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

*Davide Califano [a] ‡ Marco A. S. Kadowaki [b]‡ *, [1]Vincenzo Calabrese [a], Rolf Alexander Prade [c], Davide Mattia [d], Karen J. Edler [a], Igor Polikarpov [b], Janet L. Scott.[a]*

[a.] Department of Chemistry, University of Bath, BA27AY, UK.

[b.] São Carlos Institute of Physics, Av. Trabalhador São-carlense, 400, University of São Paulo, São Carlos, SP, 13566-590, Brazil.

[c.] Departments of Microbiology & Molecular Genetics and Biochemistry & Molecular Biology, Oklahoma State University, OK, USA.

[d.] Department of Chemical Engineering, University of Bath, BA27AY, UK.

‡ These authors contributed equally

* Corresponding author

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

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

1. INTRODUCTION

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

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

resource and sustainable processes.[9] Cellulose dissolution can be achieved at room temperature using ionic liquids (IL)[10] such as 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) and 1-ethyl-3-methylimidazolium acetate ([EMIm][OAc]) which can be recovered and recycled subsequently in the regeneration process.[11,12] Regenerated cellulose hydrogels (in both pure and derivatized forms) are considered biocompatible materials, hence, employable in a wide range of biomedical applications including tissue engineering [13,14] However, the effective design of an enzyme-based material requires an understanding of the issues associated with the immobilization method used, as it may strongly affect its functionality.[15,16] Physical absorption and electrostatic binding may induce enzyme "leakage", particularly upon changes in ionic strength, pH and temperature [17], leading to a loss of activity. Covalent binding may decrease enzyme activity due to changes in protein conformation.[18] Specific interactions often exploit binding units already developed by nature, i.e. protein regions with specific binding properties towards cellulose called carbohydrate binding modules (CBM) found on enzymes secreted by cellulose lytic organisms.[19] The binding strength of CBM toward cellulose, and specificity to particular types of cellulose (e.g. amorphous versus crystalline), may vary depending on the biological source from which the CBM is extracted.[20]

In this study, cellobiohydrolase from *Trichoderma reesei* (*Tr*CBHI) and cellobiose dehydrogenase from *Thermothelomyces thermophilus* M77 (formerly *Myceliophthora thermophila*) (*Mt*CDHA), both possessing a CBM, were immobilized separately onto regenerated cellulose films (RCF). This enzyme dual system allows the production of H_2O_2, which is triggered by stacking the two RCF together and maintained over time. The cellulose degrading enzyme *Tr*CBHI provides the cellobiose to *Mt*CDHA which in turn produces H_2O_2. The basic properties

of the material, relevant to its potential use as self-degradable H_2O_2 producing material, such as enzyme adsorption and activity, resistance to compression and reusability are herein reported.

2. MATERIAL AND METHODS

2.1 Materials

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

2.2 Cellulose dissolution and film preparation

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

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

2.3 Cellulose films deformation rheology

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

2.4 Cloning, expression and purification of *Mt*CDHA

The *Mt*CDHA gene (MYCTH_111388) from *Myceliophthora thermophila* M77 was PCR amplified from genomic DNA without the original signal peptide. The PCR product was amplified using the oligonucleotide primers: forward (5'-gggttggcaCAGAACAACGCGCCGGTAACCTTCACCGAC-3') and reverse

(5'gtcccgtgccggttaTCACAAGCACTGCGAGTACCACTCGTTCTGCATCTGGCACGT-3') and was cloned into the pEXPYR vector using the Ligation-Independent Cloning protocol (LIC).[21] The expression plasmid was transformed in *A. nidulans* A773 (*pyrG*89; *wA*3; *pyroA*4) as described earlier.[22] Approximately 10^7 spores/mL were inoculated in liquid minimal medium at pH 6.5, containing 50 mL/L Clutterbuck salts (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4 \cdot 7H_2O$ and 30.4 g/L of KH_2PO_4), 1 mL/L trace elements (22 g/L of $ZnSO_4 \cdot 7H_2O$, 11 g/L of H_3BO_3, 5 g/L of $MnCl_2 \cdot 4H_2O$, 5 g/L of $FeSO_4 \cdot 7H_2O$, 1.6 g/L of $CoCl_2 \cdot 5H_2O$, 1.6 g/L of $CuSO_4 \cdot 5H_2O$, 1.1 g/L of $Na_2MoO_4 \cdot 4H_2O$ and 50 g/L of Na_2EDTA), supplemented with 5% maltose and incubated in static culture at 37 °C for 40 h. The culture medium was filtered using Miracloth membranes (Calbiochem, San Diego, CA, USA) with a pore size of 22-25 μm, and the secreted proteins were concentrated 10-fold by tangential flow filtration using a hollow fibre cartridge with 5,000 NMWC cut-off (GE Healthcare, Uppsala, Sweden).

The concentrated protein solution was applied to a 10 mL DEAE-Sephadex column (GE Healthcare) pre-equilibrated with 20 mM Tris/HCl buffer pH 8.0. *Mt*CDHA was eluted with a stepwise gradient (200, 300, 400, and 500 mM) NaCl in 50 mM Tris/HCl pH 8.0. The purified samples were concentrated by ultrafiltration (50 kDa cut-off Centricon-Millipore, Billerica, MA, USA) and further purified using size exclusion chromatography on a HiLoad 16/60 Sephadex75 column (GE Healthcare) with a running buffer consisting of 150 mM NaCl and 20 mM Tris–HCl pH 8.0. Protein concentration was determined spectrophotometrically at 280 nm using a molar extinction coefficient of 157510 $M^{-1}cm^{-1}$. Protein purity was analysed by SDS-PAGE [23] using Coomassie Brilliant Blue G-250 staining (Sigma, Deisenhofen, Germany).

2.5 Purification of cellobiohydrolase from *Trichoderma reseei* (*Tr*CBHI)

*Tr*Cel7A was purified from a *T. reesei* cellulase cocktail (Sigma-Aldrich) using three chromatographic steps. The commercial aqueous solution (1 mL) containing ~1.3 g of total protein was diluted tenfold in 50 mM Tris/HCl buffer (pH 8.0) and desalted through two connected 5-ml HiTrap desalting columns (GE Healthcare) and loaded onto a 20-mL home-packed DEAE/Sephadex column pre-equilibrated with the same buffer. *Tr*Cel7A was eluted using a stepwise gradient (200, 300, 400, and 500 mM) NaCl in 50 mM Tris/HCl buffer (pH 8.0) and concentrated by ultrafiltration (50 kDa cut-off Centricon-Millipore, Billerica, MA, USA). The protein sample was next loaded onto a Q-Sepharose high-performance column (GE Healthcare) equilibrated with 50 mM Tris/HCl buffer (pH 8.0). The enzyme was eluted using a linear gradient from 0 to 100% buffer B (50 mM Tris-HCl pH 8.0 and 1 M sodium chloride solution). The fractions containing *Tr*Cel7A were combined, concentrated, and loaded onto a size exclusion chromatography in HiLoad 16/60 Sephadex75 column (GE Healthcare) with a running buffer consisting of 150 mM NaCl and 20 mM Tris/HCl buffer pH 8.0. Protein concentration was determined spectrophotometrically at 280 nm using a molar extinction coefficient of 86760 M^{-1} cm^{-1}. Protein purity was analysed by SDS-PAGE using Coomassie Brilliant Blue G-250 staining (Sigma, Deisenhofen, Germany).

2.6 Preparation FITC-*Mt*CDHA conjugates and confocal imaging

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

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

2.7 Product profile of *Mt*CDHA and *Tr*CBHI

The soluble products released from cellobiose by *Mt*CDHA and from regenerated cellulose disks by *Tr*Cel7A were analysed by high-performance anion-exchange chromatography. The enzymatic reaction was carried out in 50 mM sodium phosphate buffer (pH 6.0) containing 1 µM *Mt*CDHA and 1 mM cellobiose and incubated for 30 min at 50 °C. For comparison, 1 µM *Tr*CBHI was incubated in the same buffer with one 12 mm (and ~0.4 mm thick) cellulose disk for 16 h at 50 °C. The enzymatic products were centrifuged, and the soluble products loaded on a CarboPac PA1 column (2×250 mm) with a CarboPac PA1 guard column (2×50 mm) connected to a Dionex ICS 5000 instrument equipped with pulsed amperometric detection. The column was pre-equilibrated with 100 mM NaOH for 5 min at 1 mL/min. The saccharides were resolved using a gradient from 100 mM NaOH/0 mM NaOAc to 100 mM NaOH/150 mM NaOAc over 20 min followed by a wash step in 100 mM NaOH/1M NaOAc for 2 min and equilibration with 100 mM NaOH for 5 min. Cello-oligosaccharide standards (Megazyme) standards from DP 1 to 6 were used to quantify the hydrolysis products.

2.8 Absorption kinetics and binding isotherm of BSA, GOx, *Mt*CDHA and *Tr*CBHI

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

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

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

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

Protein	**MW**	**Extinction coefficient (ε)**
BSA	66400 Da	43824 M^{-1} cm^{-1}
AnGOx	64004 Da (subunit)	96845 M^{-1} cm^{-1} (subunit)
MtCDHA	86000 Da	86760 M^{-1} cm^{-1}
TrCBHI	61000 Da	157510 M^{-1} cm^{-1}

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

$$\textbf{(eq.2)}\quad C_s = K_h C_f$$

where C_s is µg of protein adsorbed per mg of cellulose in dry weight, K_h is the adsorption constant in µL/mg and C_f is concentration of free protein in µg/µL.

2.9 *Tr*CBHI activity - cellobiose production

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

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

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

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

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

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

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

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

(excitation wavelength of 540 nm and emission of 590 nm). Calibration curves were prepared by reproducing the same conditions by adding known amounts of H_2O_2 previously adsorbed in the gel. The long-term activity of H_2O_2 was quantified by measuring absorbance at 350 nm after the reaction with iodide ion (I^-) which generates a yellowish colour due to the presence of the triiodide ion (I_3^-) (Figure S3a). Specifically, aliquots of 50 µL were withdrawn from a buffered solution containing the stacked films (phosphate buffer, 0.1M, pH 6), mixed with 50 µL of a 1 M sodium iodide solution and incubated for 30 minutes before reading absorbance. Calibration curve were produced in the same way with H_2O_2 concentrations ranging between 12.25 µM to 1.225 mM (Figure S3b).

3. RESULTS AND DISCUSSION

Design rules for the development of a self-degrading H_2O_2 producing films were developed prior to preparation of the enzyme containing, multi-layer constructs. These included material requirements as well as functionality. Specifically, the support material was selected to be: (i) biocompatible - cellulose has been widely used in biomedical applications[27] (including wound dressings, ocular and tissue engineering applications[28]); (ii) biodegradable post-use - cellulose (and the enzymes used) are readily biodegraded if released to the environment[29] and (iii) a substrate for the enzyme machinery – cellobiohydrolase and cellobiose dehydrogenase have been widely described for degradation of the cellulosic component of biomass.[30]

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

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

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

3.1 Composite film production and characterization

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

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

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

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

3.2 *Mt*CDHA and *Tr*CBHI purification and activity evaluation

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

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

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

3.3 Enzyme immobilization on RCF

The efficacy of using enzymes bearing CBMs to functionalise the RCF is demonstrated by the extent of adsorption of the proteins during RCF loading. Adsorption of *Mt*CDHA and *Tr*CBHI were compared with that of bovine serum albumin (BSA) and a glucose oxidase derived from *Aspergillus niger* (*An*GOx) (Figure 5).

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

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

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

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

	Adsorption kinetic			**Binding isotherm**	
Protein	$\boldsymbol{L_{max}}$ (%)	$\boldsymbol{b}$	$\boldsymbol{R^2}$	$\boldsymbol{K_h}$	$\boldsymbol{R^2}$
***Tr*CBHI**	90.4 ±2.2	3.2 ±0.3	0.98	102.6 ±8.2	0.99
***Mt*CDHA**	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
***An*GOx**	27.5 ±3.1	1.4 ±0.5	0.82	6.7 ±0.8	0.94

3.4 Activity and continuous H_2O_2 production

To determine the effect of immobilisation on enzyme activity, the RCF/enzyme composite films were evaluated separately. In order to mimic the skin physiological environment (in view of potential use as a wound dressing), cellulose digestion by *Tr*CBHI (adsorbed overnight at 4°C) was conducted at pH 6.0 and 37 °C, rather than the optimal conditions for this enzyme which is 50 °C and pH 5.[36] Cellobiose release from the disks into the liquid media showed some dependence on disk cellulose content (Figure 6) even though the amount of enzymes adsorbed were equal.

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

Nonetheless, in all samples cellobiose was produced at sufficient concentrations (1.4 ± 0.2 nmol/min) to ensure that *Mt*CDHA, placed on its adjacent layer, would be working at a satisfactory rate to reach an H_2O_2 concentration of 150 µM in about 20 min (0.15 ± 0.02 nmol/min·cm^2). In addition, cellobiose present in the medium after adsorption and storage at 4°C resulted to be undetectable, suggesting a negligible cellulose hydrolysis during *Tr*CBHI immobilization. For the *Mt*CDHA free enzyme, the influence of pH and temperature were determined using 2,6-Dichlorophenolindophenol (DCPIP) as an electron acceptor. The optimal values of pH and temperature were found to be 6.0-6.5 and 60 °C, respectively (Figure S1). This elevated temperature does not reflect physiological conditions in the skin environment, so the production of H_2O_2 by free and adsorbed *Mt*CDHA was compared at 37 °C to evaluate changes in enzymatic 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 H_2O_2/min·mg compared to the free
390 enzyme activity of 8.9 nmol H_2O_2/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 *Mt*CDHA disks was studied
398 to assess stability for biomedical and industrial processes. In all three consecutive cycles (1 cycle
399 per day), *Mt*CDHA maintained the same enzymatic activity (Figure 7b). Thus, despite the non-
400 covalent attachment of *Mt*CDHA 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 H_2O_2 (≈ 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 *Mt*CDHA upon immobilisation and storage. a) Effect of cellulose
408 concentration in the films on *Mt*CDHA specific activity. b) Reusability of *Mt*CDHA 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.

To assess the effectiveness of the combined enzyme machinery for H_2O_2 production, layers of RCF containing *Tr*CBHI and *Mt*CDHA were stacked on the top of a "reporter layer" comprised of HRP and Amplex red adsorbed onto a RCF. Since no meaningful effects on enzymatic activity were detected as a function of cellulose wt.% (Figure 7), only 8 wt.% cellulose disks were used to evaluate activity in the stacked RCF. In order to calculate the exact amount of H_2O_2 produced, the calibration curve was determined for the same conditions as for the enzyme adsorbed RCF, resulting in a linear dependence (Figure 8).

Figure 8. Stacking experiment results. a) Calibration curve with known concentrations of H_2O_2, increase in resorufin concentration caused by the presence of H_2O_2 (inset). Shadow refers to the standard deviation derived from 3 different independent samples. b) *Tr*CBHI/*Mt*CDHA composite film kinetics (blanks are subtracted). Development of fluorescent resorufin in composite films after the stacking *Tr*CBHI and *Mt*CDHA, controls without *Tr*CBHI or *Mt*CDHA do not produce resorufin (inset).

The *Tr*CBHI/*Mt*CDHA stacked RCF system produced 0.15 ± 0.02 nmol/min·cm^2 of H_2O_2. Kinetic curves using RCF with immobilised *Tr*CBHI/*Mt*CDHA showed a short lag phase (Figure S2) which may be caused by cellobiose diffusing from the *Tr*CBHI to *Mt*CDHA containing film. Nevertheless, the reaction quickly reaches the linear regime (in about 1 minute). Using this construct (Figure 9), it was estimated that the concentration of H_2O_2 reaches 150 µM in about 20 minutes and ~ 2 mM in 24 hours (Figure S3c).

Figure 9. Schematized **s**elf-degradation process of functionalised RCF for continuous production of H_2O_2. RCF containing *Tr*CBHI (a), RCF containing *Mt*CDHA (b) and a generic surface to disinfect (c).

The same concentration produced a complete bacteriostatic and bactericidal effect towards *E.coli* and *S.aureus* in an *in vitro* study on embryonic fibroblasts [42] suggesting a potential use of the material herein produced as antimicrobial agent. In addition, having designed the release device in the form of a film would allow a homogeneous diffusion of H_2O_2 onto a surface (e.g. skin), making the product cost-effective and controllable.

Despite the satisfactory levels of H_2O_2 produced, further investigations of the antimicrobial effects of the *Tr*CBHI/*Mt*CDHA stacked RCF on bacteria still need to be conducted. In the first instance, *in vitro* experiments such as agar diffusion tests, growth curves using different strains (e.g. *P.aeruginosa* and methicillin resistant *S. aureus*) and cell biocompatibility tests are required to assess the real antimicrobial potential of the material herein designed.

4. CONCLUSION

The goal of preparing readily manufacturable materials that actively generate H_2O_2, as a potential *in situ* sterilant, was realized by constructing multi-layer cellulose-enzyme composites. The paired enzymes were chosen for their complementary activity [30] and for good binding to the cellulose support. Specifically, composites were prepared as layered RCF bearing adsorbed cellobiose dehydrogenase (CDH) from *Thermothelomyces thermophilus* M77 (*Mt*CDHA) and cellobiohydrolase from *Trichoderma reesei* (*Tr*CBHI) to provide a coupled enzymatic H_2O_2-producing machinery immobilized on regenerated cellulose films. The use of cellulose provides

both the support (for anchoring the enzymes) and the substrate to initiate the continuous H_2O_2 production without the use of chemical modifications or enzyme-support cross-linking. The CBM bearing enzymes *Tr*CBHI and *Mt*CDHA showed a high affinity for cellulose, demonstrating that the presence of appropriate binding module protein constructs led to efficient immobilization, which was further confirmed by reusability experiments. Continuous production of H_2O_2 was achieved upon stacking films containing *Tr*CBHI, which slowly degraded the cellulose into cellobiose, with films containing *Mt*CDHA, which in turn produced H_2O_2. The production rate of H_2O_2 was almost constant and sustained for at least 24 hours. In conclusion, this work provides the foundational understanding for sustainable manufacturing of a self-degradable material with potential antimicrobial applications.

ASSOCIATED CONTENT

Supporting information

Additional information on *Mt*CDHA enzyme activity, changes over temperature and pH used to determine the optimal enzyme activity; Initial lag phase during resorufin fluorescence measurements in the RCF stacked films; Validation of the iodine H_2O_2 quantification method (increase of adsorption at 350 nm, linearity over the range of concentrations measured), evolution of H_2O_2 over 24 hours.

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

https://researchdata.bath.ac.uk/id/eprint/736

Conflicts of interest

There are no conflicts to declare.

ACKNOWLEDGEMENTS

D. Califano and V. Calabrese thank the University of Bath for PhD studentship funding. M. Kadowaki thanks the University of São Paulo and financial support from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) via grant 2011/20505-4. The authors also acknowledge funding from the UK EPSRC (EP/P027490/1).

REFERENCES

(1) Craig W. Jones and James H. Clark. *Applications of Hydrogen Peroxide and Derivatives*; 1999.

(2) Linley, E.; Denyer, S. P.; McDonnell, G.; Simons, C.; Maillard, J. Y. Use of Hydrogen Peroxide as a Biocide: New Consideration of Its Mechanisms of Biocidal Action. *J. Antimicrob. Chemother.* **2012**, *67*, 1589–1596.

(3) Seymour Stanton. *Disinfection, Sterilization, and Preservation*; Lea & Febiger, Ed.; Philadelphia, 2001.

(4) Thomas, E. L.; Milligan, T. W.; Joyner, R. E.; Jefferson, M. M. Antibacterial Activity of Hydrogen Peroxide and the Lactoperoxidase- Hydrogen Peroxide-Thiocyanate System against Oral Streptococci. *Infect. Immun.* **1994**, *62*, 529–535.

(5) Loo, A. E. K.; Wong, Y. T.; Ho, R.; Wasser, M.; Du, T.; Ng, W. T.; Halliwell, B. Effects

of Hydrogen Peroxide on Wound Healing in Mice in Relation to Oxidative Damage. *PLoS One* **2012**, *7*.

(6) Thallinger, B.; Prasetyo, E. N.; Nyanhongo, G. S.; Guebitz, G. M. Antimicrobial Enzymes: An Emerging Strategy to Fight Microbes and Microbial Biofilms. *Biotechnol. J.* **2013**, *8*, 97–109.

(7) Datta, S.; Christena, L. R.; Rajaram, Y. R. S. Enzyme Immobilization: An Overview on Techniques and Support Materials. *3 Biotech* **2013**, *3*, 1–9.

(8) Huber, D.; Tegl, G.; Mensah, A.; Beer, B.; Baumann, M.; Borth, N.; Sygmund, C.; Ludwig, R.; Guebitz, G. M. A Dual-Enzyme Hydrogen Peroxide Generation Machinery in Hydrogels Supports Antimicrobial Wound Treatment. *ACS Appl. Mater. Interfaces* **2017**, *9*, 15307–15316.

(9) Luís J. del Valle, A. D. and J. P. Hydrogels for Biomedical Applications: Cellulose, Chitosan, and Protein/Peptide Derivatives. *Gels* **2017**, *3*, 27.

(10) Wang, H.; Gurau, G.; Rogers, R. D. Ionic Liquid Processing of Cellulose. *Chem. Soc. Rev.* **2012**, *41*, 1519–1537.

(11) Swatloski, R. P.; Spear, S. K.; Holbrey, J. D.; Rogers, R. D. Dissolution of Cellulose with Ionic Liquids. *J. Am. Chem. Soc.* **2002**, *124*, 4974–4975.

(12) Earle, M. J.; Seddon, K. R. Ionic Liquids. Green Solvents for the Future. *Pure Appl. Chem.* **2000**, *72*, 1391–1398.

(13) Courtenay, J. C.; Filgueiras, J. G.; Deazevedo, E. R.; Jin, Y.; Edler, K. J.; Sharma, R. I.; Scott, J. L. Mechanically Robust Cationic Cellulose Nanofibril 3D Scaffolds with Tuneable

Biomimetic Porosity for Cell Culture. *J. Mater. Chem. B* **2019**, *7*, 53–64.

(14) Courtenay, J. C.; Johns, M. A.; Galembeck, F.; Deneke, C.; Lanzoni, E. M.; Costa, C. A.; Scott, J. L.; Sharma, R. I. Surface Modified Cellulose Scaffolds for Tissue Engineering. *Cellulose* **2017**, *24*, 253–267.

(15) Sirisha, V. L.; Jain, A.; Jain, A. *Enzyme Immobilization: An Overview on Methods, Support Material, and Applications of Immobilized Enzymes*, 1st ed.; Elsevier Inc., 2016; Vol. 79.

(16) Brady, D.; Jordaan, J. Advances in Enzyme Immobilisation. *Biotechnol. Lett.* **2009**, *31*, 1639–1650.

(17) Homaei, A. A.; Sariri, R.; Vianello, F.; Stevanato, R. Enzyme Immobilization: An Update. *J. Chem. Biol.* **2013**, *6*, 185–205.

(18) Secundo, F. Conformational Changes of Enzymes upon Immobilisation. *Chem. Soc. Rev.* **2013**, *42*, 6250–6261.

(19) Arola, S.; Linder, M. B. Binding of Cellulose Binding Modules Reveal Differences between Cellulose Substrates. *Sci. Rep.* **2016**, *6*, 1–9.

(20) Campos, B. M.; Liberato, M. V.; Alvarez, T. M.; Zanphorlin, L. M.; Ematsu, G. C.; Barud, H.; Polikarpov, I.; Ruller, R.; Gilbert, H. J.; De Mattos Zeri, A. C.; Squina, F. M. A Novel Carbohydrate-Binding Module from Sugar Cane Soil Metagenome Featuring Unique Structural and Carbohydrate Affinity Properties. *J. Biol. Chem.* **2016**, *291*, 23734–23743.

(21) Camilo, C. M.; Polikarpov, I. High-Throughput Cloning, Expression and Purification of Glycoside Hydrolases Using Ligation-Independent Cloning (LIC). *Protein Expr. Purif.* **2014**, *99*, 35–42.

(22) Segato, F.; Damásio, A. R. L.; Gonçalves, T. A.; de Lucas, R. C.; Squina, F. M.; Decker, S. R.; Prade, R. A. High-Yield Secretion of Multiple Client Proteins in Aspergillus. *Enzyme Microb. Technol.* **2012**, *51*, 100–106.

(23) Gasteiger, E. et al. Protein Identification and Analysis Tools on the ExPASy Server. In *The Proteomics Protocols Handbook*; 2009.

(24) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(25) Oberholzer, M. R.; Lenhoff, A. M. Protein Adsorption Isotherms through Colloidal Energetics. *Langmuir* **1999**, *15*, 3905–3914.

(26) Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428.

(27) Picheth, G. F.; Pirich, C. L.; Sierakowski, M. R.; Woehl, M. A.; Sakakibara, C. N.; de Souza, C. F.; Martin, A. A.; da Silva, R.; de Freitas, R. A. Bacterial Cellulose in Biomedical Applications: A Review. *Int. J. Biol. Macromol.* **2017**, *104*, 97–106.

(28) Patchan, M. W.; Chae, J. J.; Lee, J. D.; Calderon-Colon, X.; Maranchi, J. P.; McCally, R. L.; Schein, O. D.; Elisseeff, J. H.; Trexler, M. M. Evaluation of the Biocompatibility of Regenerated Cellulose Hydrogels with High Strength and Transparency for Ocular Applications. *J. Biomater. Appl.* **2016**, *30*, 1049–1059.

(29) Zhang, L.; Liu, H.; Zheng, L.; Zhang, J.; Du, Y.; Feng, H. Biodegradability of Regenerated Cellulose Films in Soil. *Ind. Eng. Chem. Res.* **1996**, *35*, 4682–4685.

(30) Langston, J. A.; Shaghasi, T.; Abbate, E.; Xu, F.; Vlasenko, E.; Sweeney, M. D. Oxidoreductive Cellulose Depolymerization by the Enzymes Cellobiose Dehydrogenase and Glycoside Hydrolase 61. *Appl. Environ. Microbiol.* **2011**, *77*, 7007–7015.

(31) Zhu, S.; Wu, Y.; Chen, Q.; Yu, Z.; Wang, C.; Jin, S.; Ding, Y.; Wu, G. Dissolution of Cellulose with Ionic Liquids and Its Application: A Mini-Review. *Green Chem.* **2006**, *8*, 325–327.

(32) Dibble, D. C.; Li, C.; Sun, L.; George, A.; Cheng, A.; Çetinkol, Ö. P.; Benke, P.; Holmes, B. M.; Singh, S.; Simmons, B. A. A Facile Method for the Recovery of Ionic Liquid and Lignin from Biomass Pretreatment. *Green Chem.* **2011**, *13*, 3255–3264.

(33) Peng, J.; Calabrese, V.; Ainis, W. N.; Scager, R.; Velikov, K. P.; Venema, P.; van der Linden, E. Mixed Gels from Whey Protein Isolate and Cellulose Microfibrils. *Int. J. Biol. Macromol.* **2019**, *124*, 1094–1105.

(34) Blakytny, R.; Jude, E. The Molecular Biology of Chronic Wounds and Delayed Healing in Diabetes. *Diabet. Med.* **2006**, *23*, 594–608.

(35) Ayawei, N.; Ebelegi, A. N.; Wankasi, D. Modelling and Interpretation of Adsorption Isotherms. *J. Chem.* **2017**, *2017*.

(36) Boer, H.; Koivula, A. The Relationship between Thermal Stability and PH Optimum Studied with Wild-Type and Mutant Trichoderma Reesei Cellobiohydrolase Cel7A. *Eur. J. Biochem.* **2003**, *270*, 841–848.

(37) Brown, B. Y. W. Y. N. Solute Diffusion in Hydrated Polymer Networks. *Cellulose* **1973**.

(38) Weber, J.; Petrović, D.; Strodel, B.; Smits, S. H. J.; Kolkenbrock, S.; Leggewie, C.; Jaeger,

585 K. E. Interaction of Carbohydrate-Binding Modules with Poly(Ethylene Terephthalate).
586 *Appl. Microbiol. Biotechnol.* **2019**, *103*, 4801–4812.

587 (39) Abouhmad, A.; Mamo, G.; Dishisha, T.; Amin, M. A.; Hatti-Kaul, R. T4 Lysozyme Fused
588 with Cellulose-Binding Module for Antimicrobial Cellulosic Wound Dressing Materials. *J.*
589 *Appl. Microbiol.* **2016**, *121*, 115–125.

590 (40) Ong, E.; Gilkes, N. R.; Warren, R. A. J.; Miller, R. C.; Kilburn, D. G. Enzyme
591 Immobilization Using the Cellulose-Binding Domain of a Cellulomonas Fimi
592 Exoglucanase. *Bio/Technology* **1989**, *7*, 604–607.

593 (41) Cabiscol, E.; Tamarit, J.; Ros, J. Oxidative Stress in Bacteria and Protein Damage by
594 Reactive Oxygen Species. *Int. Microbiol.* **2000**, *3*, 3–8.

595 (42) Hyslop, P. A.; Hinshaw, D. B.; Scraufstatter, I. U.; Cochrane, C. G.; Kunz, S.; Vosbeck, K.
596 Hydrogen Peroxide as a Potent Bacteriostatic Antibiotic: Implications for Host Defense.
597 *Free Radic. Biol. Med.* **1995**, *19*, 31–37.

598

599

600

601

602

603