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2,5-Furandicarboxaldehyde as a bio-based crosslinking agent replacing glutaraldehyde for covalent enzyme immobilization†

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In the quest for a bio-based and safer substitute for glutaraldehyde, we have investigated 2,5 diformylfuran (DFF) as bifunctional crosslinking agent for the covalent immobilization of glucoamylase on amino-functionalized methacrylic resins. Immobilization experiments and systematic comparison with glutaraldehyde at four different concentrations for the activation step showed that DFF leads to comparable enzymatic activities at all tested concentrations. Continuous flow experiment confirms a similar long term stability of the immobilized formulations obtained with the two crosslinkers. The NMR study of DFF in aqueous solution evidenced a much simpler behaviour as compared to glutaraldehyde, since no enolic forms can form and only a mono-hydrated form was observed. Unlike in the case of glutaraldehyde, DFF reacts covalently with the primary amino groups *via* imine bond formation only. Nevertheless, the stability of the covalent immobilization was confirmed also at acidic pH (4.5), most probably because of the higher stability of the imine bonds formed with the aromatic aldehydes. In terms of toxicity DFF has the advantage of being poorly soluble in water and, more importantly, poorly volatile as compared to glutaraldehyde, which displays severe respiratory toxicity. We have performed preliminary ecotoxicity assays using *Aliivibrio fischeri*, a marine bacterium, evidencing comparable behaviour (below the toxicity threshold) for both dialdehydes at the tested concentrations.

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Introduction

The use of enzymes as biocatalysts has several advantages: they have high selectivity, perform reactions in mild conditions and often are able to catalyse transformations not viable through conventional chemistry. Immobilized enzymes, which are insoluble enzyme formulations, present advantages such as applicability in low-water media or continuous flow settings.^{1–3} For this reason, immobilized enzymes are used in industry in pharmaceutical synthesis, in the food sector, in the cosmetic sector and for the synthesis of fine chemicals.^{1–4}

The immobilization of enzymes can be achieved with various techniques, including carrier-free direct cross-linking of protein conglomerates (CLECs, CLEAs), adsorption on solid carriers or covalent binding to solid carriers.⁵ In this last case, the enzyme

is bound to a carrier through residues on the protein surface, usually amine residues.⁵ Likewise, the carrier needs to have active groups to which the enzyme can bind either directly (such as epoxy groups) or after activation using a bifunctional reagent. Amino-functionalized carriers are examples of this latter case. The most common bifunctional reagent is glutaraldehyde, but several alternatives have been proposed, including carbohydrate-derived dialdehydes.⁶

Other industrial applications of glutaraldehyde include its use as disinfectant, hardener in X-ray film processing, fixative in tanning, biocide in water treatment, preservative in industrial oils, biocide in sanitary solutions for aircraft and portable toilets, in small quantities as a disinfectant for air ducts, tissue fixative in electron and light microscopy and in histochemistry and biocide in aquaculture. Conversely, the glutaraldehyde market revenue was valued at US\$ 450 million in 2021.⁷

However, the exact behaviour of glutaraldehyde in water, as well as the chemical nature of the bonds it forms with the enzyme and the carrier, is not fully understood.⁸ In fact, it displays a very complex behaviour in aqueous solution. Thirteen different forms, either hydrated, cyclic, oligomeric or polymeric have been identified, and it is still unclear which of these react with lysine side chains of the protein in enzyme immobilization. In most scientific papers, imine bonds are depicted as responsible for enzyme immobilization with glutaraldehyde,

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but this could raise questions about the expected reversibility of imine bond at acidic pH. In fact, practice shows that glutaraldehyde yields viable, irreversible, protein cross-linking, applicable in a wide range of conditions. In one study a pyridinium ion form was isolated after reaction of glutaraldehyde with amines, and it was hypothesized that this structure could also be formed during enzyme immobilization.⁹

In spite of its wide use, the industrial use of glutaraldehyde raises increasing concerns due to its widely documented toxicity.^{10,11} It has been classified as a candidate substance of very high concern (SVHC) by the European Chemicals Agency,¹² and it is regarded fatal if inhaled, toxic if swallowed, and toxic to aquatic life.¹³ It can also cause severe long-term effects, such as respiratory and skin sensitization. Its highly toxic and environmentally hazardous nature is anticipated to act as a major restraint for the market growth, since the use of this chemical is highly regulated by the government in respective regions, owing to health risks associated with it. Even a 1% solution of glutaraldehyde is poisonous for humans and animals, and products containing more than 0.1% glutaraldehyde solution are labelled as hazardous. Therefore, market players are focusing to lower dependency on glutaraldehyde and to find suitable substitutes, as high level of precaution is needed to reduce occupational and environmental exposure to glutaraldehyde.

In the general quest for finding greener and safer molecules, we have identified 2,5-diformylfuran (DFF) as a bio-based alternative for glutaraldehyde. DFF is a derivative of 5-hydroxymethylfurfural (HMF), which is obtained from the dehydration of carbohydrates. The oxidation of HMF to DFF can be achieved either by chemical routes¹⁴ or by biotransformations, including the use of isolated enzymes or whole cells.^{15,16}

In the present study, we analysed its property as a replacement of glutaraldehyde for enzyme immobilization. We have investigated and directly compared the efficiency of glutaraldehyde and DFF in the covalent immobilization of glucoamylase, an enzyme of large industrial use, on amino-functionalized methacrylic resins. We employed the two di-functional reagents in a wide range of concentrations, up to very low concentrations at which differences in behaviour would be magnified. The resulting immobilized preparations have been compared in a continuous flow experiment, to simulate industrially relevant conditions. The reactivity of DFF towards primary amino groups was investigated by means of NMR spectroscopy, shedding light on the bonds formed in aqueous solution. Moreover, we compared the two crosslinkers in an ecotoxicological study, using marine micro-organisms, since at the present moment only few papers are known dealing with DFF toxicity.^{17–19} With this work we intend to pave the way for future studies and potential applications of this bio-based difunctional agent.

Experimental

Materials

Chemicals. 5-(Hydroxymethyl)furfural (HMF, $\geq 99\%$ food grade, CAS 67-47-0); glutaraldehyde 25% w/w solution (CAS 111-30-8), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

(ABTS, CAS 30931-67-0), *p*-nitrophenyl- β -D-glucopyranoside (CAS 2492-87-7), D-glucose (CAS 50-99-7), KH_2PO_4 (CAS 7778-77-0), K_2HPO_4 (CAS 7758-11-4), NaOH (CAS 1310-73-2), Na_2SO_4 (CAS 7757-82-6), Bradford reagent (product no. B6916), HCl 37% (CAS 7647-01-0) were from Sigma Aldrich. K_2CO_3 (CAS 584-08-7) was from Carl Roth. *n*-Butylamine 98% (CAS 109-73-9) and KBr were from JT Baker. Ethyl acetate (CAS 141-78-6), diethyl ether (CAS 60-29-7) and petroleum ether 40–60 (CAS 8032-32-4) were from VWR Chemicals International. Dimethyl sulfoxide (DMSO, CAS 67-68-5), D-maltose monohydrate (CAS 6363-53-7) were from TCI Europe. Citric acid (CAS 77-92-9) was from Fisher Scientific.

DFF was chemically synthesized by oxidation of HMF based on a literature procedure.¹⁴ The product was obtained with a 99% purity determined by ^1H NMR in CDCl_3 (see ESI†).

Carriers. Carrier ReliZyme HA403/M (lot IP18-061) was acquired from Resindion (Binasco, Milano). Product features as from the producer: matrix poly(methacrylate); functional group hexamethylenamino; ion exchange capacity min 600 $\mu\text{mol g}_{\text{wet}}^{-1}$; median pore diameter 40–60 nm; water retention 60–70%; particle size range 200–500 μm .

Enzymes. Glucoamylase from *Aspergillus niger* (EC 3.2.1.3, CAS 9032-08-0, liquid solution, activity according to the here described assay: 240 U g^{-1} , protein content: 83 mg mL^{-1} , commercial name Dextrozyme GA) was purchased from Novozymes (Denmark). Peroxidase from horseradish (HRP, EC 1.11.1.7, CAS 9003-99-0, type II, lyophilized powder, 150–250 U mg^{-1}) and glucose oxidase from *Aspergillus niger* (EC 1.1.3.4, CAS 9001-37-0, type X-S, lyophilized powder, 100 000–250 000 U g^{-1} solid) were purchased from Sigma Aldrich.

NMR characterization. NMR spectra were acquired with a JEOL 60 MHz MY-NMR spectrometer, a Varian 400 (400 MHz) and a Varian 500 (500 MHz). Deuterated solvents, CDCl_3 , DMSO-d_6 and D_2O , were purchased from Eurisotop and Sigma Aldrich. Tetramethyl silane was purchased from Eurisotop.

Other equipment. Roti-Spin Mini-10 columns were purchased from Carl Roth. Samples were centrifuged in a Heraeus Biofuge 13 centrifuge. UV-Vis spectra were acquired with a PerkinElmer Lambda 2 UV/Vis Spectrophotometer, connected to a data acquisition/switch unit Agilent 34972A, and a Shimadzu UV-2450. pH measures were conducted with a Metrohm 632 pH-meter, equipped with a VWR SJ225 pH electrode.

Methods

Immobilization of glucoamylase on PMMA carrier. 10 g of carrier ReliZyme HA403/M were suspended in 16 mL of potassium phosphate buffer (25 mM, pH 7, 1.6 $\text{mL g}_{\text{carrier}}^{-1}$). Then, 200 $\mu\text{mol}_{\text{dialdehyde}} \text{g}_{\text{carrier}}^{-1}$ were added to the mixture. The mixture was shaken at 25 $^\circ\text{C}$ for 2 hours. The supernatant was then removed, and the activated carrier was rinsed 3 times with 20 mL of demineralized water.

The activated carrier was resuspended in 16 mL of potassium phosphate buffer (25 mM, pH 7, 1.6 $\text{mL g}_{\text{carrier}}^{-1}$). 5 g of Dextrozyme GA (commercial glucoamylase solution; 120 $\text{U g}_{\text{carrier}}^{-1}$ referred to the amount of wet carrier as provided by the manufacturer) were added to the reaction mixture. The mixture



was kept shaking at 25 °C for 24 hours. The supernatant was then removed and tested for residual enzyme activity. The immobilized enzyme was rinsed 3 times with 20 mL of demineralized water.

The immobilized enzyme was stored at 4 °C in potassium phosphate buffer (25 mM, pH 7).

Glucosylase activity assay. One Immobilized Glucosylase Unit (IGU) is defined as the amount of enzyme that hydrolyses one μmol of maltose at the following conditions: 25% maltose in 10 mM citrate buffer, pH 4.5, at room temperature (20 °C).

For the assay, 100 mg of immobilized glucosylase were suspended in 10 mL of the maltose solution; the mixture was shaken at room temperature for 1 hour. Every 10 minutes, a sample (50 μL) of the supernatant was taken and diluted 10 times with 0.1 M HCl before glucose analysis.

Glucose concentration assay. The glucose assay solution was obtained by dissolving 55 mg of ABTS in 50 mL of 25 mM potassium phosphate buffer pH 7; then, to the solution were added 0.2 mL of a glucose oxidase aqueous solution (1 mg mL^{-1}) and 0.2 mL of a horse radish peroxidase aqueous solution (1.5 mg mL^{-1}).

50 μL of the 10 \times diluted glucosylase assay sample were mixed with 2.95 mL of the glucose assay solution in a 4 mL UV-Vis cuvette. The cuvette was mixed by inversion, and the absorbance at 405 nm was monitored for 5 minutes. The variation in absorbance over time was compared to that of a reference solution of 5 mM D-glucose in water. The glucose concentration in each sample was obtained by:

$$[\text{Glu}] \text{ (mM)} = \frac{\Delta A_{405,\text{sample}} \text{ (min}^{-1}\text{)}}{\Delta A_{405,\text{ref}} \text{ (min}^{-1}\text{)}} \times \frac{[\text{glu}]_{\text{ref}} \text{ (mM)} \times V_{\text{ref}} \text{ (mL)}}{V_{\text{sample}} \text{ (mL)}} \times \text{df}$$

where $\Delta A_{405,\text{sample}}$ and $\Delta A_{405,\text{ref}}$ are the variations in absorbance of the sample and the reference solution, respectively; $[\text{glu}]_{\text{ref}}$ is the glucose concentration of the reference solution; V_{ref} is the volume of reference solution; V_{sample} is the volume of the glucosylase solution sample; df is the dilution factor.

Activity calculation. The glucose concentration data were plotted in a [glucose] vs. time graph. Glucosylase activity was calculated by:

$$\text{Activity (U g}^{-1}\text{)} = \frac{\Delta[\text{glucose}] \text{ (mM min}^{-1}\text{)}}{2} \times V_{\text{assay}} \text{ (L)} \times 1000$$

$$g_{\text{sample}}$$

where: $\Delta[\text{glucose}]/2$ is the rate of maltose hydrolysis; V_{assay} is the volume of the assay; 1000 is the conversion factor from mmol to μmol ; g_{sample} is the weight of dried immobilized enzyme used for the assay.

After the last activity assay cycle, the supernatant maltose solution was removed by decanting, and the immobilized enzyme sample was rinsed with 3 \times 10 mL H_2O . The water was then removed, and the enzyme preparation was dried in the vacuum oven (100 °C, 6 h). The weight of the anhydrous immobilized enzyme was used for the activity calculations.

Continuous flow activity assay. 150 mg of wet weight immobilized enzyme were introduced in a 10 mL glass column. The column was filled with a 25% maltose solution in 10 mM citrate buffer, pH 4.5. A continuous flow of maltose solution was supplied to the column at a rate of 0.15 mL min^{-1} over the course of 13 days. Every day, a sample (3–4 mL) of effluent was collected. Of this sample, 50 μL were immediately diluted 10 times with HCl 0.1 M, to be used for glucose concentration analysis; the rest was used for glucosylase leaching evaluation.

At the end of the experiment, the effluent samples were analysed to determine: (1) glucose concentration (see previous section), (2) enzyme leaching (see following section). After the experiment, the enzymatic preparation was recovered and dried under high vacuum to express all results relating to dry enzyme preparation weight.

For glucose concentration, a negative measure was performed on 50 μL of 10 times diluted maltose assay solution. The glucose concentration in the negative is subtracted from that of all measured samples.

Glucosylase leaching evaluation – p-nitrophenyl- β -D-glucopyranoside assay. 600 μL of effluent from the continuous flow column were concentrated to about 60 μL using a Roti-Spin Mini-10 column and centrifuging the sample at 7000 rpm for about 10 minutes. 20 μL of the concentrated sample were then mixed with 500 μL of a 1 mg mL^{-1} solution of p-nitrophenyl- β -D-glucopyranoside in sodium citrate buffer (0.1 M, pH 4.5); the mixture was then incubated at 56 °C for 4 h. After incubation, 500 μL of a 0.5 M potassium carbonate solution were added to the mixture. The absorbance of the final mixture at 400 nm was directly measured and compared with a p-nitrophenol calibration curve, to calculate the product concentration in the samples.

Reaction of DFF with n-butylamine. 500 mg of DFF (4.03 mmol) were mixed with 20 mL of potassium phosphate buffer (0.1 M, pH 7.0). The mixture was kept under magnetic stirring. Then, 0.797 mL of n-butylamine (free base, 8.06 mmol) were added dropwise to the reaction medium using a syringe. The reaction was stirred at 22 °C for 24 hours. The aqueous phase was extracted 3 times with 20 mL of diethyl ether. The collected organic phases were dried with anhydrous Na_2SO_4 . The organic layer was evaporated. The product was analysed with ^1H NMR and ^{13}C NMR in CDCl_3 .

Reaction of DFF with isopropylamine. 6.2 mg of DFF (0.05 mmol) were dissolved in 0.1 mL of potassium phosphate buffer (1 M, pH 7.0) and 0.9 mL of D_2O . The final buffer concentration is 0.1 M. Then, 8.6 μL (5.9 mg, 0.1 mmol) were added to the solution. The reaction solution was stirred at 22 °C for 3 days. The reaction was directly monitored by ^1H NMR after 1 hour, 5 hours, 1 day and 3 days.

Protein modelling. The crystallographic structures of glucosylase from *Aspergillus niger* were obtained from Protein Data Bank (PDB ID: 3EQA, 5GHL). The structures were analysed with the visualization software UCSF Chimera (University of California), and a prediction of glycosylation sites was



conducted with the servers NetOGlyc and NetNGlyc (Technical University of Denmark).

Methods for toxicological studies. Ecotoxicological tests were performed on *Aliivibrio fischeri* (bacteria, UNI EN ISO 11348-1:2019), based on normalized methods following standardized conditions and positive controls reported by the cited methods. Tests on bacteria were performed using MICROTOX® instrument (Modern Water) and internal cultured bacterial stocks. Responses obtained in tested solutions are set respect to natural bioluminescence of tested bacteria. A solution of DMSO (0.5%) added to an aqueous NaCl solution in ultrapure water (20 g L⁻¹) was used as solvent for hydrophobic substances and tested to evaluate the natural toxicity of the solubilizing solution. Results are considered as negative control and used for the normalization of obtained results on the molecules of interest solubilized using DMSO. Scalar dilutions starting from the maximum concentration of 5 mg L⁻¹ were tested. Both solubilized DFF and glutaraldehyde were tested after 15 and 30 minutes of exposure and results were normalized by the natural toxicity of DMSO-made negative controls. Natural toxicity of DMSO-made negative controls were always below 5% of bioluminescence inhibition after 30 min of exposure. To evaluate bacterial activity and responsiveness to the exposure, both negative controls (NaCl 20 g L⁻¹ solution in ultrapure water) and positive controls were also performed, using 3,5-dichlorophenol at the standard concentration of 4.5 mg L⁻¹. Obtained responses on positive controls (71.3%, SD 0.5%) were included within the range of acceptability for the performed test (20–80%).

Results and discussion

Behaviour and reactivity of DFF in aqueous solution

In sharp contrast with glutaraldehyde, which has a very complex behaviour in aqueous environment, the behaviour of DFF in water appears to be very simple. DFF has no alpha hydrogen on the aldehyde functions, thus avoiding the formation of enols, whereas GA undergoes aldol-reactions giving rise to aldol-form intermediates leading to different reactivity and contributing to toxic effects.²⁰

As evident from the ¹H NMR spectrum in D₂O (Fig. 1), only two forms are present in aqueous solution. There are two series of signals: two peaks (a and b) correspond to DFF, as expected, while four peaks (1 to 4) belong to its monohydrated diol. Interestingly, only one of the two aldehyde groups is hydrated to diol, as there are no signals from a hypothetical di-hydrated form. The assignments were confirmed by comparison with literature.²¹

The NMR spectrum in CDCl₃ (see ESI†) presents signals only from DFF, confirming that no impurities were present in the starting sample.

On the light of the NMR evidence that the hydration equilibrium is asymmetric and that only one of the two aldehyde groups is hydrated to diol, it was necessary to investigate whether the reaction of DFF with primary amine groups occurs in a symmetrical way. That is the prerequisite for achieving the

covalent binding of superficial lysine residues of an enzyme to an amino-functionalized carrier.²²

The amine selected for the reaction was *n*-butylamine (Scheme 1a), which models the Lys side chains of the amino acid residues. The conditions of the model reaction reproduce the protocols typically used for enzyme immobilization: potassium phosphate aqueous buffer, 0.1 M, pH 7; an oily product started forming almost immediately, and the reaction was let to continue for an additional 24 hours. The precipitation of the imine product has similarities with the behaviour of the bond between the enzyme and the solid carrier, which does not participate in the equilibrium of the chemical species in solution. It must be underlined that previous studies found that the thermodynamic equilibrium of chemical reactions can significantly change in favour of the synthetic product when a soluble compound binds to molecules linked to a solid support. That is the case of the formation of amide bonds in solid phase synthesis of peptides catalysed by proteases.²³

In these conditions the di-imine derivative (pictured in Scheme 1a), which separated from the solution, was isolated and characterized *via* ¹H NMR (in CDCl₃) and ¹³C NMR (in CDCl₃) (see ESI†). The spectra proved that the di-imine structure is symmetrical, further confirming the applicability of DFF as an enzyme crosslinker.

In addition, isopropylamine was chosen as another model amine, because it forms a water-soluble product with DFF. This allows to monitor the reaction in solution for longer times, in contrast to the *n*-butylamine product that directly separates from the solution. The reaction was conducted in potassium phosphate buffer diluted with deuterated water, in order to follow the reaction *via* ¹H NMR.

After 1 hour, the reaction reached the equilibrium. The ¹H NMR spectrum (see ESI†) indicates that the ratio between the mono- and di-imine is 1.2 : 1.0. Interestingly, the hypothetical hydrated mono-imine is not present, indicating that the formation of an imine bond shifts the equilibrium of the other aldehyde group towards the non-hydrated form. The spectrum did not change during the following 30 days (see ESI†), reporting the signals from the di-imine derivative and the mono-imine derivative (pictured in Scheme 1b), residual signals of isopropylamine (18% of the initial amount of amine is still free in solution) and no signal of residual DFF in solution. These observations lead to the following conclusions: (i) the formation of the products between isopropylamine, a primary amine, and DFF happens within the first hour of reaction; (ii) after reaching the equilibrium, the only products present in solution at 22 °C are the imine products, even after 1 week; (iii) the di-imine product shows high stability over long storage times, not displaying the formation of side (oxidation) products.

These findings suggest that the immobilization of enzymes with DFF, that is conducted in similar conditions at room temperature, only involves the formation of Schiff bases between DFF and the protein, and that the resulting imine is stable under immobilization conditions, most probably due to the conjugation of the imine bond with the furan ring and the resonance stabilization. The higher stability of the aromatic imines and the consequent shift of the equilibrium towards the



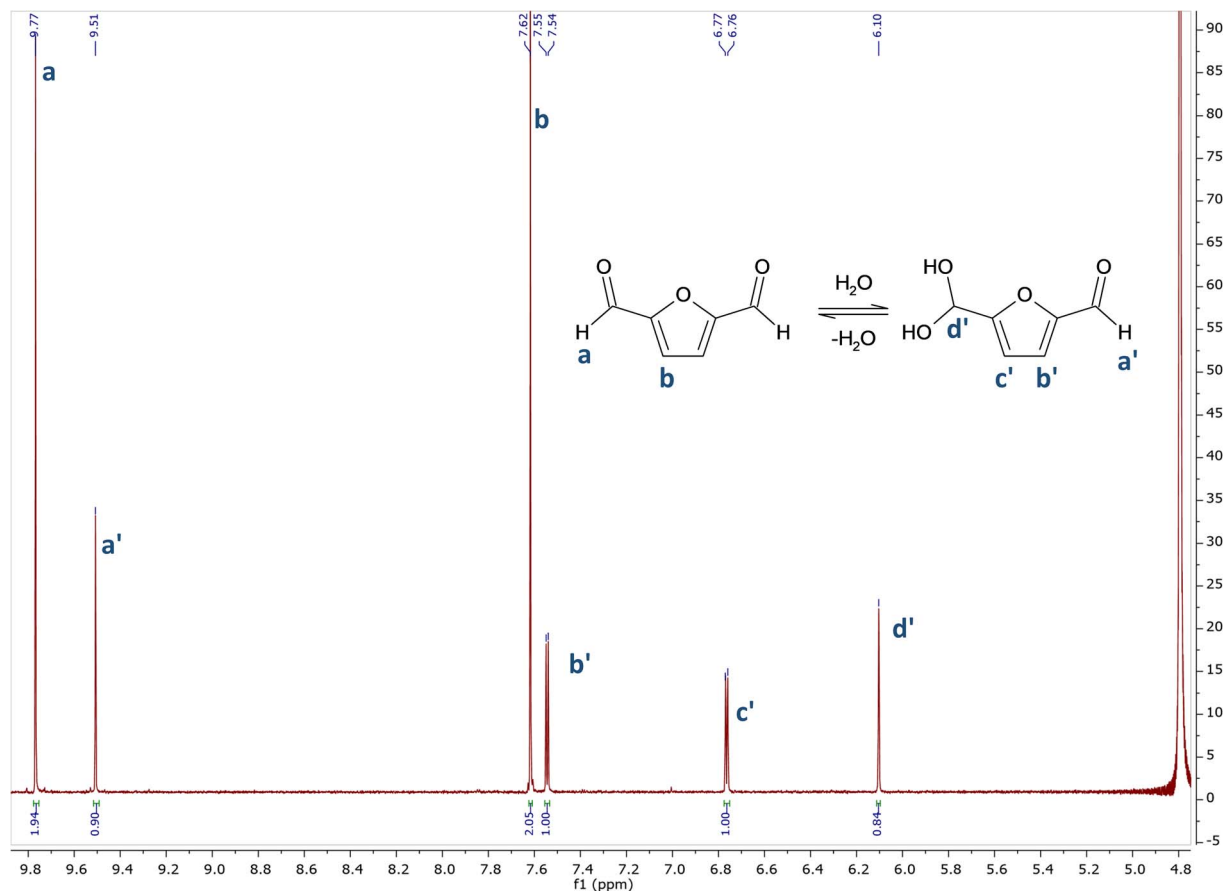


Fig. 1 ^1H NMR spectrum of DFF in D_2O . ^1H NMR (400 MHz, D_2O) δ 9.77 (s), 9.51 (s), 7.62 (s), 7.55 (d, $J = 3.6$ Hz), 6.77 (d, $J = 3.7$ Hz), 6.11 (s).

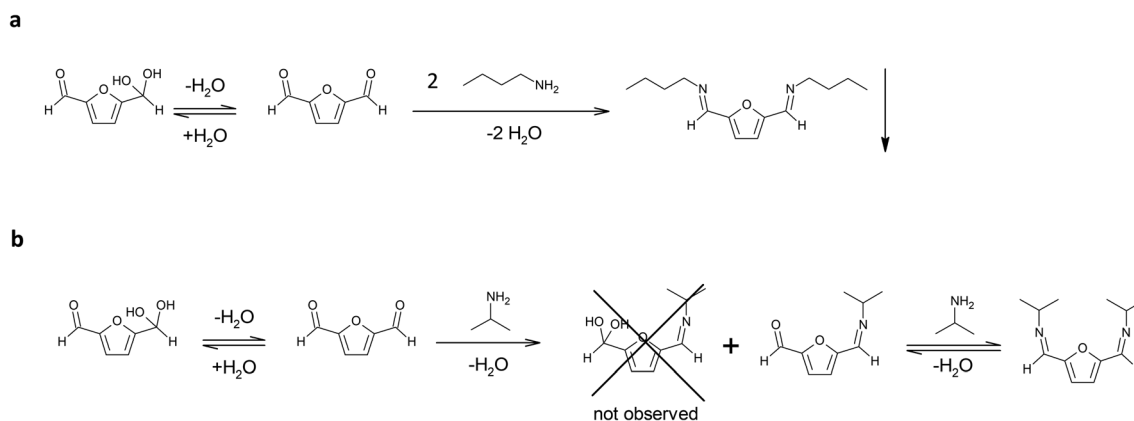
imine products have been already investigated and documented.²⁴ For instance, terephthalaldehyde, has been used as crosslinking agent to form stable gelatine membranes.²⁰

Conveniently, the behaviour of DFF in aqueous environment is more predictable than that of glutaraldehyde, facilitating further studies towards the nature of the stable chemical bond. Moreover, the dosage of this crosslinking agent can be easily determined and any excess prevented because the clearer

elucidation of the chemistry of the binding process. Finally, the formation of toxic side products can be excluded due to the absence of enolic equilibria, as in the case of glutaraldehyde.

Glucoamylase from *Aspergillus niger*

The immobilization study employed glucoamylase from *Aspergillus niger* as the model enzyme. Glucoamylase belongs to the



Scheme 1 (a) Reaction between DFF and *n*-butylamine in potassium phosphate buffer, 0.1 M, pH 7. (b) Reaction of DFF with isopropylamine in potassium phosphate buffer, 0.1 M, pH 7.



family of glycoside hydrolases (E.C. 3.2.1.3), which catalyses the cleavage of α -1,4- and β -1,6-glycosidic bonds, starting from the non-reducing end of the polysaccharide chain.^{25,26} This enzyme was chosen due to its employment on a large scale in industry,²⁷ as well as its easy immobilization on methacrylic carriers.

Structurally, glucoamylases are composed by an N-terminal catalytic domain, highly conserved between different organisms, and a smaller, C-terminal starch-binding domain; the two domains are linked by a highly flexible linker region.²⁸

The crystallographic structure of glucoamylase from *A. niger* was analysed *in silico*, using existing structures of the catalytic domain (PDB ID: 3EQA) and the starch binding domain (PDB ID: 5GHL), to establish whether covalent binding to the carrier is possible. No structures were found of the whole protein, as the flexible linker portion that connects the two domains breaks during the crystallization process;²⁶ however, for the present study it was deemed sufficient to analyse the isolated domain structures. The analysis concerned the presence of lysine residues on the enzyme surface; both the catalytic (Fig. 2A) and starch binding domain (Fig. 2B) present superficial Lys residues. It is therefore possible, in principle, to covalently immobilize the protein. In conclusion, glucoamylase from *Aspergillus niger* is suitable for covalent immobilization due to the presence of lysine residues on its surface.

Comparison of DFF and glutaraldehyde: immobilization of glucoamylase on PMMA carrier

Glucoamylase was immobilized on an amino-functionalized poly(methylmethacrylate) carrier, using the following general procedure: (a) activation of the carrier by incubation with the dialdehyde, (b) immobilization by incubation of the activated resin with the enzyme preparation; both steps were carried out in aqueous potassium phosphate buffer (25 mM, pH 7).

The activation step was studied using four different amounts of each dialdehyde (200, 20, 2 and 0.2 $\mu\text{mol g}_{\text{carrier}}^{-1}$), in order to directly compare the efficiency of DFF and glutaraldehyde over

a wide range of concentrations. The highest concentration of dialdehyde used for the activation step corresponds to one third of the concentration of amine groups of the methacrylic resin (600 $\mu\text{mol g}_{\text{resin}}^{-1}$), as declared by the producer.

The resulting activated carriers were then incubated with a fixed amount of enzyme (120 U $\text{g}_{\text{wet carrier}}^{-1}$). An excess of enzyme was used in the immobilization process, leading to a 20% of residual activity in the supernatant after the procedure (data not shown). Therefore, the observed differences in recovered activity are not ascribable to a shortage of enzyme in the procedure.

The activity of the resulting enzymatic preparations was measured in a standard assay, by recycling the enzyme for four consecutive assays, until a plateau activity value was reached. This plateau value is regarded as the enzymatic activity immobilized on the carrier. The results are presented in Table 1.

The decrease in activity between the first and the following assay cycles can be explained by the presence of adsorbed, non-covalently bound enzyme on the carrier, and it is comparable between the two crosslinkers.

By comparing the plateau values of the last two cycles, we concluded that the binding efficiency of glutaraldehyde and DFF for enzyme immobilization is comparable at all tested concentrations.

Concerning the different crosslinker concentration, the results (Table 1) show how the immobilized activity remains similar throughout the range of tested concentrations, even with a 1000-fold decrease in the dialdehyde concentration. With such a low concentration of crosslinker, and an excess of enzyme, any difference in crosslinker efficiency would be evident. The activity values are very similar between the two different crosslinking agents, glutaraldehyde and DFF. This observation further supports the conclusion that the binding efficiency of the two crosslinkers is comparable.

In order to test the behaviour of the immobilized enzyme preparations for longer times, the operational stability of

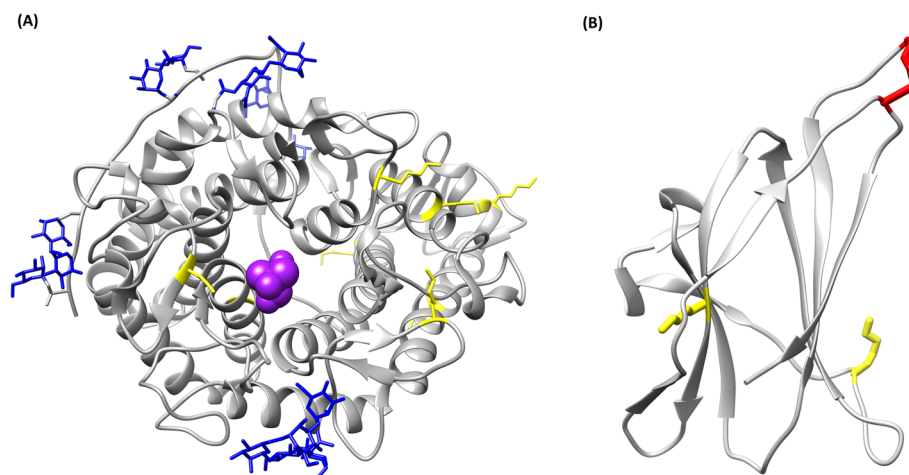


Fig. 2 Tridimensional models of the structure of glucoamylase from *Aspergillus niger*. In yellow, lysine residues; in blue, glycan residues; in purple, TRIS inhibitor in the catalytic pocket; in red, disulfide bonds. (A) Catalytic domain (PDB ID: 3EQA), (B) starch binding domain (PDB ID: 5GHL).



Table 1 Measured recovered activity for glucoamylase covalently immobilized on a PMMA carrier using decreasing concentrations of DFF and glutaraldehyde in the carrier activation step^a

Crosslinker amount ($\mu\text{mol g}_{\text{carrier}}^{-1}$)	Glutaraldehyde		DFF	
	Assay cycle	Activity ($\text{U g}_{\text{dry}}^{-1}$)	Assay cycle	Activity ($\text{U g}_{\text{dry}}^{-1}$)
200	1	105	1	137
	2	90	2	107
	3	86	3	107
	4	87	4	107
20	1	214	1	124
	2	121	2	90
	3	107	3	83
	4	90	4	80
2	1	170	1	118
	2	120	2	96
	3	77	3	70
	4	76	4	66
0.2	1	113	1	119
	2	84	2	95
	3	77	3	77
	4	75	4	73

^a DFF and glutaraldehyde comparison – continuous flow experiment.

glucoamylase immobilized on a PMMA carrier using either DFF or glutaraldehyde was analysed in a continuous flow experiment, over the course of 13 days. The immobilized enzyme was introduced in a glass column, which was filled with a 25% maltose solution in 10 mM citrate buffer, pH 4.5. A continuous flow of solution was supplied to the column at a rate of 0.15 mL min⁻¹ over the course of the experiment. The enzyme samples used for the experiment are those immobilized with the lowest amount of crosslinker (0.2 $\mu\text{mol}_{\text{aldehyde}} \text{g}_{\text{wet carrier}}^{-1}$).

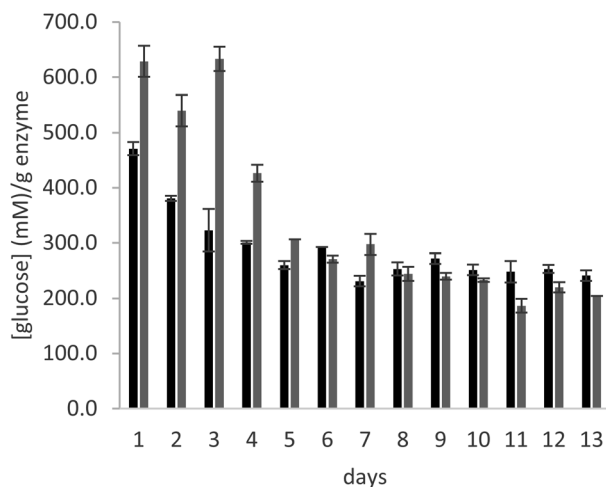


Fig. 3 Glucose concentration per g of dry enzyme preparation in the effluent of the continuous flow column experiment for glucoamylase immobilized with 0.2 $\mu\text{mol g}_{\text{carrier}}^{-1}$ of glutaraldehyde (in grey) and DFF (in black). Reaction conditions: 25% maltose solution in 10 mM citrate buffer, pH 4.5, 25 °C.

As can be seen in Fig. 3, the productivity of the enzyme decreases significantly in the first three days, stabilizing for later measurements. The trend is comparable in the samples immobilized with both crosslinkers. The cause of this behaviour is elucidated by Fig. 4, which reports the measures of enzymatic activity in the effluent as a consequence of the detachment of some residual non-covalently bound enzyme. Notably, after 2 days of the reaction, no detached enzyme is found in the effluent, and the productivity in glucose remains constant.

In conclusion, the data obtained for long term stability with the continuous flow experiment further demonstrate the efficiency of DFF for the covalent immobilization of glucoamylase on the selected PMMA carrier and confirm its potential use as a more sustainable replacer of glutaraldehyde for this purpose.

Ecotoxicological studies

To the best of our knowledge, the scientific literature reports only three papers dealing with DFF toxicity.^{17–19} In 2014, Frade *et al.*¹⁷ analysed the toxicity of HMF and twenty among its derivatives, including DFF, on human skin fibroblast cells of line CRL-1502. This is a non-tumour cell line, chosen for its resemblance with human healthy tissues. The cells were incubated with 100–500 μM of the tested compounds, DFF resulted in a cell viability of $32 \pm 2\%$ after 72 hours.

Another study from the same research group¹⁹ examines the toxicity of various *platform chemicals*, including DFF, in a Microtox assay, following the decrease in luminescence of marine bacteria *Aliivibrio fischeri*. The assay consisted in exposing *A. fischeri* to nine different concentrations of the analysed compounds, at a temperature of 15 °C, for 15 and 30 minutes. After the exposure, the luminescence of the sample is analysed, and the EC₅₀ is determined as the concentration

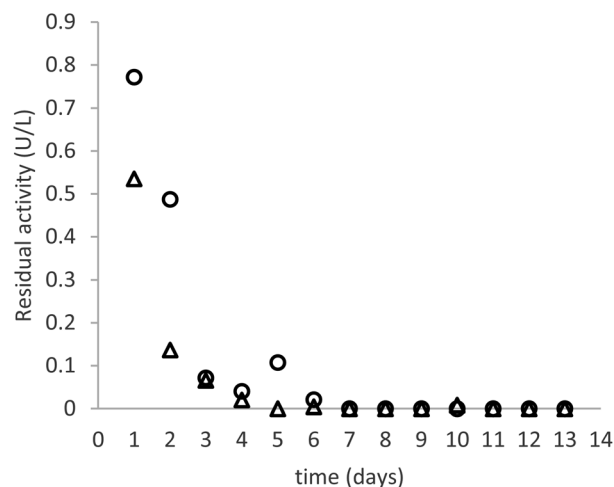


Fig. 4 Residual glucoamylase activity in the effluent of the continuous flow column experiment for glucoamylase immobilized with 0.2 $\mu\text{mol g}_{\text{carrier}}^{-1}$ of glutaraldehyde (circles) and DFF (triangles). Reaction conditions: 25% maltose solution in 10 mM citrate buffer, pH 4.5, 25 °C.



Table 2 Inhibition of natural bioluminescence at the exposure time normalized compared to negative controls

	15 min		30 min	
	Mean (%)	SD (%)	Mean (%)	SD (%)
DFF	2.31	1.13	3.76	0.32
Glutaraldehyde	0.41	0.35	4.37	0.33

corresponding to a 50% decrease in luminescence of the bacteria.

For most of the analysed compounds, the time of exposure does not influence significantly the resulting EC₅₀; an exception is DFF, for which EC₅₀ halves (in other words, toxicity doubles) going from 5 to 30 minutes of exposure. Among the analysed compounds, the authors regard DFF as moderately toxic (EC₅₀ = 100–10 mg L⁻¹).

Lastly, DFF toxicity is briefly mentioned in a work by Martins *et al.*,¹⁸ which examines toxicity of HMF and some of its derivatives towards *Aspergillus nidulans*. In this paper, IC₅₀ is calculated as the concentration of compound that can inhibit of 50% the growth of *A. nidulans*, and DFF is briefly mentioned compared to other molecules. Interestingly, in this study it is less toxic (higher IC₅₀) than HMF, unlike what observed in the other papers.

In this paper, we examined the ecotoxicology of DFF, directly comparing it to that of glutaraldehyde. Ecotoxicological tests were performed on *Aliivibrio fischeri*, in order to compare marine toxicity of DFF as compared to that of glutaraldehyde. Inhibition of natural bioluminescence at the maximum concentration tested was reported in Table 2 as mean value (standard deviation, SD) of experimental replicates. Reported results were corrected according to the DMSO natural toxicity at the concentration used to solubilize tested chemicals (0.5% DMSO in 20 g L⁻¹ NaCl ultrapure water).

Results highlighted that toxicity of tested chemicals are comparable at the maximum dose tested of 5 mg L⁻¹, closer together and lower of 5% of bioluminescence inhibition after 30 minutes of exposure. Effects lower than 15% are considered not toxic by the Italian law²⁹ and the specific literature.^{30,31} This is an encouraging result as it demonstrates that DFF can be a valid substitute for traditional aldehyde in terms of eco-compatibility. Although bacteria constitute the first link in the trophic web of aquatic ecosystems, the ecotoxicological assay with *A. fischeri* is widely used to evaluate the eco-compatibility of chemicals in both freshwater and marine ecosystems as it is highly standardized and repeatable and largely used to evaluate ecotoxicity of chemicals of possible industrial interest.^{32,33} These results are important to highlight that DFF shows an absence of toxicity under tested conditions for the standardized species used (*A. fischeri*).

Overall, in terms of toxicity DFF also has the advantage of being poorly soluble in water (about 5 mg mL⁻¹) which decreases its harmful potential in aqueous environment as compared to the fully miscible glutaraldehyde. More importantly, the high boiling point of DFF (276.8 °C at 760 mmHg)

makes this crosslinking agent considerably less harmful for human handling as compared to the volatile glutaraldehyde (boiling point 187 °C) that causes severe respiratory toxicity.

Conclusions

DFF was successfully employed as cross-linker for glucoamylase immobilization on amino-functionalized methacrylic carriers. Immobilization experiments and systematic comparison with glutaraldehyde at four different concentrations show that the efficiency of the two crosslinkers is very similar, giving comparable activities at all tested concentrations, even at very low crosslinker concentration for the activation step. Continuous flow experiment confirms that the glucoamylase immobilized with the two crosslinking agents displays comparable activity and long-term stability, with the leaching of residual adsorbed protein during the first three days of the continuous process and then reaching a plateau for the remaining 9 days.

NMR studies show that the formation of covalent bonds between DFF and primary amino groups occurs *via* imine bond formation only, unlike the case of glutaraldehyde where different mechanisms of reaction are possible.⁸ It is widely known that the formation of an imine – from an amine and an aldehyde – is a reversible reaction which operates under thermodynamic control such that the formation of kinetically competitive intermediates are, in the fullness of time, replaced by the thermodynamically most stable product.³⁴ However, when the glucoamylase was immobilized on DFF activated amino-carriers the stability of the covalent immobilization was confirmed also at acid pH (4.5). The shifting of the equilibrium towards the imine product is probably ascribable to the higher stability of the imine bonds formed with the aromatic aldehydes, as already documented in investigations dealing with terephthalaldehyde.²⁴ The ecotoxicology study of DFF against *Aliivibrio fischeri* showed a decrease in bioluminescence below the toxicity threshold for both dialdehydes. In terms of toxicity DFF has the advantage of being poorly soluble in water and, more importantly, poorly volatile as compared to glutaraldehyde, which causes severe respiratory toxicity.

The present study paves the way for further investigations aiming at the replacement of glutaraldehyde as crosslinking agent in an array of industrial applications, with the bio-based, less volatile, easy to handle DFF, which has the additional advantage of reacting according to clear and simple reaction mechanisms. The latter feature enables its easier dosage as crosslinking agent while minimizing the chemical routes that might cause toxic effects.

Author contributions

Conceptualization: Chiara Danielli, Luuk van Langen, Lucia Gardossi. Methodology: Chiara Danielli, Luuk van Langen, Deborah Boes, Monia Renzi. Validation: Chiara Danielli, Deborah Boes, Serena Anselmi, Francesca Provenza. Investigation: Chiara Danielli, Deborah Boes, Serena Anselmi, Francesca Provenza, Fioretta Asaro. Writing – original draft preparation: Chiara Danielli, Monia Renzi. Writing – reviewing and editing:



Chiara Danielli, Luuk van Langen, Lucia Gardossi, Fioretta Asaro, Monia Renzi. Supervision: Luuk van Langen, Lucia Gardossi. Funding acquisition: Luuk van Langen, Lucia Gardossi.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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