and G' , expresses the relative contribution of viscous response to the viscoelastic properties of materials [141]. Solid-like properties show a $\tan \delta$ lower than 1 whereas liquid-like properties exhibit a $\tan \delta$ greater than 1.

In order to investigate the viscoelastic properties of inks, several dynamic tests should be considered:

- 1) Strain sweep could help to find the linear viscoelastic range of a network, in which the values of G' and G'' for a range of strain are constant.
- 2) Frequency sweep illustrates the dependency on frequency of G' , G'' and complex viscosity, with which the four main different types of materials could be distinguished (Figure 2.15) [142]: diluted solution (e.g. milk [143], juice [144]), entangled solution (e.g. polybutadiene

solution [145], poly (vinyl alcohol) solution [146]), strong gel (e.g. concentrated micellar casein [147], jelly) and weak gel (e.g. mayonnaise). Regarding strong gels, G' and G'' are almost independent of frequency, and the magnitude of G' is often much higher than G'' (~10 times). In contrast, the two moduli are little dependent of frequency for weak gels, and G' is < 10 times higher than G'' .

- 3) Temperature sweep helps to provide useful information for some temperature-responsive materials, such as the helix-coil transition temperature of gelatin [142].

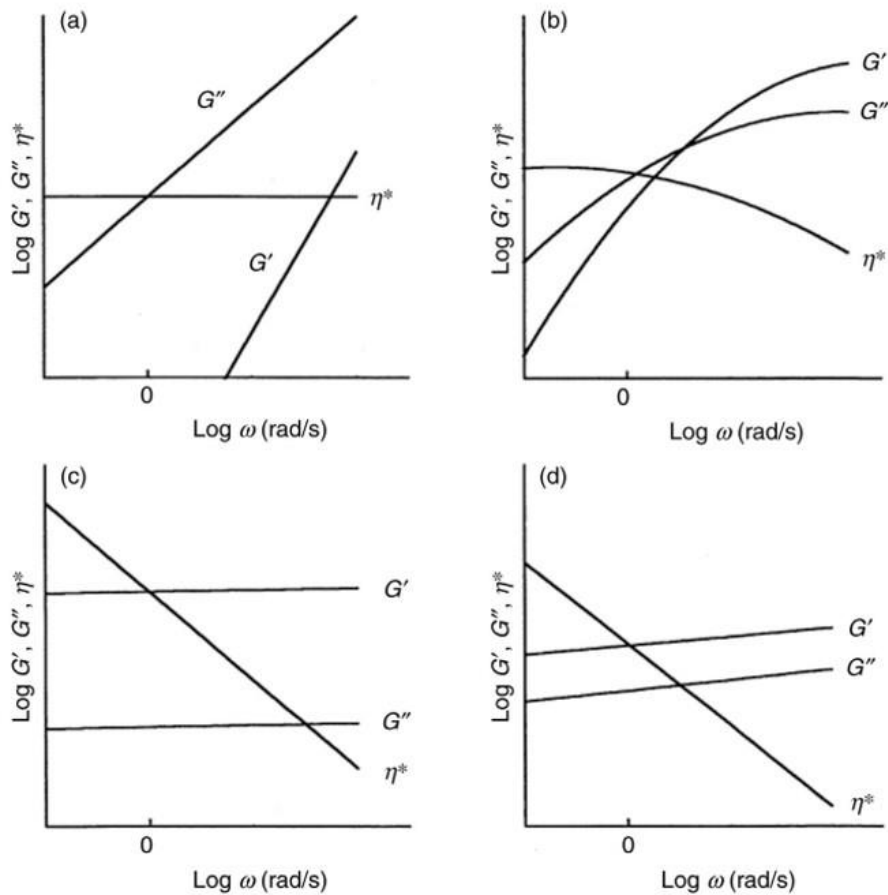


Figure 2.15 The relationship among G' , G'' and complex viscosity of four main type of materials: a) dilute solution, b) entangled solution, c) strong gel and d) weak gel [142]

Table 2.4 summarizes the latest published works reporting the effects of rheological properties of different ink materials on 3D food printing.

Table 2.4 Summary of latest studies of rheological properties of various ink materials for 3D food applications (abbreviations defined below)

Raw materials	Rheometer settings	Effect of rheological properties	Reference
Egg yolk	Parallel plates, diameter of 60 mm, gap of 1 mm, 25°C	Egg yolk showed $G' > G''$ and shear-thinning behavior when heating at 76°C for 8 min	L.Xu <i>et al.</i> [148] (2020)
Gluten wheat flour, sugar, butter and potato granules	Parallel plates, diameter of 25 mm, gap of 1 mm, 20-35°C	When K was 1000-1800 Pa.s and n was 0.15-0.3, food inks showed good printability	Q.Liu <i>et al.</i> [149] (2020)
SPI, soybean oil, GG and XG	Parallel plates, diameter of 40 mm, gap of 1 mm, 25°C	SPI ink with 0.5 wt% XG addition showed a higher viscosity and a higher $G' > 1000$ Pa than with 0.5 wt% GG addition, leading to a better flow behavior and self-supporting property	J.Yu <i>et al.</i> [150] (2022)
PPP, SPI and PPC	Serrated parallel plates, diameter of 20 mm, gap of 1 mm, 20°C	$G' < 103$ Pa or > 105 Pa would be too weak to self-support or too stiff to be printed	E.Alvarez-Castillo <i>et al.</i> [151] (2021)
Porcine longissimus muscles and back fat, KG	Parallel plates, diameter of 40 mm, gap of 1 mm, 24°C	KG addition improved the rheological properties with higher viscosity, a more significant shear-thinning behavior with lower n and higher G'	J.Xu <i>et al.</i> [152] (2021)

SPI: soy protein isolate, XG: xanthan gum, GG: guar gum, PPP: porcine plasma protein, PPC: pea protein concentrate, KG: κ -carrageenan

2.3 Summary

As reviewed above, using the 3D printing technique in food application has attracted growing attention from scientists and food industries. 3D food printing provides opportunities for the improvement of food design, personalized ingredients and texture control. Several 3D printed food materials have already entered the food market (confectionery market), such as sugar [153], cheese [154] and chocolate [10]. Figure 2.16 shows some examples of commercial customized dessert designs by 3D food printing. 3D food printing also shows great potential in food applications in some special areas. The first beneficiary group is the elderly people, who need to consume special diet based on age, physical condition and nutrition requirements. As discussed in Section 2.4.1, a few studies have already been focused on 3D printing for the dysphagia diet. There is also a strong potential for the integration of 3D food printer with other blood sensors to adapt the food or medications according to the patient needs in real-time [155, 156]. In addition, 3D printing in military and space food (e.g. US Army and NASA (National Aeronautic and Space Administration)) have also attracted a great deal of attention, especially for the fundamental food ingredients such as proteins, carbohydrates and fat [10, 157]. Two of the important problems for the traditional military and space food are the short shelf-life and unstable nutrition. With the help of 3D food printing, the micronutrient have easier conditions to be stored in raw material form. It can be then mixed with water or other food additive in order to modulate the texture, mouthfeel and flavor before printing [10].



Figure 2.16 Customized dessert designs using 3D food printing technique: a) chocolate cats; b) candies; c) sugar designs; e) chocolate roses [10].

Hydrocolloids based on proteins and polysaccharides are interesting model food systems and enable to control the nutritional and textural functions. Extensive investigations have already concentrated on the gelation properties of aqueous mixtures of protein/polysaccharide systems. Also, different foods have already been considered for 3D printing. However, there is still a gap in 3D food printing of protein/polysaccharide mixtures due to insufficient research on their rheological properties. Hence, a systematic investigation of the relationships between processing and ink material properties should be carried out to progress further in 3D food printing, in order to propose process guidelines to assess printability.

CHAPTER 3 RESEARCH OBJECTIVES AND COHERENCE OF ARTICLES

3.1 General objective

The main objective of this thesis is:

To develop novel inks based on protein/polysaccharide mixed gel systems for 3D food printing and to explore the relationship between the rheological properties, printability as well as the post-deposition behavior.

3.2 Specific objectives

To achieve the main objective, the following three specific objectives are addressed:

- 1) To develop a gelatin B/xanthan gum aqueous mixture-based food ink for DIW 3D printing towards food applications.
- 2) To establish a comprehensive investigation on properties of protein/polysaccharide mixed gel inks, in order to derive a guideline to predict their printability.
- 3) To fabricate CNC-enhanced protein/polysaccharide 3D printed complex structures that can be used for dysphagia diet design.

3.3 Presentation of articles and coherence with research objectives

The scientific contributions of the current work are presented in the form of three peer-reviewed research journal papers, which cover the research objectives and correspond to Chapters 4, 5 and 6.

Chapter 4 presents the first paper “*Development of aqueous protein/polysaccharide mixture-based inks for 3D printing towards food applications*” that has been published in *Food Hydrocolloids* (Vol. 131, October 2022, 107742, IF=11.504 in 2023). This journal was chosen because it is one of the top journals focusing on innovative research on food hydrocolloid materials. In this work, a food-grade aqueous GB/XG mixture was successfully developed to print 40-layers 3D scaffolds with defined features by the DIW technique (specific objective 1) based on the interactions between GB and XG. The effects of calcium ion (Ca^{2+}) addition and

storage temperature on rheological properties of the inks, and dimensional stability and textural properties of printed scaffolds were investigated.

Chapter 5 presents the second paper “*Processing and properties of aqueous protein/polysaccharide mixture-based inks for additive manufacturing*” that has been submitted to *Additive Manufacturing* (IF=11.632 in 2023) in June, 2023. This journal was chosen because it offers a broad platform for novel high quality research on additive manufacturing. In this work, a comprehensive study of two water-based protein/polysaccharide food inks, i.e., GB/XG and SP/XG, for DIW printing was carried out (specific objective 2). The processing maps, which were supported by the rheological properties of the inks, were derived for both GB/XG and SP/XG systems by considering nozzle diameters and XG content for the fabrication of different geometries (specific objective 2). A shape fidelity map was also generated to show the proper ranges of viscoelastic properties of inks for the fabricated 3D structures with high shape fidelity. Furthermore, the effect of protein type and XG content on the textural properties was also characterized in the context of food applications.

Finally, Chapter 6 presents the third paper “*3D printed protein/polysaccharide food simulant for dysphagia diet: impact of cellulose nanocrystals*” that was under review by *Food Hydrocolloids* (IF=11.504 in 2023) in June, 2023. This journal was chosen because it publishes advanced research concerned with rheological and textural properties of functional food hydrocolloids. In this work, two CNC-based protein/polysaccharide food inks, i.e., GB/XG and WPI/XG, were developed towards 3D printed dysphagia diet design (specific objectives 3). The effect of CNC concentration and post-processing temperature on shape fidelity, dimensional stability and textural properties was thoroughly studied, and was correlated with the rheological properties of the food inks. Most importantly, the suitability of these food inks for the dysphagia diet was investigated.

CHAPTER 4 ARTICLE 1 : DEVELOPMENT OF AQUEOUS PROTEIN/POLYSACCHARIDE MIXTURE-BASED INKS FOR 3D PRINTING TOWARDS FOOD APPLICATIONS

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4.1 Abstract

Three-dimensional (3D) food printing is a promising technique as it allows to create elaborated food constructs or customized food for elderly people and other sensitive population (i.e. children, pregnant women or athletes). The structural and textural properties of food constructs depend strongly on the ingredients used and the shape produced. Two important biological macromolecular components, namely protein and polysaccharides, are the most important in terms of nutritional content. In this work, we developed a water-based protein/polysaccharide food ink from a mixture of gelatin B (GB) and xanthan gum (XG) for 3D printing. A 40-layer 3D scaffold with defined features was successfully printed at room temperature using an aqueous mixture of 3 wt% GB and 10 wt% XG. Adding 37.5 mM calcium ions (Ca²⁺) or lowering the storage temperature to 4 °C improved the rheological properties of the ink, which endowed the scaffold with higher shape fidelity right after printing and good shape retention for at least 96 h.

Texture profile results revealed that the infill percentage of the scaffold is critical to textural properties of the scaffold, highlighting the advantage of 3D printing in tuning sensory food perception.

Keywords: 3D food printing; Gelatin; Xanthan gum; calcium salt; rheological properties; textural profile results

4.2 Introduction

Three-dimensional (3D) food printing is an emerging manufacturing technology using the additive principle through computer-controlled layer-by-layer deposition of materials [1]. It enables the production of customized food designs and creative food flavors with personalized nutrition according to individual health conditions [2, 3]. Additionally, 3D food printing technology is a convenient production process [4]. It allows to fabricate complex food design with precise temperature control during the printing process [5] and with relatively less material loss [6]. Therefore, it has the potential to replace some of the more cumbersome processes of traditional food production methods [4, 7].

The first reported 3D printer goes back to 2007 at Cornell University where the researchers developed the “Fab@Home” model [8]. Since then, researchers have developed different printing techniques, such as extrusion-based printing, selective laser sintering and inkjet printing etc.[9]. Direct-ink writing (DIW) is a 3D printing technique employing a computer-controlled translation stage, which moves an ink-deposition micro-nozzle, to generate materials with controlled design and composition. It is an adaptable approach which offers flexibility requirement for soft materials, such as colloidal suspensions, fugitive ink and hydrogels. So far, several food inks including chocolate [10], pasta [11], mushroom [12], cheese [13] and fish gel [14] have been successfully printed using the DIW technique. Different mechanisms have been employed to facilitate shape retention during post extrusion, including solvent evaporation, gelation, temperature control and UV polymerization. However, there is still a lack of fundamental knowledge on 3D printing of essential constituents of food [1, 4, 15].

Proteins and polysaccharides are two important biological macromolecules in food systems, which perform complementary nutritional, structural and textural functions [16, 17]. Attractive (biopolymers with opposite charges) and repulsive interactions (biopolymers with same or no charge) can occur depending on the environmental pH and ionic strength [18]. Electrostatic

forces and hydrogen bonding are usually the dominant interactions between the two components, while other weak interactions should also be considered such as van der Waals forces and hydrophobic interactions [19]. Different functional attributes can be provided by these interactions, such as the ability to thicken solutions, form gels or emulsions [20].

Gelatin, a linear protein, is obtained by either acid (type A) or alkaline (type B) hydrolysis of the parent protein collagen, which is derived from animal skin and/or bones [21]. It has a helix-to-coil transition temperature, below which the random coils assemble into triple helices, resulting in gelation via forming a 3D network [22]. Xanthan gum (XG) is an anionic food-grade polysaccharide produced by aerobic fermentation, which is composed of a β -(1-4)-D-glucose main chain and a trisaccharide branch (D-glucose, D-mannose and D-gluconate) as side chains [23]. The XG molecules undergo a thermal-reversible coil-helix transition in solution, and the transition temperature can be shifted to higher temperatures by the addition of electrolytes [24] or proteins [25]. XG is widely used in food engineering owing to its shear-thinning properties as well as the resistance against heat, acids, bases, salts and even enzymes [26, 27]. Moreover, required food properties can be easily adjusted by XG addition, such as viscosity, texture appearance, etc. [28, 29]. Although XG is a non-gelling polysaccharide, it can form a gel in the presence of trivalent ions or when mixed with other polysaccharides, or even proteins [17, 25, 27, 30, 31].

Several food inks have been developed so far. For example, gel formed by low methoxylated pectin (an anionic polysaccharide) has been used to print edible objects with DIW [6]. The printed 3D structure can be greatly stabilized with the addition of CaCl_2 during and after printing, due to ion bridging effects. The 3D printability of a mixture of gelatin and kappa-carrageenan was investigated by Warner et al.[32]. It was found that the addition of kappa-carrageenan enabled better control of the printing process compared to pure gelatin and they attribute it to the increase of the gelling temperature.

Synergistic gelation properties have been previously reported for gelatin B (GB) with XG in aqueous solution at a pH around the isoelectric point of GB ($\text{pI} \sim 5.3$) [26]. In addition, the gel preparation process does not involve thermal, enzyme or any other denaturing treatment, which is favorable for protecting micronutrients in food. It provides a promising way to fabricate group-

specific food. However, up to now, there is no specific study in the literature on 3D printing of GB/XG mixed gels with careful parameter control and detailed structure analysis.

In this work, we aim to shed light on the 3D printing of a protein/polysaccharide mixed gel system for food application. 3D printing of a mixture of GB and XG based on the DIW method is presented and analyzed in terms of the interactions between GB and XG. The effects of Ca^{2+} addition and storage temperature after printing on shape retention, gel strength and textural properties are investigated in order to assess the printed object quality.

4.3 Materials and methods

4.3.1 Materials

Gelatin (type B, G6650, bloom index ~ 225 , molecular weight: 20-25 kDa, pI = 4.7-5.3) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were purchased from Sigma-Aldrich Canada. XG (KELTROL RD, food-grade quality, molecular weight: $\sim 10^3$ kDa) was supplied by CP Kelco U.S. All other reagents and chemicals were of analytical grade and used without further purification.

4.3.2 Food ink preparation

GB solutions (3 wt%) were prepared by allowing the GB to swell in Milli-Q water (18.2 Ω) for 15-20 min, followed by gentle magnetic stirring at 40 $^\circ\text{C}$ for 15 min. The solutions were used on the same day. 10 wt% XG dry powder was then added to the GB solution. The GB/XG mixed solution was mechanically stirred at a speed of 600-700 rpm for at least 12 h at 40 $^\circ\text{C}$ to ensure complete dissolution. The final pH of prepared solution was 5.8. For comparison purposes, a neat XG solution of 10 wt% concentration was also prepared. Ca^{2+} was added to the GB/XG aqueous mixture at concentrations that varied from 0, 12.5, 37.5, 62.5 to 75 mM, within the recommended intake by the Institute of Medicine (IOM) [33]. The pH did not change with Ca^{2+} addition. The ink was then centrifuged (5000 rpm, 1 h) at room temperature (22 $^\circ\text{C}$) to eliminate air bubbles.

4.3.3 3D printing process and 3D scaffold fabrication

The food ink was inserted into a syringe (3 mL, Nordson EFD) linked with a cylindrical micronozzle (exit inner diameter $D = 200$ μm , capillary length $L = 12.7$ mm, Nordson EFD). Then the loaded syringes were fixed to a high-pressure adaptor (HP-7x Nordson EFD), which was installed on a computer assisted 3-axis positioning stage (I&J2200-4, I&J Fisnar Inc.). The

40-layer 3D scaffold was printed by depositing filaments in a layer-by-layer manner with the GB/XG food ink, as well as with different concentrations of Ca^{2+} solution. All scaffolds were printed at a speed of 7 mm/s and under appropriate applied pressures at room temperature (22 ± 1 °C). The speed of 7 mm/s was chosen based on preliminary experiments as a compromise between shape fidelity and printing time. Specific applied pressures are reported in the legend of Figure S1.

4.3.4 Rheological measurements

In the low shear rate region ($0.01\text{-}10\text{ s}^{-1}$), steady-shear measurements of GB/XG aqueous mixtures were performed using a rough surface parallel plate flow geometry with 25 mm diameter with a stress-controlled rheometer (MCR-502, Anton Paar, Graz, Austria). The gap between the plates was 1 mm. A rough surface was chosen to prevent wall slip. Mineral oil was used to cover the edge of the sample in the parallel plate flow geometry to prevent water evaporation. The measurements were carried out at room temperature (22 °C).

In the high shear rate region, the process-related viscosity was calculated by capillary flow analysis (CFA) [34, 35]. Different food inks were loaded into the syringe (3 mL, Nordson EFD). A 60 mm filament was extruded onto a glass substrate through a micronozzle (exit inner diameter $D = 200\text{ }\mu\text{m}$, capillary length $L = 12.7\text{ mm}$, Nordson EFD) at a speed of 0.5 mm/s for 120 s with each food ink. The different pressures were set under a range between 70 and 900 kPa. The weight of each extruded filaments was then measured with a high-precision balance (GH-202, A&D Engineering) at room temperature, which was used to calculate the process-related viscosity according to the method reported by Bruneaux et al.[34].

The effects of Ca^{2+} and storage temperature after printing on the dynamic viscoelastic properties of the food inks were also investigated using the same geometry. Linear viscoelastic (LVE) regime was determined by strain sweeps in the range from 0.01 % to 100 % at angular frequency $= 1\text{ rad/s}$. Frequency sweeps were performed over a range of 0.1 to 10 rad/s within the identified LVE regime (strain = 0.2-5%). The storage modulus (G') and loss modulus (G'') were recorded using Rheocompass software (Anton Paar).

For samples stored at 4 °C, the lower plate was pre-cooled down at 4 °C using the temperature-controlled Peltier system before the test. All the rheological experiments were performed three times.

4.3.5 Morphological characterization

Optical images of 2D and 3D structures were captured using an optical microscope (BX-61, Olympus) and a digital camera (EOS TRBRL T4I, Canon). The images were then analyzed with Image Pro Plus 6.0 (Media Cybernetics).

4.3.6 Determination of dimensional stability of 3D-printed structures

The printed dimensional stability obtained using the different food ink formulations was evaluated by directly following the thickness variation of the as-printed 3D scaffolds up to 96 h. The images of the samples were recorded at 0, 24 and 96 h after printing, respectively. The thickness of the samples was measured using a caliper. All samples were put in petri dishes and then stored under a hood in order to maintain constant room temperature and humidity, or in a refrigerator at 4 °C.

4.3.7 Compression tests for textural profile analysis

Mach-1TM Micromechanical tester (Biomomentum Inc.) was used for a double-cycle compression tests to obtain load-time curves. A 25 mm diameter cylindrical probe was used to compress the printed 40-layers 3D scaffolds (18 mm × 18 mm) composed of GB/XG food inks with different Ca²⁺ contents and different infill levels (Figure 4.1a). The main test setting parameters are listed in Table 4.1. Triplicate measurements of samples were carried out. Figure 1b shows how the various properties of the textural profile test are measured and calculated. More explanations on the various properties measured are given in the Results and discussion section.

Table 4.1 Parameters used for the compression tests

Setting parameters	Value	Unit
Contact force	1	g
Contact speed	0.1	mm/s
Test speed	0.5	mm/s
Withdrawal speed	0.5	mm/s
Compression distance	2	mm

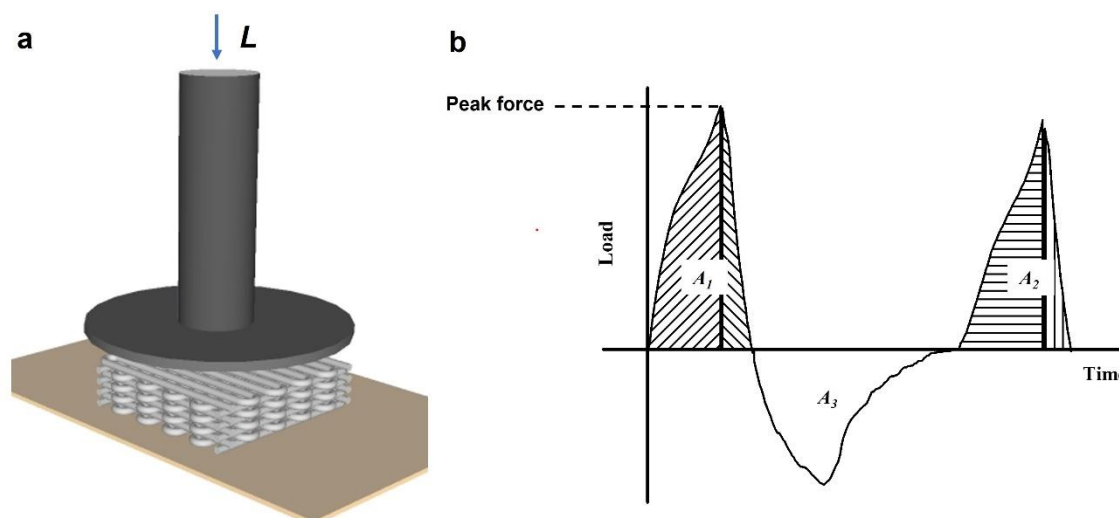


Figure 4.1 (a) Schematic representation of the compression test using the Mach-1TM Micromechanical tester (b) Textural profile diagram for gels. (Hardness: Peak Force; Cohesiveness: A_2/A_1 ; Adhesiveness: A_3 ; Gumminess: Hardness $\times$ Cohesiveness.)

4.3.8 Statistical analysis

Triplicate tests have been performed for all the tests mentioned above. The mean of the results $\pm$ standard deviation (SD) was used to present the data and analyzed by SPSS software (SPSS 20.0, IBM, Chicago, IL, USA). Significant differences ($p < 0.05$) between mean values of samples were determined using Duncan's Multiple Range test.

4.4 Results and discussion

4.4.1 3D printed structures

Figure 4.2a and b show the 3D printing process using the food ink based on GB/XG aqueous mixture and the scaffold model used, respectively. The effect of salt addition is observable when XG concentration is higher than 0.3% [36-38]. Considering a gel which can flow under applied pressure and maintain its structures after deposition, 10 wt% XG has been selected. Hence, a formulation of ink with 3 wt% GB and 10 wt% XG aqueous mixture has been obtained after carefully adjusting the composition. It allowed printing a scaffold with accurate features (Figure 4.2c). In comparison, the scaffold printed with a neat 10 wt% XG aqueous solution does not retain its structure after deposition, exhibiting a poor self-supporting behavior (Figure S4.1a). Hence, it is clear that the addition of GB imparts improved printability to the food ink. The

addition of Ca^{2+} enhances the shape retention of the scaffold printed by the food ink of GB/XG mixtures, as shown by the sharper edges (Figure 4.2c and d).

The top-view printability (Pr) (Equation 4.1) defined by Ouyang et al. [39] is used to quantitatively describe and compare the gelation condition and shape fidelity of the food inks. The Pr is calculated based on the circularity (C) (Equation 4.2) estimated by the perimeter L and pore area A (schematically shown in Figure 4.2e). When the Pr value is equal to 1, the interconnected channels of the filaments show a square shape, indicating an ideal gelation. Larger Pr value presents a greater gelation degree. Otherwise ($Pr < 1$), a lower gelation degree is noted. Figure 4.2e and f show the top-view images of the as-printed GB/XG scaffold with 0 and 37.5 mM of Ca^{2+} along with their Pr values. These values show that the printability of both food inks is within the proper range, while the one with Ca^{2+} addition is slightly closer to 1, indicative of better printability with this content of Ca^{2+} in the GB/XG food ink.

$$Pr = \frac{\pi}{4} \cdot \frac{1}{C} = \frac{L^2}{16A} \quad (4.1)$$

$$C = \frac{4\pi A}{L^2} \quad (4.2)$$

However, 62.5 mM Ca^{2+} addition (Figure S4.1d) causes irregular extrusion with structure defects. The poor structure quality is probably due to an over-gelation of the printed filament. Among the tested Ca^{2+} concentrations, the scaffold with 37.5 mM Ca^{2+} shows the best appearance (Figure 4.2d and Figure S4.1c).

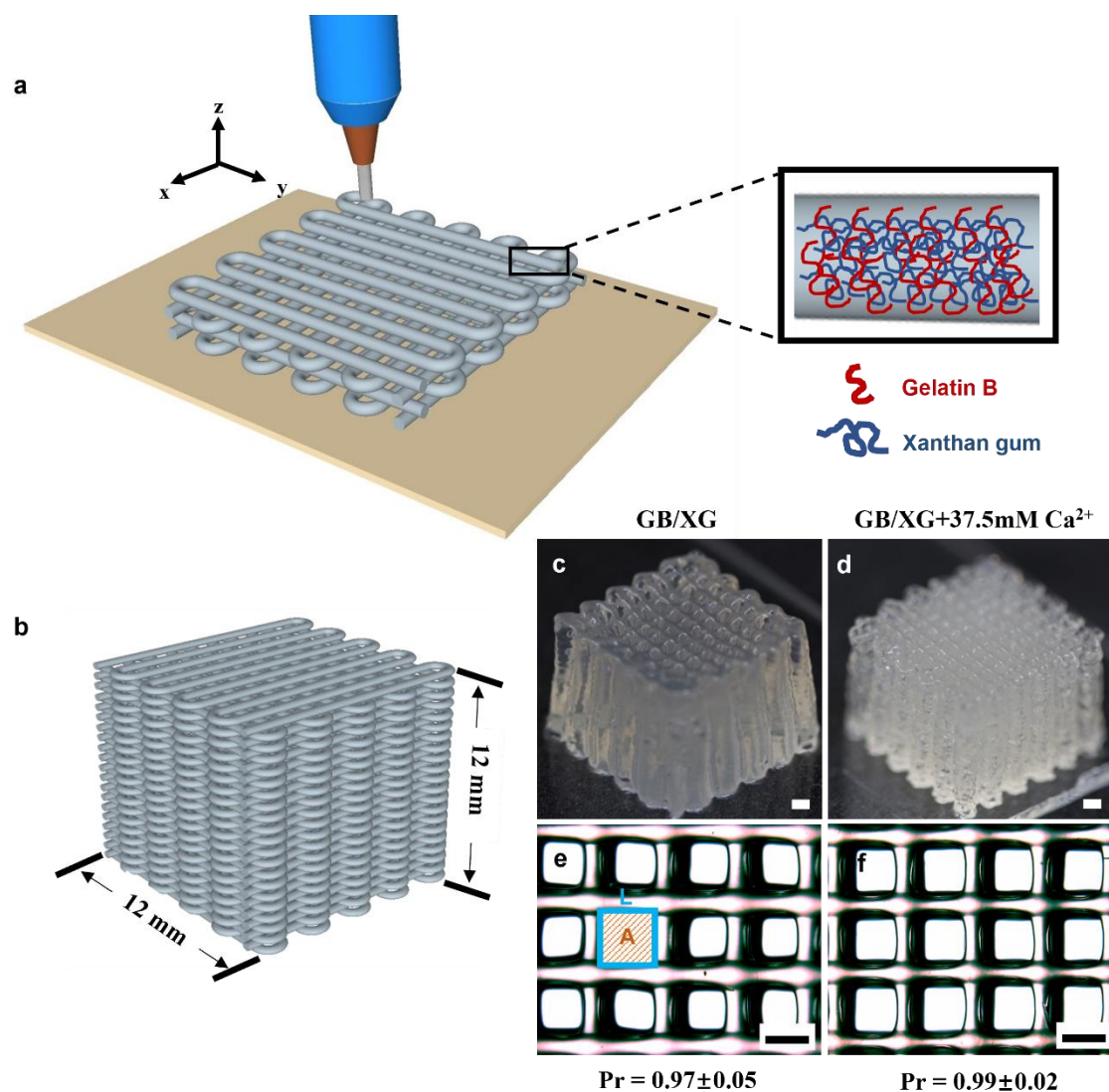


Figure 4.2 (a) Schematic representation of the 3D printing process with the food ink; (b) Model of the 40-layers 3D scaffold, (c) As-printed 40-layers GB/XG scaffold and (d) GB/XG + 37.5 mM Ca^{2+} scaffold using 200 μm nozzle; (e) Representative optical top-view image of a GB/XG scaffold and (f) GB/XG + 37.5 mM Ca^{2+} scaffold. All the photos and images were taken at room temperature right after printing. Scale bar is 1 mm. The printing time for a 12 mm $\times$ 12 mm scaffold is around 10 min.

4.4.2 Rheological properties of food inks

The rheological properties of the GB/XG ink are of critical importance since they are directly related to the extrusion process and ensure successful flow through the micronozzle and good post-deposition properties.

Figure 4.3 shows the shear viscosity of neat XG and the 3 wt GB/10 wt% XG aqueous mixture with different Ca^{2+} concentrations. The solid symbols in the low-to-moderate shear rate region ($\sim 0.01\text{-}10\text{ s}^{-1}$) represent the steady-shear viscosity of the food inks measured by rheometer at room temperature. The open symbols in the higher shear rate region ($> 10\text{ s}^{-1}$) show the process-related apparent viscosity of the food inks as a function of the process-related shear rate. These results were calculated from capillary flow analysis (CFA) [35] data obtained using a nozzle diameter of $200\text{ }\mu\text{m}$ when extruding the material at room temperature. All food inks demonstrated a shear-thinning behavior, which is favorable for the printing process. The viscosity decreases significantly with increasing shear rate, with a relatively low power-law index (n) (Table 4.2), typical of soft solids such as XG [40]. The data measured by rotational rheometer (RR) is generally coherent with the data calculated from CFA. However, as seen in Table 4.2, n values calculated from CFA are relatively higher than the results measured using the rheometer. The decreased shear rate dependency indicates that the structure of the food inks is already rather oriented or stretched in capillary flow [41, 42].

Compared with XG alone, the GB/XG food ink shows higher viscosity, which might be due to their synergistic interactions, which benefits the gelation of hydrocolloids. The electrostatic repulsions between GB and XG enhance the local concentration of XG. In addition, XG also interacts with the negative patches on GB by electrostatic attractions [26]. As shown in Table S4.1, the viscosity increased by approximately 46 % for food ink with GB addition at 1 s^{-1} . On the other hand, an increase in the viscosity of the GB/XG food ink was observed in the presence of Ca^{2+} , which is probably due to the bridging effect of Ca^{2+} with XG molecules [43-45] and/or negative patches on GB. With the increase of Ca^{2+} concentration, the viscosity at 1 s^{-1} of different food inks ranged from 158 ± 30 to $720 \pm 91\text{ Pa}\cdot\text{s}$ (Table S4.1). A maximum viscosity is obtained when Ca^{2+} concentration is 62.5 mM . Further increasing the Ca^{2+} concentration to 75 mM does not lead to a significant change in the viscosity. Therefore, an appropriate improvement in viscosity benefits the extruding ability of food ink and thus the printability.

Table 4.2 Power-law index of different food inks within two specific shear rate regimes (two methods)

Food ink type	Power-law index (n)	
	Low to moderate shear rate	High shear rate
	(RR)	(CFA)
Pure 10 wt% XG gel	0.18±0.02	0.27±0.07
GB/XG gel with no salt	0.16±0.03	0.25±0.07
GB/XG gel with 12.5 mM Ca^{2+}	0.14±0.03	0.24±0.04
GB/XG gel with 37.5 mM Ca^{2+}	0.12±0.03	0.22±0.02
GB/XG gel with 62.5 mM Ca^{2+}	0.12±0.02	0.21±0.03
GB/XG gel with 75 mM Ca^{2+}	0.12±0.02	0.21±0.03

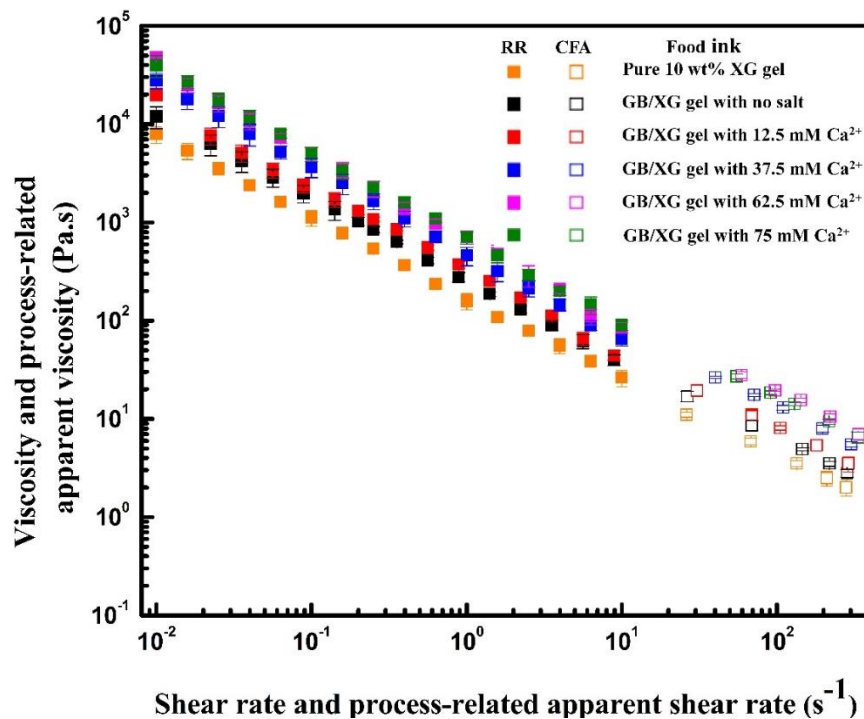


Figure 4.3 Viscosity as a function of shear rate for XG and GB/XG food ink with different Ca^{2+} concentrations at room temperature (22 °C) (solid symbols: data measured using a parallel plate flow geometry in steady shear (RR); open symbols: data obtained by capillary flow analysis (CFA)).

Linear viscoelastic properties of neat XG solution and GB/XG food ink with 0–37.5 mM Ca^{2+} addition were also measured to infer the post-deposition quality, and the results are shown in Figure 4.4. For all food inks, the linear viscoelastic regime is identified in-between a strain amplitude of 0.2–5% (Figure 4.4a). The storage modulus G' dominates the loss modulus in all the investigated strain and frequency ranges (Figure 4.4a and b). In addition, G' of GB/XG food ink without salt is almost 1.5 times larger than G' of neat 10 wt% XG. Adding Ca^{2+} to the ink leads to an increase of G' , possibly due to the bridging effects of Ca^{2+} [43–45], which enhances the gel strength of the gel formulation and also allows the corresponding ink to retain a high shape fidelity after deposition (Figure 4.2f). Comparing Ca^{2+} concentration ranging from 0 to 37.5 mM, G' gradually increased from 452 ± 26 to 1720 ± 68 Pa (380%) (Table S4.2). Similar observations of gel properties enhancement have been found for the gelatin-transglutaminase (TG) system [46]. The increases were almost 2 times in G' at 1 rad/s and viscosity at 1 s^{-1} at low content of TG solution (with a mass ratio of 0.002 (w/w) to gelatin powder) due to strong chemical crosslinking

between gelatin and TG as opposed to weaker physical crosslinking between gelatin and Ca^{2+} . Regarding the power-law parameter a on frequency dependency of G' (Table S4.3), it decreased when lowering the temperature, which resulted in a strengthening in gel structure. The decrease in frequency dependence indicated a more solid-like behavior when decreasing the temperature. Lowering the temperature to 4 °C favors the intra- and intermolecular hydrogen bonding formation [47], which boosts the formation of stiffer structures, such as triple helical structure of GB [48] and double helical structure of XG [49]. This results in a higher G' and enhances the gel strength of the food ink. For example, the G' of the GB/XG food ink with 12.5 mM Ca^{2+} addition at frequency of 1 rad/s is 2040 ± 97 Pa at 4 °C, which is about twice higher than the G' measured at room temperature (Table S4.2).

Table S4.2 also summarizes the $\tan \delta$ of different food inks at an angular frequency of 1 rad/s. The presence of GB in XG, the addition of Ca^{2+} and lower temperature resulted in lower $\tan \delta$ values, which were in a range from 0.08 to 0.15. Those gels can be qualified as weak gels [50], which benefited the printing process.

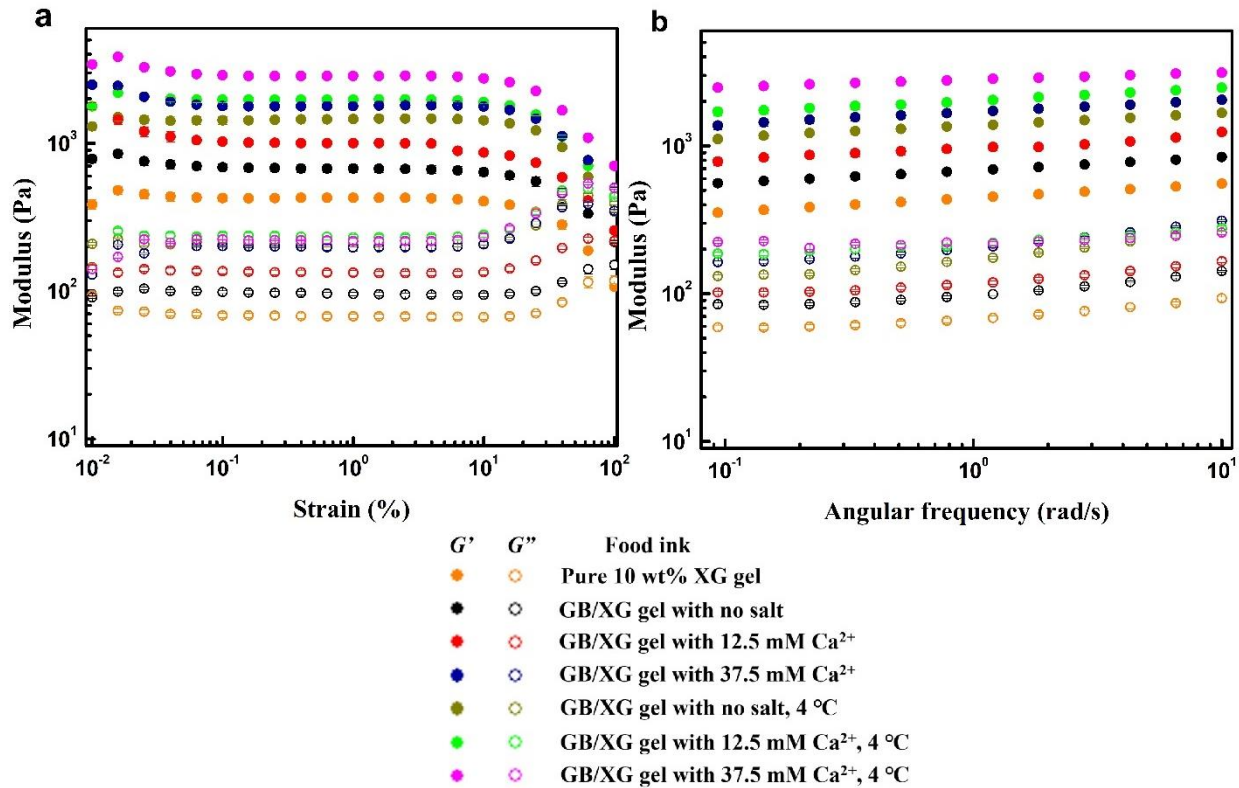


Figure 4.4 Storage modulus G' (solid symbols) and loss modulus G'' (open symbols) for XG gel alone and GB/XG mixed gels with different Ca^{2+} addition at room temperature (RT) and 4 °C: (a) as functions of strain amplitude (%) at $\omega = 1$ rad/s (b) as functions of angular frequency at strain = 1%.

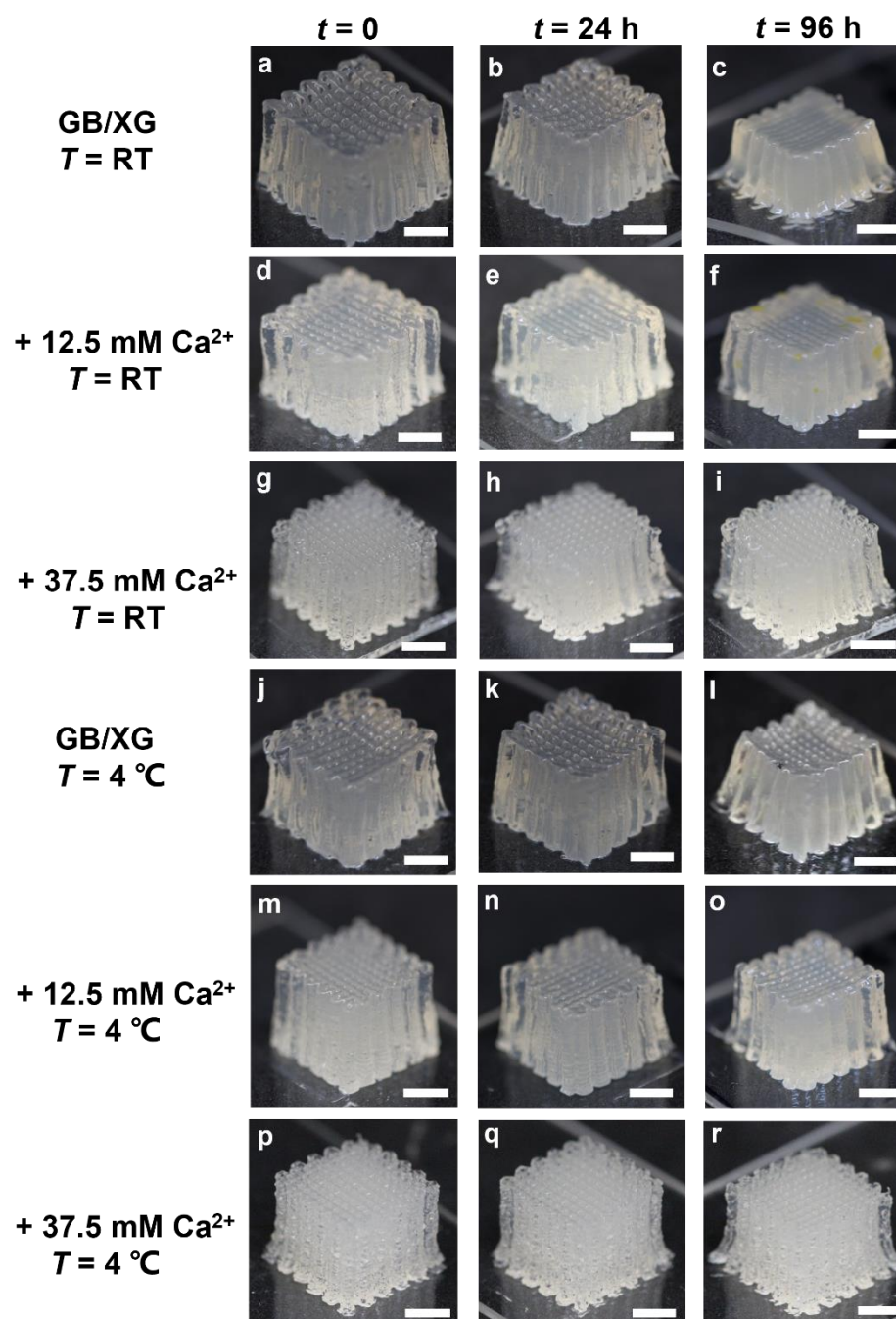
4.4.3 Dimensional stability of 3D-printed structures

The dimensional stability of 3D-printed structures was investigated as it is essential for food storage. A 4 day duration was chosen as a goal for ready-to eat food storage. Figures 4.5a-r presents the structure images showing the effect of Ca^{2+} addition and storage temperature after printing on the dimension of as-printed 3D scaffolds with respect to time. In general, adding Ca^{2+} or decreasing temperature improves the dimensional stability. Printed scaffold with sharper outline can be obtained for the GB/XG ink with added Ca^{2+} (Figure 4.5d and g) compared to the salt-free ink (Figure 4.5a). Besides, shape retention improves when increasing Ca^{2+} concentration (Figure 4.5d and g). The same phenomena has been reported based on the gelation quality of calcium-enhanced alginate gels [51] and pectin gels [6]. As the shape fidelity after deposition is closely related to the yield stress, the apparent yield stress of different food inks was calculated

based on the method by Bruneaux et al. [34], and the results are shown in Figure S4.2. The apparent yield stress of food inks increases with a linear concentration dependence on Ca^{2+} in the range of 0 to 62.5 mM, which explains the better shape retention after printing. The high apparent yield stress is probably due to the reinforced gel network with Ca^{2+} addition. The latter modifies the flow properties but also helps to induce gelation [43-45] and maintain the 3D structure during and after printing. On the other hand, lowering the storage temperature results in good shape retention ability of the 3D printed scaffolds (Figure 4.5m-r). The increased G' at low temperature from the rheological results (Figure 4.4) can also justify the superior shape retention. Besides, low temperature slows down the solvent evaporation, hence a higher shape fidelity of the hydrogel is retained.

Figure 4.5s shows the dimensional stability of the as-printed scaffolds by monitoring the evolution of the relative thickness variation to quantify the structure slumping. All the samples retain at least 75 % of their initial thickness after 96 h, indicating a satisfactory shape stability with food ink using the GB/XG pair. Comparably, thickness reduction using the food ink with 12.5 mM and 37.5 mM Ca^{2+} is 3% and 8% slower than the one with salt-free ink. In addition, the thickness reduction for scaffolds with both GB/XG and GB/XG/ Ca^{2+} food ink is quite similar at 4 °C, showing around 11% reduction after 96 h, indicating that the storage temperature effects dominate dimensional stability compared to the salt effect.

Overall, the dimensional stability agrees well with the fact that the rheological properties are enhanced when adding Ca^{2+} or decreasing temperature.



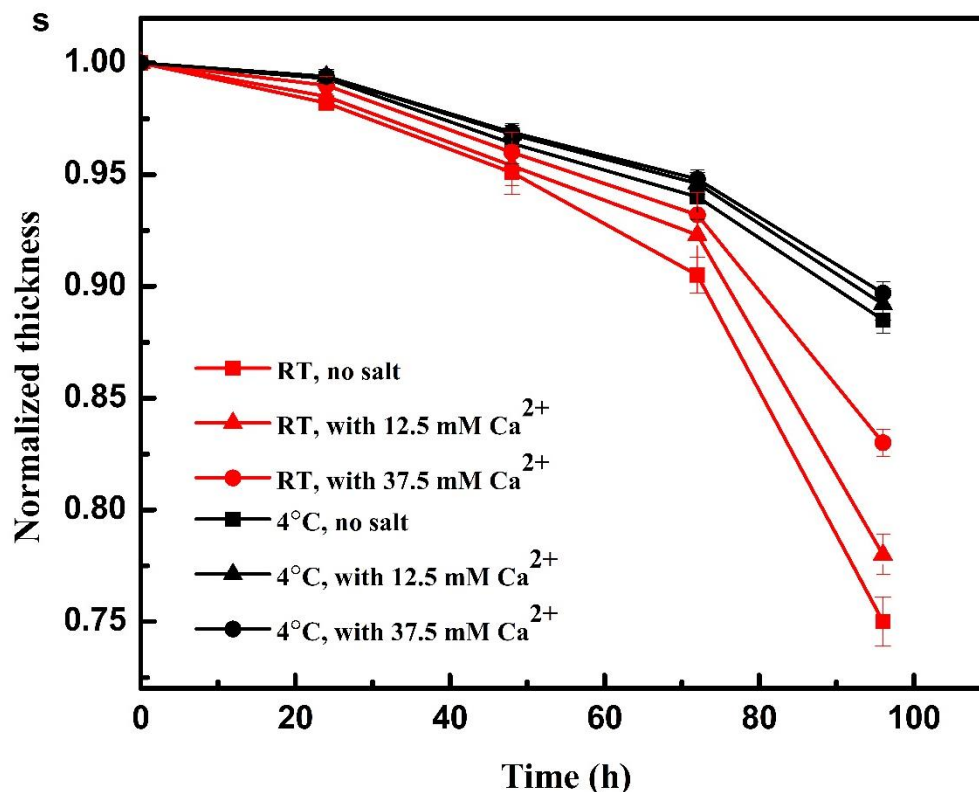


Figure 4.5 As-printed 40-layer scaffold using GB/XG mixed gels (a-c) with no salt addition (d-f) with 12.5 mM Ca^{2+} and (g-i) with 37.5 mM Ca^{2+} addition after printing 0, 24 and 96 h stored at room temperature ($22 \pm 1^\circ\text{C}$). As-printed 40-layer scaffold using GB/XG mixed gels (j-l) with no salt addition (m-o) with 12.5 mM Ca^{2+} addition and with 37.5 mM Ca^{2+} addition (p-r) after printing 0, 24 and 96 h at stored at 4°C . (s) Normalized thickness of the scaffolds fabricated using GB/XG food ink with 0, 12.5 mM and 37.5 mM Ca^{2+} addition stored at room temperature and 4°C over a period of 96 h. Scale bars are 5 mm.

4.4.4 Compression testing for textural profile analysis (TPA)

TPA is one of the most common measurement method for describing the characteristics of gels [52], which includes hardness, adhesiveness, cohesiveness, springiness and gumminess. These characteristics are strongly related with the sensory perception of food [53]. Scaffolds with different infill percentage were first printed (Figure 4.6) and were then tested for TPA. The calculated textural parameters are shown in Figure 4.7 (Table S4.4 for more details) in order to illustrate the effect of infill percentage of 3D scaffolds, Ca^{2+} concentration in the GB/XG food ink and storage temperature of printed scaffolds on basic textural properties.

Hardness is related to the sensory perception of the first bite in the mouth, which is the most direct mouthfeel reaction [54]. It is represented by the peak force of the first compression cycle. The effects of infill percentages, salt concentrations and storage temperature on the hardness of the samples are shown in Figure 4.7a. Increasing the infill percentage causes an increase in the hardness. Specifically, the samples with 96% infill percentage have a significant higher hardness value compared to the ones with lower infill percentage ($p < 0.05$). The scaffolds with 25% infill percentage collapsed easily upon compressing because of the small contact filament area with the probe and thus high pressure. One interesting point to mention here is that the infill percentage can be well controlled during the 3D food model design process, so it can meet any specific requirements in terms of food texture, highlighting the potential of 3D printing. A significant difference in hardness was also observed for the samples containing different Ca^{2+} concentration ($p < 0.05$). GB/XG food ink with 62.5 mM Ca^{2+} addition has the highest hardness value of 57.3 g. Regarding the effect of storage temperature, it has an inverse relationship with hardness. That is, a decrease in storage temperature leads to an increase in the hardness, which is probably due to the formed stiffer structure, as discussed above.

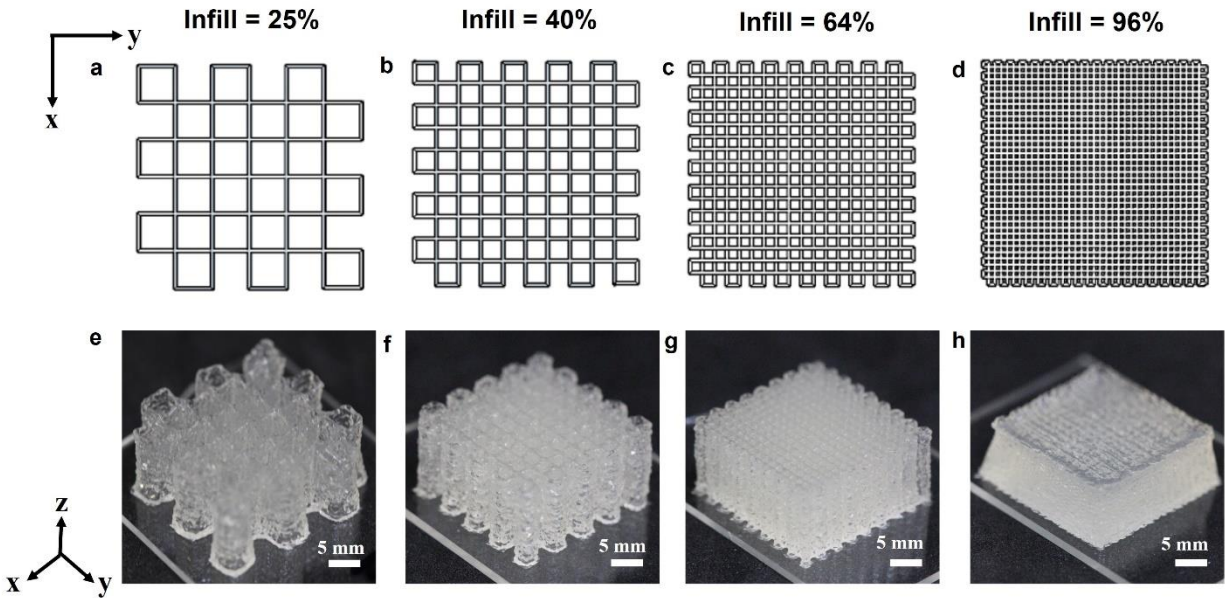


Figure 4.6 (a-d) Top-view models of scaffold with different infill percentage. (e-h) Representative images of 3D printed scaffold with different infill percentage using GB/XG with 37.5 mM Ca^{2+} food ink at room temperature right after printing. The printing times for scaffolds with different infill percentages were varying between 8 and 30 min.

When retracting the probe, a negative force arises from the attraction between the sample surface and the probe. Adhesiveness is defined as the negative area of force when the probe withdraws after the first compression. It can be interpreted as a sticky mouthfeel [55]. Appropriate adhesiveness benefits the taste and flavor releasing. However, too strong adhesiveness may cause the food sticking to the inner packaging, with potential damage when removing the packaging [54]. The adhesiveness for samples with different infill percentages, salt concentrations and storage temperatures are shown in Figure 4.7b. The scaffold with the densest structure has a significant higher adhesiveness of 183 ± 3 g.s, because of the larger contact surface ($p < 0.05$). Adding Ca^{2+} to the ink also results in an increase of adhesiveness, with the sample containing 62.5 mM having a significant higher value ($p < 0.05$), which might be explained by the increased G'' after Ca^{2+} addition. Similar observations for XG-based food gels have been reported before [56]. As for storage temperature, lower adhesiveness is observed at 4 °C because the structures containing GB become more elastic, as mentioned before.

The cohesiveness indicates the strength of internal bonds making up the body of food and the degree to which a food can be deformed before it breaks. It is defined as the ratio of the positive force area during the second compression to that of the first compression [55]. Cohesiveness comparison of infill percentages, salt concentrations and storage temperatures effects are shown in Figure 4.7c. Samples with higher infill percentage show stronger cohesiveness because of a relatively more stable structure against compression. The higher apparent yield stress for the food ink with higher Ca^{2+} provides better resistance against the deformation and thus benefits the cohesiveness of the printed scaffold. Moreover, food ink at low temperature demonstrates a higher gel strength, which translates into a larger tensile strength and higher cohesiveness of the printed scaffolds.

Gumminess is defined as the product of hardness and cohesiveness, which indicates the required energy to disintegrate food for swallowing [57]. It is another important textural characteristics, especially for semisolid food with a low degree of hardness and high degree of cohesiveness [54]. In our case, gumminess exhibits a similar trend to hardness, regardless of the effects of infill percentage, salt concentration and storage temperature, as shown in Figure 4.7d. This indicates that the gumminess of printed samples is dominated by their hardness [56].

Overall, food texture can be tuned by the rheological properties of the food ink, infill percentage during 3D printing and storage temperature. By carefully controlling the food ink properties and the designed form, it is possible to create group-specific food constructs according to their requirements. Food model designed with 100% infill are more popular in visual sight, while it is more acceptable in terms of mouthfeel with 25% infill [58]. Some special groups of people such as dysphagic patients with swallowing difficulties are usually not able to consume food with high adhesiveness [59]. 3D printing shows its ability to control food properties for a large range of needs. For food applications, the 3D printed scaffolds using our food inks have a similar range of adhesiveness and cohesiveness as cheddar [60], while their hardness and gumminess values can be referred to that of Jello™ [61] and pudding [62, 63]. This shows the great potential to use the mixture of GB and XG to simulate the texture of soft food, well in agreement with a previously published work by Cohen et al.[64].

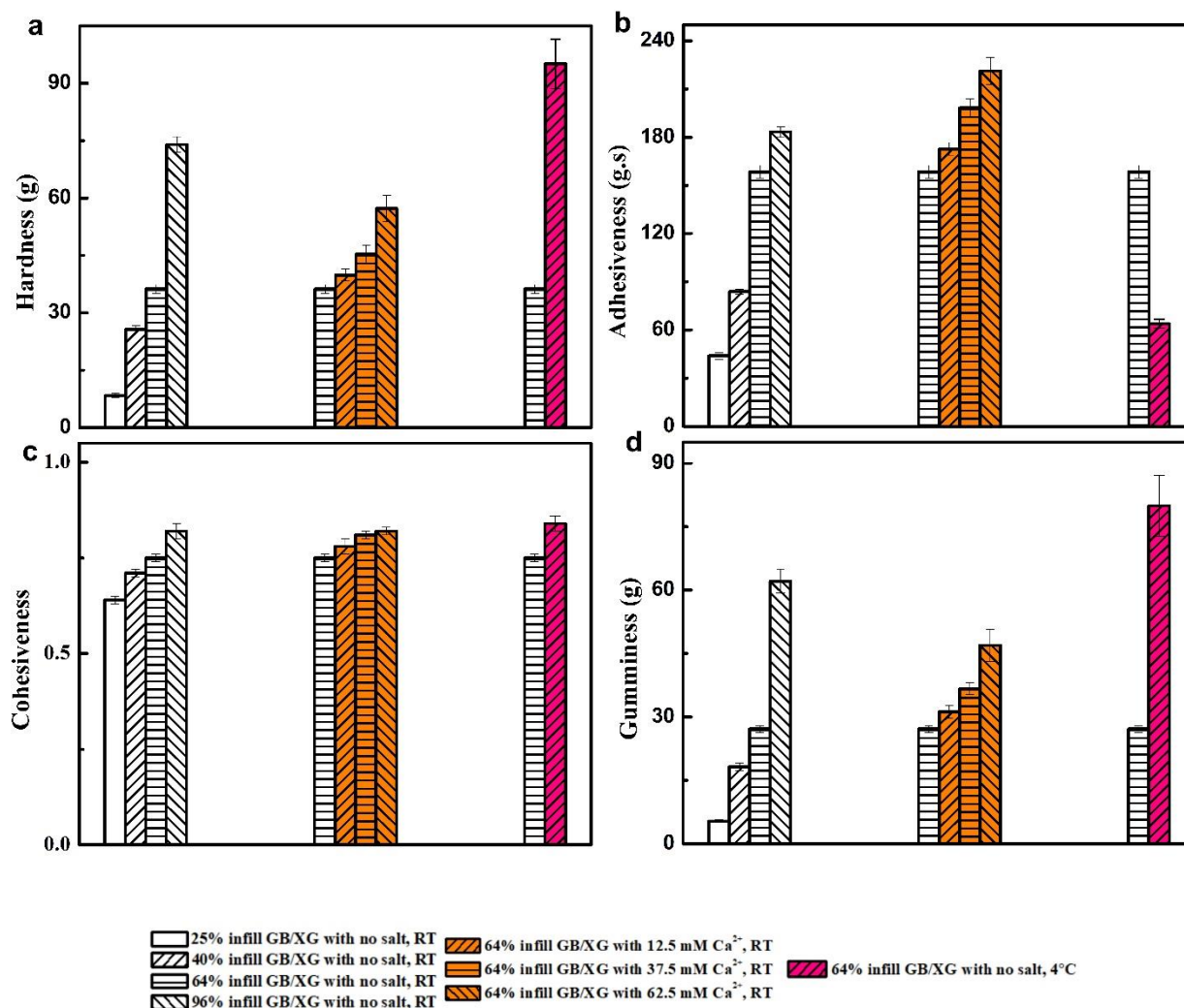


Figure 4.7 Ca^{2+} concentration, infill percentage and storage temperature effects on (a) hardness (b) adhesiveness (c) cohesiveness and (d) gumminess.

4.5 Conclusions

We successfully 3D printed food-grade scaffolds with ink composed of GB mixed with XG. The GB/XG ink exhibits a shear-thinning behavior, which allows the food ink to be easily extruded from the printer nozzle. GB is a key component as it endows the ink with improved rheological properties and the scaffold with better dimensional stability compared to that of XG alone. The addition of Ca^{2+} to GB/XG ink improves the printing results due to its bridging effect. Scaffolds exhibited the best structure and shape retention using food ink with the addition of 37.5 mM Ca^{2+} . However, ink with 62.5 mM Ca^{2+} can cause irregular filaments and structure defects possibly

because it causes over-gelation of the food ink. All printed scaffolds using GB/XG can retain more than 75% of their original thickness after 96 h of storage at room temperature, regardless of the presence of Ca^{2+} , exhibiting a high shape fidelity retention. Scaffolds with both GB/XG and GB/XG/ Ca^{2+} food ink show the lowest thickness reduction - around 11% - after 96 h at 4 °C. Finally, the textural properties of the printed food scaffold can be modulated by adding Ca^{2+} to the ink, controlling the infill percentage through the 3D printing process and using different storage temperature after printing. This provides the possibility to use the 3D printing technique to shape group-specific food formulations according to different needs, such as those of elderly people or dysphagic patients.

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4.7 References

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4.8 Supporting information

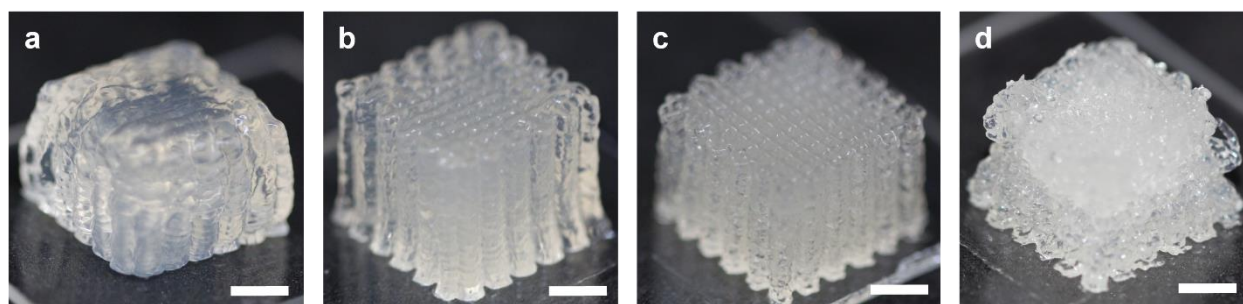


Figure S4.1. Comparison of the shape retention of as-printed 40-layers scaffolds using a 200- μm nozzle using various gel formulations right after printing at room temperature: with (a) 10 wt% pure XG ink under 224 kPa, (b) GB/XG ink with 12.5mM Ca^{2+} under 504 kPa, (c) GB/XG ink with 37.5mM Ca^{2+} under 588 kPa and (d) GB/XG ink with 62.5mM Ca^{2+} under 784 kPa. Scale bars are 5 mm.

Table S4.1 Viscosity at $\dot{\gamma} = 1 \text{ s}^{-1}$ for different food inks at room temperature (22 °C)

Food ink type	Viscosity (Pa.s)
Pure 10 wt% XG gel	158 ± 30
GB/XG gel with no salt	231 ± 27
GB/XG gel with 12.5 mM Ca^{2+}	313 ± 24
GB/XG gel with 37.5 mM Ca^{2+}	460 ± 67
GB/XG gel with 62.5 mM Ca^{2+}	720 ± 91
GB/XG gel with 75 mM Ca^{2+}	700 ± 94

Table S4.2. G' and $\tan \delta$ at $\omega = 1 \text{ rad/s}$ for different food inks

Food ink	G' (Pa)		$\tan \delta$	
	RT	4 °C	RT	4 °C
Pure 10 wt% XG gel	452 ± 26	-	0.15	-
GB/XG gel with no salt	692 ± 32	1390 ± 46	0.14	0.12
GB/XG gel with 12.5 mM Ca^{2+}	980 ± 51	2040 ± 97	0.13	0.12
GB/XG gel with 37.5 mM Ca^{2+}	1720 ± 68	2839 ± 73	0.11	0.08

Frequency dependence of G' . The angular frequency dependence of G' was presented by the power-law equation:

$$G' = K\omega^a$$

with the linear trend of G' on log-log scale as the function of angular frequency, where a is the slope and K is the y-intercept of the trendline.

Table S4.3 Power-law parameter a comparing food inks at different temperature (RT and 4 °C) within angular frequency dependence

Food ink type	Power-law parameter (a)	
	RT	4 °C
GB/XG gel with no salt	0.095 ± 0.006	0.084 ± 0.003
GB/XG gel with 12.5 mM Ca^{2+}	0.084 ± 0.005	0.081 ± 0.006
GB/XG gel with 37.5 mM Ca^{2+}	0.083 ± 0.003	0.050 ± 0.002

Apparent yield stress determination. The process apparent yield stress was determined while extruding the ink by the 3D printer. The results were calculated based on the equation [1]:

$$\tau_{app} = \frac{P_{flow} - P_a}{2\left(\frac{L}{R}\right)}$$

where τ_{app} is the apparent yield stress, P_{flow} is the minimum pressure required for flow, P_a is the atmospheric pressure, L and R are, respectively, the length inner radius of the nozzle.

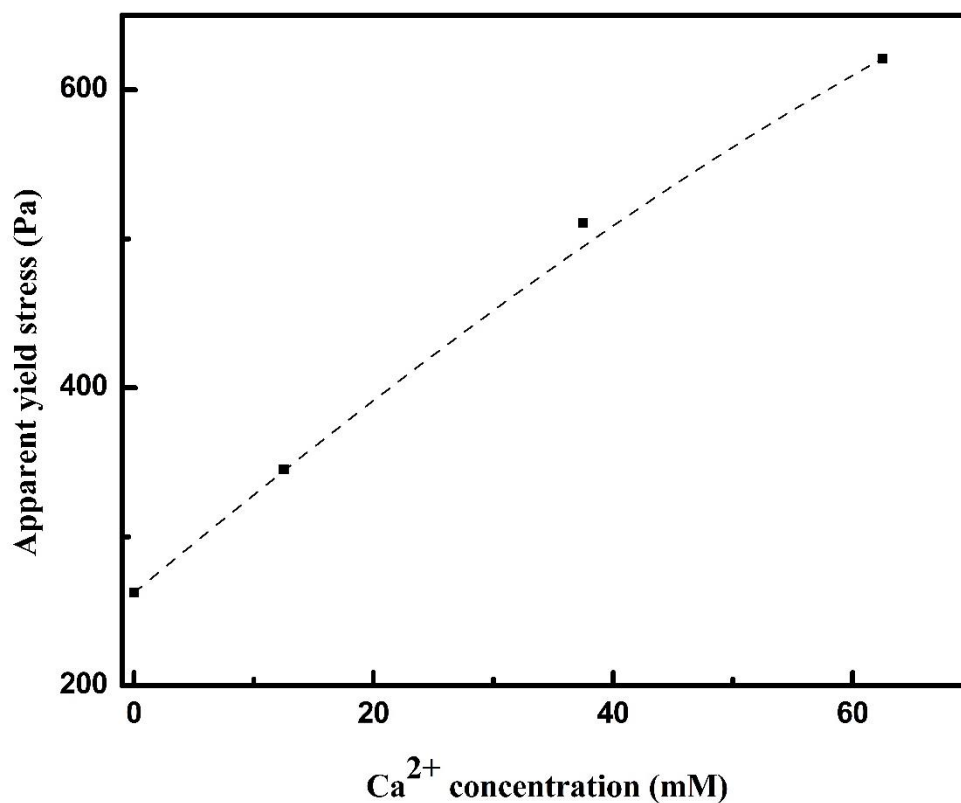


Figure S4.2. Apparent yield stress as a function of Ca²⁺ concentration.

Table S4.4. Textural properties of the 3D printed scaffolds made of different gel formulations *.

Effect	Food ink	Hardness (g)	Adhesiveness (g.s)	Cohesiveness	Gumminess (g)
Shape effect	25% IP GB/XG	8.3 (0.5) ^d	43.9 (2.2) ^d	0.64 (0.01) ^d	5.4 (0.3) ^d
	40% IP GB/XG	25.6 (1.1) ^c	84.0 (1.5) ^c	0.71 (0.01) ^c	18.2 (0.9) ^c
	64% IP GB/XG	36.2 (1.2) ^b	159 (4) ^b	0.75 (0.01) ^b	27.2 (0.8) ^b
	96% IP GB/XG	74.0 (2.1) ^a	183 (3) ^a	0.82 (0.02) ^a	60.7 (2.8) ^a
Ca ²⁺ addition effect	64% IP no salt	36.2 (1.2) ^c	159 (4) ^d	0.75 (0.01) ^c	27.2 (0.8) ^d
	64% IP 12.5 mM Ca ²⁺	39.9 (1.6) ^c	173 (4) ^c	0.78 (0.02) ^b	31.1 (1.6) ^c
	64% IP 37.5 mM Ca ²⁺	45.3 (2.4) ^b	199 (6) ^b	0.81 (0.01) ^a	36.7 (1.4) ^b
	64% IP 62.5 mM Ca ²⁺	57.3 (3.5) ^a	221 (8) ^a	0.82 (0.01) ^a	47.0 (3.8) ^a
Temperature effect	RT 64% IP GB/XG	36.2 (1.2) ^b	159 (4) ^a	0.75 (0.01) ^b	27.2 (0.8) ^b
	4 °C 64% IP GB/XG	95.1 (6.5) ^a	64.0 (2.7) ^b	0.84 (0.02) ^a	79.9 (7.3) ^a

*: Values in parenthesis are standard deviation $n = 3$. Mean values with the same lowercase superscript letter are not significantly different ($p > 0.05$) between the row.

IP = infill percentage

Supporting references

- [1] J. Bruneaux, D. Therriault, and M.-C. Heuzey, "Micro-extrusion of organic inks for direct-write assembly," *Journal of Micromechanics and Microengineering*, vol. 18, no. 11, 2008, doi: 10.1088/0960-1317/18/11/115020.

CHAPTER 5 ARTICLE 2 : PROCESSING AND PROPERTIES OF AQUEOUS PROTEIN/POLYSACCHARIDE MIXTURE-BASED INKS FOR ADDITIVE MANUFACTURING

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5.1 Abstract

Three-dimensional (3D) food printing shows its enormous potential in customization of food designs and personalization of nutritional contents. Herein, two aqueous protein/polysaccharide food inks composed of gelatin B (GB)/xanthan gum (XG) and soy protein acid hydrolysate (SP)/XG, respectively, were investigated for material extrusion additive manufacturing. For both ink systems, processing maps were generated based on the relationship between nozzle diameter and XG concentration for different types of printed geometries including filament, 40-layer scaffolds and 3D complex structures (i.e., starfish). XG concentration was found to be strongly linked to ink rheological properties, thus influencing not only the flow behavior during the printing process, but also the shape fidelity and shape retention after deposition. Inks with

3GB/11XG (3 wt% GB/11 wt% XG) and 8SP/15XG (8 wt% SP/15 wt% XG) demonstrated the highest printability. Shape fidelity map for the post-deposition assessment of printed 3D structures proposed the appropriate range of viscoelastic properties through a well-defined shape fidelity index. Texture profile results also revealed that both polysaccharide concentration and protein type are critical to textural properties of the printed objects, guiding the choice of a suitable ink for an individual 3D food printing. Finally, according to the processing and shape fidelity maps previously defined, an 18-layer two-color 3D complex dolphin shape was successfully fabricated using both 3GB/11XG and 8SP/15XG food inks by a multi-material extrusion-based printing technique in one manufacturing process. This work provides comprehensive guidelines to design and fabricate 3D structures of aqueous protein/polysaccharide mixture-based inks to serve the purpose of accurately personalizing and diversifying nutrition for food applications.

Keywords: Material extrusion additive manufacturing; Protein/polysaccharide inks; Processing and shape fidelity maps; Rheological properties; Textural properties

5.2 Introduction

Three-dimensional (3D) food printing gained increasing interest from researchers and industries [1, 2] due to its potential advantages in customizing flavors and nutritional design, high precision and relatively less material loss compared to traditional manufacturing methods [3-6]. It follows the additive principle through computer-controlled layer-by-layer deposition of materials [7]. The most common additive manufacturing techniques include extrusion-based printing, selective laser sintering (SLS) and inkjet printing [8]. Direct-ink writing (DIW) is an extrusion-based printing process widely adopted in food applications, forcing the food ink through a micro-nozzle along with the motion of the stage in a layer-by-layer manner to generate materials with controlled design and composition [9-11]. Until now, the DIW technique has been successfully applied for several food product inks such as fresh vegetables [12], fruit [13], meat materials [14, 15] and cookie dough [16]. However, the lack of fundamental knowledge on how essential food constituents behave during 3D printing is a barrier to bring this technique into widespread food applications [7, 17].

Proteins and polysaccharides are two important natural biopolymers widely used in food systems and they can contribute to the functional properties of food products [18-20]. Electrostatic

interaction and hydrogen bonding are the main driving forces influencing the interactions between these two components [20-24], which can be tuned depending on pH and ionic strength [22, 25, 26].

Gelatin and XG are two commonly used protein and polysaccharide, respectively, in food applications that provide necessary nutritional, structural and textural functions [27]. Gelatin is a linear animal protein hydrolyzed from acid (type A) or alkaline (type B) treatment of the parent protein collagen [28]. It can form physical thermo-reversible gels below the helix-to-coil transition temperature (20-30°C) [19, 29], due to the conversion from random coils to triple helices structure [30]. XG is an anionic microbial polysaccharide fermented from *Xanthomonas campestris* [31]. Owing to the unique and rod-like conformation, it exhibits a weak gel-like and strong shear-thinning behavior in solution [32]. Adequate food properties such as rheological behavior and texture profile can be easily controlled by XG concentration [33-35]. Another important protein is soy protein acid hydrolysate (SP). It is a plant-based globular protein composed of low molecular weight peptides of soy protein isolate. It has high nutritional value and provides essential amino acids for human daily requirements [36]. Compared to soy protein isolate, SP can be more easily digested and absorbed [37]. It is regarded as an alternative to animal proteins [38] as it is able to generate a meat-like behavior [39], which benefits special population such as vegans and milk protein intolerant people [40]. Up to now, a few SP food inks have been developed. For example, the SP-enriched potato puree ink has been successfully used in DIW food printing [41]. The addition of SP not only improved the rheological properties of ink to ensure a fine printed shape, but also modified the texture which was suitable for dysphagia diets. The 3D printing ability of SP gels with different NaCl concentrations was also investigated [42]. The rheological properties of the mixed gel were strongly related to the major peak relaxation time and peak area in low-field nuclear magnetic resonance (LF-NMR) spectroscopic, which can be used to predict the 3D printability of the mixture.

In our previous work, a food ink based on a mixture of Gelatin B (GB) and XG was developed for DIW 3D printing [43]. Herein, we further investigate the properties of the GB/XG ink for the DIW method, and diversify the protein/polysaccharide food ink system by considering a plant protein, namely SP, in combination with XG. Processing maps are derived for the GB/XG and SP/XG systems by considering processing parameters for different structures fabrication. The rheological properties of inks with different XG concentrations are analyzed to guide successful

DIW 3D printing. The effects of XG concentration and protein type on the post-deposition assessment of printed scaffolds are conceptualized in a shape fidelity map. Textural properties are also characterized in order to clarify how the quality of printed objects is affected by the protein type and XG concentration. As a demonstration, the generated processing and shape fidelity maps are used to fabricate a 3D dolphin using a single multi-material DIW printing process.

5.3 Materials and methods

5.3.1 Materials

Gelatin (type B from bovine skin, G9382, bloom index ~ 225 , molecular weight (M_w): ~ 50 kDa, pI ~ 4.7 - 5.3) and SP (S1674, M_w : 800-10,000 Da, pI = 4.5-5.0) were purchased from Sigma-Aldrich Canada. XG (KELTROL RD, food-grade quality, M_w : $\sim 2 \times 10^3$ kDa) was kindly offered by CP Kelco U.S. Food dye was bought from Altius Spices & Seasonings Inc., Canada.

5.3.2 Food ink preparation

In order to meet the requirements for appropriate flow properties and shape fidelity for a printable gel, the respective concentrations of GB and SP were selected based on our preliminary experiments, as well as the works reported by Cohen et al. [44] and Phuhongsung et al. [42]. GB powder was swelled in Milli-Q water (18.2Ω) for 15-20 min, and then magnetically stirred gently at 60°C for 15 min to 3 wt% GB solution. SP solutions (8 wt%) were prepared by dissolving the powder into Milli-Q water at a stirring speed of 300 rpm for 1 h at room temperature. A light brown color was obtained for the SP solution, which may be due to the Maillard reaction during the fabrication process of the SP powder [45]. The solutions were used on the same day in order to avoid any aging effect. XG dry powder was then added to the GB and the SP solutions, respectively. The added XG concentrations were varied from 2-19 wt%, within the XG intake guideline as a food additive by the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) [46]. The GB/XG and SP/XG mixed solutions were mechanically stirred for at least 12 h at 40°C to ensure homogeneous mixing. The final pH of the prepared GB/XG and SP/XG solutions were 5.8 and 5.5, respectively, hence slightly above the pI of both proteins. For comparison purposes, neat XG solutions of 3 and 4 wt% concentrations were also prepared. All the inks were then centrifuged at 5000 rpm for 1 h at 22°C to remove air bubbles.

5.3.3 Single-material DIW

The food inks were firstly transferred into a syringe barrel (3 mL, Nordson EFD) fixed to a high-pressure adaptor (HP-7x, Nordson EFD), which was connected with a computer assisted three-axis positioning stage (I&J2200-4, I&J Fisnar Inc.). The food inks were extruded through various cylindrical micronozzles (Nordson EFD, exit inner diameter $D = 100 - 510 \mu\text{m}$) and deposited onto a glass substrate under different applied pressures.

5.3.4 Multi-material DIW

Multi-material DIW process was performed using a custom-made three-axis positioning gantry stage (Aerotech, USA) connected with two independent air-operated dispensing systems. Two different inks were loaded into the barrels (3 mL, Nordson EFD). The applied pressures were tailored on the piston of the dispensers (HP-7x, Nordson EFD) using custom-made pneumatic pressure controller (Mëkanic, Canada). The two inks were then extruded independently from cylindrical micronozzles (Nordson EFD) along the print paths.

5.3.5 Microstructures fabrication

All the structures were fabricated by layer-by-layer depositing with the prepared GB/XG and SP/XG food inks at room temperature ($22 \pm 1^\circ\text{C}$). The original STL files for the digital 3D models of the starfish and dolphin were downloaded from internet (<http://www.thingiverse.com/>). The codes for printing paths were generated initially with Simplify 3D slicing software. In the case of microstructures with single materials, the initial codes were translated to the language of the printing software (JR Point for Dispensing, Janome Sewing Machine) using a Python program. In the case of microstructures made of multi-materials, a robotic simulation software, RoboDK 4.2.4 (RoboDK Inc.) was used to translate the initial codes to the language of Aerotech system (Aerobasic). Specific applied pressures, nozzle diameters and printing speeds are reported in the Results section.

5.3.6 Rheological measurements

The steady-state shear viscosity (shear rate $\sim 0.01\text{-}10 \text{ s}^{-1}$) of GB/XG and SP/XG ink formulations was characterized using a stress-controlled rheometer (MCR-502, Anton Paar, Graz, Austria) at room temperature (22°C) with a rough surface parallel plate geometry (25 mm diameter and 1.0

mm gap) to avoid wall slip. In order to prevent water evaporation, the edge of the sample was covered with mineral oil.

The process-related viscosity (shear rate $>10 \text{ s}^{-1}$) of GB/XG and SP/XG aqueous mixtures was evaluated by capillary flow analysis (CFA) as presented in the authors' previous research [43].

The process apparent yield stress was also determined while extruding the ink by the 3D printer at room temperature. The results were calculated based on the equation [47]:

$$\tau_{\text{app}} = \frac{P_{\text{flow}} - P_0}{2 \left(\frac{L}{R} \right)} \quad (5.1)$$

where τ_{app} means the apparent yield stress, P_{flow} means the minimum pressure required for flow, P_0 means the atmospheric pressure, L and R are the length and the inner radius of the nozzle, respectively.

In addition, recovery measurements were performed using the parallel plate flow geometry in order to mimic the materials shear behavior before, during and after extrusion (recovery) through the nozzle. A low shear rate of 0.1 s^{-1} for 180 s was applied, followed by a higher shear rate at 50 s^{-1} for 120 s to mimic the extrusion process and again by a low shear rate of 0.1 s^{-1} for 180 s. The rheological measurements were performed at room temperature (22°C) for all samples.

5.3.7 Morphological characterization

Optical images of all structures were obtained using a stereo microscope (SZX12, Olympus) and a digital camera (EOS TRBRL T4I, Canon), then analyzed with the software Image Pro Plus 6.0 (Media Cybernetics).

5.3.8 Compression tests for textural profile analysis

The textural properties of printed structures were analyzed by a double-cycle compression tests using the Mach-1TM Micromechanical tester (Biomomentum Inc.). The printed 3D scaffolds ($18 \text{ mm} \times 18 \text{ mm} \times 10 \text{ mm}$) with 64 % infill percentage composed of GB/XG and SP/XG food inks with different XG concentrations were compressed by a 25 mm diameter cylindrical probe (Figure 5.1). Hardness, adhesiveness, cohesiveness and gumminess were measured referring to the method and the main setting parameters reported in authors' previous work [43].

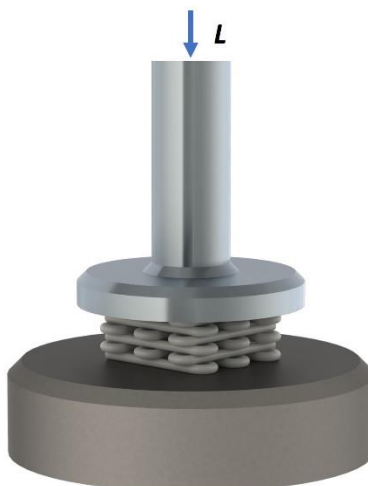


Figure 5.1 Schematic illustration of the Mach-1TM Micromechanical apparatus for compression test.

5.3.9 Statistical analysis

All the tests mentioned above have been performed in triplicate. Data were expressed as mean of the results $\pm$ standard deviation (SD) and analyzed by SPSS software (SPSS 20.0, IBM, Chicago, IL, USA). Duncan's multiple range test was applied to determine the significant differences ($p < 0.05$) between samples for the textural profile results.

5.4 Results and discussion

5.4.1 3D printed structures

Figure 5.2a and b show the 3D printing process and the designed 3D scaffold model, respectively. Figure 5.2c-n present the images of the printed structures, showing the effect of XG addition on GB/XG and SP/XG systems, respectively. The XG concentration was of major importance for the printability of the mixture ink as it is directly related to viscosity, as will be shown later. The tested XG concentration range was 3-13 wt% and 4-19 wt% for the GB/XG and SP/XG inks, respectively. Printed 3D scaffolds with apparent highest shape fidelity were achieved using food inks with 3GB/11XG (Figure 5.2g) and 8SP/15XG (Figure 5.2l). The ink viscosity was not suitable for printing when the XG concentration was too low or too high. The scaffold printed with 3GB/3XG ink exhibited a poor self-supporting behavior (Figure 5.2c), as well as the one printed with 8SP/4XG ink (Figure 5.2i). Increasing the XG concentration improved the

printability of the mixed systems, as evidenced by the sharper edges and more regular shape. However, structure defects due to the inconsistent printing behavior appeared when the XG concentration was above a critical value due to the high viscosity of the food ink, e.g. 13 wt% for the GB/XG system (Figure 5.2h) and 19 wt% for the SP/XG system (Figure 2n), respectively.

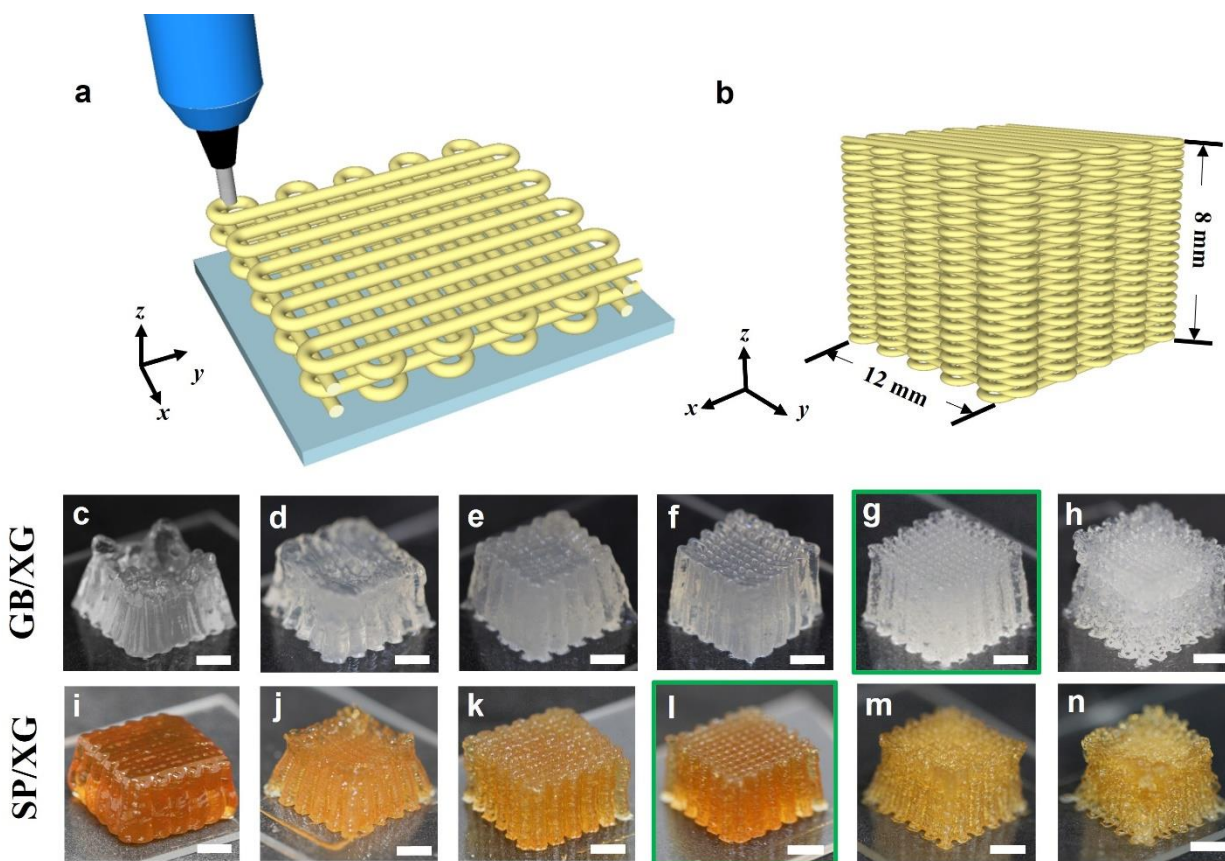


Figure 5.2 (a) Schematic representation of the DIW 3D printing process; (b) 3D model of the 40-layers scaffold. (c-h) As-printed 40-layers GB/XG scaffold (3 wt% GB) with 3, 5, 8, 10, 11 and 13 wt% XG and (i-n) SP/XG scaffold (8 wt% SP) with 4, 8, 12, 15, 17 and 19 wt% XG using a 200 μm nozzle. All scaffolds were printed at a robot velocity of 7 mm/s. Different applied pressures were set under a range between 140 to 980 kPa. All the photos were taken at room temperature right after printing. Scale bars are 4 mm. Images with green boxes represent the 3D scaffolds printed with the optimal ink formulations.

5.4.2 Processing maps

In order to further evaluate the ink printability, the processing maps of both GB/XG (Figure 5.3a) and SP/XG (Figure 5.3b) inks were generated to show the different printing zones according to

the quality of the printed objects. These maps illustrate the effects of nozzle diameters ($D = 100, 150, 200, 250, 330, 410$ and $510 \mu\text{m}$) and XG concentrations (from 2 to 19 wt%) on different types of printed geometries including 1D filaments, 2D arrays and 3D structures. The applied pressure was in a range of 0.11-1.1 MPa and the robot velocity varied from 4 to 9 mm/s for the printing process. Food inks in Zone I are too dilute for continuous filament extrusion, forming only droplets at the tip of the nozzle or the filament spreads immediately onto the substrate. In Zone II, the food inks can be readily extruded from the nozzles and form continuous cylindrical 1D filaments (Figure 5.3c and d). However, it is difficult to build a 3D structure due to the insufficient shape retention (Figure 5.2c-d and i). Zone III indicates the range where large nozzle hinders 3D shape formation, where the printed structures have difficulty in maintaining their shape after deposition. In Zone IV, the food inks have good flow properties, thus can be used to print stable 3D scaffolds (Figure 5.2e-g and 2j-m) and more complex structures (Figure 5.3e and f) with sufficient self-supporting properties. In Zone V, the inks are too viscous, often leading to unpredictable nozzle clogging during the printing process.

SP/XG has a significantly larger printable region than GB/XG (Figure 5.3a and b). However, GB/XG is more likely to form 3D structure (Zone IV) when using a large nozzle diameter ($\geq 330 \mu\text{m}$). For example, the minimum XG concentration allowing the formation of 3D structures using $330 \mu\text{m}$ nozzle in GB/XG system was 10 wt%, while the value was 12 wt% in SP/XG. The difference may come from the conformational difference between the two proteins. GB molecules occupy more space due to the extended and rigid triple helical structures compared to SP, which is known to be a compact globular protein [48-50]. The softer ink structure allows the SP/XG food inks to have a better fluidity, thus a larger printable window. Nevertheless, the stiffer structure of GB improves the gelation properties of food inks, which benefits the formation of 3D structures with large nozzle diameters ($\geq 330 \mu\text{m}$).

Consequently, the DIW 3D printing can be considered as a flexible manufacturing process to personalize the structures from aqueous GB/XG and SP/XG inks. Also, the processing maps with the diverse printed images can provide guideline for the suitable selection of printable materials and process parameters.

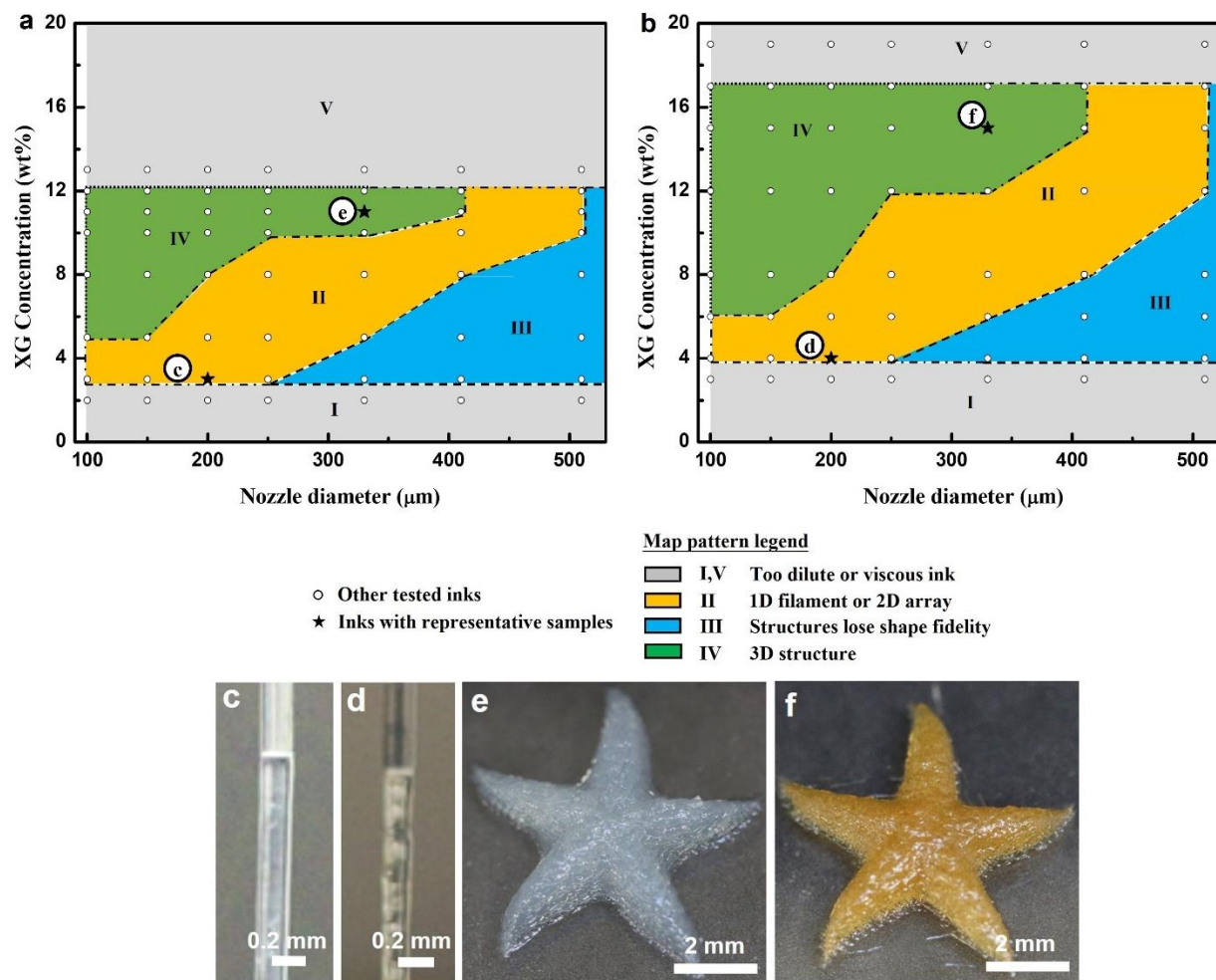


Figure 5.3 Processing maps of (a) GB/XG and (b) SP/XG food inks showing the ranges of nozzle diameter and XG concentration compatible for printing various microstructures. Printability zones are indicated by roman numbers from I to V. (c) As-printed 3GB/3XG filament using a 200 μm nozzle under 116 kPa and (d) 8SP/4XG filament using a 200 μm nozzle under 124 kPa. (e) As-printed 3GB/11XG 3D starfish using 330 μm nozzle under 490 kPa at 9 mm/s. (f) As-printed 8SP/15XG 3D starfish using 330 μm nozzle under 518 kPa at 9 mm/s.

5.4.3 Rheological properties of food inks

Characterization of inks rheological properties such as shear viscosity, recovery and apparent yield stress was performed, in order to link the food inks behavior during the extrusion process and post-deposition.

Suitable inks for 3D food printing process should have an appropriate viscosity which enables smooth flow during extrusion [51]. The shear viscosity of food ink samples with different formulations was obtained by a combination of rotational rheometry (~ 0.01 - 10 s^{-1}) and CFA ($> 10 \text{ s}^{-1}$), as shown in Figure 5.4. The dashed lines present the fitted power-law models. As the neat XG, 3GB/13XG and 8SP/19XG inks are inappropriate for printing, they were not able to be modelled. The remaining printable food inks exhibit a significant shear-thinning behavior, with a relatively low power-law index below 0.2, indicating a typical soft solid character such as XG [52] and also demonstrating the inks adequacy for the printing process. Compared with neat 3 and 4 wt% XG solution, the viscosity increases by $\sim 458 \%$ and $\sim 307 \%$ with the addition of GB and SP, respectively, at 0.1 s^{-1} (Figure 5.4a and b). This increase can be explained by the synergistic effect resulting from the electrostatic repulsion between the corresponding protein and XG above pI, as well as the electrostatic attraction between the remaining positive patches on the protein and XG [36, 43, 53-55]. The inks 3GB/3XG and 8SP/4XG exhibit the lowest viscosity of the mixtures, which correspond to their locations in Zone II and III in the processing maps (Figure 5.3a and b). The printed 3D structures using either inks were not able to hold their shapes due to an insufficient viscosity. Increasing the XG concentration leads to an increase in the viscosity of the food ink, which affects the extruding ability and the printability of the inks. With the increase of XG concentrations from 3 to 11 wt%, the viscosity at 0.1 s^{-1} of GB/XG food inks ranged from 1090 ± 47 to $4962 \pm 130 \text{ Pa.s}$. Similarly, an increase of viscosity from 1245 ± 83 to $6194 \pm 296 \text{ Pa.s}$ is observed at 0.1 s^{-1} for SP/XG food inks with the XG concentrations ranging from 4 to 15 wt%. However, further increasing the XG concentrations to 13 wt% for GB/XG ink and 19 wt% for SP/XG ink leads to an inconsistent flow behavior due to the high material viscosity, which causes poor printability and shape fidelity of 3D scaffold (Figure 5.2h and n).

Compared to the different systems with same XG concentration, GB/XG ink has a relatively higher viscosity. For example, at a shear rate of 0.1 s^{-1} , the viscosity of the 3GB/8XG ink is $3081 \pm 96 \text{ Pa.s}$, which is ~ 1.4 times of 8SP/8XG ink ($2194 \pm 68 \text{ Pa.s}$). At a higher shear rate ($\sim 50 \text{ s}^{-1}$) within the printing process region (dashed box in Figure 5.4a and b), a 1.3 times higher viscosity can be also found in 3GB/8XG ink than 8SP/8XG ink. Additionally, regarding the optimal ink formulations, SP/XG system can support a higher XG concentration with 15 wt% than 11 wt% in GB/XG system. This is coherent with the wider processing map for SP/XG ink as discussed in Figure 5.3a and b.

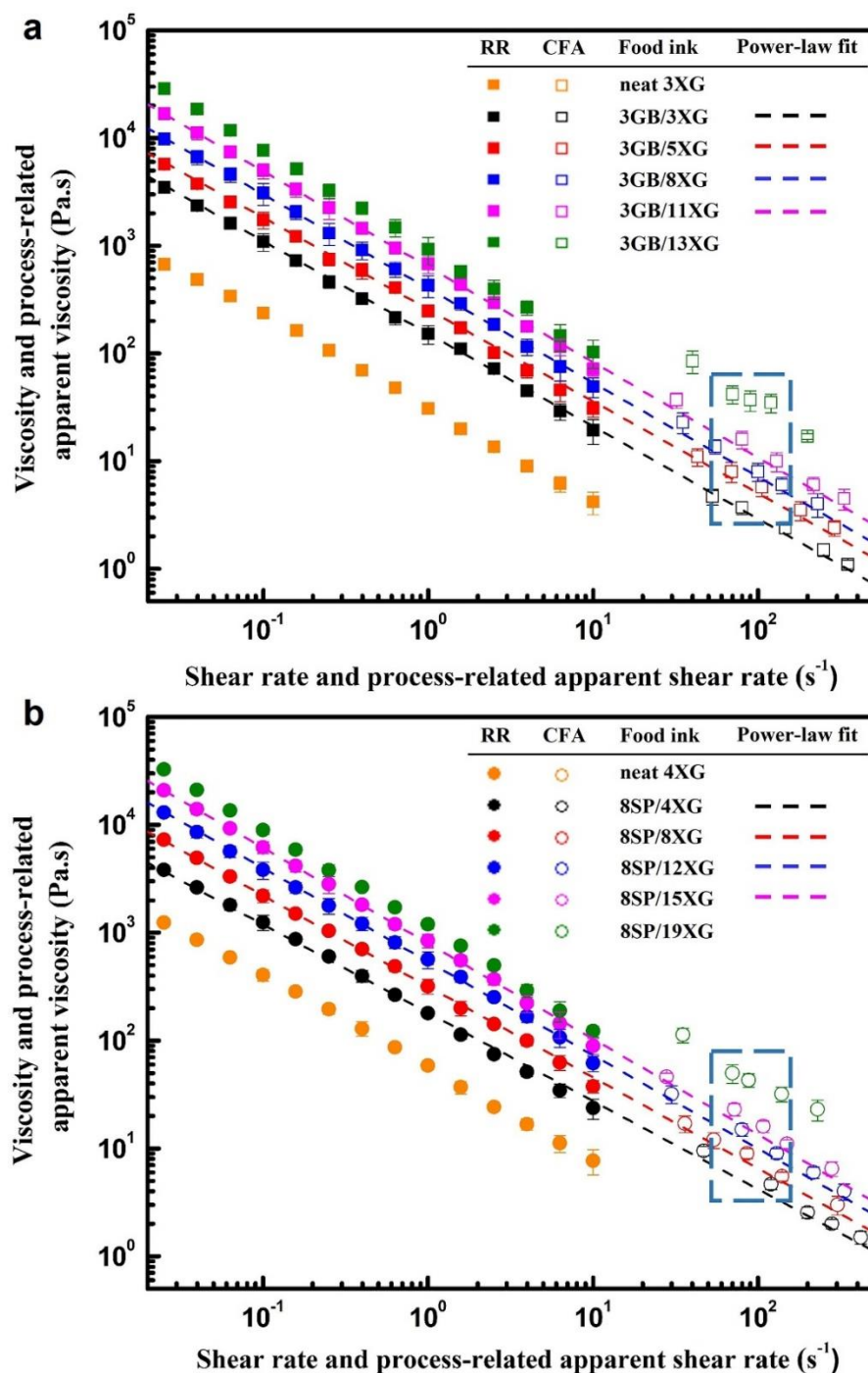


Figure 5.4 Viscosity as a function of shear rate for (a) neat 3XG and GB/XG (b) neat 4XG and SP/XG food inks with different XG concentrations at room temperature (22°C) (solid symbols: data obtained through rotational rheometry (RR); open symbols: data obtained by CFA). Typical process-related shear rate used during the printing process is shown by the dashed box ~ 50 - $150 s^{-1}$.

The post-printing behavior of materials can be probed by examining the recovery of viscosity of the food ink [12, 56]. A fast recovery after strong shear is necessary for a satisfying shape retention of the printed filaments [57, 58]. Figure 5.5a and b illustrates the recovery behavior of different ink formulations upon successive low and high shear rate intervals. Here, three different steps were used to mimic the shear conditions of the DIW process, including the inks storage in the syringe before printing, the inks extruded through the micronozzle and the recovery of the inks after deposition. This three-step method has also been used to investigate the recovery of fresh vegetables food inks [12]. As expected, the viscosity is high in the first step and decreases in the second step due to the increase of the shear rate (from 0.1 to 50 s^{-1}). In the third step, when the shear rate decreases back to 0.1 s^{-1} , a fast but partial recovery was observed for all inks. Figure 5.5c summarizes the percentage of recovery after the removal of high shear rate. The viscosity recovery is only $\sim 50 \%$ for the inks of 3GB/3XG and 8SP/4XG, respectively, an indication of poor shape retention, as shown previously Figure 5.2c and i. On the other hand, increasing the XG concentration improves the recovery properties (up to 85%). However, an obvious time-dependent decrease in viscosity of 3GB/13XG and 8SP/19XG food inks was observed during the high shear rate period. This thixotropic phenomenon demonstrated that the materials viscosity for inks with high XG concentration needs a relatively longer time ($20 - 50 \text{ s}$) to stabilize. In addition, the lower recovery value may indicate a permanent network breakage caused by the applied high shear rate [56, 59], which leads to an inconsistent ink behavior during the printing process and thus results in poor shape fidelity (Figure 5.2h and n).

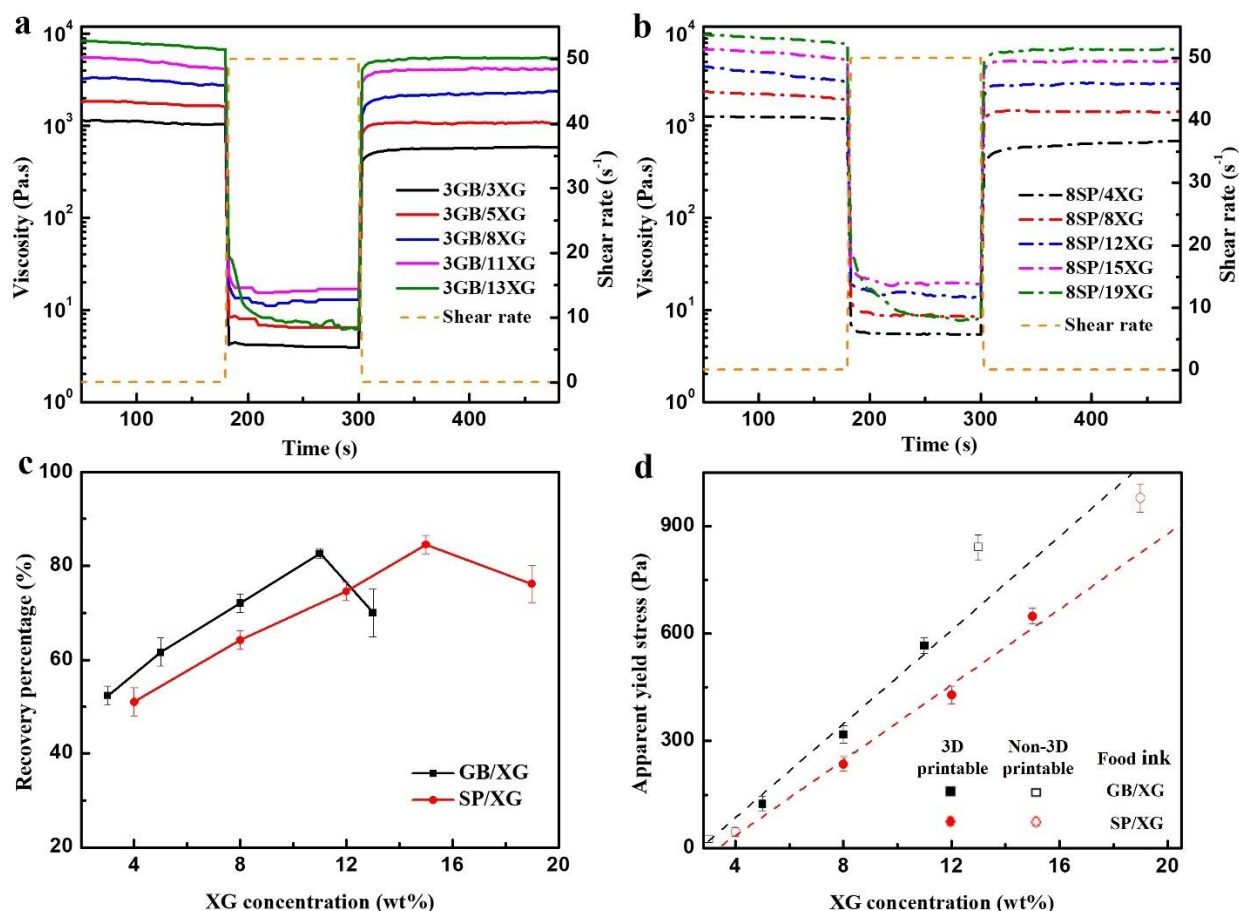


Figure 5.5 Recovery test of viscosity for (a) GB/XG and (b) SP/XG food inks with the following successive shear rates: 0.1 s⁻¹ (before 180 s), 50 s⁻¹ (180-300 s) (within the printing process region) and 0.1 s⁻¹ (after 300 s). (c) Shear recovery percentage of ink formulations calculated by the ratio of the average viscosity after high shear rate (50 s⁻¹) and the average viscosity obtained in the first low shear rate step (0.1 s⁻¹). (d) Apparent yield stress of GB/XG and SP/XG food inks with respect to the XG concentration (solid symbols: inks enables the 3D structures formation; open symbols: inappropriate inks for 3D structure printing).

The apparent yield stress is directly related to the minimum force applied to initiate a continuous flow of ink [60]. Since it also reflects the mechanical strength of inks, a sufficient yield stress can also guarantee the formation of a self-supporting structure after deposition [59, 61]. Figure 5.5d shows the apparent yield stress determined according to the method reported by Bruneaux et al. [47]. The apparent yield stress of food inks using 3GB/3XG and 8SP/4XG is <50 Pa, corresponding to a collapsed structure due to insufficient self-supporting properties, as previously

shown in Figure 5.2c and i. The apparent yield stress increases linearly with XG concentrations in the range of 3-11 wt% for GB/XG inks and 4-15 wt% for SP/XG inks with respective slopes of 65.5 and 52.7. As a higher yield stress can ensure the stacking of subsequent layers, it explains the better shape retention after printing at higher XG concentration. However, the 3GB/13XG and 8SP/19XG ink exhibit the high apparent yield stress > 800 Pa, which are beyond the linear fitting region due to the extrusion difficulty created by high XG concentration. In addition, the apparent yield stress shows a consistent result with the printable region discussed in the processing maps (Figure 5.3a and b). Specifically, the moderate apparent yield stress region of SP/XG ink is larger, which can support up to 15 wt% XG.

5.4.4 Shape fidelity map

In order to further study the post-printing behavior, shape fidelity maps were established. The shape fidelity map is plotted as a function of viscoelastic properties, namely $\tan \delta$, and as a defined shape fidelity index (SFI) Φ for the post-deposition assessment of printed 3D structures. Based on the circularity (C), the filament smoothness index Φ_f defined originally by Ouyang et al. [62] is used to quantitatively represent and distinguish the gelation conditions and filament formation of the inks. The Φ_f is determined by the perimeter l and pore area A_F for a square interstitial zone (schematically shown in Figure 5.6c) (Eq. (5.2)):

$$\phi_f = \frac{\pi}{4} \cdot \frac{1}{C} = \frac{l^2}{16A_F} \quad (5.2)$$

Regarding an ideal gelation and printability, the interconnected channels of the printed filaments display a perfect square shape with $\Phi_f=1$. Larger Φ_f value indicates a higher gelation degree (may lead to over-gelation), while a lower gelation degree is presented with $\Phi_f < 1$. In addition, the pore area ratio index Φ_p describes the effect of shadow created by multi-layer deposition, quantifying the self-supporting behavior of the food inks. The Φ_p can be defined as the ratio A_I/A_F (Figure 5.6c), where A_I represents the average pore area of the first layer from a top view, and A_F indicates the average pore area of the last printed layer. When $\Phi_p = 1$, it indicates that there is ideal self-supporting behavior of the food ink without pronounced collapse between layers, resulting in practically no shadow from the top view.

The SFI Φ was then calculated combining the arithmetic average of the filament smoothness index (Φ_f) and the pore area ratio index (Φ_p).

The SFI is plotted as a function of $\tan \delta$ for the different food inks at a given angular frequency to represent the effect of the viscoelastic properties of the materials. An angular frequency of 1 rad/s was chosen in order to observe the relative contributions of viscous component to the viscoelastic properties of the food inks [63]. An appropriate $\tan \delta$ range enables good solid-liquid balance of the ink formulations to build regular interconnection between filaments and layers, thus resulting in good shape fidelity [64]. Figure 5.6a shows the shape fidelity map for printed 3D scaffolds of both ink systems located in Zone IV of the processing maps (Figure 5.3a and b). The representative top-view images of printed 3D scaffolds within each zone are also presented in Figure 5.6b-g. In Zone A, the filaments in Figure 5.6b and e demonstrate an irregular morphology, which have a relatively higher $\Phi_f > 1.0$. In addition, the lower $\tan \delta$ value in this zone < 0.11 indicates a stronger solid-like behavior of the ink (over-gelation), which may hinder smooth filament formation during the printing process. In comparison, the fusion phenomenon between filaments and layers was found in Zone C (under-gelation), as indicated in Figure 5.6d and g, resulting in an insufficient Φ_f of < 0.6 . Also, the relatively high $\tan \delta$ value compared to other inks explains the relatively poor shape fidelity of the 3D scaffold in this zone. An appropriate range of $\tan \delta$ (0.12-0.17) of the inks in Zone B is needed to ensure good shape fidelity. It guarantees good filament quality as well as a favorable self-supporting property with just a slight deformation between layers, where the SFI is in the range of 0.6 to 1.0 (Figure 5.6c and f).

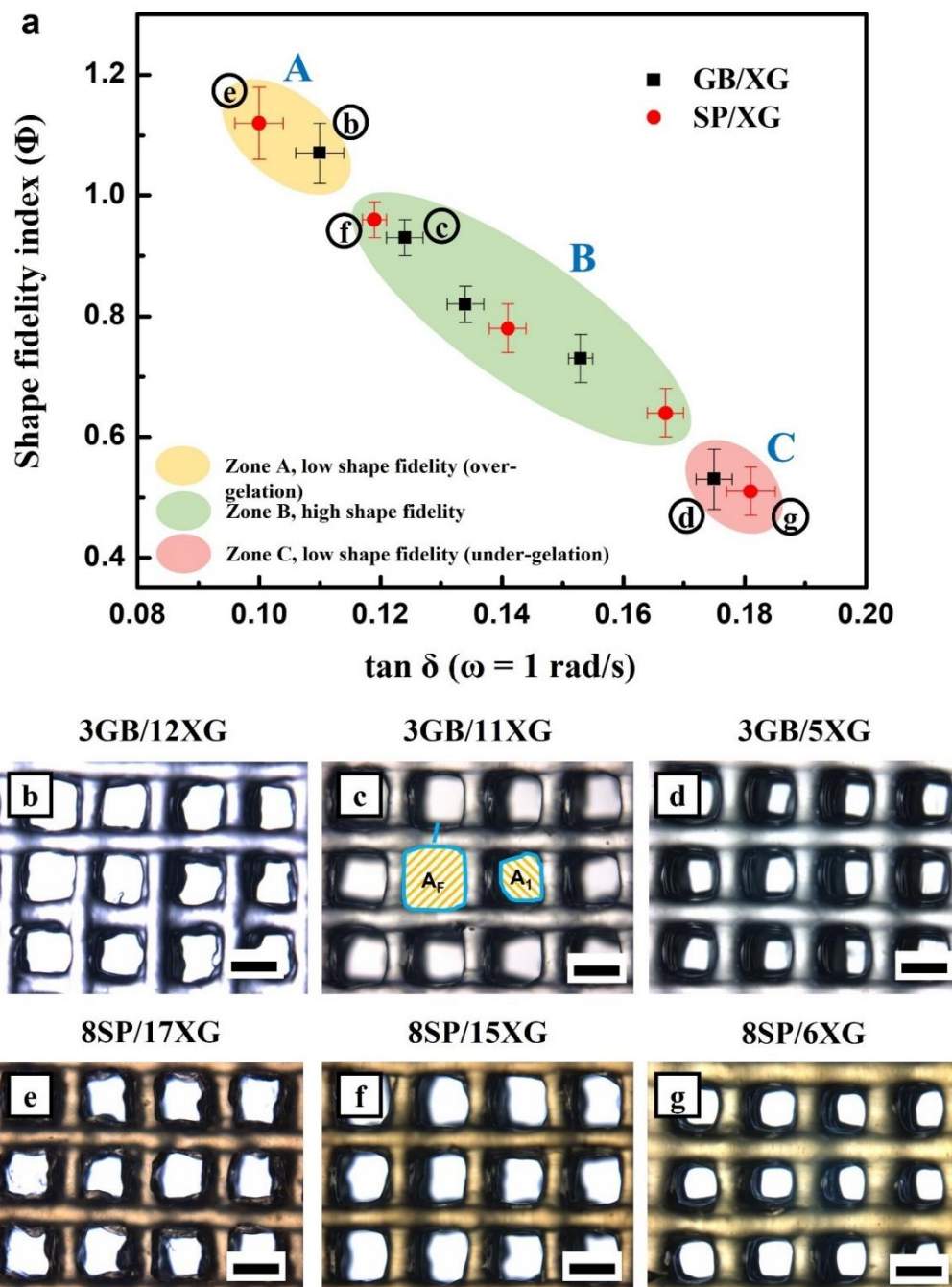


Figure 5.6 (a) Shape fidelity map of GB/XG and SP/XG food inks showing the ranges of $\tan \delta$ and SFI value for different as-printed 3D scaffold quality. Shape fidelity categories are indicated by letters from A to C. Representative optical top-view image of (b) 3GB/12XG (c) 3GB/11XG (d) 3GB/5XG (e) 8SP/17XG (f) 8SP/15XG and (g) 8SP/6XG scaffolds corresponding to different zones on the shape fidelity map using a 150 μm nozzle at 7 mm/s. All the photos and images were obtained at room temperature right after printing. Scale bars are 1 mm.

Besides the nature of the components and their content in the food ink, the visual appearance of the printed structure is also extremely critical for 3D food printing in order to attract consumers. A nutritious, appetitive and safe meal could definitely improve people's living quality [12, 65]. Compared to traditional food preparation methods, 3D food printing can create superior presentation with diverse designs, ingredients and colors within a single process. In addition, the printing process without using a mold and the ability to achieve more complex forms also makes 3D food printing more appealing to enter the future kitchen. Based on the information provided from the processing and shape fidelity maps, a multi-material printing process with both 3GB/11XG and 8SP/15XG food inks was carried out and a two-color dolphin-shape has been successfully printed and is shown in Figure 5.7, which highlights the usefulness of the generated maps.

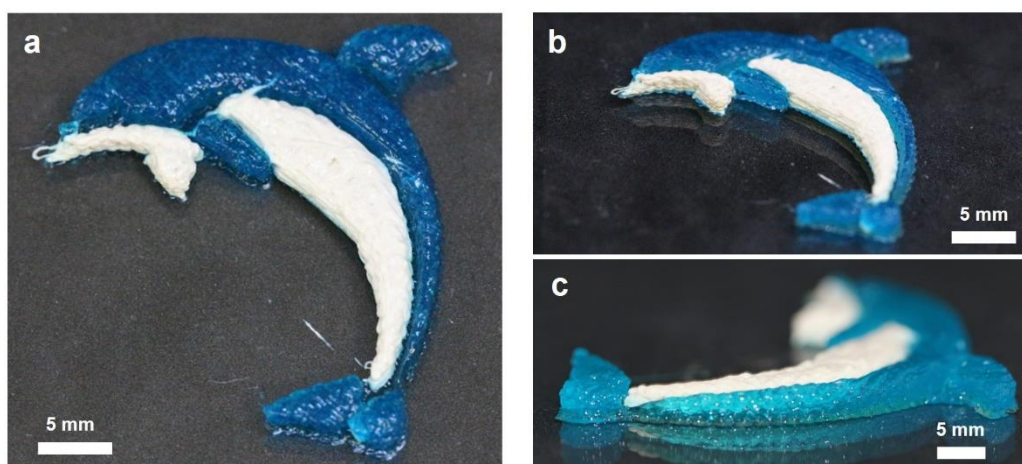


Figure 5.7 (a) Top-view, (b) front-view and (c) side-view of a 3D dolphin with the multi-material printing method using a 200 μm nozzle under 504 kPa for 3GB/11XG ink and 567 kPa for 8SP/15XG ink at 7 mm/s.

5.4.5 Compression testing for textural profile analysis (TPA)

On the basis of guaranteed high shape fidelity, the texture is also a vital assessment criteria of customers' acceptance and favor of printed food structure [66]. In order to mimic the mastication process, the TPA was conducted to investigate the textural characteristics related to food sensory perception (i.e. hardness, adhesiveness, cohesiveness and gumminess) [67] for the printed 3D scaffolds located in Zone B of the shape fidelity map (Figure 5.6a). Figure 5.8 shows the effect of

XG concentration and two different protein types in the food inks on the calculated textural parameters.

Hardness is associated with the force required for food to reach a given deformation [68]. The effect of XG concentration on the hardness of the printed scaffolds is presented in Figure 5.8a. For both GB/XG and SP/XG food inks, the hardness value increases at higher XG concentration. Specifically, the samples using 3GB/11XG and 8SP/15XG food inks have significant higher hardness values of 48.5 ± 2.2 g and 58.8 ± 1.3 g compared to the ones with lower XG concentrations within the same type of inks ($p < 0.05$). This may attribute to the denser network structure formed by higher XG concentration [69]. In order to evaluate the effect of protein types (GB and SP), samples with the same XG concentration (8 wt%) were also compared. Scaffold using the 3GB/8XG food ink has a significant larger hardness value of 32.5 ± 2.2 g than the one printed with 8SP/8XG, which is only 19.9 ± 3.1 g, even though SP/XG ink has a higher protein concentration. It can be attributed to the stiffer network formed by the triple helix structure of GB [70, 71].

A well-controlled adhesiveness results in an appropriate sticky mouthfeel, which provides the favorable taste and flavor perception [72]. Figure 5.8b shows the adhesiveness of printed samples with different XG concentrations. Consistent with a previously published work [73], the adhesiveness increases with the polymer concentration. Samples using 3GB/11XG and 8SP/15XG food inks have a significant higher value ($p < 0.05$) compared to lower XG concentration. In addition, lower adhesiveness ($p < 0.05$) is exhibited for scaffolds using the SP/XG system. It was previously reported that the soy protein isolate contributes to an improved lubrication to hydrogel samples, resulting in the decrease in the friction between the surfaces, then lowering the adhesion [74].

The cohesiveness indicates how a food retains its structure between the first and second bite. Figure 5.8c shows the cohesiveness for different XG concentrations in the different food inks. GB/XG food ink with 11 wt% XG and SP/XG ink with 15 wt% XG have the highest values of 0.76 ± 0.02 and 0.78 ± 0.02 , respectively ($p < 0.05$). Samples with higher XG concentration show larger cohesiveness, which may be attributed to the relatively stronger internal bindings, providing better resistance of 3D structures to compression [43, 75]. Moreover, regarding the stiffer structure of GB as mentioned before, 3GB/8XG food ink demonstrates a higher gel

strength than 8SP/8XG ink, which indicates a higher compressive strength, thus larger cohesiveness of the printed samples.

Gumminess illustrates the energy required to disintegrate a semi-solid food to a stable state for swallowing [76]. Coherent with our previous work [43], the trend of gumminess is similar to hardness for all tested samples, as shown in Figure 5.8d.

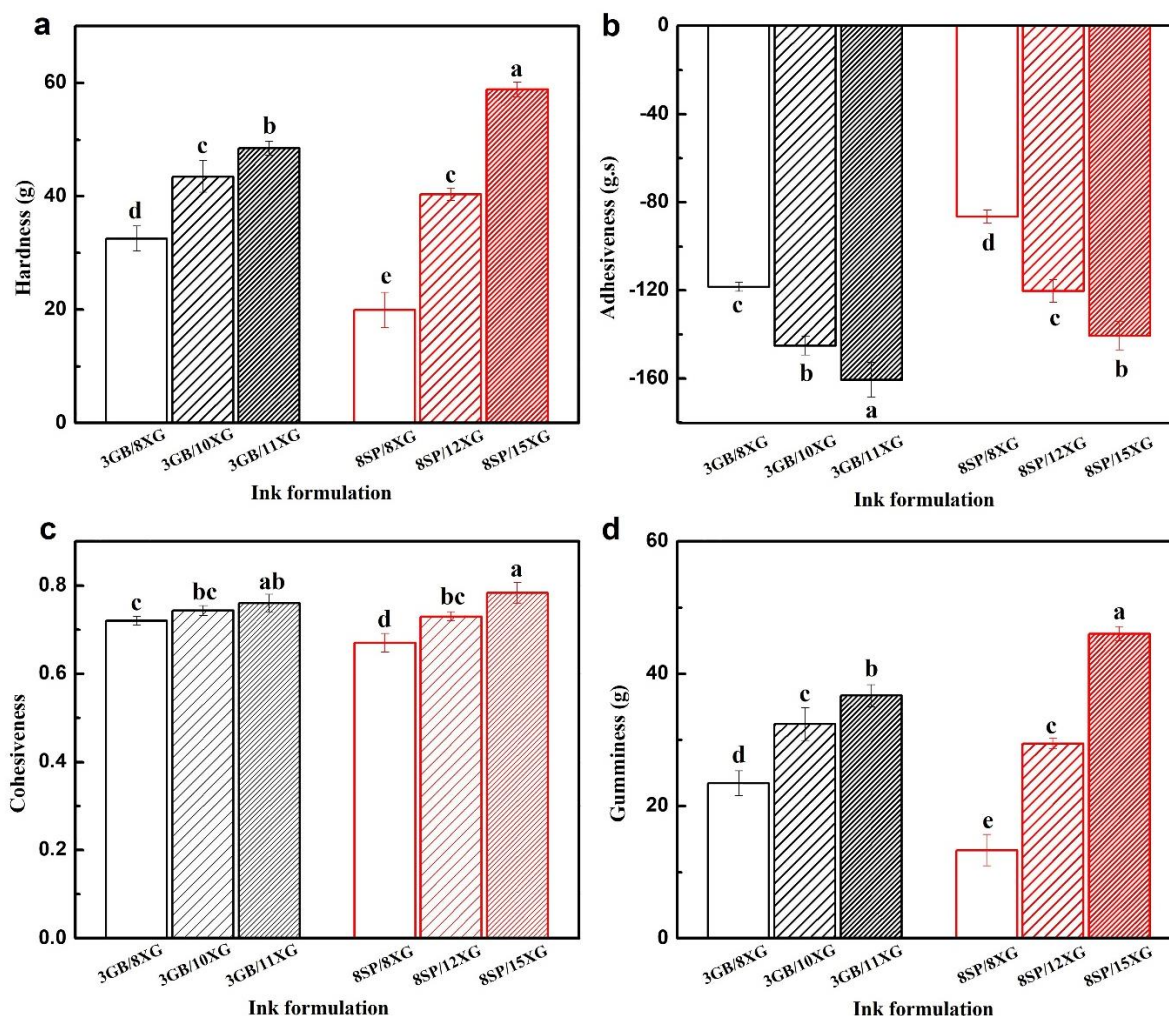


Figure 5.8 Texture properties of printed scaffolds using different inks located in Zone B of the shape fidelity map (Figure 5.6a): (a) hardness (b) adhesiveness (c) cohesiveness and (d) gumminess. Different lowercase letters above each error bar present a significant difference ($p < 0.05$).

Food texture is largely affected and can be tuned through food ink content, such as ingredient type and concentration, which can be easily controlled in 3D food printing. Group-specific food constructs could be created by carefully adjusting the ink properties according to the individual requirements. For example, food structures with low hardness are more acceptable for elderly people, especially for those who have chewing difficulties due to the problems such as tooth loss and decreasing bite force [77]. In this case, food inks with relatively lower XG concentration could be a satisfying choice. High adhesiveness increases the risk of choking for dysphagic

patients [51]. Thus, they would be more inclined to take SP/XG food products. In addition, inks with relatively higher gumminess benefits a more complex food structure fabrication, owing to the guaranteed self-supporting properties [51]. Protein/polysaccharide mixed gel inks such as GB/XG and SP/XG show great potential to be applied for the fabrication of soft food structures with individual textural requirement. However, further stringent tests should be carried out, focusing on the specific food applications such as the International Dysphagia Diet Standardization Initiative test (IDDSI) for dysphagia patients and sensory tests for food taste evaluation [78].

5.5 Conclusions

This work thoroughly investigated the inks composed of GB or/and SP mixed with XG for the purpose of the successful DIW 3D printing. SP/XG system demonstrated a wider printable region in the processing maps, while GB/XG ink facilitated forming 3D structures. Compared to XG alone, inks fabricated with mixed solutions showed improved rheological properties with efficient recovery of viscosity up to 85%. By controlling the XG concentration, scaffolds printed with 3GB/11XG and 8SP/15XG inks demonstrate the best structures. Too high viscosity caused an inconsistent ink behavior and a structure with poor shape fidelity. A general shape fidelity index was also established, which illustrates that the zone with higher shape fidelity was located for a $\tan \delta$ value of inks between 0.12 and 0.17, while a SFI value of printed scaffolds between 0.6 to 1.0. A precise multi-material complex dolphin shape using 3GB/11XG and 8SP/15XG food inks was successfully printed in one manufacturing step for the first time. Finally, by controlling the protein type (i.e., GB and SP) and XG concentration, the textural properties of the printed food scaffolds can be easily modulated according to personalized requirement. This work provides a valuable guidance for the selection of printable materials and process parameters, which can be applied to predict the printability of protein/polysaccharide-based food inks, as well as the well-controlled shape fidelity and shape retention of the printed structure. Furthermore, this feasible fabrication and prediction approach offers possibilities to extend to other soft hydrogel-based inks for additive manufacturing towards varied food applications.

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CHAPTER 6 ARTICLE 3 : 3D PRINTED PROTEIN/POLYSACCHARIDE FOOD SIMULANT FOR DYSPHAGIA DIET: IMPACT OF CELLULOSE NANOCRYSTALS

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6.1 Abstract

With the intensified aging population, the demand for dysphagia diet is growing rapidly. The food texture in a dysphagia diet needs to be adjusted to be easy-to-swallow. Thus, most food is transformed in mashed form, which is not visually appealing and reduces patients' appetite, resulting in a malnutrition risk. Three-dimensional (3D) food printing is an efficient technique to fabricate food designs with an attractive appearance and tailored nutritional content. In this work, we developed cellulose nanocrystal (CNC)-based protein/polysaccharide aqueous food inks using two commonly used proteins, namely gelatin B (GB) and whey protein isolate (WPI) for 3D printing, in combination with a polysaccharide frequently found in dysphagia diet, i.e. xanthan

gum (XG). Adding 1.0 wt% CNC in GB/XG and 1.5 wt% in WPI/XG improved the viscosity, yield stress and self-supporting ability of the inks, leading to higher shape fidelity right after printing and better shape retention for at least 96 h when stored at 4°C. Additionally, the thermal stability of GB/XG mixed gels was also largely enhanced by CNC addition, resulting in a high shape retention with ~90% of its original side area even at 50°C. As a demonstration, some complex 3D shapes fabricated using both GB/XG/1.0CNC and WPI/XG/1.5CNC inks by single and multi-material DIW printing could also well retain their structures after heating at 50°C for 5 min. Texture profile can also be tailored by both CNC concentrations and post-processing temperatures. Holding the temperature at 4°C on the GB/XG/1.5CNC sample turned it from a non-approved classification into a level 5-minced and moist dysphagia diet formulation, according to the International Dysphagia Diet Standardization Initiative (IDDSI) test framework. This work provides rheological and processing guidelines for designing attractive food creations for dysphagic patients using 3D printing.

Keywords: 3D food printing; Protein/polysaccharide food inks; Cellulose nanocrystals; Rheological properties; Texture profile analysis; Dysphagia diet

6.2 Introduction

As an emerging manufacturing technology, extrusion-based three-dimensional (3D) printing, and more precisely the direct-ink writing (DIW) is promising for food applications [1]. It provides numerous advantages for creating customized food designs, less material loss during processing and high precision of unconstrained appearance complexity by a computer-controlled layer-by-layer additive deposition principle [2-4]. For addressing the population with special dietary needs (e.g. elderly, children or athletes), 3D food printing also allows the personalization of food constructs according to their preference and specific requirement, including nutrition, texture and structure design [5-7]. Therefore, the DIW has the potential to be used in hospitals, nursing facilities, space stations, etc.[8].

The ever-growing aging population brings dysphagia as a major challenge in geriatric care [9, 10]. Dysphagia is a medical condition of swallowing difficulties [6]. It commonly happens in elderly people due to teeth loss and weakened tongue muscles [11]. As food residues remain in the buccal cavity, they cause a series of oropharyngeal problems such as coughing, aspiration pneumonia and even choking [8, 12]. Therefore, dysphagia-oriented diets need to prescribe food

that can be easily swallowed with soft, moist and cohesive textures [13]. However, most of the texture-modified foods are in mashed or puree forms, which are unappetizing for the patients, exacerbating the malnutrition problem caused by the swallowing difficulty [6]. Consequently, the development of dysphagia diets with adequate nutritional content, safe swallowing textures and appetizing appearances becomes a key challenge [8, 14]. 3D food printing shows great potential to fabricate therapeutic food for those with swallowing disorders. So far, a limited number of scientific contributions focused on 3D food printing aiming at dysphagia diet. Different types of fresh vegetables (e.g., bok choy, carrot and garden peas) [8], fruit (e.g., apple) [10] and edible fungi (e.g., black fungus and shiitake) [15] combined with various hydrocolloids were evaluated as inks for the preparation of dysphagia diet. Also, texture-modified meats such as pork [16], fish [17] and beef [18] have also been developed to create a diet for patients with swallowing difficulties. The majority of these studies were related to the finished product. There is still a lack of fundamental knowledge on 3D food printing of essential food constituents (e.g., proteins and polysaccharides), especially targeting at the dysphagia diet.

Proteins and polysaccharides are two essential natural biopolymers widely used to prepare mixed hydrogels in food systems [19-21]. The non-covalent interactions between them such as electrostatic interaction and hydrogen bonding play a major role in the structural and textural properties of food hydrocolloids [22, 23].

Gelatin B (GB), whey protein isolate (WPI) and xanthan gum (XG) are common daily-consumed proteins and polysaccharide, respectively. GB is a denatured linear protein derived from the alkaline hydrolysis of collagen from bovine skin [24], which has the advantages of low-cost, biodegradability and biocompatibility [25, 26]. Moreover, its aqueous solution exhibits a thermo-reversible behavior with a helix to coil transition temperature of $\sim 25^{\circ}\text{C}$ [27]. WPI consists of a combination of globular proteins, including large amount of β -lactoglobulin (60–70 %), few α -lactalbumin and bovine serum albumin [21, 28]. Owing to its high nutritional content and bioactivity, WPI has been highly considered in food processing [28]. Xanthan gum (XG) is an anionic microbial polysaccharide [29]. It exhibits high stability to both temperature and pH and is widely used in food products [30]. Since XG also exhibits a pronounced shear-thinning behavior, it has traditionally been used in dysphagia diet [31].

The unique physicochemical features of cellulose nanocrystals (CNC) such as their high aspect ratio, abundant hydroxy groups to form hydrogen bonds, and biocompatibility, make them ideal for a wide range of applications in various fields, such as food packaging, Pickering emulsion stabilizer, and so on [28, 32, 33]. Up to now, nanocellulose has not been approved yet by the FDA (Food and Drug Administration) [34], but based on the positive studies focused on the CNC safety these years, such naturally sourced polysaccharide has great potential for the food industry [34, 35]. For example, CNC has been used to improve heat-induced WPI gels by promoting 3D network formation [28]. The quality of meat sausages with pre-emulsified soybean oil was also enhanced by the presence of CNC by reducing cooking loss and increasing water holding capacity and emulsion stability [36].

Here, aiming for the 3D printed dysphagia diet design, we developed the novel food ink based on GB/XG mixed gel incorporated of CNC. As a comparison, another protein/polysaccharide system using a different widely consumed protein, namely WPI, in combination with XG and CNC is also thoroughly studied. We investigate the effect of CNC addition and post-processing temperature on shape fidelity, dimensional stability, and textural properties of 3D printed samples. The rheological properties of the food inks are also examined to better understand their effect on printing and post-deposition behavior. Finally, we also evaluate the suitability of the food inks in the context of dysphagia diet using the International Dysphagia Diet Standardisation Initiative (IDDSI) test.

6.3 Materials and methods

6.3.1 Materials

Gelatin from bovine skin (type B, G9382, bloom index ~ 225 , molecular weight (M_w): ~ 50 kDa, $pI = 4.7-5.3$) was purchased from Sigma-Aldrich Canada. XG (KELTROL RD, food-grade quality, M_w : $\sim 2 \times 10^3$ kDa) was supplied by CP Kelco U.S. WPI (BiPRO 9500, protein content ≥ 99.1 , on dry basis, M_w : 14-160 kDa, $pI = 5.3-5.5$) used in this research was provided by Agropur Ingredients U.S. CNC (density: ~ 1540 kg.m $^{-3}$, sulfur over carbon S/C atomic ratio is ~ 0.0057 [37] or 211 mmol.kg $^{-1}$, mean length and width of the primary rod-like particle size are: 76 nm and 3.4 nm) was acquired in a spray-dried form from CelluForce Canada. The food-grade color was purchased from Altius Spices & Seasonings Inc., Canada. All the chemicals were used as received without further purification.

6.3.2 CNC suspension preparation

CNC suspensions were prepared according to the protocol of Girard et al. [38]. Specific amounts of dried powder of CNCs were dispersed in Milli-Q water (18.2 Ω) to prepare different concentrations of CNC suspensions (0.5, 1 and 1.5 wt%). The pre-mixed suspensions were then sonicated with an energy around 167 kJ.g⁻¹.L⁻¹ (weight of CNC and volume of suspension), immersing the probe off-centered near the liquid surface and up to 1/3 of the suspension height, while being in an ice bath. The prepared CNC suspensions were stored at room temperature (RT) (22 ± 1°C) for further characterization.

6.3.3 Food-ink preparation

Based on our preliminary experiments, the respective GB, WPI and XG concentrations were selected on meeting the requirement of appropriate consistency for a printable gel. GB powder (2 wt%) was firstly swelled in different concentrations of CNC suspensions for 20 min and then dissolved at 60°C with magnetic stirring at 300 rpm for 30 min at 60°C. WPI/CNC suspensions were prepared by dispersing the WPI powder (8 wt%) into CNC suspensions by magnetically stirring at 200 rpm for 2 h at RT. 8 wt% XG dry powder was then added to the GB/CNC and the WPI/CNC suspensions, respectively. The GB/XG/CNC and WPI/XG/CNC suspensions were obtained under mechanical stirring for 12 h at 40°C. The suspensions were then placed in a mixer (DAC 515-200 SE, FlackTeck SpeedMixer®) at 2750 rpm for 10 min to ensure homogeneity. The final pH of the prepared GB/XG/CNC and SP/XG/CNC suspensions were 5.8 and 6.3, respectively, hence slightly above the pI of both respective proteins. The air bubbles in food inks were removed via centrifugation at 2000 rpm for 1h at 22°C.

6.3.4 Single-material DIW process

Single-material DIW process was performed using an extrusion-based 3D printer (I&J2200-4, I&J Fisnar Inc.) consisting of an air-operated dispensing system and a computer assisted three-axis positioning stage (Figure 6.1a). Before printing, the ink materials were poured into a syringe barrel (3 mL, Nordson EFD), linked with a cylindrical micronozzle (exit inner diameter $D = 200$ μ m, capillary length $L = 12.7$ mm, Nordson EFD). The food inks were then extruded in a layer-by-layer manner under appropriate applied pressures controlled by a high-pressure adaptor (HP-7x, Nordson EFD) along the print paths at RT.

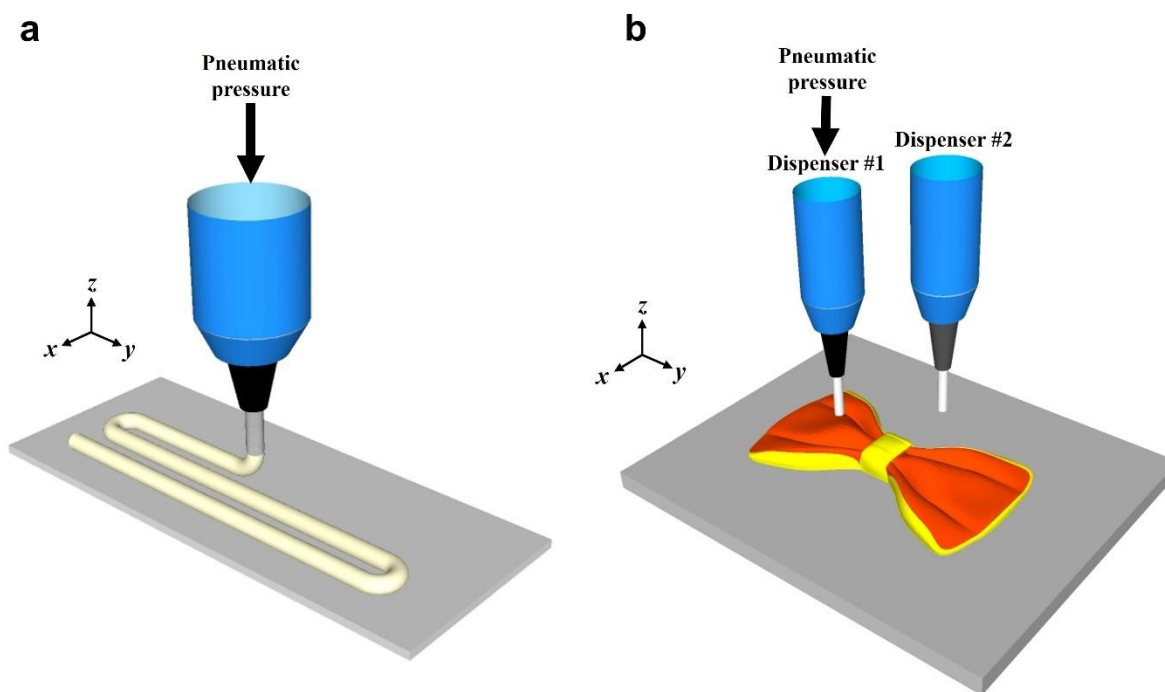


Figure 6.1 Schematic representations of the (a) single-material with a protein/polysaccharide mixed gel ink and (b) multi-material DIW 3D printing process with two different protein/polysaccharide mixed gel inks.

6.3.5 Multi-material DIW process

The multi-material DIW system is located on a custom-made multi-functional three-axis positioning gantry stage (Aerotech, USA), as shown in Figure 6.2. It is connected to two independent air-operated dispensing systems (Figure 6.1b) loaded with different food inks into the barrels (3 mL, Nordson EFD). The relative position of the two dispensers were adjusted by the calibration cameras. A pneumatic-driven controller (Mëkanic, Canada) tailored the applied pressures on the piston of the dispensing apparatus (HP-7x, Nordson EFD), in order to extrude the two food inks independently from cylindrical micronozzles (Nordson EFD) along the print paths.

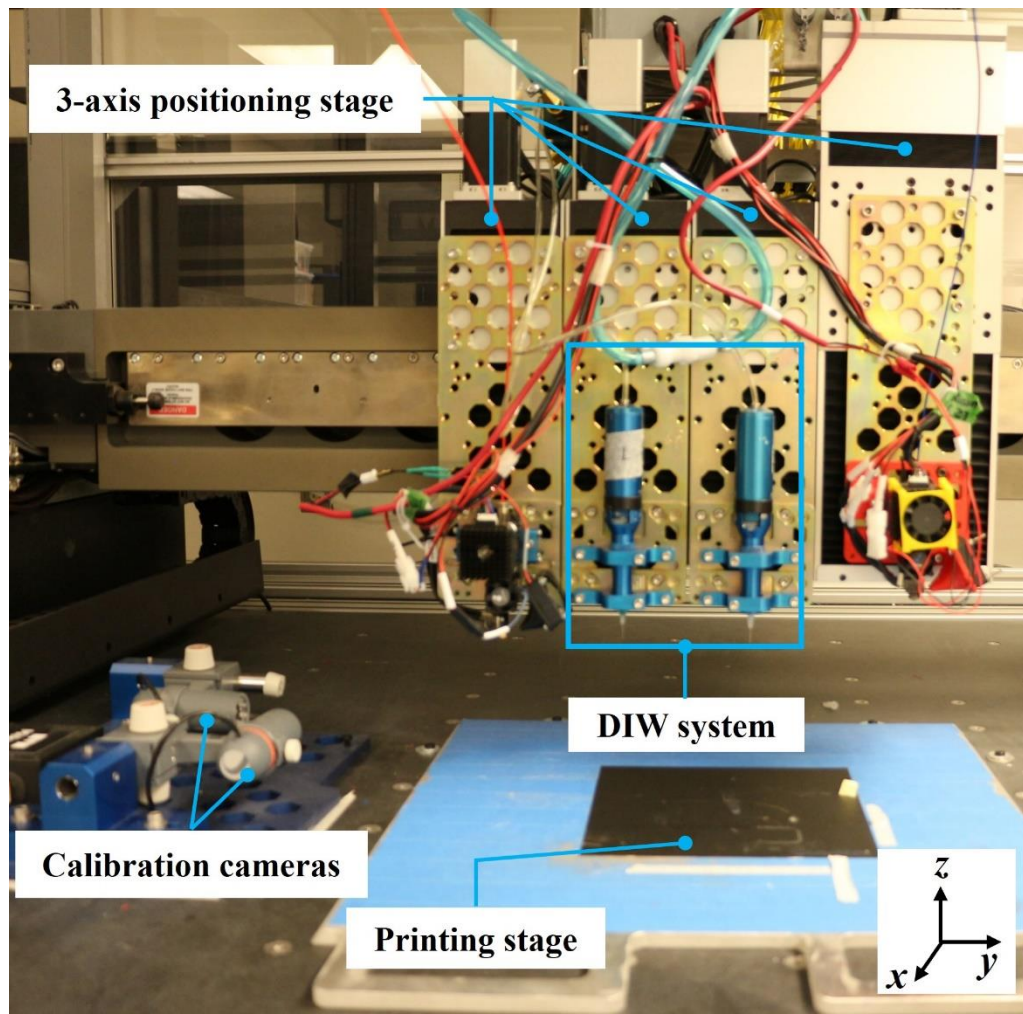


Figure 6.2 Picture of the multi-process three-axis positioning gantry system including the two DIW printing heads.

6.3.6 3D structures fabrication

The original 3D models (i.e. STL files) of the dolphin, bowtie, magic fish and double heart presented later were obtained from Thingiverse (<http://www.thingiverse.com/>). The slicing software, Simplify 3D, was used to generate the initial G-codes for printing paths. Regarding the 3D structures with single material, a Python program was used to translate the initial codes into the language of the printing software (JR Point for Dispensing, Janome Sewing Machine). For the multi-material microstructure fabrication, the initial G-codes were translated into the language of Aerotech system (Aerobasic) by RoboDK 4.2.4 (RoboDK Inc.), a robotic simulation software.

6.3.7 Rheological measurements

The rheological properties of the different food ink formulations were measured using a stress-controlled rheometer (MCR-502, Anton Paar, Graz, Austria) fitted with a rough surface parallel plate flow geometry (25 mm diameter, 1 mm gap) to avoid wall slip in three replicates. Mineral oil was slightly added to cover the edge of the sample and prevent water evaporation. The following different rheological tests were performed:

- (1) Rotational shear-viscosity measurements were carried out in the low-to-moderate shear rate region ($0.01\text{-}10\text{ s}^{-1}$) at RT (22°C).
- (2) The process-related viscosity in the high shear rate region was measured by capillary flow analysis (CFA) as presented in our previous work [39] based on the method reported by Bruneaux et al. [40].
- (3) Frequency sweep tests were conducted to investigate the viscoelastic properties of the food ink formulations. The sweeps were carried out over a range of 0.1 to 10 rad/s at 1% strain amplitude (determined within the linear viscoelastic (LVE) region by strain sweep). Rheocompass software (Anton Paar) was used to record the shear storage modulus (G'), loss modulus (G'') and also the resulting loss tangent ($\tan \delta$).

Regarding the tests at 4°C and 50°C , a temperature-controlled Peltier system was used to pre-cool or pre-heat the lower plate at the desired temperatures.

- (4) According to the method reported by Bruneaux et al. [40], the process apparent yield stress τ_{app} (Eq. 6.1) was calculated based on the minimum pressure required for the flow of the food ink extruded by the 3D printer at RT.

$$\tau_{\text{app}} = \frac{P_{\text{flow}} - P_0}{2 \left(\frac{L}{R} \right)} \quad (6.1)$$

where P_{flow} is the minimum pressure required for flow, P_0 is the atmospheric pressure, R is the inner radius of the nozzle.

- (5) Constant strain of 1% and angular frequency of 1 rad/s were used to perform temperature sweeps. The changes in storage modulus (G') and loss modulus (G'') were monitored from 10°C to 70°C at a heating rate of 1°C/min , in order to investigate the effect of temperature on the

gelation properties of the food inks. As a comparison, 5 wt% GB solution and 20 wt% WPI were also tested with the MCR 501 Rheometer (Anton Paar, Anton Paar, Graz, Austria) using the rough surface Couette flow geometry with the same controlling parameters.

6.3.8 Morphological characterization

The top-view images of 3D scaffolds were observed using a stereo microscope (SZX12, Olympus Corporation). A digital camera (EOS TRBRL T4I, Canon) was used to capture the images of 3D printed structures. All the images were analyzed using an image processing software (Image Pro Plus 6.0, Media Cybernetics).

6.3.9 Printability and dimensional stability evaluation of 3D-printed scaffolds

6.3.9.1 Printability evaluation

In order to evaluate the printability of the food inks, the shape fidelity index (SFI) and the printing accuracy of side area were calculated based on the optical top-view images and the photographs of the printed scaffolds. The details of SFI definitions and calculations are discussed later in Section 6.4.1. The side area of printed scaffold was measured using Image Pro Plus 6.0 (Media Cybernetics), and the printing accuracy of the side area was evaluated with the following Eq. (Eq. 6.2).

$$\text{Printing accuracy of side area} = \frac{S_0}{S} \quad (6.2)$$

where S is the target side area of the scaffold and S_0 is the side area of the printed scaffold determined right after printing.

6.3.9.1 Determination of dimensional stability of 3D-printed structures

The printed 3D scaffolds were put in an oven at 50°C for 5 min to ensure a complete heating of the whole structure (12 mm × 12 mm × 8 mm). The thermal dimensional stability was then estimated by directly measuring the side area evolutions of the printed 3D scaffolds before and after heating. The thermal stability was determined by calculating the normalized side area with Eq.6.3.

$$\text{Thermal stability of side area} = \frac{S_T}{S_0} \quad (6.3)$$

where S_T is the side area of the printed scaffold determined after heating at 50°C for 5 min.

6.3.10 Compression tests for textural profile analysis

Texture profile analysis (TPA) of printed samples were performed using the Mach-1TM Micromechanical tester (Biomomentum Inc.). Hardness, adhesiveness, cohesiveness and gumminess of the printed samples were determined in accordance with the description in our previous work [39]. The 3D printed scaffolds (18 mm × 18 mm × 10 mm) composed of GB/XG or WPI/XG inks with different CNC concentrations were used for the TPA. A cylindrical probe with a diameter of 25 mm was used to compress the samples to obtain the load-time curves. A double-cycle compression test was performed as follow: a contact force of 1 g, a contact speed of 0.1 mm/s, the test and withdrawal speeds of 0.5 mm/s and compression distance of 2 mm. All measurements were performed at RT.

6.3.11 IDDSI tests methods

The IDDSI (<https://iddsi.org/Framework>) classifies into 8 levels the modified drinks (levels 0-4) and foods (levels 4-7) according to their texture characteristics [41]. A combination of tests is proposed to confirm the final category of the texturally modified food product. Based on the physical characteristics of inks samples and preliminary experiments, level 4-pureed/extremely thick or level 5-minced and moist were the most probable classes within the IDDSI framework for our samples. The fork pressure test which evaluates the hardness of the samples was performed where the 3D printed cubes (15 × 15 × 15 mm³) were pressed by the thumb using a house fork. The fork drip test which assesses the thickness of the samples was carried out by placing the food sample on the fork and then observing the flow behavior of the sample. Regarding the spoon tile test, firstly, a scoop of ink sample was held on the spoon to check whether it can keep steady. Then the spoon was tilted or shaken lightly to check the sample's state, in order to evaluate the performance of the cohesiveness and adhesiveness of the samples.

6.3.12 Statistical analysis

All the measurements were performed in triplicates, and the results were presented as mean values ± standard deviation (SD). SPSS software (SPSS 20.0, IBM, Chicago, IL, USA) was used to evaluate the data. Significant differences were analyzed using Duncan's Multiple Range test at $p < 0.05$.

6.4 Results and discussion

6.4.1 3D printed structures and shape fidelity analysis

Printability characteristics are crucial to evaluate the ability of food inks to fabricate 3D structures. Here, we investigated the impact of CNC concentration on the printability of GB/XG and WPI/XG inks for the fabrication of 3D scaffolds. Figure 6.3a-h show the as-printed 3D scaffolds of both ink systems with different CNC concentrations (0-1.5 wt%), along with their corresponding optical top-view images (Figure 6.3a_i-h_i).

The optical images show that CNC concentration has a significant effect on the appearance of the printed 3D scaffolds. The scaffolds printed without CNC addition exhibit a collapsed zone at their centers due to their poor self-supporting behavior (Figure 6.3a and e). Moreover, the top-view images of these scaffolds show rounded corners at the interconnections of channels and large shadow areas created by multilayer stacking (Figure 6.3a_i and b_i). In contrast, better shape fidelity and sharper outlines were achieved for both GB/XG and WPI/XG inks with CNC addition (Figure 6.3b-c and f-h). The 3D scaffolds printed using GB/XG/1.0CNC ink and WPI/XG/1.5CNC ink exhibit the most regular shapes, and their top-view images show the desired square shape with very narrow shadow areas (Figure 6.3c_i and h_i).

However, further increasing CNC concentration to 1.5 wt% for GB/XG ink resulted in an unstable extrusion behavior during the printing process, which is characterized by rough filaments and irregular shapes in the printed scaffolds (Figure 6.3d_i), leading to structure defects (Figure 6.3d) with a bad appearance.

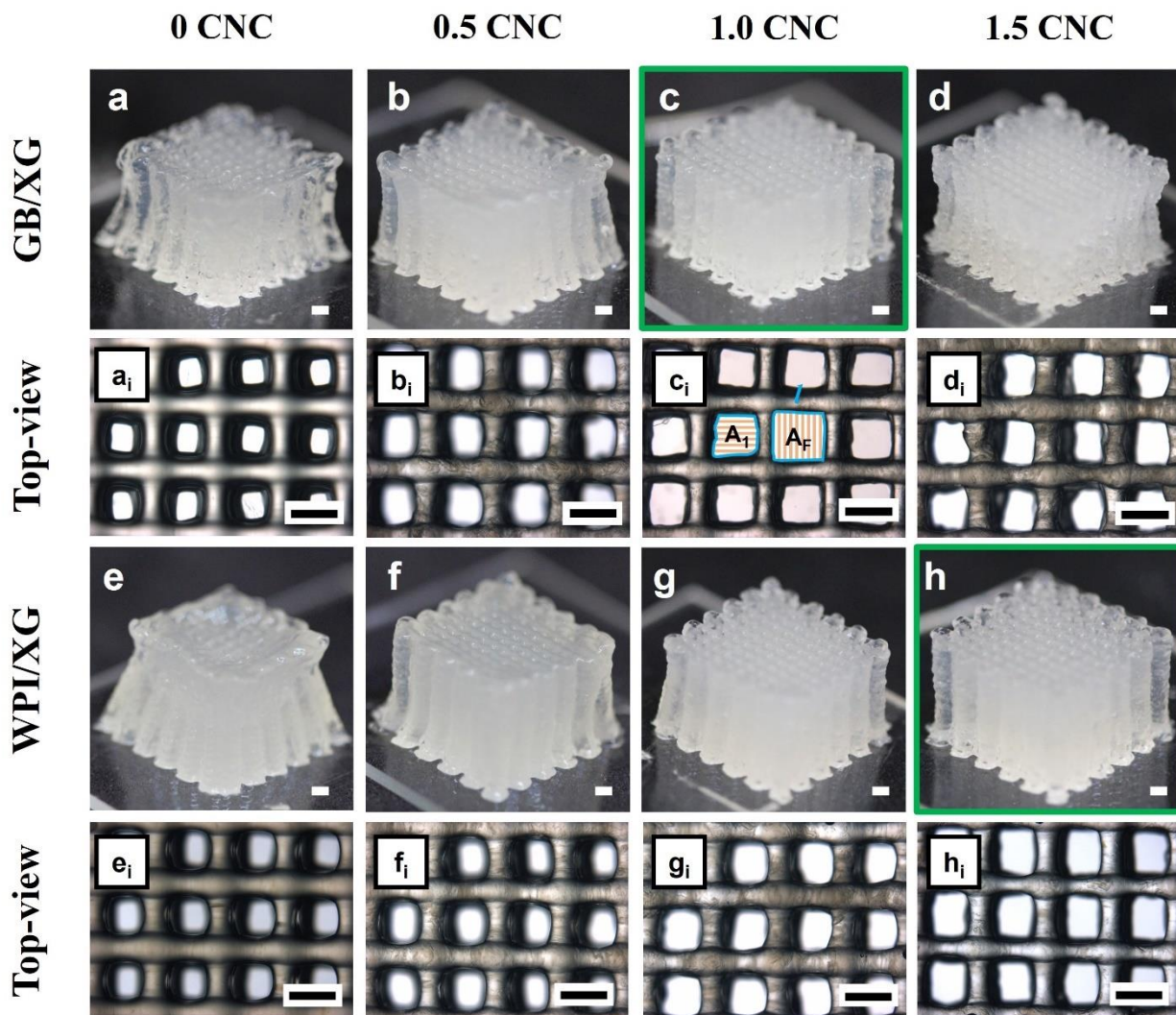


Figure 6.3 As-printed 40-layers (a-d) GB/XG scaffolds and (e-h) WPI/XG scaffolds with 0, 0.5, 1 and 1.5 wt% CNC addition using a 200 μm nozzle; Corresponding optical top-view images of (a_i-d_i) GB/XG scaffolds and (e_i-h_i) WPI/XG scaffolds. All the photographs and images were taken at RT right after printing. Scale bars are 1 mm. Images with green contours represent the 3D scaffolds featuring the highest shape fidelity.

In order to better quantify the printability of both GB/XG and WPI/XG systems with different CNC concentrations, a shape fidelity index (SFI) and printing accuracy (Eq. 2) were calculated and evaluated. The SFI of top-view images (Figure 6.3a_i-h_i) was defined based on the research of Ouyang et al. [42] and our previous work [39]. It is calculated by the arithmetic average of the

filament smoothness index (Φ_f) and the pore area ratio index (Φ_p). As shown in Figure 6.3c_i, the Φ_f is determined using the perimeter l and pore area A_F for an interconnected channel (Eq. 6.4).

$$\phi_f = \frac{l^2}{16A_F} \quad (6.4)$$

A perfect square shape of the interstitial zone of the filaments results in a Φ_f value equals to 1, indicating an ideal gelation state for the ink. Both Φ_f larger or smaller than 1 represents a greater or a lower gelation degree.

Different degrees of shadow may be created by multi-layer deposition, which can be defined as Φ_p or the ratio of the average pore area of the first layer A_I and the last printed layer A_F (Figure 6.3c_i). The ideal self-supporting behavior has a Φ_p value of 1, exhibiting no shadow from the top view.

Figure 6.4a shows the effect of CNC concentration on the SFI value of 3D scaffolds made with both GB/XG and WPI/XG inks. The printed GB/XG and WPI/XG scaffolds without CNC had relatively low SFI values of 0.52 ± 0.07 and 0.49 ± 0.05 , respectively. With the addition of CNC, the SFI value gradually increased, indicating an improved shape fidelity. The best ink formulations, i.e. GB/XG/1.0CNC ink and WPI/XG/1.5CNC ink (Figure 6.3c and h), resulted in similar SFI values ~ 1.0 . When the CNC concentration in GB/XG ink was further increased to 1.5 wt%, a larger SFI value of ~ 1.1 was obtained, which was mainly caused by a higher Φ_f value, representing rough filaments due to the over-gelled ink [42]. Figure 6.4b depicts the effect of CNC concentration on printing accuracy of 3D scaffolds made with both GB/XG and WPI/XG inks. The lowest values of 0.81 ± 0.05 and 0.72 ± 0.08 were observed for the printed GB/XG and WPI/XG scaffolds without CNC addition, respectively. A representative irregular shape of side area was shown in Figure S6.1a. Compared to the CNC-free ink, the printed scaffolds using the best ink formulations (i.e., GB/XG/1.0CNC ink and WPI/XG/1.5CNC ink) showed an increase printing accuracy of 18% and 29%, respectively. In addition, the shape of the side area was more uniform and square (presented in Figure S6.1b). However, a decrease in printing accuracy was observed for the scaffold using GB/XG/1.5CNC ink, because this concentration caused inconsistent printing behavior due to the high viscosity of the ink. The irregular filaments affected the multi-layer deposition and resulted in relatively lower printing accuracy.

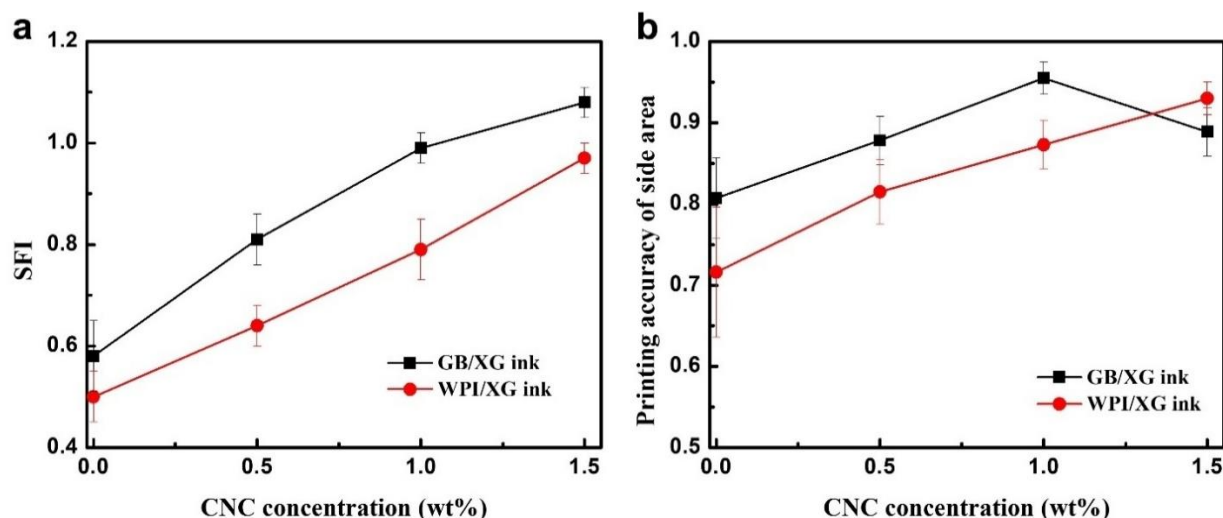


Figure 6.4 (a) SFI value and (b) printing accuracy (Eq. 6.2) of side area for 3D scaffolds printed with both GB/XG and WPI/XG food inks with respect to CNC concentration.

6.4.2 Rheological properties of food inks

Rheological properties are crucial for determining the performance of 3D food printing, as they are directly related to the flow behavior during the printing process and ensure desirable post-deposition behavior of printed structures. Additionally, there is a strong correlation between the rheological properties of food inks and the design of dysphagia-oriented products, which aim to ensure a safe swallowing process and enjoyable texture and mouthfeel [43, 44].

Viscosity is an important rheological indicator ensuring smooth flow during the printing process and safe swallowing by dysphagic patients [15, 45]. Figure 6.5a and b report the effect of shear rates on the viscosity of the GB/XG and WPI/XG inks with different CNC addition using shear viscosities measured by rotational rheometry (RR) (0.01 - 10 s^{-1} , solid symbols) and the process-related apparent viscosity calculated from capillary flow analysis (CFA) ($> 10 \text{ s}^{-1}$, open symbols). The shear-thinning behavior observed in all food inks is well-suited for the printing process, enabling smooth extrusion during deposition under high shear rate. Additionally, it is beneficial for dysphagic patients, as the low viscosity under high shear applied by the tongue allows for easy and safe chewing of the food bolus during the swallowing process [15, 44]. The data points in low and high $\dot{\gamma}$ regions are generally consistency. The power-law model (Eq. 6.5) can be used to fit the data:

$$\eta = K\dot{\gamma}^{(n-1)} \quad (6.5)$$

where η is the viscosity, $\dot{\gamma}$ is the shear rate, K is the consistency index and n is the power-law index.

Table 6.1 lists the respective fitted power-law parameters n and K for the GB/XG and WPI/XG inks with different CNC concentrations. All the inks show a relatively low n below 0.2, which indicates a strong shear-thinning behavior which corresponds to a typical soft solid character such as XG alone [46]. Moreover, as the CNC concentration increases in both GB/XG and WPI/XG systems, there is a decrease in n , which shows an enhanced shear-thinning behavior. It is beneficial for dysphagia patients as it provides a more pleasant texture perception with a less slimy feeling and makes the swallowing/chewing process easier [44]. Therefore, the addition of CNC in our mixed gel systems can be considered as a promising method for designing dysphagia diets. Furthermore, K increases with increasing CNC concentration in both systems (Table 6.1). For instance, with CNC concentration increased from 0-1.5 wt%, K ranged from 214.25 ± 1.57 to 777.88 ± 6.18 Pa. s ^{n} for GB/XG food inks, and from 170.49 ± 0.49 to 456.12 ± 3.20 Pa. s ^{n} for WPI/XG food inks. However, a too high K may cause an inconsistent extrusion behavior, leading to an rough or discontinuous filaments [15, 45, 47]. A high K of 777.88 ± 6.18 Pa. s ^{n} for GB/XG/1.5CNC ink might explain the irregular scaffold structure in Figure 6.3d.

The increase in the viscosity of both GB/XG and WPI/XG systems was also observed with the CNC addition in Figure 6.5a and b. Specifically, the viscosity of GB/XG inks with different CNC (0-1.5 wt%) concentrations increased from 217 to 780 Pa.s at 1 s⁻¹. Similarly, a 2.7 times increase in viscosity was noted in WPI/XG/1.5 CNC ink compared to CNC-free WPI/XG ink. The GB/XG system has a higher viscosity than the WPI/XG gel system, even with a lower protein content. It is likely due to the better gelation properties of GB/XG inks, which are a result of the extended and rigid triple helical structures of GB [48, 49]. At the most representative reference shear rate for normal swallowing processes, which is ~ 50 s⁻¹ [44], the GB/XG ink had a 1.26 times higher viscosity than the WPI/XG ink without CNC addition.

Table 6.1 Power-law parameters of different ink formulations

Ink formulation	Power-law fit	
	Power-law index, n (-)	Consistency index, K (Pa. s ^{n})
GB/XG	0.15 ± 0.02	214.25 ± 1.57
GB/XG/0.5CNC	0.11 ± 0.03	320.60 ± 2.79
GB/XG/1.0CNC	0.09 ± 0.03	473.49 ± 4.73
GB/XG/1.5CNC	0.07 ± 0.01	777.88 ± 6.18
WPI/XG	0.16 ± 0.01	170.49 ± 0.49
WPI/XG/0.5CNC	0.15 ± 0.02	230.37 ± 1.13
WPI/XG/1.0CNC	0.13 ± 0.02	305.36 ± 1.69
WPI/XG/1.5CNC	0.12 ± 0.02	456.12 ± 3.20

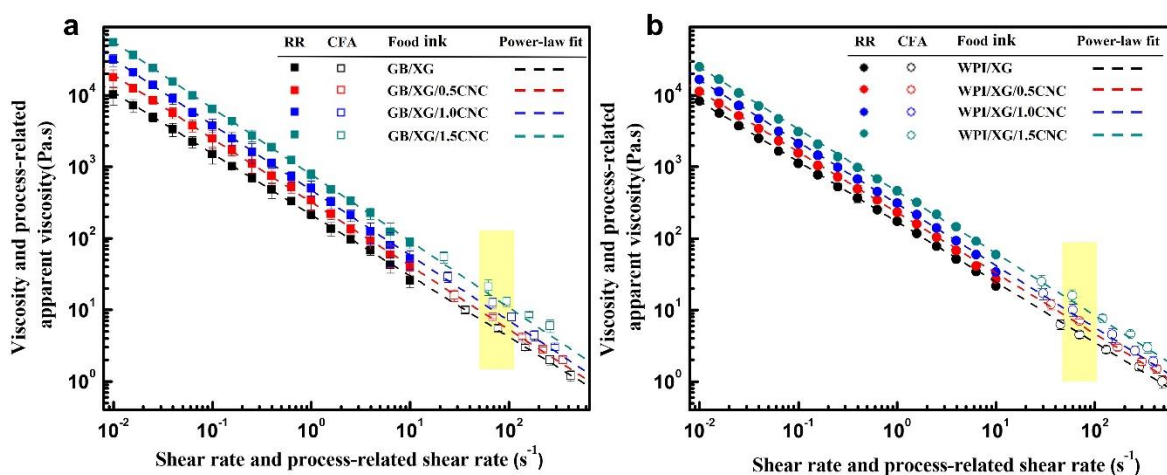


Figure 6.5 Viscosity as a function of shear rate for (a) GB/XG and (b) WPI/XG food inks with different CNC concentrations at RT (solid symbols: data measured using RR; open symbols: data obtained by CFA). The fitted shear-thinning power-law model are showed with dashed lines. Typical processing window is shown by the yellow zone ~ 50 - 100 s^{-1} .

The apparent yield stress represents the minimum shear stress required to initiate the flow of the ink [44] and is directly related to the efforts of the tongue in initiating the flow of the food bolus

for the dysphagia diet [10]. On one end an adequate apparent yield stress is necessary to ensure a safe swallowing process [44], and on the other hand it also qualifies the self-supporting behavior of the food ink, which is essential for DIW printing [50, 51]. Figure 6.6 shows the apparent yield stress of both GB/XG and WPI/XG food inks evaluated using the method reported by Bruneaux et al. (Eq. 6.1) [40]. The inks without CNC addition showed a minimum apparent yield stress below 200 Pa, indicating poor self-supporting behavior leading to collapsing structures. Increasing CNC concentration ranging from 0-1.5 wt%, the apparent yield stress of both inks showed a linear increase, with slopes of 361 Pa/wt% and 254 Pa/wt% for GB/XG and WPI/XG inks, respectively. The best ink formulations, GB/XG/1.0CNC, and WPI/XG/1.5CNC, showed an apparent yield stress of ~ 500 Pa, resulting in satisfactory self-supporting characteristics and regular 3D structures (Figure 6.3c and h). High apparent yield stress leads to a non-uniform printed 3D scaffold shown in Figure 6.3d, as shown by GB/XG/1.5CNC ink with an apparent yield stress of ~ 800 Pa.

The increased viscosity and apparent yield stress of both GB/XG and WPI/XG food inks containing CNCs is attributed to the strengthened 3D network formed by hydrogen bonding and van der Waals forces between CNCs-protein chains and/or XG chains [28, 52-55], as well as between CNCs [56].

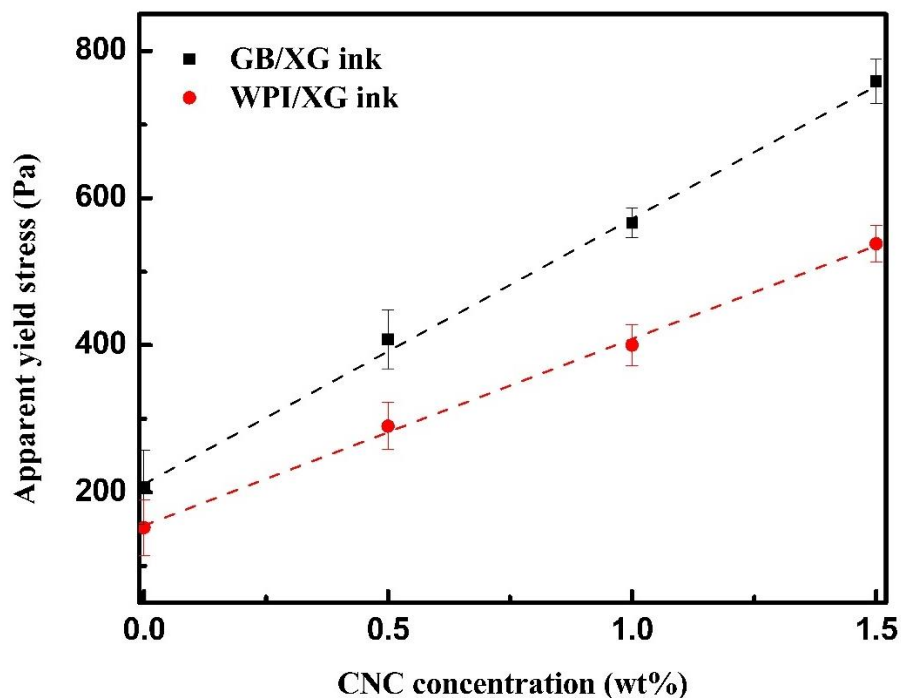


Figure 6.6 Apparent yield stress of GB/XG and WPI/XG food inks with respect to CNC concentration.

To evaluate the post-deposition quality of the printed structures, the linear viscoelastic properties of both GB/XG and WPI/XG systems with 0-1.5 wt% CNC addition were measured. As illustrated in Figure 6.7a and b, the storage modulus G' dominated the loss modulus G'' over the entire angular frequency range for all the food inks, indicating a solid-like behavior. Adding CNC to both GB/XG and WPI/XG inks leads to a gradual increase of G' and G'' , which could be attributed to the reinforced intermolecular network by the hydrogen bond formed through proteins-CNC, XG-CNC and among CNCs [28, 53, 54, 56]. G' gradually increased from 840 to 2550 Pa (300%) for GB/XG ink, and 484 to 1830 Pa (370%) for WPI/XG ink at 1 rad/s when increasing the CNC concentration from 0 to 1.5 wt%, which could explain the enhanced shape fidelity by CNC addition after deposition in Figure 6.3b-c and f-h.

In addition to G' and G'' , $\tan \delta$ is another important viscoelastic property that can reflect the post-printing properties of food inks. The $\tan \delta$ values of the GB/XG and WPI/XG systems with different CNC concentrations are summarized in Table S6.1 at an angular frequency of 1 rad/s. The addition of CNC led to a decrease in $\tan \delta$ values for both GB/XG and WPI/XG systems,

indicating an increase in rigidity and a more solid-like structure [28, 55, 57]. The $\tan \delta$ values ranged from 0.10 to 0.18, which suggested that the food inks exhibited weak gel behavior, and were suitable for DIW applications [58].

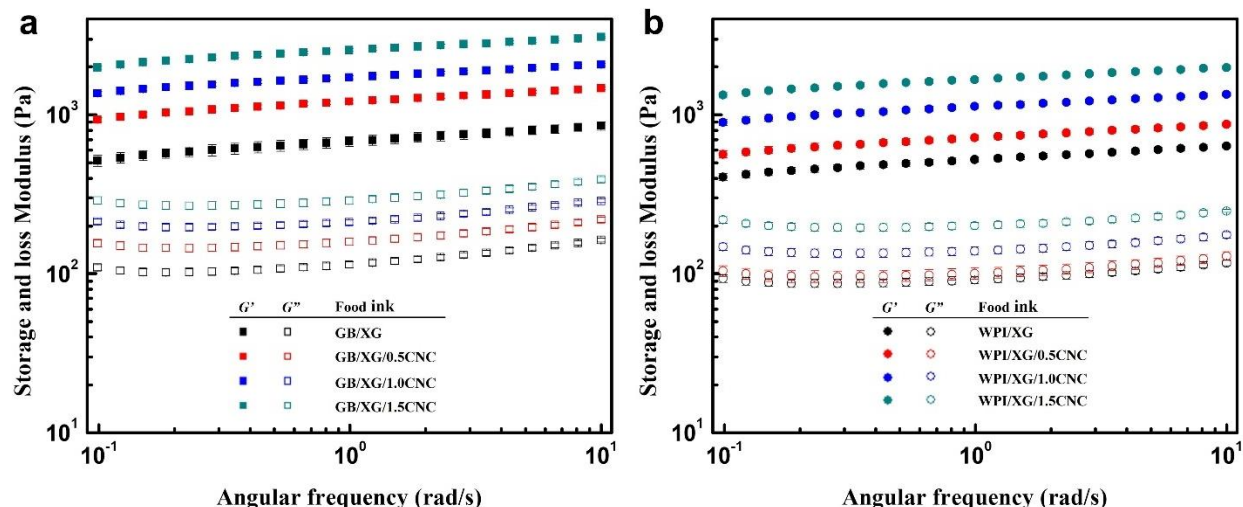


Figure 6.7 Storage modulus G' (solid symbols) and loss modulus G'' (open symbols) for (a) GB/XG and (b) WPI/XG mixed gels with different CNC addition at RT as functions of angular frequency at a strain of 1%.

Temperature sweep tests were also performed to evaluate the thermal stability of the ink formulations. As presented in Figure 6.8a, G' was always larger than G'' within the tested temperature range (10–70°C) for GB/XG inks, indicating a gel-like behavior. The characteristic drop in G' of all GB/XG inks was observed during the heating process, which can be considered as the helix-to-coil transition of GB in the mixture. The effect of CNC on the transition temperature (T_{hc}) was evaluated using the peak of the curve of the first derivative of G' vs. T [59] (Figure S6.2a), and the results are summarized in Table S6.2. The neat GB gel has a T_{hc} of 22.9°C, which was consistent with previous research [20]. The T_{hc} of CNC-free GB/XG mixed gel increases to 24°C, which can be explained by the synergistic effect between GB and XG [39, 60]. The T_{hc} of all GB/XG mixed gels increases from 24°C to 29.6°C as the CNC concentration increases in the range of 0–1.5 wt%. It can be attributed to the formation of the strengthened 3D network by hydrogen bonds between CNCs and GB chains, leading to an improved thermal stability of GB/XG/CNC mixed gels. In contrast, as demonstrated in Figure 6.8b and Figure

S6.2b, the G' of WPI/XG system and neat WPI solution was almost independent of temperature in the tested range (10–70°C), indicating their thermal stability below 70°C.

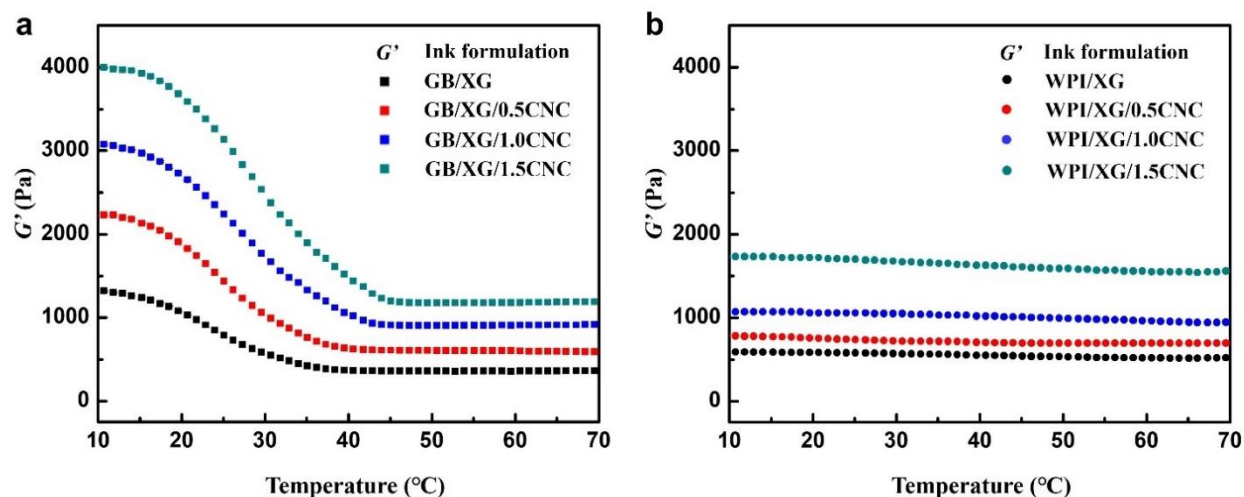


Figure 6.8 Measurement of storage modulus (G') of (a) GB/XG and (b) WPI/XG mixed gel systems with different CNC contents as a function of temperature. Heating rate: 1°C/min. G' of neat GB, WPI and XG solution are also shown in Figure S6.2b for a comparison purposes.

6.4.3 Dimensional stability of 3D-printed structures

The effect of CNC on thermal stability was also evaluated by analyzing the printed scaffolds after heating at 50°C, and the results are shown in Figure 6.9a-h. A temperature at 50°C was selected as the tested temperature because ~48°C was considered to be a comfortable food temperature in the mouth [61] while for food temperature above 50°C, a sensation of pain on the tongue is encountered [62, 63]. The heating process had a significant effect on the structures of GB/XG-based inks (Figure 6.9a-d vs Figure 6.3a-d). The scaffold printed with CNC-free GB/XG inks exhibits an obvious structure slumping (Figure 6.9a) and a fusion of the filaments and layers, which may be because the test temperature was above the helix-to-coil transition temperature of the GB/XG ink (~25.2°C). Adding CNC reduced the structure collapse phenomenon and enhanced the thermal stability (Figure 6.9b-d). This is coherent with the temperature sweep results in Figure 6.8a. Different from GB/XG based inks, the printed scaffolds using WPI/XG inks showed excellent resistance against temperature variations (Figure 6.9e-h), since the viscoelastic properties of WPI/XG system are almost independent within the temperature range from 10°C to 70°C.

Figure 6.9i quantifies the side area change of scaffolds fabricated with GB/XG and WPI/XG based inks after heating at 50°C for 5 min. The side area of the scaffolds fabricated using CNC-free GB/XG inks was reduced by ~75 % after heating. In contrast, shape retention was improved by adding CNC (0.5-1.5 wt%) with the side area being ~ 83 % to ~ 90 % of its original value at RT. On the other hand, there was just a slight side area reduction for WPI/XG scaffolds ranging from 9 % to 4 %, which may come from water evaporation during the heating process. Additionally, the side area reduction gradually decreased with CNC addition from 0.5-1.5 wt%. This is attributed to the hinderance of water evaporation by the intermolecular hydrogen bonding formation between WPI-CNCs and XG-CNCs, resulting in a better shape retention after heating.

The effect of CNC addition on the time stability of the as-printed 3D scaffolds over 96 h at 4°C was also investigated. As presented in Figure S6.3a-p, compared to the CNC-free 3D scaffolds, samples with CNC addition had a better shape retention for both GB/XG and WPI/XG mixed systems. Quantified results based on the variation of the side area of printed scaffolds with time are shown in Figure S6.3q and r. Structures with smaller side area reduction of ~7 % and ~10 % than the CNC-free scaffolds were obtained after 96 h by using 1.0 wt% CNC in the GB/XG ink and 1.5 wt% CNC in the WPI/XG ink, respectively. More details along with the methodology are presented in the SI.

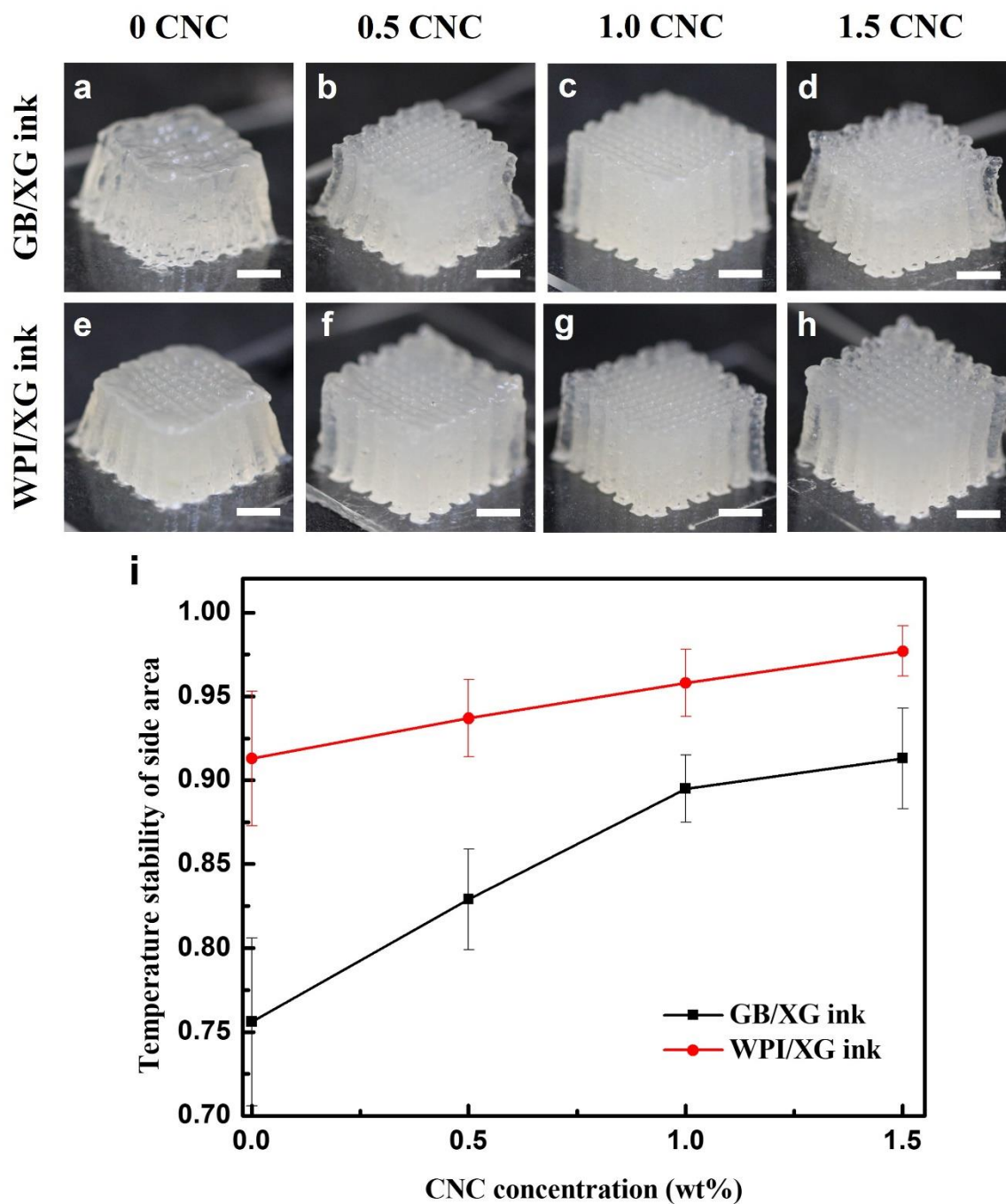


Figure 6.9 As-printed 40-layers (a-d) GB/XG scaffolds and (e-h) WPI/XG scaffolds with 0-1.5 wt% CNC addition after heating at 50°C for 5 min. (i) Temperature stability of side area of 3D scaffolds fabricated using GB/XG and WPI/XG food inks with 0-1.5 wt% CNC addition after heating at 50°C. Scale bars are 5 mm.

One of the main challenges of the traditional dysphagia diet is the unappealing appearance [64] of puree forms. 3D food printing, especially multi-material 3D printing, brings more opportunities for the fabrication of customized diet with flexible designs and diverse ingredients in a single manufacturing process. Figure 6.10 shows different colorful 3D printed complex shapes by single-material and multi-material DIW using the optimal ink formulations (i.e. GB/XG/1.0CNC and WPI/XG/1.5CNC) after a thermal storage at 50°C for 5 min. All the structures demonstrate a good resolution even after high temperature holding, revealing the excellent thermal stability of the inks with CNC addition. Additionally, these attractive shapes and visual appeals could greatly enhance the patients' acceptance of dysphagia diet, thus potentially upgrade their quality of life.

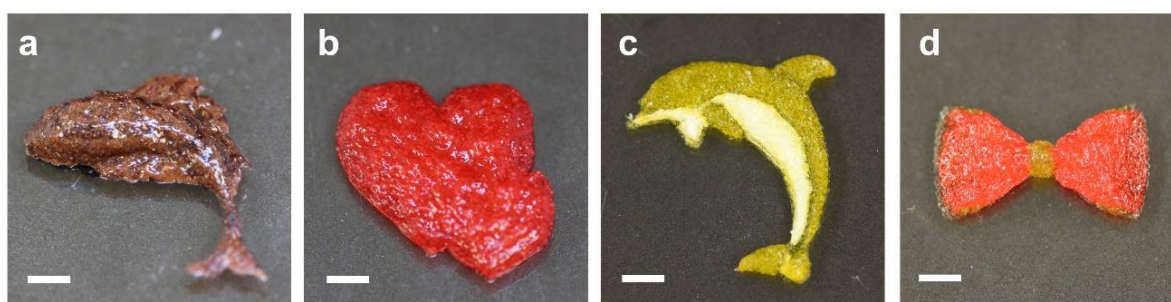


Figure 6.10 As-printed (a) GB/XG/1.0CNC 3D fish ($D = 200\ \mu\text{m}$, $V = 9\ \text{mm/s}$, $P = 420\ \text{kPa}$) and (b) WPI/XG/1.5CNC 3D double heart ($D = 330\ \mu\text{m}$, $V = 9\ \text{mm/s}$, $P = 308\ \text{kPa}$) with the single-material DIW method. As printed (c) 3D dolphin and (d) 3D bowtie (Video S6.1) with the multi-material DIW method using a $200\ \mu\text{m}$ nozzle under $469\ \text{kPa}$ for GB/XG/1.0CNC ink (yellow part) and $378\ \text{kPa}$ for WPI/XG/1.5CNC ink (white and red part) at $7\ \text{mm/s}$. Scale bars are $5\ \text{mm}$. All the printed structure images were taken after a thermal storage at 50°C for $5\ \text{min}$.

6.4.4 Compression testing for textural profile analysis (TPA)

Texture is closely related to consumers' preference and acceptance for food products [10]. The TPA tests were carried out for the printed structures to mimic the chewing process, while the calculated characteristics closely related to the sensory perception of food in terms of hardness, adhesiveness, cohesiveness and gumminess [65] are presented in Figure 6.11 (see Table S6.3 for more details). In this test, the effect of CNC addition on textural properties was studied for printed structures using both GB/XG and WPI/XG mixed gel systems. Moreover, the effect of

post-deposition temperature on the printed scaffolds using the optimal ink formulation (i.e. GB/XG/1.0CNC and WPI/XG/1.5CNC) was also discussed.

Hardness represents the most direct reaction mouthfeel, as it simulates the first bite in the mouth [66]. While adhesiveness results from the negative force created from the attraction between the probe and the sample surface during the probe retraction process, it is considered as a sticky mouthfeel [67], which is closely related to the lingual force to drive the food through the pharynx [15]. The effect of CNC addition and temperature on the hardness and adhesiveness of printed samples are shown in Figure 6.11a and b. The hardness and adhesiveness increase significantly ($p < 0.05$) with increasing concentration of CNC for both GB/XG and WPI/XG mixed gels. GB/XG/1.5CNC food ink had the highest values with 53.9 g and -158.7 g.s, respectively, while a 1.5 wt% CNC concentration in WPI/XG mixed gel showed the maximum hardness and adhesiveness values with 39.3 g and -118.1 g.s, respectively. The significant correlation ($p < 0.05$) between G' and hardness for semi-solids has already been reported by Maruyama et al. [68]. Additionally, the CNC-based gels had an improved denser network, the reason why the adhesiveness increased with higher CNC concentration. It can also be observed that with the same CNC concentration, the hardness and adhesiveness values of GB/XG ink were significantly higher than for the WPI/XG ink, which was probably caused by the triple helix structure of GB leading to a stronger 3D network in the GB/XG mixed gel [69, 70].

Decreasing the temperature leads to an increase in the hardness of GB/XG/1.0CNC scaffold, with a value of 75.3 g at 4°C compared to 46.4 g at RT, which is probably due to the stiffer gel structure. Further increasing the temperature to 50°C leads to a lower hardness value of 35.6 g, which might be due to the random coil structure of GB above its transition temperature. An opposite trend was observed for the temperature response of adhesiveness. A lower adhesiveness was obtained at 4°C, which is consistent with our previous work [39]. When the temperature rises to 50°C, the hydrogen bonds inside the gel structure are broken, causing more exposed free groups that can interact with the surface and thus an increase in the adhesion [71, 72]. Regarding the WPI/XG system, there was no significant difference ($p > 0.05$) of hardness and adhesiveness with the effect of temperature, as the rheological properties were almost independent of temperature (Figure 6.8b).

Figure 6.11c shows the comparison of cohesiveness with CNC concentration and temperature effects. Samples with higher CNC concentrations showed stronger cohesiveness for both GB/XG and WPI/XG systems, owing to a stronger strength of internal bonds formed by CNC addition [67]. Thus, food pieces can be better bonded together to prevent the decomposition into small granules, which guarantees a safe swallowing process. Also, higher apparent yield stress with CNC addition (Figure 6.5) for the food ink provides better resistance against the structure deformation, which can explain the higher cohesiveness when increasing the CNC concentration. Moreover, food ink at low temperature resulted in a stronger solid-like behavior of GB/XG system, which translates into a larger gel strength and higher cohesiveness of the printed scaffolds. Higher temperature weakened the gel network within the GB/XG system, explaining the decrease in cohesiveness measured.

Gumminess is another interesting food textural characteristic, especially for semisolid with a low degree of hardness and high degree of cohesiveness [66]. The gumminess is defined as the product of hardness and cohesiveness [73]. As all the samples had a relatively high cohesiveness value, the gumminess shown in Figure 6.11d demonstrated a similar trend for hardness with the effect of CNC concentration and temperature on both GB/XG and WPI/XG systems.

The textural properties play a vital role in terms of dysphagia diet, which could be controlled by the rheological properties of food inks, as well as the post-deposition temperature. Food aiming at swallow-impaired patients should not have a high adhesiveness value, in order to avoid the risk of choking, and let the food separate from palate easily to ensure a safe swallowing process [10, 14, 15]. Hence, the food structures that exhibit high adhesiveness may not be suitable for the dysphagia diet, such as those printed with GB/XG/1.5CNC ink or heating at 50°C. On the other hand, adequate cohesiveness is another important criterion for a dysphagia diet, because food with low cohesiveness will be easily disintegrated into small particles, raising the oral residue of food leading to increased risk of aspiration [14]. Food structures without CNC addition might be inappropriate for both GB/XG and WPI/XG systems. It has also been reported that the key parameters of semisolid for ideal dysphagia diet should have a low hardness/gumminess and minimal chewing effort [14].

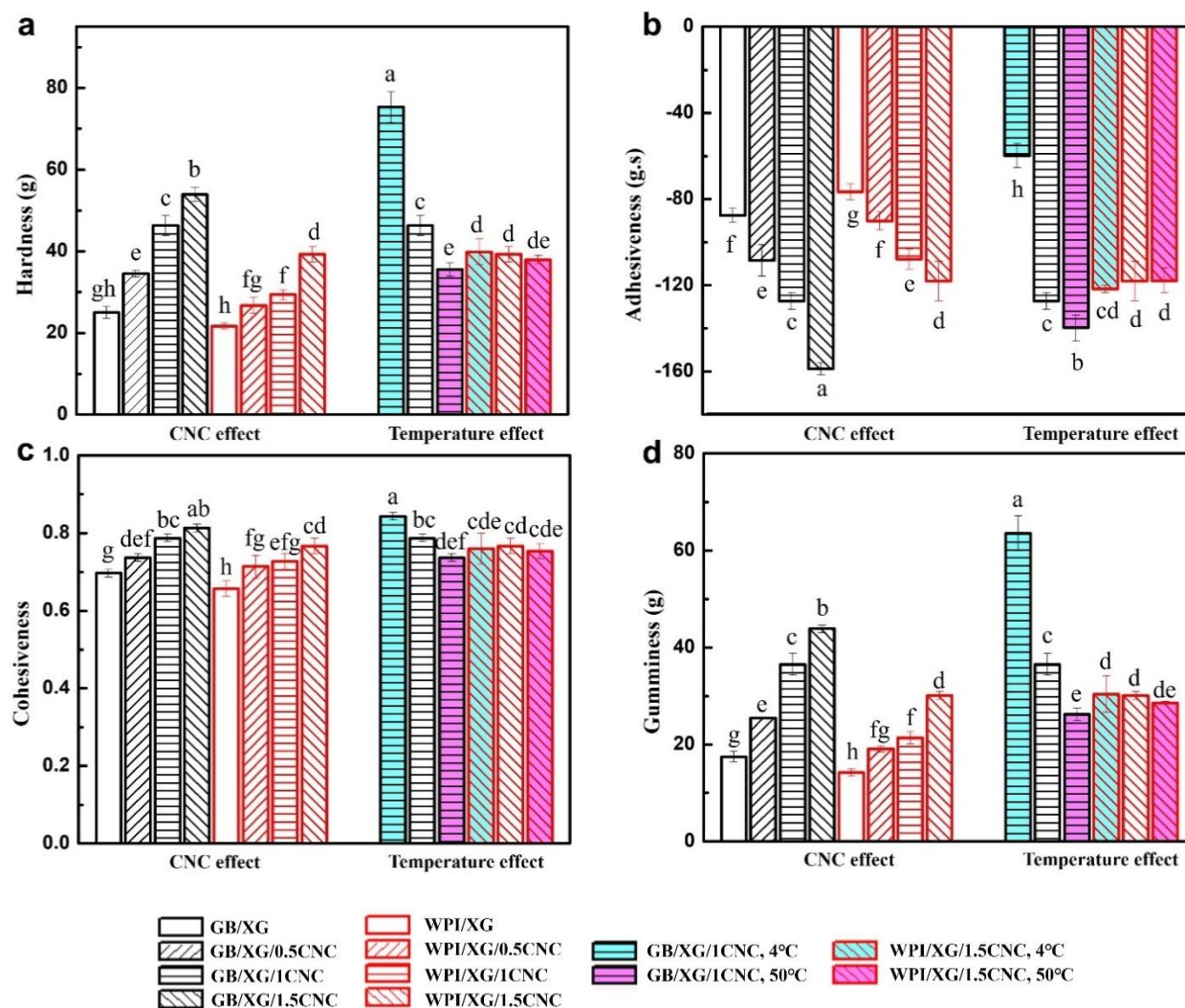


Figure 6.11 CNC concentration and post-deposition temperature effects on (a) hardness (b) adhesiveness (c) cohesiveness and (d) gumminess of samples printed with different ink formulations. Different lowercase letters above each error bar indicate a significant difference ($p < 0.05$).

6.4.5 IDDSI tests

The IDDSI tests provide a global and convenient assessment of modified food texture for dysphagic patients. Figure 6.12 and Figure 6.13 show the representative IDDSI results based on the fork pressure test, the fork drip test and the spoon tilt test for the CNC-enhanced GB/XG and WPI/XG samples according to the IDDSI guidelines [41].

The IDDSI test results for the samples without any post-deposition treatment are shown in Figure 6.12. Fork pressure test was evaluated with a 3D printed $15 \times 15 \times 15 \text{ mm}^3$ cuboid sample, which was considered as the maximum size for the adult dysphagic patient to reduce the choking risks [10, 15]. The safe swallowing range of food particle size is 2-4 mm based on the standard requirement within the IDDSI framework. Therefore, the gap between the prongs of fork used in this test was 4 mm. It can be observed that all GB/XG and WPI/XG samples were squashed easily with a clear indent pattern on the samples' surface by the fork. All cubes can be passed through with the 4 mm prong gap, indicating the safe range of particle size of our samples. Moreover, the applied pressure by the fork was below the value for which it can whiten the thumb nail ($\sim 17 \text{ kPa}$), equivalent to the tongue pressure exerted during swallowing [8, 74]. The observations from the fork pressure test indicated that all the samples were located within the characteristics of “level 4-pureed” and “level 5-minced and moist” dysphagia diet range.

In the fork drip test, all samples could be piled on the fork without any ink flow or falling through the prongs of the fork, which conforms to the description of level 5-minced and moist dysphagia diet. The cohesiveness and adhesiveness of the samples were evaluated using the spoon tilt test, in order to further confirm the IDDSI level. All samples demonstrated enough cohesiveness to retain their shapes on the spoon. Furthermore, when the spoon was lightly shaken, GB/XG samples with 0.5-1 wt% CNC addition and all WPI/XG/CNC samples can be easily slid off the spoon, confirming the level 5-minced and moist dysphagia diet obtained from the previous two tests. However, the GB/XG sample with 1.5 wt% CNC addition exhibited a high degree of adhesiveness, resulting in a large amount of food residue on the spoon, making it difficult to be used in a dysphagia diet. The sticky property of the GB/XG/1.5CNC sample could increase the choking risks for dysphagic patients, as stronger lingual effort would be required to push such sticky food through the pharynx, which could be highly challenging for individuals with chewing or swallowing problems [15, 75].

The effect of temperature on GB/XG/CNC samples were further evaluated for IDDSI tests due to their sensitivity to the post-deposition temperature, and the results are shown in Figure 6.13. Interestingly, the results show that all samples stored at 4°C pass the fork pressure test, fork drip test and the spoon tilt test, even the GB/XG/1.5CNC sample which fails the spoon tilt test at RT due to the high adhesiveness. Hence, the sticky properties of GB/XG/1.5CNC sample were reduced at 4°C , and became suitable for the “level 5-minced and moist” dysphagia diet. When the

temperature of the sample increased, the IDDSI test results did not have any obvious change for the 3 tests. Even when higher temperature increased the adhesiveness, both GB/XG/0.5CNC and GB/XG/1.0CNC sample could still pass the spoon tilt test and be classified as “level 5-minced and moist” dysphagia diet.

Overall, IDDSI test is an efficient method to qualify the swallowability of a texture modified food product with some simple measurements [41]. By carefully controlling the food ink properties, it is possible to create a dysphagia diet within the IDDSI framework. It is interesting to notice that the post-deposition temperature can also modify the food texture aiming to the dysphagia diet. For example, the GB/XG/1.5CNC food ink can become suitable for the dysphagia diet when stored at 4°C. Additionally the 3D food printing technique allows bite-size food design with desired shapes, illustrating its advantage for dysphagia diet fabrication. However, the IDDSI tests can only evaluate qualitative properties. Further studies combining the IDDSI framework with other tests should be considered in the future, such as sensory and oral tribological tests, in order to better characterize and quantify the swallowability of food products [5].

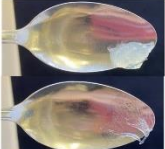
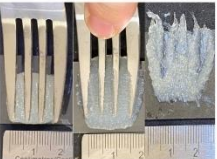
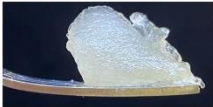
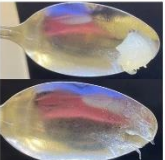
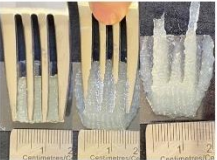
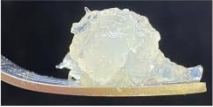
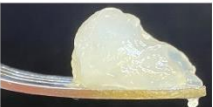
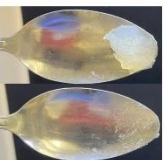
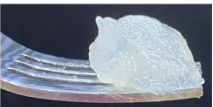
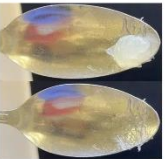
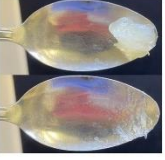
Ink formulation	Fork pressure test	Fork drip test	Spoon tilt test	Comments
GB/XG/0.5CNC				Level 5-Minced and Moist
GB/XG/1CNC				Level 5-Minced and Moist
GB/XG/1.5CNC				Not classified
WPI/XG/0.5CNC				Level 5-Minced and Moist
WPI/XG/1CNC				Level 5-Minced and Moist
WPI/XG/1.5CNC				Level 5-Minced and Moist

Figure 6.12 IDDSI test results of different ink samples at RT. Images with red contours represent samples that failed in the IDDSI test.

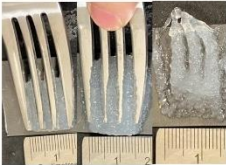
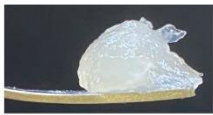
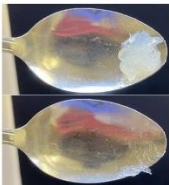
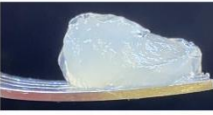
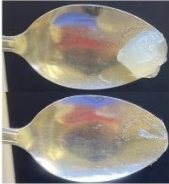
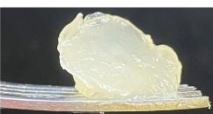
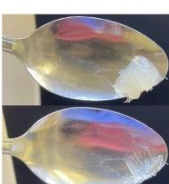
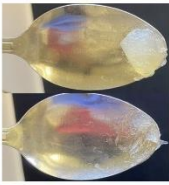
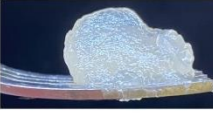
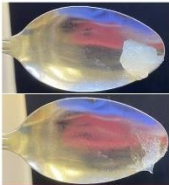
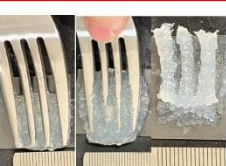
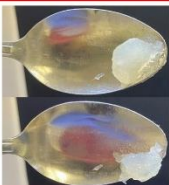
T (°C)	Ink formulation	Fork pressure test	Fork drip test	Spoon tilt test	Comments
4	GB/XG/ 0.5CNC				Level 5-Minced and Moist
	GB/XG/ 1CNC				Level 5-Minced and Moist
	GB/XG/ 1.5CNC				Level 5-Minced and Moist
50	GB/XG/ 0.5CNC				Level 5-Minced and Moist
	GB/XG/ 1CNC				Level 5-Minced and Moist
	GB/XG/ 1.5CNC				Not classified

Figure 6.13 IDDSI test results of GB/XG ink samples with different CNC concentrations after different post-deposition temperatures. Images with red contours represent samples that failed in the IDDSI test.

6.5 Conclusion

In this work we firstly developed novel CNC-enhanced food inks composed of GB or/and WPI mixed with XG, suitable for a 3D printed dysphagia diet design. The addition of CNC to GB/XG and WPI/XG mixed gels generally improved their rheological properties which provided the appropriate flowability and self-supporting properties, illustrating enhanced printing results due to the strengthened 3D network by hydrogen bond between CNCs and protein/polysaccharide. The increase in printing accuracy of 18% and 29% were found for the printed 3D scaffolds with the best ink formulations (i.e., 1 wt% CNC addition in the GB/XG system and 1.5 wt% CNC addition in the WPI/XG system) than the CNC-free inks. Additionally, CNC addition improved the thermal stability of GB/XG scaffolds with nearly a 90 % shape retention even after a heating at 50°C for 5 min. Finally, the textural properties of the printed food scaffolds could be modulated by CNC addition and different post-deposition temperatures. Samples with best ink formulations were confirmed to comply with “level 5-minced and moist” dysphagia diet according to the IDDSI tests. Also, the storage of the GB/XG/1.5CNC food ink sample at 4°C turned it from a non-approved product for dysphagia diet at RT to a “level 5-minced and moist” dysphagia food. 3D food printing enables not only properly controlled and personalized food texture and nutrition for the dysphagia diet requirement, but also realizes the appealing food design to potentially improve patients’ appetite. The findings of this study provide guidelines to use the 3D printing technique to shape dysphagia food formulations with protein/polysaccharide mixed systems adequate for patients in hospital, space station and even for home kitchens. In future research, more emphasis should be placed on multi nutrition ink formulations (e.g. vitamins, microelements, nutraceuticals) based on protein/polysaccharide mixed gels towards dysphagia diet. Moreover, the effect of structures and shape designs (e.g. infill percentage and infill type) on the food texture should be further investigated to discover the full potential of 3D food printing on dysphagia diets.

6.6 Acknowledgements

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6.8 Supporting information

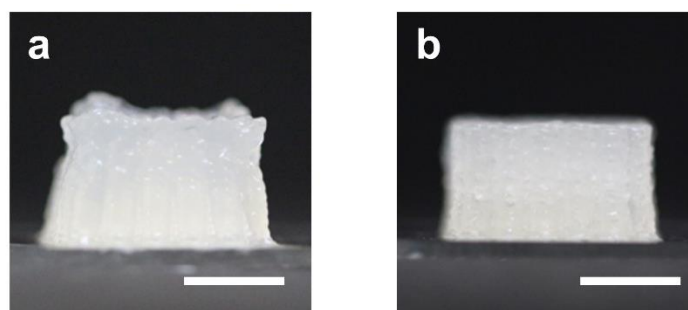


Figure S6.1 Representative side view of as-printed 40-layers 3D scaffolds of (a) CNC-free WPI/XG ink with a poor shape fidelity, and (b) GB/XG/1CNC with a higher shape fidelity using a 200 μm nozzle. Scale bars are 5 mm. Photos were taken right after printing.

Table S6.1 Values of $\tan \delta$ at $\omega = 1$ rad/s for different food inks at 22°C.

Food ink	$\tan \delta$
GB/XG	0.17
GB/XG/0.5CNC	0.13
GB/XG/1.0CNC	0.12
GB/XG/1.5CNC	0.11
WPI/XG	0.18
WPI/XG/0.5CNC	0.14
WPI/XG/1.0CNC	0.13
WPI/XG/1.5CNC	0.12

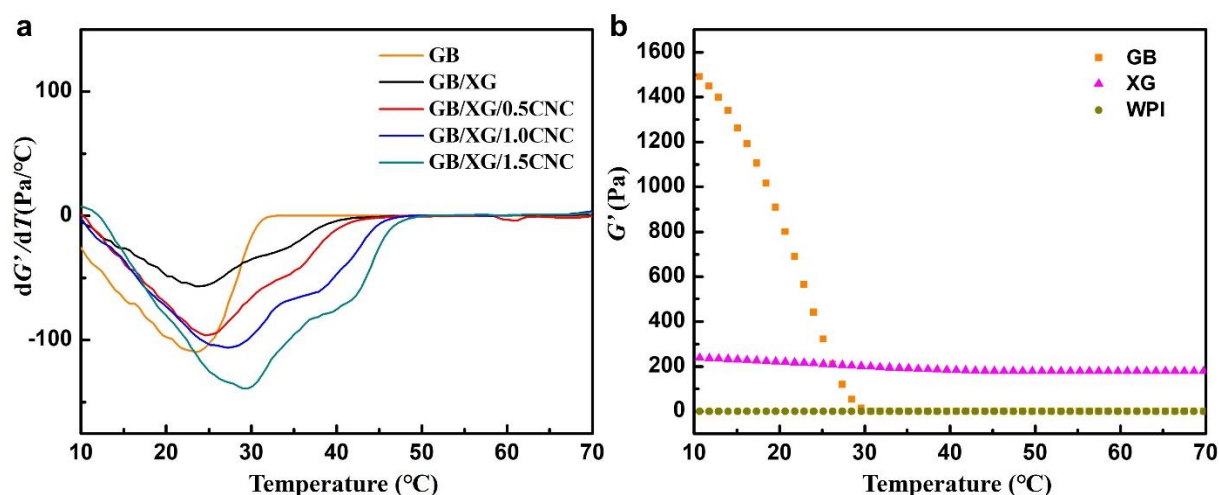


Figure S6.2 (a) dG'/dT of GB/XG systems with different CNC concentrations with respect to temperature. (b) Evaluation of G' for neat 5 wt% GB, 20 wt% WPI and 8 wt% XG solutions with respect to temperature.

Table S2 Transition temperature (T_{hc}) of GB in GB/XG mixed gels with different CNC concentrations.

Ink formulation	T_{hc} (°C)
GB	22.9
GB/XG	24
GB/XG/0.5CNC	25.7
GB/XG/1.0CNC	27.9
GB/XG/1.5CNC	29.6

Time stability of 3D printed scaffolds. The side area variation of the printed 3D scaffolds was monitored and evaluated up to 96 h. The images of the samples were recorded 0, 24 and 96 h after printing, respectively. All samples were put in Petri dishes and then stored in a refrigerator at 4°C, in order to prevent the protein degradation and/or microbial growth [1]. The side areas were measured by Image Pro Plus 6.0 (Media Cybernetics).

The time stability was determined by calculating the normalized side area with Eq. S6.1.

$$\text{Time stability of side area} = \frac{S_n}{S_0} \quad (\text{S6.1})$$

where S_n is the side area of the printed scaffold determined at n hours ($n = 24, 48, 72$ and 96) after printing, and S_0 is the side area of the printed scaffold determined right after printing.

Maintaining the shape of printed food structures over time is crucial for their shelf-life and also for longer fabrication time. The time frame of 4 days (96 h) was chosen as a typical storage time for ready-to-eat food. The effect of CNC addition on the time stability of the as-printed 3D scaffolds over 96 hours at 4°C is presented in Figure S6.3a-p. The addition of CNC improves the shape retention of both GB/XG and WPI/XG mixed systems. Without CNC, the printed scaffolds show distinct center collapse and shrinkage, which became worse with longer storage time, as observed in Figure S6.3a-b and 8i-j. In contrast, the CNC addition results in better shape retention of the 3D printed scaffolds, as observed in Figure S6.3c-h and k-p, where the structures maintain their shape with only slight changes compared to those right after printing. The improved shape retention may be due to the higher gel strength resulting from CNC addition.

Figure S6.3q and r shows the quantified values based on the variation of the side area of printed scaffolds with time for both GB/XG and WPI/XG inks, respectively. All the samples show a good shape stability with at least 80 % retention of their initial side area after 96 h. Comparably, the side area reduction using the food inks with the optimal ink formulations (e.g. GB/XG/1.0CNC and WPI/XG/1.5CNC) is ~7 % and ~10 % smaller than the inks without CNC addition. As discussed above, the hydrogen bonding formation between protein-CNCs and XG-CNCs results in a stronger gel structure by the intermolecular 3D network. However, no significant change on the shape retention was observed between GB/XG and GB/XG/1.0CNC inks when further increasing the CNC concentration to 1.5 wt%. The irregular deposition between layers affected the structure coherence, preventing better time stability expected for a higher CNC concentration.

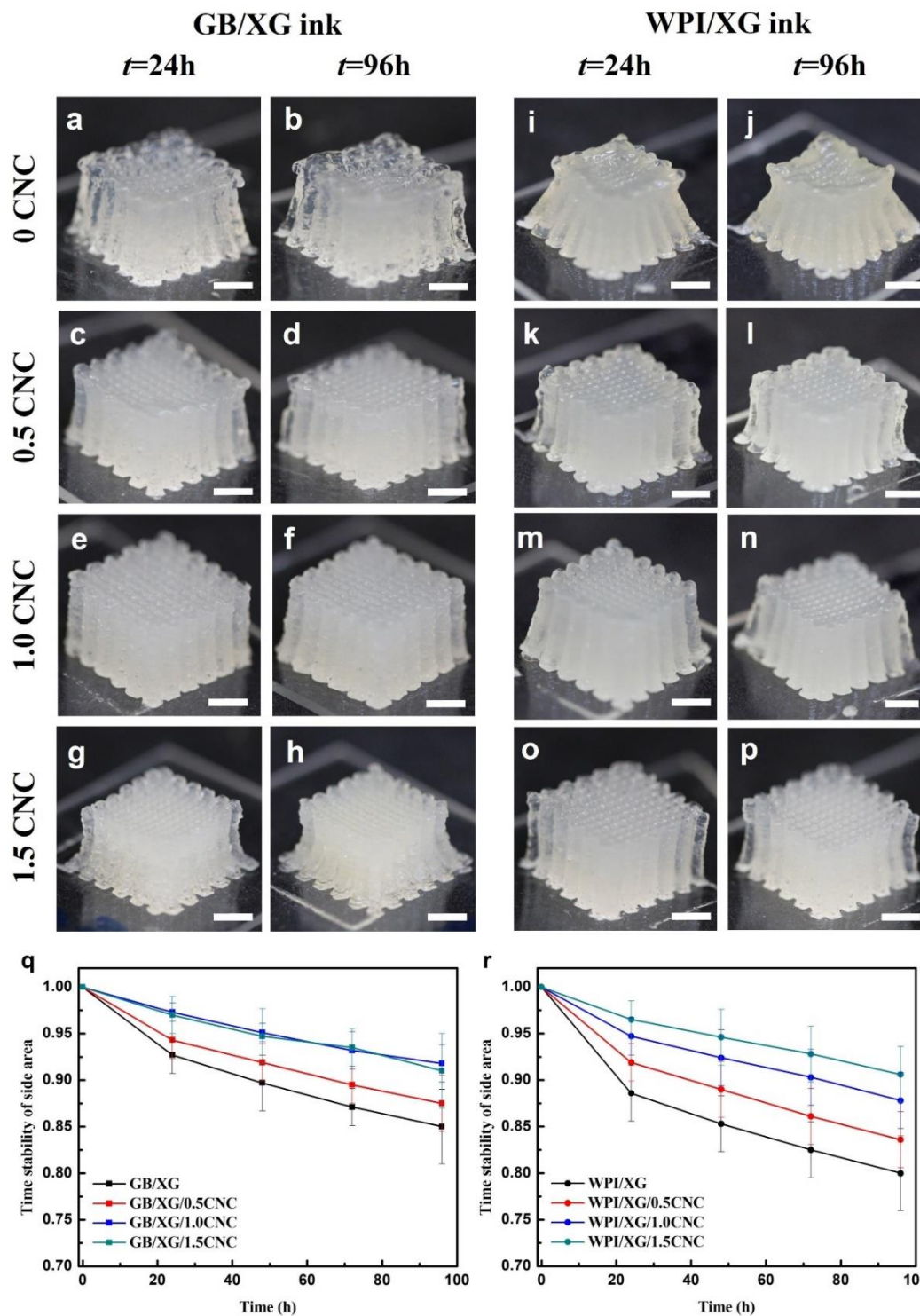


Figure S6.3 As-printed 40-layers (a-h) GB/XG scaffolds and (i-p) WPI/XG scaffolds with 0, 0.5, 1 and 1.5 wt% CNC addition after printing and stored for 24 and 96 h at 4°C. Time stability of side area of 3D scaffolds fabricated using (q) GB/XG and (r) WPI/XG food inks with 0-1.5 wt% CNC addition stored at 4°C over a period of 96 h. Scale bars are 5 mm.

Table S3 Textural properties of the 3D printed scaffolds made of different gel formulations *.

Effect	Food ink	Hardness (g)	Adhesiveness (g.s)	Cohesiveness	Gumminess (g)
CNC addition	GB/XG	25.1 (1.5) ^d	-87.6 (3.3) ^f	0.70 (0.01) ^g	17.4 (1.1) ^g
	GB/XG/0.5CNC	34.6 (0.9) ^c	-108.5 (7.2) ^e	0.74 (0.01) ^{def}	25.4 (0.2) ^e
	GB/XG/1.0CNC	46.4 (2.5) ^c	-127.3 (3.8) ^c	0.79 (0.01) ^{bc}	36.5 (2.2) ^c
	GB/XG/1.5CNC	53.9 (1.7) ^a	-158.7 (2.8) ^a	0.81 (0.02) ^{ab}	43.9 (0.8) ^b
Temperature	4°C GB/XG/1.0CNC	75.3 (3.8) ^a	-59.8 (5.5) ^h	0.84 (0.01) ^a	63.5 (3.6) ^a
	RT GB/XG/1.0CNC	46.4 (2.5) ^c	-127.3 (3.8) ^c	0.79 (0.01) ^{bc}	36.5 (2.2) ^c
	50°C GB/XG/1.0CNC	35.6 (1.7) ^e	-139.8 (5.7) ^b	0.74 (0.02) ^{def}	26.2 (1.3) ^e
CNC addition	WPI/XG	21.7 (0.8) ^h	-76.56 (3.8) ^g	0.66 (0.01) ^h	14.2 (0.7) ^h
	WPI/XG/0.5CNC	26.8 (2.0) ^{fg}	-90.2 (4.2) ^f	0.71 (0.03) ^{fg}	19.0 (0.6) ^{fg}
	WPI/XG/1.0CNC	29.4 (1.2) ^f	-107.8 (4.6) ^e	0.73 (0.02) ^{efg}	21.3 (1.3) ^f
	WPI/XG/1.5CNC	39.3 (1.9) ^d	-118.1 (9.3) ^d	0.77 (0.02) ^{cd}	30.1 (0.8) ^d
Temperature	4°C WPI/XG/1.0CNC	39.8 (3.2) ^d	-121.7 (1.7) ^{cd}	0.76 (0.04) ^{cde}	30.4 (3.7) ^d
	RT WPI/XG/1.0CNC	39.3 (1.9) ^d	-118.1 (9.3) ^d	0.77 (0.02) ^{cd}	30.1 (0.8) ^d
	50°C WPI/XG/1.0CNC	37.9 (1.1) ^{de}	-117.7 (5.8) ^d	0.75 (0.02) ^{cde}	28.6 (0.3) ^{de}

*: Values in parenthesis are standard deviation $n = 3$. Mean values with the same lowercase superscript letter are not significantly different ($p > 0.05$) between the row.

Supporting references

[1] C. L. Kielkopf, W. Bauer, and I. L. Urbatsch, "Purification of Polyhistidine-Tagged Proteins by Immobilized Metal Affinity Chromatography," Cold Spring Harb Protoc, vol. 2020, no. 6, p. 102194, Jun 1 2020, doi: 10.1101/pdb.prot102194.

CHAPTER 7 GENERAL DISCUSSION

Additive manufacturing enables the on-demand creation of complex constructs without molds, and has become an emerging manufacturing technique widely adopted in biomaterials and biomedical applications [158]. In recent years, customized foods which present an attractive appearance, delectable flavor and contain healthy ingredients, have attracted much interest [6]. Due to the importance of protein/polysaccharide mixed gel systems in nutrition, they have been systematically developed as inks in this thesis as a proof of concept.

Herein, the general discussion of this thesis is divided mainly into two parts. First, the behavior of the selected protein/polysaccharide ink during and after 3D printing process is discussed. Second, the adaptation and applications of our protein/polysaccharide inks to the 3D food printing are also examined.

7.1 3D printing of protein/polysaccharide mixed systems

As an inspiration, the research by Cohen *et al.* [159] proposed that the GB/XG aqueous mixtures were able to mimic different types of food mouthfeel. However, their work was too empirical without a systematic investigation of ink properties and printing behavior. In Chapter 4, we successfully developed an aqueous GB/XG food ink which enabled 40-layer 3D scaffolds fabrication with defined features using the DIW technique. The proper printability mainly came from the enhanced gel properties by the synergistic interactions between GB and XG. In order to further enhance the printing performance, calcium ions (Ca^{2+}) were considered due to their bridging effect. On one hand, the more significant shear-thinning behavior and higher viscosity of inks with Ca^{2+} addition benefited the printing process. On the other hand, the bridging effect also improved the self-supporting properties of the mixed gels, resulting in a higher shape fidelity and dimensional stability over 96 h. To the best of our knowledge, this was the first time a protein/polysaccharide mixed gel systems was used for 3D printing in terms of food application. Also, this part of the work provided the basic information for the following research.

A comprehensive guideline to design and fabricate 3D structures using protein/polysaccharide inks needed to be laid down. Based on the study of Chapter 4, the processing and properties of the GB/XG ink for DIW were investigated in Chapter 5. SP/XG was also studied as a comparison in terms of a vegetarian alternative. Two different types of maps guiding ensured a successful 3D

printing performance. On one hand, processing maps provided the adequate conditions (i.e., nozzle diameter and XG concentration) to achieve the 3D structure region. On the other hand, shape fidelity map oriented towards 3D structure with high precision and shape fidelity. Eventually, this work allowed to print a more complex shape, a 3D dolphin, with high precision using two optimised inks by a multi-material DIW technique in a one-step manufacturing process.

Two things must be considered but were neglected in the first two parts. The first one was to enhance the thermal stability of the protein/polysaccharide inks. All our printing was achieved at room temperature, which may affect the behavior of temperature-sensitive ink materials such as GB/XG mixed gels. The second one was to make our protein/polysaccharide inks adequate for specific food applications. To address the first challenge, CNC, a natural source polysaccharide, is an ideal choice for our systems due to its known excellent properties in nanocellulose-reinforced food-grade hydrogels [160]. In Chapter 6, we developed a CNC-based GB/XG aqueous food ink for DIW. The third protein/polysaccharide pair which was used in 3D printing, WPI/XG, was also explored and compared with the GB/XG system. Besides the improvement of shape fidelity and shape retention of the printed scaffold by CNC addition in both systems, the thermal stability of the printed GB/XG scaffold has also been enhanced. It made the CNC-enhanced GB/XG scaffold exhibits ~90% side area retention after heating at 50°C for 5 min, which was supported by the rheological temperature sweep results. Furthermore, the CNC addition to the mixed gel systems allowed various complex 3D structures to be printed by the multi-material DIW process using both GB/XG/1.0CNC and WPI/XG/1.5CNC that retained their shapes after a thermal treatment at 50°C for 5 min.

For the first time, protein/polysaccharide mixed gels have been systematically investigated in the scope of the DIW technique, which allowed to generate 3D printed structures with high shape fidelity, high shape retention and good thermal stability. Some highlights need to be addressed for our work: (1) Stable 3D structures can be obtained by DIW without any post-processing treatment (e.g. curing process). (2) Compared to the existing 3D food printing, our strategy was able to fabricate the 3D structure with high shape fidelity and controlled resolution, which can be evaluated with our original defined SFI. Unlike the common method to determine the shape fidelity using the ratio of actual printed size (e.g. length, width or height) and the theoretical size, our SFI presented not only the filament smoothness, but also the self-support between layers. (3)

The protein/polysaccharide inks were used for the first time in multi-material DIW in a single process to fabricate 3D complex shapes.

However, there still exist some limitations in our work: (1) The ink preparation time needed ~13 h, which is time-consuming. (2) The maximum printing speed in our work was 9 mm/s, which is relatively slow. (3) All the studies were done with XG, a good shear-thinning material which is beneficial for the printing process. In order to establish a complete study for 3D printing of protein/polysaccharide mixed gel inks, other type of polysaccharides should also be considered.

7.2 Towards food applications

In this section, the discussion is mainly focused on two directions including the food adaptation and applications of our current work.

7.2.1 Food adaption

All the raw materials and additives used in our work are food-grade proteins, polysaccharide and microelements for human body. Ca^{2+} plays an essential role in several physiological processes, such as the formation of bone, blood clotting and enzyme functionalization, etc. [161]. Although the CNC has not been approved by FDA, several publications have shown that it does not give rise to cytotoxic or genotoxic concerns [160]. Secondly, the essential texture properties related to food of the printed structures (i.e. hardness, adhesiveness, cohesiveness and gumminess) can be well controlled by the processing related parameter (i.e. infill percentage), material related parameters (i.e. protein types and XG concentrations) and post-processing temperature. In addition, the heating temperature 50°C used in the thermal stability of printed structures tests is the comfortable food temperature for consumption [162].

7.2.2 Food applications

This work first reported CNC-based protein/polysaccharide inks that have great potential in 3D food printing for dysphagia diet in Chapter 6, Article 3. On one hand, the CNC-enhanced protein/polysaccharide food inks showed good printability to fabricate various appealing 3D structures with high shape fidelity and thermal stability, in order to raise dysphagic patients' appetite. On the other hand, the texture of 3D printed samples with CNC addition complied with the level-5 minced and moist dysphagia food within the IDDSI framework.

Generally, our current work shows great potential to bring the 3D food printing to the kitchen with food-grade ingredients, clean and simple fabrication process and food-related texture. In addition, our research shows a good suitability for dysphagia food design which opens new prospects for 3D food printing of protein/polysaccharide inks. Nevertheless, concerns still exist: (1) In order to have a complete nutritious system, other food additive should be added in future studies, which may change the pH of the food inks and affect their rheological properties and printability. For example, vitamin C is acidic and baking soda is alkaline. (2) For the real utilization of CNC in food inks, the approval by FDA is necessary.

CHAPTER 8 CONCLUSION AND RECOMMENDATIONS

8.1 Conclusion

In this thesis, protein/polysaccharide aqueous mixtures were successfully developed for single and multi-material 3D printing. The effect of ink properties (i.e., protein type, XG concentration and additive content) and post-deposition temperature on the printing performance was investigated, and linked to the rheological properties of the inks. The conclusions drawn from this work are divided into 3 parts.

- 1) Compared to neat XG solution, GB/XG mixed gel had improved rheological properties which endowed a better printability. Due to the bridging effect, adding Ca^{2+} to GB/XG inks resulted in enhanced rheological properties with more significant shear-thinning behavior, higher viscosity and better self-supporting properties. Thus, the printed structures exhibited higher shape fidelity and shape retention. GB/XG food inks with 37.5 mM Ca^{2+} addition showed the best structure with a defined *Pr* value ~ 1 , and the highest thickness retention $\sim 83\%$ and $\sim 89\%$ after 96 h at room temperature and 4°C , respectively. Besides Ca^{2+} addition and different storage temperature, the infill percentage could also help controlling the textural properties of printed food structures, which was modulated through the 3D printing process (Chapter 4, Article 1).
- 2) The zones to produce 1D filaments, 2D arrays and 3D structures were well defined in respective processing maps showing the relationship with XG concentrations and nozzle diameter for both GB/XG and SP/XG systems. SP/XG had a significantly larger printable region than GB/XG due to its softer ink structure. Increasing XG concentration in a range of 3-12 wt% for GB/XG inks and 4-17 wt% for SP/XG inks improved the rheological properties. 3GB/11XG and 8SP/15XG demonstrated the best structures owing to the higher recovery of viscosity $\sim 85\%$ and higher apparent yield stress ~ 600 Pa. Further increasing the XG concentration caused an unstable ink behavior with unsmooth filaments and irregular structure. A shape fidelity map was generated for the 3D structures zone from the processing maps, demonstrating the range of $\tan \delta$ (0.12-0.17) and SFI value (0.6-1.0) for a printed structure with high shape fidelity. As a demonstration, the 3GB/11XG and 8SP/15XG inks were used to print a 3D dolphin with fine resolution by a multi-material DIW process based on the respective processing maps (Chapter 5, Article 2).

- 3) Due to the strengthened 3D network induced by CNC addition, CNC-based GB/XG and WPI/XG inks showed better rheological properties with more significant shear-thinning behavior and enhanced self-supporting properties, which benefited the printing results and also the swallowing process. GB/XG/1.0CNC and WPI/XG/1.5CNC were the best ink formulations, providing higher printing accuracy ~ 0.95 and side area retention ~ 0.93 at 4°C . Moreover, the thermal stability of GB/XG inks was also improved by CNC addition due to the higher transition temperature compared to neat GB. The printed GB/XG/1.0CNC scaffold had almost 90% shape retention after heating at 50°C for 5 min. The CNC-enhanced GB/XG and WPI/XG food inks allowed the fabrication of various 3D complex shapes with good thermal stability using a multi-material DIW process. With the combination of texture profile analysis and IDDSI tests, GB/XG samples with 0.5-1.0 wt% CNC and WPI/XG samples with 0.5-1.5 wt% CNC showed a good suitability for dysphagia diet in level 5-minced and moist. Storing the GB/XG/1.5CNC sample at 4°C achieved the transition from a non-dysphagia food to a level 5-minced and moist dysphagia appropriate food (Chapter 6, Article 3).

8.2 Recommendations

The following aspects are recommended for exploration in future work:

- 1) Other food grade proteins and polysaccharides such as casein, pea protein, curdlan, pullulan and others may be considered as new ink formulations to diversify the 3D printed protein/polysaccharide database.
- 2) Food additives such as vitamins, microelements and nutraceuticals may be employed in the mixed gel systems.
- 3) In Articles 1 and 3, one of the goal was to use a food additive to enhance the ink gel properties for a suitable printing process. However, in an opposite strategy, other food additives may be considered to weaken the gel strength as required (e.g. mixtures with GB at high concentrations ≥ 5 wt%).
- 4) Effect of infill patterns on textural properties should be further investigated.

- 5) Sensory and oral tribological tests should be carried out in order to better characterize the printed structure for real food applications.
- 6) High speed (≥ 20 mm/s) printing should be considered for the reported systems, in order to increase the printing efficiency.
- 7) Inks properties should be further improved in order to work with larger nozzles.
- 8) Larger 3D structures need to be considered by using dispenser with higher volume, which may need a longer time to fabricate. Thus, the time stability of food inks should be further investigated.

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