HHS Public Access

Author manuscript

Biofabrication. Author manuscript; available in PMC 2020 February 24.

Published in final edited form as:

Biofabrication. ; 12(2): 022003. doi:10.1088/1758-5090/ab6f0d.

Assessment Methodologies for Extrusion-Based Bioink Printability

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Abstract

Extrusion-based bioprinting is one of the leading manufacturing techniques for tissue engineering and regenerative medicine. Its primary limitation is the lack of materials, known as bioinks, which are suitable for the bioprinting process. The degree to which a bioink is suitable for bioprinting has been described as its “printability.” However, a lack of clarity surrounding the methodologies used to evaluate a bioink’s printability, as well as the usage of the term itself, have hindered the field. This article presents a review of measures used to assess the printability of extrusion-based bioinks in an attempt to assist researchers during the bioink development process. Many different aspects of printability exist and many different measurements have been proposed as a consequence. Researchers often do not evaluate a new bioink’s printability at all, while others simply do so qualitatively. Several quantitative measures have been presented for the extrudability, shape fidelity, and printing accuracy of bioinks. Different measures have been developed even within these aspects, each testing the bioink in a slightly different way. Additionally, other relevant measures which had little or no examples of quantifiable methods are also to be considered. Looking forward, further work is needed to improve upon current assessment methodologies, to move towards a more comprehensive view of printability, and to standardize these printability measurements between researchers. Better assessment techniques will naturally lead to a better understanding of the underlying mechanisms which affect printability and better comparisons between bioinks. This in turn will help improve upon the bioink development process and the bioinks available for use in bioprinting.

Keywords

Bioprinting; bioink; hydrogel; extrusion; printability; tissue engineering; regenerative medicine

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1. Bioprinting and bioinks

Three-dimensional (3D) printing is a manufacturing process used in regenerative medicine and tissue engineering, commonly chosen for its advantages in automation, reproducibility, speed, cost, and ability to manufacture complex and patient-specific shapes.[1–6] Many traditional 3D printing modalities require conditions lethal to cells such as high temperature, toxic chemicals, or a dry environment.[3] These techniques can be utilized by seeding cells onto scaffolds after fabrication; however, this strategy has limitations including difficulty controlling cell distribution and concentration, especially with multiple cell types, and difficulty reaching the deepest regions in scaffolds which are large or contain very small pores.[7] Acellular scaffolds, which instead uses a strategy of recruiting a patient's own cells to migrate into the scaffold, may be ideal in the short-term due to their accelerated regulatory approval pathway. Concurrently, in many applications, the patient's cells are impaired due to the disease being treated, senescence, and/or old age. Nevertheless, many studies have shown the inclusion of cells to result in improved biological outcomes.[7] Lastly, a majority of the materials used in traditional 3D printing are much stiffer than soft tissue, limiting their application to hard tissues such as bone.

When 3D printing technologies print with cells, it is referred to as bioprinting[2,3,8,9] and the materials are referred to as bioinks.[10] Bioprinting has targeted a variety of applications including trauma, congenital defects, whole organ diseases, and tumor removal. These constructs have been successfully applied to *in vitro* drug testing and disease modeling.[3] The ultimate goal of bioprinting is clinical engraftment. While a complete, functional organ has yet to be printed, the field is optimistic towards its eventual feasibility.

Of the four major bioprinting technologies which incorporate cells directly (extrusion, inkjet, stereolithography, and laser-assisted printing methods), extrusion bioprinting has several distinct advantages. Inkjet and laser-assisted have excellent resolutions but are limited by the speed at which they can print and size of constructs which can be manufactured.[3] Stereolithography (including digital light processing, DLP) can match extrusion bioprinting in these areas but requires a large number of cells as the entire bath volume must be filled. Stereolithography is also limited to only photo-crosslinkable materials, a single bioink per construct, and a uniformity (both density and distribution of phenotype) of included cells.[11]

Extrusion-based bioprinting is the most commonly used type of 3D printing in tissue engineering applications.[8,12–17] Most extrusion bioprinters contain multiple print heads for printing multiple materials within a single construct. This allows researchers to manufacture constructs with regional differences in biomaterials, cell types, cell densities, and signaling molecules.[8] Relative to other bioprinting processes, extrusion bioprinters can also operate at higher cell densities.[18] Its biggest drawback is that cells are exposed to shear stress when passing through the nozzle and pressure while in the syringe prior to extrusion, both of which can decrease cell viability and function.[11]

While extrusion bioprinting may have more biomaterials available than other printing modalities, its most frequently cited limitation is the lack of available biomaterials, known as bioinks.[2,19,20] A bioink is a "formulation of material(s) and biological molecules or cells processed using bioprinting technologies."[3] For extrusion bioprinting, bioinks are the mixture of cells, signaling molecules, and biomaterials which are extruded by the bioprinter. Because of their high water content and ability to suspend cells, hydrogels are the primary biomaterial utilized for this purpose. They may be either natural (e.g., gelatin, alginate, agarose, chitosan, dextran, fibrinogen, gellan gum, hyaluronic acid, etc.) or synthetic [e.g., poly(ethylene glycol)s (PEGs), Pluronics, polyacrylamide, poly(2-hydroxyethyl lmethacrylate) (PHEMA), etc.). Natural hydrogels have improved bioactivity while synthetic hydrogels are cheaper, more easily modified (i.e., tunable degradation and mechanical properties, functional modification), and have a greater batch to batch consistency.[5,19–21]

Presumably, a specific shape is desired when a construct is bioprinted; however, because these materials are mostly water, achieving adequate shape fidelity upon deposition is challenging.[22] Overhangs, microchannels, and tall structures (greater than 1 centimeter) are especially difficult to create with these materials. Adding to this difficulty, bioinks with too high of viscosity cannot be used as the pressure required for extrusion (and subsequent shear stress placed on the cells) can cause permanent cell damage and death.[18] As a result, most bioinks are not self-supporting without some secondary intervention. Most commonly, one or more forms of crosslinking are applied. Physical crosslinking, such as with thermoresponsive gelatin, is employed for systems which use different temperatures between the material and printing space. This can be achieved either through a heating/cooling sleeve around the syringe, temperature control of the air in the printing environment, printing onto a hot/cold plate, or some combination thereof.[20] Ionic crosslinking, such as with alginate hydrogels, can be used either through partial crosslinking prior to printing, applying an ionic mist, printing directly into an ionic solution, or simply submerging into an ionic solution after the print has finished.[20] Finally, ultraviolet (UV) crosslinking, such as with methacrylated hydrogels, can help maintain the material's shape by applying a UV light and photoinitiator. This is done either at the conclusion of the print, in between each layer, or directly at the filament as it is being extruded.[20] Support materials such as melt extrusion polymers [e.g., poly(ε-caprolactone) (PCL)] and sacrificial materials (e.g., Pluronic F127) have also been used.[8] Finally, submerged bioprinting into a solution of similar density (e.g., a perfluorocarbon bath) also has shown to improve the shape fidelity of a final construct.[23] In addition to increasing the complexity, print time, and cost of the system, each of these strategies also has limitations and drawbacks. Additionally, while different types of crosslinking and support elements have improved the shape fidelity of final constructs, there is still room for improvement beyond these methods, and ultimately, a self-supporting material would be highly desirable.

2. Bioink evaluation and printability

When selecting a hydrogel for a particular application, many parameters are considered including gelation time, crosslinking mechanism, biocompatibility, bioactivity, mechanical strength, swelling characteristics, and degradation profile. With the long-term applications in mind, hydrogels may be evaluated on their commercialization potential and ability to gain

regulatory approval.[5] When new bioinks are presented in the literature, their performance in these categories is typically reported and discussed. With regard to biological activity, standard evaluation techniques such as proliferation assays, quantitative real-time polymerase chain reaction (qPCR), histology, and immunohistochemistry are well established. Rarely is an article published without degradation and swelling ratio experiments. Strain sweeps, frequency sweeps, compression tests, and tensile tests are also typically carried out when appropriate and with similar methodology from paper to paper.

These methodologies have been established through their wide usage in the field of tissue engineering and regenerative medicine. When looking specifically at extrusion bioprinting applications, the printability of bioinks has only recently garnered attention. Researchers have used a wide range of definitions for printability, but in general, it has been used to describe the ability of a bioink or a set of printing parameters to be used for extrusion bioprinting. The word “printability” initially came into existence to describe the relative ability of paper to take printed ink. It has since been adopted by 3D printing to describe the ability of materials to be used in various 3D printing modalities. As each modality has a specific set of requirements for its materials, the word has come to have many different meanings depending on the type of 3D printing being used. These requirements may also vary depending on the intended application, complicating the term’s usage even further (Figure 1).

There is an immense need for further bioink development within bioprinting, with an acute requirement for improved bioink printability.[6,22] Recently, many researchers have proposed sets of criteria for bioink properties in order to guide the development of new bioinks specifically for extrusion-based bioprinting.[19,24–28] Unfortunately, most of these have been broad objectives a material should aim to achieve instead of applicable engineering specifications and guidelines. Hydrogels must be able to suspend cells and cells must be able to survive the printing process.[16] Constructs must be able to be printed in a reasonable timeframe. Bioinks should be shear thinning in order to flow easily during extrusion. Final shape fidelity is also important as patent microchannels within a scaffold play a crucial role in cell survival and function by facilitating oxygen transfer, the delivery of nutrients, and the removal of metabolic waste.[8,12] Specific geometries are desirable for many applications especially load-bearing tissues (articular cartilage, muscle), “patch” implants (scaffolds replacing infarcts, removed tumors, trauma), and those with an aesthetic function (ear, skin). Printability has also been linked to rheological properties, viscosity, extrusion line dimensions, the ability to print sharp angles, filament shape, filament fusion, filament collapse, structural stability, and degradation rate.[4,7,21]

Murphy and Atala defined printability as “the properties that facilitate handling and deposition by the bioprinter.” Viscosity, gelation methods, rheological properties, print time, cell viability, and nozzle gauge are discussed as factors which can impact printability.[4] Kyle et al.[29] and Mandrycky et al.[7] define printability as “the relationship between bioinks and substrates that results in printing an accurate, high-quality pattern.” Meanwhile, Gopinathan and Noh[21] describe printability as dependent on, “different parameters such as viscosity of the solution, the surface tension of the bioink, the ability to crosslink on its own, and surface properties of the printer nozzle.” While these reviews have briefly discussed the meaning of

the word printability amongst their broader topics, their considerations to printability have been minimal, and the ambiguity of these definitions is reflected by the usage of the term in the rest of the literature.

For the purposes of this review, a broad, generic definition of printability will be defined as,

> "The ability of a material, when subjected to a certain set of *printing conditions*, to be printed in a way which results in *printing outcomes* which are desirable for a given application."

"Printing conditions" refers to process parameters—the user-controlled settings for the 3D printer and its environment—and "printing outcomes" refers to the measures of success for the print. In the case of extrusion bioprinting, the printing conditions include feed rate, pressure, path design, nozzle height, temperature, and many others. Printing outcomes; however, have been poorly defined by the literature. Ultimately, establishing a quantitative definition of printability and improving its outcome measurements will aid dramatically in bioink development, which in turn will advance extrusion bioprinting closer towards its long-term applications and goals.

A wide variety of tests have been used in the literature in an attempt to describe printability and printing outcomes. Oftentimes, these are qualitative; a researcher will simply take a photograph of their final construct to include in their publication. It is not uncommon for papers presenting newly developed bioinks[30–35] to exclude any quantitative or qualitative descriptions of printability, even among recent publications. Researchers have also used classification systems based on several possible printing outcomes.[36–40] While these evaluation techniques provide some value and information regarding printability, quantitative measurements are less subjective and allow for comparison between bioinks and printing conditions. The next several sections will cover the various requirements of bioinks as related to printability and a discussion of their outcome measurements which have been used in the literature thus far.

3. Extrudability

A basic requirement of printability is the capability to achieve extrusion. The amount of pressure required to extrude a given bioink using a given printing system can be referred to as that system's extrudability (Figure 2). Extrudability primarily affects cell viability and total print time. The more pressure required to extrude a material, the more shear stress the cells will experience and the more damage will be inflicted upon the cells during extrusion.[27,41,42] Additionally, materials which extrude easily may be printed at a faster rate, which in turn results in shorter print times.

Extrudability has been linked to several factors in the literature: printing parameters, bioink viscosity, and bioink shear-thinning abilities. Bioinks are easier to extrude when the diameter of the nozzle is increased when the length of the nozzle is decreased, and for conical relative to cylindrical nozzles.[18] In terms of material properties, viscosity is the primary measure of a bioink's resistance to extrusion forces. However, most hydrogels are shear thinning, meaning their viscosities decrease with increasing shear strain.[7,20,43] Some

hydrogels also exhibit thixotropic behavior, meaning that under shear their viscosity also decreases over time.[22]

A bioink's viscosity, shear-thinning, and thixotropic behaviors can be measured through rheology, and several mathematical models relating these measurements to extrudability and cell damage have been well-established.[26,44,45] As such, rheological characterizations can be successfully used as indirect measures of extrudability. Most notably, frequency sweeps have been used to measure the viscosity of bioinks across different shear rates. To compare between bioinks, these values can be plotted as is or fitted to the power law equation to derive their consistency index (K) and flow index (n). The consistency index is related to the initial, or zero shear, viscosity of the bioink with lower values indicating higher extrudability. Meanwhile, the flow index relates to the shear-thinning abilities of the bioink, with a flow index of one indicating Newtonian behavior and values closer to zero indicating a higher degree of shear-thinning and therefore extrudability.[26,46–51]

Extrudability has been characterized in several other manners by researchers. Utilizing a piston-driven bioprinting system, Chung et al. held strain rate constant and measured the extrusion force.[52] Gao et al. used the pressure required to achieve a given flow rate as the defining measurement.[53] Examining several bioinks, Paxton et al. used pressure and flowrate to describe a *printability window* by setting a minimum flowrate which was needed to achieve a reasonable print time and a maximum pressure to prevent extensive cellular damage.[26] Roehm et al. used extrudability to determine how certain processing steps affected their chitosan-gelatin hydrogel's printability. In their system, prolonged, high pressure would eventually detach the extrusion needle. Bioinks were deemed unprintable if they could not be extruded through the needle or if the needle detached prior to the completion of the print.[42] Zhu et al. categorized their bioinks as unextrudable if they were not able to achieve flow at a maximum of 380 kPa.[54] Wilson et al. labeled certain concentrations of their bioink unprintable if they underwent "premature gelation" and were unable to be extruded.[55]

Webb et al. looked at theoretical shear stress as a way to account for extrudability. While not calculating shear stress directly, the authors used the ratio of nozzle diameter to pressure as a proxy for theoretical shear stress. This ratio was then used to optimize printing conditions as a part of their Parameter Optimization Index (POI).[56] Giuseppe et al. used a modified version of POI, including extrusion pressure directly in their optimization calculations.[57] Extrudability may also be inferred by live/dead assays with poorer cell viability correlating with poorer extrudability.[27,39,41,42,58] However, it is important to note that extrudability, as it relates to printability, should be limited to cell viability immediately following extrusion. Extrudability represents one of the most important and thoroughly studied components of printability and as a result is one of the more understood components.

4. Filament classification

As a way to prescreen bioinks for their use in bioprinting, several researchers have identified the shape of extruded filaments. Different methods to measure extrusion shape include categorizing the filament just after extrusion, but before deposition (filament drop test),

categorizing filament shape upon deposition, and quantifying filament shape upon deposition.

The filament drop test involves extruding a filament into the air rather than on a substrate (Figure 3A).[26,27,37,41] If a bioink is too weak, a droplet will form at the nozzle tip, and the bioink will not be capable of printing 3D structures. Rather, a bioink should form a smooth, uniform filament.[26,37] Ouyang et al.[27] appended the filament drop test classification system to include a category for filaments which result in a bumpy, non-uniform geometry referred to as “over-gelled” filaments.

These types of filaments have also been described after deposition. Habib et al. categorized their filaments as either straight or curvy.[41] Zhu et al. described similar filaments as ‘irregular’ fibers and deemed the materials unprintable if they exhibited this behavior.[54] Wilson et al. also described filaments with inconsistent diameter for their bioink with feedrates above 8 mm/s, although no quantifiable measurements were made.[55] Using their submerged bioprinting system, Jin et al. categorized their filaments by the filament diameters relative to the nozzle size (swelling, equivalent diameter, and stretched filaments) and also had subcategories for types of failure (rough surface, over-deposited, compressed, and discontinuous filaments).[59]

Some researchers have attempted to quantify the extent to which these filaments are non-uniform (Figure 3B). Gao et al. measured the perimeter of extrusion lines normalized by the length of a perfectly straight line to quantify the uniformity of a given filament. A value of 1.0 represents maximum uniformity and higher values indicate less uniformity.[53] Meanwhile, Ouyang et al. developed a parameter, *Pr*, where the perimeter of a square pore is measured and compared to that of a perfectly square pore. By the author’s criteria, a *Pr* value equal to 1 indicates perfect extrusion line uniformity and *Pr* values greater than 1.1 had unacceptable uniformity.[27] Lastly, Gohl et al. looked at the standard deviation of the heights and widths of their extruded filaments. While this was done in order to compare between their experimental data and computational model, it could also serve as a measurement of filament uniformity.[60]

When these non-uniform bioinks are used for scaffold manufacturing, the extrusion lines may deviate from the intended path significantly or even curl up and stick to the dispensing nozzle. However, bioinks of this nature are commonly used in tissue engineering applications. At this point in time, only simple shapes and small constructs are being fabricated. This material characteristic may prove unusable for more complex geometries (where small inaccuracies can have a large impact) and larger constructs (where inaccuracies can accumulate and increase the error with each layer). While it has been shown that hydrogels can transition from droplet to uniform filaments and from uniform to non-uniform filaments with increasing concentration or degrees of crosslinking,[27,53] little research has been aimed at this phenomenon otherwise.

5. Shape fidelity

Shape fidelity has been a vague, but an important term used to describe the printability of bioinks (Figure 4). In general, shape fidelity is the ability of a bioink to maintain its shape upon deposition. Bioinks with poor shape fidelity may have extrusion lines which spread too wide with very limited height. Geometries may sag under their own weight as more layers are added. Vertical pores may close as spreading occurs and lateral pores may collapse due to the inability of the bioink to maintain an unsupported fiber.

The most common design for this type of evaluation is the crosshatch where extrusion lines are rotated 90 degrees every other layer for at least two layers.[28,39,61–71] This could also be an anatomical shape based on the application such as the knee meniscus,[67,72] an arterial bifurcation[23,73,74] or quadfurcation,[59,74] the liver,[75] the pancreas,[41] the ear,[58,67] and other interesting shapes including tubular[23,41,66,68,70,73,74,76] and cuboidal structures,[66,75] trees,[23] stars,[66] and honeycomb geometries.[77] In these examples, shape fidelity is either categorized (e.g., ‘low fidelity,’ ‘defined,’ ‘high fidelity’[38]) or simply shown qualitatively through a photograph. Bioink shape fidelity has been quantified by measuring several commonly printed features. These measurements can be categorized by the phenomenon they are addressing, including filament spreading, height maintenance, and filament collapse.

5.1. Filament spreading

Shape fidelity is most commonly analyzed using a single layer, such as by measuring the dimensions of printed filaments. Line width has been utilized to compare different bioink compositions,[57,60,78–82] concentrations,[38,40,55,75] temperatures,[42,58] and degrees of crosslinking.[52] Daly et al. normalized the line width by the nozzle diameter and named this term ‘spreading ratio,’[79] while Chung et al. used an indirect measurement of the filament width by measuring the distance between fibers.[52] In general, successful criteria in these studies stated that a smaller filament width (and therefore less spreading) was more desirable. Therefore, bioinks which resulted in the thinnest fibers were deemed to have the best printability,[40,55–57,79,80] and one study even equated line width as the inverse of ‘accuracy.’[56]

The height of printed filaments has also been used to compare among bioink compositions,[60,81] among bioink concentrations,[55,75] and printing with or without a fluorocarbon support fluid.[23] These studies either identified the most desirable shape fidelity as a larger height-to-width ratio (also known as the aspect ratio) or did not establish a preferred outcome.[23] In addition to the height and width of individual filaments, the *Pr* value developed by Ouyang et al. can also be used to quantify filament spreading. Just as a value greater than 1 is the result of filament non-uniformity (as discussed in the ‘Filament Classification’ section), a value of less than 1 is the result of material spreading and poor shape fidelity. Values lower than 0.9 were concluded to have poor shape fidelity by their criteria.[27,41,83] Habib et al. modified the *Pr* measurement by varying the pore sizes instead of using a consistent cross-hatch.[41] While this modified *Pr* value was easy to measure, bioprinted constructs are rarely only a single layer, and as a result, these measurements may not capture the entire behavior of the bioink.

5.2. Height maintenance

The ability of a bioink to maintain its height upon deposition of multiple layers is also a key factor in its printability. The larger a construct becomes, the more critical this aspect of shape fidelity is. As more layers are added to a single construct, the weight experienced by the lower layers increases. By printing alginate/agarose in a perfluorocarbon bath, Blaeser et al. described a 'critical height' as the highest vertical height a filament could be printed successfully.[23] Duarte Campos et al. measured the heights of hand-extruded cylindrical structures and compared them to the designed dimensions.[36] Gao et al. printed cylindrical structures and measured their heights, referred to as 'structural integrity', to compare different concentrations of gelatin-alginate bioinks.[53] While filament spreading looks at a single layer, multi-layer tests challenge the bioink significantly more. Additionally, a multi-layer analysis may minimize the effects seen from the printing substrate, which otherwise have a greater impact on the filament spreading analyses.

5.3. Filament collapse

A common feature among bioprinted constructs is the unsupported filament which spans between filaments from the previous layer. This type of design can create lateral pores which allow for diffusion into the deepest parts of the construct. By printing a filament across gaps of increasing distances, researchers have measured the deflection of the filament to quantify shape fidelity. Ribeiro et al. quantified this phenomenon with a mechanical model, using the angle of the partially collapsed or sagging filament. Materials which resulted in a higher angle of collapse were considered to have poorer shape fidelity.[84] Habib et al. used a similar test using a measurement they called the 'collapse area factor' by measuring the area underneath the filament and standardizing it by the theoretical area had there been no sagging. The researchers repeated this test both on a plastic model with varying gap sizes as well as from the lateral pores of a 3-layer hydrogel cross-hatch print.[41]

Quantifying shape fidelity is still a relatively novel concept in bioink development and improvements to the methodologies are needed, especially in isolating measurements which are not affected by the printing parameters. Improved measurements would assist in testing for and identifying the underlying mechanisms affecting shape fidelity. Additionally, many different architectures may be desired from bioprinting outcomes depending on the application. Researchers may want to tailor their shape fidelity measurement for their particular application, but this should also be weighed against the benefits of standardization. Having similar testing methods among research labs would significantly improve bioink comparisons, scientific communication, and bioink development overall.

6. Printing accuracy

While the shape fidelity of a bioink may limit the type of structures which can be bioprinted, the printing parameters play a major role in the outcome of a print. Even an ideal bioink will result in an unacceptable printing outcome with improper printing conditions. Outcome measurements which attempt to capture the various ways a print can fail due to the printing conditions have been referred to with the catch-all term printing accuracy (Figure 5). Printing accuracy can be thought of as the degree to which printed filaments, features, and

constructs match their intended size, shape, and location with respect to the printing parameters used.

For every bioprinted construct, a specific geometry is desired. If the final construct strays too far from this shape, it may be unusable for the given application. For example, the anatomical shape is often important for function, or a specific defect may need to be filled. If the construct is delivering a drug or implanting cells to the injury site, the size, and therefore dose, is of paramount importance. Unfortunately, due to the layer-by-layer nature of 3D printing, even small errors early in the print may propagate over time causing the overall print to fail.

Printing inaccuracies can manifest in several different ways. For any given printing condition, there are ranges of inappropriate settings which correspond to specific failure mechanisms. Over-deposition of material can result in closed pores (when patent ones are desired), merged filaments, and significant deviations from the designed shape. Instead of filaments being deposited on top of the previous layer, it is possible for the nozzle to be submerged and extrude directly into the previous layer. The nozzle tip may also run into the construct and displace material which was previously in the proper location. Under-deposition can result in discontinuous filaments and in constructs which are smaller than desired. Filaments may also stick to the nozzle tip instead of the desired location. Many researchers have devised outcome measurements to describe these failures.

The most basic of these measurements qualitatively categorize the final printing outcomes. Duarte Campos et al. used a rating scale from 0–2 for both the uniformity and stability of their printed structures.[36] Ng et al. printed a grid pattern and categorized the outcome as either excessive material deposition (merged filaments, closed pores), optimal printing parameters (desirable outcome), or incomplete printing (discontinuous filaments).[40] Several studies have described printing conditions which resulted in discontinuous/broken filaments.[39,42,70,78] If filament breaking occurred, those conditions were considered to have poor printability.

The most common quantitative measure of printing accuracy is the width of extruded filaments. While filament width does not account for the height, cross-sectional area, or mass of the deposited material, width is positively correlated with these measures. Many relationships have been examined between line width and printing conditions such as pressure,[38,40,41,54,56,75,81] flowrate,[42,85] feedrate,[38,40,41,54–56,58,60,75,76,81,86] nozzle height,[42,60,76,81] and nozzle diameter.[56,57,81,85] A smaller filament width was considered more desirable in most of these studies.[40,55–57,79,80] However, a few studies considered a line width equal to the designed width as the most desirable outcome.[52,82] Filament height is another common measurement and has been examined in relation to pressure,[75,81] feedrate,[55,60,75,81] nozzle height,[60,81] and nozzle diameter.[81] These studies either identified the most desirable printability as a height which was equal to the predetermined nozzle height, or they did not specify an ideal outcome.[41,55,75] There is a key difference between these experiments and those which used similar measurements to study shape fidelity. Experiments which evaluated printing accuracy utilized a single bioink and varied the

printing parameters. Studies which evaluated shape fidelity, meanwhile, compared their results among various bioinks.

Several researchers have also used the area of horizontal pores as an outcome measurement and normalized this relative to their design, termed either “diffusion rate”[41,76] or simply “accuracy.”[12,57,87,88] Instead, some measured the total area occupied by the construct viewed from above rather than the size of its pores. This measurement has been referred to as both “printing accuracy”[89–91] and “square area error.”[82] Several studies have also used micro-computed tomography (μCT) to quantify the overall similarity of their final constructs to the designed computer-aided design (CAD).[34,92]

The merging of filaments is another printing outcome which has been used previously to describe undesirable levels of deposition. Wüst et al. printed a rectangular spiral and measured the minimum distance at which the filaments did not fuse together.[93] Ng et al. described lines which merged together due to over-deposition but did not make any quantitative measurements.[40] He et al. provided a measurement where extrusion lines are printed at acute angles, and the overlap region between the two segments was quantified.[76] Ribeiro et al. conducted a robust filament fusion test where filaments were printed in a zig-zag pattern, increasing the distance between filaments by 0.05 mm at each turn-around. Filament merging was measured by the length of the merged portion of parallel lines (denoted as ‘fused segment length’). Fused segment length was also normalized by filament thickness. Notably, the authors also dismissed the first two layers and made measurements on the third layer to decrease the effect the printing substrate had on the results.[84]

Finally, extrudate weight can also be used to quantify the total amount of bioink deposited.[53] This is a simple measurement and isolates material deposition regardless of other conditions and outcomes. Measuring the weight of the extrudate, however, also ignores the location of the deposited material, which is an important aspect of bioprinting. As such, weight may be better suited as a control variable for other printability measurements than as a measure of printability itself.[53]

7. Other Measures

7.1. Cell mixing and suspension

The ability to mix and suspend cells is fundamental to hydrogels for bioprinting, but these are not simple tasks.[6] Bioinks must be thin enough to homogenously mix a cell pellet throughout the gel. If a bioink is too viscous, it cannot be mixed thoroughly via pipetting which can result in a poor yield, air bubbles trapped within the bioink, and a non-homogenous cell distribution.[61] This cell clumping can also cause clogging at the nozzle tip. However, if printed at a viscosity which is too low, cells can slowly sink to the bottom during printing and lose their homogenous distribution. Therefore, bioinks must also be viscous enough in their subsequent state to maintain the desired cell distribution. If cells are not homogeneously distributed, then cell concentrations will vary in different regions of the construct and even from construct to construct if multiple are being printed. This problem is commonly addressed by using thermoresponsive bioinks. Cells are mixed at one temperature while the bioink has a very low viscosity and printed and then transferred to another

temperature which increases the bioink's viscosity and locks the cells in place. While this concept is commonly accepted within the field, neither the maximum viscosity at which mixing cells homogenously is possible nor the minimum viscosity at which cells can remain suspended has been established in the literature. Bioink development would benefit significantly from identifying a measurable requirement for cell encapsulation which all bioinks must satisfy.

7.2. Homogeneity

Another feature of printable materials is homogeneity. Most bioprinters apply constant extrusion pressure during printing. If the printed material lacks sufficient homogeneity, a constant extrusion pressure will result in broken filaments, non-uniformity, and/or over-extrusion. Cohen et al. quantified the variability of extrusion force overtime of alginates which had been mixed to varying degrees,[72] while Chung et al. used similar methods to quantify consistency using alginate bioinks with varying degrees of crosslinking.[52] This is not problematic for most bioinks, but inconsistency is another way in which a bioink could fail and, therefore, should be considered during bioink development.

7.3. Printing resolution

Resolution is another term which has been used to quantify the printability of bioinks. In general, resolution refers to the smallest dimension produced by a process, but the bioink literature has used several definitions. Resolution in extrusion bioprinting is a complicated parameter because resolution is affected by many things. Printing conditions (such as pressure, feedrate, nozzle shape, and nozzle diameter) have the greatest impact on resolution and have been studied in detail as discussed in the material deposition section. The material properties may also impact resolution. Non-uniform filaments may decrease resolution as the extrusion path becomes wider when the filament deviates from the designed path. Shape fidelity may also play a role. A gel which holds its shape well upon deposition will maintain a relatively cylindrical shape, while bioinks with poor shape fidelity tend to sag and spread out, creating flatter and wider filaments. Additionally, it is possible that stronger gels may be able to tolerate higher feedrates before breaking and forming discontinuous filaments. A bioink's extrudability may also play a role in resolution by limiting nozzle diameter. Decreasing nozzle diameter and decreasing extrudability both result in decreased cell viability. Therefore, the minimum nozzle diameter which can be used for a given material is limited by its extrudability.

Die swell is a phenomenon in which polymer chains disentangle during extrusion and return to their natural, entropy-maximizing state after exiting the nozzle tip.[22] This causes extrusion filaments to have a larger diameter than the nozzle from which they are extruded and has been studied in melt polymers. While the phenomenon has been observed in hydrogels, little research has been done to quantify this behavior for bioprinting. Lastly, resolution may also be limited by total print time. The smaller the filament being used, the longer it will take to print a given construct. All these factors make the resolution a difficult and uncommon parameter to use for comparisons of printability. Nevertheless, these factors are more useful when comparing different manufacturing techniques than the printability of given bioinks or printing conditions.[3]

8. Current limitations

8.1. Printability terminology

One of the primary goals of this review was to define the term printability. This term has been obscured by its usage to discuss bioink evaluations which are unrelated to the bioprinting process, such as a bioink's degradation profile, bioactivity, and many other factors. Many of these factors are important in the overall assessment of a bioink. No matter how excellent a material's shape fidelity and extrudability are, it is of little use as a bioink if it cannot facilitate tissue growth and regeneration. While success during the printing process is essential for the success of a bioink, printability is better viewed as the first hurdle a bioink must clear rather than its primary purpose. Nevertheless, the term printability naturally reads as the "ability to print." As such, for the sake of clarity, the term should be limited to outcomes during or at the immediate conclusion of the printing process. On the other end of the spectrum, some research uses printability to refer to only one aspect of the printing process, such as the ability to achieve extrusion. Since the term has already been used to refer to many aspects of bioprinting and because each of these aspects must be successful for a print to be successful, it is logical that each aspect should be considered within the scope of printability. This review has attempted to provide clarity to these different aspects by referring to them by their more specific terminology such as 'extrudability' and 'shape fidelity.'

We expect that the definition and boundaries of printability outlined in this review shall continue to evolve. Some factors which were not included in this review or covered only briefly include resolution, crosslinking ability, material homogeneity, and surface tension. Resolution is affected by nearly every material property and printing parameter in some way, but resolution impacts few printing outcomes other than total print time. Resolution is rarely a factor which needs optimization and is more of a passive outcome of other more important decisions. For extrusion bioprinting applications, the resolution is generally in a similar range, between 150 μm and 600 μm. This is not to say resolution is without use, but in this review it has been only superficially discussed. Crosslinking ability has also been largely omitted from this review. There are different types of crosslinking and methods for inducing them either before, during, or after the printing process. Crosslinking tends to increase shape fidelity, increase mechanical properties, and slow the degradation process. The outcome measurements outlined in this review are applicable regardless of what kind or if any crosslinking is used. In this way, bioink printability may still be compared between bioinks which crosslink and those which do not. In fact, crosslinking bioinks will likely outperform those without. However, the acceptable material properties and printing conditions required in these cases will likely be specific to the crosslinking mechanism. For example, if a bioink is crosslinked immediately upon extrusion, it may have excellent shape fidelity despite having much weaker rheological properties prior to printing. In this way, crosslinking acts as both a printing condition and a material property.

Material homogeneity is important to printability and has been quantified by piston-driven bioprinters.[52,72] With pneumatic bioprinters, clumping, over-deposition, under-deposition, print inaccuracies, clogging, and filament discontinuities can occur in non-homogenous

bioinks. However, material non-homogeneity is a relatively rare failure mechanism and somewhat difficult to quantify using the more common pneumatic bioprinters. As such, it has only briefly been discussed here. Lastly, surface tension has been identified as potentially impacting printability.[21] However, a surface tension measurement of viscoelastic materials is controversial, and little is known on the role it plays within bioprinting.[22,94,95] As such, surface tension is another factor which merits further study and potential inclusion in future discussion regarding printability.

8.2. Relationships between printing outcomes

While each of the main aspects of printability outlined in this review was discussed separately, they are not entirely independent of one another. There are tradeoffs among aspects, especially in regards to “strong” (e.g. high viscosity) and “weak” (e.g. low viscosity) bioinks. Strong bioinks typically have poor extrudability and are more likely to form non-uniform filaments. They have excellent shape fidelity, and their print accuracy is more easily controlled.[5,19,25] Conversely, weak bioinks typically have excellent extrudability and are more likely to form droplets when extruded. They have poor shape fidelity, and their print accuracy is more difficult to control.[5,19,25] What exactly defines a strong versus a weak bioink is not straightforward, but nonetheless a tradeoff exists between the various aspects of printability. Whether bioinks can be improved in the various aspects of printability without sacrificing other aspects will be a determining factor in the ultimate success of extrusion bioprinted constructs.

The relationships among printability outcomes also come in to play when selecting the control variables for printability experiments. In many of the studies in this review, it was clear qualitatively that certain materials spread, lost their height, or collapsed more than others. However, in many cases, it is difficult to parse how much of these differences were attributable to what factor. Line width appears to be a simple outcome but incorporates printing parameters, die swell, material deposition, shape fidelity, and substrate interactions all into one measurement. One of the biggest limitations of current printability assessments has been a lack of controlling for material deposition. When attempting to assess some aspect of a bioink’s shape fidelity, researchers will often control for either feedrate or extrusion pressure (or worse, both) and vary the other in an indirect attempt to control for material deposition. However, without directly measuring and controlling for material quantity, it is difficult to parse whether the measured effects were due to the material properties or simply more material being deposited. For example, in a recent paper,[41] the outcome measurement was termed the ‘diffusion rate’ and calculated with the following equation:

$$Df_r = \frac{A_t - A_a}{A_t} * 100\%$$

where A_t is the theoretical area of a pore (meaning the designed area) and A_a is the actual area of a printed pore. Their results after testing 5 different bioinks are shown in Figure 4C.

From this it can be qualitatively seen that the two bioinks on the far right had much worse filament spreading than the other bioinks. However, the outcome measurement (pore size) is the result of both material spreading and over-deposition, so it is unclear if material spreading was the sole cause of this print failure or if these bioinks might show better printability if appropriate printing parameters were set.

The *Pr* value is one measurement which inherently controls for material deposition. It does so by normalizing the perimeter of the pore by its area. While over- or under-deposition might change the size of the pore, the resulting pore shape impacts the *Pr* value.[27] Shape fidelity can also be measured independently of material deposition by controlling for the weight of the material deposited for any given test.[53] Notably, Jin et al. used a term *speed ratio* (feedrate/flowrate) to control for the quantity of material deposition.[59] However, the units for *speed ratio* are ($1/mm^2$). Instead, if the reciprocal of this ratio was used (flowrate divided by feedrate), it would have the unit mm^2, which is the theoretical cross-sectional area of the printed filaments.

Selecting the control variables is difficult when evaluating printing outcomes due to a large number of variables in play and the many interactions among them. In general, when comparing different printing conditions, the bioink should be controlled for. When comparing bioinks, one strategy is to maintain the same feedrate between all bioinks and change the printing pressures to achieve the same flowrate/material deposition in each experiment. This may not always be feasible, in which case both feedrate and flowrate may be varied so long as the speed ratio is maintained and similar printing accuracies are achieved. Of course, each study's objectives are unique, so these strategies may not be suitable for every situation.

9. Future directions

A byproduct of printability's novelty is a lack of research which has been done on the topic. Many techniques for evaluating printability only examine a single printed layer. This layer is disproportionately impacted by the bioink's interactions with the printing substrate, which may vary among research labs and is not necessarily indicative of the printing outcomes after several layers. Other evaluation techniques are flawed in that they do not set clear success and failure criteria. The minimum outcome required to be considered successful or the ideal outcome is not obvious in a majority of the articles cited in this review. Simultaneously, some methodologies may simply benefit from more widespread usage and standardization. Prevalent adoption of methodologies would facilitate cross-study comparisons, improve scientific communication, and cultivate a better understanding of techniques and results. However, more work is needed to compare and improve these evaluation methodologies at this early stage.

Several outcomes have also been described in the literature without the development of a corresponding measurement. No test for filament discontinuity has been reported beyond simple observation for whether any filament fracture occurred during the print. Other failures related to printing accuracy, such as errors in the initiation and termination of filaments and in the creation of sharp corners, have been described, but evaluation methods

have not been developed.[41,76] Techniques are also lacking for quantifying the ability of a bioink to suspend cells and maintain that cell distribution throughout the printing process.[61]

In addition, the underlying mechanisms which impact these printability outcomes need further experimentation. Except for extrudability, the rheological properties which impact the different aspects of printability are poorly understood. Viscosity, storage modulus, loss tangent, and yield stress have all been put forth as predictors of shape fidelity. Yield stress has been implicated in the ability to encapsulate cells, but only with a single material, tested over a limited range, and not using quantifiable outcomes. No rheological studies have been performed on the minimum requirements to maintain the distribution of suspended cells. Neither have any rheological studies investigated the transition of filaments from droplets to smooth fibers to non-uniform fibers, although hydrogel concentration and the extent of gelation have been implicated. Identifying rheological measurements which can be used as proxies instead of the more labor-intensive printability assessments would be of great benefit. A better understanding of what causes bioinks to fail or succeed in each aspect of printability also could lead to improved bioink design.

As for the printing conditions, their impact on different aspects of printability is better understood, although this is largely as a result of individual experience rather than published research. The field of extrusion bioprinting could benefit from research into the maximum feedrate in which excellent printing accuracy can still be expected. The ideal nozzle height is generally accepted to be equal to the nozzle diameter, although no study has shown this, and minimum and maximum tolerable nozzle heights have also not been identified. The impact of ambient humidity and air pressure on printability, particularly printing reproducibility, has not been quantified. Nozzle shape and size may also impact the flow of the bioink prior to extrusion and therefore what type of filament forms at the nozzle tip. With so many parameters and so many aspects of printability, there is no shortage of research to be done in this area.

10. Conclusions

Recently, printability has been introduced to the field of bioprinting as both a new term and research topic. Many aspects of printability have been identified during this time. Evaluation methodologies have been developed for many of these aspects resulting in outcome measurements for quantifying printability. In turn, these outcome measurements have been used to examine the impact printing conditions and bioink material properties can have on printability. This review has presented a discussion on the topic of printability in an attempt to reduce ambiguity in the term’s usage, identify key areas where further research is needed, promote quantitative printability evaluations of bioinks, and aide researchers in the bioink development process.

Acknowledgments

This study was supported by National Institutes of Health (1P41EB023833-346 01).

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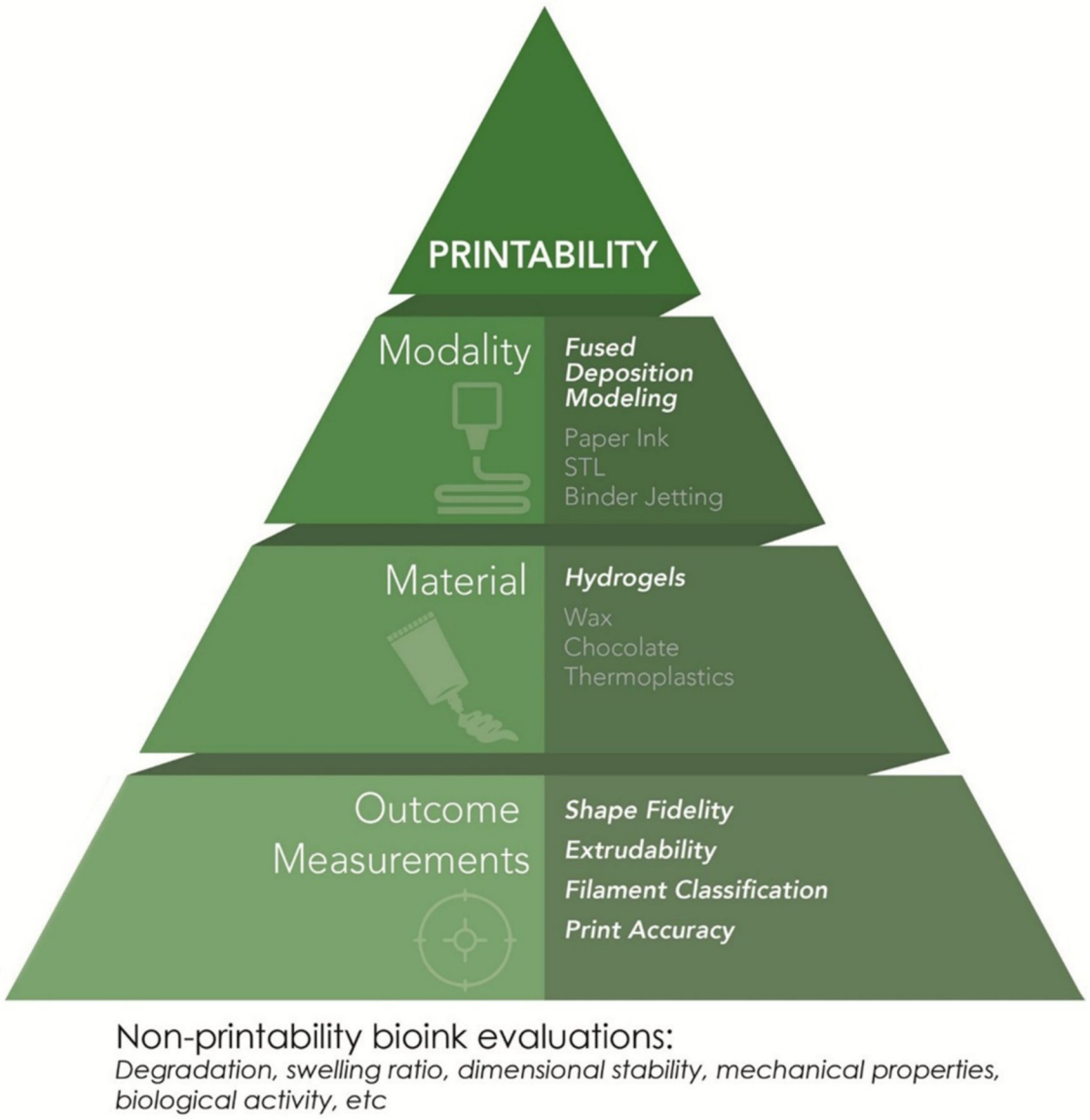

Figure 1.

Printability can have many different definitions depending on the modality and material being used. Even for a set definition within a modality and material, some aspects of printability may be more important than others depending on the application.

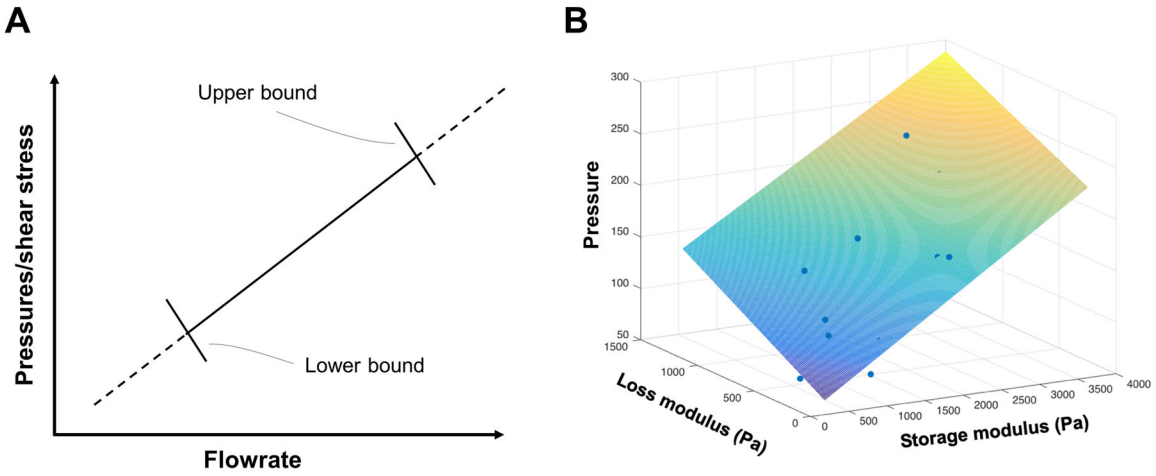

Figure 2.

Extrudability. (A) Extrudability during the printing process is an essential characteristic of any bioink. Bioinks must both be extruded above a minimum flow rate to achieve a reasonable print time and under a maximum pressure/shear stress to achieve reasonable cell viability. (B) Extrudability in Gao et al.[53] was measured as the pressure required to extrude a given amount of bioink per construct and related to the storage and loss moduli of that bioink.

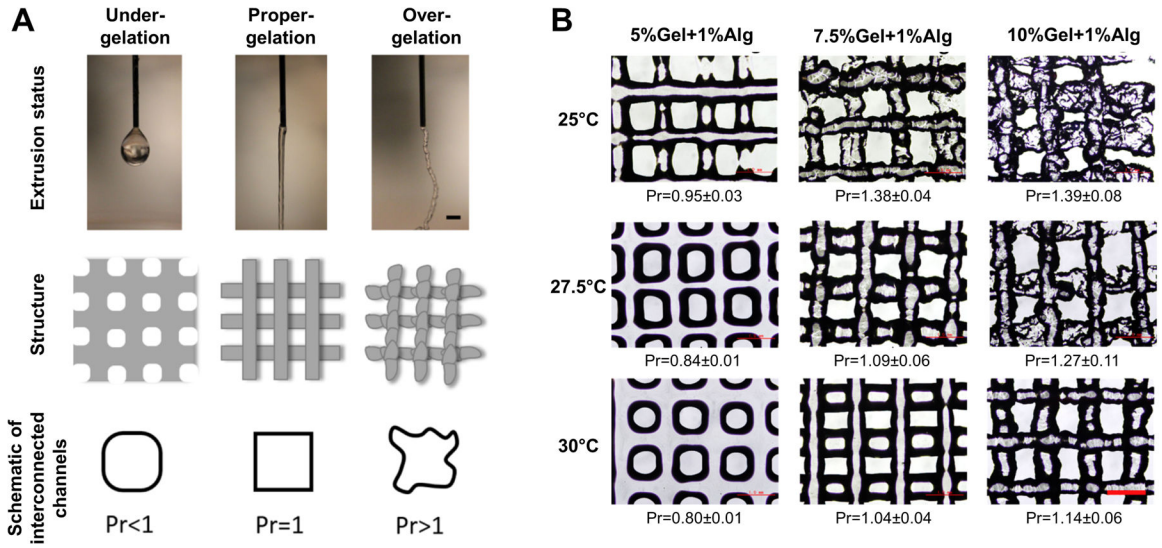

Figure 3.

Filament classification from Ouyang et al.[27] demonstrating the (A) filament drop test used to prescreen bioinks and (B) quantitative evaluation of different types of filaments.

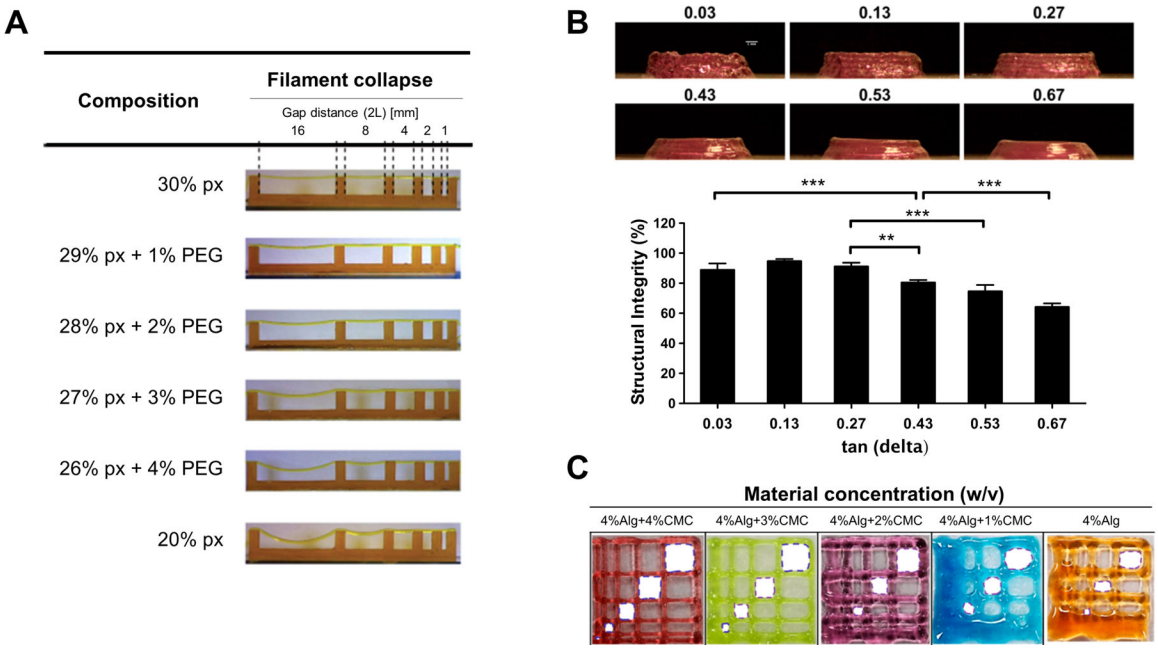

Figure 4.

Shape fidelity has been measured in various ways including the bioink's ability to (A) span gaps unsupported to create vertical pores,[84] (B) maintain its height by stacking multiple layers,[53] and (C) avoid filament spreading to create horizontal pores.[41]

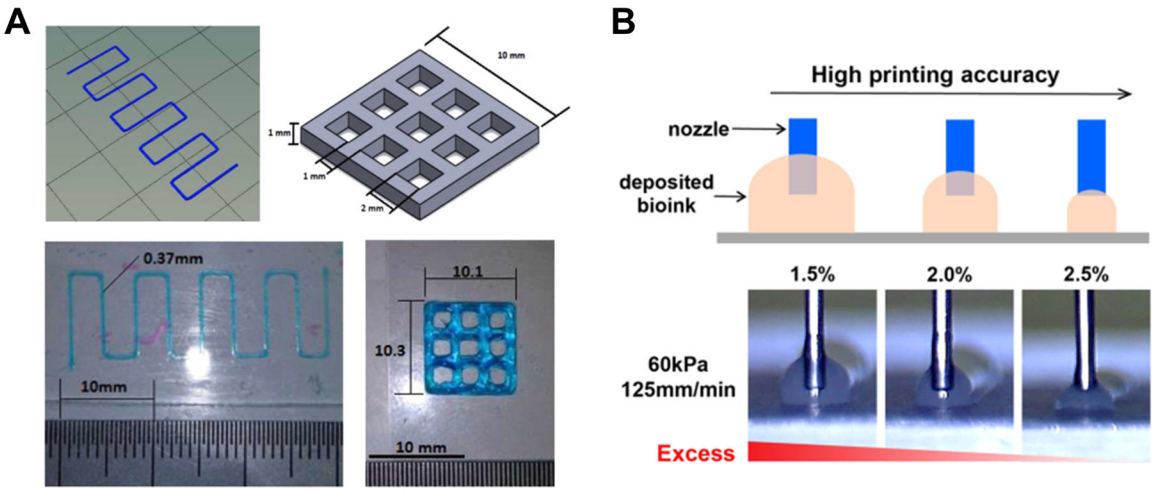

Figure 5.

Printing accuracy typically refers to how similarly a printed construct resembles the intended design. This has been measured in many ways such as by quantifying (A) various feature dimensions[57] and (B) material deposition.[75]

Table 1.

Representative printability measurements

Extrudability			
Type	**Measurement**	**Limitation/ Condition**	**Citation(s)**
Binary Classification	Bioinks “unprintable” if flow could not be achieved at a maximum acceptable pressure	Nozzle detachment	42
	Bioinks “unextrudable” if flow could not be achieved at a maximum acceptable pressure	380 kPa	54
	Bioinks “unprintable” if a flowrate of 0.3 mL/hr could not be achieved by their system	Unspecified	55
Presssure under Given Conditions	Pressure generated by plunger displacement at a constant speed	0.2 mm/s plunger speed	52
	Pressure required to achieve a given amount of material deposition per construct	100 mg construct	53
	Pressure required to achieve a minimum acceptable flowrate (lower bound) & pressure required to achieve a maximum acceptable flowrate (upper bound)	Varied with nozzle size	26
	Minimum pressure required to achieve consistent flow	Unspecified	57
Theoretical shear stress	Proportion of nozzle diameter to extrusion pressure	n/a	56
Filament Classification			
Type	**Classifications/Measurements**		**Citation(s)**
Submerged	Swelling, equivalent diameter, stretched, rough surface, over-deposited, compressed, discontinuous		59
Filament Drop Test	Droplet, filament		37
	Droplet, smooth filament, over-gelled filament		27
Qualitative Deposition	Straight, curvy		41
	Regular, irregular		54,55
Quantitative Deposition	Uniformity ratio (filament perimeter normalized by length)		53
	Pr (pore perimeter normalized by pore area)		27
	Standard deviation of filament heights and widths		60
Shape Fidelity			
Type	**Measurement**		**Citation(s)**
Qualitative	Cross hatch, anatomical shapes		23,28,39,41,58,59,61–77
Filament Spreading (single layer)	Filament width		38,40,42,52,55,57,58,60,75,78–82
	Filament height		55,60,75,81
	Spreading ratio (filament width divided by nozzle diameter)		79
	Aspect ratio (filament height divided by width)		24
	Pr (pore perimeter normalized by pore area)		27
Height Maintenance	Critical height (maximum acheivable height)		24
	Height of cylindrical structure		36
	Height of 5-layer tubular structure		53
Filament Collapse	Angle of deflection of unsupported filament		84

	Pore area below an unsupported filament		41
Printing Accuracy			
Type	**Measurement**		**Citation(s)**
Filament Dimensions	Observation of broken filaments		39,40,42,70,78
	Filament width		38,41,42,54–58,60,75,76,81,85,86
	Filament height		55,60,75,81
	micro-CT		34,92
Pore Dimensions	Pore area		12,41,57,76,82,87–91
Filament Merging	Minimum distance required between filaments without merging		93
	Overlap distance of a filament printed at an acute angle		76
	Length of fused segment between adjacent filaments with increasing distance between filaments		84
Other			
Type	**Measurement**		**Citation(s)**
Construct Size	Weight of construct		53
Cell Mixing	Whether cells could be mixed with pipette		61
Homogeneity	Variability of extrusion force over time		52,72