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1 Multi-Enzyme Cellulose Films as Sustainable and
2 Self-Degradable Hydrogen Peroxide Producing
3 Material

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13 **ABSTRACT:** The use of hydrogen peroxide releasing enzymes as component to produce
14 alternative and sustainable antimicrobial materials has aroused interest in the scientific
15 community. However, the preparation of such materials requires an effective enzyme binding
16 method which often involves the use of expensive and toxic chemicals. Here, we describe the
17 development of an enzyme-based hydrogen peroxide producing regenerated cellulose film (RCF)
18 in which a cellobiohydrolase (*TrCBHI*) and a cellobiose dehydrogenase (*MtCDHA*) were
19 efficiently adsorbed, $90.38\% \pm 2.2$ and $82.40\% \pm 5.7$ respectively, without making use of cross-
20 linkers. The enzyme adsorption kinetics and binding isotherm experiments showed high affinity
21 of the proteins possessing cellulose-binding modules for RCF, suggesting that binding on
22 regenerated cellulose via specific interactions can be an alternative method for enzyme
23 immobilization. Resistance to compression and porosity at a μm scale were found to be tunable by
24 changing cellulose concentration prior to film regeneration. The self-degradation process,
25 triggered by stacking *TrCBHI* and *MtCDHA* (previously immobilized onto separate RCF),
26 produced $0.15 \text{ nmol}/\text{min}\cdot\text{cm}^2$ of H_2O_2 . Moreover, the production of H_2O_2 was sustained for at least
27 24 hours reaching a concentration of $\sim 2\text{mM}$. The activity of *MtCDHA* immobilized on RCF was
28 not affected by reuse for at least 3 days (1 cycle/day) suggesting that no significant enzyme leakage
29 occurred in that timeframe. In the material herein designed, cellulose (regenerated from a 1-ethyl-
30 3-methylimidazolium acetate/DMSO solution) serves both as support and substrate for the
31 immobilized enzymes. The sequential reaction led to the production of H_2O_2 at a μM - mM level
32 revealing the potential use of the material as a self-degradable antimicrobial agent.

33 **Keywords:** Cellulose, biodegradable, antimicrobial material, cellulose film, hydrogen peroxide,
34 H_2O_2 , CDH, CBH, cellobiohydrolase, cellobiose dehydrogenase.

35

36 1. INTRODUCTION

37 The discovery of hydrogen peroxide (H_2O_2) in the early 19th century, followed by its use
38 as potent oxidant represented an important breakthrough in many applied fields. ¹ Thanks to its
39 oxidative damage towards biological macromolecules (e.g. proteins and DNA), H_2O_2 can be used
40 as antimicrobial agent. ² H_2O_2 has a wide spectrum of efficacy against bacteria, fungi and viruses
41 which makes it one of the most commonly used antiseptic substances. ^{3,4} However, repeated topical
42 applications of diluted H_2O_2 commercial products (0.03 wt.% aqueous solutions) may exhibit
43 adverse effects caused by the generation of harmful oxygen reactive species (ROS).⁵ For this
44 reason, the use of H_2O_2 producing enzymes, capable of releasing appropriate levels of H_2O_2 , as
45 novel antimicrobial agents are under investigation.⁶ This class of enzymes includes glucose
46 oxidase (GOx) and cellobiose dehydrogenase (CDH) which are able to steadily produce H_2O_2 in
47 the presence of glucose and cellobiose respectively. Despite their ability to produce H_2O_2 in
48 solution, enzyme immobilization into a polysaccharide network is essential for the design of
49 advanced materials such as antimicrobial films and food preservatives.⁷ Specifically, the use of
50 cellulose in combination with its corresponding degrading enzymes (i.e. cellulases) would allow
51 the release of the required glucose or cellobiose substrates for GOx or CDH which in turn produce
52 H_2O_2 .

53 The use of CDH coupled with cellulases in a cross-linked carboxymethyl cellulose/succinyl
54 chitosan gel has been reported as an example of coupled enzyme reaction for the design of a
55 promising wound dressing material.⁸ However, polymer cross-linking and the use of different
56 polymers can make the design of such materials rather intricate, thus not easily reproducible.
57 Cellulose regenerated from solution instead, represents an excellent enzyme support since it
58 provides access to materials with a tunable shape and porosity produced using a widely available

59 resource and sustainable processes.⁹ Cellulose dissolution can be achieved at room temperature
60 using ionic liquids (IL)¹⁰ such as 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) and 1-ethyl-
61 3-methylimidazolium acetate ([EMIm][OAc]) which can be recovered and recycled subsequently
62 in the regeneration process.^{11,12} Regenerated cellulose hydrogels (in both pure and derivatized
63 forms) are considered biocompatible materials, hence, employable in a wide range of biomedical
64 applications including tissue engineering^{13,14} However, the effective design of an enzyme-based
65 material requires an understanding of the issues associated with the immobilization method used,
66 as it may strongly affect its functionality.^{15,16} Physical absorption and electrostatic binding may
67 induce enzyme “leakage”, particularly upon changes in ionic strength, pH and temperature¹⁷,
68 leading to a loss of activity. Covalent binding may decrease enzyme activity due to changes in
69 protein conformation.¹⁸ Specific interactions often exploit binding units already developed by
70 nature, i.e. protein regions with specific binding properties towards cellulose called carbohydrate
71 binding modules (CBM) found on enzymes secreted by cellulose lytic organisms.¹⁹ The binding
72 strength of CBM toward cellulose, and specificity to particular types of cellulose (e.g. amorphous
73 versus crystalline), may vary depending on the biological source from which the CBM is
74 extracted.²⁰

75 In this study, cellobiohydrolase from *Trichoderma reesei* (*TrCBHI*) and cellobiose
76 dehydrogenase from *Thermothelomyces thermophilus* M77 (formerly *Myceliophthora*
77 *thermophila*) (*MtCDHA*), both possessing a CBM, were immobilized separately onto regenerated
78 cellulose films (RCF). This enzyme dual system allows the production of H₂O₂, which is triggered
79 by stacking the two RCF together and maintained over time. The cellulose degrading enzyme
80 *TrCBHI* provides the cellobiose to *MtCDHA* which in turn produces H₂O₂. The basic properties

81 of the material, relevant to its potential use as self-degradable H₂O₂ producing material, such as
82 enzyme adsorption and activity, resistance to compression and reusability are herein reported.

83

84 **2. MATERIAL AND METHODS**

85 **2.1 Materials**

86 Hydrogen peroxide 30 wt.% (VWR Chemicals), horseradish peroxidase ~150 U/mg
87 (77332, Sigma-Aldrich), absolute ethanol (VWR Chemicals, ≥99.8), microcrystalline cellulose
88 (435236, Sigma-Aldrich, LOT #MKCF1486), ethyl-3-methylimidazolium acetate [EMIm][OAc]
89 (BASF Basonics, ≥95%), DMSO (Alfa Aesar, ≥99%), glucose oxidase from *Aspergillus niger*
90 (G7141, Sigma-Aldrich) (*An*GOx), bovine serum albumin (05470, Sigma-Aldrich, ≥96%),
91 Sodium iodide (383112, Sigma-Aldrich, ≥99.5%), sodium phosphate dibasic (S9763, Sigma-
92 Aldrich, ≥99%), sodium phosphate monobasic (S3139, Sigma-Aldrich), sodium carbonate (S7795,
93 Sigma-Aldrich, ≥98%), sodium bicarbonate (S5761, Sigma-Aldrich, ≥99.5%), sodium sulfite
94 (S0505, Sigma-Aldrich, ≥98%), Phenol (328111, Sigma-Aldrich, ≥99%), Amplex[®] Red reagent
95 (Invitrogen), 2,6-Dichloroindophenol (D1878, Sigma-Aldrich), Sephadex G-50 Medium (Sigma-
96 Aldrich), 3,5-Dinitrosalicylic acid (D0550, Sigma-Aldrich, ≥98%), Coomassie Brilliant Blue G-
97 250 staining (Sigma, Deisenhofen, Germany), fluorescein isothiocyanate (F7250, Sigma-Aldrich,
98 ≥90%).

99 **2.2 Cellulose dissolution and film preparation**

100 To prepare cellulose solutions (6, 8 and 10 wt.%), microcrystalline cellulose (MCC) was
101 dried at 75 °C under reduced pressure and dispersed in DMSO with an overhead stirrer (900 rpm)
102 at room temperature. Subsequently the IL [EMIm][OAc] (previously dried under reduced pressure

103 for 6h at 60 °C) was added dropwise into the dispersion to reach a ratio of 70:30 w/w
104 DMSO:[EMIm][OAc]. The mixture was stirred for 4 h at room temperature to achieve complete
105 dissolution. Cellulose solutions were cast using an Elcometer 4340 Automatic Film Applicator
106 with a distance of 600 µm between the blade and glass plate and then precipitated into an ethanol
107 bath. Films were Soxhlet extracted with absolute ethanol for 4 h to remove the residual DMSO
108 and [EMIm][OAc]. Solvent exchange was achieved by soaking films in abundant deionised (DI)
109 water with at least 3 solvent replacements. Wet cellulose disks were prepared by punching out
110 circles from films with 12 mm and 6 mm round shape cutters and stored in DI water at 4 °C.

111

112 **2.3 Cellulose films deformation rheology**

113 Uniaxial compression was performed using a stress-controlled rheometer (Discovery HR3,
114 TA Instruments) equipped with a plate-plate geometry (12 mm). The gels were uniaxially
115 compressed at a constant deformation rate of 1 µm/s. The apparent strain (γ_a) was calculated as the
116 percentage of sample deformation. The distance corresponding to the sample height was obtained
117 at the point where the axial force abruptly increased. The apparent stress (σ_a) was calculated as the
118 axial force (N) over the area of a 12 mm diameter gel disk. An average of three independent
119 measurements is reported.

120

121 **2.4 Cloning, expression and purification of *MtCDHA***

122 The *MtCDHA* gene (MYCTH_111388) from *Myceliophthora thermophila* M77 was PCR
123 amplified from genomic DNA without the original signal peptide. The PCR product was amplified
124 using the oligonucleotide primers: forward (5'-
125 gggttgcaCAGAACAACGCGCCGGTAACCTTCACCGAC-3') and reverse

126 (5'gtcccgtgccggttaTCACAAGCACTGCGAGTACCACTCGTTCTGCATCTGGCACGT-3')

127 and was cloned into the pEXPYR vector using the Ligation-Independent Cloning protocol (LIC).²¹

128 The expression plasmid was transformed in *A. nidulans* A773 (*pyrG89*; *wA3*; *pyroA4*) as described

129 earlier.²² Approximately 10^7 spores/mL were inoculated in liquid minimal medium at pH 6.5,

130 containing 50 mL/L Clutterbuck salts (120 g/L NaNO₃, 10.4 g/L KCl, 10.4 g/L MgSO₄·7H₂O and

131 30.4 g/L of KH₂PO₄), 1 mL/L trace elements (22 g/L of ZnSO₄·7H₂O, 11 g/L of H₃BO₃, 5 g/L of

132 MnCl₂·4H₂O, 5 g/L of FeSO₄·7H₂O, 1.6 g/L of CoCl₂·5H₂O, 1.6 g/L of CuSO₄·5H₂O, 1.1 g/L of

133 Na₂MoO₄·4H₂O and 50 g/L of Na₂EDTA), supplemented with 5% maltose and incubated in static

134 culture at 37 °C for 40 h. The culture medium was filtered using Miracloth membranes

135 (Calbiochem, San Diego, CA, USA) with a pore size of 22-25 µm, and the secreted proteins were

136 concentrated 10-fold by tangential flow filtration using a hollow fibre cartridge with 5,000 NMWC

137 cut-off (GE Healthcare, Uppsala, Sweden).

138 The concentrated protein solution was applied to a 10 mL DEAE-Sephadex column (GE

139 Healthcare) pre-equilibrated with 20 mM Tris/HCl buffer pH 8.0. *MtCDHA* was eluted with a

140 stepwise gradient (200, 300, 400, and 500 mM) NaCl in 50 mM Tris/HCl pH 8.0. The purified

141 samples were concentrated by ultrafiltration (50 kDa cut-off Centricon-Millipore, Billerica, MA,

142 USA) and further purified using size exclusion chromatography on a HiLoad 16/60 Sephadex75

143 column (GE Healthcare) with a running buffer consisting of 150 mM NaCl and 20 mM Tris-HCl

144 pH 8.0. Protein concentration was determined spectrophotometrically at 280 nm using a molar

145 extinction coefficient of $157510 \text{ M}^{-1}\text{cm}^{-1}$. Protein purity was analysed by SDS-PAGE²³ using

146 Coomassie Brilliant Blue G-250 staining (Sigma, Deisenhofen, Germany).

147

148

149 **2.5 Purification of cellobiohydrolase from *Trichoderma reesei* (TrCBHI)**

150 *TrCel7A* was purified from a *T. reesei* cellulase cocktail (Sigma-Aldrich) using three
151 chromatographic steps. The commercial aqueous solution (1 mL) containing ~1.3 g of total protein
152 was diluted tenfold in 50 mM Tris/HCl buffer (pH 8.0) and desalted through two connected 5-ml
153 HiTrap desalting columns (GE Healthcare) and loaded onto a 20-mL home-packed
154 DEAE/Sephadex column pre-equilibrated with the same buffer. *TrCel7A* was eluted using a
155 stepwise gradient (200, 300, 400, and 500 mM) NaCl in 50 mM Tris/HCl buffer (pH 8.0) and
156 concentrated by ultrafiltration (50 kDa cut-off Centricon-Millipore, Billerica, MA, USA). The
157 protein sample was next loaded onto a Q-Sepharose high-performance column (GE Healthcare)
158 equilibrated with 50 mM Tris/HCl buffer (pH 8.0). The enzyme was eluted using a linear gradient
159 from 0 to 100% buffer B (50 mM Tris-HCl pH 8.0 and 1 M sodium chloride solution). The
160 fractions containing *TrCel7A* were combined, concentrated, and loaded onto a size exclusion
161 chromatography in HiLoad 16/60 Sephadex75 column (GE Healthcare) with a running buffer
162 consisting of 150 mM NaCl and 20 mM Tris/HCl buffer pH 8.0. Protein concentration was
163 determined spectrophotometrically at 280 nm using a molar extinction coefficient of 86760 M⁻¹
164 cm⁻¹. Protein purity was analysed by SDS-PAGE using Coomassie Brilliant Blue G-250 staining
165 (Sigma, Deisenhofen, Germany).

166

167 **2.6 Preparation FITC-*MtCDHA* conjugates and confocal imaging**

168 *MtCDHA* was FITC labelled - the isothiocyanate group forms a new covalent bond with
169 the amine groups of lysine residues of the proteins at pH 9.0. FITC in DMSO (1 µg/µL) was mixed
170 with the protein solution to a final concentration of 100 ng of FITC per µg of protein in 0.1 M

171 carbonate buffer (pH 9.0) and incubated at 37 °C for 90 min under mild agitation in darkness.
172 Labelled *MtCDHA* was separated from the free probe by gel filtration chromatography on a
173 Sephadex G-50 M column (Sigma-Aldrich) equilibrated with phosphate buffer (pH 6.0) and 150
174 mM NaCl also used for elution. The ratio of fluorescein to protein after labelling was calculated
175 by dividing the absorbance of FITC at 495 nm over the absorbance of the protein at 280 nm. RCF
176 were soaked in a 0.8 mg/mL FITC-*MtCDHA* solution and confocal laser scanning microscopy
177 (CLSM) images were taken with a ZEISS LSM 880 microscope.

178

179 **2.7 Product profile of *MtCDHA* and *TrCBHI***

180 The soluble products released from cellobiose by *MtCDHA* and from regenerated cellulose
181 disks by *TrCel7A* were analysed by high-performance anion-exchange chromatography. The
182 enzymatic reaction was carried out in 50 mM sodium phosphate buffer (pH 6.0) containing 1 μ M
183 *MtCDHA* and 1 mM cellobiose and incubated for 30 min at 50 °C. For comparison, 1 μ M *TrCBHI*
184 was incubated in the same buffer with one 12 mm (and ~0.4 mm thick) cellulose disk for 16 h at
185 50 °C. The enzymatic products were centrifuged, and the soluble products loaded on a CarboPac
186 PA1 column (2 \times 250 mm) with a CarboPac PA1 guard column (2 \times 50 mm) connected to a Dionex
187 ICS 5000 instrument equipped with pulsed amperometric detection. The column was pre-
188 equilibrated with 100 mM NaOH for 5 min at 1 mL/min. The saccharides were resolved using a
189 gradient from 100 mM NaOH/0 mM NaOAc to 100 mM NaOH/150 mM NaOAc over 20 min
190 followed by a wash step in 100 mM NaOH/1M NaOAc for 2 min and equilibration with 100 mM
191 NaOH for 5 min. Cello-oligosaccharide standards (Megazyme) standards from DP 1 to 6 were
192 used to quantify the hydrolysis products.

193

194 2.8 Absorption kinetics and binding isotherm of BSA, GOx, MtCDHA and TrCBHI

195 To examine protein adsorption kinetics into RCF, 200 μL of protein solution at a
196 concentration of 0.8 mg/mL were incubated with two 12 mm wide RCF (5 mg in dry weight) for
197 24 h at 4 $^{\circ}\text{C}$ in a static manner. The time course of absorption was monitored by measuring protein
198 concentration in the supernatant using the Bradford method.²⁴ Samples were withdrawn at 0.25,
199 0.5, 0.75, 1, 2, 4 and 24 h, values of absorbed protein (expressed as a percentage) were plotted and
200 the curves fitted with a single-term exponential:

$$201 \quad (\text{eq.1}) \quad E\% = L_{max}(1 - e^{-bt}) \times 100$$

202 Where $E\%$ stands for percentage of enzyme adsorbed, L_{max} is the maximum loading in %,
203 b is the exponential coefficient and t is time in hours. For binding isotherm experiment 50 μL
204 enzyme solutions at different concentration (approximated to 0.5, 1, 2, 3 and 4 $\mu\text{g}/\mu\text{L}$) were
205 incubated with two 6 mm wide RCF (~ 1.3 mg in dry weight) for 16 h at 4 $^{\circ}\text{C}$ in a static manner.
206 Protein concentrations were determined before and after incubation by comparison of the
207 absorption measured at 280 nm with a NanoDropTM spectrophotometer (Thermo ScientificTM One
208 Microvolume). Extinction coefficients and molecular weights used are shown in Table 2.

209

210 **Table 2.** Molecular weights and extinction coefficients of the proteins used.

<i>Protein</i>	MW	Extinction coefficient (ϵ)
<i>BSA</i>	66400 Da	43824 $\text{M}^{-1} \text{cm}^{-1}$
<i>AnGOx</i>	64004 Da (subunit)	96845 $\text{M}^{-1} \text{cm}^{-1}$ (subunit)
<i>MtCDHA</i>	86000 Da	86760 $\text{M}^{-1} \text{cm}^{-1}$
<i>TrCBHI</i>	61000 Da	157510 $\text{M}^{-1} \text{cm}^{-1}$

211

212 The adsorption constants were calculated by fitting a linear equation (eq. 2) after plotting
213 μg of protein adsorbed into RCF versus concentration of free proteins subsequent to the absorption.
214 A linear equation was chosen due to the low cellulose surface coverage expected.²⁵

215
$$\text{(eq.2) } C_s = K_h C_f$$

216 where C_s is μg of protein adsorbed per mg of cellulose in dry weight, K_h is the adsorption constant
217 in $\mu\text{L}/\text{mg}$ and C_f is concentration of free protein in $\mu\text{g}/\mu\text{L}$.

218

219 **2.9 *Tr*CBHI activity - cellobiose production**

220 The enzymatic activity of *Tr*CBHI was assayed by measuring the increase in absorbance
221 at 575 nm subsequent to the reaction of reducing sugars with 3,5-dinitrosalicylic acid (DNS).²⁶
222 Aliquots of cellobiose, released from 12mm diameter cellulose disks (6, 8 and 10 wt.%) in the
223 presence of *Tr*CBHI (previously adsorbed overnight at 4°C from 500 μL of a 0.8 mg/mL solution
224 in 0.1 phosphate buffer, pH 6), were withdrawn from the supernatant and mixed with DNS reagent
225 in a 1:1 volume ratio and incubated for 5 min at 90°C before measuring absorption. DNS reagent
226 was prepared by mixing 3,5-Dinitrosalicylic acid (10 g) in water together with phenol (2 g), sodium
227 hydroxide (10 g), sodium sulfite (0.5 g) in a total volume of 1L of DI water. One unit (U) of
228 enzyme activity was defined as the amount of enzyme that catalyses the production of 1.0 μmol
229 glucose/min. All experiments were performed in triplicate samples.

230

231 **2.10 *Mt*CDHA activity - H_2O_2 production in liquid media**

232 Hydrogen peroxide (H_2O_2) production catalysed by *Mt*CDHA was measured using the
233 enzymatic-coupled reaction of horseradish peroxidase (HRP) and Amplex[®] Red reagent
234 (Invitrogen). Initially, RCF containing *Mt*CDHA were placed in a 24 well plate (1 disk per well)

235 containing 480 μL of 50 mM phosphate buffer (pH 6), then the reaction was triggered with 20 μL
236 of cellobiose (0.5M) to reach a final concentration of 20 mM. H_2O_2 aliquots (20 μL) were
237 withdrawn every 5 min for 30 min for subsequent reaction with HRP and Amplex red. For the
238 reaction, 180 μL containing 7.5 U/mL horseradish peroxidase, 50 μM Amplex Red and 50 mM
239 phosphate buffer (pH 6.0) were mixed with 20 μL of H_2O_2 and the fluorescence developed was
240 measured with FLUOstar Omega Microplate Reader (BMG LABTECH). The peroxidase converts
241 the non-fluorescent Amplex Red to fluorescent resorufin by reaction in 1:1 stoichiometry with
242 H_2O_2 . All measurements were performed in triplicate in a 96 black microtiter plate reader.
243 Fluorescence was followed at an excitation wavelength of 540 nm and emission of 590 nm. The
244 rate of H_2O_2 production was determined based on the slope of the increase in fluorescence
245 compared to standard curves.

246

247 **2.11 Continuous production of H_2O_2 in RCF - self-degradation process**

248 In order to mimic the working conditions of *TrCBHI/MtCDHA* RCF, H_2O_2 release rates
249 were measured after stacking 3 wet cellulose disks (produced from an 8 wt. % cellulose solution)
250 containing from top to bottom *TrCBHI*, *MtCDHA* and Amplex Red/HRP, respectively. Blanks
251 were prepared without *TrCBHI* in the upper disk (Figure 1).

252

253 **Figure 1.** Graphical representation of disks arrangement for H_2O_2 production in semi-dry
254 environment (self-degradation experiment).

255 *TrCBHI* provides cellobiose to *MtCDHA* which is converted into H_2O_2 ; then
256 HRP/AmplexRed (AR), used as reporter, generated fluorescence by oxidising AR into resorufin.
257 Fluorescence intensity in RCF was measured with a Microplate Reader in a 24 well plate at 37°C

258 (excitation wavelength of 540 nm and emission of 590 nm). Calibration curves were prepared by
259 reproducing the same conditions by adding known amounts of H₂O₂ previously adsorbed in the
260 gel. The long-term activity of H₂O₂ was quantified by measuring absorbance at 350 nm after the
261 reaction with iodide ion (I⁻) which generates a yellowish colour due to the presence of the triiodide
262 ion (I₃⁻) (Figure S3a). Specifically, aliquots of 50 μL were withdrawn from a buffered solution
263 containing the stacked films (phosphate buffer, 0.1M, pH 6), mixed with 50 μL of a 1 M sodium
264 iodide solution and incubated for 30 minutes before reading absorbance. Calibration curve were
265 produced in the same way with H₂O₂ concentrations ranging between 12.25 μM to 1.225 mM
266 (Figure S3b).

267

268 3. RESULTS AND DISCUSSION

269 Design rules for the development of a self-degrading H₂O₂ producing films were developed
270 prior to preparation of the enzyme containing, multi-layer constructs. These included material
271 requirements as well as functionality. Specifically, the support material was selected to be: (i)
272 biocompatible - cellulose has been widely used in biomedical applications²⁷ (including wound
273 dressings, ocular and tissue engineering applications²⁸); (ii) biodegradable post-use - cellulose (and
274 the enzymes used) are readily biodegraded if released to the environment²⁹ and (iii) a substrate for
275 the enzyme machinery – cellobiohydrolase and cellobiose dehydrogenase have been widely
276 described for degradation of the cellulosic component of biomass.³⁰

277 To facilitate manufacturing at scale, the bulk components used were required to be easily
278 formed into a variety of shapes, thicknesses and densities. The forming and setting of cellulose by
279 anti-solvent induced phase inversion of cast or molded solutions in organic electrolyte solutions
280 comprised of polar aprotic solvents and ionic liquids is now widely established.³¹ The components

281 were also required to comprise of cost-effective, readily available ingredients. Purified plant
282 cellulose is inexpensive and ionic liquids and solvents can be recycled due to large differences in
283 boiling points and vapor pressures that facilitate separation by distillation.³² The enzymatic
284 components needed to be: (i) efficiently used, i.e. absorbed as completely as possible into the
285 porous cellulose films to avoid enzyme wastage; (ii) not likely to allow significant enzyme
286 leaching and, (iii) active at appropriate levels for sustained periods of time.

287 A comprehensive characterization of the films produced as well as their self-degradation
288 mechanism is described below.

289

290 **3.1 Composite film production and characterization**

291 The RCF produced were thinner than the casting blade height (set at 600 μm), but constant
292 for all cellulose concentrations ($410 \pm 50 \mu\text{m}$), suggesting that some shrinkage occurred during the
293 regeneration process (precipitation and solvent exchange). However, as the thickness was similar
294 for all films, irrespective of the initial MCC concentration in solution, materials with different
295 macroporosity were obtained. Uniaxial compression of the RCF was performed to assess their
296 rheological properties, an important feature to evaluate the material for potential applications and
297 to allow selection of a suitably robust material. The RCF were found to exhibit strain-hardening
298 behaviour (Figure 2), as recently observed for protein and cellulose composite gels.³³

299

300 **Figure 2.** Rheological properties of cellulose films. Apparent stress (σ_a) as function of the apparent
301 strain (γ_a) for the 6, 8 and 10 wt.% cellulose gels. Shadows refer to the standard deviation ($n=3$).

302

303 The increasing slopes with increased cellulose concentrations, in the low σ_a -range, indicate
304 a proportional toughening of the gels, in agreement with the network densification visible in Figure
305 3. CLSM images of fluorescein isothiocyanate (FITC) labelled *MtCDHA* adsorbed by soaking
306 RCF in the FITC-labelled protein solution, show variation of the surface structure. In fact,
307 fluorescence emission is detected only in the dense cellulose regions whilst it is absent in the pores.
308 The films prepared from 8 wt. % solutions maintained an appreciable macroporosity as observed
309 in CLSM microphotographs (absent in 10 wt. % films) and yet exhibited higher mechanical
310 strength than films prepared from 6 wt. % solutions due to the presence of a denser network, which
311 is less susceptible to the stress.

312
313 **Figure 3.** Confocal laser scanning microscopy (CLSM) pictures of *MtCDHA* adsorbed on RCF
314 showing gel network porosity at a microscopic scale. Films precipitated from 6, 8 and 10 wt.%
315 cellulose solutions respectively a, b and c) displaying difference in porosity. Scale bars 10 μm .

316 317 **3.2 *MtCDHA* and *TrCBHI* purification and activity evaluation**

318 The enzymes to be adsorbed onto RCF were both prepared in-house. *MtCDHA* was cloned
319 and expressed in *A. nidulans* and *TrCBHI* purified directly from a commercial cellulase cocktail.
320 The enzyme preparations were homogeneous and > 95% pure as judged by SDS/PAGE (Figure
321 4a) after the purification steps. As further proof of purity, the product profile of both enzymes was
322 evaluated: *MtCDHA* completely oxidised cellobiose to cellobionic acid (Figure 4b) with only a
323 trace peak representing glucose observed in high performance anionic exchange chromatography
324 (HPAEC) analysis, indicating negligible contamination with β -glucosidase. A similar result was
325 observed for *TrCBHI*, showing cellobiose as the main product of hydrolysis and only a small peak

326 corresponding to glucose (Figure 4c). The lack of β -glucosidase contamination is crucial because
327 the eventual production of glucose could promote the growth of microorganisms.³⁴

328

329 **Figure 4.** Purification and product profile of *MtCDHA* and *TrCBHI*. a) SDS-PAGE of the purified
330 enzymes (MW: molecular weight of markers in kDa). The theoretical MW of the respective
331 enzymes (indicated by the arrows) corresponds to the obtained bands (gels were stained with
332 Coomassie blue). (b, c) HPAEC chromatograms showing the products released by *MtCDHA* (b)
333 and *TrCBHI* (c) after incubation with cellobiose and cellulose disks, respectively. Peaks were
334 assigned based on cello-oligosaccharide standards.

335

336 **3.3 Enzyme immobilization on RCF**

337 The efficacy of using enzymes bearing CBMs to functionalise the RCF is demonstrated by
338 the extent of adsorption of the proteins during RCF loading. Adsorption of *MtCDHA* and *TrCBHI*
339 were compared with that of bovine serum albumin (BSA) and a glucose oxidase derived from
340 *Aspergillus niger* (*AnGOx*) (Figure 5).

341

342 **Figure 5.** Impact of CBM on adsorption kinetic and binding affinity. a) Proteins absorption
343 kinetics on cellulose films. The proteins at 0.8 mg/mL were incubated with 6 wt.% cellulose films
344 (5 mg in dry weight) at 4 °C. b) binding isotherm adsorption. The proteins, at different
345 concentrations, were incubated for 18h with 6 wt.% cellulose films (~ 1.3 mg in dry weight).
346 *TrCBHI* (empty red circles), *MtCDHA* (solid blue circles), BSA (empty orange triangles) and
347 *AnGOx* (solid green triangles). Shadows refer to the standard deviation (n=3).

348

349 While all four proteins tested reached equilibrium after 4 h, the two groups of proteins
350 showed distinct maximum adsorption, L_{max} values (see eq.1 in experimental section). The
351 enzymes bearing CBM reached 85-95% adsorption on RCF, while only 37% and 27% of the BSA
352 and *AnGOx* were adsorbed, respectively (Figure 4a). Binding isotherm experiments confirmed the
353 high affinity of CBM-possessing proteins for the RCF supports, with higher adsorption constants
354 for *TrCBHI* and *MtCDHA* adsorption compared with BSA and *AnGOx* (Figure 5b and Table 1).
355 After a first attempt at fitting binding isotherm curves with a Langmuir model, a linear equation
356 was employed (eq.2 in experimental section).

357 In fact, the affinity of the proteins for RCF displays a first order behaviour because of low
358 surface coverage, where lateral protein/protein interactions (e.g. electrostatic repulsion) are likely
359 to be negligible.^{25,35} Both kinetics and binding isotherm analyses indicated that the CBM-
360 possessing proteins ensured efficient immobilisation onto RCF, with most proteins transferred
361 from solution into the films over the range of concentrations investigated.

362

363 **Table 1.** Adsorption kinetics and binding isotherm parameters. obtained by fitting eq.1 and eq.2
364 (see experimental section).

Protein	Adsorption kinetic			Binding isotherm	
	L_{max} (%)	b	R^2	K_h	R^2
<i>TrCBHI</i>	90.4 ±2.2	3.2 ±0.3	0.98	102.6 ±8.2	0.99
<i>MtCDHA</i>	82.4 ±5.7	1.7 ±0.4	0.90	56.7 ±3.2	0.98
BSA	37.2 ±1.5	3.3 ±0.6	0.95	11.2 ±0.9	0.98
<i>AnGOx</i>	27.5 ±3.1	1.4 ±0.5	0.82	6.7 ±0.8	0.94

365

366 3.4 Activity and continuous H₂O₂ production

367 To determine the effect of immobilisation on enzyme activity, the RCF/enzyme composite
368 films were evaluated separately. In order to mimic the skin physiological environment (in view of
369 potential use as a wound dressing), cellulose digestion by *Tr*CBHI (adsorbed overnight at 4°C)
370 was conducted at pH 6.0 and 37 °C, rather than the optimal conditions for this enzyme which is
371 50 °C and pH 5.³⁶ Cellobiose release from the disks into the liquid media showed some dependence
372 on disk cellulose content (Figure 6) even though the amount of enzymes adsorbed were equal.

373

374 **Figure 6.** Hydrolysis of cellulose disks by *Tr*CBHI over time. The amount of released cellobiose
375 product was calculated by measuring the release of reducing sugars from a 12mm cellulose disk
376 on which *Tr*CBHI was adsorbed. Shadows refer to the standard deviation (n=3).

377

378 Nonetheless, in all samples cellobiose was produced at sufficient concentrations (1.4 ± 0.2
379 nmol/min) to ensure that *Mt*CDHA, placed on its adjacent layer, would be working at a satisfactory
380 rate to reach an H₂O₂ concentration of 150 μM in about 20 min (0.15 ± 0.02 nmol/min·cm²). In
381 addition, cellobiose present in the medium after adsorption and storage at 4°C resulted to be
382 undetectable, suggesting a negligible cellulose hydrolysis during *Tr*CBHI immobilization. For the
383 *Mt*CDHA free enzyme, the influence of pH and temperature were determined using 2,6-
384 Dichlorophenolindophenol (DCPIP) as an electron acceptor. The optimal values of pH and
385 temperature were found to be 6.0-6.5 and 60 °C, respectively (Figure S1). This elevated
386 temperature does not reflect physiological conditions in the skin environment, so the production
387 of H₂O₂ by free and adsorbed *Mt*CDHA was compared at 37 °C to evaluate changes in enzymatic
388 activity upon immobilisation. The specific activity was found to decrease approximately 3.5-fold,

389 the immobilised enzyme yielded an activity of 2.4 nmol H₂O₂/min·mg compared to the free
390 enzyme activity of 8.9 nmol H₂O₂/min·mg (Figure 7a). This reduced specific activity may be
391 attributed to several factors such as (i) enzyme confinement, (ii) substrates/products diffusion and
392 (iii) enzyme conformational changes upon immobilisation.^{7,18} Undoubtedly, the immobilisation
393 process confines the enzyme on the cellulose film, thus lowering surface contact area with the
394 substrate if compared with free enzyme. The reduced diffusion of the soluble cellobiose, which is
395 likely to occur because of the interactions with cellulose,³⁷ may also affect the enzyme activity. In
396 contrast, conformational changes upon immobilisation should not be relevant considering the
397 flexible nature of CMB-linker binding system.³⁸ The reusability of the *MtCDHA* disks was studied
398 to assess stability for biomedical and industrial processes. In all three consecutive cycles (1 cycle
399 per day), *MtCDHA* maintained the same enzymatic activity (Figure 7b). Thus, despite the non-
400 covalent attachment of *MtCDHA* onto the cellulose disks, no enzyme loss from the material was
401 observed upon storage between each cycle. Accordingly, similar studies in which CBM was
402 employed to immobilize enzymes on cellulose demonstrated an efficient binding and no enzyme
403 leakage.^{39,40} Moreover, contact with a relatively high concentration of H₂O₂ (≈ 2 mM) did not
404 cause any enzyme deactivation, suggesting that no relevant oxidative damage occurred in the
405 protein.⁴¹

406
407 **Figure 7.** Specific activity of *MtCDHA* upon immobilisation and storage. a) Effect of cellulose
408 concentration in the films on *MtCDHA* specific activity. b) Reusability of *MtCDHA* films, after
409 each cycle disks were stored overnight in phosphate buffer at 4 °C and then reused in next cycle.
410 Error bars refer to the standard deviation derived from 3 different independent samples.

411 To assess the effectiveness of the combined enzyme machinery for H₂O₂ production, layers
412 of RCF containing *TrCBHI* and *MtCDHA* were stacked on the top of a “reporter layer” comprised
413 of HRP and Amplex red adsorbed onto a RCF. Since no meaningful effects on enzymatic activity
414 were detected as a function of cellulose wt.% (Figure 7), only 8 wt.% cellulose disks were used to
415 evaluate activity in the stacked RCF. In order to calculate the exact amount of H₂O₂ produced, the
416 calibration curve was determined for the same conditions as for the enzyme adsorbed RCF,
417 resulting in a linear dependence (Figure 8).

418
419 **Figure 8.** Stacking experiment results. a) Calibration curve with known concentrations of H₂O₂,
420 increase in resorufin concentration caused by the presence of H₂O₂ (inset). Shadow refers to the
421 standard deviation derived from 3 different independent samples. b) *TrCBHI/MtCDHA* composite
422 film kinetics (blanks are subtracted). Development of fluorescent resorufin in composite films after
423 the stacking *TrCBHI* and *MtCDHA*, controls without *TrCBHI* or *MtCDHA* do not produce
424 resorufin (inset).

425
426 The *TrCBHI/MtCDHA* stacked RCF system produced 0.15 ± 0.02 nmol/min·cm² of H₂O₂.
427 Kinetic curves using RCF with immobilised *TrCBHI/MtCDHA* showed a short lag phase (Figure
428 S2) which may be caused by cellobiose diffusing from the *TrCBHI* to *MtCDHA* containing film.
429 Nevertheless, the reaction quickly reaches the linear regime (in about 1 minute). Using this
430 construct (Figure 9), it was estimated that the concentration of H₂O₂ reaches 150 μM in about 20
431 minutes and ~ 2 mM in 24 hours (Figure S3c).

432

433 **Figure 9.** Schematized self-degradation process of functionalised RCF for continuous production
434 of H₂O₂. RCF containing *TrCBHI* (a), RCF containing *MtCDHA* (b) and a generic surface to
435 disinfect (c).

436
437 The same concentration produced a complete bacteriostatic and bactericidal effect towards *E.coli*
438 and *S.aureus* in an *in vitro* study on embryonic fibroblasts ⁴² suggesting a potential use of the
439 material herein produced as antimicrobial agent. In addition, having designed the release device in
440 the form of a film would allow a homogeneous diffusion of H₂O₂ onto a surface (e.g. skin), making
441 the product cost-effective and controllable.

442 Despite the satisfactory levels of H₂O₂ produced, further investigations of the antimicrobial
443 effects of the *TrCBHI/MtCDHA* stacked RCF on bacteria still need to be conducted. In the first
444 instance, *in vitro* experiments such as agar diffusion tests, growth curves using different strains
445 (e.g. *P.aeruginosa* and methicillin resistant *S. aureus*) and cell biocompatibility tests are required
446 to assess the real antimicrobial potential of the material herein designed.

447

448 **4. CONCLUSION**

449 The goal of preparing readily manufacturable materials that actively generate H₂O₂, as a
450 potential *in situ* sterilant, was realized by constructing multi-layer cellulose-enzyme composites.
451 The paired enzymes were chosen for their complementary activity ³⁰ and for good binding to the
452 cellulose support. Specifically, composites were prepared as layered RCF bearing adsorbed
453 cellobiose dehydrogenase (CDH) from *Thermothelomyces thermophilus* M77 (*MtCDHA*) and
454 cellobiohydrolase from *Trichoderma reesei* (*TrCBHI*) to provide a coupled enzymatic H₂O₂-
455 producing machinery immobilized on regenerated cellulose films. The use of cellulose provides

456 both the support (for anchoring the enzymes) and the substrate to initiate the continuous H₂O₂
457 production without the use of chemical modifications or enzyme-support cross-linking. The CBM
458 bearing enzymes *TrCBHI* and *MtCDHA* showed a high affinity for cellulose, demonstrating that
459 the presence of appropriate binding module protein constructs led to efficient immobilization,
460 which was further confirmed by reusability experiments. Continuous production of H₂O₂ was
461 achieved upon stacking films containing *TrCBHI*, which slowly degraded the cellulose into
462 cellobiose, with films containing *MtCDHA*, which in turn produced H₂O₂. The production rate of
463 H₂O₂ was almost constant and sustained for at least 24 hours. In conclusion, this work provides
464 the foundational understanding for sustainable manufacturing of a self-degradable material with
465 potential antimicrobial applications.

466

467 **ASSOCIATED CONTENT**

468

469 **Supporting information**

470

471 Additional information on *MtCDHA* enzyme activity, changes over temperature and pH used to
472 determine the optimal enzyme activity; Initial lag phase during resorufin fluorescence
473 measurements in the RCF stacked films; Validation of the iodine H₂O₂ quantification method
474 (increase of adsorption at 350 nm, linearity over the range of concentrations measured), evolution
475 of H₂O₂ over 24 hours.

476 Data supporting this article have been made freely available via the University of Bath Research
477 Data Archive system at:

478 <https://researchdata.bath.ac.uk/id/eprint/736>

479

480 **Conflicts of interest**

481 There are no conflicts to declare.

482

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488

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