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Physical characterization and *in silico* modeling of inulin polymer conformation during vaccine adjuvant particle formation

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Abstract

This study combined physical data from synchrotron SAXS, FTIR and microscopy with *in-silico* molecular structure predictions and mathematical modeling to examine inulin adjuvant particle formation and structure. The results show that inulin polymer chains adopt swollen random coil in solution. As precipitation occurs from solution, interactions between the glucose end group of one chain and a fructose group of an adjacent chain help drive organized assembly, initially forming inulin ribbons with helical organization of the chains orthogonal to the long-axis of the ribbon. Subsequent aggregation of the ribbons results in the layered semicrystalline particles previously shown to act as potent vaccine adjuvants. γ -inulin adjuvant particles consist of crystalline layers 8.5 nm thick comprising helically organized inulin chains orthogonal to the plane of the layer. These crystalline layers alternate with amorphous layers 2.4 nm thick, to give overall particle crystallinity of 78%.

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Keywords

Inulin; Polysaccharide; SAXS; FTIR; Modeling; Adjuvant

1 Introduction

Inulin is a natural biopolymer comprising linear chains of 2–100 fructose units capped at the reducing end with glucose (Figure 1) (Barclay, Ginic-Markovic, Cooper & Petrovsky, 2010; Stevens, Meriggi & Booten, 2001). This polysaccharide is biocompatible and biodegradable and specific semicrystalline particulate forms of inulin have immunomodulatory properties (Cooper & Petrovsky, 2011). The utility of γ - and δ -inulin isoforms as vaccine adjuvants has been demonstrated in both animal (Feinen, Petrovsky, Verma & Merkel, 2014; Honda-Okubo, Kolpe, Li & Petrovsky, 2014; Saade, Honda-Okubo, Trec & Petrovsky, 2013) and human (Gordon, Kelley, Heinzl, Cooper & Petrovsky, 2014; Gordon, Sajkov, Woodman & Honda-Okubo, 2012) studies. However, to fully realize the vaccine adjuvant benefit of inulin particles there is a need to better understand how inulin polymer chains assemble to form the adjuvant-active inulin particles.

The use of inulin in both the food and pharmaceutical industries means its physicochemical properties are well studied (Barclay, Ginic-Markovic, Cooper & Petrovsky, 2010). Crystalline inulin adopts a regular helical conformation, with six fructose units forming one helix turn (Andre et al., 1996), solid inulin materials often containing a mixture of semicrystalline forms (isoforms) distinguished by their physical properties (Hébet et al., 1998; Ronkart, Deroanne, Paquot, Fougny & Blecker, 2010). To date, seven of these different semicrystalline forms of inulin have been identified and isolated by precipitation from aqueous solution under conditions specific to each form, yielding discoid particles of 1–10 μm diameter made up of lamellar sheets (Figure 1) (Cooper, Barclay, Ginic-Markovic & Petrovsky, 2013).

It is the semicrystalline inulin forms that are insoluble in water at 37 °C, such as γ - and δ -inulin, which have the immunomodulatory properties useful in vaccine formulations. As dissolved and amorphous inulin have no adjuvant activity (Silva, Cooper & Petrovsky, 2004), it is apparent that the nanostructured surface of the inulin particles determines their interaction with the immune system, and so structural investigations are integral to a better understanding of their biological properties. Furthermore, this understanding should assist design of optimized methods for their large-scale manufacture, a prerequisite for their broad application as vaccine adjuvants. Consequently, in this work we apply physical characterization in combination with *in-silico* structural and mathematical modeling to build a better understanding of the meso-scale organization of adjuvant-active inulin particles and the way they form.

2 Experimental

2.1 Materials

AdvaxTM was prepared by Vaxine Pty Ltd, Adelaide, Australia as described previously (Cooper & Petrovsky, 2011). A freeze-thaw cycle was used to regress the particles to γ -inulin (Supplementary Information, Figure S1), to provide a better match to particles formed in SAXS and FTIR ATR analysis at 15 °C (Cooper, Barclay, Ginic-Markovic, Gerson & Petrovsky, 2014; Cooper, Barclay, Ginic-Markovic & Petrovsky, 2013). Phosphate buffered saline (PBS) solutions were prepared from Sigma-Aldrich tablets using pure water (18.2 M Ω).

2.2 FTIR ATR

Attenuated total reflectance Fourier transform infrared (FTIR ATR) spectroscopy used a Thermo Electron Corporation Nicolet 6700 spectrophotometer with a Harrick FastIR ATR attachment employing a liquid flow-through accessory to pass fluid samples over the ZnSe crystal. For analysis, γ -inulin (100 mg/ml) was dissolved in PBS at 85 °C and then was left to precipitate whilst stirring at 15 °C over 24 h. Samples at various times were injected into the flow-through accessory then measured with a DGTS TEC detector using 64 scans at a resolution of 4 cm⁻¹. A sample of undissolved γ -inulin (100 mg/ml in PBS) was also analyzed. Fresh PBS background samples were run before each inulin sample and the data was subtracted from the inulin data. All data manipulation was conducted using OMNIC© software.

2.3 SAXS

Small angle X-ray scattering (SAXS) measurements were made at the Australian Synchrotron (Kirby, Boldeman, Gentle & Cookson). An energy resolution (E/E) of 10⁻⁴ was obtained from a cryo-cooled Si(111) double-crystal monochromator with X-ray beam wavelength of 0.06199 nm (20 keV). SAXS images were collected using a Pilatus 1 M detector (active area 169 × 179 mm with pixel size of 172 μ m²) located 1480 mm from the sample position, yielding a range of $q = 0.0171 - 0.8197$ Å⁻¹. For static analysis, γ -inulin particles (100 mg/ml) were dispersed or dissolved in PBS, passed through a capillary tube in the beamline at 15 °C with scattering patterns acquired using a 1 s exposure. For dynamic analysis of particle formation, γ -inulin (100 mg/ml) was dissolved in PBS at 85 °C and then was left to precipitate at 15 °C while being stirred and pumped through a capillary using a peristaltic pump at 50 ml/min. Scattering images were acquired using 1 s exposure times every minute over 8 h. For all SAXS data, radial integration, plotting of scattering profiles and solvent subtraction was carried out using ScatterBrain software provided by the Australian Synchrotron. Analysis of the subsequent scattering profiles were conducted using the Mathematica Software Package (Version 10) (Wolfram Research, 2014) and the Small-Angle Diffraction tool that is part of the Irena macro for Igor Pro (Ilavsky & Jemian, 2009). Correlation function analysis was performed using the “FindFit” command of the Mathematica Software Package, following a development of the Hosemann model (Hosemann & Bagchi, 1962) published by Fatnassi (Fatnassi, Ben Cheikh Larbi & Halary, 2010).

2.4 TEM

Transmission electron microscopy (TEM) was conducted on γ -inulin samples that were exchanged into pure water and diluted to 0.25 mg/ml before depositing 3 μ L onto formvar coated copper grids. The samples were air-dried before imaging using a FEI Tecnai G2 Spirit TEM operating at 100 kV.

2.5 AFM

Atomic force microscopy (AFM) was conducted on aqueous solutions of inulin (50 mg/ml) gently agitated at 5 °C. Samples were taken over time, diluted to 0.1 mg/ml and then 5 μ L was air-dried on a silicon wafer for imaging. Full details of the imaging process have been published previously (Cooper, Rajapaksha, Barclay, Ginic-Markovic, Gerson & Petrovsky, 2015) and are also presented in the Supplementary Information.

3 Theoretical calculations

3.1 MD simulation method

To prepare the system for molecular dynamic (MD) simulation an optimised 8DPN molecule, consisting of either inulin (1 glucose end group capping the reducing end of a 7 fructose unit chain) or polyfructose (8 fructose units), was parameterised using AMBER ff99SB force field parameters (Hornak, Abel, Okur, Strockbine, Roitberg & Simmerling, 2006) using the wrapper script, acpype (Sousa da Silva & Vranken, 2012). Partial atomic charges were calculated using the YASARA server (Krieger, Koraimann & Vriend, 2002). Then each molecule was placed in a cubic box ($x=4$ nm, $y=4$ nm, $z=4$ nm) of simple point charge (SPC) water (Toukan & Rahman, 1985) and 100 mM NaCl added. MD simulation was conducted using GROMACS 4.5.6 with 2 fs time steps and periodic boundary conditions. Following steepest descent minimisation, the system was equilibrated in two steps. Initially, a 50 ps simulation was carried out under the NVT ensemble where inulin and solvent were separately attached to temperature coupling baths. The temperature of the system was maintained at 278 K using the Berendsen weak coupling method (Eslami, Mozaffari, Moghadasi & Muller-Plathe, 2008). Next, the system was equilibrated to the NVT ensemble for 50 ps with weak coupling to maintain the pressure isotropically at 1.0 bar (Eslami, Mozaffari, Moghadasi & Muller-Plathe, 2008). Production simulation was conducted for 10 ns with no restraints applied. Short-range nonbonded interactions were cut off at 1.4 nm. Long-range electrostatics were calculated using the particle mesh Ewald (PME) algorithm (Cerutti, Duke, Darden & Lybrand, 2009).

The structures from the end of each simulation were used for steered MD simulation. Each molecule was placed at ($x=4$ nm, $y=2$ nm, $z=2$ nm) in a rectangular box ($x=12$ nm, $y=4$ nm, $z=4$ nm) SPC water and 100 mM NaCl was added. Then 100 ps NPT equilibration was conducted as described above. Following equilibration, restraints were applied on chain-B while the chain-A was pulled in the X direction (Figure 5) over 400 ps. Pull rate was 0.01 nm/ps and the spring constant was 1000 kJ/mol. Snap shots were taken from the trajectories and sampling windows were selected at 0.1 nm spacing up to 1 nm COM separation and at 0.2 nm spacing for the remaining distance. All together 23 windows were selected for umbrella sampling. In each window, 10 ns of MD simulations were performed. Results were

analysed using the weighted histogram analysis method (WHAM) (Zhu & Hummer, 2012). Molecule visualisation was done with Visual Molecular Dynamics (VMD) package (Humphrey, Dalke & Schulten, 1996). H-bonding analysis was done with the GROMCS built in script `g_hbond`. Graphs were plotted using the plotting tool Xmgrace.

3.2 DFT computational simulation

Density functional theory (DFT) computational simulation was carried out at absolute zero (0 K) using the *Vienna Ab-initio Simulation Package* (VASP) code with a projector-augmented wave method and a plane wave basis set (Kresse & Joubert, 1999), and the general gradient approximation-type Perdew-Burke-Ernzerhof functional. The plane wave basis was truncated with an energy cut-off at 480 eV for the VASP calculation (*i.e.* all plane waves with kinetic energies < 480 eV are included in the basis set for calculation). The inulin unit cell structure was constructed based on André's hemihydrate model, having a regular sixfold helix of (2→1) β-D-fructofuranose rings in the 4T_3 conformation (Andre et al., 1996). Inulin hydroxyl hydrogens (not provided by André) were added to the unit cell, with O-H bond lengths and directions initially set to be at 0.0942 nm and parallel to the *b* axis of the unit cell respectively. Similarly, hydrogen atoms not given in the original publication for H₂O molecules were created according to the H₂O's geometry (*i.e.* OH bond length at 0.0957 nm and H-O-H angle at 104.5 °), with the H-O-H planes initially kept parallel to the *ac* plane. All atoms were allowed to relax/move during the simulation changing the conformation of the fructofuranose rings having variable transitional conformations in the range $^2T_3 \leftrightarrow E_3 \leftrightarrow ^4T_3$, amongst the four geometries most often favored in β-D-fructofuranose systems (French, Mouhous-Riou & Perez, 1993; Immel, 1995), and generating lattice parameters of: *a* = 1.7177 nm, *b* = 0.9308 nm and *c* = 1.4223 nm. The fractional coordinates are listed in Table S1 and also presented as a .cif file, the torsion angles and conformations are presented in Table S2 and an image of a super cell is shown in Figure S13 of the Supplementary Information).

4 Results

4.1 FTIR ATR

Time resolved FTIR ATR spectra of γ-inulin particles and the precipitation of dissolved inulin at 15 °C are shown in Figure 2. The peaks at less than 950 cm⁻¹ are due to diagnostic carbohydrate skeletal modes (Synytsya & Novak, 2014), the band at 935 cm⁻¹ being characteristic of inulin and attributed to the β2→1 glycosidic bond (Bekers, Grube, Upite, Kaminska, Danilevich & Viesturs, 2008). This band and others occurring at 880 cm⁻¹, 992 cm⁻¹, 1037 cm⁻¹, 1120 cm⁻¹ and 1143 cm⁻¹ all sharpen over time, indicative of increased molecular organization as inulin precipitates (Shao, Chen, Wang, Chen & Du, 2012). Of these bands, all but the one at 935 cm⁻¹ also shift position, illustrating that the chemical environment changes with precipitation.

The main peak for dissolved inulin at 1037 cm⁻¹ and its shoulders at 992 cm⁻¹ and 1060 cm⁻¹ can all be attributed to the C-O-H deformation and C-O stretch of the hydroxyl groups (Grube, Bekers, Upite & Kaminska, 2002; Max & Chapados, 2007). The shoulders change character significantly upon precipitation; with the one at 992 cm⁻¹ becoming defined as a

distinct peak for the precipitate, while the shoulder at 1060 cm^{-1} slowly disappears and is replaced by a new peak forming at 1082 cm^{-1} . This indicates a significant change in the character of the hydrogen bonding of the hydroxyl groups as aggregates form. There is also significant change to the character of two associated peaks between 1120 cm^{-1} and 1143 cm^{-1} , attributed to C-O stretching within the sugar ring (Grube, Bekers, Upite & Kaminska, 2002; Max & Chapados, 2007), illustrating changing character of the sugar rings as the semicrystalline material forms.

4.2 SAXS

SAXS has previously been successfully applied to resolve fine detail of complex multi-aspect polysaccharide structures (Schuster, Cucheval, Lundin & Williams, 2011), and hence we pursued its use in the analysis of inulin particle formation. Firstly, the time-resolved SAXS curves (Figure 3a) were analyzed using the Guinier-Porod model (Hammouda, 2010) (Supplementary Information, equations S1 and S2). The radius of gyration (R_g) and Porod exponents were calculated for all curves for which a successful fit could be made; the fit breaking down beyond 300 min as large semi-crystalline aggregates start to dominate. The Porod exponent ($p = 1.68\text{--}1.66$ for $t < 125$ min) indicates that dissolved inulin has a swollen random coil conformation with R_g of 11 Å (Hammouda, 2010). The changing nature of the Porod region upon precipitation is illustrated in Figure 3b, which shows an increase in the R_g to 29 Å between 150 and 300 min, attributable to the formation of nanoscale structures. As this change in R_g occurs, the changing Porod exponent (Figure 3c) indicates that the randomly coiled inulin ($t < 125$ min; $p = 1.68\text{--}1.66$) aggregates into high-axial-ratio ribbons ($t = 215$; $p = 1.36$) previously identified in time-resolved AFM studies (Cooper, Rajapaksha, Barclay, Ginic-Markovic, Gerson & Petrovsky, 2015), which then transform to fully-formed nanostructured inulin microparticles ($t > 290$ min; $p > 2$) (Hammouda, 2010).

Inulin particle formation also results in emergent peaks in the SAXS curves shown in Figure 3a. These peaks were analyzed from the static SAXS curve for γ -inulin using the Small-Angle Diffraction tool from the Irena macro for Igor Pro (Supplementary Information, Figure S2) (Ilavsky & Jemian, 2009). The peaks at $q > 0.5$ are attributed to crystalline d -spacings of the unit cell (101 , 11.15 Å ; 011 , 7.84 Å ; 111 , 7.28 Å) previously identified using X-ray diffraction analysis (Cooper, Barclay, Ginic-Markovic, Gerson & Petrovsky, 2014). The main peak at $q = 0.06\text{ Å}^{-1}$ is due to the lamellar spacing (L_p) in the particles, calculated to be 109 Å . Correlation function analysis was also used to characterize the lamellar arrangements that result in the main peak at $q = 0.06\text{ Å}^{-1}$ using a development of the Hosemann (Hosemann & Bagchi, 1962) theoretical model outlined by Fatnassi et al. (Fatnassi, Ben Cheikh Larbi & Halary, 2010) (Supplementary Information, Equation S3) and achieved close fits to the SAXS data (Supplementary Information, Figure S3). This model was used to confirm the lamellar spacing ($L_p = 109\text{ Å}$), and calculate the crystalline layer thickness ($l_c = 85\text{ Å}$) and the amorphous layer thickness ($l_a = 24\text{ Å}$), from which a calculated crystallinity of 78% was derived. The crystalline layer thickness correlates well with that calculated for γ -inulin helices from DFT data for the unit cell of 81 Å comprising 5 helical turns plus glucose and fructose end groups (Cooper, Barclay, Ginic-Markovic & Petrovsky, 2013).

The final significant feature of Figure 3a is the clear iso-scattering point at $q = 0.09 \text{ \AA}^{-1}$. Such iso-scattering points are poorly understood, but they have been observed previously in the analysis of phase change processes for a range of materials including polymer films (Liu et al., 2012), proteins (Nicolai, Pouzot, Durand & Weijers, 2006), liquid crystals (Pereira et al., 2008) and fats (Dewettinck, Foubert, Basiura & Goderis, 2004). In this instance the iso-scattering point is attributed to the change in symmetry that the inulin chain undergoes upon adopting the helical conformation found in the aggregated structures (Dewettinck, Foubert, Basiura & Goderis, 2004; Liu et al., 2012).

4.3 Microscopy

Inulin particles formed during dynamic SAXS and FTIR ATR analyses were imaged using TEM. Based on the temperature of formation these are γ -inulin particles (Cooper, Barclay, Ginic-Markovic & Petrovsky, 2013). Whilst showing more variation in density and size, these particles have similar morphology to γ -inulin formed under optimized conditions (Supplementary Information, Figure S4) and consequently the analysis for SAXS and FTIR ATR applies to particles having biological adjuvant action.

Time-resolved AFM studies of the formation of inulin particles have been described recently by us (Cooper, Rajapaksha, Barclay, Ginic-Markovic, Gerson & Petrovsky, 2015). Early morphologies include small, round structures that have an average height of 5.5 nm (std = 0.9 nm) and diameter of 53 nm (std = 12 nm) when dried on a flat surface (Figure 4a). These structures have also been observed using TEM (Supplementary Information, Figure S5). Appearing with the round structures are high-axial-ratio ribbons that have clearly stepped increases in height correlated with increasing width as illustrated in the scatterplot in Figure 4b. The distinct steps in ribbon height strongly suggest the addition of discrete organized units rather than random and disordered accumulation of chains from solution. This provides evidence that the helical organization of inulin chains is present in these earliest organized structures. If the helical organization of ribbons is accepted, then the smallest step in the AFM height measurements (1.5 nm; std = 0.3 nm) can only be attributed to helical chains lying parallel to the surface as the length of helical γ -inulin is greater than 8 nm long according to the SAXS data analysis and DFT calculations. Comparison of the measured height ranges for each step (Figure 4b) and the calculated heights for layers of helices lying flat on the surface based on the DFT-optimized the unit cell dimensions are shown in Table 1. The correlation of this data provides confirmation of the helical arrangement. Further, Table 1 shows that 3 and 5 helical layers fall between the measured steps and so do not exist. While Table 1 shows that a single layer could exist, the fact that only multiples of double-layers exist suggests that inulin ribbons initially form only as double-layers, and that the increase in ribbon height only occurs through the addition of complete double layers. This arrangement is supported by the fact that a double-layer is stabilized via four of the available six fructose groups in each helical turn interacting through hydrogen bonding, while for each helix in a single-layer the structure would be stabilized by only two interactions, which may not support a helical conformation.

The increase in ribbon width does not occur in discrete steps, in contrast to the ribbon height, though ranges of width are correlated with the ribbon height steps (Figure 4b). For

the smallest ribbons the width measurements correlate with between 2 and 3 helical chains of inulin arranged orthogonally to the long axis, showing that the semicrystalline lamellar arrangement is established in the earliest aggregates.

The high-axial-ratio ribbons tend to aggregate in aligned bundles (Supplementary Information, Figure S6) and spherulite-like structures comprised of relatively short ribbons were also observed using AFM and TEM (Figure 4c; Supplementary Information, Figures S7 and S8). The spherulites are a possible transitional structure between ribbons and particles; the particles appearing as a spherulite or axialite composed of layers rather than ribbons. The SEM image in Figure 1 illustrates this spherulite character and AFM measurements demonstrate that the central height of particles on a flat surface is less than 50% (47%; std = 17) of the edge height. Indeed, spherulites constructed from polymers are usually considered to form from lamellar-based ribbon structures (Tien, Nishikawa, Hashimoto, Tosaka, Sasaki & Sakurai, 2014), and spherulites are known to form from the aggregation of dispersed high-axial-ratio structures (Tamhane, Zhang, Zou & Fang, 2010), and not just by direct growth from melts. However, further investigations are required to define the role of spherulites in inulin assembly. Ribbon-based structures (linear and spherulite) co-exist with particles for some time (Figure 4d), before eventually being completely replaced with stable inulin particles (Supplementary Information, Figure S9).

4.4 Molecular modeling

In-silico structural modeling was performed to try and bring together the physical results from FTIR ATR, SAXS and microscopy. For computational efficiency, inulin with a degree of polymerization of eight sugar rings was used as this is the minimum size able to assume a helical arrangement comprising a six fructose unit cell with additional glucose and fructose end groups (Cooper, Rajapaksha, Barclay, Ginic-Markovic, Gerson & Petrovsky, 2015; Oka, Ota, Mino, Iwashita & Komura, 1992). The simulation commenced with either two antiparallel inulin helices or two antiparallel polyfructose helices. In each case the system was equilibrated and then subjected to a steered MD simulation in which chain-A was pulled in the X direction (Figure 5) and the free energy calculation performed with umbrella sampling. The use of pulling force alters the equilibrium of the system and as a result thermodynamic data can be obtained from the steered MD trajectories without large error. Weighted histogram analysis method (WHAM) is commonly used to extract free energies from the steered MD trajectories for a more direct comparison of two systems.

Umbrella sampling and free energy calculation (Figure 6a) shows associated inulin helices have a lower energy state ($G_{\text{Bind}} 50.7 \text{ kJ.mol}^{-1}$) than associated polyfructose helices ($G_{\text{Bind}} 41.7 \text{ kJ.mol}^{-1}$), illustrating the effect of glucose end groups to increase the strength of the interaction between otherwise identical chains. This difference can be attributed to increased hydrogen bonding opportunities provided by the glucose end group, as shown using hydrogen bonding analysis (Figure 6b). While quantitative measurements of carbohydrate binding energies are difficult to find, (Çarçabal, Cocinero & Simons, 2013) similar calculations made for glucose and short cellulose oligomers (glucose 4.2 kJ.mol^{-1} , cellobiose 13.8 kJ.mol^{-1} , cellotetraose 31.4 kJ.mol^{-1}) (Bergenstråhle, Wohler, Himmel &

Brady, 2010) show that the calculated association energies for inulin and polyfructose helices are reasonable and that the binding between inulin helices is relatively strong.

Force versus time profiles for pulling apart the polysaccharide chains illustrates the differences in the force required to dissociate the pairs of inulin compared to pairs of polyfructose (Supplementary Information, Figure S10). Dissociation of inulin pairs resulted in four peaks of force with a highest peak of 405 kJ/mol/nm, 65 ps in to the steered MD. Trajectory analysis for inulin showed that at this time glucose and the 7th fructose at the end of one chain are hydrogen bonded to the two fructose residues (1st and 2nd) at the end of the adjacent chain (Supplementary Information, Figure S11). Contrastingly, there were only three peaks of force in the dissociation of polyfructose with a highest force of 260 kJ/mol/nm after 75 ps. For polyfructose, the highest peak force occurs when fructose residues at the end of one chain are hydrogen bonded with the 4th and the 5th fructose residues located in the middle of the adjacent chain (Supplementary Information, Figure S12). Thereby, taking both the umbrella calculations and the force versus time profiles into account, overall the MD simulations support our previously theorized importance of the glucose and fructose end groups in the formation of stable semi-crystalline structures for inulin (Cooper, Rajapaksha, Barclay, Ginic-Markovic, Gerson & Petrovsky, 2015).

5 Discussion

The collected physical data together with the MD and DFT modeling data provide good agreement and the most complete picture yet obtained of the structural changes and mechanisms as dissolved inulin polymers progress through to fully assembled crystalline particles. The SAXS data confirmed that dissolved inulin chains exist as swollen random coils (Figure 7a) (Hammouda, 2010), consistent with images of round nanostructures in both AFM and TEM (Figure 4a; Supplementary Information, Figure S5). This also agrees with previous structural analyses that show intermolecular hydrogen bonding is required for stable inulin helix formation (Andre et al., 1996).

The molecular dynamic modeling confirmed that inulin helices are more strongly associated than polyfructose helices and that this can be attributed to the strength of the hydrogen bonding between the glucose end group of one chain and a fructose group of an adjacent chain (Figure 7b). Experimentally this is supported by the loss of stability of adjuvant particles subjected to gamma irradiation (Cooper, Barclay, Ginic-Markovic & Petrovsky, 2014), ascribed to the hydrolysis of inulin glycosidic linkages and resulting in glucose-free polyfructose chains. These modeling and experimental data therefore are aligned with the previous prediction that a bidentate hydrogen bond interaction between glucose and fructose end groups initiates organized inulin aggregation (Cooper, Rajapaksha, Barclay, Ginic-Markovic, Gerson & Petrovsky, 2015). Such information has major significance to the manufacture of inulin adjuvant particles as it demonstrates the importance of avoiding hydrolysis during inulin particle formation, as this could otherwise generate polyfructose chains unable to form adjuvant-active particles.

Microscopy images (Figure 4 and Supplementary Information, Figures S6–S8) show the first visibly-organized inulin aggregates are high axial ratio, one-dimensional ribbons. We

postulate that an antiparallel arrangement of the inulin chains is the basis for ribbon growth, with inulin chains oriented orthogonally to the long axis of the ribbon and having helical organization (Figure 7c). This proposal is supported by the AFM evidence and corresponds best with the established molecular organization in inulin particles. It also agrees with the suggested orientation of chains in other polysaccharide ribbons, such as amylose (Leloup, Colonna, Ring & Roberts, 1992; Nordmark & Ziegler, 2002). The helical organization in organized inulin aggregates is supported by intermolecular hydrogen bonding, confirmed by distinctly changed hydrogen bonding environment upon inulin precipitation provided by FTIR ATR (Figure 2). Logical progression of the organized aggregation of the one-dimensional ribbons in both height and width as described in the AFM results could clearly result in two-dimensional semicrystalline layers (Figure 7d). Of course, the process isn't necessarily this simple as the ribbons are much longer than any dimension of the particles and there appears to be a transition through spherulites to the layered axillite-like structure of the particles, with this aspect currently being pursued with further synchrotron SAXS experiments.

The accepted model of inulin semicrystalline materials as lamellar arrangements of antiparallel chains with helical conformation has been derived from scanning electron microscopy (Cooper & Petrovsky, 2011), SAXS (Hébet et al., 1998), X-ray diffraction (Cooper, Barclay, Ginic-Markovic, Gerson & Petrovsky, 2014) and electron diffraction data (Andre et al., 1996). This arrangement for γ -inulin is also supported by the current SAXS data (Figure 3a), with peaks that correlate with the helical unit cell of inulin and a peak assigned to the lamellar repeat calculated at 10.9 nm. The crystalline layer thickness of 8.5 nm calculated from SAXS data using correlation function analysis is also consistent with an estimation of the length of γ -inulin helices of 8.1 nm based on DFT data for the unit cell.

6 Conclusions

By combining physical data and modeled results we have been able to demonstrate that inulin particles assemble from dissolved swollen random coil chains. The precipitation of inulin is helically organized through hydrogen bonding interactions between adjacent chains supporting initial formation into one-dimensional ribbons, and then into two-dimensional nanolayers that make up the three-dimensional inulin microparticles. This study has solved a longstanding manufacturing issue in production of inulin adjuvant, explaining why even limited hydrolysis of inulin during crystallization results in dramatically reduced adjuvant yield. It further demonstrates the power of bringing together physical data and *in-silico* modeling approaches to decipher complex polymer behavior. It has provided the most complete picture yet of the nature of inulin particle formation and structure, important information that will assist its ongoing development as a novel vaccine adjuvant platform. Whilst this work represents a major advance in understanding of the nature of formation of γ -inulin lamellae, the nature of the external chemistry of the γ -inulin particles remains to be determined. This will be important to better understand the biological interactions between inulin adjuvant particles and the immune system and we are currently conducting further quantum modeling and other approaches to characterize the nature of the surfaces formed by the crystallized inulin particles and how these might be interacting with and activating immune cells, thereby explaining their immune-modulatory actions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

SAXS	small angle X-ray scattering
FTIR ATR	attenuated total reflectance Fourier transform infrared
AFM	atomic force microscopy
TEM	transmission electron microscopy
PBS	phosphate buffered saline
MD	molecular dynamics
DFT	density functional theory

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Highlights (for review)

- Physical analyses and modelling reveal key features of inulin particle formation.
- Inulin polysaccharide chains adopt random coil in solution.
- Glucose end groups contribute to organised self-assembly of inulin particles.
- Inulin fibers comprise inulin helices perpendicular to the long axis.
- γ -Inulin particles comprise 11 nm semi-crystalline layers with 78% crystallinity.

Figure 1.
Inulin molecular structure and SEM image of inulin adjuvant particles

Figure 2.

Time-resolved FTIR ATR spectra of the formation of inulin particles over 1 day at 15 °C. Each spectrum is enlarged to full scale to remove concentration artifacts induced by the aggregation into large particles.

Figure 3.

(a) Time-resolved SAXS curves recorded during the formation of inulin particles over 8 h at 15 °C. Each SAXS curve is an average of 5 curves taken over 5 min and each displayed curve is separated from the previous one by ~ 40 min. (b) Plot of R_g versus time and (c) plot Porod exponent (p) versus time.

Figure 4.

(a) AFM image of small round inulin structures and an inulin ribbon; (b) Scatterplot of inulin ribbon heights and widths measured using AFM; (c) TEM image of spherulite-like aggregation of inulin ribbons (some examples highlighted by arrows); (d) AFM image of inulin particles and ribbons

Figure 5.
Anti-parallel assembly of inulin chains

Figure 6.
Comparison of the Potential Mean Force (PMF) and number of hydrogen bonds between end groups as paired helices of inulin or polyfructose are pulled apart.

Figure 7. Schematic representation of inulin particle formation: (a) Inulin chains with random coil; (b) formation of glucose-fructose link; (c) antiparallel arrangement of inulin helices in ribbons, red arrow indicates the long axis of the ribbon; (d) inulin ribbons combine, likely through spherulite intermediates, forming semicrystalline particles; (e) inulin ribbons lying flat on a surface; blue rings accentuate inulin helices.

Table 1

Comparison of AFM measured inulin ribbon heights with those calculated from the unit

Ribbon Height (nm)		Helical Layers
AFM measured	DFT Calculated ^a	
0.9–2.1	0.88–1.2	1
	1.4–1.9	2
	2.2–2.8	3
2.7–3.8	3.0–3.5	4
	3.8–4.4	5
4.7–7.7	4.6–5.1	6

^aDFT calculated ribbon heights are presented as a range dependent on the flexibility of noninteracting fructose groups external to the ribbon structure.