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Mechanical properties of TEMPO-oxidised bacterial cellulose-amino acid biomaterials

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Abstract

Amino acid functionalised bacterial cellulose is a non-toxic biocompatible material, which can be further modified with active groups and nanoparticles for various biomedical applications. Many studies have focused mainly on the chemical and biomedical characterisation of modified bacterial cellulose; however, the mechanical performance of these materials remains undetermined. In this paper, we investigate the mechanical performance of amino acid modified TEMPO-oxidised bacterial cellulose (TOBC-AA). Highly crystalline bacterial cellulose was initially oxidised via TEMPO-mediated oxidation and amino acids – specifically glycine, alanine and proline- were grafted to TOBC via EDAC/NHS coupling agent. Manufactured materials have been tested and compared with pure bacterial cellulose and our recently studied monomeric amino acid bio-glues. Under tensile loading, the rigid crystalline structure of the copolymer has a slightly higher strength and toughness compared to pure BC, however its adhesive properties were significantly lower than those of monomeric amino acids. The side chains on TOBC-AA physically interlock under the conditions of shear; however amino acids grafted to BC lack mobility and the ability to form H-bonds, in contrast to monomeric amino acid such as glycine and alanine.

Table 1 – List of abbreviations

List of abbreviation	
BC	Bacterial cellulose
TOBC	TEMPO oxidised bacterial cellulose
AA	Amino Acid
Ala	Alanine
Gly	Glycine
Pro	Proline
BC-AA	Bacterial cellulose based composite with monomeric amino acid
TOBC-AA	Amino acid modified TEMPO oxidised bacterial cellulose
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
NHS	N-hydroxy-succinimide
EDAC	N-ethyl-N-(3-dimethylaminopropyl)-carbodiimide hydrochloride

Introduction

Cellulose is one of the most abundant materials in nature, existing predominantly as the structural component of plant and algal cell walls. Cellulose nanofibers are also exuded by a range of bacteria, of which *Acetobacter xylinum* produces the highest yield. These bacterial celluloses (BC) possess a significantly higher crystallinity, fibre length, and strength than plant celluloses. Moreover, they can be grown into films with essentially, any shape and thickness [1] [2]. BC exhibits respectable mechanical properties in both dry and wet states for a biopolymer which can be further adjusted by chemical modification. The compression modulus of unmodified BC is slightly lower than articular cartilage which can be increased by phosphorylation or increasing the solid content of BC [3]. Furthermore, tensile strength of BC is on par with articular cartilage that can be improved by increasing hydrogen bond formation by adhesive materials like monomeric alanine and glycine. The specific strength and specific stiffness of BC is in the same region of collagen and compact bone [4].

BC based biomaterials are also of high purity, have a high capacity for water retention and the nano-scale arrangement of BC fibrils provides added benefit in applications where biocompatibility is of importance [1]. Currently, BC based materials have a wide range of potential applications in biomedical fields including; bone tissue engineering [3], artificial blood vessels [5], controlled drug delivery [6] wound dressings [7] [8] and cartilage replacement [9].

BC molecules create a network of micro fibrils which interact by hydrogen bonding through the presence of active OH sites on BC molecular chains. There are various cellulose treatment methods used to improve functionality and mechanical performance such as; silylation [10], mercerisation [11], phosphorylation and sulfation [3], polymer grafting and bacterial modification [12].

A recent method for treating BC has been to use a TEMPO-mediated oxidation process. In this treatment process, 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) oxidises the C6 primary hydroxyl on the cellulose chains to form C6 carboxylate groups [13]. These carboxylic groups allow for the chemical (covalent) functionalisation of BC by bio-active molecules such as amino acids. There are plenty of reports on the TEMPO oxidation of plant cellulose. TEMPO oxidised plant cellulose typically has high crystallinity, uniform and ultrafine width, and a high aspect ratio. This process can be performed under moderate aqueous conditions similar to that of enzymatic or biological reactions [2]; however, considering the advantage bacterial cellulose has over plant cellulose in terms of purity, crystallinity and other factors previously mentioned, TEMPO oxidised BC (TOBC) has the potential to be a better candidate in targeted applications. Luo et al. [14] reported that TEMPO oxidation causes a negligible loss in mechanical strength of bacterial cellulose; however it maintains BC's physical attributes e.g. morphology and nanofiber arrangements. Consequently, TOBC retains the advantage of a 3D nanofibrous structure over plant cellulose.

The main advantage of oxidised BC is that various functionalities can be added to the material via carboxylic groups [15] [16] including; hydrophilic, aliphatic and aromatic amino acid groups [17]. Primary amino acid groups provide access for further modification such as preparing nanoparticles without any transfection reagents for easy cell uptake [18], strong antimicrobial activity that can be incorporated by attaching silver nanoparticles (AgNPs) [19] or amino alkyl groups [20] to the carboxylate groups. These modifications are beneficial in a variety of medical applications, in particular as wound dressing materials. Cateto et al. [21] reported the functionalisation of BC with L-leucine using Fmoc-L-leucine and cellulose whiskers to enhance cell-adhesive structures, hydrophobic drug carriers and gas separation membranes. Kalaskar et al. [17] reported the characterisation of cellulose fibre surfaces by modification using three different amino acids with variable side chain groups using solid phase peptide synthesis. Kalaskar and co-workers concluded that the size of the side chains of the amino acid has a direct effect on the final surface properties and chemical modification of cellulose fibres. The majority of previous research on normal

and modified oxidised cellulose has tended to focus on the characterisation of different functionalities resulting from the side chains. Significantly fewer reports have considered the mechanical behaviour of TEMPO-oxidised bacterial cellulosic materials. The mechanical properties of manufactured materials play a decisive role in its final biomedical or industrial application and form a topic of this paper.

In our previous work [22] we identified key amino acids in flagelliform silk contributing to 'stickiness' and subsequently used monomeric amino acid to bind bacterial cellulose. We found that the adhesive properties of the amino acids glycine and alanine raised quite respectably the mechanical performance of bacterial cellulose sheets. Monomeric alanine and glycine acted as bioglues in BC biomaterial sheets, and molecular dynamics simulations confirmed they have similar characteristics to flagelliform silk in terms of bond strength. The key characteristic of these amino acids is that they readily form hydrogen bonds with neighbouring bacterial cellulose molecules. Since they are small mobile molecules, they can effectively glue together the bacterial cellulose network in areas where the bacterial cellulose cannot connect.

In this paper, we compare the molecular characteristics and mechanical properties of TEMPO-oxidised bacterial cellulose nanofibers, covalently bonded by the same monomeric amino acids used in our initial work [22]. Our hypothesis is that covalent bonding arising through the TEMPO-oxidation process will be inferior to secondarily bonded bioglues. Our objective is to provide clear mechanistic insight behind the molecular level biomechanics of TEMPO-oxidised bacterial cellulose covalently bonded by monomeric amino acids, and to the way in which this affects the global macro-mechanical properties.

Materials and Methods

1. Manufacture of TEMPO-oxidised bacterial cellulose sheets covalently grafted by amino acids

Bacterial cellulose was extracted from nata de coco (obtained in Bantul, Indonesia). The nata was treated initially by boiling in water and rinsing several times to remove basic chemical impurities as well as acetic acid arising from the culturing process. The nata was mixed with caustic soda solution and then stored in a freezer (-18°C). Prior to use, stored BC was washed and then crushed by hand mixer and the gel produced was treated further with 0.1 M NaOH solution at 80°C for 20 min in order to purify the bacterial cellulose of microorganisms and of any final residues from the media. The gel was then neutralised in deionised water and filtered using a Buchner filtration funnel to obtain higher solid content (up to 10%) of bacterial cellulose [7]. Amino acid monomers and other chemicals for TEMPO-mediated oxidation and amino acid modification were purchased from Sigma Aldrich and used as received without any further purification. Methanol (99% purity) and ethanol (>95%, laboratory grade) was purchased from Sigma Aldrich.

TEMPO-mediated oxidation of bacterial cellulose was conducted according to the methods reported in [19] and [23]. The change in chemical structure from the initial cellulose glucopyranose ring is illustrated in Figure 1. Bacterial cellulose sheets (0.648 g solids, i.e. 4 mmol of anhydroglucose units) were initially dispersed in $Na_2CO_3/NaHCO_3$ buffer solutions (80 ml, pH = 10) inside a reactor. After a quick dispersion with moderate stirring, the reactor was placed in ice bath to keep the reaction temperature at 0°C and TEMPO (10 mg, 0.065 mmol) and NaBr (0.20 g, 1.9 mmol) was added to the suspension. To start the reaction, sodium hypochlorite solution, which was stored at 0°C (21%, 4.1 ml, 13.2 mmol) was added to the reactor and the solution pH was adjusted 10 by 0.5 M aqueous NaOH. The reaction was stirred continually overnight and finally the oxidation process was quenched by adding 10 ml of methanol. The oxidised cellulose was washed with water and then ethanol solution, centrifuged several times and finally dialysed in distilled water overnight to remove impurities and unreacted monomers. Final product was then oven-dried at 65°C. Based on the method published in [23], carboxylate content under reaction conditions was determined using the conductometric titration method and found to be approximately one carboxylate group per seven anhydro-glucose units (approximately 0.8 mmol/g).

Grafting amino acids to the oxidised cellulose was carried out following a method described in [19]. 10 mg of dried oxidised bacterial cellulose was added to 50 ml of deionised water. The mixture was stirred in the reactor at a moderate stirring rate to form a homogeneously dispersed solution. Following this, 23 ml (10 mmol) of N-hydroxy-succinimide (NHS) aqueous solution (50 mg ml^{-1}) was added. 12 ml (0.62 mmol) fresh N-ethyl-N-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) aqueous solution (10 mg ml^{-1}) was quickly added to the suspension and stirred vigorously for 30 minutes at room temperature. The pH was adjusted to close to neutral by addition of NaOH or HCl. The excess EDAC, NHS and by-product urea was removed by dialysis in distilled water for 8h. Finally, 100 mg of glycine, alanine or proline was added individually to separate beakers of 100 ml of suspension and subsequently dialysed for 2 days to remove un-reacted amino acids and other residues. The final product was partially dried in a pressure filtration rig to form composite sheets for mechanical testing and FTIR characterisation. The grafted amino acids, which are the three predominating amino acids in flagelliform silks; glycine, alanine and proline, are the same as in our previous work [22].

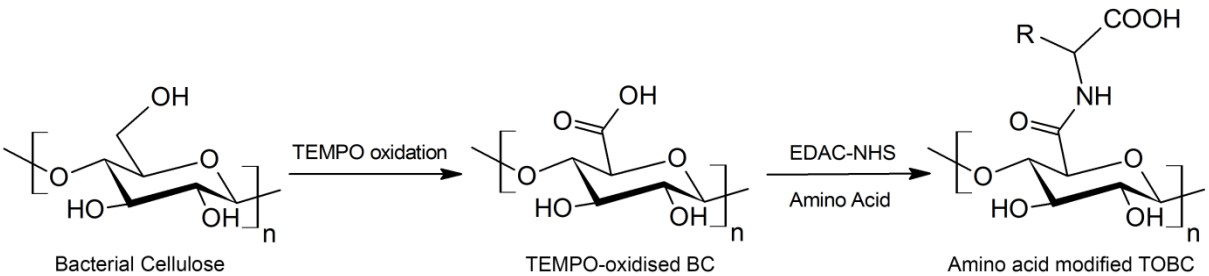

Figure 1 – preparation of amino acid modified bacterial cellulose by grafting of glycine, alanine or proline into oxidised bacterial cellulose.

The synthesised materials were poured into a custom built rectangular pressure filtration rig. Compressed air at 1 MPa pressure was applied to manufacture composite sheets. The volume of bulk material is measured in order to achieve specific thickness of c.a. 200 mm after removal of excess water and further drying. The mixture was poured in the pressure filtration rig after mixing, therefore the orientation of fibres is completely random. The sheets were further dried in oven at 80°C for 4 hours, Figure 2.

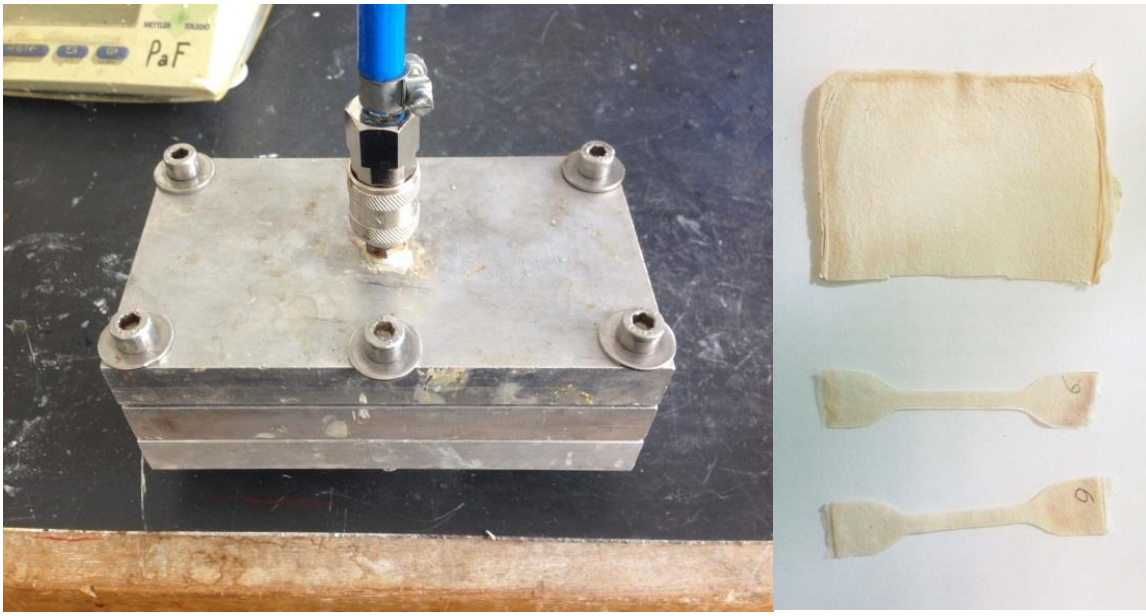

Figure 2 – (a) Pressure filtration rig and (b) prepared sample with cut out dog-bone shape sample for tensile testing on the right

2. Mechanical testing of TEMPO-oxidised amino acid grafted biomaterials

Dried sheets were cut into dog-bone form with a gauge length of 50 mm and a width of 4 mm. Density of each sample were determined by measuring the thickness (c.a. 200 μm) and weight of the manufactured samples [24]. An Instron 8872 was used for tensile testing with the crosshead speed of 1mm min^{-1}. The tests were conducted at room temperature (25°C ± 2°C) and a relative humidity of 55 %. Wet samples were tested after conditioning in a humidity chamber at 30°C and 90% relative humidity for 5 hours. Tests on wet samples were conducted immediately upon removal from the humidity chamber. For each sample (wet and dry) at least 5 specimens were tested and mean average was reported.

3. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR was used to investigate the outcome of TEMPO-mediated oxidation of BC, amino acid modification of TOBC and H-bond formation in the final product. FTIR was performed on

Nicolet iS50 ATR mode and 218 scans were recorded for each sample. We conducted analyses on pure BC, TEMPO oxidised BC with non-reacted amino acid and finally amino acid (Alanine, Glycine and Proline) modified bacterial cellulose.

4. Molecular dynamics modelling

Molecular dynamics simulations were conducted using Ascalaph Designer to study the interaction energies of amino acid grafted bacterial cellulose. Ascalaph Designer has pre-defined quantum mechanical calculations for partial charge distributions in amino acids. However, for cellulose molecules partial charges were calculated using the Firefly/PC GAMESS package [25] via *ab initio* simulations. In the *ab initio* simulations, electrostatic potential derived charges were determined using the second Møller–Plesset [26] perturbation theory alongside the 6-311+G(2d,p) Gaussian basis sets.

Molecular dynamics simulations were carried out using an AMBER94 force field. The potential energy using an AMBER94 force field is determined by four terms; energy of the covalently bonded atoms, their angles, torsion and non-bonded energy between all atom pairs (which have a basis in 6–12 potentials and Coulomb's law respectively) [27]. Molecules and mapping of hydrogen bonds in the simulated molecules was visualised with Visual Molecular Dynamics VMD [28].

For each model, three adjacent molecules of bacterial cellulose with grafted amino acids were positioned in parallel with an initial separation distance of 10Å. Each molecule consisted of 36 glucopyranose repeats and 12 amino acids. Glycine, alanine and proline were attached to the C6 primary carboxyl group, to mimic the TEMPO-oxidised amino acid covalent grafting from the experimental work. Furthermore, the adhesive properties of simulated TOBC with amino acid (TOBC-AA) were compared to monomeric amino acid-BC composite wherein 24 amino acids were distributed between BC strands. In the first model for three strands, 12 grafted amino acids on the outer sides are not engaging in molecular interaction with central strand, therefore for monomeric acid BC composite system, 24 amino acid were engaged in intermolecular interactions between the strands. Pure BC with the same characteristics was simulated as reference.

Results and Discussion

1. Molecular modelling

The intermolecular and potential energy for each sample was monitored during the process of molecular self-assembly. The simulations were continued until the molecules reached an energetic steady state, Figure 3. The amino modification of TEMPO oxidized BC is rather fast reaction where EDAC/NHS coupling has been used. This coupling reaction is an

efficient reaction that works by activating carboxyl groups for direct reaction with primary amines via amide bond formation. In our simulations hydrogen bonding would typically reach steady state between 300-400 fs. Table 2 shows the lowest intermolecular energy at steady state and the number of hydrogen bonds at steady state. In this table, BC composites with monomeric amino acids retain higher intermolecular energies, while equivalent TOBC-AA molecules have lower intermolecular energies. Both are nevertheless higher in energy than pure BC. The higher energy of TOBC-AA (specifically TOBC-Ala) compared to pure BC is due to a small increase in hydrogen bond formation, and a subsequent increase in van der Waals interactions due to extra amino acids on the side chains. In TOBC-AA, the number of hydrogen bonds formed was slightly higher than in pure BC; however, the high stiffness of bacterial cellulose crystalline structures retards any evident changes in the molecular conformation of packed bacterial cellulose molecules. Unlike amino acid modified bacterial cellulose, monomeric amino acids have the advantage of being mobile between BC strands and are able to create a network of hydrogen bonds that induce self-healing effects under the conditions of shear.

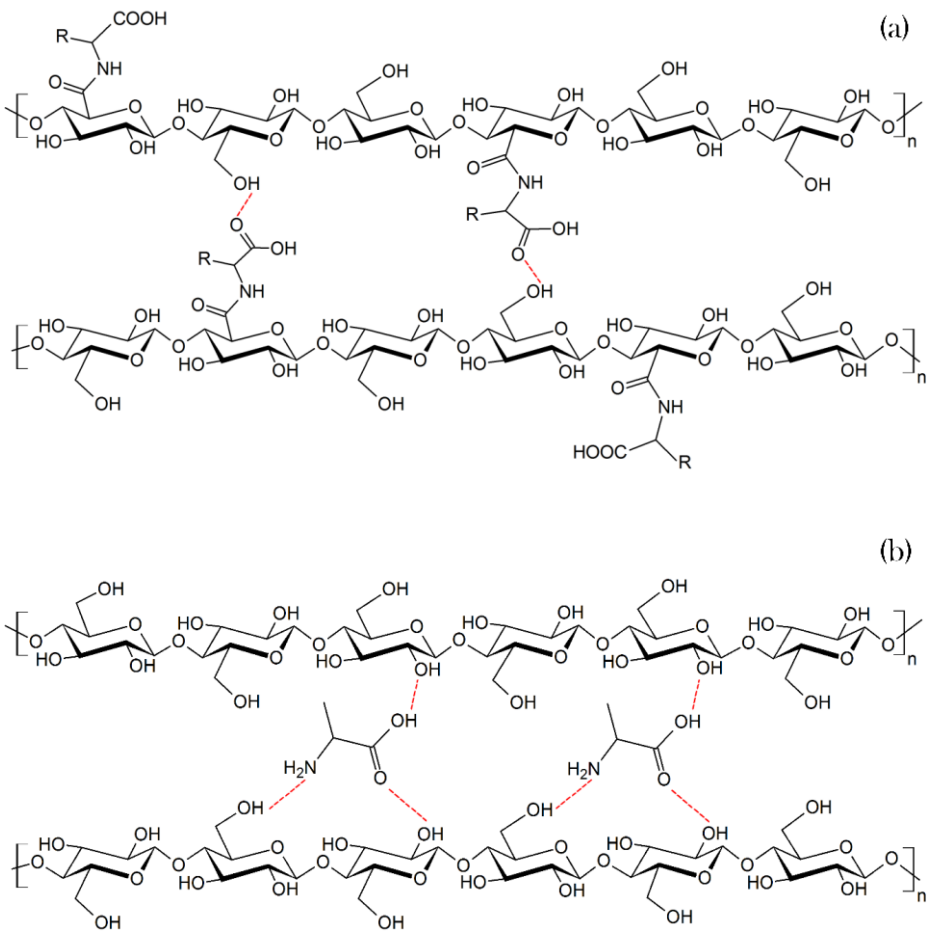

Figure 3 – Schematic illustration of (a)TOBC-Ala and (b) BC-monomeric Ala after self-assembly. Red hashed lines represent possible hydrogen bond formation sites. Monomeric amino acid in bacterial cellulose has higher potential of forming hydrogen bonds.

Amongst the amino acids used (TOBC-AA), TOBC-Ala yielded the highest intermolecular energies and a greater number of hydrogen bonds at steady state. All groups of proteins are essentially capable of forming hydrogen bonds. Hydrogen bonding dictates the secondary structure of proteins like alpha helices and beta sheets. Alanine, glycine and proline are non-polar amino acids. Contrary to polar amino acid, where their side chains act as hydrogen donor or acceptor, the carbonyl and carboxyl groups are hydrogen acceptor and amine and amide nitrogen are the donor in hydrogen bond formation. As a stronger acceptor, the carboxyl oxygen generally forms hydrogen bonds with the amines while the carbonyl oxygen remains unbounded. The difference in hydrogen bonding formation potential is due to size, mobility and side chains of the amino acids. Alanine and glycine have similar quantities of hydrogen bonds forming due to their similar characteristics e.g. in terms of electronegativity, therefore similar affinity to form hydrogen bonds. However, alanine is a bigger molecule and this slightly reduces atomic mobility, thus increasing the van der Waals interaction energies and resulting in an overall higher intermolecular energy than any other combination of TOBC-AA. The large ring structure of proline prevents close range interactions and hydrogen bonding with BC. As such, the intermolecular energy at steady state in TOBC-Pro is lower than those of TOBC-Ala and TOBC-Gly.

Table 2 - Number of hydrogen bonds between molecules in simulated samples and the intermolecular energies for each system after self-assembly. The intermolecular energy is a summation of both the electrostatic and van der Waals interactions. TOBC-Ala, TOBC-Gly and TOBC-Pro values are from the present work and are compared against values for BC, BC-Ala, BC-Gly and BC-Pro (with free moving amino acids) from our previous work [19].

	Intermolecular energy kcal/mol	Maximum number of H-bond
Pure-BC	-218	7
TOBC-Ala	-307	11
TOBC-Gly	-255	10
TOBC-Pro	-245	8
BC-Ala	-357	16
BC-Gly	-348	14
BC-Pro	-324	12

FTIR spectroscopy Error! Reference source not found.(a) shows the FTIR ATR transmittance spectra of bacterial cellulose before and after TEMPO-mediated oxidation and amino acid grafting. In BC the main spectral peaks appear at 3340 cm^{-1} for OH stretching, two peaks indicating CH group; peak at 2893 cm^{-1} for CH stretching in CH_2 groups and at 1428 cm^{-1} for CH_2 symmetric bending and peak at 1162 cm^{-1} corresponds to antisymmetric bridge C-O-C stretching which is from glycosidic linkages within cellulose [29]. Compared to pure BC, TOBC sodium salt has a clear peak at 1573 cm^{-1}, Figure 4(a), which corresponds to a C=O vibration on a carboxylate group and confirms the successful oxidation of bacterial cellulose [30]. The carboxylate group attaches on the C6 position of the glycopyranose ring structure of cellulose.

In Figure 4(a), the vibrational peak from the NH_3^+ group in amino acid appears in the wavenumber range of 1480 cm^{-1} to 1550 cm^{-1} and following amino acid grafting on the carboxylic group, the peak at 1515 cm^{-1} on non-reacted TOBC is diminished. This peak corresponds with the amine group in the monomeric glycine molecule and confirms amino acid coupling to oxidised BC. A carboxylic COO^- asymmetric (ν^{as}) stretch from the amino acid typically arises between 1540 cm^{-1} and 1680 cm^{-1} [31] [32]. A strong peak is visible in TOBC with unreacted alanine, TOBC-Ala, TOBC-Gly and TOBC-Pro at 1605 cm^{-1}. H-bond formation usually appears in the range of 1610 cm^{-1} to 1620 cm^{-1} and on the shoulder of the COO- band [32]. These peaks can be identified by deconvolution of the spectra within this range.

Figure 4(b), shows the clear peak at 1616 cm^{-1}, which corresponds to H-bond formation in some of the TOBC-AA composites. In this spectrum, the intensity of the TOBC-Ala peak is the highest relative peak, evidencing hydrogen bonding presence in this biomaterial. A smaller relative peak is noted in TOBC-Gly biomaterial and no peak is seen at all in the TOBC-Pro biomaterial. Table 3 summarises noteworthy peaks in these biomaterials.

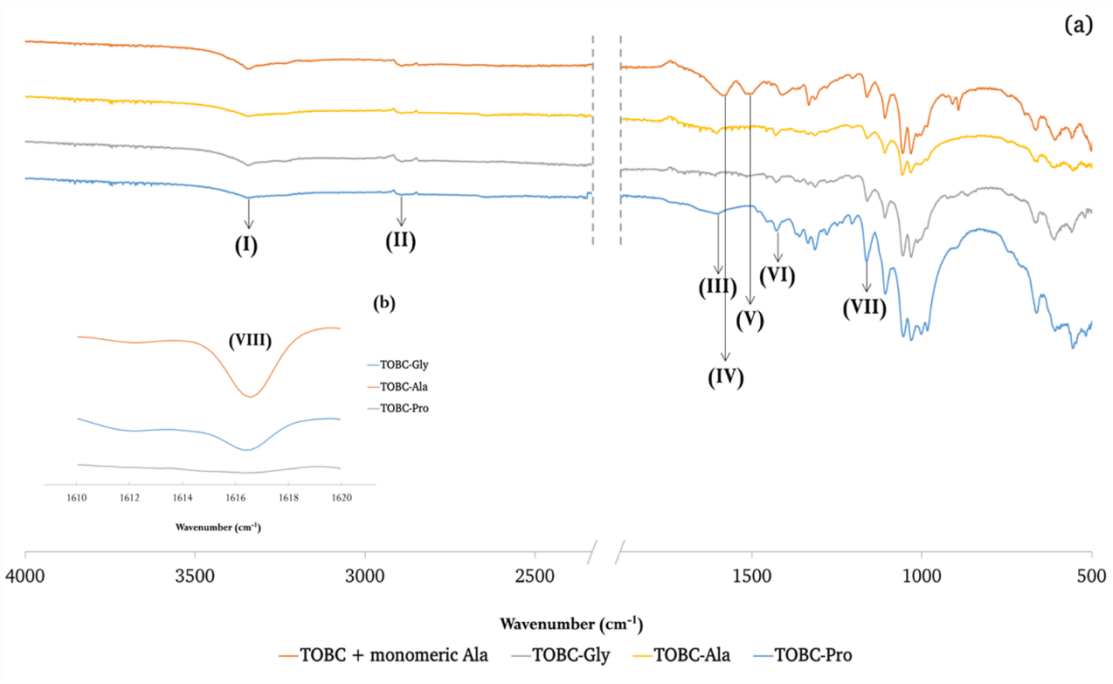

Figure 4 - FTIR spectra for (a) bacterial cellulose peaks present in BC based composite samples; non-reacted TOBC with monomeric alanine, TOBC-Gly, TOBC-Ala and TOBC-Pro, and (b) H-bond peaks for TOBC-AA after spectral deconvolution.

Table 3 - Summary of relevant FTIR spectral peaks during amino acid modification of TEMPO oxidised bacterial cellulose chemical reaction process.

	Chemical bond	Absorption wavenumber
(I)	OH stretching on BC	3340 cm^{-1}
(II)	CH groups on BC	2893 cm^{-1}
(III)	Asymmetric carboxylic COO- stretch on the amino acids	1605 cm^{-1}
(IV)	C=O vibration of carboxylate anion on TOBC-Na	1573 cm^{-1}
(V)	NH_3+ on amine group present with unreacted amino acid and disappears after grafting to TOBC	1515 cm^{-1}
(VI)	CH_2 group on BC	1428 cm^{-1}
(VII)	Anti-symmetric bridge C-O-C stretching	1162 cm^{-1}
(VIII)	Hydrogen bond formation on TOBC-AA	1616 cm^{-1}

2. Experimental mechanical tests

Initial mechanical test results suggest that amino acid modified TOBC sheets possess similar toughness values as compared to pure BC sheets, Figure 5(a). BC sheets based on their production method has tensile strength around 100 to 300 MPa [33] [34] [35] [36].

Although the elastic moduli and the ultimate strengths of the TOBC materials are either slightly higher or equal to those of the pure BC, Figure 5 (b) and (c) respectively, they show nevertheless lower elasticity as compared to pure BC, Figure 5(d). The mechanical strength of TOBC-AA samples against load is higher compared to pure BC which is related to the initial hydrogen bonding and physical obstruction of amino acid side chains. However, the structure weakens once the initial resistance is broken. TOBC-AA crystalline molecular structure lacks flexibility to provide effective intermolecular interactions, and lower hydrogen bonding potential results in relatively similar ultimate strength of TOBC-AA samples and pure BC.

Based on our modelled molecular structures, the side chains of TOBC-AA have the advantage of higher hydrogen bond formation and van der Waals interactions over pure BC. While shearing, side chains will interlock and entangle that in turn will increase the intermolecular resistance to shear deformation, which gives rise to elastic modulus of synthesised TOBC-AA samples, Figure 5(c). Specifically, TOBC-Ala, exhibits marginally superior mechanical properties to TOBC-Pro and TOBC-Gly, which presumably relates to their molecular structures and interactions. This amino acid gives rise to the highest levels of hydrogen bonding (Table 2), and as a consequence, yields the highest strength and stiffness values of all the TOBC-AA biomaterial. Nevertheless, TOBC-Ala biomaterials also exhibit low ultimate strain, which in turn decreases their overall toughness values such that they are similar to TOBC-Gly and TOBC-Pro. Glycine has a similar affinity to form hydrogen bonds as does alanine, but the alanine, being a larger molecule, has stronger van der Waals interactions as compared to glycine. Due to its large ring structure and size, proline has the least opportunity to form hydrogen bonds. As such, it does not reach the same levels of strength and stiffness as TOBC-Ala.

The carboxylic group on the amino acid has higher affinity to water molecules compared to pure BC which increases the plasticisation of TOBC-AA samples. Here, the amino acid side chains connect to water molecules via electrostatic attraction, therefore unlike pure BC, the toughness of TOBC-AA samples will decrease when wet. The elastic modulus of wet samples exhibits opposite trends to dry samples, and TOBC-Pro is noted to have a higher E-modulus compared to TOBC-Gly and TOBC-Ala. Here, water molecules compete over potential hydrogen bonding sites with the amino acid molecules, which reduces biomolecular interactions and weakens the biomaterials. We hypothesise that the large ring on the molecule of proline effectively blocks incoming water, which results in an elevated elastic modulus in TOBC-Pro samples. The mechanical properties of all dry and wet samples are compared in Figure 5. The mean values, standard deviations and coefficients of determination for each test series are provided as numerical values as Electronic Supplemental Material. Representative stress-strain curves of selected manufactured samples is depicted in Figure 6.

In our previous work, we studied the adhesive properties of monomeric amino acid within BC biomaterials [22]. Some of these results have been reproduced with permission from Elsevier, for the purpose of comparison (in Figure 5 and Table 4). BC-Ala was compared with TOBC-AA samples for its similar mole fraction of BC to amino acid. From Table 4, we see that monomeric alanine and glycine give rise to higher strength, stiffness and toughness than the TOBC-AA biomaterials. The reason for this is that they contribute to significantly higher adhesion in the BC sheet because they are mobile and as such, can more easily develop intermolecular hydrogen bonds. Proline is the exception of the three monomeric free-moving amino acids since it has a large ring structure that acts as an obstruction to H-bond formation. BC-Ala has a 40% higher toughness and ultimate strength when compared to TOBC-Ala, and a notably higher elastic modulus. BC biomaterials glued by free monomeric amino acids take advantage of the high mobility of the monomeric amino acids and their ability to create hydrogen bonds with bacterial cellulose. The higher potential for hydrogen bond formation in monomeric amino acid strengthens the composite more effectively than the TOBC-AA samples, which are strengthened primarily through molecular interlocking and entanglements. Self-healing mechanisms increase the elasticity of BC-AA biomaterials where, when subjected to stress, one hydrogen bond breaks, another one can form with the neighbouring bacterial cellulose repeat. Contrarily, we posit that the TOBC-AA biomaterials are unable to self-heal as effectively as BC-AA biomaterials since they are not as easily able to electrostatically reconnect (see Figure 3.) Other factors can also be considered in determining the mechanical performance of TOBC-AA such as modification of pH, which can be further investigated in the future study.

The densities of the TOBC-Ala and TOBC-Gly sheets are slightly lower compared to those of pure bacterial cellulose. That is due to the free volumes that form between the molecules due to the presence of the side chains. Proline, being the larger molecule fills the free volumes between the molecules more effectively than glycine and alanine and increases the final density of the material, Figure 7. Table 4 provides values for the density, specific stiffness, specific strength and mechanical properties normalised by BC values for different samples. These values confirm that the properties of the TOBC-AA biomaterials are inferior to the BC-AA biomaterials also with respect to weight. Based on the Ashby plot in [22], we note that synthesised TOBC-AA biomaterials have specific strength values similar to bone and specific stiffness values close to that of collagen.

Table 4 - Dry density, specific stiffness, specific strength and mechanical properties normalised by BC values for bacterial cellulose with amino acids and amino acid modified TEMPO oxidised bacterial cellulose and pure BC as reference. RH95 - relative humidity at 95°C. The values for BC-Ala, BC-Pro and BC.

	(ρ) - g/cm³	E/ρ - MNm/Kg	σ_{ult}/ρ - kNm/Kg	E/E(BC) %	G/G(BC) %	σ/σ(BC) %
pure BC	0.969	3.7	60.6	1.0	1.0	1.0
pure BC_RH95		2.0	56.2	0.5	1.3	0.9
BC-Gly	0.834	7.1	101.1	1.7	1.1	1.4
BC-Gly_RH95		6.4	98.5	1.5	1.1	1.4
BC-Ala	0.862	6.8	111.0	1.6	1.5	1.6
BC-Ala_RH95		5.1	87.9	1.2	1.2	1.3
BC-Pro	1.026	3.1	60.0	0.9	1.2	1.0
BC-Pro_RH95		1.7	52.0	0.5	1.1	0.9
TOBC-Gly	0.915	4.5	66.1	1.2	1.0	1.1
TOBC-Gly_RH95		3.3	52.2	0.9	0.7	0.9
TOBC-Ala	0.932	4.5	72.2	1.2	1.0	1.2
TOBC-Ala_RH95		2.5	51.0	0.7	0.9	0.8
TOBC-Pro	0.988	3.6	56.9	1.0	1.0	1.0
TOBC-Pro_RH95		3.2	50.8	0.9	0.8	0.9

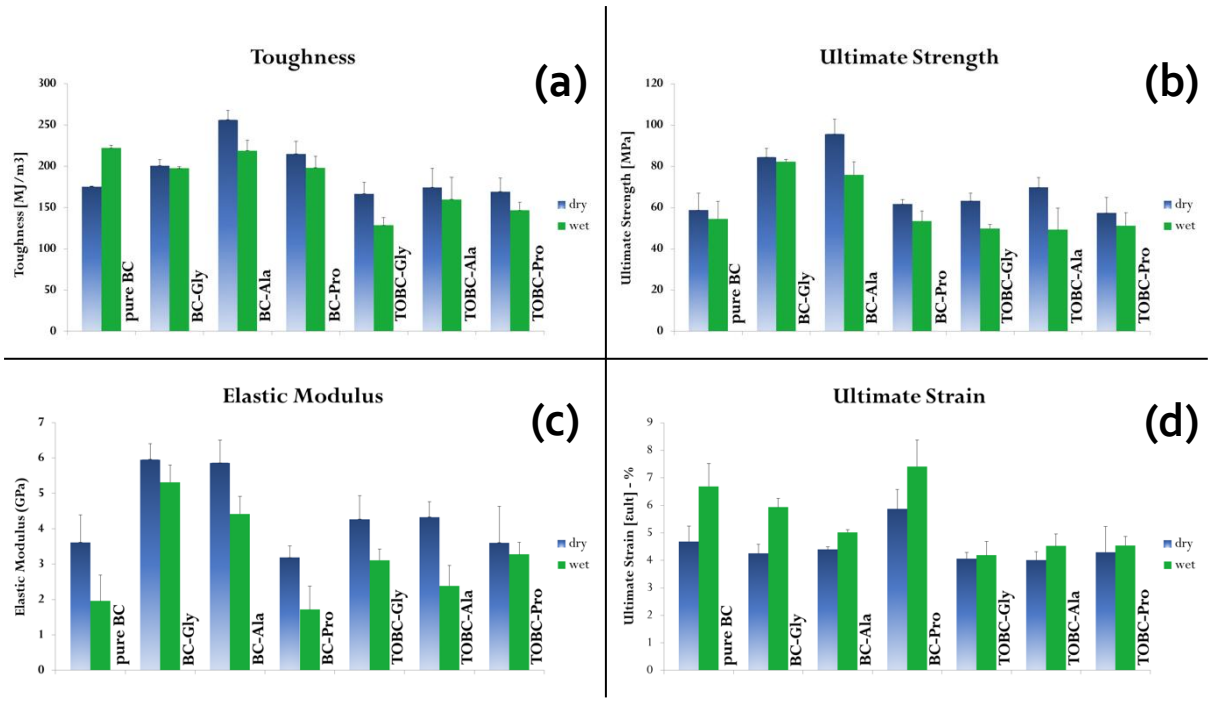

Figure 5 - Histograms showing a) ultimate strength, b) toughness, c) elastic modulus and d) ultimate strain comparison for pure BC, TOBC-AA and BC- monomeric amino acid in both dry and wet states. Error bars indicate the standard deviation from an arithmetic mean.

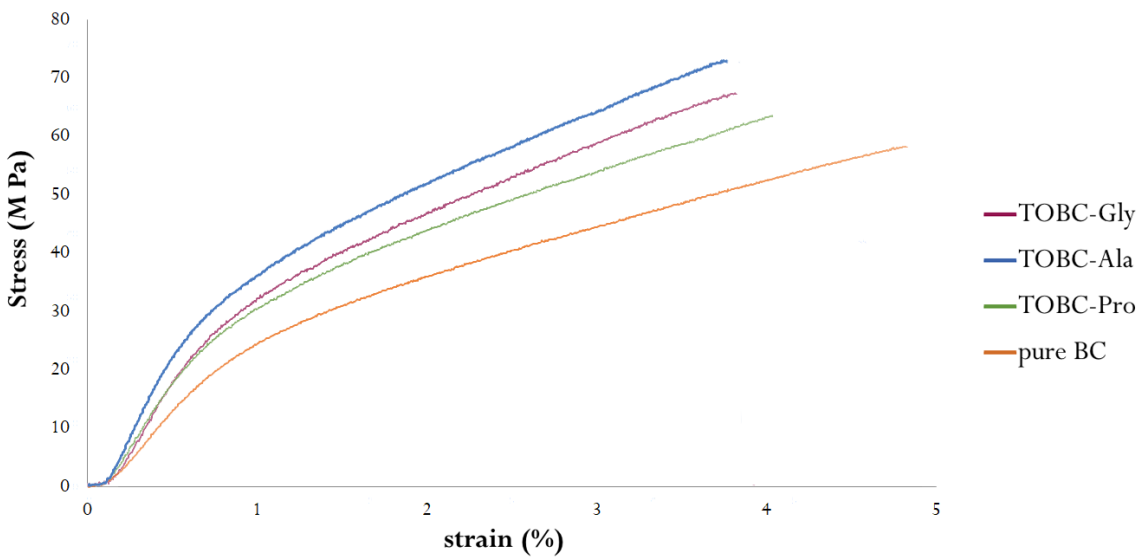

Figure 6 – Stress-strain curve for three samples for the TOBC-Gly, TOBC-Ala and TOBC-Pro samples and pure BC as reference

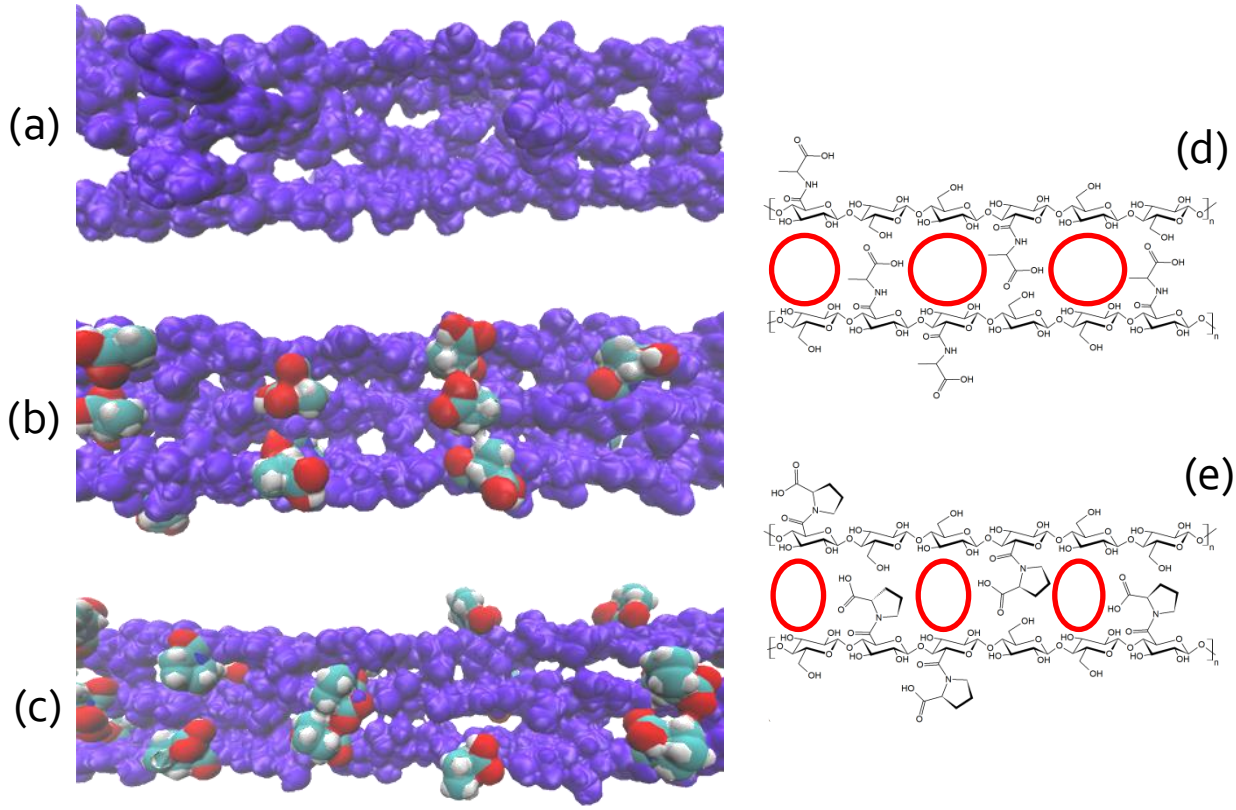

Figure 7 - Surface plots at steady state of (a) pure BC (b) TOBC-Ala and (c) TOBC-Pro. BC molecules were assigned with violet colour, whereas amino acid molecules are identifiable as non-violet colouration.

Conclusions

We studied the mechanical properties of TEMPO-mediated bacterial cellulose-amino acids sheets. These biomaterials can be readily functionalised for various biomedical applications. Here, synthesised samples were tested and compared against pure BC and against BC biomaterials glued using monomeric amino acids. The structures were modelled using molecular dynamics simulations and at the steady state, which showed that TOBC-AA samples developed more hydrogen bonds than pure BC. As a result, these biomaterials were of a higher intermolecular energy as compared to pure BC. Mechanical testing confirmed a modest improvement in TOBC-AA samples over pure BC in terms of ultimate strength and elastic modulus. However, the synthesised samples fall short in mechanical performance when compared to our previously published monomeric amino acid bio-glues in BC biomaterials. The higher stiffness of TOBC-AA samples compared to pure BC comes down to slightly higher hydrogen bonding and the molecular entanglement of side chains. Nevertheless, the lack of mobility and the large size and rigidity of TOBC-AA molecules concurrently results in lower mechanical properties when compared to BC-AA, which develop a more thorough hydrogen bonded network that can self-heal on shearing. In synthesised dry TOBC-AA biomaterials, alanine was seen to have a marginally superior mechanical strength when compared to glycine and proline. In its wet state nonetheless, proline has the highest elastic modulus because its large ring creates a physical obstruction

to water ingress, reducing therefore, the extent of plasticisation noticed in the biomaterial as compared to TOBC-Ala and TOBC-Gly.

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