The Current State of 3D Bioprinting and Embedded 3D Bioprinting (EMB3D) as Novel Methodology for Tissue Engineering

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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The Current State of 3D Bioprinting and Embedded 3D Bioprinting (EMB3D) as Novel Methodology for Tissue Engineering

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Abstract:

3D Bioprinting is an emerging field within tissue engineering. With four major techniques, extrusion, inkjet, stereolithography, and laser-based transfer, and a wide variety of different compatible materials the field has grown considerably in recent years and shows the potential to continue expanding. This review presents the major advantages and disadvantages of the major techniques and provides a broad overview of the most common and promising biomaterials for tissue engineering. A novel approach called Embedded 3D Bioprinting (EMB3D) is also explored as a promising alternative to conventional methods. Preliminary results show great cell viability for bacteria printed as an embedded structure. Results suggest that EMB3D is a viable alternative to increase cell viability, proliferation, and tissue functionality in a well-defined and ordered tissue construct.

Keywords: Embedded 3D Bioprinting (EMB3D), Carbomer, Carbopol, Review, Techniques, Materials

Introduction:

Bioprinting is a rapidly growing and changing field in medicine as it has wide reaching clinical and commercial value for both *in vitro* and *in vivo* methods.[1,2] The field has the potential to revolutionize the medical industry with the abilities to fabricate new tissues and organs, customize prosthetics and implants, and provide alternative anatomical models for drug delivery and discovery.[3] It is projected that the bioprinting industry and the medical devices associated with it will be a multi-billion dollar industry in the near future.[4,5]

Currently a large portion of *in vitro* biological study is done on two-dimensional (2D) cell cultures, but 2D cultures are limited in that they do not necessarily mimic how tissues would respond to stimulus in a three-dimensional (3D) *in vivo* environment.[6] Live organs and tissues have very well-defined and ordered cellular structures that are not simulated completely in 2D structure. This lack of proper tissue mimics and models derived from them has slowed drug discovery and modeling of diseases. Often times proposed drugs show promise in a 2D plate, but upon clinical testing they fail. The same can be seen with cancer models, as what we have learned from 2D cancer research does not align with what we see in real tissues. As such, there is a growing niche for viable 3D tissue constructs that can properly integrate and model the cell-cell interactions and overarching hierarchy of real tissue.[6–8]

One of the main challenges to reproducibility and viability of bioprinted tissues comes from the need to create a highly ordered structure, that can provide structural support while also creating an environment where cells can proliferate and migrate as they would *in vivo*.[9,10] This is solved, to varying degrees of success, by printing cells embedded in a biocompatible gel, called a bioink, on to a base material, that is either a natural or synthetic structure or some form of decellularized matrix.[11,12] Once the cells have grown and developed as they are intended, another major problem is finding a way to remove the initial base matrix. Some hydrogels are degradable over time or can be digested by the body in an *in vivo* environment, yet these may leave behind byproducts.[13]

There are several different types and methods of bioprinting and each of these methods show promise, but they all have their own advantages and disadvantages. Some methods can provide high resolution and accurate positioning, but are limited in their structural integrity. Others are very useful in maintaining vertical configuration and creating transport networks, but have very low cell viabilities due to the stress of the printing procedure. Combining multiple different approaches and optimizing for different cell types and applications has shown promise, but no single technique is optimal as the most feasible, reproducible, and viable printing procedure.[1,2,5] Through a thorough review of the major bioprinting techniques and common biomaterials used in bioprinting as well as an analysis of the developing methodology of embedded bioprinting, this paper will provide an overview of the current status of the field and a novel direction that it could take.

Results:

Extrusion Printing

Extrusion-based bioprinting is an additive technique in which filaments of bioink are deposited in specific patterns as they are passed through a printing nozzle.[14,15] As shown schematically in Figure 1, biomaterials are loaded into a syringe with a fine needle and forced out pneumatically, with piston or by screw. It is very similar to conventional fused-deposition modeling (FDM), which is the most common and recognizable form of 3D-printing in all fields. Many groups have used this similarity to their advantage as they perform this technique by first obtaining a commercially available FDM printer and modifying it with syringes for bioprinting.[16–19] As the demand has grown, a field dedicated to extruders designed specifically for bioprinting has also sprung up. It is because of this commonality, ease of use, and relatively low start-up cost that extrusion-based bioprinting has become very common in recent years. To

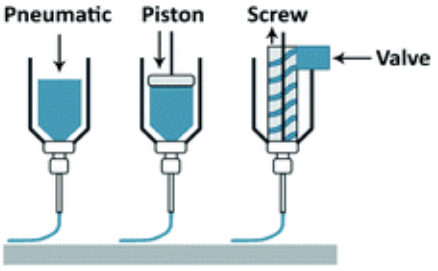


Figure 1: *Extrusion Printing, sourced from*

this point extrusion-based bioprinting has been used to create constructs for skin[20], cartilage[21], liver[22,23], bone[24,25], and vasculature[26,27] tissues among many others.

Coupled with its low cost and simplicity, extrusion bioprinting can create large structures with multiple materials built into one construct.[14,28] Typically built into the system are controls for print speed and nozzle pressure which allow for a high variability in the types of materials that can be used. A wide range of materials with viscosities from $30 - 6 * 10^7\ mPa * s$[29] are thus viable. This is by far the widest range for any of the techniques. Where extrusion really outpaces other techniques is the ability to print with very high cell densities. Cell spheroids loaded into bioinks before the printing process self-assemble into the desired structure increasing density and potentially mimicking the *in vivo* environment.[30,31]

A major cost of the high variability in printing parameters and low cost is the relatively low speed and limited resolution that these printers can provide. Many printers that have been modified from standard FDM printers and even high-end commercial bioprinters do not possess resolutions better than ~100 μm.[15,32] Printing rapidly through a nozzle as is performed with extrusion techniques may also present dangerous amounts of shear stress on the cell-laden gel. It has been shown that this high pressure and shear has noticeably negative effects on the viability of cells after the print.[33] This highly variable effect from the nozzle parameters is a major reason that extrusion-based techniques have a lower and much larger range of cell viabilities (40 – 85%) reported in the literature.[29,34–37]

Ink-Jet Printing

Ink-Jet printing is another fairly common, low cost method of printing that has emerged over recent years.[29,34,36] As shown in Figure 2, instead of physically being forced out of a nozzle, bioink is loaded and rapidly separated and deposited in uniform droplets with diameters lower than 30μm[38] typically by either thermal or piezoelectric methods. Thermal ink-jet printers rely on a mechanism that heats up and generates air bubbles at a controlled frequency. When these bubbles pop, they pulse the ink releasing a controlled amount of substance. Varying the frequency of bubble generation can be used to create different sized bubbles that can be used to control the size of the released ink.[39,40] Piezoelectric printers rely on a polycrystalline ceramic that pressurizes the ink when an electrical signal is applied to it. Similar to thermal printers, a longer signal duration is associated with larger droplet deposition. [41,42]

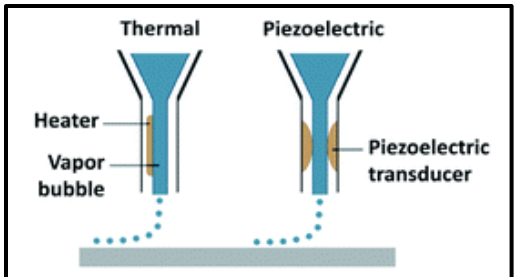


Figure 2: *Ink-Jet Printing, sourced from Gungor-*

With thermal and piezoelectric firing frequencies on the order of 5 – 10kHz[43] these printers can eject a large amount of droplets in a fraction of second, making this one of the fastest printing techniques available. Notably, the high speed has allowed ink-jet printers to be used *in situ* at certain lesions as a means of regenerating both skin[44] and cartilage[45]. As mentioned above, droplets can get smaller than 30μm in diameter, meaning ink-jet printers can typically have very good resolution. Many ink-jet printers can get to resolutions as fine as 50μm.[15] Ink-Jet printers are also very capable of using very low viscosity inks during the printing process, most reporting lower than $10\ mPa * s$.[29,34,36,43] The high temperatures and potentially high pressure are brief, so despite being essential to droplet formation, it has not been shown that there are any adverse effects on cell viability after the print.[46,47] Of the four general methods, ink-jet ranks fairly high with cell viabilities from 85 – 95%.[40,48]

High speed and resolution and good viability comes with certain disadvantages however. To prevent clogging and ensure proper droplet formation, inks must have very low viscosity and generally are liquids.[36,43] This severely limits the potential inks that can be used with this approach and thus limits the viability of certain applications. The need to prevent clogging and to lower overall shear stress also generally requires very low cell density in the initial solution, typically no more than a few million cells per milliliter.[29,46,48] The rapid release of small droplets allows for high speed and resolution, but by being individual droplets it is never really one full construct. Initial barriers between the droplets must be overcome by cells as it is not one continuous strand like extrusion-based methods.

Stereolithography

Stereolithography (SLA) is a layer-by-layer method that utilizes certain photosensitive polymers to build scaffolds from pre-determined splices.[9,49] The process, shown in Figure 3, is actually fairly simple, as the main requirement is a UV or visible light source. The whole technique works by programming the light source to activate photosensitive polymers in a sequence that allows each layer to crosslink in an additive manner. Changing the intensity of the light and duration of exposure can be used to adjust how the polymers develop.[50] Typically, the scaffold is grown on a mobile platform that can descend within a large vat of the photosensitive polymer ink.

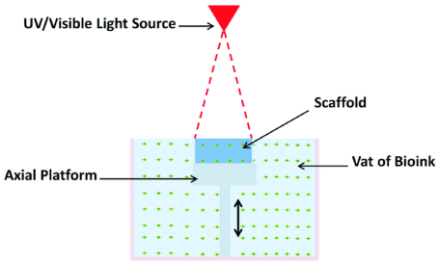


Figure 3: *Stereolithography, sourced from*

Where both extrusion and inkjet printing are limited by the necessity in using a tight nozzle for release of ink, stereolithography has the advantage of being independent of nozzle system. This means that the potentially dangerous shear stresses cells are exposed to in these processes are non-existent. As it is not reliant on the use of a translating printhead and nozzle the technique is also faster than extrusion methods. Another great advantage of printing without a nozzle is there is no longer a limit on the viscosity of the inks used. Since nothing must be extruded or passed through anything the viscosity of the material is not of great importance.[51,52] While it does require an external light source, the technique as a whole is fairly cheap as there is an abundance of photosensitive polymers and photoinitiators that can be interfaced with cells.[37,49] Cells printed in this manner are also very viable, with researchers boasting viability greater than 90%.[53]

While stereolithography generally has great cell viability, cells are at a disadvantage as some polymers require extended exposure to UV light. UV light is known to damage cells and specifically DNA.[54,55] While not always prevalent this can limit viability and damage certain cell types. Stereolithography is generally very accurate and quick, but the use of an unfocused light source limits the resolution of the final printed product. Values around 100µm, similar to extrusion printing, are typically reported.[15,53,56] Cell density is much better than with ink-jet printing, but stereolithography can not be used with cell densities as high as extrusion printers. If high cell densities are a requirement, then extrusion based printing remains the better choice.[29,34]

Laser-Assisted Printing

Laser-assisted printing comes in several different varieties, but the most common devices rely on a high energy laser, a thin metallic absorbing layer, typically gold or silver, and a thin bioink on its underside like in Figure 4. First introduced in the 1980s it was initially used as a means of writing on a metal substrate using a metal film.[57] The laser pulse heats up the metallic surface, which then heats up the bioink layer. As the ink heats up, it will bubble and depending on the viscosity, angle, and radius of the bubble, will eject forward and down towards the substrate or print surface.[58,59] Wavelength of the laser, duration of the pulse, divergence in the beam, and adjustments to focusing mirrors have major effects on the overall resolution of the print and the ability for the device to be used in a high throughput manner.

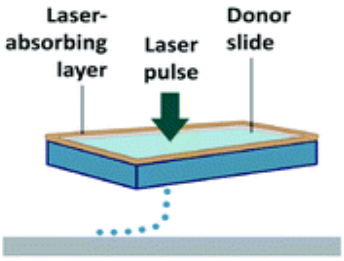


Figure 4: *Laser-Assisted, sourced from Gungor-*

One of the greatest advantages of laser-assisted printing, is the incredibly high level of resolution it can provide. In the literature, values lower than 10µm have been reported.[29,60,61] The focused nature of the laser pulse and the interaction with the ink that results is very accurate and allows for resolution on the scales of single mammalian cells. On top of having a fairly high resolution, laser-based printing methods can typically function with fairly high cell densities, some reporting more than 1×10^8 cells/ml, without

losing any viability. Like stereolithography, laser-based printing does not rely on a nozzle, so it is not limited by shear stresses inherent to a nozzle and will not be slowed by clogs. At a laser firing speed 5kHz, high-end devices can rapidly deposit droplets.[52,59,61,62]
Laser-based printing techniques, while showing many advantages are by far the most expensive of the techniques discussed. The reliance on a laser, with various mirrors and focusing implements, and the need to use a metallic absorbing surface drives the price up.[29,34,62] The reliance on heated metal which interacts with the bubbling ink has also been shown to lead to detrimental metal particles ending up in a tissue engineered product.[63,64] The potential for metallic particles and brief laser-ink interactions contribute to cell viability that is consistently reported lower than 85%.[34,65] In order for proper bubbling and interaction with the metallic absorbing surface, the inks are also limited to viscosities in the range of 1 – 300 mPa*s.[62,65,66]
A summary of the major facets of these four techniques is provided below in Table 1.

TABLE 1: Techniques	*Extrusion*	*Ink-Jet*	*Stereolithography*	*Laser-Assisted*
Advantages	Simple, wide range of applications and gels and cells	High speed and resolution at low cost	Nozzle printing is unnecessary, high cell viability	High resolution, high throughput, fairly high cell density
Disadvantages	Slow and limited resolution, high shear damages cell-laden gel	Must use low viscosity ink, very low cell density	Limited to photosensitive polymers, UV damage is possible	Expensive, potential damage from laser, limited viscosity range
Speed	Slow	**Fast**	Average	**Fast**
Cost	**Low**	**Low**	**Low**	High
Cell Density	**Very High**	Low	Average	**High**
*Viscosity (mPa*s)*	$\mathbf{30 - 6 * 10^7}$	<10	**Any**	1 - 300
Cell Viability	40 – 85%	80-90%	**>90%**	75-85%

Biomaterials Design

To accompany a wide array of growing and developing techniques, both naturally-derived and synthetic biomaterials are explored for bioprinting. Natural biomaterials are typically favored for their biocompatibility, biodegradability, and non-toxicity, whereas they can be limited by mechanical strength and batch to batch variability.[67] Synthetic biomaterials are typically favored for their mechanical strength, ease of use, and ability to functionalize in many different ways, while they can at times be limited by biocompatibility and the presence of potentially toxic byproducts.[68] In many cases researchers have successfully combined multiple different materials, including mixing natural and synthetic polymers.[69–74]

Natural Materials

Collagen

Collagen is one of the most common proteins in animals and exists in at least 28 different varieties in the human body.[75] It is a major structural protein in the extracellular matrix (ECM) and is typically found in fibrous tissues like skin, tendons, and ligaments, as well as many other structures that

require elastic strength like blood vessels, bone, and cartilage.[76,77] Due to its prevalence and fundamental role in biology it has been heavily researched. As such, on top of being very easy to isolate and purify, it has well documented physical, chemical, and immunological properties.[67] Collagen is also known to be very stable, biocompatible, biodegradable, and without any cytotoxic properties.[78] In the body, collagen is formed from a triple helix of protein chains that combine in long strands. Under tensile stress, this helix will stretch and unwind giving collagen fibers their characteristic strain stiffening tensile strength.[79,80] Hydrogels are typically formed by raising temperature and pH to initiate fibril self-assembly. The temperature has a profound effect on the size of fibrils and overall architecture of the gel.[81,82] This change in architecture can be utilized to alter overall function of the gel as seen in Figure S1. As a characteristic protein of the ECM, collagen has also been shown to play an important role in cell differentiation, adhesion, migration, and proliferation.[83,84] Despite its essential mechanical properties in the body, hydrogels formed by collagen are not very stable and its mechanical properties do not necessarily mimic the ECM.[85–87]

Due to this vital biological role, collagen has been extensively used in studies surrounding cell growth, tissue repair, and wound healing. Specifically, the areas of skin, bone, and cartilage have seen a large amount of research.[88–92] One of the greatest advantages collagen has is that the body can enzymatically target it allowing for rapid and non-toxic degradation.[86,87] Unlike other natural and synthetic polymers, collagen is also readily able to interact positively with cells without any further processing.[86] Its prevalence and importance within the ECM has also made collagen a prime target to be included as a component of scaffolds used for cell seeding and matrix simulation. Recently researchers have used collagen coated decellularized matrices,[93] in 3D models of gut endothelium,[94] as a drug delivery system,[95] and for heart valve structures.[77,96] Collagen has been used as a support structure and as a modified bioink for extrusion[88], ink-jet[91], and laser-assisted prints.[89] On a few occasions, collagen molecules have been modified with photocrosslinkable molecules for applications in stereolithography.[97,98]

Gelatin

Gelatin is formed from the controlled hydrolysis of collagen.[99] In this hydrolyzed form, gelatin can swell in water forming a network structure.[100] Like its parent protein, gelatin is cheap, readily available, very stable, biocompatible, and biodegradable, but with the added benefit of being less antigenic than collagen.[101] A major advantage of gelatin is that it also abundantly forms arginine-glycine-aspartate (RGD) motifs which have been shown to be vital in cell adhesion.[102–104] In its non-crosslinked form gelatin has limited shear elastic modulus and is susceptible to lowered structural support with temperature fluctuations,[99,105] so it is typically crosslinked using a secondary molecule or combined with other biocompatible proteins. Gelatin's cross-linked gel-like structure also allows it to provide adequate structural support as a scaffold while also being porous to allow for blending of other included factors.[74,106,107]

Like collagen, gelatin's interesting structural and biological properties have made it a prime candidate as a potential scaffold to replace to the ECM. It fact it has been shown that ultrafine gelatin fibers have improved cytocompatibility and cell infiltration characteristics that point to it being an adequate replacement for ECM in certain cell studies.[108] Gelatin has been shown to have potential applications in cardiovascular[109], bone[110], skeletal muscle[111], and wound healing.[112] In many cases, researchers have found that gelatin can be combined with other biomaterials for added effect. Gelatin is very easy to modify and in the case of bioprinting is commonly methacrylated to create GelMA.[113,114] The process is shown in Figure S2. GelMA shows great promise for bioprinting as it can be crosslinked with the addition of UV light and the structure more gel-stability at body temperatures.[115] Gelatin is also commonly mixed with alginate as the two show promise when used to create a combined hydrogel.[105,116,117] Again like collagen, gelatin has been used with extrusion[19], ink-jet[118], laser-assisted[119], and stereolithography[37] methods.

Alginate

Alginate is a polymer typically derived from brown algae. It is quickly becoming one of the most popular natural polymers in tissue engineering due its excellent biocompatibility, low toxicity, and low cost.[67,120] Like both collagen and gelatin it is easily processible, with its most typical formation being a hydrogel. Shown in Figure S3, shear thinning alginate gels are very easily formed as the polymer will readily crosslink and swell when exposed to a diprotic cation such as Ca^{+2}, and as such $CaCl_2$ is commonly used as a cheap and effective crosslinking agent.[121] A major advantage inherent to the ion-based crosslinking with alginate is that concentration of both alginate and ion can be used to fine tune certain mechanical and time-dependent properties of the final gel.[85,86,122] The properties of an alginate hydrogel very closely mimic those of the ECM leading alginate-based treatments to be widely used for wound treatment.[123] In addition to its known biocompatibility alginate has been reported to be very durable flexible.[124] Despite its many advantages, alginate is limited by reportedly poor cell adhesion and must be modified with RGD motifs to improve adhesion.[125] It has also been reported that alginate may experience slow degradation *in vivo* depending on quality of material and the amount of oxidation it experiences.[126]

Alginate-based tissue engineering solutions have been documented for bone[127], eye[128], brain[129], ear[130], skin[131], heart[84], cartilage[132], and liver[23] tissues. Bioinks made from alginate have become increasingly common as not only do they show high cell viability, but also good cell mobility, differentiation, and proliferation rates amongst known hydrogel constructs.[120,133] Notably, alginate bioinks have also shown a lot of promise in the delivery of growth factors for vascularization.[134,135] Alginate-based bioinks are very commonly used with extrusion[15] and ink-jet[46,136] based printing methods, but UV crosslinkers have been utilized for stereolithography methods[133,137]

Chitosan

Chitin is a naturally occurring polysaccharide found in the exoskeletons of plants and arthropods. Chitosan, which is easily derived from chitin via exposure to the chitin-deacetylase enzyme, is a commonly used molecule in scaffold preparation.[138] This process is presented in Figure S4. Much of the interest in chitosan is found by those researching bone regrowth.[139,140] Chitosan's main advantage is its inherent antibacterial nature.[141] Coupled with being porous and moldable to many different geometries it is an ideal candidate for cell growth specifically in the area of osteoconduction.[140] It is difficult to further capitalize on this antibacterial property because chitosan forms very viscous liquids so it not necessarily suitable for being a bioink. Typically, chitosan is combined with another molecule like alginate, collagen or hyaluronic acid if it is intended to be a bioink.[69,74,142,143] When used as a bioink, studies have been performed under extrusion and ink-jet methods[92,139], but it is mainly used as a scaffold material.

Hyaluronic Acid

Hyaluronan is an abundant polysaccharide found in the ECM of many different tissues. Hyaluronic acid is a molecule derived from this polysaccharide and is typically sourced from connective tissues and certain bodily fluids.[67,83] Hyaluronic acid is very soluble and has easily accessible functional groups which make it ideal for chemical modification. Similar to Gel-MA, hyaluronic acid is commonly methacrylated for photocrosslinking, increased mechanical stiffness, and improved long-term stability.[144] Methacrylated hyaluronic acid has been shown to be subject to radical polymerization, so other groups will modify hyaluronic acid with thiol-norbornene which allows for more controlled photocrosslinking.[145] The easy modification and wide range of chemical activity of hyaluronic acid can also be a disadvantage however. Hyaluronic acid interacts with many different cell types and protein pathways so it is possible to generate a negative pathological response if one of these pathways is triggered.[86,87]

Synthetic Materials

Polyethylene oxide

Polyethylene oxide (PEO), also commonly known as polyethylene glycol (PEG) is a commonly used polymer across the field of biomedical engineering. It is known to have very good biocompatibility and low toxicity.[146,147] Two of the major advantages of using PEO based systems are that the polymer is very hydrophilic, which allows for easy diffusion and gas exchange, and its inherent non-immunogenicity. PEO shows very little protein absorption *in vivo* and is generally inert making it a prime target for bioinks, scaffolds, and drug delivery systems.[34,86] Like many other synthetic polymers, PEO has easily accessible functionable groups that allow for a large amount of further functionalization to allow for tailor-made mechanical and chemical properties.[86,148] PEO's main hydroxyl groups have been modified with acrylate, amine, azide, carboxyl, thiol, and many other groups.[149] PEO is so easily modified that it is often referred to as a "blank slate" material. PEO's versatility allows it to be incorporated into bioinks where PEO is the main backbone[150], or even where PEO is used to crosslink other polymers.[45]

While PEO's basic inertness is appreciated in many tissue engineering and drug delivery applications because it typically allows the polymer to go unnoticed, this can also be a severe disadvantage for bioprinting where often the goal is to encourage cell interactions.[146] Due to this, it is almost always essential to add in an RGD sequence to the PEO chain to promote cell adhesion. However, this can limit further functionalization.[151] Pure PEO hydrogels have very low viscosity and are closer to liquid solutions than gels. A low viscosity lowers the viability of PEO bioinks for extrusion-based printing unless they are combined with other polymers of interest.[152]

Pluronic acid

Pluronic acid is a triblock copolymer composed of the hydrophobic polypropylene oxide (PPO) sandwiched between two PEO domains.[146,153] Pluronic is a thermo sensitive polymer with reversible gelation properties.[154] This means that at low temperatures pluronic is stable in its liquid structure and as temperature increases it swells to form a gel. This is a great advantage for pluronic as cells and other materials can be incorporated and homogenized at low temperatures, then as temperatures rise towards body temperature, it can form a viable gel construct. The gelation properties are dependent on the temperature and chain lengths of each of the domains.[155–157] Typically, shear-thinning gels can form at body temperature at ratios above 25% weight/volume.[153] Pluronic has shown to have very good resolution in 3D-printed structures with researchers reporting strut sizes about equal in size to the diameter of the printing nozzle.[158,159] Further photocrosslinking of pluronic acid chains has been shown to reduce toxicity as cells are prevented from internalizing the structure.[158]

Interesting thermoresponsive qualities and great resolution come at the cost of poor mechanical stability and cell support. [158,160] Pluronic lacks any cell affinity domains, so it is not known to effectively mediate cell adhesion.[146] Inadequately crosslinked pluronic structures do not have the strength to hold cells and will collapse. These non-crosslinked structures show very poor cell viabilities as researchers have reported nearly 90% cell death by the end of day 3 of culture after printing. Even photocrosslinked pluronic structures struggle with viability as these struggle to keep cell death below 40% after printing.[155,159] This is much worse than other hydrogel structures which can boast upwards of 80% viability.

Polyacrylamide

Polyacrylamide (PA) is a synthetic polymer that has shown a lot of promise and growth recently due to its many derivatives and good biocompatibility. Similar to collagen, it has a long history of use and very simple structure, so it is well understood and has been heavily studied.[161] It typically is formed from the chemical crosslinking of acrylamide monomer with bisacrylamide crosslinker. However, changes to monomers and the crosslinker can change properties like hydrophilicity and permeability of the gel, making PA gels heavily modifiable.[86,162,163] Because these gels have been so heavily studied, there are very specific and tunable procedures for generating hydrogels with desired properties.[164] This is

advantageous over other polymers, especially natural polymers, because it can be much easier to fine tune gel stiffness, protein affinity, and permeability. With greater control over the gel's properties, studies can be much more conclusive and clearer about what cellular mechanisms are actually at work. However, PA gels cannot be used in 3D due to the toxicity of acrylamide precursors.[86,165,166] This limitation on 3D culture, typically eliminates PA from being a viable bioink on its own, but it has been used in conjunction with other bioinks and scaffolds.[34,146,167]

Polycaprolactone

Polycaprolactone (PCL) is a semi-crystalline biodegradable polyester. At physiological temperatures, PCL forms a rubbery structure in its semicrystalline state leading to high toughness and great mechanical properties.[168] PCL degrades very slowly and can last up to 2 to 3 years in physiological conditions making it great for things like medical sutures, but eliminates it from technologies like *in situ* injection and typically means it is limited to being a scaffold material.[169] High strength and toughness couple with low toxicity and tissue compatibility has made PCL a strong target for bone regeneration based therapies. PCL has served as a scaffold and mixed with other polymers like hyaluronic acid and collagen, as well as hydroxyapatite and has shown improved cellular response and osteoconduction.[170,171] PCL has also been successfully integrated with alginate to show improved cellular response and regrowth in cartilage tissue.[172]

Embedded 3D Bioprinting as a Novel Approach

Innovation

From the discussion thus far, it is evident that there are a multitude of different combinations of techniques, biomaterials, and cell types that show promise in the field of bioprinting. However, one of the main barriers to continuing success is the reported difficulty in reliably building environments that provide adequate ordered and structural support while also stimulating the same levels of proliferation and migration that would be seen *in vivo.*[2,10,15,34,86,146] To date, attempts to solve this issue have been made, to varying degrees of success, by printing cells on to a base material, such as a natural or synthetic bioink or a decellularized matrix. Once the cells have grown and developed as they are intended, another major barrier has been finding a way to remove the initial base matrix. Some hydrogels are degradable over time or can be digested by the body in an *in vivo* environment, yet these typically leave behind byproducts.[11,13]

The proposed methodology for Embedded 3D Bioprinting (EMB3D) aims to solve these issues. Shown schematically in Figure 5, EMB3D is a modified version of extrusion based bioprinting where its main difference from is that instead of depositing material *on* the surface of a matrix, bioink is deposited *within* a matrix hydrogel in specific orientations. By depositing inside the hydrogel instead of on top, the hydrogel can be used to provide support and structural orientation for cells while also limiting the amount

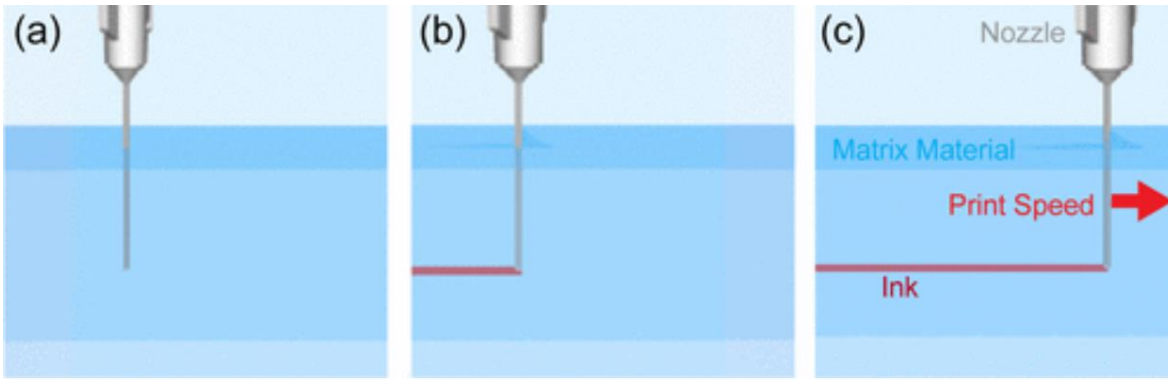


Figure 5: *Schematic of Embedded 3D Bioprinting (EMB3D)*. Instead of depositing material on the surface of a base layer or matrix, the print nozzle sticks inside and moves within the base layer. Material is deposited in the interior, as the nozzle moves. The fluidity and

of shear stress that cells would be subjected to from layered printing. Previously, lower layers of cells would have to hold up the next layer during the printing process which has limited cell viability after printing is completed. Recently, promising results from Lewis and Stone following the use of EMB3D have made it a new avenue for further development of tissue mimics.[173–176]
Alone, EMB3D is very promising, but it can become even more interesting when it is combined with a viscoelastic hydrogel that can be removed easily following printing. This hydrogel is called a "sacrificial hydrogel." Sacrificial hydrogels provide the same structural support and advantages of any other hydrogel, but when they are subjected to a specific stimulus or wash, they lose their cross-linkage and return to their liquid forms. They are used in a wide variety of applications, some as the base for cosmetics and others as a means of creating tubing for vascularization in other polymer gels.[177–179] A sacrificial hydrogel could be incredibly important when used with EMB3D as once the hydrogel has served its purpose of providing structure and orientation control, the gel can be removed leaving only the printed material unaltered.

Filler Fluid Methods

Printing inside the matrix material provides unique challenges and mechanical requirements for support gel and bioink alike. Notably, as the printhead passes through the support structure it will leave voids and fissures that can disrupt the integrity of the gel or cause unwanted motion of printed materials. To combat this issue, some groups have used a system consisting of an ink, a support bath, and a filler fluid.[153,180] As the nozzle deposits ink and translates through the support bath, the filler fluid, which is on top of the support bath slides in behind the nozzle and fills in the gaps. Figure S5 provides visual context. Pluronic F127 has been successfully used in this capacity to create 3D microvasculature.[153] Pluronic's PEO-PPO-PEO structure naturally forms micelles with known diameter between 20 and 80nm that under standard temperature conditions will hydrate to form a shear-thinning gel of optimal qualities for a bioink.[181] When lowered below standard conditions, the gel liquifies allowing for removal. To create their support gel, Pluronic F127 was modified with terminal diacrylate groups to allow for chemical crosslinking after printing. For the filler fluid, acrylate-modified Pluronic F127 was chosen because of its near liquid-like properties that allow the nozzle to pass through unperturbed and for its chemical similarity to the rest of the support gel. After printing, the fluid that filled in the gaps can be photocured with the UV light. With similar methodology, EcoFlex 00-30 has been used to build embedded strain sensors.[180] EcoFlex is a silicone based elastomer that can be modified to the obtain optimal rheological properties for the support gel and the filler fluid using, Thivex and Silicone Thinner respectively.

While filler fluid methods have capitalized on the abundance of research into photocurable support matrices and have the advantage of high resolution, they are severely disadvantaged by the fact that the nozzle cannot safely revisit locations it has already passed through. If the nozzle were to pass through its own path multiple times, it could potentially disrupt un-cured filler fluid and cause further damage to printed structures. While exposure to UV light is only required for a few minutes during the photocuring phase, the process is further disadvantaged for biological prints as limiting the extent of UV light contacting cells is ideal.

Granular and Jammed Microgel Materials

An approach that eliminates the need for a filler fluid involves the use of shear-thinning, non-thixotropic, and microgel forming materials. EMB3D has also been studied using intriguing soft granular media.[176,182–185] Non-thixotropic gel materials have very specific properties. The matrix gel must have a sufficiently high shear elastic modulus to support the printed structure while also having a yield stress low enough to allow the printing nozzle to pass through it. It has been shown that both the shear modulus and yield stress of the ink must be around an order of magnitude higher than those of the matrix.[175,180] If the shear modulus of the ink is too large, the ink has a tendency to be dragged along by the nozzle, while if it is too small, the ink has a tendency to bead up and potential break apart. When the yield stress of the matrix is too low, the gel can lose its integrity and printed structures may slide around within it as the nozzle moves through. However, when the matrix yield stress is too large, the nozzle may leave cracks or

fissures in the material as it passes through. With the correct mix of these two properties, the gel has the ability to "self-heal" around printed cells and in the wake of the moving printhead.

Carbomer 940, also known as Carbopol, is mostly composed of polyacrylic acid (PA) specially cross-linked with pentaerythritol to maintain biocompatibility. PA is composed of a carbon backbone with attached carboxyl groups. As seen in the literature, these carboxyl groups exhibit an important pH response.[186,187] When a solution containing Carbomer 940 powder is slowly made more basic through the addition of a strong base like sodium hydroxide, the carboxyl group is ionized. The now negatively charged groups repel each other leading to a transition from a more globular structure to a more rod-like coiled structure as seen in Figure 6. In this conformation, the solution will be noticeably more viscous and exhibits the elastic and yield properties of a non-thixotropic material. Using a pH probe, it was found that 0.1 wt% Carbomer 940 consistently exhibits this gel property between pH values of 6.4 and 8.9.

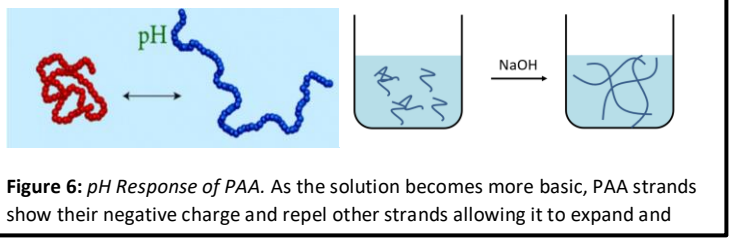


Figure 6: *pH Response of PAA.* As the solution becomes more basic, PAA strands show their negative charge and repel other strands allowing it to expand and

An important factor of Carbomer 940 gels is that this pH-dependent reaction is reversible. As detailed in Figure S6, when the pH is lowered back towards being acidic or the charge interactions of the polymer chains are otherwise influenced, the gel will collapse and lose its mechanical properties.[182,187] This relationship is important because it will allow for the retrieval of printed material from within the hydrogel. This pH dependence can also be a severe disadvantage for Carbomer 940, as it eliminates any possibility of using ionic crosslinkers and any material that can interrupt the charge separation required for the gel to hydrate.

Other materials like, gelatin[188], polystyrene-*block*-ethylene/propylene (SEP) diblock, polystyrene-*block*-ethylene/butylene-*block*-polystyrene (SEBS) triblock[185], and polyethylene-oxide (PEO/PEG)[183] have been used to create packed microgels with similar properties to Carbomer 940. Granular microgels such as these are at a great advantage over filler fluid methodologies because they only require one material for the print and do not need to be reprocess or cured after printing. They also have been successfully and repeatably used to create tissue mimics with great biomimetic properties. Simplicity and ease of use does come with the potential for a wide range of potential resolutions that are varied by the order of microgel size and quality of equipment being used.[176,188,189] Gelatin while promising is also thermo-responsive so it limits the printing of other materials with similar thermo-sensitivity ranges.

Nanoclays

Another recent development surrounding EMB3D is the increased use of nanoclays. Laponite has recently emerged as a potential nanoclay of interest for EMB3D.[190] In its dry form Laponite will clump up, but in an aqueous solution it will form a colloid suspension with well-defined mechanical properties.[191,192] At different grades and concentrations, a Laponite suspension will have known yield stresses and moduli that make it tunable for a variety of applications. Laponite has been shown to function very similarly to microgel materials, as the nozzle can pass through regions of the suspension repeatedly without disturbing the overall stability of the matrix. As the nozzle passes through the clay, it disrupts local charge interactions that allow for material deposition and once the nozzle has move on, the clay rapidly reforms its stable structure. Jin et al has shown that the disruption is only on the order of seconds and the disruption field is only twice the radius of the nozzle.[190] Also shown is that the higher the Laponite concentration, the less sensitive the support and that the size of the disruption region is independent of the nozzle speed. On top of the fantastic properties of the material, when combined with a gelatin-alginate bioink, the structure boasts greater than 90% cell viability. However, this nanoclay comes with the potential disadvantage of having difficulties printing materials with a pH lower than 7. The Laponite suspension has a pH of or above 7, and it is possible that nozzle and gel clogging can occur with inks of more acidic nature.

EMB3D with Gelatin-Alginate

To further demonstrate the potential of EMB3D, an experiment was run in a modified gelatin-alginate structure. As previously discussed, gelatin provides excellent mechanical properties for bioprinting and the combination of gelatin and alginate has been shown to provide excellent cell viability. Thus, a support matrix of gelatin and a bioink made from alginate were selected. Alginate's ionic gelation is heavily dependent on the concentration of Ca^{+2} ion, so typically researchers use a fairly high concentration to ensure rapid creation of spherical hydrogels.[193,194] However, in this case a lower concentration was selected to slow the gel formation and allow channels to form between the droplets. In theory, connecting the droplets will ensure the structure will be one unit and will allow embedded cells to communicate and potentially proliferate between the initial droplets. A calcium concentration of ~6mM has been selected to achieve this goal.

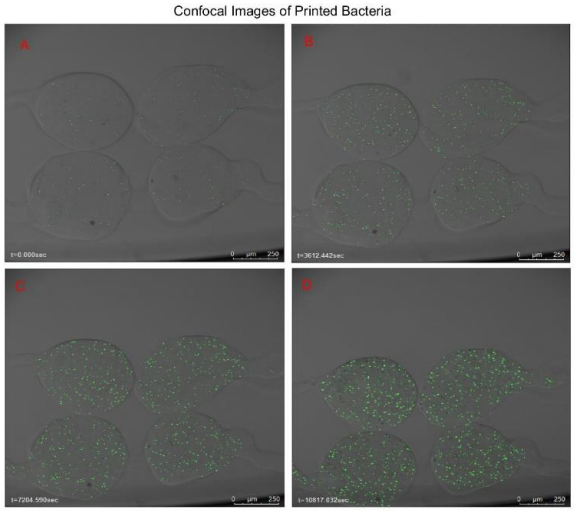


Figure 7: *Printed Bacteria in a 2x2 Structure over 3 Hours.* A) Bacteria at t = 0s. B) Bacteria at t = 1 hr. C) Bacteria at t = 2 hr. D) Bacteria at t = 3hr.Printed bacteria cells not only survive the printing process, but also is able to thrive in its new

To create the proper environment for alginate gel formation, gelatin powder was dissolved in a 6mM $CaCl_2$ solution before vigorous blending. This solution is then heated to 40°C before being allowed to cool overnight in a 4°C refrigerator. Finally, more of the 6mM solution is added in as the gelatin is blended until it is homogenized.

Printed Bacteria

Fluorescently tagged bacteria were combined with an alginate and bacterial media solution and printed inside the gelatin structure. Following the print, a 2x2 segment of the structure, shown in Figure 7, was removed from the gelatin support gel and plated on petri dish. The petri dish was loaded into a confocal microscope and conditions were set to 37°C with a constant CO_2 supply in order to provide an optimal growth environment for bacteria. The culture was allowed to grow over several hours with the results shown above in figure 3. Over just three hours, rapid proliferation and clustering of bacteria cells is apparent. The cells continued to proliferate in this manner until the hydrogel eventually dried out. Fantastic cell growth shows that not only do the cells survive the harsh shear stresses they are subjected to by the nozzle deposition process, but also that the cells can survive and thrive embedded into a matrix. As shown in Figure 8, on top of a general increase in fluorescence, which indicates an increase in the number of cells, there is also an increase in the number of individual clusters which suggests that the bacteria are able to move through their gelatin/alginate bioink even several hours after the printing process.

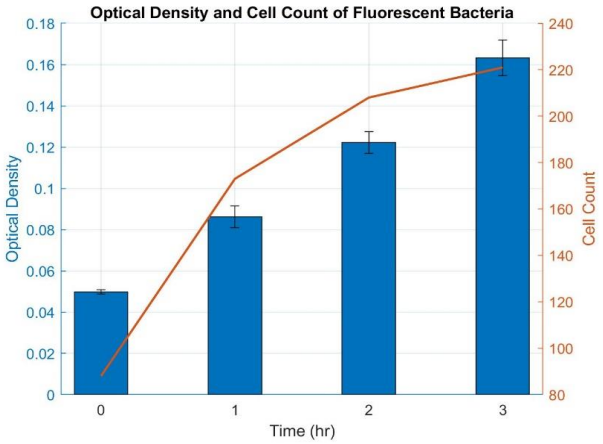


Figure 8: Printed bacteria cells show great viability after EMB3D as both overall fluorescence and cluster count increase for several hours

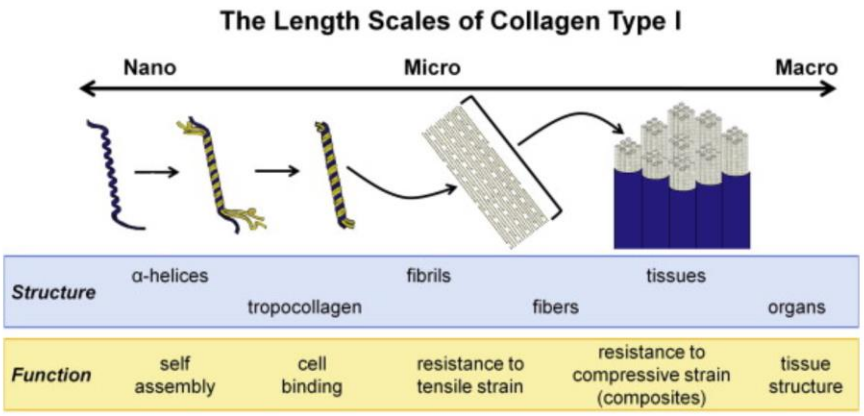
Discussion:

The field of bioprinting sits in a position where not only are there numerous researched options, but also a multitude of new and interesting techniques that could be potentially viable. Decades of research has taken the field from purely theoretical to a world where *in situ* bioprinted tissues are a reality[195,196] and exceeding expectations. While the field has progressed so much, it does face some major barriers. Today, no one technique can combine the necessary viability, density, and motility necessary to reproducibly and effectively print optimally every time. Some techniques can provide high cell density but have limited throughput and low resolution. Others can obtain fantastic resolutions and fast speeds, but risk damage to cells and run at high costs.

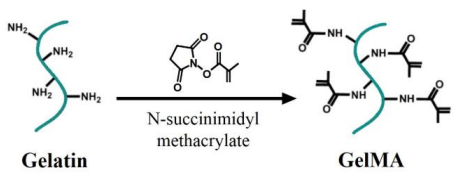
As the field grows these challenges will continue to be addressed by the addition of better printers and more modified materials for bioinks and cell scaffolds. In recent years, an entire industry devoted to developing fast, accurate, and high resolution bioprinters has emerged. Commercially available printers can only serve to improve research in the field as better constructs of multiple cell types can be built at lower costs. Commercial systems also increase the scope of the field as researchers are not forced to build or modify their own printers for bioprinting studies.[197,198] As discussed, there are a wide variety of materials used in the field that are far beyond the scope of those discussed in this paper. Everyday researchers discover new material configurations and build upon previous results to show impressive results within the field of bioprinting. Continued research into modification of bioinks and support structures with factors like RGD[17,103,104] show a lot of promise and will continue to be utilized as a means to improve the viability and tissue growth of cell constructs. Interesting new ways to use materials with well-understood properties evident in other fields like Carbopol[176,182] and Laponite[190,199] also continue to be viable future methods.

New techniques, such as EMB3D, built upon existing knowledge and infrastructure also present an interesting path forward. New methods can help to solve the challenges facing the field without requiring extensive new research. As an example, EMB3D accomplishes the goal of providing optimal structural support, cell viability, and potential for cell motility all in the one monolithic structure. This is a major advantage over conventional methods where highly specific orientation can not be accomplished in a single structure. While it shows promising results, EMB3D is also very limited in the types of materials, crosslinking chemicals, and high-throughput methodologies. As a newer facet of the field, not many materials have been identified as viable. Carbopol is great as it combines low cost with good biocompatibility, but its reliance on charge separation for gelation limits the use of charged species in the prints and during cross-linking. Laponite as a nanoclay shows similar promise, but repeated prints may become increasingly expensive. Nonetheless, more research into intriguing materials and better equipment has the potential to expand the potential of not only EMB3D, but the entire field of bioprinting.

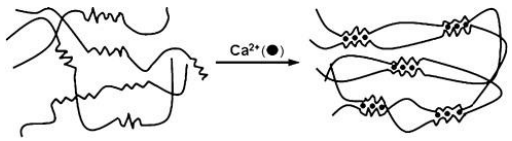
Supplemental



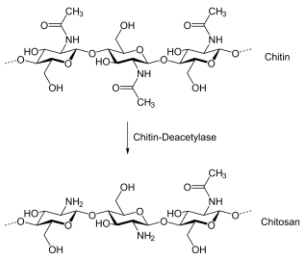
S1: *Architecture of Collagen Matrix*, changing temperature and pH will influence the growth of fibrils into fibers and overall tissues leading to modifications to function. Sourced from Walters et al.



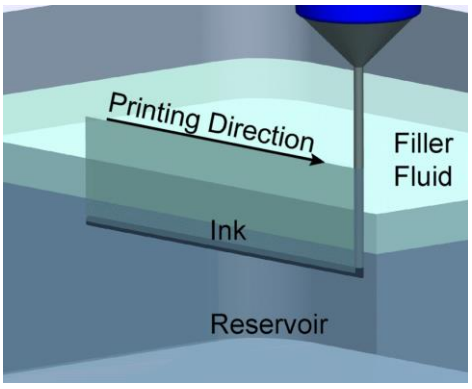
S2: *Formation of GelMA. from Jiashing, Yu et al.*



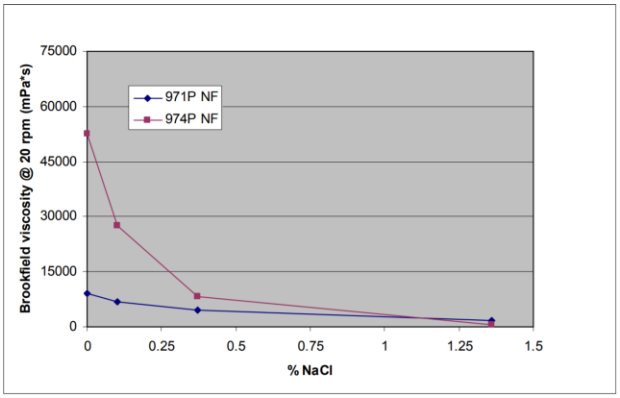
S3: *Alginate Crosslinking. from Yong Lee et al.*



S4: *Formation of Chitosan*



S5: Filler Fluid Methodology. Muth et al



S6: *Viscosity Response of Carbomer 940.* Dilute NaCl solutions completely restore the liquid structure of the solution. From Lubrizol Health

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