Electronic Supplementary Information

Bioinspired design of DNA in aqueous ionic liquid media for sustainable packaging of horseradish peroxidase under biotic stress

Diksha Dhiman,[a,Ŧ] Aaftaab Sethi,[b,Ŧ] Rakesh Sinha,[a] Sagar Biswas,[a] Gregory Franklin,[a] and Dibyendu Mondal*[a,c]

[a]*Institute of Plant Genetics (IPG), Polish Academy of Sciences, Strzeszyńska 34, 60-479 Poznań, Poland.*
[b]*Laboratory of Biomolecular Interactions and Transport, Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University, Uniwersytetu Poznanskiego 6, Poznan 61-614, Poland.*
[c]*Centre for Nano and Material Sciences, Jain (Deemed-to-be University), Jain Global Campus, Kanakapura, Bangalore, Karnataka 562112, India.*
[Ŧ]*Equal contribution.*
Email: DM: m.dibyendu@jainuniversity.ac.in; dmon@igr.poznan.pl

1. Materials

Peroxidase from horseradish (HRP), trypsin from porcine pancreas, deoxyribonucleic acid (DNA), sodium salt, salmon testes, choline bicarbonate (80 wt% in water), phosphonoacetic acid, hydrogen peroxide, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), sodium phosphate monobasic monohydrate and sodium phosphate dibasic were purchased from Merck. Ultrapure Milli-Q water was used for preparing assay solutions.

2. Synthesis of Ionic Liquid (IL)

Choline phosphonoacetic acid ($[Cho]_2[PAA]$; 2:1) was synthesized following a standard protocol through a simple acid-base reaction.[1] Phosphonoacetic acid was added slowly in portion to choline bicarbonate with continuous stirring at 75 °C. The reaction mixture was kept stirring for 12 h. The ILs was dried in rotavapor as well as under reduced pressure using a vacuum line. ILs with 3±1 wt% of water were then collected and stored in air tight vessel inside a desiccator for further use to study the enzymatic reaction. The pH of synthesized $[Cho]_2[PAA]$ (50%) was found out to be 5.5±0.2. The structure of the IL is shown in Figure S1.

$[Ch]_2[PAA]$

Figure S1. Chemical structure of the IL used in the present study.

3. **Enzymatic assays preparation and peroxidase activity of HRP**

(a) Optimization of final concentration of IL, DNA, and IL+DNA

The concentration of HRP was quantified using Shimadzu UV-1900i spectrophotometer with a quartz cuvette of 1 cm path length. The extinction coefficient of HRP at 403 nm is 1.0×10^5 M^{-1} cm^{-1}. Further, to optimize the final concentration of formulations, the activity studies of HRP was conducted by combining, 10 µL of 100 nM HRP, and 50 µL of 40 mM ABTS solution to 930 µL of phosphate buffer (100 mM, pH 7.4) and to all the IL solutions (0 to 20 wt%), DNA (0 to 0.75 mg/mL) and IL (0 to 20 wt %) + DNA (0.75 mg/mL), DNA (0 to 0.75 mg/mL) + IL (15 wt%) based formulations. The assay solution was incubated for 15 minutes at 37 ℃ before adding 10 µL of 10 mM H_2O_2, and the reaction progress was monitored using UV-vis spectroscopy at 420 nm for 120 seconds. The enzymatic activities were calculated by observing the changes in absorbance at 420 nm using the spectrophotometer considering the final product $ABTS^{+\cdot}$ (ε_{420} nm=3.6×10^4 M^{-1} cm^{-1}). Subsequently, the HRP activity was calculated from the following equation with the first 60 seconds duration.

$$\%\ Relative\ activity = \frac{Absorbance(t = 60\ sec) - Absorbance(t = 0\ sec)\ in\ IL/DNA}{Absorbance(t = 60\ sec) - Absorbance(t = 0\ sec)\ in\ buffer} * 100$$

(b) Activity of HRP after trypsin digestion

To profile the activity of HRP in the presence of a biological denaturant, HRP was further incubated in the presence of 6 µM of trypsin at 37°C for 24 h along with phosphate buffer (pH=7.4) and all optimized formulations (IL (15 wt%), DNA (0.75 mg/ mL) and IL(15 wt%)+ DNA(0.75 mg/mL)). For relative activity calculations, the activity of HRP in buffer incubated in the presence of trypsin at time 0 was taken as 100%, and the activity of other systems was calculated relative to that.

4. **Stability Studies of HRP in the presence of phosphate buffer (pH =7.4), IL, DNA and IL+DNA**

The conformational and colloidal stability of HRP in the presence of IL, DNA and IL+ DNA was analyzed using UV-visible, circular dichroism (CD) spectroscopy and zeta potential.

The UV absorbance spectra of HRP (0.5 mg/mL) at 403 nm were recorded using a Shimadzu UV-1900i spectrophotometer using 1 nm resolution, utilizing matched quartz cuvettes with a 1 cm path length in the presence of phosphate buffer (pH=7.4), IL (15 wt %), DNA (0.75 mg/ mL) and IL(15 wt %)+DNA (0.75 mg/ mL). The average of three spectra after eliminating the relevant blank from the tentative spectrum was considered for analysis. Further, HRP was digested in trypsin (6 µM) in the presence of all four optimized formulations for 24 h and the UV spectra of HRP was recorded.

CD spectrum of DNA, HRP and trypsin was recorded on Jasco-1500 spectrophotometer, equipped with a Peltier system for temperature control. The wavelength range taken was 190-350 nm. The response time of 1 s and 1 nm bandwidth was used with a scan speed 20 nm/min. The concentration of DNA taken was 0.75 mg/ mL in the presence of phosphate buffer and IL (15 wt %). Whereas, the HRP and trypsin concentration was 2 mg/mL in the presence of phosphate buffer (pH=7.4), IL (2 wt %), DNA (0.75 mg/mL) and IL (2 wt %) + DNA (0.75 mg/mL). The samples were equilibrated for 15 min. All spectra were procured after blank subtraction.

Zeta potential (ζ) studies were carried out using Anton Paar Litesizer 500 device in which prior to the analysis, the samplings were made similar to that of UV-Vis spectra. ζ potential analysis was performed in the Omega cuvette Mat. No. 225288 and all the studies were recorded in the protein mode with 200 cycles of run.

5. **SDS-PAGE of HRP before and after trypsin digestion**

HRP stability before and after digestion with trypsin in PBS and different solvent systems namely $[Ch]_2[Dhp]$ IL, DNA, and DNA+IL were investigated using denatured SDS-PAGE. HRP was individually dissolved in 15 wt% of IL, 0.75 mg/mL DNA, and IL+DNA, respectively and were incubated with trypsin for 24 h at 37 ^{o}C. HRP solutions before and after digestion with trypsin were added with 2x laemmlie sample buffer was mixed with β-mercaptoethanol (50 µL in 950 µL 2x laemmlie sample buffer) for denatured SDS PAGE. Prior addition of the sample to the sample well the samples were heated to 90 ^{o}C for 5 minutes for all the denatured SDS PAGE. After that 6 µg of enzyme sample was added to the sample well of 12% (PolyAcrylamide Gel) precast Mini-Protein TGX gel. Subsequently, electrophoresis was conducted at 100 V for 4 h with 10x-tris-glycine/SDS buffer. Thereafter, it was stained with silver nitrate solution for 20

seconds to develop the electrophoretic bands. The bands were analyzed comparing with pre-stained protein ladder (PageRuler, Thermo Scientific).

6. **Computational Study**

The structures of horseradish peroxidase (HRP), Trypsin (TRY) and B-DNA were obtained from the Protein Data Bank (PDB) with the corresponding PDB IDs: 1HCH, 1S0Q and 1BNA, respectively. The initial preparation of these structures involved the removal of water molecules and other heteroatoms. Hydrogen atoms were subsequently added using the Discovery Studio Visualizer,2 to ensure proper geometry and charge distribution. To accurately model the protonation states of the HRP and TRY residues at the experimental pH of 5.5, the H++ server was employed.[3,4] This is crucial for achieving realistic docking simulations. HRP-DNA and HRP-TRY docking simulations were conducted using the HDock server.[5] For further validation of docked complexes, AlphaFold's structural predictions were employed.[6] The structure of the ionic liquid (IL) was initially drawn in ChemDraw.[7] It was then optimized and minimized using Maestro,[8] part of the Schrödinger Suite, to ensure a conformation suitable for docking. The docking of the IL with the DNA, protein and HRP/DNA complex was performed using the SwissDock.[9,10] For DNA-IL docking: the box size was set to 40, 40, 40 and the box center to 15.0, 21, 9.0. For IL-HRP docking: the box size was set to 30, 30, 30 and the box center to -2.0, -2.0, 7.0. For IL-HRP/DNA complex docking: the box size was set to 40, 37, 33 and the box center to -9.0, -2.0, 1.0. The analysis of the docked complexes was carried out using PyMOL.[11]

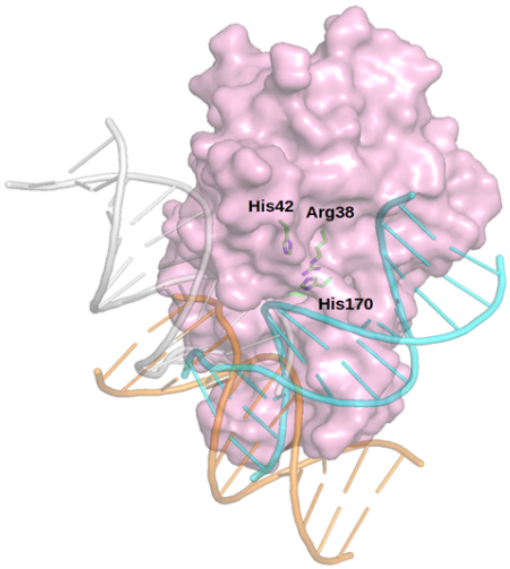

Figure S2. HRP-DNA complex highlighting three distinct DNA conformations from the top 10 docked models. HRP is shown in magenta, while the DNA conformations are represented as orange (top pose), gray (second-best pose), and cyan (third-best pose). These conformations, although distinct, bind around the HRP catalytic site without obstructing it. Catalytic residues are highlighted as green sticks and labelled.

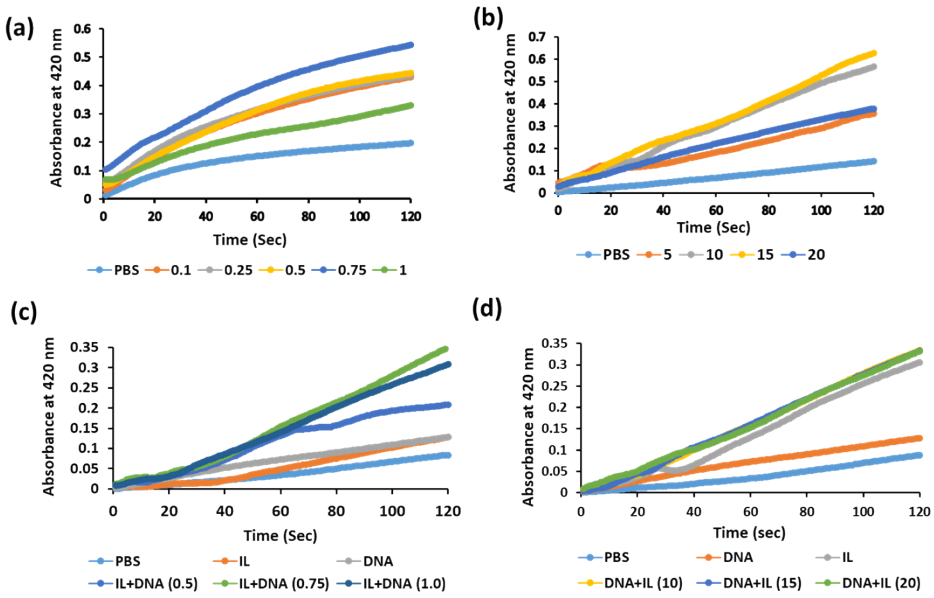

Figure S3. (a-b) Absorbance vs. time plot of HRP activity assay in presence of various concentration of DNA (a), different concentration of $[Ch]_2[PAA]$ IL (b), at various concentration of DNA with fixed IL concentration (15 wt%) (c), and at various concentration of IL and fixed DNA concentration (0.75 mg/mL) (d).

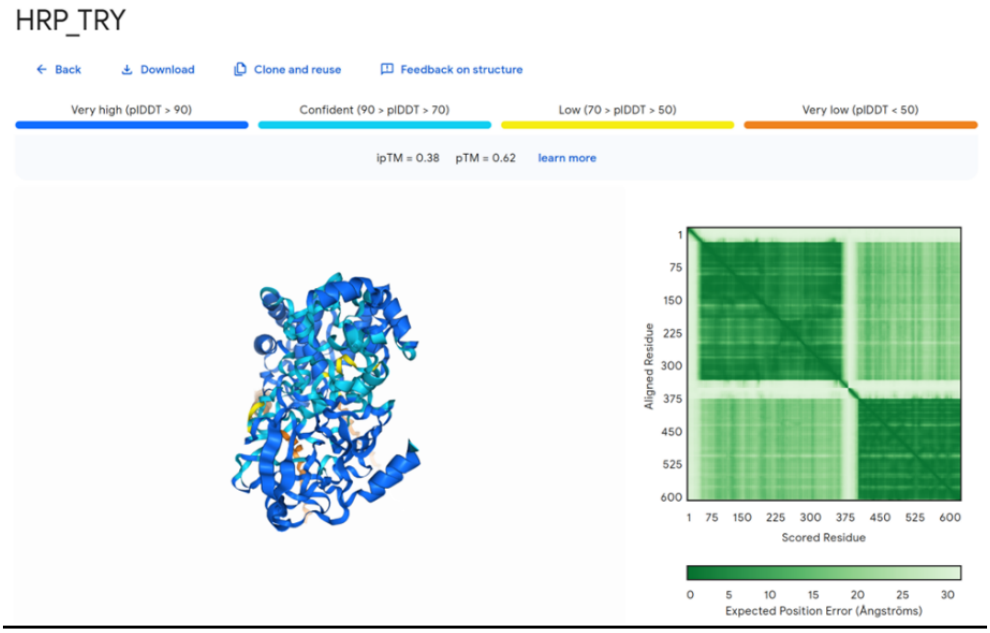

Figure S4. AlphaFold-predicted structure of the HRP-TRY complex with an ipTM score of 0.38, indicating very low reliability in predicted relative positions of the sub-units. Thus, these results were not considered for analysis.

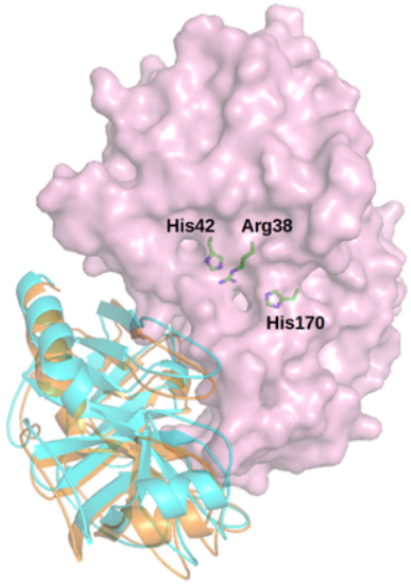

Figure S5. Validation of the HRP-TRY complex docking results. HRP is depicted in magenta, with catalytic residues shown as green sticks. The top pose generated by GRAMM[12] (orange), is in agreement with the corresponding pose from HDock (cyan). For clarity and presentation purposes, the loops have been smoothed in PyMOL.

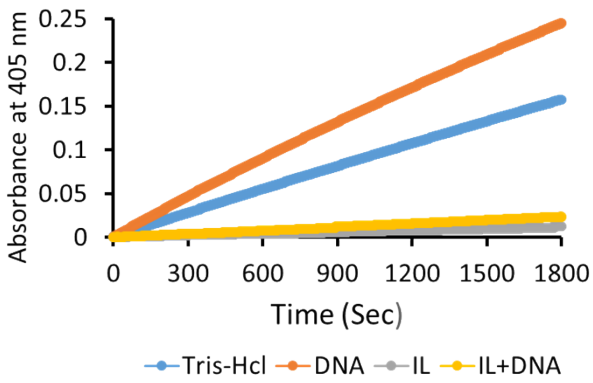

Figure S6. Activity of trypsin in presence of different solvent systems using N-α-benzoyl-DL-arginine 4-nitroanilide as substrate.

Activity of trypsin: We investigated the effect of IL/DNA-based formulations on the activity of Try. A mixture of N-α-benzoyl-DL-arginine 4-nitroanilide (BAPNA, 1 mM) and Try (0.5 µM) was prepared at 37 °C in Tris-HCl buffer (50 mM, pH 7.8) containing $CaCl_2$ (10 mM).[13] The activity was studied under different conditions: in the presence of Tris-HCl alone, and in the presence of our studied systems—IL, DNA, and IL+DNA. The reaction progress was monitored using UV-vis spectroscopy at 405 nm over a duration of 1800 seconds.

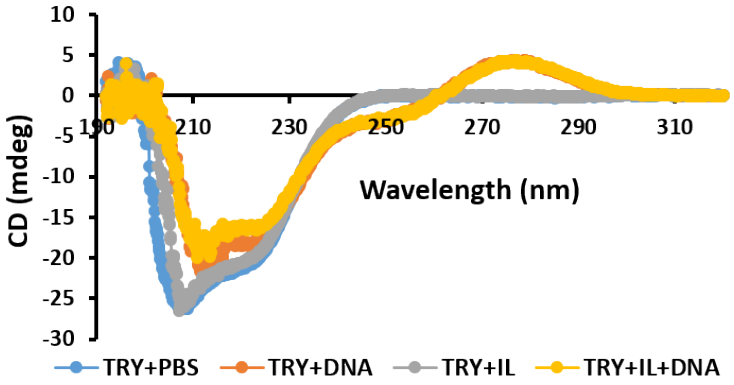

Figure S7. CD spectra of trypsin in the presence of different solvent systems.

References

1. C. Mukesh, D. Mondal, M. Sharma and K. Prasad, Rapid dissolution of DNA in a novel bio-based ionic liquid with long-term structural and chemical stability: successful recycling of the ionic liquid for reuse in the process. Chem. Commun., 2013, 49, 6849–6851
2. *Discovery Studio*. Dassault Systèmes. https://www.3ds.com/products/biovia/discovery-studio (accessed 2024-08-25).

3. Anandakrishnan, R.; Aguilar, B.; Onufriev, A. V. H++ 3.0: Automating pK Prediction and the Preparation of Biomolecular Structures for Atomistic Molecular Modeling and Simulations. *Nucleic Acids Research* **2012**, *40* (W1), W537–W541. https://doi.org/10.1093/nar/gks375.
4. Gordon, J. C.; Myers, J. B.; Folta, T.; Shoja, V.; Heath, L. S.; Onufriev, A. H++: A Server for Estimating p Ka s and Adding Missing Hydrogens to Macromolecules. *Nucleic Acids Research* **2005**, *33* (suppl_2), W368–W371. https://doi.org/10.1093/nar/gki464.
5. Yan, Y.; Tao, H.; He, J.; Huang, S.-Y. The HDOCK Server for Integrated Protein–Protein Docking. *Nat Protoc* **2020**, *15* (5), 1829–1852. https://doi.org/10.1038/s41596-020-0312-x.
6. Abramson, J.; Adler, J.; Dunger, J.; Evans, R.; Green, T.; Pritzel, A.; Ronneberger, O.; Willmore, L.; Ballard, A. J.; Bambrick, J.; Bodenstein, S. W.; Evans, D. A.; Hung, C.-C.; O'Neill, M.; Reiman, D.; Tunyasuvunakool, K.; Wu, Z.; Žemgulytė, A.; Arvaniti, E.; Beattie, C.; Bertolli, O.; Bridgland, A.; Cherepanov, A.; Congreve, M.; Cowen-Rivers, A. I.; Cowie, A.; Figurnov, M.; Fuchs, F. B.; Gladman, H.; Jain, R.; Khan, Y. A.; Low, C. M. R.; Perlin, K.; Potapenko, A.; Savy, P.; Singh, S.; Stecula, A.; Thillaisundaram, A.; Tong, C.; Yakneen, S.; Zhong, E. D.; Zielinski, M.; Žídek, A.; Bapst, V.; Kohli, P.; Jaderberg, M.; Hassabis, D.; Jumper, J. M. Accurate Structure Prediction of Biomolecular Interactions with AlphaFold 3. *Nature* **2024**, *630* (8016), 493–500. https://doi.org/10.1038/s41586-024-07487-w.
7. *ChemDraw*|*Revvity Signals Software*. https://revvitysignals.com/products/research/chemdraw (accessed 2024-08-25).
8. *Maestro*. Schrödinger. https://www.schrodinger.com/platform/products/maestro/ (accessed 2024-08-25).
9. Grosdidier, A.; Zoete, V.; Michielin, O. SwissDock, a Protein-Small Molecule Docking Web Service Based on EADock DSS. *Nucleic Acids Research* **2011**, *39* (suppl_2), W270–W277. https://doi.org/10.1093/nar/gkr366.
10. Bugnon, M.; Röhrig, U. F.; Goullieux, M.; Perez, M. A. S.; Daina, A.; Michielin, O.; Zoete, V. SwissDock 2024: Major Enhancements for Small-Molecule Docking with Attracting Cavities and AutoDock Vina. *Nucleic Acids Research* **2024**, *52* (W1), W324–W332. https://doi.org/10.1093/nar/gkae300.
11. *PyMOL | pymol.org*. https://www.pymol.org/ (accessed 2024-08-24).
12. Singh, A., Copeland, M.M., Kundrotas, P.J., Vakser, I.A. GRAMM Web Server for Protein Docking. In: Gore, M., Jagtap, U.B. (eds) Computational Drug Discovery and Design. Methods in Molecular Biology 2024, 2714. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-3441-7_5
13. K. Okuro, M. Sasaki, T. Aida, *J. Am. Chem. Soc.* 2016, **138**, , 5527–5530. https://doi.org/10.1021/jacs.6b02664