VARPA: In Silico Additive Screening for Protein-Based Lighting Devices

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Protein optoelectronics is an emerging field facing implementation and stabilization challenges of proteins in harsh non-natural environments, such as dry polymers, inorganic materials, etc., operating at high temperatures/irradiations. In this context, additives promoting structural and functional protein stabilization are paramount to realize highly performing devices. On one hand, trial-error experimental assays based on previous knowledge of classical additives in aqueous solutions are effort/time-consuming, while their translation to water-less matrices is uncertain. On the other hand, computational simulations (molecular dynamics, electronic structure methods, etc.) are limited by the system size and time. Herein, ligand-binding affinity and atomic perturbations to create a day-fast computational method combining Vina And Rosetta for Protein Additives (VARPA) to simulate the stabilization effect of sugars for the archetypal enhanced green fluorescent protein embedded in a standard dry polymer color-converting filter for bio-hybrid light-emitting diodes is merged. The VARPA's sugar additive prediction trend for protein stabilization is nicely validated by thermal and photophysical studies as well as lighting device analysis. The device stability followed the predicted enhanced stability trend, reaching a 40-fold improvement compared to reference devices. Overall, VARPA can be adapted to a myriad of additives and proteins, driving first-step experimental efforts toward highly performing protein devices.

1. Introduction

Protein-hybrid technologies are an emerging field fueled by the large toolbox to program proteins' bio-functionalities and hierarchical structures that could replace unsustainable components/systems without affecting device performance.[1–4]

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However, many technologies require the need of foreign environments (e.g., water-free or water-less materials, organic solvent deposition technique, etc.), unfriendly structures (e.g., inorganic and/or metal interfaces, etc.), and harsh working conditions (e.g., high temperature/irradiation, mechanical stress, etc.).[2,3] As a leading example in protein-based lighting systems, fluorescent proteins (FP) have been applied as color down-converting systems in dry polymer matrices,[1,2] showing stabilities of thousands of hours under low applied currents as well as <5 min under high applied currents. As a standard polymer matrix, a mixture of branched and linear polyethylene oxides dried upon gentle vacuum results in a water-less self-standing polymer filter that fairly stabilizes the FP structure and functionality over time. What is more, their preparation allows an easy implementation of additives to enhance the thermal- and photo-resilience of the FPs. However, additives testing is very demanding to screen in terms of time and human effort as no prior knowledge of their effect on water-less FP-polymer coatings is available.[2] Even when reasonable general hypotheses about the mechanism by which specific additives for aqueous buffers act on FPs are available, the stabilization effect of, for example, sugars is different depending on the drying method,[5,6] protein type, and environmental conditions.[6] In this context, computational methods (e.g., molecular dynamics, electronic structure, etc.) would offer strong working hypotheses coming with the tradeoff regarding time consumption and the need for large computational resources.

Herein, we propose VARPA, Vina, And Rosetta for Protein Additives, a quick and cheap computational method to autonomously predict the stabilization of FPs in the presence of an excess of additives placed all over the surface receptors of FPs. VARPA relies on the robust capacity of i) Rosetta to evaluate energy and conformational changes of FPs in either different oligomeric states or in the presence of single ligands,[7] and ii) Autodock[8] to quickly explore the interaction of single ligands on multiple spots at the protein structure. Thus, their combination in an automatic pipeline method allows us to predict the most

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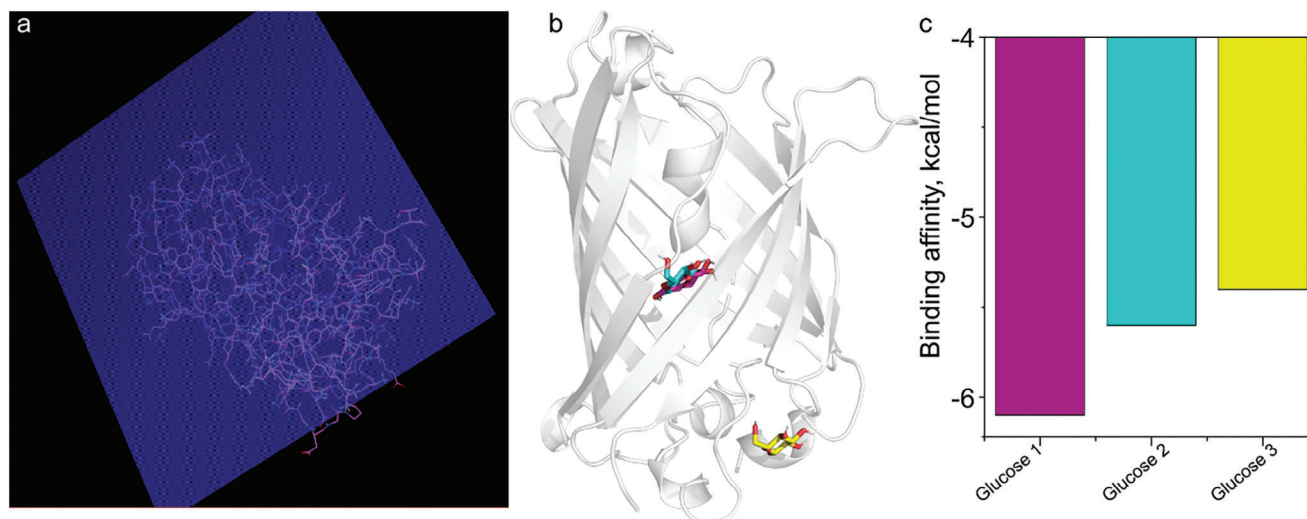**Figure 1.** a) Whole protein (eGFP) grid box (126 × 126 × 126) dockings. b) Three independent docking poses for glucose docking. c) The best dockings are predicted at the chromophore cavity with predicted binding affinities of −6.1 (purple) and −5.6 (cyan) kcal mol^{-1}, while the third best is on the protein surface with −5.4 kcal mol^{-1} (yellow). Each solution was the best per independent run.

likely interaction spots for the different additives, minimizing the newly generated protein structures saturated by them. This led to a scoring function to determine the most stable protein structure by the type of additive docked on the protein surface. When VARPA was applied to determine the best sugar stabilizers (library of 20 most commonly reported) for the archetypal FP-enhanced green fluorescent protein (eGFP), the prediction indicated that the large differential stabilization effect goes from trehalose to sucrose and sorbitol as best. These in silico results were experimentally validated in standard eGFP-polymer filters applied to lighting devices. In short, the predicted sugar trend is confirmed by the enhanced thermal and photoluminescence features and, in turn, the device stabilities. What is more striking, the use of the sorbitol additive led to 40-fold enhanced device stability compared to the pristine reference devices, highlighting the high potential of VARPA. Overall, this method represents a solid basis for elucidating additives to stabilize FP-polymers for optoelectronics in silico, while it could further grow toward more sophisticated methods upon experimental feedback.

2. Results and Discussion

2.1. Computational Model

The VARPA approach divides the above problem into three main tasks: i) independent localized dockings of a large number of small molecules (additive) on the surface of the protein receptor of interest, ii) the preparation of a new structure file that contains the receptor and all the ligand molecules that could simultaneously bind, that is, the so-called complex, and iii) a complex relaxation and score comparison with other complexes with different additives. In this way, we can alleviate the limitations from the docking step, such as i) the rigidity of the receptor, ii) the fixed bond angles and lengths of the additive, and iii) the prediction of the free energy binding. Here, using the simple scoring function of the docking algorithm and the Rosetta relaxation step for the

complex is enough to get valuable trends–vide infra. Upon a reasonable request, we can provide the set of scripts to perform the VARPA approach and the analysis of its results–see Supporting Information.

In order to develop the computational workflow, we decided to focus on screening sugar additives to improve the performance of the archetypal eGFP in the standard polymer matrix applied to lighting systems–vide supra. Sugar additives were of interest since i) they are abundant and highly effective in protecting proteins from desiccation effects,[5,6] ii) they are highly compatible with the family of polymers applied in device fabrication, and iii) they offer cost-effective and straightforward processability.

Traditionally, protein-ligand docking is performed by considering one single ligand molecule per protein. Thus, it cannot reflect the interaction at a saturated concentration of small molecules (additives) with one single protein. For example, AutoDock-Tools and AutoDock Vina (Vina) docking is achieved by defining a box that contains either the complete protein or a portion of it (**Figure 1a**), if prior knowledge of a binding cavity is available.[8–10] Thus, three issues are encountered: i) the best accommodation to maximize the total inclusion of the protein surface might not consider some residues that are key for the docking process, ii) low molecular weight additives can be artificially docked inside the FP occupying the chromophore cavity as AutoDock Vina does not consider the chromophore–vide supra, and iii) independent runs would only reveal a few different binding pockets for a single additive molecule since they are considered as the best answers based on the predicted binding affinity during the stochastic search.[8,9] As an example, Figure 1b,c shows three independent glucose docking with the biggest possible grid box for AutoDock Tools (126 × 126 × 126) containing as much as possible of the eGFP structure. The most energetically favorable dockings are for binding poses in the chromophore cavity (−6.1 and −5.6 kcal mol^{-1}). At the same time, only one falls on the surface with an affinity of −5.4 kcal mol^{-1}. Finally, we are more interested in the simultaneous interaction of a protein
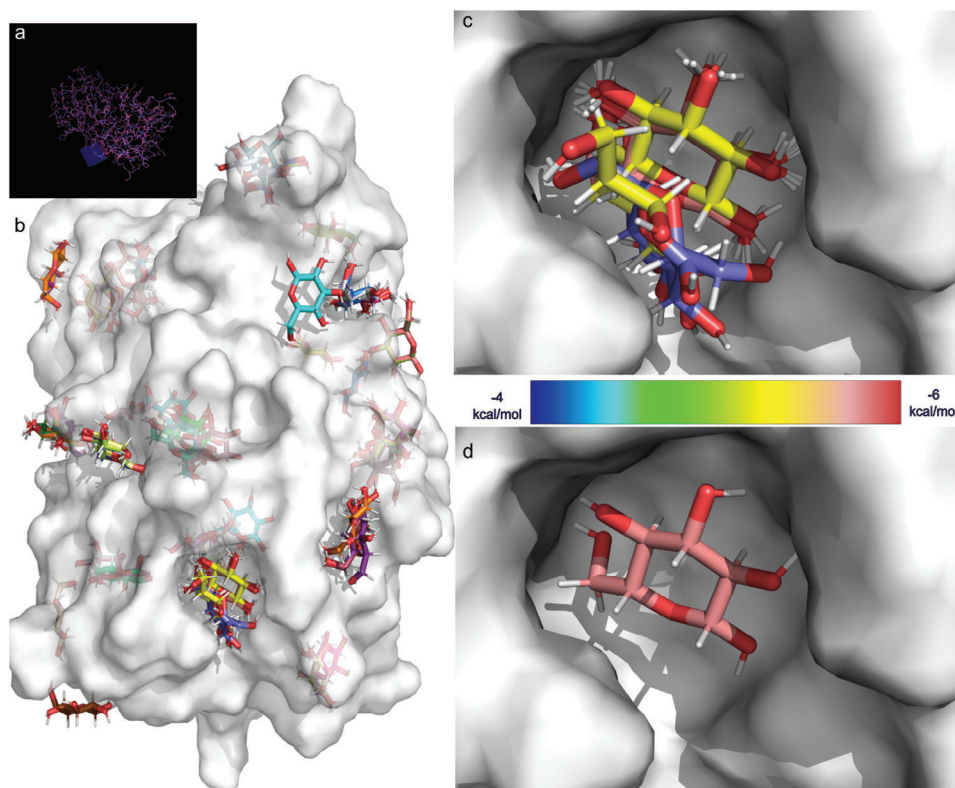
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Figure 2. Best dockings of glucose on the surface of eGFP according to Vina and clash removal. One of many surface 20 × 20 × 20 grid boxes is depicted in a), while the result at the end of the docking process is shown in b). c) Close-up of a cavity with the best dockings before clash removal, carbon colors go from blue to red according to predicted binding affinity. d) Top one docking after clash removal.

with all the possible additive molecules placed all over the protein surface, regardless of the magnitude of the predicted binding affinity. In other words, we look for global stabilization of the protein when an excess of a particular compound is present, a more realistic scenario for an additive rather than a natural ligand (**Figure 2**).

To this end, a merged collection of dockings per protein offers enough information on a total predicted binding affinity and, eventually, approximates how stable a saturated complex with a candidate additive is compared to others. This implementation relies on defining i) small grid boxes that are just big enough for an additive candidate (e.g., 20 × 20 × 20 for glucose) to move freely inside – Figure 2a, and ii) the all-over key atoms of the protein surface, such as side chain atoms of residues that face out the surface for additive interaction – Figure 2b. This approach significantly reduces the chances of artificial binding inside a buried protein cavity, allowing hierarchies by binding affinity of the solutions per small box. Since the exploration volume has been reduced by 250-fold, this approach also offers docking reproducibility in energy and conformation for the small additive molecules. As displayed in Figure 2b,c, most of the protein surface positions can accommodate more than one binding pose, overcrowding the surface. This scenario is not chemically realistic. Indeed, if this saturated complex is relaxed in Rosetta, the result would not be informative enough, as many clashes among the ligands are happening at the same time and cannot be alleviated with the small perturbations that take place with the relax protocol.[11] This re-

sults in a rise to unreasonable high Total Scores (i.e., positive values). Thus, only the docked additive molecules with the best binding affinity per spot must be preserved, as judged by a threshold distance from other docking solutions, carbon-carbon single bond (>1.5 Å)–Figure 2c,d. Thus, these constraints were implemented in VARPA to automatically filter the best poses once the dockings are finished–Figure 2d.

At this point, a library of 20 traditional sugars and sugar derivatives (e.g., sialic acid) was considered–**Figure 3**. This choice is based on i) their well-known protein stabilization effect upon dehydration,[6,12] and ii) their different features covering a large distribution of molecular weights, 3D arrangements, and hydrophobicity/hydrophilicity characters. As the docking step takes place considering the protein as a rigid body, only the binding affinity of the additive molecules can be considered, while we want to evaluate if the whole complex is more or less favorable in comparison with other complexes, regardless of the additive affinity. For example, docking results indicated that trehalose is among the sugars with the highest total affinity for the protein – **Figure 4** (left y-axis, red triangles), regardless if the average affinity is not significantly different from other sugars if we consider the error bars–Figure 4 (right y-axis, black circles). This is in line with the abundant literature that points out trehalose as one of the best-stabilizing sugars in solution or freeze-drying.[5,13–15] However, there is abundant literature that states that ligand affinity is a controversial parameter for determining stabilization capacity.[14,16,17] For example,
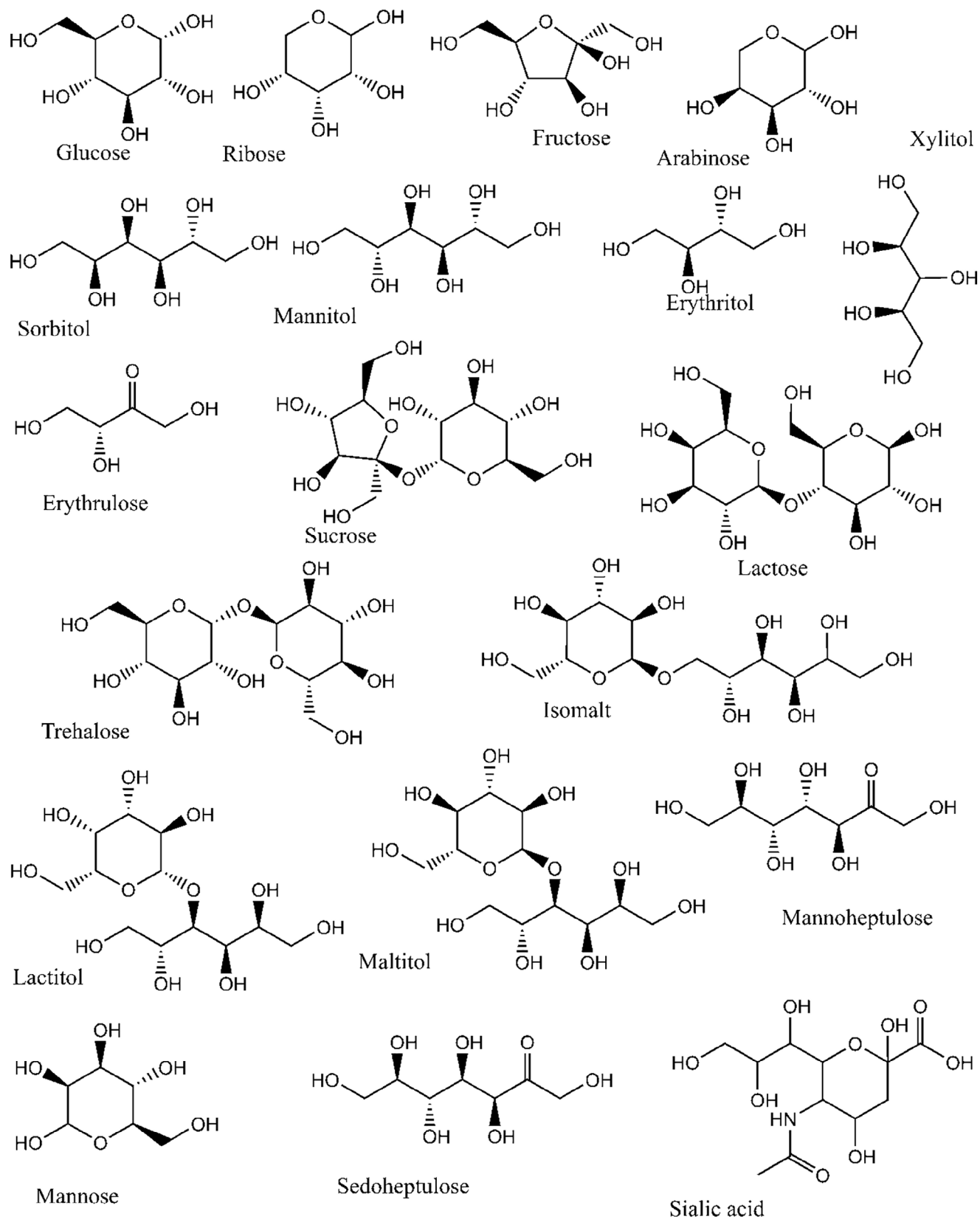
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Figure 3. Chemical structures of the library of sugars.

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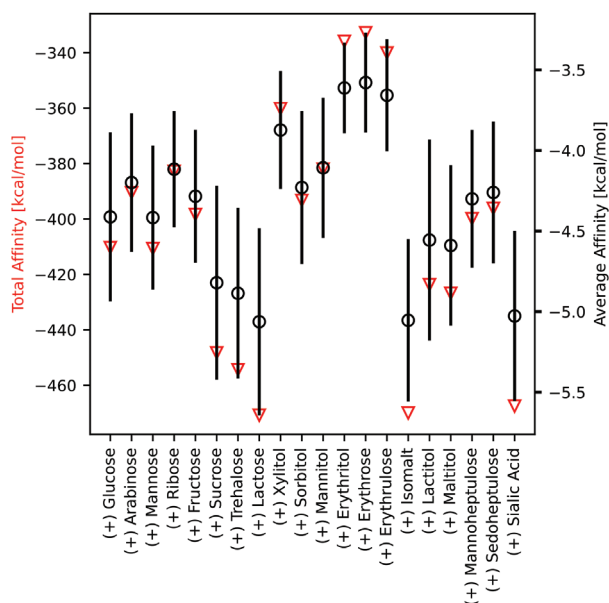
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Figure 4. Global Vina Affinity of Sugar Molecules on eGFP. Total affinity (left *y*-axis) on the best-docked sugar molecules on the surface of eGFP in red triangles, and Average affinity (right *y*-axis) on the best-docked sugar molecules on the surface of eGFP. Average values are on the empty black circles, with standard deviations as black error bars.

Arakawa & Timasheff's groups, while trying to establish the mechanism behind the preferential hydration of proteins in the presence of aqueous amino acid solutions, also considered the fact that molecules like urea, which feature a high affinity for protein that results in preferential binding, does not lead to protein stabilization.[16,18] Later on, Timasheff's group pointed out that there is no rigid shell of water around a protein, but rather a fluctuating cloud of water molecules that are thermodynamically affected by the protein. If trehalose is at high concentrations, it will act as an osmolyte with a higher preferential exclusion from the protein surface than, for example, sorbitol.[17] This behavior is critical, as the FPs will function in a hybrid solid–gel-like environment, such as the above polymer mixture matrix.[2] Hence, more than affinity to replace water for protein stabilizing interactions, we need to determine if the protein's overall structure in the complex is more stable thanks to the bound sugar additives. This aspect should be reflected in enhanced photoluminescence and thermodynamic features of the proteins in the polymer matrix–vide infra.

A fast way to approximate the complex stability is the Rosetta relax tool,[11] where local perturbations give rise to populations of 1000 models, in which the atoms of both protein and additives are allowed to move. The relaxed populations can then be compared, depending on the different sugars present by their Total Score distributions. This is not precisely a canonical energy indicator but rather a way to estimate the impact of intramolecular clashes on how likely a protein is going to fold in a particular way–**Figure 5**.[19] If we only consider the docking results, sugars like trehalose, lactose, isomalt, and sialic acid tend to be stronger binders than erythritol, erythrose, glucose, sorbitol, and mannitol. This contrasts with the Rosetta relax results, showing that the binding affinity is inversely related to the Total Score of the com-

plexes after relaxation–Figure 5a. In order to maximize the differences for the 20 molecules in our library, we normalized the Total Scores per population according to the population with the lowest Total Score, which in this work turned out to be the eGFP-sorbitol complex–Figure 5b. This is in line with literature regarding protein hydration, where different osmolytes promote hydration versus exclusion of water, being trehalose the most extreme case, while sorbitol is in the middle way to neutrality.[17] It also turns out that, according to our normalization, trehalose is the other extreme of the sugars library–Figure 5b, which is interesting considering that trehalose i) promotes storage stability of freeze-dried proteins due to its lack of reducing groups,[14,20] and ii) trehalose was the original proposed natural way in which the resurrection plant Selaginella lepidophylla survives desiccation,[21] regardless that sorbitol and xylitol are more abundant in this plant than in its desiccation susceptible relatives.[13] Based on our normalization and the reported facts just mentioned, sorbitol would be the best candidate to be used as an eGFP additive, followed by glucose and trehalose (i.e., ≈5 times worse than sorbitol). After having settled the predicted trend, we proceed with the experimental validation in the series of the best three sugars (sorbitol > glucose > trehalose), determining whether the Total Score predictor reflects on the thermal and photostability trends of the proteins in dry polymers and, in turn, on the device performance.

2.2. Experimental Validation

We followed the same protocol to prepare the FP-polymers as reported elsewhere.[3,4] The different amounts of sorbitol, trehalose, and glucose were added to the eGFP solution prior to addition to the mixture with the polymer matrix – **Figure 6**.

The first parameter to evaluate the effect of the additives is the photoluminescence figures-of-merit of eGFP in the presence of additives after its incorporation in the polymer coatings. In line with the prior art,[1,22] the changes of the emission and extinction spectra as well as fluorescence excited state lifetimes (τ) and photoluminescence quantum yields (ϕ) going from solution to the polymer matrix can be summarized by a slightly red-shifted emission band and a slight reduction of τ and ϕ values – Figure 6a,b and **Table 1**. This has been attributed to structural rearrangements in the protein backbone that change the polarity of the chromophore cavity due to the polymer-protein interaction and the slow dehydration process.[14,16–18] This is, indeed, confirmed by the increase of the τ of the tryptophan (emission centered at ≈320 nm) and the lack of a new emission band centered at 450 nm, indicating that the ionic form of the chromophore is preserved.[4]

The incorporation of any of the additives did not impact the emission and excitation spectra compared to the reference eGFP-polymers–Figure 6c–e. However, the τ and ϕ values change depending on the type of additive. In detail, trehalose and reference coatings share similar figures –, i.e., τ of 2.7 ns and ϕ of 66%; Figure 6f and Table 1, while the addition of glucose and sorbitol increases the τ to ≈2.9 ns and 3.5 ns and ϕ to 73% for both samples. Notably, the use of sorbitol leads to similar figures to those of eGFP in solution (i.e., τ of 3.3 ns and ϕ of 74%), suggesting that sorbitol preserves the native conformation of eGFP in the polymer matrix –Table 1. This experimental trend perfectly

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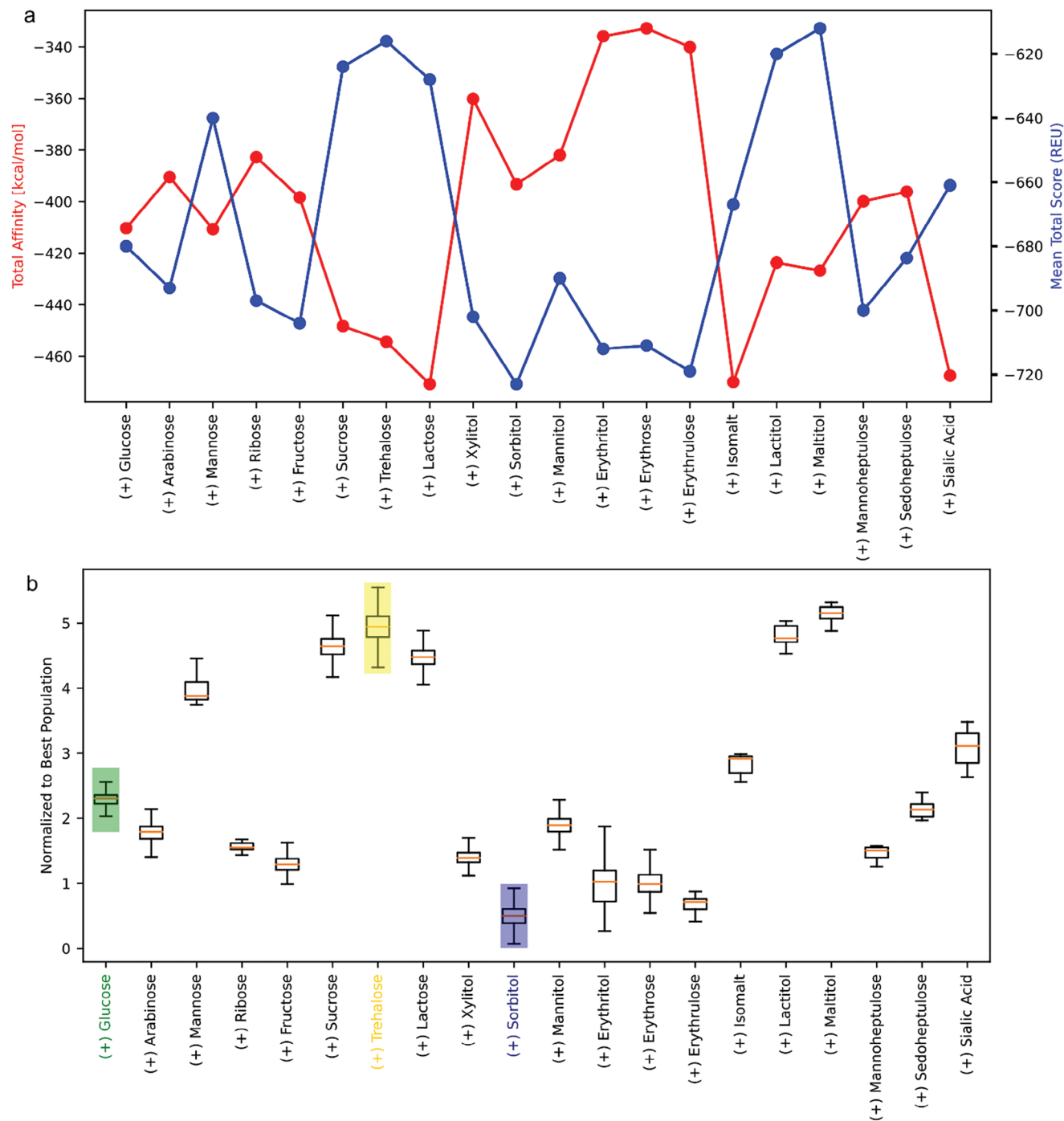
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Figure 5. Affinity and Total Score comparisons. a) Total Affinity (kcal mol^{-1}) versus Mean Total Score (Rosetta Energy Units (REU)) for the 20 sugars in this study. b) Populations of Total Scores Normalized on eGFP-sorbitol population.

agrees with our prediction that sorbitol should provide a more marked effect, as its Total Score is the best, followed by glucose and trehalose−Figure 5b.

Next, the thermodynamic stability of eGFP in solution and polymer matrices was investigated through modulated scanning fluorimetry to determine the temperature of nonreversibility of folding (T_{nr}) − Figure 6g,h.[23] In line with the literature,

the addition of both sorbitol and trehalose results in a slight increase in the temperature of nonreversibility caused by the preferential hydration of eGFP in the presence of additives in solution,[15,24] while the addition of glucose does not provide any evident change. This contrasts with the eGFP polymer coatings, in which the T_{nr} increases going from 41 °C (reference coating) up to 46 °C (trehalose coating) to 60 °C (glucose coating) and to
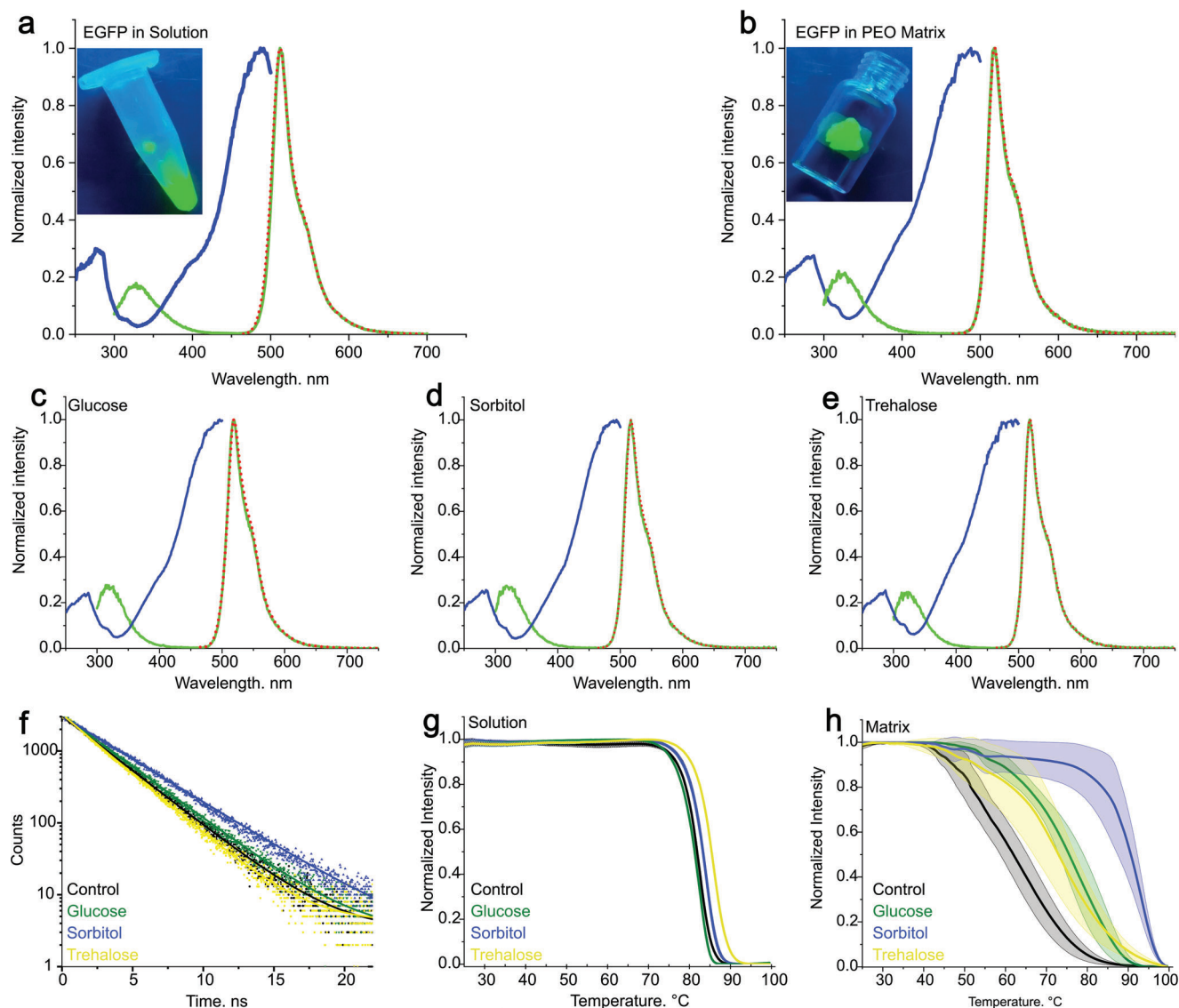
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Figure 6. Top: Excitation at λ_{max} emission (blue line) and emission at λ_{exc} of 280 nm (green line) and at $\lambda_{exc} = 450$ nm (dotted red line) spectra of eGFP in solution a) and eGFP in the polymer matrix b) as shown in the embedded pictures under UV irradiation. Middle: Excitation at λ_{max} emission (blue line) and emission at $\lambda_{exc} = 280$ nm (green line) and $\lambda_{exc} = 450$ nm (dotted red line) spectra of eGFP polymer coatings with the same amount of glucose c), sorbitol d), and trehalose e) additives. Bottom: Tests of changes of different treatments to eGFP using glucose (green), sorbitol (blue), and trehalose (yellow) additives compared to a control without additives. Excited state lifetime f) and thermocycling in solution g) and matrices h) of eGFP embedded in the polymer matrix with different additives using eGFP with the same amount of additives in aqueous phosphate buffer solution for reference purposes.

90 °C (sorbitol coating) due to the stabilization effect of the sugars upon solidification and dehydration in the presence of the polymer environment, regardless of the amount of sugar—Figure 6h. The denaturing effect of the applied polymers is ascribed to their direct interaction with hydrophobic patches at the protein backbone that forces a change in protein conformation.[24] In line with the above photophysical studies, only sorbitol leads to a full recovery of the T_{nr} values as measured in buffer aqueous solution, indicating that the native conformation of eGFP is preserved in the polymer matrix as predicted by VARPA.

As a final step, Bio-HLEDs were prepared by covering a commercial blue-emitting LED chip (450 nm) with a dome-shaped eGFP-polymer coating (i.e., on-chip architecture) with and without the additives – see Experimental Section. We monitored the emission intensity of the eGFP at high applied currents of 200 mA (150 mW cm^{-2}) – **Figure 7**. Regardless of the presence of additives, all the devices reached a working temperature of 75 °C. As expected from the thermal and photoluminescence characterizations of the polymer coatings, the device stability with trehalose is similar to that of reference devices (<40 s), while those with glucose and sorbitol additives featured enhanced stabilities up to 99 and 1725 s (≈30 min), respectively – Figure 7. This significant difference is related to the improved thermodynamic stability of eGFP in the presence of sugar additives. As
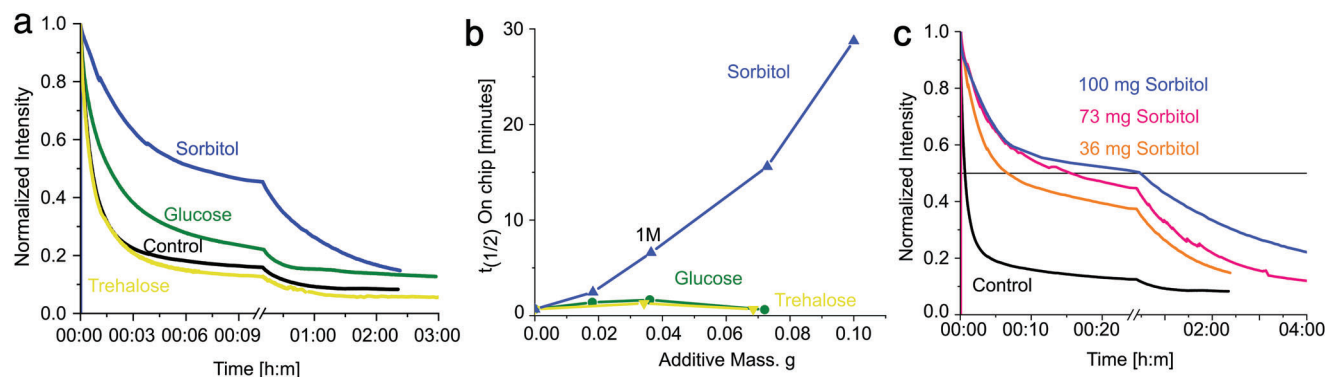
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Figure 7. a) Emission intensity decay profiles of the eGFP Bio-HLEDs in the presence of the different additives at 1 M, b) Bio-HLEDs half-life in the presence of increasing amounts of the additives. c) Emission intensity decay profiles of the eGFP Bio-HLEDs in the presence of the sorbitol at increasing concentration.

Table 1. Photophysical characteristics of eGFP in buffer aqueous solution and polymer matrix changing the type of additives.

Composition	Amount [mg]	λ_{em} [nm]	Φ [%]	eGFP $< \tau >$ [ns]
Solution control (PBS)	–	512	73	3.3
Reference coating	0	519	66	2.8
1 M Sorbitol	36	518	74	3.5
1 M Glucose	36	519	73	2.9
1 M Trehalose	68	520	66	2.7

the final test, we decided to compare the stability gain versus increasing amounts of all the additives–Figure 7b,c. The positive effect of sorbitol increases with concentration, while no clear concentration-dependent effect can be observed for glucose and trehalose–Figure 7b. Here, the use of sorbitol retains the native conformation of eGFP, as reflected by the similar photoluminescence figures-of-merit to those of solution and the increased resilience against temperature during device operation conditions. Thus, we can conclude that a sugar additive to act as a stabilizer in a solid state needs interactions that lead the whole eGFP-sugar complex to achieve a low Total Score, as predicted by VARPA.

2.3. Statistical Analysis

As mentioned, the experimental raw data was normalized according to the highest value. When errors are included, the sample size (n) was six. No statistical methods were required, other than the data being expressed as a mean with the corresponding standard deviation.

3. Conclusion

VARPA has been demonstrated as an effective predictive methodology to set the first steps toward exploring small molecule additives to stabilize FPs in polymer matrices with, for example, high interest for photon down-conversion purposes in lighting devices. This relies on the automatic massive docking algorithm recognizing the best-relaxed structures of the additives on the most sensitive protein surface positions, followed by score calculations upon protein structure relaxation in the so-called protein-additive complex. The powerful combination of methodologies implemented in VARPA agrees with previous suggestions that the binding affinity of a small molecule should be strong enough to achieve receptor stability through water replacement, but not so much to denature the receptor.[6,17] This hypothesis was confirmed by deciphering the potential of using sugar additives to stabilize FPs in water-less polymer environments, requiring a day analysis per additive to reach a trustable predictive output trend. In short, a large family of sugars was studied, reaching a series, in which the best additives follow the trehalose < glucose < sorbitol trend. This is rationalized considering the binding affinity and the changes in the protein structure with respect to the native X-ray structure. Indeed, photophysical and thermal studies of the eGFP-polymer coatings nicely support the prediction, since i) sorbitol preserves the photoluminescence and thermal features of eGFP in the polymer as that in aqueous solution, reaching similar τ, ϕ, and T_{nr} values, and ii) trehalose does not enhance τ, ϕ, and T_{nr} values compared to both: those in solution and polymer without additives. This resulted in devices with enhanced stability going from 40 s (device without additive and with trehalose) to ≈30 min (device with sorbitol). All-in-all, these findings outline the possibilities to quickly explore in silico the prospect of using small molecule additives, such as sugars to stabilize FPs in polymer matrices. Ongoing work in our laboratory concerns the use of other FPs with a different structural topology as well as other types of additives, such as ionic liquids, amino acids, etc.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

additives, fluorescent protein stabilization, in-silico prediction, lighting sources, protein-based optoelectronics

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