Special Issue on
Industrial Biotechnology-Made in Germany: The path from policies to sustainable energy, commodity and specialty products

Edited by:
Dr. Thomas Brück
Professor of Industrial Biocatalysis, Dept. of Chemistry, Technische Universität München (TUM), Germany
Shaping the Future with Industrial Biotechnology—New and Efficient Production Processes for Biopolymers

Bendig, C., Kraxenberger, T., Römer, L.*
AMSilk GmbH, Planegg/Martinsried, Germany

Abstract

The large-scale production of biopolymers has been an emerging branch of industry for decades. Besides mere substitution of oil-based polymers, biopolymers with innovative features are in the focus of research and industry. This review highlights modern high-impact biopolymers, their respective industrial production processes and relevant applications.

The vast majority of prominent commercial biopolymers are either made from sugars (such as cellulose derivatives), acids (such as poly lactic acid) or proteins (such as silk). Some of the more simple biopolymers such as γ-polyglutamic acid and bacterial cellulose are mainly produced in their native microorganisms. A more challenging trend in biopolymer production is the switch from traditional extraction or conversion of natural products to recombinant/heterologous production techniques in microorganisms. This is analyzed in detail for collagen, hyaluronic acid and silk. Despite the complexity of these biopolymers in structure and production, all share important features such as biocompatibility, adjustable shapes and slow biodegradation. The combination of properties renders these polymers ideal materials for biomedical scaffolding, surgery and wound care as well as related pharmaceutical applications and drug delivery.

ABBREVIATIONS

CG: Collagen; HA: Hyaluronic Acid; PGA: γ-Polyglutamic Acid; BC: Bacterial Cellulose

INTRODUCTION

Nowadays, green products and green chemicals gain more and more importance—not only in public discussion but also in industry’s practice. At the same time traditional chemistry is facing another challenge manifested in the rising need for substitution of oil-based polymers. For instance, for applications in medical devices, the complexity of the materials required is rising. The need to avoid risks of contaminations such as viruses.

Biopolymers are essentially different from traditional synthetic polymers in terms of their biocompatibility and biodegradability. They are generally characterized by the chemical background of their monomers: Polypeptides, polysaccharides and nucleic acids, among others. The unique properties of these polymers make them ideal materials for biomedical scaffolding, surgery and wound care as well as pharmaceutical applications.

Most available biopolymers are either isolated from nature or produced by native existing organisms such as silk, xanthan and polyhydroxybutyrate [2]. These polymers serve as cocoons, capsule or storage material, respectively, in their corresponding organisms. Since scientists elucidated the molecular and genetic basis of biosynthesis pathways, a rising number of these polymers were understood and after years of research some of them produced recombinant by genetically modified microorganisms. Productions utilizing microorganisms do not only enable industry to produce significant quantities within relatively short production times in a compact volume. It also ensures production independently of sometimes only locally available, costly and – most important – limited natural precursors. Further, recombinant biopolymers can be produced animal-free, lowering risks of contaminations such as viruses.

In this review important biopolymers which were introduced to the marked in recent years are highlighted. The focus lies on multi-billion dollar markets such as esthetic and reconstructive...
surgery as well as related biomedical and pharmaceutical applications including topical wound treatment and drug delivery. All these applications have overlapping needs for their material of choice: biocompatibility, scaffold forming capabilities and slow biodegradation.

BIOPOLYMERS

Poly-γ-glutamic acid (γ-PGA)

Polyglutamic acid (PGA) is a polymer of the amino acid glutamic acid (figure 1). In contrast to common proteins, the peptide bonds are in this case between the amino group and the carboxyl group at the end of the side chain (γ-linkage of the molecules). Three different types of γ-PGA are known, which of consist of D-glutamic acid (D-PGA), L-glutamic acid (L-PGA) or a mixture of both (DL-PGA). DL- and L-PGA can be produced by several different microorganisms, mainly Bacillus species, in different chain lengths up to 10,000 kDa. In contrast, D-PGA is only produced by Bacillus anthracis [6]. Furthermore, a chemically synthesized α-PGA with α-linked glutamic acid is available which can also be obtained by enzyme catalysis [7].

Production of PGA by Bacillus performed in classic stirred tank reactors. A steady supply of L-glutamic acid and/or ammonia sources is necessary to achieve high productivity. Yields up to 101.1 g/L [8,9] were described in the literature. The issue known for most fermentative extracellular polymer productions, namely the increasing viscosity to approximately 4 Pa·s and the correlated mass-transfer problems especially for oxygen, is also the biggest challenge to produce PGA [8].

PGA is utilized in many different fields of application. A major application is the use as floculent in waste-water treatment [10,11]. An outstanding property of PGA is its ability to bind heavy metals which is an advantage in the water treatment [12]. The food safety and its metal binding feature can be exploited to increase bioavailability of essential but not readily soluble metals in functional food [13]. Additionally the use of PGA as protectant for nutritional probiotic bacteria during freeze-drying is discussed on this background [14].

Another application is based on its hygroscopic effect. The use of PGA in cosmetics as moisturizer is more and more accepted [15]. Furthermore the use in drug-delivery is promoted by the humidity of PGA and especially the use for anti-cancer drugs is under research [16,17].

In contrast to other biopolymers discussed here, the optimization of the PGA production focuses on already
established Bacillus strains [18,19]. Additionally, new strains from natural sources are permanently identified (e.g. natto, a traditional Japanese fermented food) also by high-throughput screenings [19].

**Cellulose**

In contrast to PGA, cellulose is directly produced from natural resources. Cellulose is a polysaccharide composed of β-(1-4)-linked D-glucose molecules. As cell-walls of plants contain approx. 50% cellulose, it is the most abundant organic compound available on earth. The β-linkage of the glucose molecules forms a strand, which is more robust then the α-linkages in storage polysaccharides such as in starch. Cellulose is used in the energy sector, the pulp and paper production as well as in food and pharmaceutical applications. Furthermore, sugar derived from lignocelluloses is thought to play a rising role in microbial production processes of for instance ethanol or lactic acid. Traditionally, cellulose is extracted from plants. Derivatives like methylcellulose, cellulose acetate or nitrocellulose are enhanced by chemical modifications to obtain new properties. Besides plant cellulose, bacteria, fungi and algae also produce cellulose. Bacterial cellulose (BC) is mostly produced with Acetobacter (or Gluconacetobacter) xylinum and its microfilms are approx. 100x smaller than plant cellulose [20,21]. Secreted BC single-strains assemble to highly crystalline structures - according to specific culture methods [22]. This crystallinity is characterized by finely structured high density layers.

BC has numerous applications and thereby a high potential as industrial relevant material. The main disadvantage is, however, the relatively slow growth of the BC on the interface between substrate and the required oxygen enriched medium. A cellulose film of proper size requires 2-15 days (summarized in [21,23]). Even though different kinds of reactors (stirred tank reactors, rotating disk reactors, airlift reactors and belt-reactors (HoLiR, [24]) are available for production, all have severe disadvantages [21]. Especially the formation of non-producing mutants in aerated reactors has to be overcome [25], which can be achieved for example by optimization of production conditions. In addition, production strains were improved towards a homogenous BC producing phenotype [26] with better yield by reducing the production of metabolic side-products [27]. Further improvement of productivity can be achieved by using genetic approaches (reviewed in [21]). The use of BC for medical devices is believed to have a big potential as it is biocompatible and stable. Today, BC is already used for the coverage of heavy wounds which is a straightforward application [20]. The use as vascular grafts is a more ambitious aim which is still under research [28]. Although the main sources of cellulose are plants, the biotechnological production of cellulose has distinct advantages, especially for biomedical applications. For example the MW of the polymer, the density of aggregates and also the shape can be varied, either by production process optimization or by genetic manipulation of the producing organism. On the long run, this can also facilitate new products and applications.

**Chitosan**

At a first glance, the production and application of Chitosan is similar to cellulose. It is produced by chemical treatment of natural occurring material, in this case the chitin of crustaceans. Nevertheless, chitosan is suited for more applications with unique features, making it a versatile polymer for technical and medical applications. Chitosan is a β-(1,4)-glycosidic poly amino sugar. It is constructed from the monomer N-acetylglucosamine. The mass lies between 10 and 10,000 kDa [29]. In Chitosan less than 40 % of the monomers are acetylated [30]. In contrast to chitin, chitosan has a cationic nature in its protonated state rendering it water soluble.

Chitosan can be produced from Chitin by deacetylation with alkali or with enzymes [31]. Chitin is a major waste-product in the production of crustaceans and today mainly used for the production of Chitosan. Nevertheless, the chemical modification is not environmentally friendly [32] and has strong influence on the molecular weight and the amount of deacetylated residues [33]. To overcome these problems a biotechnological approach has been developed in which the crustacean residues are fermented by lactic acid bacteria and/or treated by proteolytic enzymes. This process dramatically reduces the use of acids and alkali and produces chitosan with a higher nutritional value, due to a higher content of protein residues in the final product [34]. Chitin can also be isolated from fungi. Even though the chitin contain in fungi is less than in crustaceans the production can be done in stirred tank reactors under controlled conditions and is thought to be economical feasible [35].

The properties of chitosan are typical for modern biopolymers and they directly imply its industrial use: it is biocompatible, biodegradable, an adsorption enhancer, antioxidant and antimicrobial among other features [29]. Its high capacity to bind heavy metals and the usability as a flocculation agent makes it a good material for waste water treatment comparable to PGA as mentioned above [36]. The ability to form aggregates can also be used for paper production [37]. The properties of chitosan to be an anion exchanger cannot only be used in waste water cleaning, but also as filling material for chromatography. Especially fungal chitosan has advantages due to the uniformity of particle size [38]. Its antimicrobial effect is exploited in water cleaning procedures [39] the beer-brewing process [40], wound coverage [41] and textile production [42]. In pharmaceutical applications a big potential in the drug delivery is claimed. Here the possibility to make chemical modifications on the material and the different solubility with changing pH has a big potential to work as a reservoir and a specific carrier [29,43].

**Hyaluronic acid (HA)**

Besides processed biocompatible material such as cellulose and chitosan, materials already present in the human body should be per se compatible, offering even more sophisticated applications. Hyaluronic acid (HA), also called hyaluronan is a simple, linear polymer of the glycosaminoglycan family (figure 1). HA is produced by all vertebrates and Streptococci group A and C [44,45]. As HA from all known sources is chemically identical, the HA from animal or microbial resources is always directly biocompatible to humans. It consists of disaccharide repeats of D-glucuronic acid (GlcUA) and N-acetylgalactosamine (GlcNAc) joined alternately by β-1,3 and β-1,4 glycosidic bond with a MW from 104 to 107 Da. The synthesis via HA synthases is
well studied and understood [46-48]. Various states of HA such as hydrogels, fibers meshes or sponges have been described [49]. The most prominent applications are currently dermal filler and regenerative medicine applications such as scaffolds for tissue engineering [50]. The world market doubled in the past years to approx. 1 billion USD [51,52].

The main commercial sources of HA are animal tissues, particularly rooster combs, and bacteria (Streptococci). These source simply contamination risks of proteins, DNA, viruses and endotoxins of animal or bacterial origin, respectively and require extensive and harsh purification strategies [53]. Improvement of Streptococci strains, optimized fermentation media and conditions lead to yields of 6-7 g HA per liter fermentation broth (reviewed in [52]).

A new trend in HA production is the optimization towards a defined molecular weight. Low MW-HA for instance stimulates the immune response [54]. Bacillus subtilis lacking HA degrading enzymes mitigates this problem. It has also been reported recently, that metabolic engineering and induction strategy lead to more uniform HA products [55]. Additionally, "generally recognized as safe" (GRAS) organisms such as Bacillus subtilis minimize risks of contamination with toxines.

Engineered B. subtilis is propagated to be the major player in recombinant HA production (reviewed in [52], [56-59]. Patents, publications and journal articles indicate, that HA produced in B. subtilis is available in sufficient yields and purity [58,60, 61]. “Hyasis”, a hyaluronic acid with improved properties produced in B. subtilis was put on the market by Novozymes and is propagated to be the new generation HA (www.hyasis.com).

New HA derivatives with defined rigidity can only be achieved by crosslinking due to the invariable core structure of HA. The challenge lies in identifying non-toxic or irradiant cross linkers. In order to form highly stable scaffolds for tissue regeneration, cross linking via di-glycidyl ether was reported. Lyophilized sponges were chemically derivatized and showed high stability against enzymatic degradation [62].

HA is an important component of e.g. the extracellular matrix in vertebrates, but it is exceeded in mass by collagen, the predominant structural polypeptide.

Collagen

Collagen (CG) and HA show similar properties and occurrence but a very different chemical background. Collagen is a polypeptide, thereby intrinsically exhibiting more variability due to its individual amino acid composition. The sequence defines the biochemical properties, leading to more complex and graduated functions in both, native as well as artificial applications. It is the predominant structural protein in various connective tissues and extracellular matrices in humans and animals and makes up 5% to 35% of their entire protein. It is the main component of fascia, cartilage, ligaments, tendons, bone and skin [63]. The CG super family comprises 28 different types of collagen proteins to date. Although this family is highly diverse, most - if not all - share a unique repeated amino acid motif Gly-Pro-X or Gly-X-Hydroxyproline, where X may be any amino acid [64,65]. CG is synthesized in a well understood procedure including cleavage of N- and C-termini and hydroxylation of prolyl- and lysyl-residues, N-linked oligosaccharides, glycosylation of hydroxylysyl residues and disulphide bond formation, which takes place in defined cell compartments. Mature CG finally assembles to fibers with diameters ranging from 0.5–3 microns [65].

Pure CG is suited for various sophisticated applications. It can be used for medical and cosmetic tissue engineering, drug delivery and wound care [66,67]. CG isolation from various animal tissues has been developed for years and includes harsh chemical and enzymatically extraction methods [68]. As discussed above for hyaluronic acid, recombinant production of CG improves yield, purity and productivity in comparison to animal sourced CG. Plants, insect cells, yeast and bacteria are used to produce recombinant CG or CG like proteins. Recombinant human collagen type I has been successfully expressed in tobacco [69]. By co-expression of a special set of human enzymes, post translational modifications are also possible. The properties of such a material are comparable to natural derived CG, yielding an optimal alternative to natural sources [70].

Pichia pastoris was engineered to produce recombinant human collagen intracellularly, featuring a hydroxylated triple helical molecule at levels up to 1.5 g/L. Hydroxylated collagen was produced by coexpression of recombinant collagen with recombinant prolyl-hydroxylase [71]. Further engineering of the yeast lead to collagen fragments with defined length and composition and high expression levels (3 to 14 g/L) [72]. The performance of the material can be seen by a cornea derived from recombinant CG which was stably integrated into human without rejection for four years [73].

Although bacteria commonly do not have the machinery for posttranslational modifications, short and cheap cultivation and production times together with easy strain/product engineering make it favorable to explore this strategy [74].

The main function und thus the main applications of CG are determined by its unique triple-helical structure, making it a versatile scaffold. Leaving the world of vertebrates, even more specialized structural proteins with extraordinary properties suited for multiple applications can be found.

Silk

Silk is a natural protein fiber traditionally obtained from insect cocoons and commonly associated with Bombyx mori fibers. Silk and its fabrics are characterized by low weight, decent tensile strength, isolating properties, among others. In hydrolyzed form silk is used in cosmetics. However, Hymenoptera (e. g. bees, wasps, ants) and especially Arthropods (e. g. spiders) produce even superior silks, which display higher toughness and ductility as well as better mechanical and chemical resilience (figure 1). Thus, spider silk is the most outstanding non-bombyx silk and in the focus of research in academia and industry for decades. Silks of the genera Araneus (European garden cross spider), Nephila (golden silk orb-weaver) and others were identified and are still studied extensively. Spiders produce different types of silk for different functions in their webs and cocoons such as scaffolding silks, capturing lines or dragline silk. The latter is used for the webs outer rim and is characterized by high tensile strength and...
The dragline of *Nephila clavipes* consists of two predominant proteins MaSp1 and MaSp2, each with a molecular size of more than 300 kDa. These proteins are comprised of highly repetitive motifs with an unusual high alanine, glycine and proline content (MaSp2). The dragline of *Nephila clavipes* contains approx. 80% MaSp1 and approx. 20% MaSp2, leading to a high tensile strength exceeding 1500 MPa [76].

As spiders are territorial, they cannot be farmed easily and silk fibers would have to be prepared manually from each single spider. Since the publishing of a (partial) spider silk amino acid sequence the work on its recombinant production in engineered host organisms began [77].

Spider silk can be produced in goats [78], plants [79], yeast [80], silkworms [81] and many more systems. None of these platforms ever reached industrial scale. The breakthrough

---

**Table 1: Summary of biopolymers, their origin, potential production organisms and features.**

<table>
<thead>
<tr>
<th>Biopolymer</th>
<th>Monomer</th>
<th>Natural origin</th>
<th>Production Microorganism</th>
<th>GMO</th>
<th>Biodegradable</th>
<th>Biocompatible</th>
<th>Hydrogel</th>
<th>Fiber</th>
<th>Commercialized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>glucose</td>
<td>Plants, Micro-organism</td>
<td><em>A. xylinum</em></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Wound covering</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>N-acetyl-glucoamin</td>
<td>Vertebrates, Streptococci Streptococci, B. subtilis</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Dermalfillers, Vis-cosupplementation</td>
<td></td>
</tr>
<tr>
<td>γ-PGA</td>
<td>Glutamic acid</td>
<td><em>Bacillus spp.</em>, Microorganism <em>Bacillus spp.</em></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Cosmetics, fertilizer, flocculant</td>
<td></td>
</tr>
<tr>
<td>Silk</td>
<td>protein</td>
<td><em>Bombyx mori</em>, spider, bee <em>E. coli,</em></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Cosmetics, cell adhesion scaffolds</td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>protein</td>
<td>Vertebrates</td>
<td>Yeasts</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Scaffolds, Cosmetics</td>
</tr>
<tr>
<td>Chitosan</td>
<td>N-acetyl-glucoamin (partly deacylated) fungi Yeast, bacteria</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Flocculant, Filter membranes, Cosmetics, wound covering</td>
<td></td>
</tr>
</tbody>
</table>

---

Figure 2 Shapes of recombinant spider silk. Possible shapes of biopolymers are presented on the example of recombinant spider silk. Other biopolymers discussed in this review are able to adapt at least one or more of these shapes. Similar to natural spider silk, recombinant silk can be converted into fibers. It is also possible to transform silk proteins in other two- or three-dimensional shapes such as films, hydrogels, foams, capsules and spheres (pictures with courtesy from AMSilk GmbH, 2014).
for industrial scale production was achieved by Scheibel and coworkers [82]. Engineered domains of the dragline proteins ADF3 and ADF4 from Araneus diadematus can now be produced in established E. coli production processes. Recombinant spider silk is an ideal material for various industrial sectors such as cosmetics, pharmaceutics or technical products. It is available in form of coatings, particles, films, nonwovens, hydrogels and silk threads (figure 2). Together with its biocompatibility the proteins offers ideal prerequisites for implant coatings. A recent study demonstrated that spider silk coated silicone implants are significantly better accepted than common, non-coated implants [83].

As silk monomers can be specially engineered and extended with other protein domains, nearly any functional protein entity can be fused to spider silk proteins, thereby adding a new functionality to the spider silk properties. Wohlrab and collegues [84] showed, that a film with a spider silk derivative comprising the integrin recognition sequence (RGD-motif) significantly improved adherence of for instance BALB/3T3 mouse cells. These findings can be assigned to applications, where specific cell lines are directed to scaffolds or tissues in reconstruction medicine. Spider silk based materials can additionally be functionalized with covalently or non-covalently bound compounds. Recent studies indicate, that enzymes and drugs can be imbedded in silk, which are later released linearly [85,86].

CONCLUSIONS

All presented biopolymers show similar properties even though they sometimes arise from totally different origins. They are biodegradable, even though they are very stable under most conditions. Furthermore, they all are biocompatible and applications in form of medical devices or in pharmaceutical products already exist or are under development. Most of them can be used as hydrogel or as a fiber independently of their original, natural appearance (table 1).

The main difference between the described biopolymers is their respective industrial production process. Bacterial cellulose, hyaluronic acid, γ-PGA and chitosan are in nature already produced by microorganisms. Their production under controlled conditions in bioreactors is thus possible without demanding changes within the production organism. Silk and collagen can in principle be directly produced from their originating animals/insects, too. Nevertheless, production from animals always holds the risk of diseases and impurities due to the nature of living higher order organisms. In the near future, technological improvements achieved by applying genetically modified organisms and/or newly designed production systems (as for instance in the case of cellulose) will further improve the availability of materials with high purity and quality.

The complexity of these production processes becomes evident after taking a closer look into the structure of the companies producing such materials. Bacterial cellulose is classically produced in simple production systems, but academia has developed a special production reactor which eventually led to the founding of Jenacell (Germany). The production of hyaluronic acid in B. subtilis has been developed by Novozymes (Denmark) one of the biggest biotech companies in the world. Kytozyme (Belgium) - the first company which started to produce Chitosan from fungi in 2007 - also originates from academical research. The same is true for the production of spider silk.

The proprietary silk platform technology of AMSilk (Germany) was also developed in a university lab. Five years after its formation, AMSilk is the first company offering commercial products made from spider silk [87]. These products include for example cosmetical bulk ingredients such as Silkbeads and Silkgel. In parallel, a new and unique medical product, the SanaSilk skin protection spray, has been developed and is scheduled to hit the market in 2014. More sophisticated medical devices such as spider silk covered silicone implants show significantly reduced post-operative inflammation as well as reduced capsule formation in comparison to untreated silicone implants [83]. Here, further development is ongoing. The first artificial spider fiber (Biosteel) was presented early 2013. Currently the production process for this fiber is evaluated on pilot-scale.

The presented examples demonstrate that innovative biopolymers are in many cases not developed by multinational corporations, but by specialized research laboratories and/or dedicated small companies. As is true for most breakthrough inventions, money and time are critical parameters. (Federal) Investment in research and universities is thus a prerequisite for commercial success in industrial biotechnology, accelerating new and sophisticated products without further exploiting our environment.

REFERENCES


