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▶ **To cite this version:**

Yu Ogawa, Jean-Luc Putaux, Yoshiharu Nishiyama. Crystallography of polysaccharides: Current state and challenges. Current Opinion in Chemical Biology, 2022, 70, pp.102183. 10.1016/j.cbpa.2022.102183 . hal-03714972

HAL Id: hal-03714972
https://cnrs.hal.science/hal-03714972

Submitted on 6 Aug 2022

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Crystallography of polysaccharides: Current state and challenges.

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Abstract

Polysaccharides are the most abundant class of biopolymers, holding an important place in biological systems and sustainable material development. Their spatial organization and intra- and intermolecular interactions are thus of great interest. However, conventional single crystal crystallography is not applicable since polysaccharides crystallize only into tiny crystals. Several crystallographic methods have been developed to extract atomic-resolution structural information from polysaccharide crystals. Small-probe single crystal diffractometry, high-resolution fiber diffraction and powder diffraction combined with molecular modeling brought new insights from various types of polysaccharide crystals, and led to many high-resolution crystal structures over the past two decades. Current challenges lie in the analysis of disorder and defects by further integrating molecular modeling methods for low-resolution diffraction data.

Keywords: Polysaccharides, Crystallography, Fiber diffraction, Electron diffraction

Introduction

The knowledge of the spatial arrangement of molecules is fundamental to a detail understanding of the properties and molecular interactions in materials and biological systems. Single crystal analysis using millimeter-sized crystallites is a standard tool to analyze such structures at atomic or subatomic resolution. However, in many practical systems, single crystals of the corresponding structure are not available. Polysaccharides, the most abundant biopolymers on Earth, are one of such systems that do not yield macroscopic single crystals but polycrystalline aggregates of smaller crystallites. This polycrystalline nature has posed a challenge and led to methodological developments in the structural determination of polysaccharide crystals. In this article, we review the recent

advances in the crystallography of polysaccharides in their native or recrystallized state (Fig. 1). In particular, we will focus on the crystallographic methods for three different sample forms of polysaccharides, namely polycrystalline fibers, microscopic single crystals and crystalline powders. Finally, we will discuss the current challenges in the field of polysaccharide crystallography.

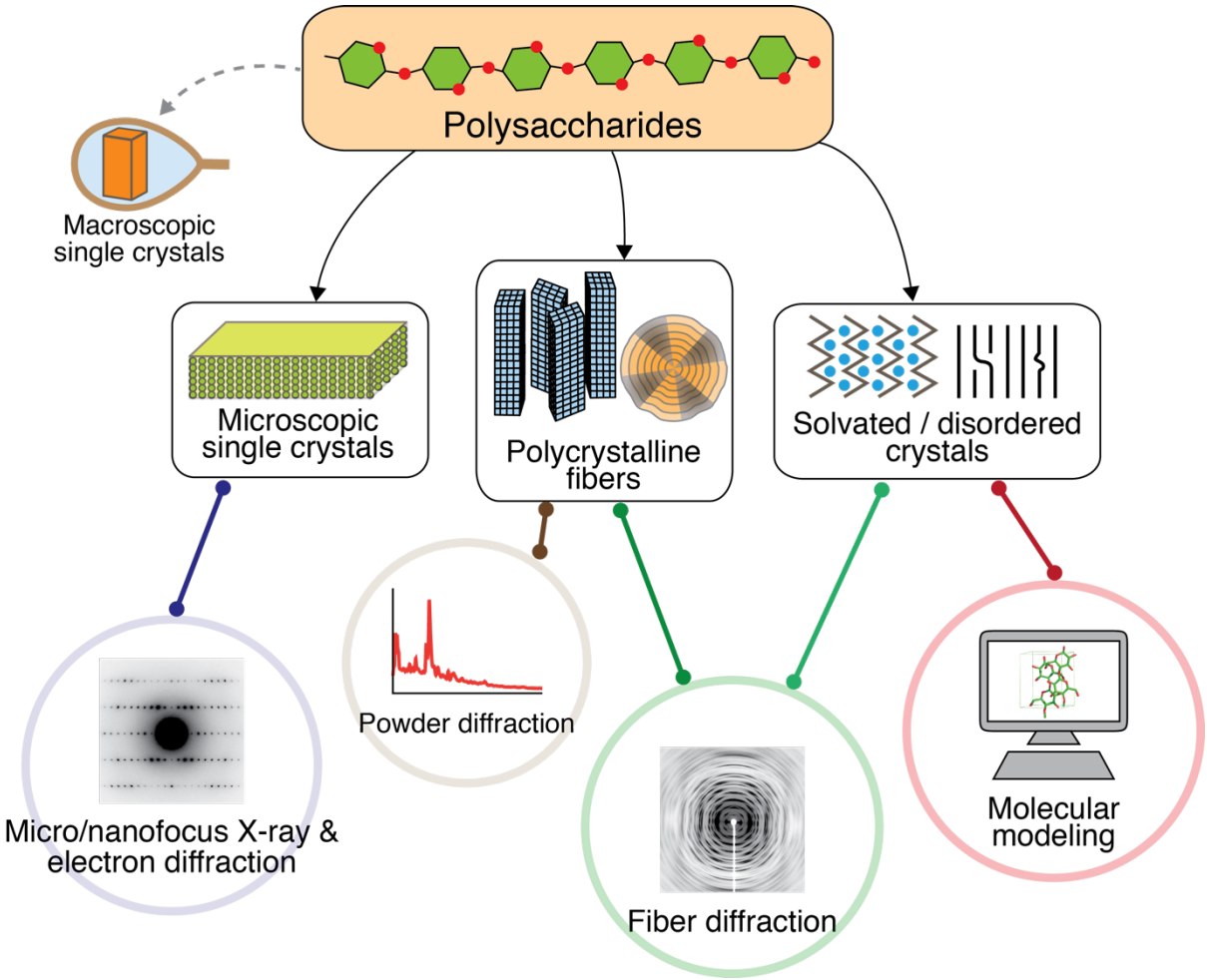


Figure 1. General scheme of polysaccharide crystallography. Polysaccharides are not crystallized into macroscopic single crystals but yield microscopic crystals with different geometries and features. Several crystallographic methods are available for structure determination of such small polysaccharide crystals.

Polysaccharides and their crystallinity

Polysaccharides are a class of molecules constituted of carbohydrate units (sugars) linked via glycosidic linkages. Potentially, the variety in molecular structures is huge, because each sugar can link at different positions, with two possible anomeric configurations, and it can contain different monomer sequences [1]. However, in terms of biomass quantity, relatively

simple homopolymers, such as cellulose and chitin, are dominant. Those are also the molecules that tend to crystallize. Such carbohydrate crystals provide rich experimental information about the details of molecular packing. In recent years, they have attracted increasing attention as renewable resources for materials, thanks to their abundance, renewability, biocompatibility, low toxicity, and interesting material properties.

Polysaccharides are traditionally categorized into two groups based on their *in vivo* functionality, namely structural and storage polysaccharides. The former includes cellulose [2] and chitin [3]. The structural polysaccharides confer the mechanical strength and support to biological tissues. For instance, cellulose is the primary load-bearing material in plant cell walls, and chitin is a structural component in crustacean/insect exoskeletons and fungal cell walls. The storage polysaccharides, on the other hand, serve as the energy storage in cells. Its representative examples are starch [4], β-1,3-glucan (paramylon) [5], and inulin [6] in plants, and glycogen in animals and fungi [7]. Mannan has a dual nature as it can be found both a structural and storage polysaccharide [8]. For instance, galactoglucomannans are one of the main structural components in soft wood cell walls, while linear mannans are found as energy storage in plant seeds such as ivory nuts. The polysaccharides in the extracellular matrix, such as hyaluronic acid, possess specific functionalities (e.g. lubricants in the joints) and are not categorized into either of the two traditional groups [9].

In nature, structural polysaccharides often occur as fibrous objects in which the chains are aligned along the long axis of the molecular assemblies. In particular, cellulose and chitin are biosynthesized into crystalline nanofibrils. Other structural polysaccharides are found naturally in amorphous matrices and sometimes associate with other fibrous components (e.g. hemicelluloses) [10]. The native state of the storage polysaccharides can vary. For instance, paramylon and starch are biosynthesized in plant cells as several micrometer-large semicrystalline granules [4], while glycogen is an amorphous nanoparticle [7].

Regardless of the *in vivo* functionality, most linear polysaccharides have a strong crystallization tendency. This characteristic has been exploited to prepare crystalline specimens suitable for the crystallographic analysis as described below. Furthermore, polysaccharides often exhibit polymorphism. Different crystalline allomorphs can be obtained under different *in vitro* crystallization conditions. The polymorphism also occurs in natural polysaccharide crystals (e.g. cellulose Iα and Iβ, α- and β-chitin) [3,11].

Crystallographic methods for polysaccharides in different geometries

Conventional single crystal analysis is not applicable to the structural determination of polysaccharides due to the lack of availability of their macroscopic crystals. Several other crystallographic methods are available for microscopic polysaccharide crystals. Each of them is applicable only to specific sample geometries as described below.

Fibrous crystals

Polycrystalline fibrous assemblies yield robust and reproducible fiber diffraction patterns that contain invaluable structural information. One famous example is the X-ray fiber diffraction pattern of DNA that led to the model of its helical structure (Nobel prize 1962) through a trial-and-error model building. Fibrous polysaccharides have been providing such fiber diffraction patterns since over a century [12]. The prerequisite of the fiber diffraction analysis is the cylindrical symmetry of the crystallites along the fiber axis. While there are some specimens that naturally possess individual crystallites in that symmetry (e.g. cellulosic fibers), there are many different methods reported to prepare such fibrous specimens from non-oriented polysaccharide crystallites. Examples include uniaxial orientation of fibrous nanocrystals in a soft matrix [13,14] and recrystallization of stretched films of polysaccharides [15]. In addition, crystalline domains in spherulites are also locally arranged in a cylindrical symmetry. They thus provide fiber diffraction patterns when probed at their periphery with small X-ray beams [16]. Fiber diffraction patterns were successfully collected from large starch granules using microfocus X-ray beams [17,18].

In the 1960-80s, crystal structures were refined based on *a priori* constraints such as interatomic distances and bond angles in the molecules (cf. linked-atom least square refinement, or LALS). This led to a series of structural models of polysaccharide crystals with limited data available at the time [19–22]. These early structures are still reliable as approximations, largely due to the low degree of freedom of polysaccharides in the dry unsolvated crystalline state, but sometimes fail to distinguish chain polarities, for example [23].

In the late 1990s, highly resolved fiber diffraction patterns became available with synchrotron X-ray sources becoming accessible, and more significantly large-area detection systems with high-spatial resolution and dynamic range (Fig. 2A). They have resulted in a

series of atomic-resolution crystal structures of cellulose [24–28] and chitin [29–31] allomorphs as well as chitosan [32,33]. Unlike the previous structures, they were refined without strong geometric constraints. The positions of flexible side groups such as hydroxymethyl groups were determined based directly on Fourier maps (Fig. 2B). In some cases, hydroxy hydrogen positions were also refined based on neutron fiber diffraction analyses coupled with intracrystalline deuteration (Fig. 2C,D) [14,25–27,33,34]. The localization of hydrogen atoms allowed the unambiguous determination of the hydrogen bonding networks in the crystal structures. These high-resolution fiber diffraction structures serve as the structural basis in the various research fields of carbohydrate science, from materials science to glycobiology.

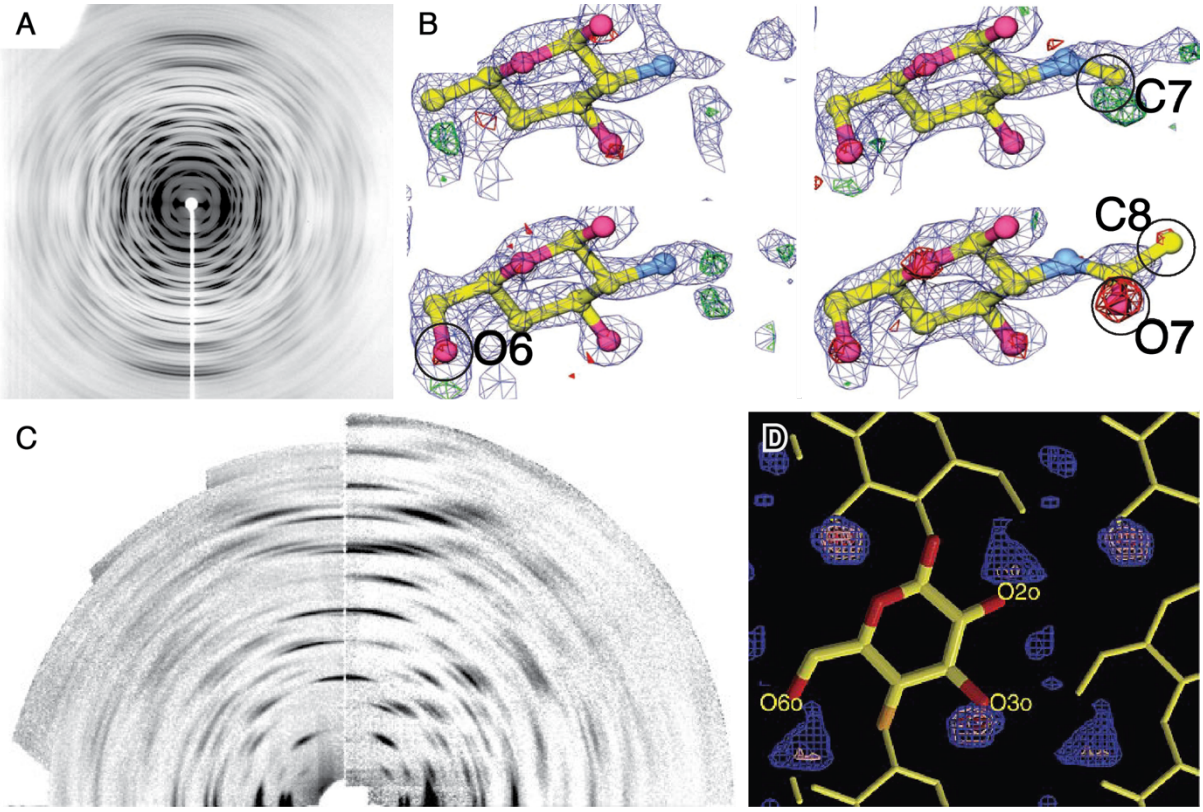


Figure 2. Fiber diffraction analysis of polysaccharide crystals. (A) X-ray fiber diffraction pattern of anhydrous β-chitin with a resolution of 1 Å. (B) Fourier OMIT map analysis of anhydrous β-chitin based on the intensity data extracted from the diffraction pattern shown in (A). Acetamide side chain structure is refined based on the Fourier maps. Adapted with permission from Nishiyama et al. [28] (C) Neutron fiber diffraction pattern of hydrogenated (left) and deuterated (right) cellulose Iβ. (D) Fourier OMIT map analysis of cellulose Iβ based on the intensity data extracted from the diffraction patterns shown in (C). The density peaks indicate the positions of deuterium. Adapted with permission from Nishiyama et al. [24]

Microscopic single crystals

When polysaccharides are crystallized into microscopic single crystals, typically with at least one dimension larger than a few hundreds of nanometers, single crystal crystallography is applicable. Such crystals can be prepared by *in vitro* crystallization. In many cases, *in vitro* crystallization of linear polysaccharides results in thin lamellar crystals with a thickness of a few to tens of nanometers, suitable for electron diffraction experiments (Fig. 3A,B) [35,36]. The acquisition of high-resolution electron diffraction data of polysaccharide crystals is challenging due to their intrinsic radiation sensitivity. Despite this challenge, several electron crystal structures of polysaccharides have been solved in the 1980-90s, including those of mannan [37], inulin [38], A-amylose[39] and anhydrous chitosan [40]. More recently, the structure of polysaccharide-solvent complexes (e.g. V-amylose) has been solved at near atomic resolution based on electron diffraction experiments carried out under cryogenic condition [41–43]. These crystallographic analyses were mostly based on single two-dimensional diffraction patterns recorded with the electron beam perpendicular to the lamellar plane, *i.e.*, the crystals were thus treated as 2D arrays of polysaccharide molecules. In some cases, three-dimensional diffraction data could be collected by rotating the crystals along selected low-index crystallographic axes [38,39,44]. Since the chain axes of polysaccharides are generally perpendicular to the lamellar plane, the analysis of base-plane diffraction data can lead to inaccurate structural refinement along the chain direction, as was demonstrated, for instance, by comparing X-ray and electron structures of anhydrous chitosan [32]. The implementation of 3D electron crystallography methods, such as microbeam electron diffraction (microED), is expected to overcome this problem [45]. The 3D ED methods enable the collection of diffraction data during sample rotation, thus covering a large reciprocal space volume.

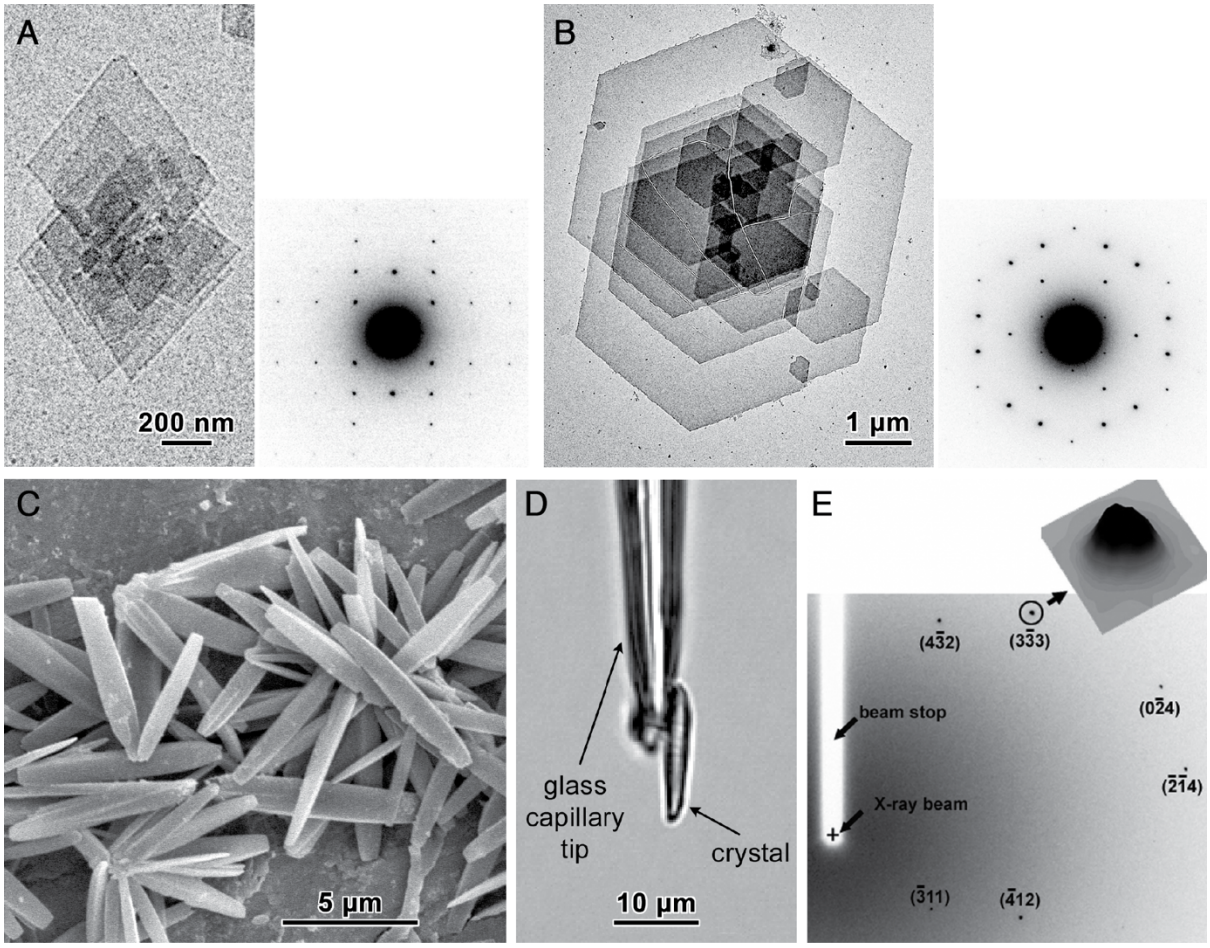


Figure 3. Single crystal analysis of polysaccharides. (A,B) Transmission electron micrographs and corresponding electron diffraction patterns of lamellar crystals of mannan I (A) (adapted from Grimaud et al. [35] with permission) and V-amylose inclusion complex with myristic acid (B) [38]. (C) Scanning electron micrograph of A-amylose single crystals. (D) A single crystal mounted on a tip of glass capillary for microfocus X-ray diffraction measurement. (E) An example of diffraction diagram collected from one A-amylose single crystal using microfocused X-rays (reproduced from Popov et al. [45] with permission).

When polysaccharides grow into larger 3D crystals, X-ray single crystal analysis can be used based on micro- or nanofocus X-ray beams. To date, the only example is the crystal structure of A-amylose with 1.3 Å resolution data using acicular single crystals probed by a synchrotron X-ray microbeam [46]. Similarly to electron diffraction experiments, the radiation damage becomes significant when using microfocus X-rays [47]. In this analysis, the complete dataset was obtained by merging data from different crystals to limit the detrimental effects of radiation damage. Despite being powerful and robust, the method is

underused largely due to the lack of availability of micron-sized 3D crystals and the accessibility to microdiffraction setups available only at synchrotron beamlines.

Crystalline powders

Fiber X-ray and single crystal electron diffraction techniques are not always available in carbohydrate chemistry laboratories. Powder X-ray diffraction measurement using a standard diffractometer is often the primary option for structural characterization. This situation resulted in many powder diffraction patterns of polysaccharide crystals in the literature whose structures are not determined so far [5,48]. Such powder diffraction patterns contain useful structural information, but the analysis is not straightforward. For an unknown structure, the unit cell determination, in particular indexing, is challenging partly due to the limited crystallite size that contributes to the line broadening and peak overlaps. Combining single-crystal or fiber methods is crucial at this step. For instance, electron diffraction has played a key role in the unit cell determination of polysaccharide crystals as demonstrated in the unambiguous identification of unit cells of two native cellulose allomorphs [11]. A similar approach can be taken for further structural investigation of these polysaccharide crystals.

Molecular simulation as a complementary technique

The use of molecular modelling, in particular force-field methods, is common practice in the protein X-ray structure refinement process since the 1990s. Examples of such methods include simulated annealing [49] and ensemble refinement (e.g. Phenix program) [50]. It was used to overcome the problem of many-minima that hinders the search of the “most likely” model. This approach has been extended to neutron and electron diffraction analyses as well as cryogenic electron microscopy (cryoEM) structures. However, so far, it has not been integrated into the atomic-resolution fiber diffraction analysis and thus hardly used in the structure refinement of polysaccharide crystals.

In recent years, the force-field-based molecular dynamics (MD) simulations and periodic density functional theory (DFT) calculations have been utilized to analyze the hydrogen bonding patterns in cellulose allomorphs and anhydrous chitosan [33,51]. This approach has provided useful insights into the geometry of hydrogen bonds in

polysaccharide structures and proved the effectiveness of computational methods in complementing the neutron fiber diffraction analysis.

Crystallographic analysis of biological systems

In addition to information about the molecular arrangement in space, the crystallographic information can provide insights into the biosynthesis mechanisms of natural polysaccharide crystals. A texture analysis of *Valonia* cell wall based on fiber diffraction revealed the existence of microfibrils running in opposite sense, suggesting a movement of cellulose synthetic complex on the cell membrane [52]. A similar texture analysis was performed on a housing tube of deep-sea tubeworm. The unipolar orientation of chitin microfibrils in the tube implied the direct connection between the chitin secretion and the motion of the worm [53].

Conversely, scientific discoveries regarding biosynthetic and/or biodegradation mechanisms have advanced our understanding of polysaccharide crystal structure. For instance, the directionality of cellulose and chitin molecules in the unit cells were experimentally determined based on the electron diffraction measurements and the knowledge of the chain elongation, by addition of monomers to the non-reducing end of the existing chain [54,55].

Current challenges and future directions in polysaccharide crystallography

Over the last two decades, a series of high-resolution crystal structures of polysaccharides were reported based on highly resolved X-ray/neutron fiber diffraction data. This led to the detailed understanding of molecular interactions in cellulose allomorphs and their analogues. However, these reliable crystal structures are mostly those of crystals exhibiting a high-symmetry and a low degree of freedom. The samples are also limited to the fibrous crystalline assemblies with large individual crystallite size. Many crystalline polysaccharides do not fall into this specific category. Further methodological developments are expected in the field of polysaccharide crystallography to tackle more complex and yet relevant crystal structures.

One of such challenging structures is that of polysaccharide-solvent crystalline complexes. These crystallosolvates are of great interest because they provide clues for the understanding of solute-solvent interactions that govern the processing of materials. The

incorporation of guest molecules is also of interest to understand and design the functionality of materials such as drug delivery or separation. Although several structures of relatively simple crystallosolvates, such as cellulose-amine complexes, have been reported [56,57], many crystallosolvates with larger and more complex guest molecules are yet to be studied. Improved experimental data have become available for many such structures in recent years [16,58,59]. They are, however, not yet producing reliable molecular models due to the high degree of freedom and dynamic nature of the system and the lack of an established method to explore all the potential molecular arrangements. In addition, some parts of the structure such as guest molecules can adopt non-crystallographic positions, and there are cases for which the crystal symmetry is only due to the spatial and time average. For instance, in the chitin-hydrazine complex, the molecular dynamics simulation showed that the positions of two nitrogen atoms were not fixed but interchangeable at room temperature [60]. To investigate the large conformational space and the dynamic aspects of the structures and the molecular interactions, it is necessary to fully integrate the molecular modelling methods into the fiber diffraction analysis. Both force-field and *ab initio* methods are useful for investigating different aspects of such dynamical structures as demonstrated in the case of the chitin-hydrazine complex [60].

The full implementation of molecular modelling in the fiber diffraction method would benefit structural analysis of polysaccharide crystals with defects and disorders. For instance, many practical cellulosic materials are composed of crystallites much smaller than model cellulose crystals used in the crystallographic analyses. These smaller crystals generally contain more disorder and defects. While small or disordered crystals would not yield high-resolution diffraction data, their fiber diffraction patterns provide rich structural information [61]. Quantitative comparison of the experimental diffraction patterns and those predicted from molecular simulations should shed light on the molecular details of the disordered systems.

Declaration of competing interest

The authors declare that they have no competing financial interest or personal relationships that influence the work reported in this paper.

Acknowledgements

The authors acknowledge Agence Nationale de la Recherche (ANR) for the financial support as part of the ECoCar project (grant number: ANR-21-CE29-0016-1).

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