Sustainable 3D Printing with Soy-derived Bioink

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Abstract: Nutritional foods and medical resources have become prohibitively expensive. As a result, many socio-economically disadvantaged populations continue to choose between long-term health or immediate survival. There is a direct correlation between the increased utilization of medical resources and disease states caused by poor nutrition. Therefore research of methods that increase access to nutritional foods while simultaneously reducing the cost of medical resources is highly advantageous. Three-dimensional (3D) printing combined with soy-derived bioinks (SBDs) offers the advantage of reducing processing waste, encouraging customization, while also lowering production costs. Fabrication of 3D food and tissue constructs is a promising solution to address nutritious food and medical resource cost and scarcity. Easily cultivated soy-derived protein is a ubiquitous resource that has been determined to be safe and contains many bioactive properties. 3D printing can create reproducible complex geometries with automated processes. This project will research and implement the development of a versatile SDBs for use in 3D printing for soy-based food applications.

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Introduction

The increasing costs of nutritious foods and medical resources become more apparent each year. The cost of health care in the United States increased to $3.5 trillion in 2017[1] while the number of undernourished people in the world has been increasing since 2014, reaching an estimated 821 million in 2017.[2] A study by Rao et al. determined that healthier food-based diet patterns cost more than less healthy patterns.[3] Furthermore, the current impact of industrial agriculture in the United States has introduced massive amounts of chemical and biological contamination into our food supply and environment.[4,5] This information establishes a cycle of malnutrition leading to long-term health problems. To address these issues, replicating popular food choices with alternative methods of food fabrication could elucidate a sustainable process that would provide a viable solution. If this process could also reduce waste while improving nutritional outcomes by using renewable resources that are inexpensive and environmentally friendly, a new method of food production could be established that would benefit the global population. To address these issues, the combination of 3D printing and soy-derived bioinks (SDBs) provides a possible path towards a solution.

In the early 1980s, Charles Hull developed a novel additive manufacturing (3D printing) process which revolutionized manufacturing and research.[6] This process has since evolved from industry practices to open-source applications that have provided access to inexpensive, desktop 3D printers giving engineers, scientists, and hobbyists a chance to make unparalleled innovation. The advent of 3D printing has reduced processing waste, encouraged customization, and lowered production costs in the food and medical industries.[7] Applying 3D manufacturing methodologies to food production using SDBs allows for customized or large scale nutritional needs in areas such as hospitals, schools, nursing homes, or low-income communities.[7,8] Numerous research

groups have made impressive progress augmenting 3D printing technology to meet unmet needs in the food industry. Kim et al. have investigated the factors affecting the properties of vegetable powders suspended in a hydrocolloid matrix provided valuable insight into methods of standardizing food inks.[9] Similarly, Holland et al. are investigating the ability to use cellulose powders to enhance the structural properties of 3D printed food objects.[10] Also, SDBs have been researched extensively for food [11,12] and medical applications.[10,13] The research presented by the groups mentioned above begins to address the primary obstacle when considering 3D printing, choosing a viable material to 3D print.

Selecting ingredients that are easily modifiable and allow for optimal printability is the initial step. The primary compound of the bioink described above will be soy protein isolate (SPI), which obtained from soybeans, and contains many essential amino acids. The most common forms of soy protein that are commercially available are soybean flour (~ 50% protein), soybean concentrate (~70% protein), and SPI (~ 90% protein). Soy protein is used in snack foods, protein powders, meat analogs to provide binding, emulsification, stabilization, moisture control, and texture control. This plant-derived protein is a ubiquitous resource that has been determined to be safe, contains many bioactive properties[14] and has been successfully extruded to construct porous scaffolds for use in tissue regeneration and create complex edible geometries in food production.[15,16] The secondary component is gelatin which is heavily used in the food industry for gelling, stabilization or thickening.[17] Gelatin is a water-soluble hydrocolloid, commercially available in two primary forms, type A and type B. Type A is derived from an acid pretreatment, while type B is derived from an alkaline pretreatment.[17] One of the most fundamental properties of gelatin is the ability to form hydrogen bonds with water molecules, which leads to the formation of a stable 3D gel structure. When placed in water, gelatin absorbs

5-10 times its volume of water. The final component is sodium alginate; a natural polysaccharide derived from brown seaweed. The primary properties of alginate, when added to a food substrate, consists of gelling, stabilizing, thickening, and film-forming.[18] Dissolving sodium alginate in aqueous solution increases the viscosity of the solution, enhancing its mechanical properties. Also, adding calcium salt to a solution containing sodium alginate creates a gelation reaction occurs without the addition of heat, forming stable gel.[19]

In the 3D printing field, designing and modeling of stereolithography (STL) files are an essential component in achieving desired outcomes. As such, the ability to model or scan objects using computer-aided design and fabricate those objects readily allows for rapid prototyping. This modeling and printing process can be translated into printability of an edible matrix in which different ingredients can be extruded layer by layer to create textures, insert flavors, and fabricate whole meals that could be comparable to that of traditional foods. Developing a motion/extrusion sequence that can be used to ensure structural and textual reproducibility will be needed for extrusion of various bioinks with different rheological properties. Furthermore, it will be necessary to optimize methodologies printing soft, gel-like materials as they have a wide range of intrinsic properties, such as viscosity, which can vary significantly between batches during extrusion.

For the scope of this project, the bioprinter extrusion system that will be utilized is the Inkredible+ (Cellink), a pneumatically driven bioprinter that extrudes material in thin strands. Fabrication of a 3D model involves extrusion of multiple strands of bioink in distinct paths such that the printed material looks like the model desired. An STL alone does not have the information needed for the bioprinter printhead to create these distinct path extrusions. Also, as a hollow mesh, the STL lacks the inner structure information needed to prevent the top of the print

from collapsing on itself. Slicing software partitions an STL into a vertical stack of paths that the bioprinter can follow to both produce the exterior of an object and the inner structure to support it. Each path is broken into a list of cartesian coordinates for the bioprinter to follow called G-codes. G-codes are a set of instructions in the form of movements and actions provided to a 3D printer.

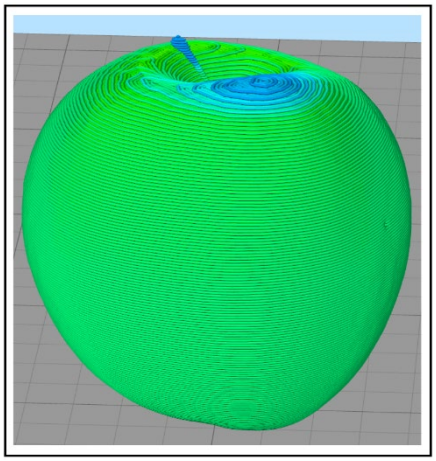

Figure 1. Post-sliced STL object

Materials and Methods

Preparation of Bioink

The bioink material is comprised of different ratios of SPI, gelatin, and sodium alginate. The composition of the bioink material consists of 20% SPI (Clairsoy Labs), 2% gelatin (AM Labs), and 0.5 % sodium alginate (Willpower Labs). Material is formed by dissolving 20 g of SPI in 100 mL of distilled/deionized water (DDW) heated to 50°C while suspended in a sand medium, mixed at 400 rpm on a heated, magnetic stir plate until for 24 hours. Next, 2 g of gelatin is dissolved in SPI mixture also at 50°C while suspended in a sand medium and mixed at 400 rpm for 2 hours. Following gelatin addition, the suspension was allowed to cool to room temperature (24°C) at which point, 0.5 g sodium alginate was added to SPI/gelatin suspension mixed at 400 rpm for an additional 2 hours. The soy/gelatin/alginate (SGA) mixture forms a paste that must be further homogenized after initial formulation due to large particulate masses formed by unevenly distributed SPI powder. To achieve a fully homogenized suspension, the SGA mixture was agitated using a handheld immersion blender (Cuisinart) for 10 minutes at 10,000 rpm.

The homogenized SGA mixture then transferred to two 50 mL conical tubes and centrifuged at 5,000 rpm for 20 minutes to remove suspended air pockets obtained during the mixing process. Food-coloring agents were added to partitioned volumes of SGA mixture to help delineate features during the printing process as seen in figure 2. The SGA mixture is then transferred to multiple 3 mL piston dispensing cartridges (EFD Nordson), capped, sealed, and centrifuged again at 5,000 rpm for 20 minutes. Cartridges loaded with SGA bioink are then stored at 4°C for later use.

Figure 2. Loaded cartridges with dyed SGA bioink

Rheological characterization

Shear viscosity data was obtained using a rotational viscometer (Black Pearl Rheometer). Three separate measurements were obtained using samples obtained from different areas of the semi-solid SGA material. The measuring system is comprised of a 30 mm parallel plate with a 1.05 mm gap between the bottom and top plate. Angular velocity parameters consisted of a starting rate of 0.2 rad/s and increasing over 10 step intervals (5 seconds per step) to the end rate of 4 rad/s.

3D Modelling and Slicing

Objects were designed using Fusion 360 (AutoCad) and exported to STL file format. The object size was limited to the size of the build plate of the bioprinter (130mm x 80mm). Objects designs were limited to small circular shapes to investigate tool path parameters and bioprinter performance. Object widths were selected based on the gauge of the extrusion nozzle. Multiple

objects were placed inside of one another to test the multiple nozzle extrusion capabilities of the bioprinter. Completed designs were exported to .STL file format and imported into slicing software (Simplify3D). A unique settings profile was created to optimize bioprinter operation and output with primary parameters seen in table 1:

Table 1. Primary printing parameters

Infill	100%		**Retraction Vertical Lift**	1mm
Nozzle Diameter	1mm		**Layer Height**	1mm
Printing Speed	5.0 mm/s		**Top Layers**	1
X/Y - Axis Speed	150.0 mm/s		**Bottom Layers**	1
Z - Axis Speed	30.0		**Perimeters**	1

After applying settings to specified objects, the file is then converted into a printable file format (.GCODE) using Simplify3D. Afterwhich, the file must be post-processed using Python in order to convert the standard 3D printing file into a format needed by the bioprinter. Briefly, typical 3D printers use plastic filaments which are extruded using a series of motors and gears which feed the filament through a heated nozzle. The bioprinter that is being used for this project uses filtered air pressure provided by a pneumatic pump and controlled through a series of micro-controlled solenoid valves. Therefore, the Python script converts all extrusion data from extrusion steps into pressure steps.

3D printing process

All 3D printing of SGA bioinks were conducted using a pneumatic pressure-based extrusion system (Cellink - Inkredible +). The selection of extrusion nozzle gauge was object-dependent. Nozzle gauges ranging from 13 – 23 gauge were chosen to print multiple single and multi-material objects. Two 3 mL cartridges loaded with SGA bioink were secured to the x-axis gantry via heated aluminum cartridge holsters. After loading of the cartridges, each print-head (PH) was lowered to calibrate the distance between the tip of the nozzle and the top of the build plate. The distance was set to 0.75 mm and the values were set in the flash memory of the bioprinter. After calibration, both the left and right cartridges were heated to 35°C for over 5 minutes. Once the cartridges were at the desired temperature, the GCODE was transferred to a memory card and loaded into the bioprinter.

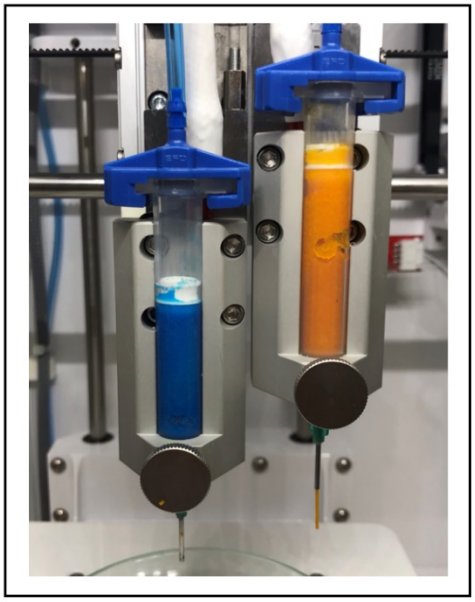

Figure 3. Loaded cartridges on X-axis gantry

Results and Discussion

Lower pressures with larger nozzle diameters allow for higher resolution with viscous materials while maintaining high structure fidelity and form. Figure 4 shows the slicing tool path output generated by Simplify 3D and the resulting extruded output. Table 2 outlines the nozzle gauges and pressures that were selected along with observations. Object A was extruded with 13 gauge nozzle and represented a portion of an artery obtained from a computerized tomography scan. Object B was extruded with a 14 gauge nozzle and was the first attempt at a multi-material extrusion. Noticeable artifacts are present thought the layers of the object due to excess material adhering to the tip of the nozzle. Object C was extruded with a 15 gauge nozzle with noticeably

fewer artifacts due to an increase in speed from 2 mm/s to 5 mm/s. Objects D-G were all extruded using a 20 gauge nozzle with structural alteration incorporated into the design. As the layers increased, the height of the object increased, and slight deformation can seen on the top most layer. This issue is related to two parameters. First, higher gauge nozzles produce fine extrusion paths. As the layers of an object increase, the fine extrusion path will tend to succumb to external forces, such as gravity. Secondly, if the pressure and speed are not optimized, over-extrusion will push the top layer into the layer beneath causing deformation.

Table 2. Selected nozzle gauges, pressures, and observations

Gauge	Pressure (kPa)	Observations
13	34 - 42	Large extrusion paths, material in cartridge quickly depleted
14	51 - 67	Object has improved resolution but the material still depleted quickly. Fast printing speeds may be possible
15	75 - 82	Pressure/speed relationship produces structures with high resolution and minimal artifact creation on edges and overall structure
20	172 - 181	Extrusion paths have improved resolution, pressure still builds on sides of cartridges creating air pockets
22	230 - 260	Globules form around parameters, pressure is too high for even print resolution on all extrusion paths
23	363	Higher pressures have greater fluctuation, air pockets formed in the cartridge, reduced resolution due to material over-extrusion

Dispensing tips which have lower gauge diameters are proving to be optimal for SGA bioink materials. The larger diameters allow for a uniform flow of materials that contain a high concentration of particulate matter, such as 20% SPI. Gauges of 13, 14, 15, have provided the best results with pressures ranging from 34 – 42 kPa, 51 -67 kPa, and 75 - 82 kPa respectively. Higher gauges have been used (20, 22, and 23 gauge), but the pressures required to extrude the SGA bioink proved to be too high resulting in air pockets forming within the cartridge. This is

due to the pressure limit for the piston/cartridge interface being exceeds and therefore, the air was able to leak into the cartridge.

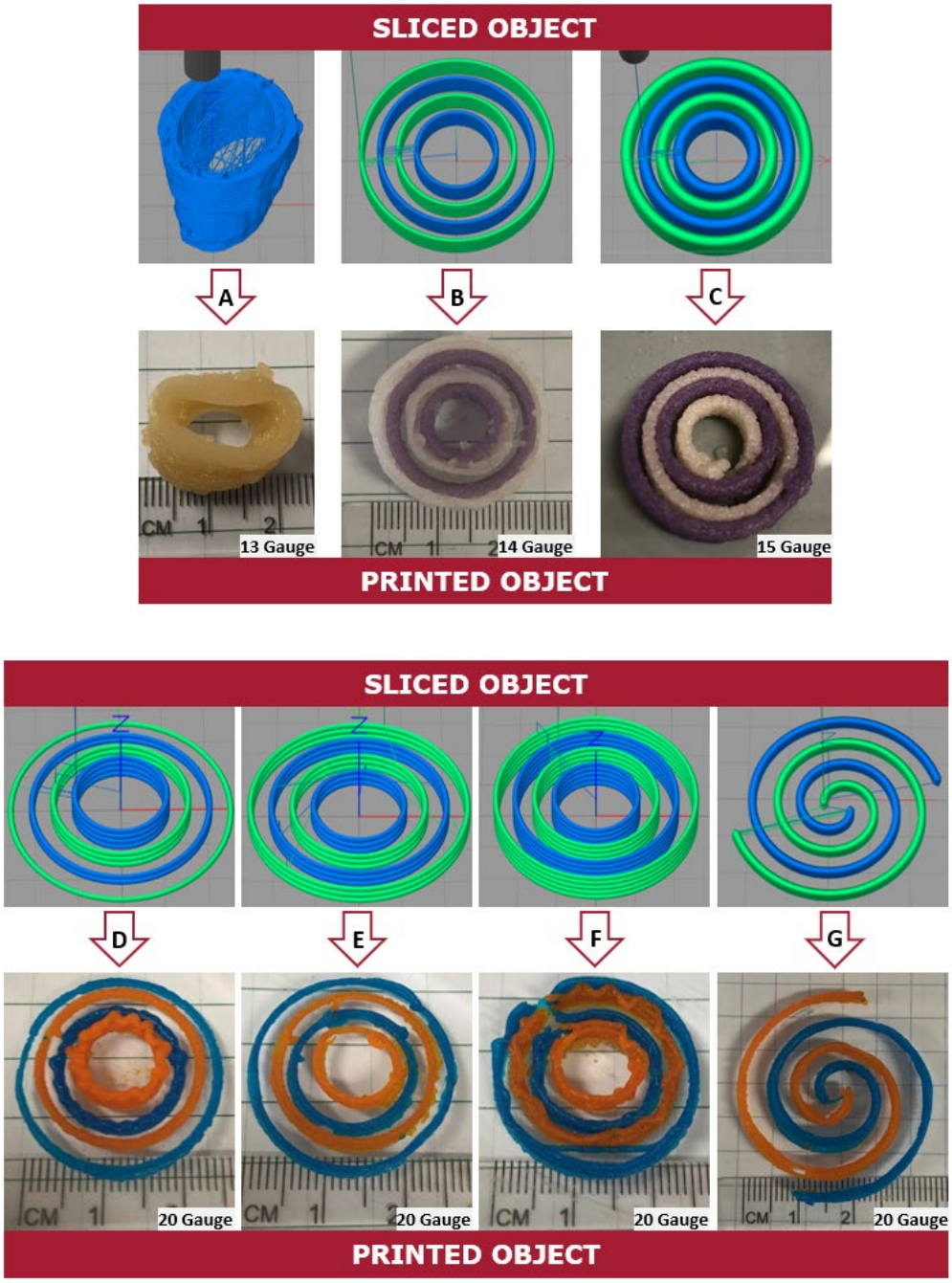

Figure 4. Sliced object files and resulting printed structures

Viscosity is a principal parameter when any rheological measurements of fluids, such as liquids, semi-solids, and gases are made and the rheological properties of a bioink are directly related to 3D printability.[20] The high concentration of SPI and gelatin substrates, 20% and 2% respectively, are associated with higher viscosity as seen in figure 5. This can be attributed to the increase in polymer chains during mechanical and heat dependent mixing protocols.[21]

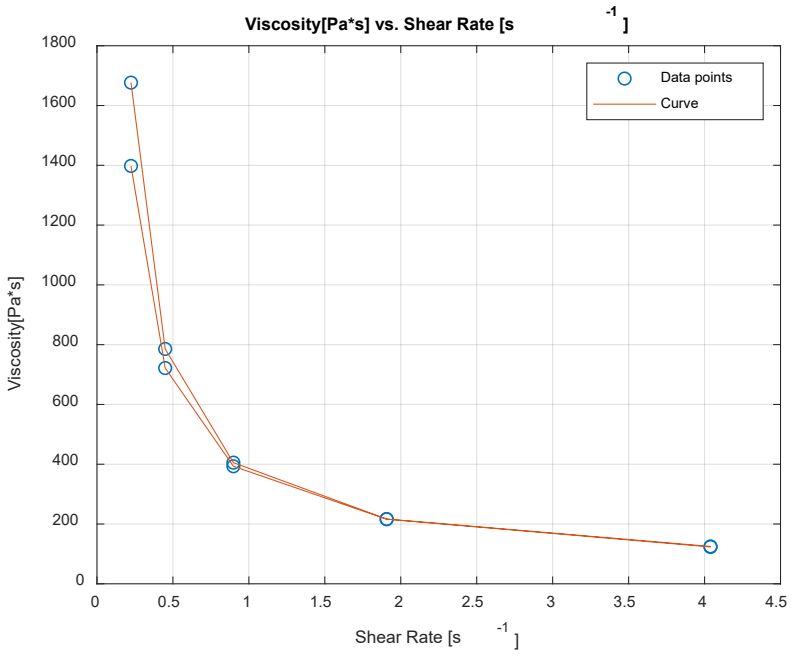

Figure 5. Rheological measurements

Limitations and Future Directions

Although this research was successful, further consideration must be taken to achieve the ability to fabricate edible food for consumers. Limitations of this experimental setup are described below to identify aspects that require further investigation. First, the 3 mL cartridge volumes are inadequate to extrude sufficient material necessary to constitute a meal. Also, if large cartridges were to be used, a more substantial, high-pressure 3D printing system would need to be devised. This system would need to be fitted with high-pressure, high-volume cartridges capable of continuous extrusion.

Furthermore, objects designed and extruded within this research are relatively simple in form and do not demonstrate the ability to control texture based on geometry. To achieve this, further knowledge and experimentation are required to produce and test a variety of object infill patterns and determine if those patterns closely recapitulate the textural properties of native foods. One solution could be realized by obtaining and analyzing topographic microscopy of a broad range of food types to be recreated into object infill patterns of a slicer.

Additional investigation and improvement to printing parameters must be explored so that printed structures are repeatable, uniform, and high resolution. Optimization of tool paths is of vital importance when working with soft materials and hard material alike. Adjustments must be made to reduce object crossing that the nozzles perform while traveling from one part of the print to the other. To achieve this, further improvement of post-processing parameters implemented by the Python script and the factory settings installed on the bioprinting is necessary to achieve tool path optimization. Furthermore, high-velocity homogenization of SGA is required so that all particulate material is dispersed evenly to ensure proper flow and continuous pressure application. This can be achieved by improving mixing protocol parameters such as heating and cooling steps and subjecting the mixture to further emulsification with a high-velocity blending apparatus. Another aspect to consider is the method in which pressure is applied to the system. Using a pneumatic pressure source is useful if constant pressure is needed. However, plastic-based 3D printing systems used a variable extrusion rate with fabricating objects. The same is needed with extruding semi-solid materials, such as SGA bioink. Without a variable pressure system, complex food geometries can not be created due to the adjustments needed in high-resolution objects.

There is also concern with using an animal-derived component in the bioink presented. Gelatin, although widely available and inexpensive, is produced from ossein, the demineralized bone, skin, and cartilage cattle, and pigs. Using ingredients that are derived from animals diminishes the population that would be able to consume 3D printed foods from the material presented in this research. However, substituting gelatin with an enzymatic protein crosslinker such as transglutaminase (TG) could provide a non-animal derived gelling solution. TG is an enzyme that is derived from the bacterium Streptoverticillium sp and routinely used in the culinary arts.[22] The primary function of TG is the ability to form crosslinks between protein molecules which enhances protein gelation capability. Considering the results obtained, it can be concluded that there is a wide range of variability in extrusion resolution based on nozzle gauge and pressure. Larger nozzle diameters produce more consistent geometry when extruding SGA bioink. Also, a high degree of homogenization is required for uniform extrusion of SGA bioinks.

Conclusion

The results described herein provide evidence that extrusion of SGA bioink using a pneumatic pressure-based 3D printing system, and fabrication of multi-material geometries using edible ingredients is possible. However, this can only be considered an initial phase to this research. Further investigation into replicating the textures, flavor, and shapes of traditional food products using 3D printer technology is necessary to fully realize the notion of an edible product designed for consumers. The continued development of a natural bioink as an alternative source for nutrition could provide an inexpensive material that is easily modifiable and therefore customizable to an individuals' taste. Further efforts to realized this goal could begin to address the lack of affordable, healthy food options that affect millions of individuals worldwide. For

these reasons, additional research of inexpensive, sustainable, and innovative solutions is of paramount importance.

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