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Multi-scale model for the hierarchical architecture of native cellulose hydrogels

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ABSTRACT

The structure of protiated and deuterated cellulose hydrogels has been investigated using a multi-technique approach combining small-angle scattering with diffraction, spectroscopy and microscopy. A model for the multi-scale structure of native cellulose hydrogels is proposed which highlights the essential role of water at different structural levels characterised by: (i) the existence of cellulose microfibrils containing an impermeable crystalline core surrounded by a partially hydrated paracrystalline shell, (ii) the creation of a strong network of cellulose microfibrils held together by hydrogen bonding to form cellulose ribbons and (iii) the differential behaviour of tightly bound water held within the ribbons compared to bulk solvent. Deuterium labelling provides an effective platform on which to further investigate the role of different plant cell wall polysaccharides in cellulose composite formation through the production of selectively deuterated cellulose composite hydrogels.

1. Introduction

Cellulose, the most abundant polymer found in nature, is currently one of the most actively studied materials due to its great relevance from both a scientific and an industrial perspective. Understanding the structure of cellulose is essential for gaining knowledge about the biosynthesis and growth mechanisms relevant to plant biology, as well as optimising manufacturing processes in both diverse industrial sectors (e.g. food, textile and paper) and in emerging areas (e.g. the production of biofuels and the synthesis of bio-based composites). Besides being the major polysaccharide in plant cell walls (PCWs), cellulose is also synthesized by several bacterial species and marine organisms. Regardless of its source, due to the sequential character of its biosynthesis process (Brown 1996; Delmer & Amor, 1995; Mutwil, Debolt, & Persson, 2008), native cellulose is formed via an assembly of hierarchically organised structural features. At the most basic structural level, cellulose chains, consisting of glucose units connected by β-1-4-linkages, are typically arranged into highly ordered crystalline domains. These crystalline fractions are combined with amorphous (i.e. regions with randomly arranged cellulose chains) and paracrystalline (i.e. regions with loose molecular packing or some degree of crystal distortion) domains, forming structural features known as cellulose microfibrils. Due to the presence of surface hydroxyl groups, strong interfibrillar interactions and/or interactions with other polysaccharides or water are established by hydrogen bonding, leading to the formation of structures often referred to as cellulose macrofibrils, bundles or, in the specific case of bacterial cellulose, ribbons.

The deconstruction of the PCW structure by means of successive component extraction and hydrolytic treatments has been extensively carried out to investigate the structural roles of cellulose and matrix components in PCWs (Jungnikl, Paris, Fratzl, & Burgert, 2008; Kent et al., 2010; Penttilä et al., 2010; Pingali et al., 2010a,b). However, this approach is of limited relevance when attempting to clarify the biosynthetic assembly of PCWs, due to the possible structural alterations induced by the applied chemical and/or enzymatic treatments. An alternative approach is the synthesis of highly hydrated (ca. 99 wt.-% H_2O) cellulose pellicles (known as cellulose hydrogels) by the cultivation of bacterial species such as *Komagataeibacter xylinus* in a culture medium rich in carbohydrates or polyols (Iguchi, Yamanaka, & Budhiono, 2000). This constructive approach is particularly promising since it enables the investigation of cellulose biosynthesis without interference of additional components, and a deliberate assessment of the influence of polysaccharides by selectively and specifically incorpo-

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rating them into the culture medium (Haigler, Brown, & Benziman, 1980; Hirai, Tsuji, Yamamoto, & Horii, 1998; Mikkelsen, Gidley, & Williams, 2011; Tokoh, Takabe, Fujita, & Saiki, 1998; Tokoh, Takabe, Sugiyama, & Fujita, 2002; Uhlin, Atalla, & Thompson, 1995; Whitney, Brigham, Darke, Reid, & Gidley, 1998; Whitney et al., 2006).

While this approach has already provided meaningful insights with regards to the interactions established between cellulose and different PCW matrix components (Gu & Catchmark, 2013; Lopez-Sanchez et al., 2014; Martínez-Sanz, Lopez-Sanchez, Gidley, & Gilbert, 2015; Mikkelsen, Flanagan, Wilson, Bacic, & Gidley, 2015; Mikkelsen et al., 2011; Tokoh et al., 2002; Uhlin et al., 1995; Whitney et al., 1998; Yamamoto, Horii, & Hirai, 1996), the mechanistic implications of these studies on PCW bio-assembly are still not completely understood. The uncertainties concerning the biosynthetic assembly path and structure of bacterial cellulose have precluded the determination of how the fermentation approach can be translated into the analogous PCW system. Based on the linear arrangement of the synthesising terminal complexes (TCs) in the *Komagataeibacter* bacterial cell membrane (Brown, 1996), the cross-section of bacterial cellulose ribbons is typically assumed to be rectangular (Astley, Chanliaud, Donald, & Gidley, 2001; Fink, Purz, Bohn, & Kunze, 1997; He et al., 2014; Tischer, Sierakowski, Westfahl, & Tischer, 2010); although this has only been supported by the observation of thick and thin regions in corresponding SEM and TEM micrographs (Tokoh et al., 1998, 2002; White & Brown, 1981). However, due to the severe effect of the drying process required for sample preparation, the observed morphologies may not be representative of the native (i.e. highly-hydrated) cellulose hydrogels.

In this context, the use of small angle X-ray and neutron scattering (SAXS and SANS) techniques is a valuable tool to probe the nanostructure of native cellulose hydrogels, as previously demonstrated (Astley et al., 2001; He et al., 2014; Koizumi et al., 2008; Martínez-Sanz, Gidley, & Gilbert, 2015; Martínez-Sanz, Gidley, & Gilbert, 2016; Martínez-Sanz, Lopez-Sanchez et al., 2015), since minimal sample preparation is required. Additionally, due to the different nature of the radiation sources (and thus, different scattering length density (SLD) contrasts generated between different components in the system), SAXS and SANS may highlight different structural features (Martínez-Sanz, Gidley et al., 2015). Furthermore, in the particular case of SANS, the different scattering length of hydrogen and deuterium opens up the possibility of selective SLD modification by D_2O/H_2O solvent exchange and deuterium labelling. Successful production of deuterated bacterial cellulose by utilising deuterated glycerol as the carbon source has already been reported (Bali et al., 2013; He et al., 2014), although it has not been definitively demonstrated whether the structure of the protiated and deuterated cellulose hydrogels in their native hydrated state are comparable across their different structural levels.

Despite the potential for scattering methods, the lack of robust models to describe the experimental data of cellulose hydrogels has precluded the full exploitation of these techniques. In contrast to conventional models, which do not account for the hierarchical organisation of the cellulose structure nor for the potentially vital role of water (Astley et al., 2001; He et al., 2014; Tischer et al., 2010), it has recently been demonstrated that SANS data of bacterial cellulose hydrogels may be described by a function characteristic of a core-shell structure (Martínez-Sanz et al., 2016; Martínez-Sanz, Lopez-Sanchez et al., 2015). According to this model, cellulose ribbons may be understood as two-phase systems with a core, composed of impermeable cellulose crystallites interacting through a network of hydrogen-bound hydrated paracrystalline cellulose, and a more accessible shell containing hydrated paracrystalline hydrated cellulose.

This study seeks to determine the structure of deuterated cellulose hydrogels in their native hydrated state and assess to what extent this structure is comparable to that of the unlabelled hydrogel across different structural levels. The approach used is necessarily multi-technique covering over three orders of magnitude in spatial resolution due to the intrinsic hierarchical architecture of the material. The complementary characterisation by spectroscopy, diffraction and microscopy aids in the successful interpretation of the small-angle scattering results, covering a broad and relevant size range of interest from the molecular to the macromolecular length-scale. Furthermore, by exploiting selective labelling and deuteration, the structural role of water in cellulose hydrogels is explored and identified.

2. Materials and methods

2.1. Preparation of protiated (H-CH) and deuterated (D-CH) cellulose hydrogels

Komagataeibacter xylinus (formerly *Gluconacetobacter xylinus*) strain ATCC 53524 (American Type Culture Collection, Manassas, VA, USA) was fermented on protiated and deuterated carbon sources to generate the protiated (H-CH) and deuterated cellulose hydrogels (D-CH). The bacterial strain was cultivated in Hestrin and Schramm (HS) medium, as previously described (Mikkelsen & Gidley, 2011), with the addition of glucose 2% (w/v) or deuterated glucose 2% (w/v) (552003–1 G—Sigma-Aldrich, Castle Hill, NSW, Australia) as the carbon source. Incubations were performed at 30 °C for 48 h under static conditions, with the medium starting pH of 5.0. At the end of incubation, cellulose hydrogels were harvested and washed in ice cold milliQ water with gentle agitation (150 rpm), with frequent rinsing to wash off excess medium and dislodge loosely-associated bacterial cells, until the harvested hydrogels were white in appearance. Post-rinsing, all samples were stored in 0.02% (w/v) sodium azide solution at 4 °C, until used for experiments.

2.2. Scanning electron microscopy (SEM)

Fully hydrated H-CH and D-CH were freeze-substituted according to the method of McKenna, Mikkelsen, Wehr, Gidley, and Menzies (2009) with minor modifications, as described in (Martínez-Sanz, Lopez-Sanchez et al., 2015). The resultant samples and corresponding air-dried specimens were placed overnight in a vacuum desiccator at 40 °C, followed by plasma cleaning for 30 s (E.A. Fishione Plasma Cleaner, PA, USA). Samples were then coated with iridium (×3 treatments) at 10 mA for 100 s (Bal-tec coater, Leica microsystems, Wetzlar, Germany) and kept in a vacuum desiccator until microscopy studies. Images were obtained using a JSM 7100F electron microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 5 kV and a working distance of 10 mm. Cross-sections of cellulose ribbons were determined using ImageJ software (Abràmoff, Magalhães, & Ram, 2004) from the SEM micrographs at their original magnification.

2.3. Small angle neutron scattering (SANS)

SANS measurements were performed on the 40 m QUOKKA instrument at the OPAL reactor (Gilbert, Schulz, & Noakes, 2006), with the three configuration described in (Martínez-Sanz et al., 2016). H-CH and D-CH were studied by placing the samples in 1 mm path length cells with demountable quartz windows and filling the cells with the required solvent (H_2O, D_2O or different H_2O/D_2O mixtures). To maximize D/H exchange, prior to the SANS measurements, the hydrogels were soaked in D_2O or H_2O/D_2O mixtures with an approximate sample/solvent ratio of 1 g/30 mL.