

# Chemically tuning dynamic networks and supramolecular assemblies to enable synthetic extracellular matrices for tissue engineering

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# Chapter X

## Valorization

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In this chapter, we discuss the need for bioink development and why 3D bioprinting of soluble bioinks can be a possible solution to defective tissues or organs. Then, we discussed which bioink products are available in the market and how the research done in this thesis could be commercialized.

### **Why bioinks and 3D bioprinting are needed**

Organ and tissue transplantation has been successful in bridging the gap between life and death. Transplantation medicine might be crucial for a healthy life; however, worldwide rising cases of end-stage failure of many organs including the heart, liver, and kidney might hamper this possibility. According to a study, in the US alone, there were approximately 95,000 patients on the waiting list in the year 2006 and over 6300 deaths were recorded of the patients on the waiting list<sup>1</sup>. According to a new survey, 76% of patients failed to receive transplants<sup>2</sup>.

For instance, the incidence of renal failure is between 140 and 160 million per year in the US and Canada, whereas the supply of organs from deceased donors (DCD) is between 20 and 22 per million per year<sup>1</sup>. In the US, the cost of dialysis over four years is three times that of a kidney transplant. Canada spent nearly Can\$100000 per year for Canadians on dialysis with chronic kidney diseases (CKD) and Can\$32 billion per year for patients with CKD but not on dialysis<sup>3</sup>. A European study found that kidney transplantation resulted in a 2 million euro savings for 1000 individuals<sup>1</sup>. Heart failure affects more than 64 million people worldwide which not only resulted in poor quality of life but also adds social and economic burden on the healthcare system<sup>4</sup>. According to a 2013 national survey by the American Heart Association, the direct and indirect expenditures associated with HF in the USA would more than double, from \$20.9 billion in 2012 to \$53.1 billion in 2030<sup>5</sup>. Cirrhosis is the end-stage chronic liver disease (CLD) and is the 11<sup>th</sup> leading cause of death worldwide<sup>6</sup>. More than 160 million people were impacted by CLD in 2017<sup>7</sup>, and around 1.32 million died as a result<sup>6</sup>. Transplantation of the liver remains only an option for end-stage liver diseases and on average 15% of the CLD patients died while awaiting the transplant<sup>8</sup>. Germany experienced a severe organ shortage in 2011; there were 1191 liver transplants performed and 1792 new patients were added to the waiting list<sup>9</sup>. Bone is another organ that can fracture due to sports injuries, traffic accidents, and osteoporosis<sup>10</sup>. Delayed bone healing or non-unions exist when the repair is not complete. Non-unions can occur because of failure of biology, failure of the host (comorbidities and other diseases such as diabetes or vascular disease), and failure of mechanics<sup>11</sup>. Total fragility fractures of 3.3 million are expected in EU6 (France, Germany, Italy, Spain, UK, and Sweden) by 2030 resulting in an annual fracture-related cost of €48 billion<sup>12</sup>. Average direct costs of treatment for long bone non-union have been reported as follows: Canada, \$11,800; the USA \$11,333; the UK £ 29,204<sup>11</sup>. There could also be indirect costs to each patient for example either via loss of earnings or through additional social care. This huge socio-economic cost demands an optimal and innovative solution to diseased tissues and the shortage of organs for benefitting humankind at large.

Tissue engineering offers a potential solution to overcome the challenges of treating defective tissues and organ transplantation<sup>13,14,15</sup>. Tissue engineering is a technique that combines cells,

materials, and biochemical molecules intending to maintain, restore or augment the function of injured tissue or organs<sup>16</sup>. Current challenges of tissue engineering include a lack of biomaterials with appropriate mechanical properties for correct tissue formation and the creation of a three-dimensional (3D) complex architecture of native tissues using biomaterials and cells in the lab. This can be achieved via 3D bioprinting. 3D bioprinting is an additive manufacturing technique that utilized bioink combined with cells and biological growth factors to create 3D complex biological constructs with living cells.

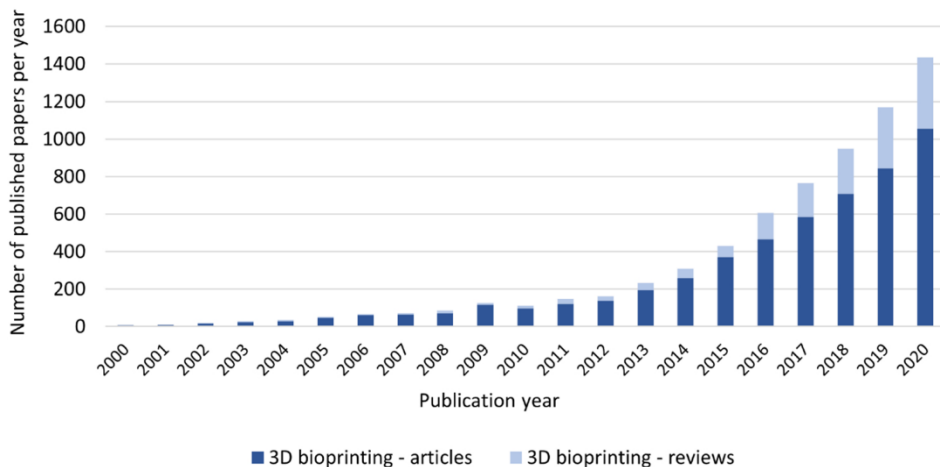
The bioink is defined as a material formulation with biological molecules and cells that can be processed using bioprinting technology<sup>17</sup>. Hydrogels are typical materials that are used as bioinks since they mimic the hydrated environment of ECM and allow tuning of mechanical and rheological properties required for correct tissue formation and 3D bioprinting<sup>17-19</sup>. With the development of new dynamic chemistries, hydrogels with reversible and dynamic bonds have been developed which advances the field of 3D bioprinting<sup>20</sup>. 3D bioprinting has the potential to revolutionize medicine and healthcare by providing on-demand and patient-specific solutions to diseased tissue or replacing artificial organs. However, achieving this milestone requires an immense amount of research for finding bioinks with appropriate mechanical and rheological properties and detailed biochemical analysis of gene and protein formation in bioprinted constructs.

## Research trends in 3D bioprinting:

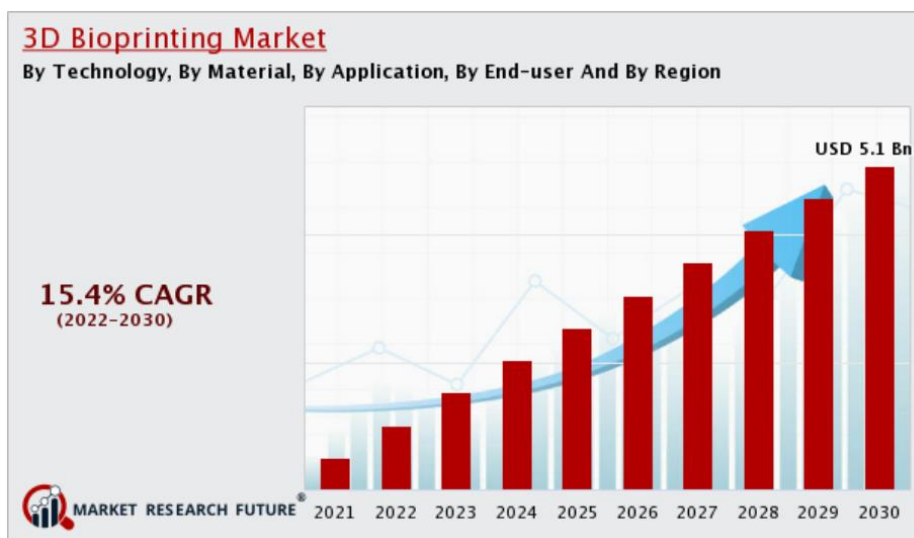
**Figure 1** shows the exponential increase in the number of publications on bioprinting and according to Santoni et al., a total of 9314 science articles were published from 2000 to 2020 which includes 7574 original research articles and 1740 review papers. A total of 79% of published articles were published after 2014. Importantly, 61% (4620 out of 7574) of research articles and 74% (1288 out of 1740) of research reviews have been published since 2016, which indicates exponential growth of research activities on bioprinting<sup>21</sup>.

## Market analysis of 3D bioprinting

The global bioprinting market was estimated to be worth USD 586.13 million in 2019 and is projected to reach USD 1,949.94 million by 2025, representing a compound annual growth rate (CAGR) of 21.91% for the duration between 2020 and 2025.<sup>21</sup> Research and Markets reported that global bioprinting market was USD 1.3 billion in 2022 and is projected to reach USD 3.3 billion by 2027<sup>22</sup>. According to Market Research Future (MRF) report on the 3D bioprinting market by technology, material, application, and end-user, the 3D bioprinting market size value was USD 1.9 billion in 2022 and revenue forecast for 2030 is USD 5.1 billion with compound



**Figure 1.** The graph shows the number of publications on 3D bioprinting. Original research articles are shown in blue and reviews are indicated in light blue color <sup>11</sup>.



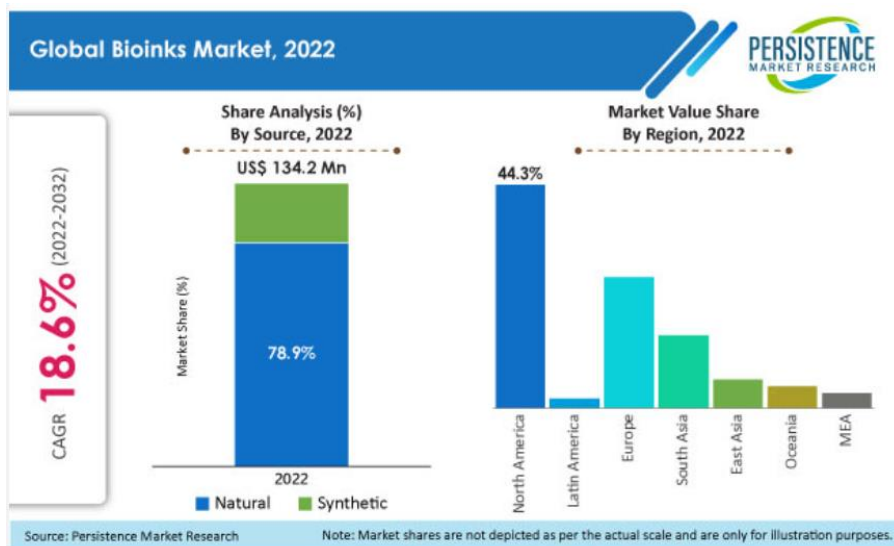
**Figure 2.** The graph displays the 3D bioprinting market forecast by technology, material, application, and ends user. The 3D bioprinting market is forecasted to reach USD 5.1 billion by 2030. The graph has been taken from Market Research Future website (<https://www.marketresearchfuture.com>).

annual growth rate (CAGR) of 15.4%<sup>23</sup> (**Figure 2**). A report of the 3D bioprinting market by component, technology, application, and end-user by Precedence Research indicates that the market was worth USD 1.8 billion in 2021 and is projected to reach USD 8.3 billion by 2030, with a CAGR of 18.51% from 2022 to 2030<sup>24</sup>.

## Market Analysis of bioinks

The global bioinks market generated USD 115.7 million in revenue in 2021, and it is anticipated that this market will grow at a CAGR of 18.6% to reach USD 738.2 million by the end of 2032.

**Figure 3** demonstrates the global bioink market of USD 134.2 million<sup>25</sup>. In 2021, sales of bioinks represented an 8.9% revenue share of the worldwide 3D bioprinting market<sup>25</sup>.



**Figure 3.** The graph demonstrates that the global bioink market in 2022 of USD 134.2 million.

## Existing bioinks

A bioink is a hydrogel polymer. Bioinks can be made from either natural or synthetic polymers. Natural polymers are widely employed due to their natural abundance and excellent biodegradability and biocompatibility. Mainly alginate, collagen, gelatin, hyaluronic acid, and agarose are commonly used natural polymers in bioinks applications. Protein-based materials such as collagen and gelatin contain cell binding sites and other polymers such as hyaluronic acid and alginate can be functionalized with cell binding sites for bioactivity to promote cell attachment, which is desirable for controlling cell spreading and differentiation. The reproducibility of mechanical and biological properties of natural bioink could be challenging due to batch-to-batch variability.

Synthetic bioinks offer the advantage of tunable mechanical and chemical properties. PEG acrylates are purely synthetic bioinks, which have been developed. PEG hydrogels with matrix metalloproteinases (MMP) cleavable crosslinkers have also been developed, which provides mimics biodegradability. Several bioinks have been designed using a combination of natural polymers and synthetic cross-links for better tuning of mechanical and biological properties.

Natural polymers offer the freedom to be modified and cross-linked easily using synthetic cross-links.

In **Table 1**, we have listed existing bioinks with their strengths and weaknesses to provide an overview of different types of bioinks available either in the market or developed in the lab.

Table 2: Bioinks developed, companies selling commercial bioinks, and bioinks strengths and weaknesses.

Bioink	Owned by	Strength	Weakness
Peg-acrylates	Not owned (Sigma, Merck, Advanced BioMatrix, etc.)	Fully synthetic, well-established, cheap(ish)	No stress-relaxation or ability for cells to remodel, low viscosity, high network density, use of UV light
Gel-MA	Not owned (Merck, Sigma, CellInk, Allevi) Ali Khademhussini,	Biodegradable, well-established biocompatibility	Cannot tune biochemistry and mechanical properties independently, low-tunability, uncontrolled degradation, non-degradable kinetic chains, Use of UV-light
Collagen Bioinks	Not owned (CellInk, Advanced BioMatrix, ALLEVI, Humabiologics)	Biocompatible Biomimetic	Largely extracted from an animal source Not reproducible Required pH changes
rhCollagen	CollPlant Holdings	Plant-based technology to produce recombinant human type I collagen (rhCollagen). Biomimetic, similar to collagen I Free of pathogens and no foreign body response Cell binding domains enabled by perfect triple helix enhance cellular attachment Controlled mechanical properties	Costly Difficult to tune the range of mechanics and stress relaxation Expensive
Methacrylated Hyaluronic Acid	Not owned (Allevi, CellInk, Advanced BioMatrix,	Tunable stiffness	No stress relaxation No remodeling by cells Non-specific interactions by cells
Methacrylated Alginate	Advanced BioMatrix	Tunable stiffness Tunable bioactivity No specific interactions with cells	No stress relaxation No remodeling by cells

Alginate-Nanocrystals	CellInk	Established market share, established in multiple cell lines, RGD and laminin conjugated for bioactivity	Not modular, non-degradable, relatively expensive (\$179 for 3mL, one use)
Ghost	Burdick Lab	Good publications and visibility Tunable viscoelasticity by secondary cross-links	No company formed yet. Limited and weak mechanical properties.
Peptide self-assembly	BioGelX	Fibrous structure	Poor mechanical properties, expensive (\$350 for 4 mL, one use)
MMP cleavable PEG systems	Not owned	Tunable initial mechanical properties Tunable degradation of the hydrogel	The local cellular environment is different than the bulk Difficult to control dynamic mechanical properties and stress relaxation Local and bulk degradation
VitroINK, Xeno-free tunable bioink	The Well Bioscience Inc.	No UV, no temperature/pH curing, or chemical crosslinking Tunable bioactivity with either RGD or IKVAV or YIGSR peptides or with Matrix Metalloproteinases (MMP)	Limited tunability of mechanical properties

**Table 1** discussed leading players including Cellink, BiogelX, Allevi, The Well Bioscience Inc, and CollPlant, which focused on developing and commercializing bioinks for 3D biofabrication. Some companies, for example, CollPlant Holdings have aimed to design a universal bioink and commercial product is driving technological advancement. Most bioinks allowed tuning stiffness and introduction of bioactivity. However, tuning of stress relaxation in ECM mimetic fibrous hydrogel is missing. Peptide-based fibrous bioinks have been developed by BioGelX and The Well Bioscience Inc.; however, these hydrogels exhibited poor mechanical properties and no stress relaxation tuning has been shown. We took on the challenge of developing bioinks with tunable stress relaxation and controlled fibrous structure utilizing dynamic covalent chemistry and supramolecular chemistry.

## Bioinks developed in this thesis

Bioinks in this thesis were developed using dynamic covalent and supramolecular synthetic chemistries (**Table 2**).



Table 3: list of bioinks developed in this thesis with their unique advantages and disadvantages

Bioink	Owned by	Strength	Weakness
Alginate with imine type crosslinks (our systems)	Chapter IV of this thesis	Commercially and cheaply available cross-linkers; Mechanical properties tunable; Different cell adhesion molecule presentations are possible	Scale-up would be difficult; Unlimited swelling might be an issue
Supramolecular BTA hydrogels (Our system)	Chapter VII of this thesis	Tunable viscoelasticity and stress relaxation across 5 orders of magnitude; ECM (collagen) mimicking the fibrous structure in synthetic bioink	Longer than 10 days cell culture not possible; Erosion in cell culture media
Supramolecular Norbornene BTA hydrogel (our system)	Chapter VIII of this thesis	Tunable stiffness and stress relaxation; Tunable toughness ECM mimicking the fibrous structure; Longer cell culture is possible; Can be made MMP degradable	Use of UV light for cross-linking

## Applicability of bioinks developed in this thesis

In this thesis research, we described the development of dynamic bioinks based on dynamic covalent and supramolecular chemistry. Owing to the increasing worldwide demand for organ replacement and tissue regeneration, there is a growing need for advanced bioinks for 3D bioprinting for replacing damaged organs or tissues with healthy and functional organs and tissues. Advanced bioinks require good control of mechanical properties post-printing for healthy tissue formation e.g., stiffness, viscoelasticity, and stress relaxation. In addition, rheological properties such as shear-thinning (decrease in viscosity upon application of shear strain) and self-healing (ability to repair broken bonds and achieve mechanical properties similar to before bioprinting) are important during and after 3D bioprinting. Therefore, we took on the challenge of developing bioinks with controlled mechanical and rheological properties.

### *Bioinks using dynamic covalent chemistry*

In **chapter IV**, we developed a series of dynamic covalent hydrogels for their applicability as bioinks. We employed imine-type cross-links with a range of equilibrium constants ( $K_{eq}$ ). Using

imine-type dynamic crosslinkers and oxidized alginate with aldehyde groups, we created alginate-based oxime, semicarbazone, and hydrazone dynamic hydrogels. We demonstrated that storage moduli can be tuned in the range of soft tissues (500–3000 Pa). We showed that all of the hydrogels were extrudable through a 25 G needle; however, semicarbazone and hydrazone demonstrated better extrudability compared to oxime. Semicarbazone and hydrazone displayed macroscopic self-healing. We demonstrated ATDC5 chondrocytes and human dermal fibroblasts exhibited good cell viability with these hydrogels. Hydrazone showed excellent 3D bioprinting and ATDC5 chondrocytes demonstrated good biocompatibility within hydrazone hydrogel. Excellent bioprintability and biocompatibility using hydrazone hydrogel showed that hydrazone hydrogel can be the future bioink used in the lab for investigating bioprintability and functional tissue formation with cell types that are more clinically relevant such as mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs).

We envision providing customized formulations in dry powder form and the user has to follow a protocol for preparing a hydrazone bioink. For example, the user has to dissolve alginate and cross-linker in the calculated amount of cell culture media. Once dissolved, the alginate solution, cross-linker solution, and cell suspension can be mixed to prepare a bioink. After a waiting time of 15–45 minutes, the bioink is ready to be bioprinted into a defined shape.

### ***Bioinks using supramolecular chemistry***

Developing dynamic bioinks with controlled fibrous structures and mechanical properties remains a challenge. Supramolecular chemistry can enable the creation of bioinks that are dynamic yet biomimetic e.g., mimic the fibrous structure of proteins in the extracellular matrix (ECM). Supramolecular bioinks can also offer shear-thinning and self-healing properties owing to supramolecular transient interactions. For designing bioinks that are dynamic yet fibrous, we chose to develop bioinks using benzene, 1,3,5-benzene tricarboxamide (BTA). BTA has been known to undergo self-assembly via a combination of hydrogen bonding and hydrophobic interactions, resulting in one-dimensional (1D) fibers of a few nanometers in diameter and micrometer-scale long. Yet, the translation of BTA hydrogelator for bioink development with controlled mechanical properties has not been explored.

In **chapter VII** of this thesis, we developed a series of BTA hydrogelators bioinks with controlled mechanical properties. We simply altered the hydrophobic length (12, 16, 18, 20, and 24) on the exterior of benzene-1,3,5-tricarboxamide (BTA), which resulted in the modulation of viscoelasticity over 5 orders of magnitude in fibrous hydrogels. Interestingly, all hydrogelators demonstrated a similar equilibrium storage modulus. ATDC5 chondrocytes and human mesenchymal stem cells (hMSCs) displayed good cell viability within BTA hydrogels. BTA hydrogelators with 16, 20, and 24 carbon atoms length demonstrated shear-thinning, self-healing, and 3D printing into multi-layers structures. We showed that increasing carbon length on BTA provides better stability and shape fidelity to 3D-printed BTA structures. ATDC5 chondrocytes displayed high cell viability in bioprinted constructs. BTA hydrogelator bioinks

developed in **chapter VII** highlight the importance of the molecular design of hydrogel network for accessing the wide range of viscoelasticity in biomimetic fibrous bioinks for bioprinting.

In **chapter VIII** of the thesis, we developed an improved version of the bioink by replacing hydrophobic on the exterior of the BTA with norbornene (NB). The design of this bioink was inspired by nature's use of covalent reinforcement of self-assembled structures for designing tough tissues. NB BTA can undergo thiol-ene chemistry and could enable intra- and inter-fiber crosslinking. NB BTA self-assembles and forms a fibrillar and viscoelastic hydrogel. We demonstrated that cross-linking of self-assembly by intra- and inter-fiber enables tuning stiffness, strength, and toughness of the hydrogel. NB BTA hydrogels were extrudable, shear-thinning, and self-healing. NB BTA ink showed excellent 3D printability with good shape fidelity and enabled toughening of 3D printed structures by covalent cross-linking. hMSCs spheroids were mixed with NB BTA ink for preparation of NB BTA bioink and bioprinted into cartilage structure. hMSCs spheroids successfully produced cartilage tissue within bioprinted NB BTA constructs. The ability of hMSCs to produce cartilage tissue in NB-BTA hydrogelator makes NB BTA a promising bioink. There are already a few existing fibrous bioinks<sup>26,27</sup> and tough hydrogels<sup>28</sup>; however, tuning toughness within the synthetic biomimetic fibrillar bioink remains a formidable challenge that we addressed by developing NB BTA bioink. Unlike existing bioinks, such as gelatin and Matrigel, NB BTA bioink has been made using fully synthetic components and provides better control over the tuning of dynamics and toughness in synthetic bioink.

We envision providing NB BTA hydrogelator kits for laboratories to conduct their research in the areas of tissue engineering and bioprinting. The NB BTA hydrogelator kit will contain NB BTA hydrogelator powder, cross-linker solution, and appropriate cell culture media for making hydrogels out of the NB BTA hydrogelator. For Bioprinting and extrusion applications, the NB BTA kit will contain a bioprinting syringe and an appropriate needle size for successful bioprinting. Collaboration with other companies can also be considered for the distribution of NB BTA kits for example such companies could be CellInk, Advanced BioMatrix, and Allevi.

We aim to provide NB BTA hydrogelator for a range of applications including cell culture, organoid growth, and drug screening for increasing the commercial potential of the NB BTA bioink. Investigation with other cell types will help establish protocols with a variety of cell types, which is beneficial for enhancing the commercialization potential of the NB BTA bioink. For example, NB BTA hydrogels can be investigated with cells from tough and fibrous tissues such as tendons and muscles. Additional research on coating two-dimensional (2D) polystyrene culture dishes or creating 2D substrates of NB BTA for investigation of cell differentiation will also enhance the commercial value of the NB BTA bioink.

## Patents

The research carried out in **chapter IV** has produced a patent entitled "Bioinks", which has been filed in Europe and US.

The research carried out in **chapter VIII** has resulted in the patent application of “Injectable tough and viscoelastic BTA hydrogel”. The european search report found our claims to be new and inventive.

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