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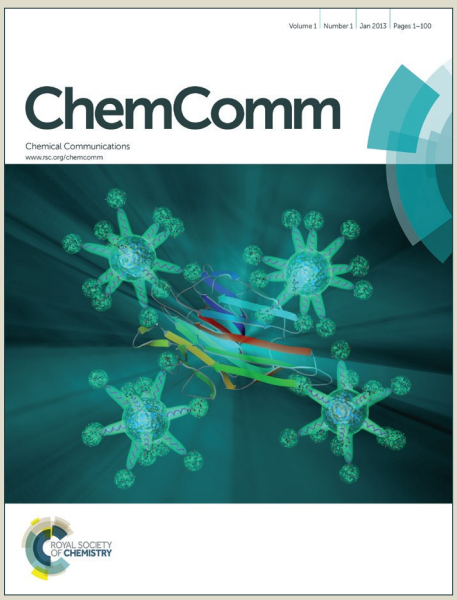

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Tailored biocatalyst achieved by the rational anchoring of imidazole groups on a natural polymer: furnishing a potential artificial nuclease by the sustainable material engineering

José G. L. Ferreira, Aline Grein-Iankovski, Marco A. S. Oliveira, Fernanda F. Simas-Tosin, Izabel C. Riegel-Vidotti and Elisa S. Orth

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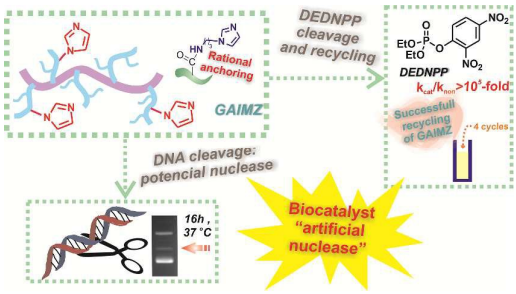

Biocatalysts were obtained by anchoring imidazole groups on gum arabic, a biocompatible polymer, with catalytic activity in dephosphorylation reactions and effective in cleaving plasmid DNA.

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Tailored biocatalyst achieved by the rational anchoring of imidazole groups on a natural polymer: furnishing a potential artificial nuclease by the sustainable material engineering

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Foreseeing the development of artificial enzymes by the sustainable materials engineering, we rationally anchored reactive imidazole groups on gum arabic, a natural biocompatible polymer. The tailored biocatalyst GAIMZ demonstrated catalytic activity (>10^5-fold) in dephosphorylation reactions with recyclable features and was effective in cleaving plasmid DNA, comprising a potential artificial nuclease.

Mimicking biological enzymes has attracted increasing interest since it furnishes promising tools to develop novel highly effective artificial catalysts, broadening applications. Specifically, designing artificial enzymes prompts many advances, *e.g.*, in gene therapy.[1, 2] In this context, dephosphorylation reactions are essential, dictating many biological functions, such as signalling processes.[3] These reactions are also involved in the damage of DNA and RNA (mutagenic) and in their repair (gene therapy), knowingly a challenge.[4] The development of promising artificial enzymes requires firstly, a complex multifunctional backbone, enzymatic-like, with desirable biocompatibility, which can be engineered by the targeted functionalization with specific reactive groups. Insofar, gum arabic (GA) comprises an interesting candidate, since it is a natural biocompatible polymer, obtained from *Acacia* trees exudates, mainly *Acacia senegal* and *Acacia seyal*.[5] GA is cheap and extensively used in the food and pharmaceutical industries. It is constituted mainly of branched complex polysaccharides and some protein fractions (low content), with an acidic nature, accounted to the glucuronic acids groups (GlcUA). Studies report GA as stabilizers of nanoparticles[6, 7] and also as excellent emulsifiers.[8] Therefore, the chemical composition and the aggregate-like assembling features of GA resemble the complex enzymatic nature and can be exploited as an artificial enzyme backbone, which to the best of our knowledge is not reported. Herein, we propose the rational anchoring on GA of highly reactive imidazole (IMZ) groups, recurrent in enzymes, comprising an innovative strategy for obtaining artificial enzymes. The tailored biocatalyst GAIMZ was fully characterized by colorimetric assays, infrared (FTIR) and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and potentiometric titration. GAIMZ was evaluated as a catalyst in the reaction with diethyl 2,4-dinitrophenyl phosphate (DEDNPP), which showed recyclable features. Finally, DNA cleavage by GAIMZ was verified, thus, enabling a potential artificial nuclease. Figure 1 summarizes the focus of this study. Moreover, our proposal offers an environmentally friendly appeal with the sustainable material engineering as a promising tool for developing optimum artificial enzymes.

GAIMZ was obtained, as shown in Figure 1, following similar procedures described in the literature,[9-11] namely forming highly stable amidic bonds on the carboxylic acid sites (GlcUA). Briefly, a solution of commercial GA from *Acacia senegal* and *Acacia seyal* (10 mg/mL) was mixed with N-hydroxysuccinimide (NHS, 13.2 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 13.2 mmol) and N-(3-Aminopropyl)-imidazole (API, 13.2 mmol) and left stirring overnight at room temperature. The mixture was dialyzed against deionized water for 3 days and then stocked in the freezer (stock solution of GAIMZ was calculated as a function of the reactive IMZ groups anchored, *i.e.*, local concentration, estimated as [IMZ^{local}]=2 mM). To standardize, we will address GA concentration as a function of [GlcUA]. Full experimental description is given in the Supplementary Information. It is noteworthy that this procedure is innovative for GA, comprising a targeted functionalization tool, which can be extended to other functionalities, foreseeing further applications.

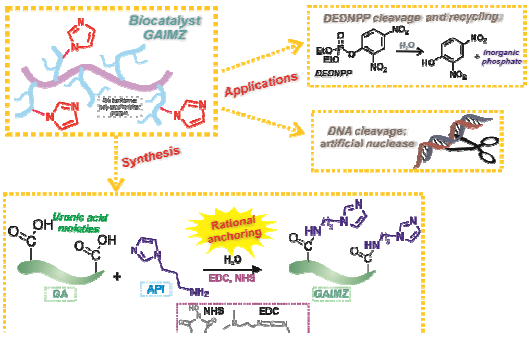

Figure 1. Representation of GAIMZ and the applications carried out herein

The degree of functionalization was determined quantifying the content of GlcUA (in mass) by a colorimetric assay, accordingly to Filisetti-Cozzi and Carpita.[12] The assay showed for GA a 27.0% content of acid moieties, while for GAIMZ decreased to 15.5%, indicating the successful functionalization with nearly 42% of efficiency. Likewise, considering GA´s total content, the degree of functionalization overall is 11.5%. In contrast to a fully functionalized sample, this discrete functionalization is desirable for prompting only some active sites, which would act synergistically with neighbouring groups, thus resembling a natural enzyme.

Analysis of the FTIR spectra obtained for GA and GAIMZ (shown in Supplementary Information) evidenced some characteristic bands: (i) 1057-1142 cm^{-1} due to arabinogalactan chains; (ii) 1420 cm^{-1}, assigned to carboxylate groups and (iii) 1615-1660 cm^{-1} attributed to the protein fraction.[13] For GAIMZ, the most evident difference was the band related to the GlcUA, which is attenuated, indicating that functionalization occurred. No further differences were found probably due to the complex nature of the sample, low degree of functionalization and overlapping of the bands. Indeed, the characteristic bands expected such as the amidic carboxylate (1640 cm^{-1}), NH of the IMZ ring (1540, 1410 and 517 cm^{-1})[14] are indistinguishable, mostly due to the numerous bands of considerable intensity from GA backbone.

^{13}C NMR spectra for GA and GAIMZ (shown in Supplementary Information) unequivocally confirmed functionalization and signals for the GlcUA carboxylic groups (-COOH, δ=173.7 ppm)[8] as well as the amide group (-CONR, δ=160.0 ppm) of the functionalized sites,[15] were distinguished. Although ^{13}CNMR spectrum is not quantitative, the intensities of these signals agree with the colorimetric assay: slightly higher intensity for the non-functionalized GlcUA moieties. Moreover, other ^{13}C signals of the polysaccharides[8] were not significantly modified upon functionalization, as expected. Additionally, signals from the anchored API were depicted and assigned to the: (i) IMZ ring at δ137.3, δ 126.0, and δ 120.5 ppm; and (ii) secondary carbons at δ 44.9, δ 42.7, and δ 24.9 ppm.[15]

Potentiometric titration were also carried out in order to confirm functionalization and further correlate the catalytic activity (*vide infra*) with the mechanism, inferring the reactive groups. Figure 2 clearly evidences a distinct titration profile for GAIMZ, in contrast to GA. The characteristic pK_a for the IMZ group (~7, -NH^+)[16] is observed for GAIMZ, consistent with the functionalization. Data were successfully fitted using the program BEST7 (solid line in Figure 2),[17] which provided for GA two expected pK_as of 4.50±0.01 and 6.49±0.01 accounted to the GlcUA and amino acid groups, respectively.[18] These pK_as were also detected for GAIMZ with an additional third pK_{a3}=7.48±0.01, assigned to the IMZ moieties, as reported in other complex systems such as enzymes with histidine side groups.[19, 20] Moreover, the relative amount of GlcUA titrated decreases going from GA to GAIMZ, suggesting a ~10% degree of functionalization, accordingly with previous colorimetric assay.

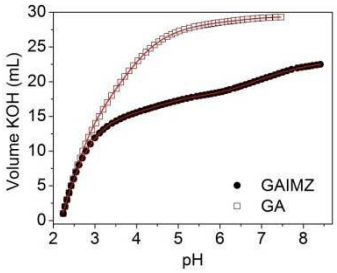

Figure 2. Titration curves for GA ([GlcUA]= 3 mM) and GAIMZ (IMZ^{local}]=0.5 mM) with KOH ($8.0x10^{-3}$ and 0.01 M for GA and GAIMZ respectively), 25°C. Solid lines correspond to the fits using the program BEST7[17]

Given the potential catalytic activity of GAIMZ due to the freely available reactive IMZ groups, its reaction with DEDNPP was evaluated by UV-Vis spectroscopy, following the appearance of the product 2,4-dinitrophenlate (DNP), under pseudo-first order conditions. Figure 3 presents the pH rate profile obtained with GA and GAIMZ, along with data for the spontaneous reaction of DEDNPP, for comparison purposes. Results show that GAIMZ is effective in accelerating the dephosphorylation reaction evaluated, which increases with pH, indicating that neutral IMZ groups are the reactive species in the reaction (correlated with titration results, *vide supra*). It is noticeable that GA cannot promote any significant rate enhancement, suggesting that the few IMZ group anchored create catalytic active sites on GAIMZ backbone, thus comprising a biocatalyst. The profile for GAIMZ was successfully fitted with equation 1 (solid line in Figure 3), which predicts three important reactions: (i) spontaneous with H_2O, k_0. (ii) alkaline hydrolysis with $^-$OH, k_{OH} and (iii) the predominant nucleophilic pathway by the neutral IMZ groups on GAIMZ (molar fraction χ_{GAIMZ}, using the pK_a determined by titration) , k_{GAIMZ}. We obtained k_{GAIMZ} =4 x 10^{-2} M^{-1} s^{-1}, which gives a prominent rate enhancement of 3 x 10^5-fold, compared to the spontaneous reaction. Further values obtained, k_0.=8x10^{-6} s^{-1} and k_{OH} =0,50 M^{-1} s^{-1}, are consistent with previous reports for DEDNPP.[16]

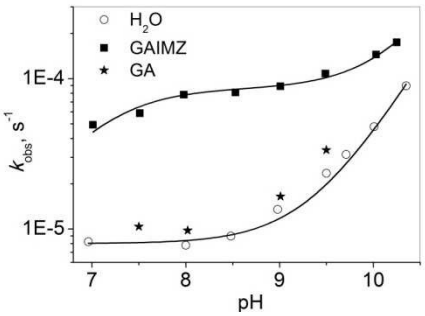

Figure 3.pH rate profile for the reaction of DEDNPP with H_2O (spontaneous),[16] GAIMZ (IMZ^{local}]=2 mM) and GA ([GlcUA]= 2 mM), 25 °C

$$k_{obs} = k_0 + k_{OH}[OH] + k_{GAIMZ}[GAIMZ]_T \chi_{GAIMZ} \quad (1)$$

Regarding the mechanism, we propose a pathway analogous to the reaction of IMZ with DEDNPP, which was thoroughly clarified previously.[16] Thusly, as shown in Figure 4, the IMZ groups anchored on GAIMZ attack the phosphorus centre, leading to a phosphorylated intermediate (GAIMZ-Phos.) and the phenolic product DNP. The intermediate is short-lived, hence, decomposes, regenerating the IMZ moiety. Therefore, GAIMZ is a genuine biocatalyst and promising artificial enzyme, since its reactive sites are recovered and can be recycled consecutively without losing activity. A possible synergistic effect that can account for the exceptional activity of GAIMZ is a hydrophobic attraction of DEDNPP on GAIMZ domains. Indeed, previous studies evidence that GA aggregates in aqueous medium giving rise to hydrophobic and hydrophilic segments in its macromolecular structure.[21] Hence, the attraction of DEDNPP is feasible, which would concentrate and approach the reactants, enhancing the overall activity of GAIMZ, as observed in micellar catalysis,[22] again resembling enzymatic catalysis. In fact, the hydrophobic effect is important for mimicking artificial enzymes not only by the binding perspective, but also by modulating the transition state geometry.[23] Additionally, possible neighbouring protein side groups can assist through multiple general acid-base catalysis.[24] Therefore, GAIMZ acts as a self-assembled bioreactor.[25] Moreover, varying the concentration of GAIMZ in the reaction with DEDNPP (given in the Supplementary Information) indicates saturation at higher concentration, suggesting an assembly feature as observed with micelles.[22]

In order to confirm the recycling features of GAIMZ, consecutive reactions with DEDNPP were followed for several cycles, as shown in Figure 4. Initially, an aliquot of DEDNPP was added to a GAIMZ solution. Upon total consumption of DEDNNP, another aliquot of DEDNPP was added to the same reaction medium, which was repeated for four cycles. The remaining DNP product after each cycle should not influence consecutive reactions and the regeneration of the active sites of GAIMZ upon each cycle (Figure 4) guarantees the recycling process. Results conclusively show that the catalytic activity of GAIMZ was maintained after several cycles, corroborating the recycling features. In addition, potentiometric titration of GAIMZ after recycling (shown in the Supplementary Information) was similar to results before recycling (Figure 2), confirming the characteristic pK_as and relative amount of the ionisable groups. Therefore, the overall functionalities of GAIMZ are preserved after reaction with no leaching of the IMZ groups. Indeed this is expected since the IMZ groups are anchored by stable amide bonds,[11] and further reiterates the potentiality of GAIMZ as an artificial biocompatible enzyme.

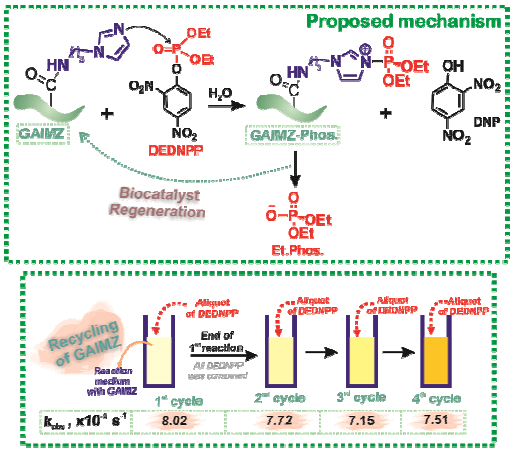

Figure 4. Proposed mechanism for the reaction of GAIMZ with DEDNPP and recycling results (IMZ^{local}]=2 mM), 25ºC, pH 8.53

Finally, the potential of GAIMZ as an artificial nuclease was verified with DNA, which comprises a far less reactive phosphate ester, compared to the activated DEDNPP. Reactions were followed by incubating varying amounts of GAIMZ and GA (for comparison) with plasmid DNA for 16 h at 37°C and pH 8.0. Full experimental description is given in the Supplementary Information. Figure 5 presents the DNA assay obtained and evidences that GAIMZ can effectively cleave DNA strands to the linear form (LD band),[26, 27] even at low concentrations. This was confirmed comparing the migration of the band with the DNA ladder. The control reactions with GA and solely buffered solution (Tris-HCl pH 8.0, shown in Supplementary Information) didn't show any plasmid cleavage. It should be noted that the higher brightness for the assay with GA is typical of super coiled DNA.

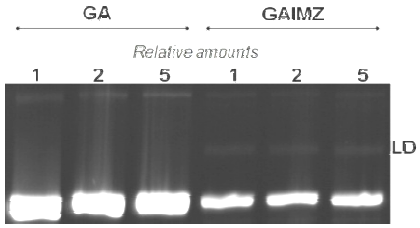

Figure 5. Cleavage of plasmid DNA with GA and GAIMZ at varying relative amounts, incubated for 16 h at 37°C and pH 8.0. LD band corresponds to the linear form of DNA.

Overall, the nuclease-like activity observed for GAIMZ suggests a possible binding with DNA strands, followed by a nucleophilic catalysis by the active sites containing IMZ groups, as proposed with the model DEDNPP (Figure 5). This binding is favoured by the complex biocompatible enzyme-like nature of GAIMZ backbone, containing polysaccharide and protein fractions. This indicates that the additional synergistic effect of GAIMZ is necessary for an efficient catalysis. Accordingly, GAIMZ configures a tailored artificial nuclease.

In conclusion, herein, we explored a natural polymeric exudate from *Acacia* trees, which is biocompatible and easily obtained (*i.e.*, is cheap). An innovative methodology by a one-pot reaction was carried out to rationally anchor reactive IMZ groups on the GlcUA sites of GA backbone. Functionalized GAIMZ was thoroughly characterized and showed an impressive catalytic activity in dephosphorylation reaction, fundamental in biological processes. Rate enhancements over 10^5-fold were observed for the reaction of the activated phosphate ester DEDNPP with GAIMZ. An elegant enzymatic-like mechanism was proposed involving firstly attraction of DEDNPP on GAIMZ domains, followed by nucleophilic catalysis by the IMZ moieties, which are regenerated after reaction, thus, GAIMZ comprises a promising biocatalyst. Recycling features were also confirmed, which showed that the catalytic activity of GAIMZ is maintained over several consecutive reaction cycles. To the best of our knowledge, this is the first report regarding functionalization of GA for catalytic purposes. The complex polysaccharide and protein fractions and micelle-like nature[21] resemble the enzymatic nature, whereby, GAIMZ was evaluated as a potential artificial nuclease in the cleavage of the less reactive plasmid DNA. Results evidence that GAIMZ can efficiently cleave DNA strands to the linear form, not observed with solely GA. Therefore, we propose that GAIMZ´s backbone binds to DNA, assisting catalysis by the IMZ groups. Overall, the tailored biocatalyst GAIMZ obtained by the rational material engineering is a promising artificial enzyme for gene therapy. It is noteworthy that GA from *Acacia mearnsii* is a by-product in the tannin industry and can be considered a residual problem.[8] Hence, by the sustainable perspective, we could extend the proposed study to these residues and consistently reuse the waist to produce environmentally friendly catalyst. Finally, the proposed catalysts are promising as detoxifying agents for organophosphorus agents, recurrent in chemical warfare and pesticides.[28] Henceforth, many efforts are concerned with eliminating stocks and contain chemical attacks, preferably seeking recyclable and sustainable non-toxic catalysts, such as GAIMZ.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [Further experimental details, FTIR and NMR spectra, kinetic data, titration profiles and DNA cleavage data.]. See DOI: 10.1039/c000000x/

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