WHITE PAPER

X-pure
ADVANCING MEDICAL SCIENCE

LOW ENDOTOXIN GELATINS AND COLLAGENS FOR (BIO)MEDICAL APPLICATIONS

INTRODUCING X-PURE®, THE FIRST FULL RANGE OF PURIFIED GELATINS AND COLLAGENS WITH UNCOMPROMISED PERFORMANCE[1]

This white paper outlines the benefits of gelatin and collagen for (bio)medical applications and highlights the importance of low endotoxin levels for optimal API (Active Pharmaceutical Ingredients) delivery and (bio)medical engineering materials. The paper also highlights the value-added expertise that Rousselot can offer to researchers, developers and manufacturers of (bio)medical applications of gelatin, hydrolyzed gelatin and collagen. Rousselot Biomedical offers world leading advice on ingredient compatibility and selection of gelatin and collagen characteristics for optimal physical properties and product stability.

By Jos Olijve, Principal scientist and R&D project manager, Rousselot
and Barbara Vanhoecke, Innovation Manager Biomedical, Rousselot. April 2019.

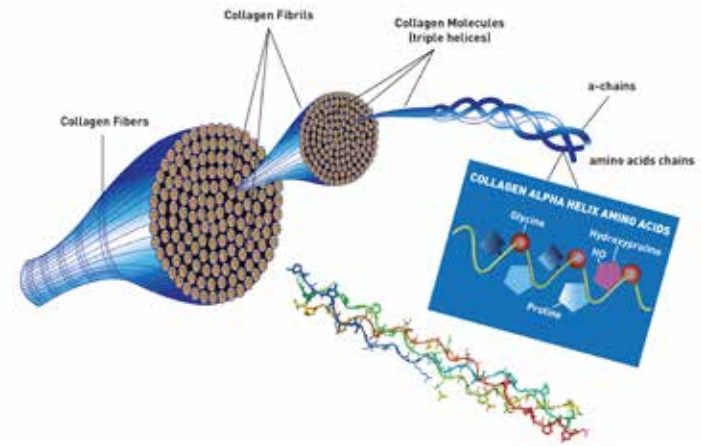
[1] Type A and B gelatin with <10 EU/g endotoxin levels vs typical standard gelatin produced and available to purchase in commercial volumes.

INTRODUCTION

With X-Pure®, new low endotoxin gelatins and collagens are now available to enable low immunogenic (bio)medical applications. X-Pure is ultrapure without compromising the unique tunable and biocompatible characteristics of natural gelatin and collagen.

Gelatin – A natural, adaptable and trusted excipient and biomaterial

Gelatin has a long history as a trusted excipient within the pharmaceutical industry, meeting the highest standards of safety and regulatory compliance.
Gelatin is formed by the partial hydrolysis of collagen, the most abundant protein in the body and the most prevalent macromolecule of the extracellular matrix (ECM).



Gelatin used within pharmaceutical applications is produced in the same way as gelatin for food applications i.e. via the breakdown of collagen into Type A (acid process) or Type B (lime process) gelatin, which enables a broad range of applications.

Due to a more extensive deamination of asparagine and glutamine in Type B gelatin, the isoelectric point (IEP) of type B gelatin is lower compared to a Type A gelatin. The IEP of Type A being 7.0-9.0 and Type B being 4.9-5.1. With further hydrolysis, so-called 'hydrolyzed collagen/gelatin' is formed. These hydrolyzed molecules or peptides have different characteristics and are no longer able to form a gel, while unique stabilizing properties remain with limited impact on viscosity.

The natural origin and tunable physicochemical properties render gelatin an ideal product for a wide range of (bio)medical applications. Examples of application areas where gelatin offers attractive product characteristics include:

- Hemostatic applications for control of bleeding using gelatin sponges, strips, powders or fibers
- Drug delivery and parenteral applications as a safe excipient for vaccines and other injectables to achieve optimal drug delivery
- Regenerative medicine applications such as implantable membranes, (stem) cell culture, 3D bioprinting, advanced drug delivery and tissue engineering.

Introducing Jos Olijve and Barbara Vanhoecke

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 author/co-author of 10 scientific papers.

Barbara Vanhoecke holds a PhD in Medical Sciences as well as a Master in Biochemistry. She joined Rousselot in 2017 and is now Innovation Manager Biomedical working in particular on the X-Pure range. Barbara Vanhoecke has published 2 patent applications and is author/co-author of more than 40 scientific papers.

ENDOTOXINS – A CHALLENGE TO THE SAFE APPLICATION OF GELATIN AND COLLAGEN IN (BIO)MEDICAL APPLICATIONS

The use of traditionally manufactured gelatin and collagen in (bio)medical applications is challenged by the presence of endotoxins (lipopolysaccharides). Endotoxins are large, highly immunogenic molecules that are the major component of the outer membrane of Gram-negative bacteria.

Endotoxins are highly heat resistant, making 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 the risk for fatal shock (Weil and Spink, 1957). Endotoxins mediate their effect via the inflammasome and the TLR4 receptor present on cells, resulting in the release of pro-inflammatory cytokines (Diamond et al., ImmunoTargets and Therapy, 2015:4 131–141 and Triantafilou et al. ERMM, 2004, 6, 1-18).

Even exposure to very low levels of endotoxin have been found to cause significant immune responses and the FDA has imposed restrictions on endotoxin content for a number of medical device applications (FDA, 2012). Endotoxin limits for medical devices are set at 2.15 Endotoxin Units (EU) per device (or 0.06 EU/ml) for devices exposed to the central nervous system via cerebrospinal fluid, and at 20 EU/device (or 0.5 EU/ml) for devices in peripheral applications reached by the cardiovascular and lymphatic systems.

Traditionally manufactured gelatin and collagen can contain endotoxin levels that are typically more than a hundred times higher than the limits recommended by the FDA.

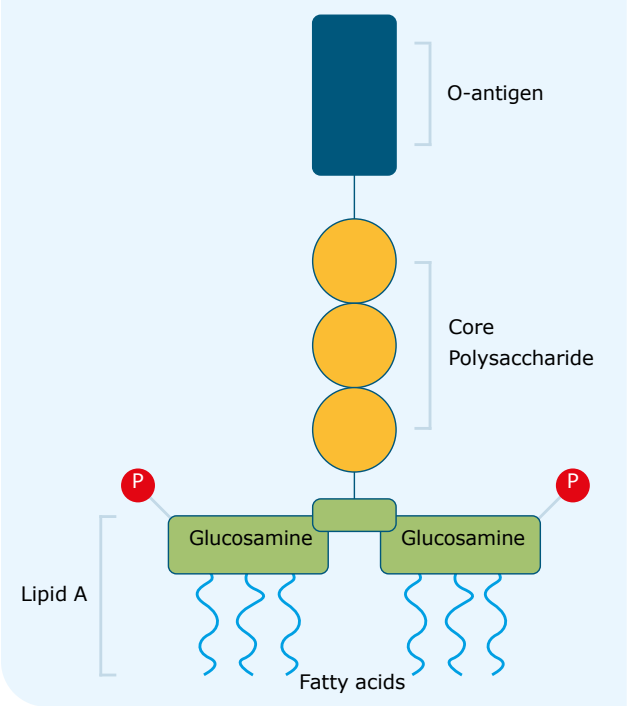


Figure 1. Structure of endotoxin - Adapted from Lieder, Petersen and Sigurjonsson (2013)

X-PURE - THE FIRST COMPLETE RANGE OF LOW ENDOTOXIN GELATINS FOR (BIO)MEDICAL APPLICATIONS

To facilitate (bio)medical application of gelatin, Rousselot has developed a patent protected process (Patent WO2016085345) to remove endotoxins. Hence, X-Pure gelatin contains very low endotoxin levels, being below 10 EU/g for the highest grade version of X-Pure. The endotoxin limit is validated for each batch using the Limulus Amoebocyte Lysate (LAL) assay, the FDA approved method for endotoxin level quantification in (bio)medical applications.

The purification process has been developed and optimized for both Type A and Type B gelatin, both hydrolyzed and non-hydrolyzed, to ensure the full spectrum of physical properties of gelatin to be available for X-Pure. The validated process is performed under controlled clean room conditions and stringent quality control with batch release conditions suitable for GMP (bio)medical applications. The resulting X-Pure gelatin is a completely pure, highly consistent gelatin with very low endotoxin levels.

X-Pure is delivered virus safe, with any viruses independently deactivated in multiple process steps including acid/lime and heat treatment, and can additionally be packaged and supplied under sterile conditions if required. In addition, Rousselot offers optional assistance with crosslinking and modification of X-Pure, e.g., for 3D bioprinting or tissue engineering purposes.
X-Pure is available with extensive documentation and full traceability, meeting the most stringent requirements for ethical and regulatory compliance.

(BIO)MEDICAL AREAS FOR GELATIN AND COLLAGEN

Hemostatic applications – Achieving effective bleeding control

Severe bleeding accounts for about 80% of deaths in the operating theatre (Gaunt and Woolley, 2014), which stresses the importance of effective control over bleeding during surgery. Gelatin can be designed to absorb more than ten times its own weight and is widely accepted as an ideal material to control blood flow through hemostatic applications, mainly in the form of sponges, strips, powder or nanofibers. In most of these products, Type A gelatins are used because of their superior stability and foaming properties. Endotoxins in hemostats can cause an exaggerated immune response and at high quantities, even septic shock (Opal, 2010). With X-Pure there is now a full range of low endotoxin gelatins and collagens available including type A gelatin, offering an unrivalled low endotoxin gelatin solution for hemostatic applications.

Drug delivery and parenteral applications – Achieving safe delivery of vaccines and injectables

The demand for excipients as safe carriers in vaccines and other injectables is higher than ever before (Kaddar, 2013) where gelatin has been found to be a highly versatile drug delivery platform (Nikkhah, et al., 2016). Hydrolyzed gelatins / collagens have been a trusted excipient for decades; however, endotoxins need to be low to avoid any unwanted immune response (Weil and Spink, 1957). With X-Pure it is now possible to minimize the risk of endotoxin-induced immune responses in drug delivery applications.

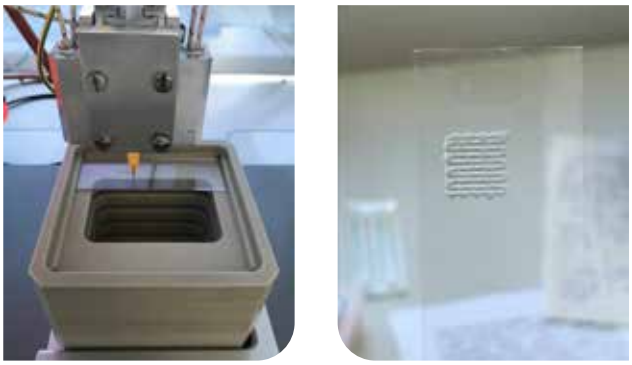
The right design and development of injectable and self-healing biomaterials is important to stimulate the regeneration of lost or damaged tissues

Prof. Dr. Sander Leeuwenburgh:
"I work on the design and development of injectable and self-healing biomaterials able to stimulate the regeneration of lost or damaged tissues. Gelatin is an ideal material for many of the applications I work on. Compared to other (bio)polymers, one of the main assets of gelatin is its easy processing into microspheres, nanospheres, coatings, nanofibers, sponges, and injectables. X-Pure is an ideal gelatin for these applications due to its low endotoxin level, tunable molecular weight and charge, and versatile chemical functionalization."

Regenerative medicine – A safe and effective basis for a range of regenerative applications

A wide range of regenerative technologies has recently been developed to repair or regenerate injured or diseased tissues and organs, or to aid in development of new targeted therapeutic treatments. All of these technologies rely on a safe and biocompatible materials as, e.g., a scaffold for cells. Gelatin and collagen are biomaterials that are highly suitable for a range of regenerative medicine applications, including:

- Implantable membranes
- Wound healing
- Stem cell and organoid culturing
- Advanced drug delivery, for small and large molecules (biologics)
- 3D bioprinting and tissue engineering



3D printing of gelatin

Implantable membranes

Implantable fibrous membranes can act as scaffolds and support to tissue following trauma or surgical procedures. However, these applications require highly biocompatible and bio-absorbable materials. Implantable membranes, woven or nonwoven, can be prepared from gelatin among others using electrospinning to provide a favorable cell growth scaffold. Residual endotoxins are a common cause of complications in surgical applications. In the United States alone 40,000 out of 500,000 annual arthroplasties require revisions due to aseptic loosening, which is often associated with the presence of endotoxins (Goveia, et al., 2016).

Wound healing

Wound healing provides one of the greatest challenges for healthcare today, with post-surgical complications and chronic wound costs greater than those for asthma, dementia or obesity (Institute for Pressure Injury Prevention, 2017).

Gelatin is a suitable material for use in skin grafts and scaffolds to enhance healing of wounds, and found to be suitable for use also in combination with other (bio)materials. By using among others cross-linking, the mechanical strength and microstructure can be precisely tuned (Nikkhah, et al., 2016). Prolonged inflammation is often a complication preventing normal wound healing and hence, minimizing additional pro-inflammatory stimuli from endotoxins within grafts and scaffolds could reduce the risk of sustained inflammation. X-Pure offers a complete range of low endotoxin gelatin and collagen suitable for scaffold, graft and wound dressing applications where low immunogenicity is desired.

Stem Cell and Organoid Culturing

When culturing cells, it is challenging to maintain cell viability and to achieve efficient differentiation. Gelatin has been shown to be a medium in which stem cells can grow and differentiate easily (Nikkhah, et al., 2016). As a recent example, scaffolds prepared from gelatin sponges incorporated with methacrylic anhydride and ß-tricalcium phosphate achieved high cell viability and osteogenic differentiation of human adipose-derived stem cells in a bone regeneration model (Lee, et al., 2019). However, stem cells have been demonstrated to elicit host defense responses even in the presence of trace amounts of endotoxins (Nomura, et al., 2018 and Lieder, et al., 2013).

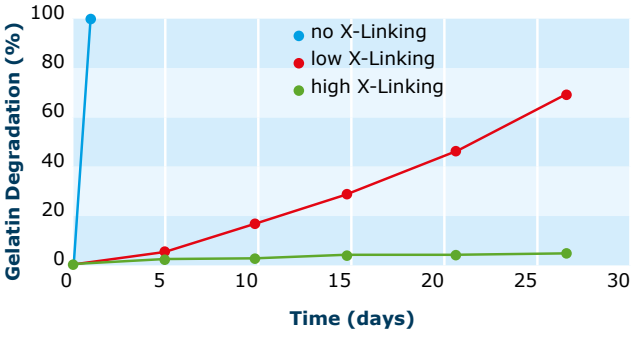
Endotoxin levels higher than 0.05 EU/ml have been found to inhibit osteoblast differentiation (Kadono, et al., 1999) and proliferation of hemotopietic stem cells (Rinehart, et al., 1997). For organ-on-a-chip, e.g. for toxicity screening, low endotoxin levels are important to avoid unwanted cell behavior and functionality which can lead to misinterpretation of results (Tarrant, 2010). With X-Pure, gelatins and hydrolyzed collagens are now available with endotoxin levels complying with highest requirements. This makes it possible to leverage the benefits of gelatin in a wide range of cell culture and organ-on-a-chip applications without any concern related to the negative impact from endotoxins.

Advanced drug delivery

Gelatin has demonstrated great utility and versatility in many advanced drug delivery systems. Examples include large and small molecules (biologics) encapsulated in micro- and nano-spheres, gel films, ocular inserts, and eye drops, thereby offering great flexibility to control drug release profiles. In addition, bio-availability of drugs from gelatin can be tuned across a wide range of chemistries, i.e., hydrophobic, hydrophilic, or neutral. Drugs that have struggled from poor bio-availability have been successfully formulated with gelatin (Nikkhah, et al., 2016).

In such drug delivery applications any remaining endotoxins can cause significant negative effects (Vallhov, et al., 2006). With X-Pure now available it is possible to minimize the risk of endotoxin induced immune responses in advanced drug delivery applications.

Gelatin particle degradation at 40°C at different crosslinking densities



Wang et al., Journal of Controlled release 166 (2013) 172-181

Tissue engineering and 3D bioprinting

Tissue engineering and bioprinting are receiving significant attention and investment, based on the prospects to create customized organs-on-a-chip for drug and cosmetics development as well as to print cell-laden scaffolds for human tissue engineering (Derakhshanfar, et al., 2018). Combined with for example methacrylamide, gelatin can be used to create UV initiated cross-linked scaffolds of virtually any shape and with a large range of mechanical properties and suitable for a range of tissue engineering applications (Malda, et al., 2013), including new bone formation (Visser, et al., 2015). Such scaffolds can additionally be engineered for controlled release of a variety of biomolecules (Nikkhah, et al., 2016). In such applications it is important to minimize the risk that the new bioengineered tissue gets rejected via the immune system. With X-Pure, it is now possible to produce such 3D printed biomaterials with a low risk of an immune reaction to endotoxins within the scaffold.

In all of these applications, gelatin offers attractive physical properties such as adjustable gel strength and melting characteristics. In addition, gelatin is highly biocompatible and biodegradable (Gelatin-based biomaterials for tissue engineering and stem cell bioengineering. Biomaterials from Nature for Advanced Device and Therapies, First Edition. ISBN-10: 1118478053. Nuon M. Neves, et al., 2016).

INFLUENCE OF AUTOCLAVE STERILIZATION OF GELATIN ON PHYSICAL PROPERTIES AND ENDOTOXIN LEVEL AND THE INFLUENCE OF ENDOTOXIN LEVEL ON ENDOTHELIAL CELLULAR ACTIVITY

In a recent poster (Vanhoecke and Olijve, 2018), the influence of autoclave sterilization of gelatin and endotoxin levels on endothelial cellular activity was evaluated. The aim of this study was to determine the influence of autoclave sterilization of gelatin on physical properties and on endotoxin levels. Additionally, the influence of endotoxins on cellular viability of an endotoxin-sensitive endothelial cell line was evaluated.

Method:
A 10% solution of a type A gelatin from Sigma-Aldrich (G1890) with endotoxin level of 20,000 EU/g was autoclave sterilized during 30 minutes at 121°C and 1 bar overpressure. The molecular weight, gel strength and the endotoxin level of the sterilized G1890 gelatin was compared to a purified (WO2016085345) X-Pure Rousselot (Sigma-Aldrich) gelatin with an endotoxin level < 10 EU/g. The endotoxin levels of the gelatins were measured using the Endozyme recombinant factor C method from Hyglos GmbH (Germany). A 1% solution was used to measure the effect of both gelatins on mitochondrial activity and cellular viability of an endotoxin-sensitive endothelial cell line.

Results:
Autoclave sterilization of G1890 gelatin gave a significant decrease in gelatin physical properties (average molecular weight and gel strength), demonstrating that autoclave sterilization is less suitable - compared to non-autoclaved Rousselot X-Pure gelatin - for applications where physical properties are important, like bioprinting. Additionally, the experiment demonstrated that autoclave sterilization is not sufficient to inactivate endotoxin in gelatin (Figure 1). Finally, a clear reduction in endothelial cellular activity (Figure 2) and viability (Figure 3) was observed with increasing endotoxin levels which indicates that low endotoxin levels are important in applications such as tissue engineering using living cells.

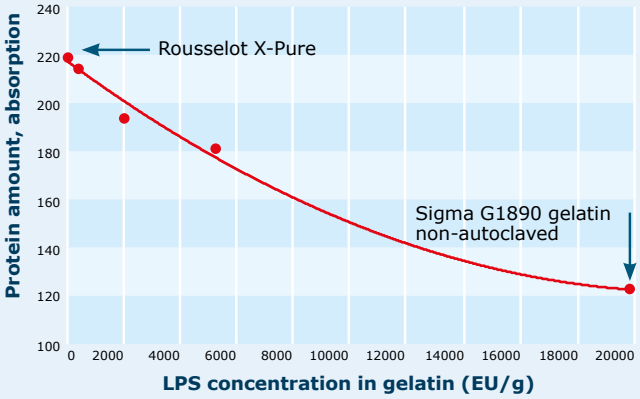


Figure 2. Effect of Sigma G1890 and Rousselot X-Pure gelatin solution (1%) on cell growth (measured by protein amount) of an endotoxin-sensitive endothelial cell line.

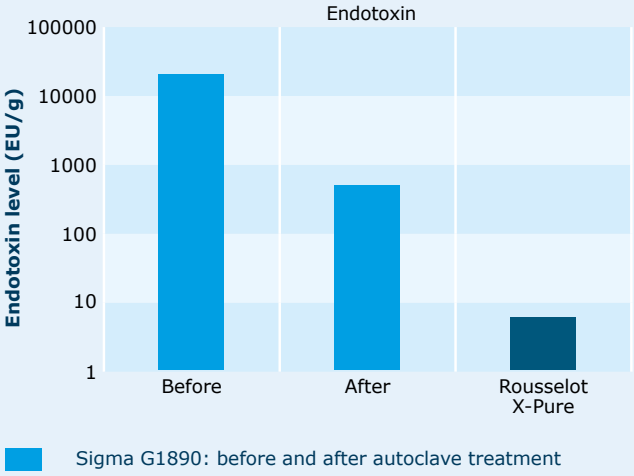


Figure 1. Endotoxin level of Sigma G1890 gelatin before and after autoclave treatment compared to Rousselot X-Pure gelatin. Autoclave treatment reduced the endotoxin level of G1890 gelatin, however the endotoxin level is still significantly higher compared to non-autoclaved Rousselot X-Pure gelatin.

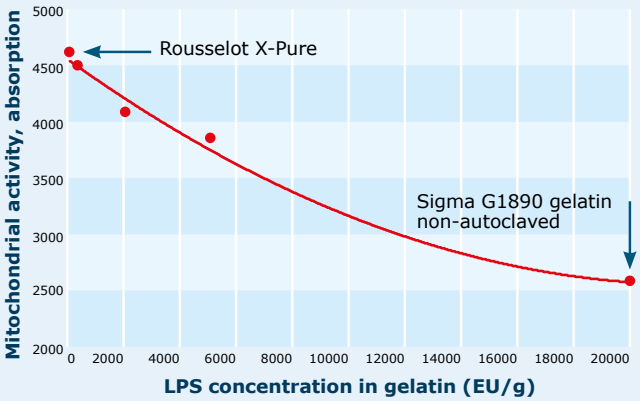


Figure 3. Effect of Sigma G1890 and Rousselot X-Pure gelatin solution (1%) on cell viability (measured by mitochondrial cell activity) of an endotoxin-sensitive endothelial cell line. Both figures (2 & 3) indicate that the cellular activity and growth of the endothelial cell line was largely influenced by endotoxin. The G1890 Sigma gelatin with endotoxin level of 20,000 EU/g gave significantly lower cellular activity compared to purified Rousselot X-Pure gelatin with endotoxin level <10 EU/g.

ROUSSELOT - YOUR PARTNER FOR ADVANCED APPLICATIONS OF GELATIN/COLLAGEN

When choosing X-Pure, you will additionally benefit from Rousselot's global expertise established from more than 125 years of gelatin manufacturing and collaborations with several leading Universities. As a Rousselot customer you will be supported by a dedicated local team in each of your markets to secure timely customer support and access to the best solutions to your product requirements. You can rely on world-class products that meet the highest global quality and safety standards, delivered in spec and on time. At Rousselot we adhere to the highest ethical standards, both in our commitment to respect the environment and through the integrity and transparency we show you.

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