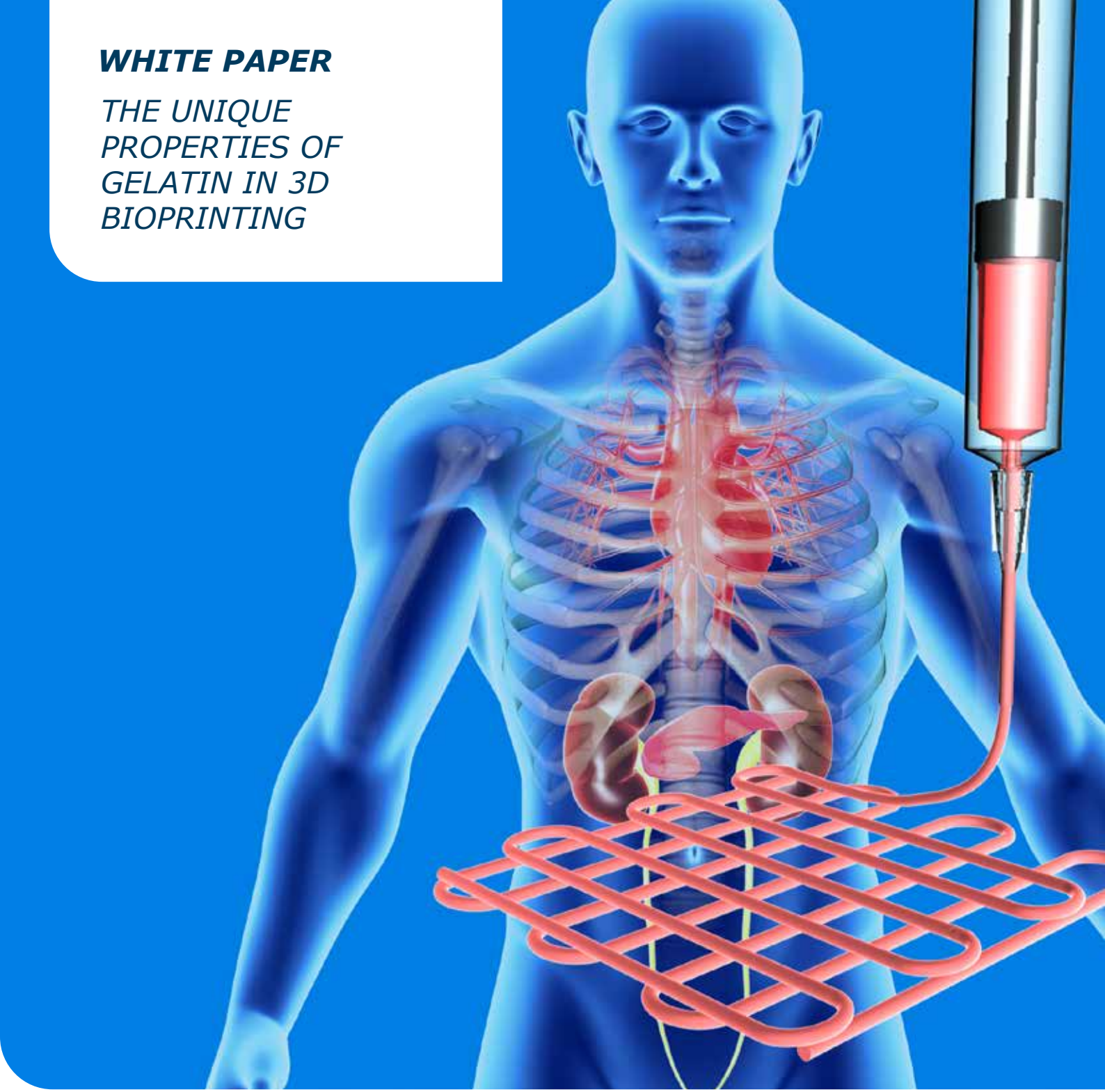
WHITE PAPER

THE UNIQUE PROPERTIES OF GELATIN IN 3D BIOPRINTING



How to select the right bioink to secure proper cellular characteristics and functionalities in engineered tissues: exploring the benefits of low endotoxin gelatins.

Gelatin has long been a trusted excipient in the pharmaceutical industry. For advanced biomedical applications like in-body use, there are strict regulations for endotoxins levels of the final product (medical device) to avoid unwanted side-effects.
3D bioprinting is a very popular tissue engineering technology used in the field of regenerative medicine. In this whitepaper, we explore the benefits of using gelatin and in particular gelMA gelatin as a bioink in various 3D bioprinting technologies, and describe how the low endotoxin gelatins in the X Pure® assortment – a range of highly purified gelatins and modified gelatins – can help secure proper cellular characteristics and functionalities, and enable (bio)medical applications with low immunogenicity.

By Jos Olijve, Scientific Support Manager, Rousselot Biomedical

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Introduction

Tissue engineering: a leading trend in regenerative medicine

Tissue engineering is one of the most fascinating and trendsetting strategies for regenerative medicine. Its aim is to replace, repair or reconstruct injured tissue or organs. Engineered functional tissue, biological implants and cell based multi-organ models have attracted great attention for clinical, diagnostic and pharmaceutical research[1,2].

Despite the emergence of tissue engineering, no functional bio artificial organs have been created in a clinic to date. However, the complexity of the parameters that influence the formation of biological tissues has been comprehensively investigated.
3D bioprinting has been promoted as a state of the art tissue engineering technology, used to fabricate biomimetic scaffolds that are structurally and functionally relevant. These 3D bioprinted scaffolds can support lineage-specific differentiation, intercellular communication between cells and their environment, and development into microstructures, such as capillaries, epithelia or organoids. All of this means that 3D bioprinting offers a great technological platform for reconstructing hierarchical tissue structures with full cellular functionality within the construct.

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After graduating in Biochemistry, Jos Olijve worked as a Researcher in the Department of Molecular Genetics at the University of Groningen. Since 2012, he has been responsible for the development of new gelatin-based products and applications at Rousselot. Jos Olijve has published 15 patent applications and is the author/co-author of 12 scientific papers.

Biomaterial gelatin

Gelatin has a long history as a trusted excipient within the pharmaceutical industry, as it meets the highest standards of safety and regulatory compliance. It is produced by partially hydrolyzing collagen, the most abundant protein in the body and the most prevalent macromolecule in the extracellular matrix (ECM).

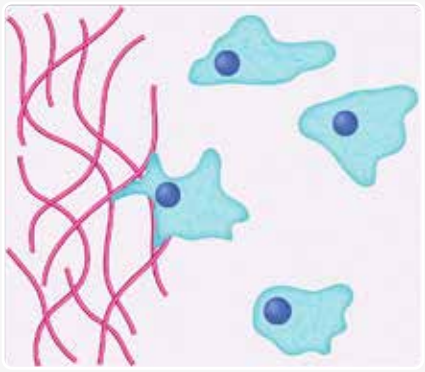
Gelatins that are used in pharmaceutical applications are produced in the same way as gelatins used in food applications, i.e. by breaking down collagen into type A (acid process) or type B (lime process) gelatin.

Due to a more extensive deamination of asparagine and glutamine in type B gelatin, the isoelectric point (IEP) of type B gelatin is lower (4.7-5.6) compared to type A gelatin (6.0-9.5)[3]. This property enables gelatin to be negatively or positively charged at neutral physiological pH.

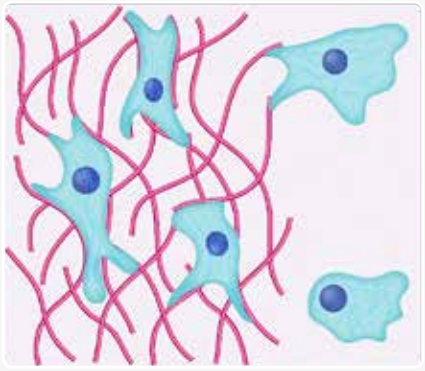
Another useful property is gelatin's chemical versatility, which allows for a broad range of applications. The existence of amine, hydroxyl and carboxylate groups enables gelatin to be chemically modified at a molecular level to achieve the desired properties for specific applications. Moreover, the abundant presence of the cell recognition sequence Arginine Glycine Aspartic acid (RGD) facilitates attachment of cells to gelatin, promoting their spreading and proliferation. These cell matrix interactions are crucial for the organization of complex tissue[4] (Figure 1).

Figure 1.

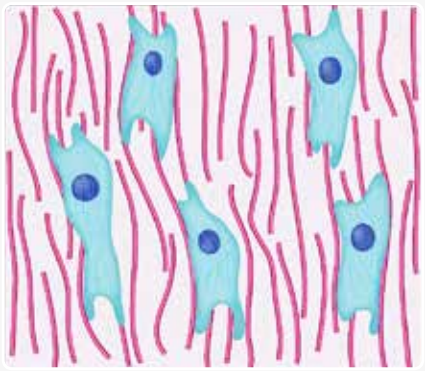
Graphical depiction of the mutual impact on the extracellular matrix and the cytoskeleton of cells during organization of tissues[5].



1. Cell attachment onto a gelatin scaffold



2. Cell migration through the gelatin scaffold



3. Tissue reorganization

Endotoxin

Despite the unique properties of gelatin, the use of traditionally manufactured gelatin in (bio)medical applications is challenged by the presence of endotoxins (lipopolysaccharides). Endotoxins are large, highly immunogenic molecules and present in the outer membrane of gram negative bacteria. They are highly heat resistant, which make them difficult to inactivate. When exposed to the immune system, endotoxins initiate an immune response, which can lead to tissue inflammation, increased sensitivity to other allergens, and in extreme cases even fatal shock[6]. Endotoxins mediate their effect via the inflammasome and the TLR4 receptor present on many cells, resulting in the release of pro inflammatory cytokines[7,8].

The **FDA** has imposed restrictions on endotoxin content for a number of medical devices. These are 2.15 Endotoxin Units (EU) (or 0.06 EU/ml) for devices exposed to the central nervous system via cerebrospinal fluid, and 20 EU (or 0.5 EU/ml) for devices for peripheral applications that can be reached by the cardiovascular and lymphatic systems[9]. The **European Pharmacopoeia**, as well as its US and Japanese counterparts, also requests compliance to endotoxin limits for parenteral administered pharmaceuticals. Endotoxin limits for injectables have to be determined in relation to the body weight and have conventionally been set to 0.2 EU/kg if introduced into the spinal canal and 5.0 EU/kg if injected elsewhere[10,11].

X-Pure and X-Pure GelMA gelatins

Offering many valuable functionalities such as gelation, crosslinking, controlled swelling and more, gelatin is an unrivalled asset to formulation.

Responding to growing concerns linked to endotoxin contamination in biomaterials that are used for tissue engineering, Rousselot®, the leading gelatin producer, has developed X-Pure®, a range of highly purified gelatins for pharmaceutical and medical applications.

Since X-Pure is highly purified without compromising the unique tunable and biocompatible characteristics of natural gelatin, these functionalities are highly suitable for sensitive biomedical applications as well. In addition, with less than 10 EU/g, X-Pure gelatins facilitate compliance with FDA specifications for endotoxin limits.

For 3D bioprinting and cell engineering applications, GelMAs (methacryloyl gelatins) are particularly popular.

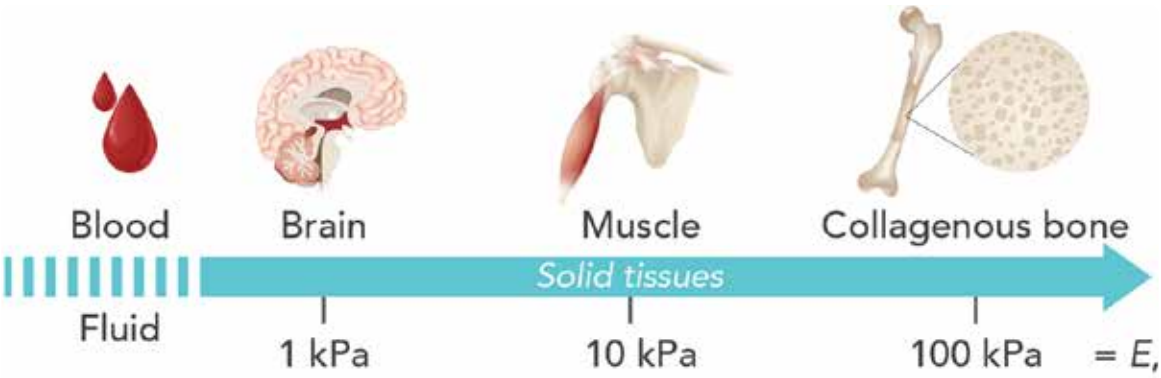
The X-Pure range also includes ultra-purified GelMAs. Low in endotoxins and other impurities, X-Pure GelMAs have a unique advantage over other standard market products due to their consistent molecular weight and degree of modification, thus guaranteeing stable mechanical properties.

All Rousselot's X-Pure and modified gelatins are produced under GMP conditions.

3D Bioprinting and bioinks

3D bioprinting is a biofabrication process that combines biomaterials with living cells to generate precisely controlled 3D cell models and tissue constructs. This technology facilitates the recapitulation of the delicate shapes and structures of targeted organs and tissues. Compared to traditional biofabrication methods, which require additional cell seeding procedures, encapsulation of the cells during 3D bioprinting allows for fine tuning of cellular attachment and the distribution of cells and biomolecules within the printed scaffold[12]. By optimally combining multiple cell types, growth factors and biomaterial compositions, highly complex and fully functional tissue constructs can be generated through self-organization and maturation. To achieve this, it is of critical importance to select suitable biomaterials for 3D bioprinting. These biomaterials should not only be biocompatible, but also have high water binding capacity and sufficient porosity to provide nutrient diffusion and cell migration[13-15]. Additionally, mechanical properties such as stiffness and geometry have a great impact on cellular functions. For example, hard and mineralized ECM is beneficial for the generation of bone constructs, whilst cartilage requires elastic hydrogels (Figure 2).

Figure 2. *Elastic properties of different tissues*



E, Elasticity of microenvironnement

Bioinks can generally be described as formulations of biomaterials containing living cells that are suitable for processing into designed shapes by 3D bioprinting technology. Bioinks are mostly composed of multiple biomaterials that mimic the ECM that supports the biological behavior of living cells, spheroids or microtissues[16]. Important bioinks requirements are[15,16]:

- Printing temperatures equal to or below physiological temperatures;
- Tunable viscoelastic properties (viscosity, shear thinning and recovery time);
- Containing bioactive components.
- Rapid gelation to obtain high structural resolution and crosslinkage;
- Suitable to support cell adhesion, proliferation and differentiation;

Gelatin in bioprinting

Gelatin is a versatile biomaterial that can be used in bioprinting to obtain biocompatible cell laden constructs with high structural resolution and shape[14]. It can be used in three main bioprinting technologies (Figure 3)[14-17]:

- Inkjet based bioprinting,
- Laser assisted bioprinting,
- Extrusion based bioprinting.

Extrusion based bioprinting is the most widespread technology for the biofabrication of 3D cell laden constructs thanks to its low cost and medium to fast printing speed. The bioink is generally inserted in disposable plastic syringes and dispended either pneumatically or mechanically onto the receiving substrate. Extrusion bioprinters do not dispense small bioink droplets but whole filaments (~100-500µm in diameter, Figure 5). The printing speed is generally slower compared to inkjet and laser assisted bioprinting, but it allows for higher cell loads and even the incorporation of spheroids[13,15,16].

Depending on the bioprinting method or technology, and the type of tissue to be printed, bioinks with low or high viscosity can be used. For example, extrusion based bioprinting relies on higher viscous bioinks, while inkjet printing and laser assisted technologies benefit more from low viscous solutions[15,16].
Gelatin's versatile and tunable physicochemical properties allow the development of both high and low viscosity gelatin based bioinks. The gelling can be controlled by temperature, which facilitates optimization of the flow behavior during bioprinting.
To create bioprinted hydrogels with a good mechanical stability at physiological temperatures, gelatin needs to be crosslinked. Crosslinking of gelatin can be easily achieved chemically, for example with genipin or 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide, or enzymatically by using transglutaminase[1,18-22].

These crosslinking agents have been shown to be more biocompatible compared to the conventional crosslinker glutaraldehyde[18,23]. With these crosslinking methods, the stiffness, swelling and degradation rate of the gelatin based hydrogels can be fine tuned and precisely controlled[18,23].

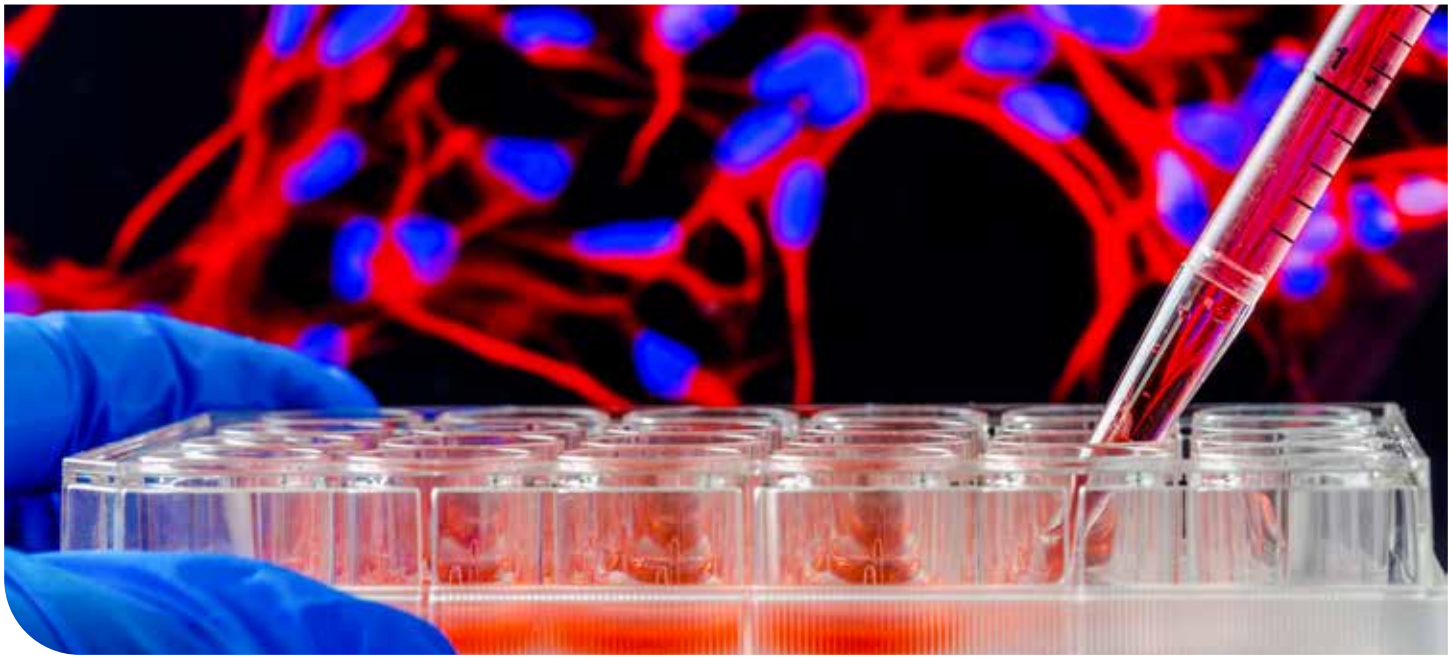
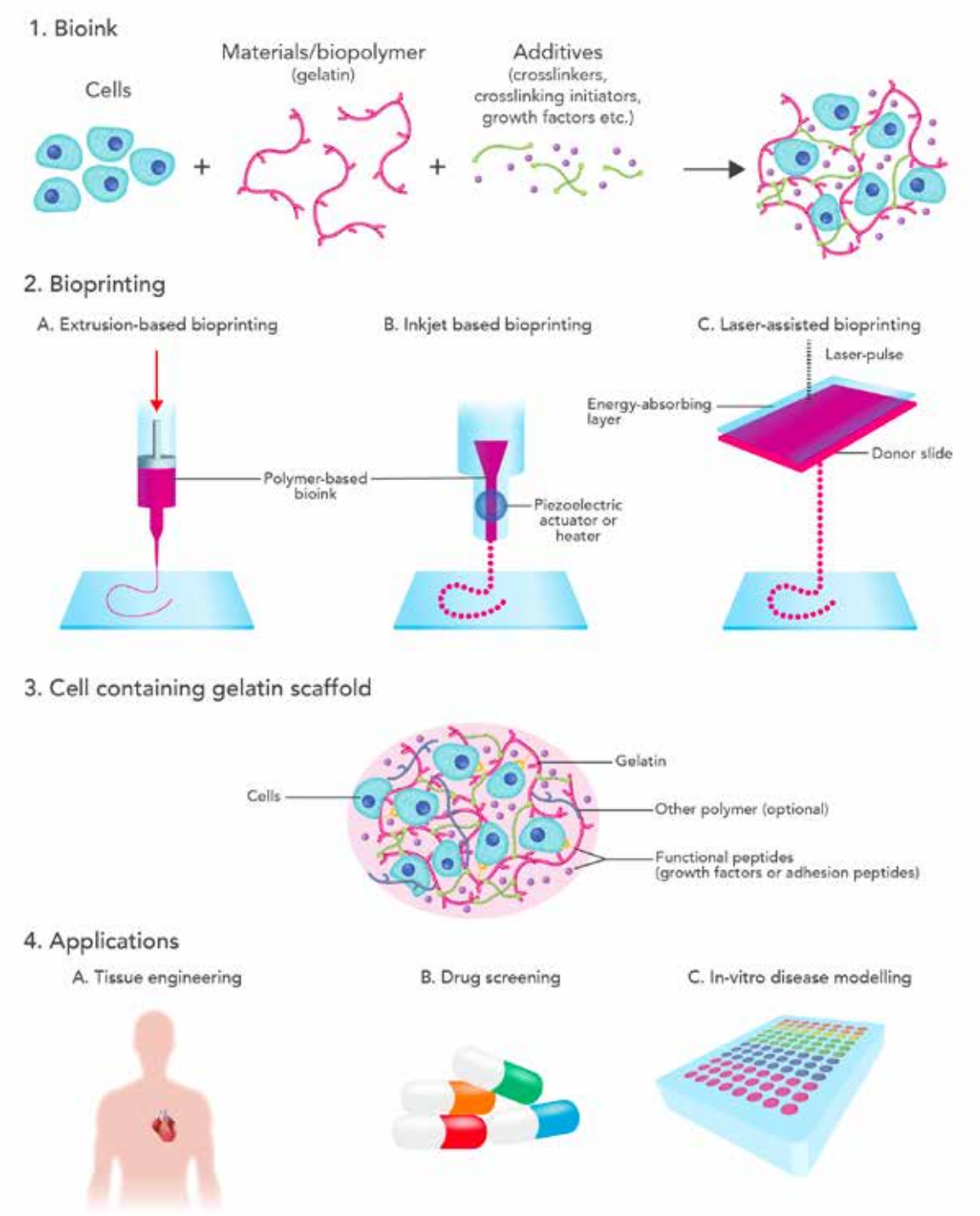


Figure 3. *Gelatin can be used in different 3D bioprinting technologies*



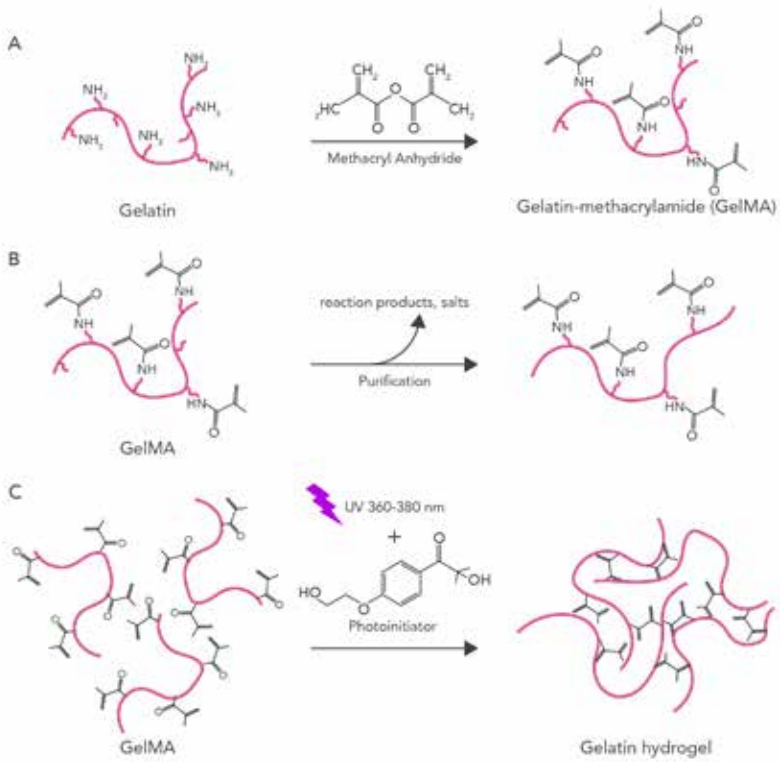
Gelatin methacryloyl (GelMA)

The most extensively used and studied modification reagent to crosslink gelatin for bioprinting is methacryl anhydride (MA). MA modified gelatin is generally referred to as GelMA[24,25]. Modification is obtained through the derivatization of gelatin with MA, resulting in the modification of lysine and hydroxyl residues with MA and methacrylate side groups. The crosslinking of GelMA is initiated by radicals, which are generated by UV or blue light - depending on the photo-initiator used (Irgacure I2959 or LAP [lithium phenyl-2,4,6-trimethylbenzoylphosphinate]) (Figure 4).
The ability to crosslink the material using UV or blue light also generates new possibilities to work with other methods of bioprinting, like digital light processing (DLP) or stereolithography (SLA) printing. These printing techniques are capable of printing with much higher resolution than extrusion printers, while also being able to print objects with a greater complexity.

Various modification degrees of GelMA are available, which provides a versatile and modular toolbox to tailor the mechanical and physical properties of gelatin based bioinks and bioprinted hydrogels. A higher modification degree reduces the intermolecular interactions between gelatin, resulting in reduced viscosity and gelling25. The decrease in viscosity and corresponding low shear stress of the bioinks are favorable for cell survival during printing[24,26].
An optimum MA modification degree is postulated at about 0.35 mmol methacrylic groups per gram of gelatin, which is equal to 100% lysine modification[25,27]. Besides the degree of MA modification, the inherent physical gelation of the gelatin before crosslinking will also increase the stiffness of hydrogels[28]. Typically, there is an inverse relationship between stiffness and water uptake of crosslinked hydrogels[3,29,30].

Figure 4.

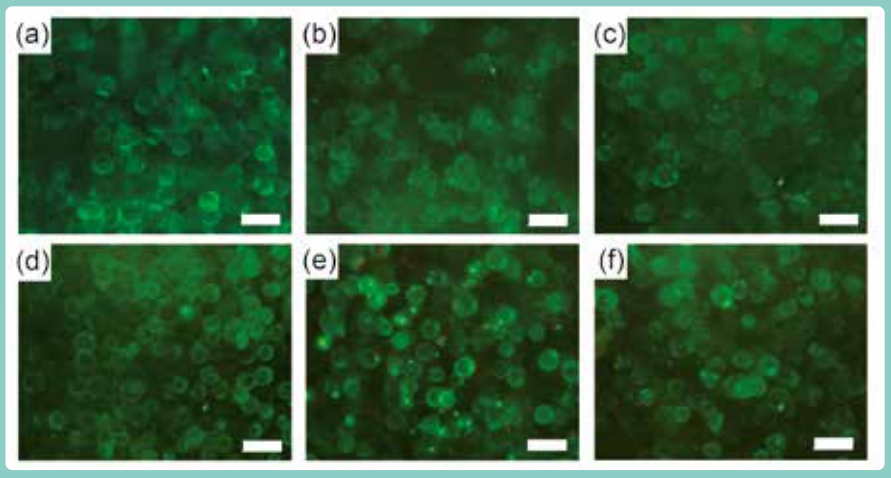
Methacrylamide modification of gelatin (a), purification (b) and the mechanism of chain growth in the radical crosslinking of gelatin methacryloyl after UV exposure in the presence of a photo initiator (c).



Cells stay viable during the photo induced crosslinking and cell recognition sites are maintained, making GelMA an ideal biocompatible material for bioink formulation[2,24,31-34] (Figure 5). Another advantage of

Figure 5.

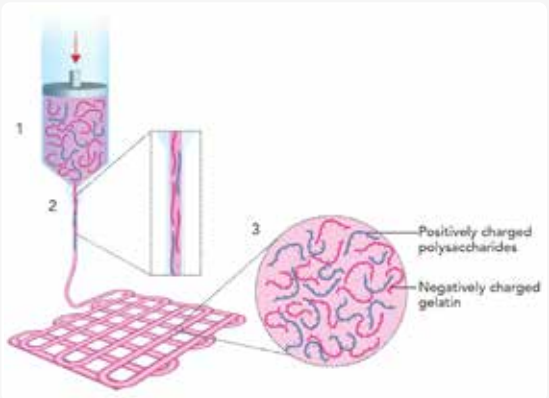
Live dead staining of primary human adipocytes encapsulated in GelMA hydrogels cured for 1 2 3 minutes and cultured for 1 (a-b-c) and 5 days (d-e-f).



GelMA based bioinks is their versatility for combining with other materials to improve the printability and biocompatibility of bioinks. Polysaccharide additives such as gellan gum, can be used to optimize the rheological properties of GelMA based bioinks (Figure 6). Gellan gum GelMA bioinks have thixotropic properties due to the reversible charge interactions between gelatin and gellan gum. These charge interactions are disrupted, and the molecules can be aligned to provide a low viscous solution, which is important to secure a high cell viability. An increase in viscosity is achieved after printing, since the charge interactions between GelMA and gellan gum are restored[15,26]. The GelMA based bioinks can further be combined with other ECM components such as proteoglycans, hyaluronic acid, growth factors, etc. to obtain a biocompatible 3D printed biomatrix[28,31,32,35].

Figure 6.

Schematic representation of shear thinning and yield stress in plotting GelMA/gellan gum based bioink.



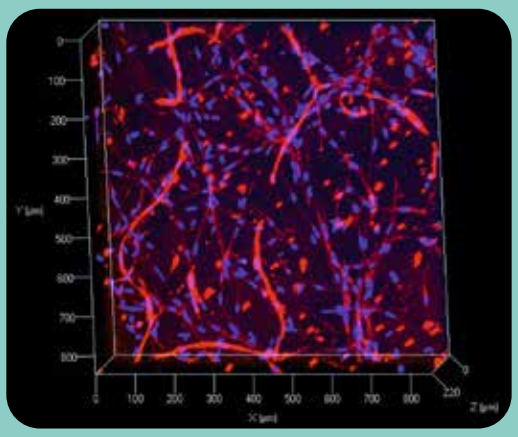
1. *In the syringe the gellan chains (blue) form a temporary network with GelMA (red) and induce a gel like viscosity;*
2. *As it is dispensed through the needle, the temporary network is broken up by shear. The polymer chains align, which results in a reduced viscosity;*
3. *Directly after removal of shear stress, the temporary GelMA gellan gum network is restored and the printed filament solidifies instantly to create high fidelity structures.*

Gelatin-based sacrificial bioinks

An interesting development in the field of bioinks are the so called 'sacrificial bioinks', which are very popular for building suitable models, for uses such as drug screening, among others[35-36]. These 'sacrificial bioinks' offer temporary support for cells and are also widely used to fabricate complex geometries to create vasculature networks within a structure[35-37] (Figure 7). When combined with other matrix biomaterials, these bioinks can be dissolved and removed after printing[35]. Ideally, a 'sacrificial biomaterial' offers high print fidelity, cytocom-patibility and is easy to remove. Gelatin is intensively used as sacrificial material, for instance, in combination with GelMA and endothelial cells for the fabrication of blood vessels. In this way, hollow channels are fabricated with an increased supply of oxygen and nutrients, which enhance cell viability in bioprinted structures[38].

Figure 7.

Hydrogel with vascular network established using sacrificial bioink.



References

1. K. Yue, G. Trujillo-de Santiago, M.M. Alvarez, A. Tamayol, N. Annabi, A. Khademhosseini, Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels, Biomaterials 73 (2015) 254-271.
2. A.I. Van Den Bulcke, B. Bogdanov, N. De Rooze, E.H. Schacht, M. Cornelissen, H. Berghmans, Structural and rheological properties of methacrylamide modified gelatin hydrogels, Biomacromolecules 1(1) (2000) 31-8.
3. GME monograph Version 12 - May 2017
4. M. Santoro, A.M. Tatara, A.G. Mikos, Gelatin carriers for drug and cell delivery in tissue engineering, Journal of controlled release: official journal of the Controlled Release Society 190 (2014) 210-8.
5. Alberts B., Molecularbiologie der Zelle, 3 auflage 1995. ISBN: 9783527304929. VCH Verlag Weinheim.
6. M.H. Weil, W.W. Spink, A comparison of shock due to endotoxin with anaphylactic shock, The Journal of laboratory and clinical medicine 50(4) (1957) 501-15.
7. K. Miyake, Endotoxin recognition molecules, Toll-like receptor 4-MD-2, Seminars in immunology 16(1) (2004) 11-6.
8. M. Triantafilou, K. Triantafilou, Sepsis: molecular mechanisms underlying lipopolysaccharide recognition, Expert reviews in molecular medicine 6(4) (2004) 1-18.
9. Transfusion and Infusion Assemblies and Similar Medical Devices, US Pharmacopoeia Chapter 161 (2011).
10. Bacterial Endotoxins, European Pharmacopoeia 5th edition (2016) Chapter 2.6.14.
11. M. Dawson, Endotoxin Limits For Parenteral Drug Products, Associates of Cape Cod Incorporated Bacterial Endotoxin test (BET) White Paper (2017).
12. Pan, C.-C, Bruyas, A; Yang, Y.P. Materials Matters. 2016 11 (2), 49-55
13. H.-W. Kang, S.J. Lee, I.K. Ko, C. Kengla, J.J. Yoo, A. Atala, A 3D bioprinting system to produce human scale tissue constructs with structural integrity, Nat Biotech 34(3) (2016) 312-319.
14. Katja Hölzl, Shengmao Lin, Liesbeth Tytgat, Sandra Van Vlierberghe, Linxia Gu and Aleksandr Ovsianikov. Biofabrication 8 (2016) 032002.
15. A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher, Matrix Elasticity Directs Stem Cell Lineage Specification, Cell 126(4) (2006) 677-689.
16. Hospodiuk, Monika; Dey, Madhuri; Ozbolat, Ibrahim (Jan 3, 2017). "The bioink: A comprehensive review on bioprintable materials". Biotechnology Advances. 35 (2): 217-239. doi:10.1016/j. biotechadv.2016.12.006. PMID 28057483
17. Elomaa, L.; Kang, Y.; Seppälä, J. V.; Yang, Y. J. Polym. Sci. A Polym. Chem. 2014, 52(23), 3307–doi: 10.1002/pola.27400. Science 136(25) (2019).
18. W.E. Hennink, C.F. van Nostrum, Novel crosslinking methods to design hydrogels, Advanced Drug Delivery Reviews 54(1) (2002) 13-36.
19. V. Perez-Puyana, M. Felix, A. Romero, A. Guerrero, Influence of the processing variables on the microstructure and properties of gelatin-based scaffolds by freeze-drying, Journal of Applied Polymer Science 136(25) (2019).
20. D.M. Kirchmajer, C.A. Watson, M. Ranson, M.i.h. Panhuis, Gelapin, a degradable genipin cross-linked gelatin hydrogel, RSC Advances 3(4) (2013) 1073-1081.
21. K. Jarquín-Yáñez, J. Arenas-Alatorre, G. Piñón-Zárate, R.M. Olivares, M. Herrera-Enríquez, B. Hernández-Téllez, A.E. Castell-Rodríguez, Structural Effect of Different EDC Crosslinker Concentration in Gelatin- Hyaluronic Acid Scaffolds, Journal of Bioengineer and Biomedical Science 6 (2016).
22. G. Yang, Z. Xiao, H. Long, K. Ma, J. Zhang, X. Ren, J. Zhang, Assessment of the characteristics and biocompatibility of gelatin sponge scaffolds prepared by various crosslinking methods, Scientific Reports 8(1) (2018) 1616.
23. Elomaa, L.; Kang, Y.; Seppälä, J. V.; Yang, Y. J. Polym. Sci. A Polym. Chem. 2014, 52(23), 3307–doi: 10.1002/pola.27400. Science 136(25) (2019).
24. J. Van Hoorick, P. Gruber, M. Markovic, M. Tromayer, J. Van Erps, H. Thienpont, R. Liska, A. Ovsianikov, P. Dubruel, S. Van Vlierberghe, Cross-Linkable Gelatins with Superior Mechanical Properties Through Carboxylic Acid Modification: Increasing the Two-Photon Polymerization Potential, Biomacromolecules 18(10) (2017) 3260-3272.
25. L. Sewald, C. Claaßen, T. Götz, M.H. Claaßen, V. Truffault, G.E.M. Tovar, A. Southan, K. Borchers, Beyond the Modification Degree: Impact of Raw Material on Physicochemical Properties of Gelatin Type A and Type B Methacryloyls, Macromolecular Bioscience 18(12) (2018) 1800168.
26. Jos Malda, Jetze Visser, Ferry P. Melchels, Tomasz Jüngst, Wim E. Hennink, Wouter J. A. Dhert, Jürgen Groll, and Dietmar W. Hutmacher. Adv. Mater. 2013, 25, 5011–5028
27. C. Claaßen, M.H. Claaßen, V. Truffault, L. Sewald, G.E.M. Tovar, K. Borchers, A. Southan, Quantification of Substitution of Gelatin Methacryloyl: Best Practice and Current Pitfalls, Biomacromolecules 19(1) (2018) 42-52.
28. E. Hoch, T. Hirth, G.E.M. Tovar, K. Borchers, Chemical tailoring of gelatin to adjust its chemical and physical properties for functional bioprinting, Journal of Materials Chemistry B 1(41) (2013) 5675-5685.
29. S. Stier, L. Rebers, V. Schönhaar, E. Hoch, K. Borchers, Advanced formulation of methacryl- and acetyl-modified biomolecules to achieve independent control of swelling and stiffness in printable hydrogels, J Mater Sci: Mater Med 30(3) (2019) 35.
30. Li Z, Huang S, Liu Y, Yao B, Hu T, Shi H, et al. Tuning Alginate-Gelatin Bioink Properties by Varying Solvent and Their Impact on Stem Cell Behavior. Scientific Reports. 2018;8:8020.
31. E. Hoch, G.E.M. Tovar, K. Borchers, Biopolymer-based hydrogels for cartilage tissue engineering, Bioinspired, Biomimetic and Nanobiomaterials 5(2) (2016) 51-66.
32. A. Wenz, K. Borchers, G.E.M. Tovar, P.J. Kluger. Bone matrix production in hydroxyapatite-modified hydrogels suitable for bone bioprinting. Biofabrication 9(14) (2017) 044103.
33. Yu Shrike Zhang, Kan Yue, Julio Aleman, Kamyar Mollazadeh Moghaddam, Syeda Mahwish Bakht, Jingzhou Yang, Weitao Jia, Valeria Dell'Erba, Pribpandao Assawes, Su Ryon, Mehmet Remzi Dokmeci, Rahmi Oklu and Ali Khademhosseini. 3D Bioprintingfor cell culture and tissue fabrication. Bio-Design and Manufacturing 1(1) (2019) 45-61.
34. B. Huber, K. Borchers, G.E. Tovar, P.J. Kluger, Methacrylated gelatin and mature adipocytes are promising components for adipose tissue engineering, Journal of Biomaterials Applications 30(6) (2016) 699-710.
35. Margaret E Prendergast, Ricardo D Solorzano1 & Danny Cabrera. J. 3D Print. Med. 10.2217/3dp-2016-002.
36. David B. Kolesky, Kimberly A. Homana, Mark A. Skylar-Scotta, and Jennifer A. Lewis. pnas.org/cgi/doi/10.1073/pnas.1521342113.
37. Kang HW, Lee SJ, Ko IK, Kengla C, Yoo JJ, Atala A. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. Nat. Biotech. 34(3), 312–321 (2016).
38. Yu Shrike Zhang, Rahmi Oklu, Mehmet Remzi Dokmeci and Ali Khademhosseini. Cold Spring Harb Perspect Med 2018; 8:a025718.
39. Wentao Shi, Ran He, Yaling Zhen. European J. of Biomedicine Research 1(3) 3-8 (2015)
40. Andrea Mazzocchi, Shay Soker and Aleksander Skardal. Appl. Phys. Rev. 6, 011302 (2019); doi 10.1063/1.5056188.

Conclusion and *future* perspectives

3D bioprinting is already a powerful technology used in tissue engineering and pharmaceutical developments[39-40]. This technology will further develop and expand to address more complex problems in regenerative medicine. As a favorable biomaterial for 3D bioprinting, gelatin is widely used as a bioink material due to its tunable physicochemical and biological properties. However, the purity of gelatin is essential to secure proper cellular characteristics and functionalities in the engineered tissue that will ultimately be transplanted into the body to restore the damaged tissue. With X-Pure® and X-Pure GelMA, low endotoxin gelatins are now available to accelerate clinical translation of developments and technologies that are still in the R&D phase such as 3D bioprinting.

Rousselot® Biomedical

As the most recent strategic segment within Rousselot, we have drawn upon Rousselot's 130+ years of worldwide expertise and proven track record of pharmaceutical gelatins and collagens to develop innovative ranges of purified, modified and non-modified gelatins and collagens for biomedical applications. Offering unique advantages to assure performance, quality and safety from bench to clinic, Rousselot® X-Pure® and Rousselot® Quali-Pure™ provide consistent quality and are backed by strong scientific data and on-going research. Rousselot Biomedical is committed to support end-to-end partnerships to help "Advancing medical science". Rousselot is Darling Ingredients' health brand.

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