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Increasing bacterial cellulose compression resilience with glycerol or PEG400 for robuster engineered living materials.

Joaquin Caro-Astorga [a,b,*], Koon-Yang Lee [c], Tom Ellis [a,b,*]

[a] Department of Bioengineering, Imperial College London, London, SW7 2AZ, UK
[b] Imperial College Centre for Synthetic Biology, Imperial College London, London, SW7 2AZ, UK
[c] Department of Aeronautics, Imperial College London, London, SW7 2AZ, UK

A B S T R A C T

Bacterial cellulose (BC) is one of the current natural materials at the edge of innovation in engineered living materials (ELMs) research due to its ease of growth and outstanding properties as a hydrogel. One of the main limitations of this material, however, is its quick dehydration in open environments as water molecules leave the porous network. Here we show that other solvents with higher evaporation temperatures, namely glycerol and polyethylene glycol (PEG), can play the same role as water within the BC structure interacting with cellulose fibres via hydrogen bonds. We demonstrate that these molecules provide up to a 130-fold improvement in the Young's Modulus of BC hydrogels to compression forces in a concentration dependent manner. To take advantage of these effects for application in BC-based ELMs produced by *Komagataeibacter rhaeticus*, we also explored the effect of glycerol and PEG400 on the survival of the BC-producing bacteria in BC pieces. PEG400 at 20% doubled the material resilience to compression forces, still allowing bacteria to survive within the material for weeks. These results open further opportunities to explore new applications and stacked storage conditions.

Introduction

Freshly grown bacterial cellulose (BC) is a hydrogel-like material produced by bacteria from the genus *Acetobacteriaceae* as the structural element of its biofilms. This material is attracting attention from a myriad of industries due to its applications as a dry or wet material in areas like food, textiles, composites, cosmetics or medicine (Gorgieva and Trček, 2019; Gorgieva and Trček, 2019; Zhong, 2020). BC is produced by growth of the bacteria within days and is generally considered as a hydrogel at the point of its production due to its high-water holding capacity, where it retains up to 99% of water [2]. As well as this, it naturally has desirable mechanical properties (3-5 GPa), a high degree of crystallinity (84-90%)(Czaja et al., 2004), a translucent nature, high purity, high porosity, biocompatibility and biodegradability (Zhong, 2020). And when fibres are aligned to make BC crystals, the mechanical properties become even more impressive (e.g. tensile strength 100–400 MPa and Young's modulus 10–20 GPa)(Wang et al., 2017), Many of these desirable properties are associated with the hydrated state of BC where interactions between water molecules and cellulose molecules define the material properties(Lupaşcu et al., 2022). On the molecular scale, water bridges BC fibres with hydrogen bonds, maintaining the fibre separation and providing outstanding material stability. Dehydration due to water loss via evaporation removes these bridges and produces hornification, a process that agglomerates and stacks the cellulose fibres and turns the material into a brittle piece(Lee et al., 2014).

Several attempts of using glycerol and PEG to improve the mechanical properties showed poor results. Previously, PEG 200 has been proposed as a candidate for long term storage of BC and hornification prevention, but offered no improvement in tensile strength (Santmarti et al., 2020). Elsewhere, glycerol, in combination with other compounds and in low concentrations has also been assessed and was shown to improved tensile strength of BC up to 10 and 20 fold in stress and in Young's Modulus measurements, respectively (Cielecka et al., 2019). Based on these past observations, we here explored the effect of soaking BC in different concentrations of glycerol and PEG 400 to investigate whether this could prevent hornification as PEG200 does and/or lead to improved material resilience.

BC is usually used as a purified material, but it has a great potential in ELMs research and particularly in applications where materials containing genetically engineered cells can provide new 'living' properties material like self-repair, sensing and responding to chemical or physical cues and antimicrobial properties (Gilbert et al., 2019; Nguyen et al., 2018; Sabio et al., 2021a, 2021b). Recently the *Komagataeibacter* Tool Kit (KTK) has been developed in our lab to engineer the high BC yield producer *K. rhaeticus* and its related species, opening up a new stage for ELMs based on BC (Goosens et al., 2021; Jin et al., 2022). Given the

* Corresponding authors.
E-mail addresses: jcaroast@ic.ac.uk (J. Caro-Astorga), t.ellis@imperial.ac.uk (T. Ellis).

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outstanding properties of BC in its hydrogel form, we investigated how glycerol and PEG 400 can improve the grown material robustness and can potentially be formulated to allow survival of engineered living cells within the material.

Materials and methods

Dehydration test

Commercially-grown BC was purchased from a retailer (Xiangsun Ltd., Lugang Township, Changhua County, Taiwan). The pellicles (approx. 1.5 cm thickness) were cut in cubic shape pieces of 2 × 2 × 1.5 cm and treated with 5 times the volume of the cube of 20%, 50%, 80% and 100% of glycerol and PEG 400 diluted with distilled water. The cubes were incubated overnight with agitation, repeating the process of exchanging the medium 6 times. The pieces were placed in a tray and incubated at 60°C for 14 h, measuring their weights every hour. The experiment was performed in triplicate.

Compression test

BC cut into cubes of 2 × 2 × 1.5 cm were treated with 5 times the volume of the cube of 20%, 50%, 80% and 100% of glycerol and PEG 400 diluted with distilled water and were incubated with agitation overnight, exchanging the medium 6 times in the process. The cubes were rolled quickly on filter paper to remove the glycerol or PEG adhered to the sample, but not belonging to the hydrogel structure. Then placed in the Zwick/Roell Z2.5 equipment to test sample performance in compression test at a speed of 10 mm/min. The experiment was performed at 20°C.

The stress was calculated as Loading Force (N) / Area (mm^2). The strain was calculated as $\Delta L/L_0$, where L_0 is the initial thickness of the sample and ΔL is the exerted compression from the starting point. Young's modulus values were calculated from the average stress/strain relationship of the 20 values following the first register for 0.002, 0.1, 0.2, 0.3 or 0.4 values of strain. The measurements were performed with between 4 and 8 replicates.

Bacteria conservation within BC samples

Small BC sample pellicles were grown by *Komagataeibacter rhaeticus* iGEM in 96 square deep-well plates at 30°C in static conditions. After 7 days of growth, pellicles were collected and soaked in 5 ml of 20% glycerol for 1 h in shaking conditions at 50 rpm to facilitate homogenization. After that, pellicle samples were stored at 4°C. Samples were collected at each time point to assess survival in triplicate. Pellicles were placed in 2 ml tubes, suspended in Hestrin–Schramm (HS) medium (Schramm et al., 1957) (5 g/L yeast extract, 5 g/L soybean peptone, 20 g/L glucose, 8 mM citric acid, 20 mM Na_2HPO_4) diluted 1 in 10 with 5% cellulase and incubated at 37°C for 3 h in shaking conditions to degrade the cellulose. Serial dilutions of each suspension were made, and these dilutions were plated in four technical replicates on HS agar plates supplemented with 2% of glucose. After 5 days of incubation at 30°C, colonies were counted from the agar plates and colony-forming units (CFUs) per cm^2 pellicle area were calculated.

Scanning electron microscopy (SEM)

Samples of BC were placed in the oven at 60°C overnight to dehydrate the samples. The samples treated with glycerol and PEG and compressed were placed in water to remove the compounds for two hours twice before dehydration. Images were taken using LEO Gemini 1525 FEGSEM equipment after coating BC with 10 nm of chrome.

Results

BC pellicles with glycerol and PEG 400

Cellulose fibres establish hydrogen bonds with each other and with the water retained in the hydrogel due to the polarity of the three C-OH groups per glucose unit (Fig. 1a) (Ek et al., 2009). These forces allow more than 99% of the weight of BC to be water when it is fully hydrated but are not strong enough to prevent the water molecules evaporating easily from BC surfaces during air contact. Glycerol is also a polar molecule able to establish three hydrogen bonds per molecule via its three OH moieties. Compared to three molecules of water, glycerol has the three polar groups linked to a 3-C-chain that gives additional stability and prevents its evaporation at room temperatures (Fig. 1a). Polyethylene glycol (PEG) shows the same properties of being soluble in water, containing polar moieties within a carbon backbone and not easily evaporating. In an initial attempt to coat BC samples to prevent evaporation, we observed that BC pieces coated with glycerol and PEG 400 lose volume due to water evaporation, but the pellicles only lose thickness, and do not get wrinkled and deformed, leading us to speculate that these molecules were diffusing into the material and taking the place of water. From this, we then studied the dehydration profile of BC samples that has been soaked in different concentrations of PEG 400 and glycerol, observing that slopes of dehydration for these samples were lower with increased concentrations of either of the solvents, clearly showing how these molecules help to slow the process of dehydration (Fig. 1b).

We also observed that glycerol and PEG 400 containing BC had a much stronger consistency compared to water-containing BC. Past studies reported an increase in tensile strength when these molecules were used as plasticizers, but poor data was found regarding compression resilience (Bella et al., 2020; Cielecka et al., 2019). To assess this ourselves, we used 2 × 2 × 1.5 cm BC pieces and exchanged the water within their matrix with glycerol or PEG 400 by serial immersions. These pellicles showed an increase in the transparency of the material (Fig. 1c), a property very desirable for many commercial applications. This effect was also reported in past work where 8% PEG 400 was added to the culture medium, obtaining more transparent BC pellicles (Beekmann et al., 2020).

As BC is a biological material that can show local variability, pellicles were measured in volume and weight before and after exchanging solvent, showing high similarity between BC pieces (Supplementary Fig. 1). The treated pellicles were then tested for their response to compression forces when containing either water, glycerol or PEG 400 within their matrix. In the compression testing of the pieces, there are several different stages. In the first stage, the piece of BC suffers a plastic deformation leading to an internal rearrangement of fibres and solvent and leading to a concave-to-convex shift in the sides of BC pieces, without squeezing out any solvent. After the rearrangement, the BC network is then in tension and unable to expand at the sides. The process then goes through a new stage in which the compression force is bigger than the force of the cellulose-solvent hydrogen bonds, squeezing the solvent out of the material as liquids are not compressible. The squeezed liquid liberates space in the structure to allow extra rearrangement of the fibres and solvent. This gradual change cannot be seen in the compression curve as a clear slope change, resulting in a soft curve instead. For this reason, it is difficult to calculate Young's Modulus and strength. In the last stage, when hydrogen bond forces are much higher than the compression force, cellulose fibres achieve maximum tension and break, allowing a much higher rearrangement which is seen in the data curves as a temporal inversion of the slope as fibres are not in tension anymore.

The mechanical properties of BC vary depending on the level of hydration (Rebelo et al., 2018). To compare the effect of different solvents in BC materials, we compared the stress at several specific strains (Fig. 1d). The strength at a strain of 0.4 (40% compression) was on

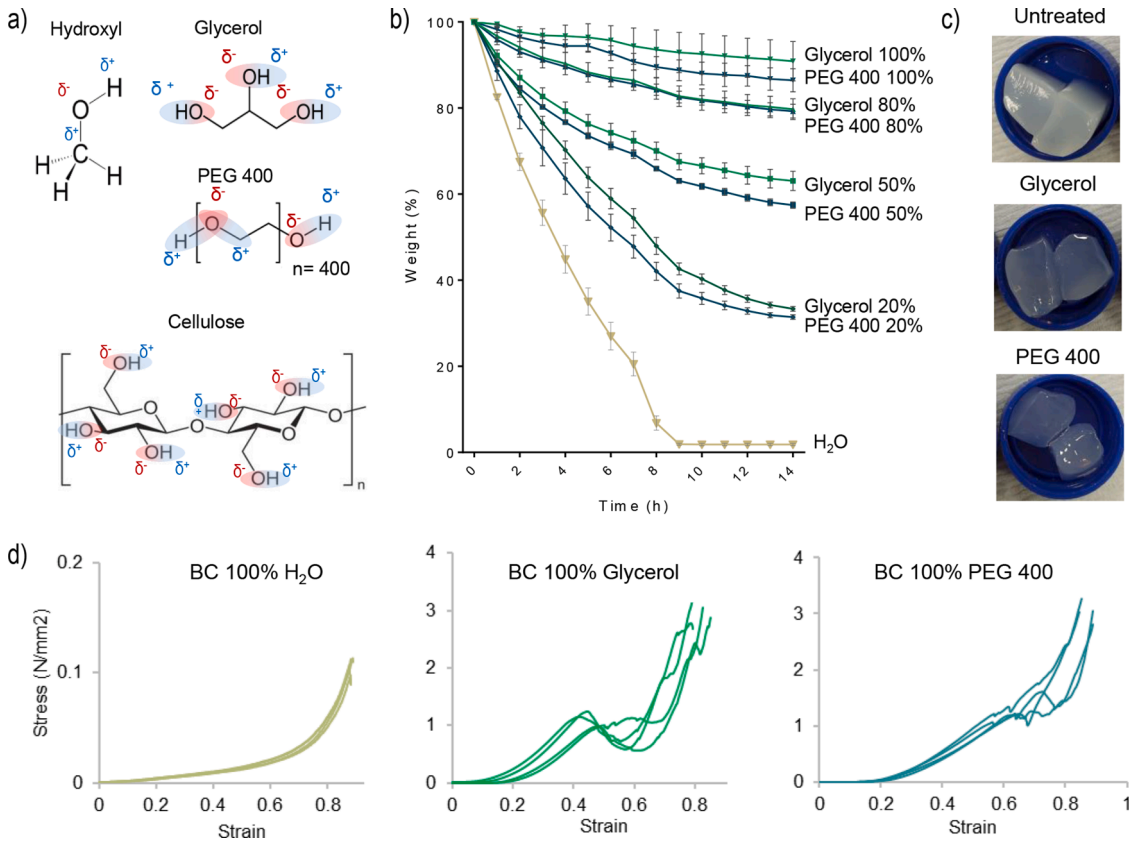

Fig. 1. Bacterial Cellulose treatment with glycerol and PEG 400. a) Polarity of the hydroxyl group, glycerol and polyethylene glycol molecules showing the position of the partial charges able to produce H-bonds. b) Dehydration profile of BC containing water and different concentrations of PEG400 and glycerol at 60°C. Error bars indicate SD. c) Images of BC pieces with glycerol and PEG 400, gaining transparency after the treatment. d) Compression stress curves of BC and BC soaked in glycerol and PEG 400 in four replicates.

average 0.009, 0.823 and 0.49 N/mm^2 (MPa) for water, glycerol and PEG 400, respectively, with glycerol and PEG 400 being approximately 90- and 50-folds higher strength than water-BC pellicles (Fig. 2a). We also calculated Young's modulus at 0.002, 0.1, 0.2 and 0.3 strains (Fig. 2b, Table 1). Samples of BC with glycerol and PEG 400 used as a solvent showed up to 130- and 75-fold higher Young's modulus, respectively than the original water-BC samples (Table 2). After full compression, the water-BC samples maintained their square shape, while the glycerol and PEG 400 treated samples expanded and acquired a hollow rounded shape (Supplementary Figure 2a-b). This can be interpreted as (1) the internal structure of water-BC is not affected; (2) the cellulose fibres have not been in maximum tension in water BC and

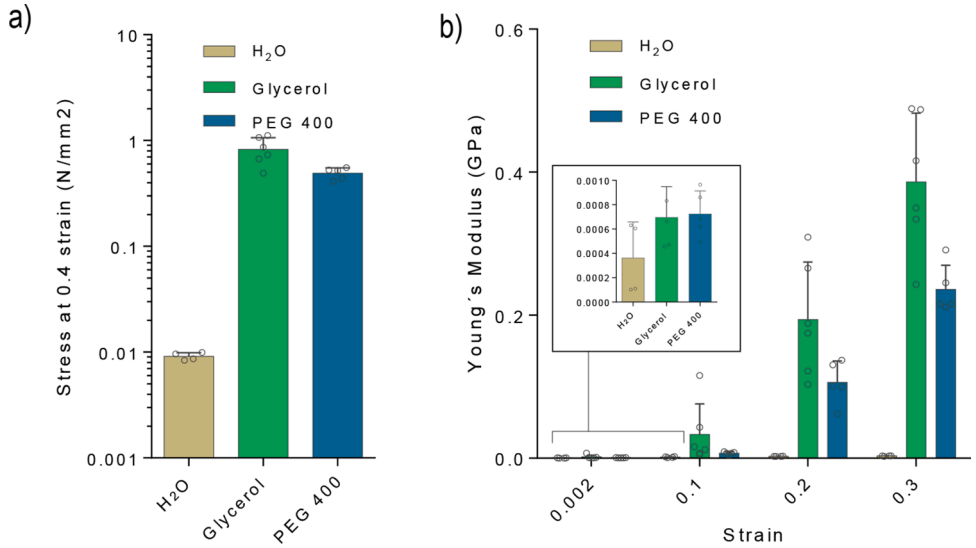

Fig. 2. Mechanical properties of BC with glycerol and PEG 400 used as solvents. a) Stress values at a strain value of 0.4 (40% compression) for BC pieces (2 × 2 × 1.5 cm) soaked in glycerol and PEG 400 and compressed at a speed of the loading cap of 10 mm/min. b) Young's modulus at different strains (2%, 10%, 20% and 30% compression) for BC pieces treated with glycerol and PEG 400. Bars indicate the average of 4 to 6 samples. Error bars indicate SD.

Table 1
Average calculated Young's modulus (MPa) at different strains of BC samples containing water, glycerol or PEG 400 as solvent, ±SD.

Strain	0.002	0.10	0.20	0.30
H_2O	0.36 ±0.26	1.62 ±0.54	2.68 ±0.19	3.14 ±0.35
Gly	1.70 ±0.24	33.26 ±15.31	193.90 ±63.04	386.54 ±87.43
PEG	0.72 ±0.13	7.10 ±0.81	106.19 ±16.85	235.91 ±30.83

Table 2
Average fold increase in Youngś modulus of BC samples containing glycerol or PEG 400 compared with water as solvent.

Strain	0.00	0.10	0.20	0.30
Gly	4.7	20.5	72.4	123.1
PEG	2.0	4.4	39.7	75.2

have just adapted to a new shape as the force is applied and water squeezes; (3) glycerol and PEG 400 give the hydrogel enough consistency to bring cellulose fibres to maximum stress up to the breaking point, maximising the material resilience; and (4) the major tension and breakage of the cellulose fibres takes place in the innermost area of the material, reshaping it into a hollow rounded form. Despite the deformation of these glycerol and PEG 400 samples, the remaining BC could rehydrate with water up to approx. 80% of the original volume. However, recovering the original squared shape was only possible in water-BC samples, as they maintained the cellulose macro structure (Supplementary Figure 2c). We used SEM imaging to visualise whether the compression forces or the treatment with glycerol or PEG produced any major changes in the fibres and their distribution, but no clear differences were observed (Supplementary Figure 2d).

The effect of glycerol and PEG 400 in different concentrations on BC resilience

We next investigated how different concentrations of glycerol and PEG 400 would affect the mechanical properties of BC. We repeated the compression testing experiments using 20%, 50% and 80% concentrations for each compound, revealing a decoupled effect in the rise of strength values in response to increased concentration (Fig. 3 and Supplementary Figure 3). While PEG 400 produced a gradual increase in the resilience to compression forces with increasing concentrations, glycerol showed little improvement at low concentrations and much better performance at high percentages of volume. It is also interesting that the difference between 80% and 100% PEG 400 is not as high as in the case with glycerol. These differences should be related to the nature of the long-chain molecules of PEG 400 versus the small molecules of glycerol, affecting the interactions with cellulose and water. At 100% concentrations, glycerol shows much better improvement in mechanical properties than PEG 400, which can be explained by the fact that glycerol can establish more hydrogen bonds per mass than PEG 400. For 80 and 100 % in both compounds, can be observed and inversion in the curves. This is normal behaviour when a material achieves the elastic limit in these tests and produces breaks in parts of the fibres. The broken fibres no longer offer resistance to the compression force and the stress drops for a period. In low concentrations of glycerol or PEG400 there is water that interacts with less energy and can be squeezed out of the material easily, allowing fibres, glycerol/PEG and water molecules to rearrange and preventing fibres from hitting the breaking limit.

Bacterial survival in grown pellicles with 20% glycerol and PEG 400 used as solvent

BC-based living materials are of increasing attention in the emerging research field of engineered living materials. We explored if we could improve the mechanical properties of BC-based materials with living cells within them by using glycerol and PEG 400 in concentrations that do not impede bacterial survival within BC. Such an improvement could enable or improve many BC-based material applications that would require hydrogel-like properties but also the actions of living cells within the material.

Glycerol is commonly used to conserve bacteria as frozen stocks in strain collections ("Long-Term Preservation of Fungus Cultures with Liquid Nitrogen Refrigeration | Applied Microbiology," n.d.), adding 10-20% final concentration of glycerol to bacterial cultures. In our experiments, the concentration of 20% glycerol or 20% PEG 400 is high enough to show a 1.15-fold and 2-fold improvement in BC Young's modulus towards compression forces at a strain of 0.2 (Fig. 4a). However, in a 16 week experiment we found that bacteria in 20% glycerol only survived for less than 8 weeks at 4°C, while bacteria in 20% PEG 400 showed much better performance, with bacteria viability possible in this condition beyond 8 weeks (Fig. 4b). PEG 400 provided a higher Young's modulus than glycerol and so is revealed as an option for doubling the resilience to compression forces for future BC-based ELMs without abolishing cell viability in the material within an 8 week timeframe. Note that to perform the survival experiment, dilutions of glycerol and PEG 400 were prepared in distilled water, which meant maintaining bacteria in a media without any nutrients present. As a control, we used Hestrin-Schramm (HS) media diluted 1:10 with water, and this showed much better survival of bacteria over time, presumably partly due to nutrients and ions being present. Therefore, using HS media to dilute PEG 400 to 20% instead of water could be a further method to increase conservation and may also allow higher concentrations of glycerol and PEG 400 to be used in BC-based ELMs, potentially also increasing the desirable mechanical properties of these cell containing hydrogel materials further.

Discussion

Glycerol and PEG solvents have previously been studied as

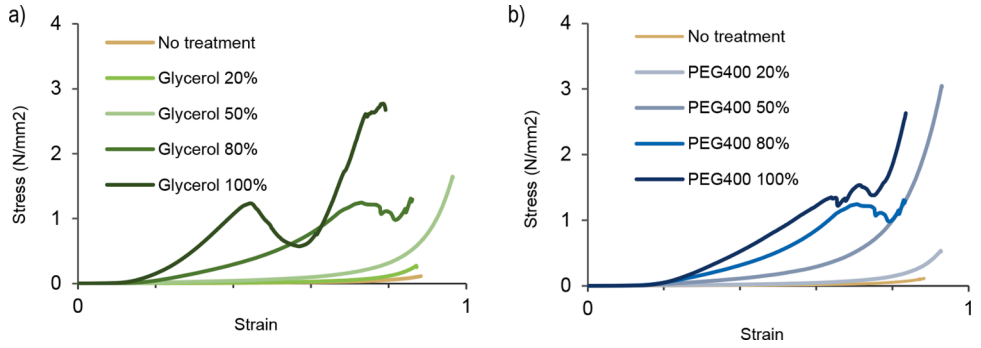

Fig. 3. Bacterial cellulose resistance to compression forces. Compression test curves of one representative sample of BC and BC soaked in 20%, 50%, 80% and 100% of glycerol (a) and PEG 400 (b). Untreated BC in brown.

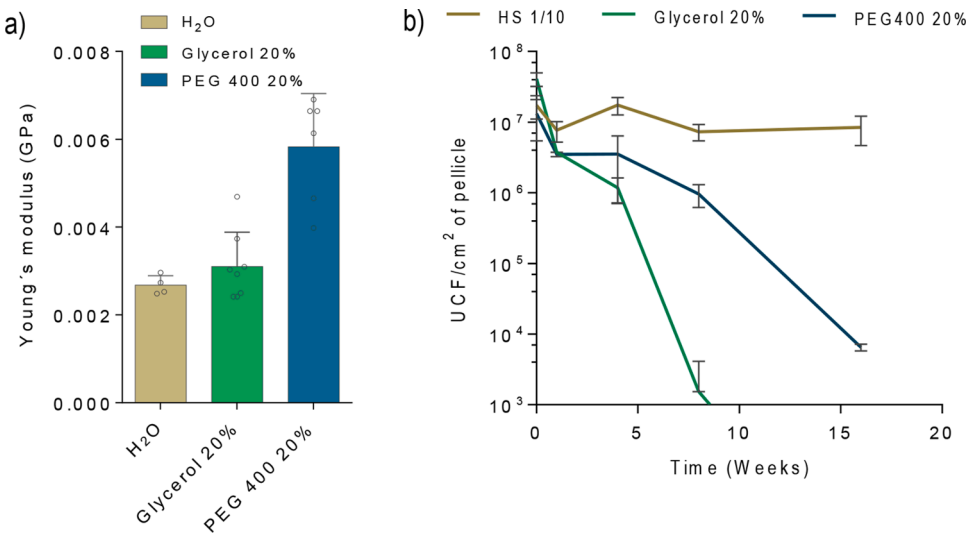

Fig. 4. Living materials soaked in 20% glycerol and PEG 400. a) Young's moduli at 0.2 strain (20% compression) for BC samples soaked in either water, 20% of glycerol and 20% of PEG 400. Bars indicate the average of between 4 to 8 samples. Error bars indicate SD. b) Survival of *K. rhaeticus* bacteria up to 16 weeks within BC pieces soaked in either Hestrin–Schramm (HS) media (diluted 1:10 with water), in 20% glycerol, or in 20% PEG 400. Pieces were stored at 4°C. Error bars indicate SD of 3 biological replicates, plated in 4 technical replicates.

plasticisers or hornification preventive agents for BC, sometimes reporting changes in mechanical properties. Unfortunately, however, there are still many gaps in our knowledge about the possible advantages of these compounds when used with BC due to the endless combinations of compounds, concentrations, combinations, their effect in tension or compression experiments and the conditions of the mechanical tests. One of the key problems is the many different molecular weights (MW) of PEG available which can show different results and can also be used in mixtures of PEG of different MW. This makes difficult for a single research work to cover the entire spectrum of possibilities. In past works, replacing water for PEG 200 in BC has been proposed for long term storage of BC at room temperature, to prevent hornification, but no significant differences were observed in tensile strength (Santmarti et al., 2020). As we report in this work, PEG 400 and glycerol provide outstanding mechanical properties improvements. These molecules have other advantages, as PEG and glycerol in high concentrations prevent the growth of microorganisms that can degrade BC due to a reduced water activity (Cox, 1966; Roger et al., 1992). Glycerol can also inhibit cellulase activity, providing an extra level of protection of the cellulose. In contrast to results reported with PEG 200 (Santmarti et al., 2020), other works showed that PEG and glycerol do improve the tensile strength and Young's Modulus of BC in different conditions. Mixtures with chitosan, carboxymethyl cellulose or hydroxyethyl cellulose plasticized with <1%, 2.5% or 10% glycerol showed improved tensile strength, but compression tests were not performed and only low concentrations of glycerol were used (Bella et al., 2020; Cielecka et al., 2019). It is worth noting that these polysaccharide mixtures, although derived from cellulose, are structurally very different and, consequently, it is reasonable that they greatly differ in their functional properties. Our work covers that gap, revealing outstanding improvements in compression forces and the effects of different concentrations in purified bacterial cellulose.

In a previous work, BC with PEG 200, PEG 1000, mixtures and polyethylene glycol diacrylate (PEGDA) polymerized with UV light (Numata et al., 2015) showed a similar performance to BC-water in compression tests and did not achieve the fracture point, but this was probably due to the settings, as they used a very slow speed during compression (10% min^{-1}), squeezing the liquid matrix slowly while fibres readjust. In our conditions, we used a faster movement of the loading cap (10 mm/min), allowing us to achieve the fracture point in high concentrations of glycerol and PEG 400. The variability produced by different speeds of the compression is explained by two factors. One is due to the time required for the rearrangement of the solvent within the material upon a continuous force, producing the adaptation of the material to the forces. Secondly, when compression forces reduce the internal space available to allocate the solvent, it needs to be displaced and flow all the way out through the pores of the material against the forces of hydrogen bonds that continuously are establishing and disappearing while the molecules are moving. This slow flow requires time to squeeze the solvent out and slow compression experiments would allow rearrangement and solvent reallocation, showing no effect. In our experiments, the substitution of 80% and 100% of water with glycerol and PEG allowed us to achieve the fracturing point. At 0.4 strain, the Young's modulus of glycerol-soaked BC was up to 130-fold higher than water-BC, measurements that may increase at a higher speed of the applied force. These results suggest that any application using BC with glycerol or PEG must take into account the nature of the forces that BC will overcome in order to harness the improvement of these treatments.

The increase of 2-fold in the compression resilience of BC when soaked in 20% PEG 400 is a great improvement for future BC-based ELM applications. In the case of glycerol, we found that the increase in compression resilience at low concentrations is poor and shows higher toxicity compared to PEG. However, there is room for improvement in bacterial survival using combinations of glycerol and PEG, the use of other MW of PEG and the addition of nutrients to the solutions. Our viability results show that bacteria are reasonably well-conserved within the material when soaked in the correct concentrations of glycerol or PEG. In fact, *Komagataeibacter* can use glycerol as a carbon source (Kose et al., 2013). The problem for the bacteria is inability to grow in high concentration of these solvents, as they produce osmotic stress that halts cell activity. This is an effect studied previously in many organisms (Ghedira et al., 2018; Roger et al., 1992; Szymanowska-Powałowska, 2015) where the high osmotic environment arrests bacterial division immediately. What we propose here is a compromise that we believe is useful for ELMs applications, where concentrations of infused solvents are high enough to give improved material properties and reduce hornification, while preserving cells for at least several weeks. Where the material requires active cells for a 'living' element (e.g., for self-repair) the infused BC could then be soaked in rich media without glycerol or PEG to awaken the cells, restart growth and begin the intended action. The addition of HS media components, such as glucose, may lead to some changes in the mechanical properties of the materials, but we expect the impact of this to be marginal. It is highly unlikely that the bacteria in the material will have had undergone any lasting changes between growth arrest and it being restarted, as it is well-established that microbes stored in high glycerol concentrations are well-preserved.

The improvement in compression resistance can be directly applied to improve the BC storage or to contribute to developing new

applications of Engineered Living Materials. It can also inspire applications for flexible electronics or for the field of Organismal Engineering, incorporating organic materials or living materials into classical robots. Bacterial cellulose materials with glycerol/PEG with or without genetically engineered bacteria could be used for joints, artificial skin or organic pseudo-organs integrated into robots (Chiolerio and Adamatzky, 2021; Knöller et al., 2020; Webster-Wood et al., 2017).

Conclusion

In this work, we have uncovered the improvement up to 130 folds in the resistance of BC materials to compression forces by exchanging the water for glycerol and PEG 400 in a concentration dependent manner. These molecules had a retardant dehydration effect for samples containing mixtures with water. As an application for engineer living materials, we demonstrate that it is possible at least to double the material resilience to compression forces using PEG 400 20% while maintaining bacteria alive.

CRediT author statement

JCA proposed and designed this research, performed the experiments and wrote the first version of the paper. KYL provided the bacterial cellulose material and gave advice on mechanical testing. TE supervised all the work, reviewed the manuscript, and wrote interactively through multiple rounds of revisions. JCA, KYL and TE discussed the results and commented on the paper.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.carpta.2022.100245.

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