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Unlocking the Potential of Field Effect Transistor (FET) Biosensors: A Perspective on Methodological Advances in Computational and Molecular Biology

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Field-effect transistor (FET)-based sensors are increasingly gaining relevance in diagnostic, healthcare, and environmental monitoring applications. A FET operates by transducing chemical interactions between a surface-immobilized bioreceptor and the target analyte into a detectable electrical signal. FET biosensors can detect and monitor molecules (i.e., biomarkers, small molecules, viruses, bacterias) present in liquid samples, making a "liquid gate" configuration of FETs the most suitable approach. However, this FET architecture presents dimensional constraints that affect bioreceptors' stability and immobilization in the liquid phase. To overcome these limitations, herein, a combination of computational and molecular biology techniques for improving bioreceptors' applicability in biosensing is proposed. This results in the optimized and problem-tailored protein receptors for specific FET biosensors applications, thus enhancing their overall performance. The interplay between the computational and experimental approaches will represent a ground-breaking solution for the development of next-generation biosensors.

1. Introduction

Biosensors are a clear example of a multidisciplinary and fascinating field. The combination of physics, chemistry, electronic engineering, biology, and nanotechnology makes them a powerful tool with a wide range of applications. Bioanalytical sensors convert a binding event between a bioreceptor immobilized on a surface and the target analyte into a measurable electrical signal.[1] A typical biosensor is essentially composed of two parts, i.e., a surface area where the biorecognition element is linked and a physico-chemical transducer (**Figure 1**). Various mechanisms can transduce biochemical or biological signals, resulting in the classification of biosensors into different types such as optical, electrochemical, electrical, and mechanical. These have been extensively reviewed in several papers, detailing the differences between them.[1–4] Biosensors can detect a wide range of analytes, including DNA and RNA molecules, proteins, small molecules, cells, viruses, and bacteria.[5–7] (Figure 1). Among the different sensing solutions, field-effect transistors (FETs) have demonstrated greater potential and numerous advantages compared to other types of biosensors.[4–7]

FET-biosensors offer several assets including tunability, high sensitivity, specificity and selectivity, low detection limits, and real-time monitoring capabilities. In addition, their ability to analyze small sample volumes makes them highly efficient, whereas characteristics like reproducibility and reusability confirms them as a top-notch diagnostic tool.[1–3] FET-based biosensors can be used for a wide range of applications including virus detection, diseases diagnosis, drug discovery, food safety, and environmental

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adsr.202300053

DOI: 10.1002/adsr.202300053

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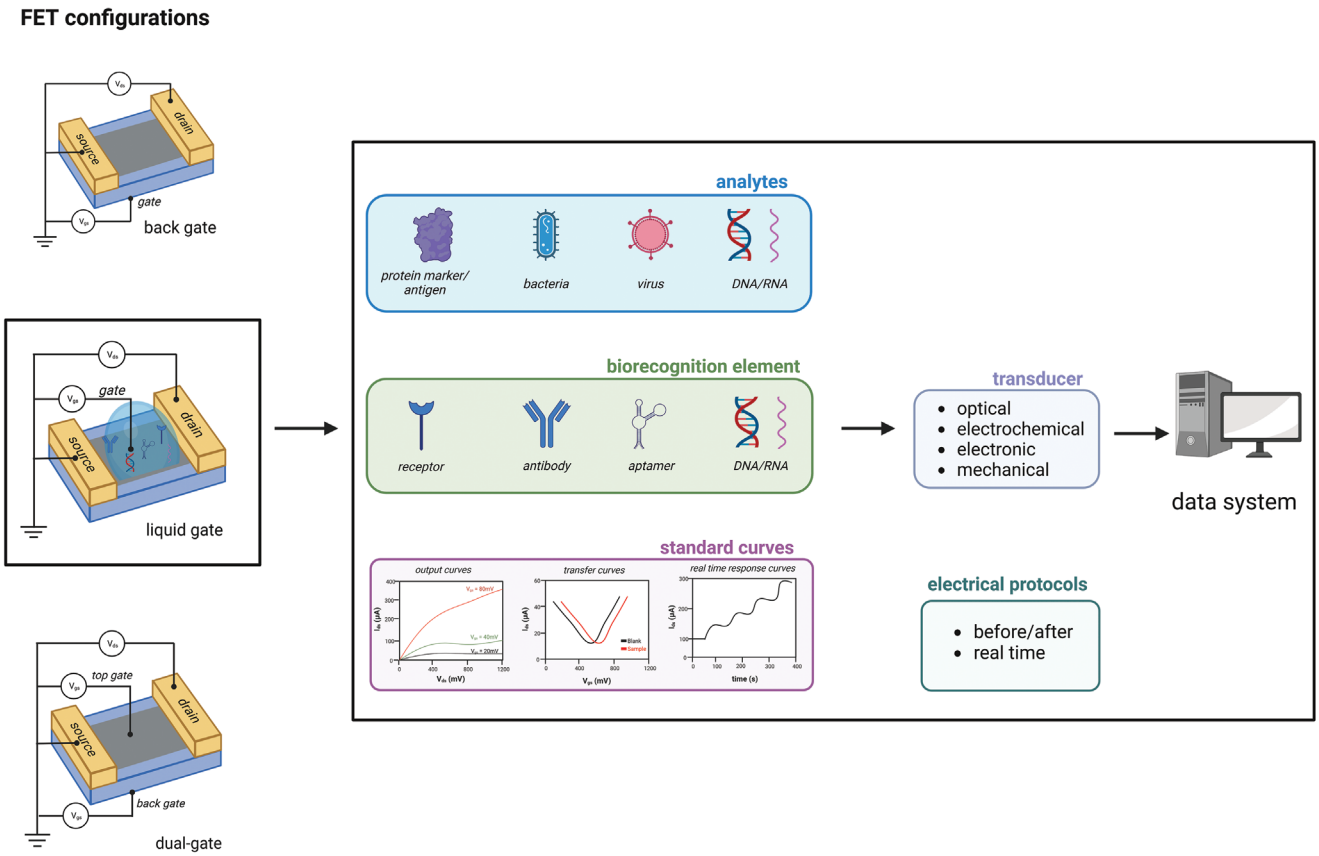

Figure 1. Schematic representation of various FETs structures: back-gate, dual-gate, and liquid-gate configurations. Additionally, a typical FET bioanalytical sensor involves specific design components (i.e., analytes, biorecognition elements, transducers). Standard curves commonly observed in 2D FET sensors are shown: output curves, transfer curve, and real-time response curve and the two different electrical metrics protocols are also cited (i.e., before/after and real-time).

monitoring.[8–10] For instance, in the healthcare field, this class of biosensors can be used for the early detection of diseases, such as cancer,[11] Alzheimer's disease,[12] and diabetes[13] by detecting specific biomarkers in bodily fluids. In particular for cancer diagnosis, several types of biosensors have been developed for the detection of different biomarkers, including prostate specific antigen (PSA) and prostate-specific membrane antigens (PSMA),[14,15] vascular endothelial growth factor (VEGF),[16] extracellular vesicles,[17] carcinoembryonic antigen (CEA),[18] and multiplex cancer markers detection.[19] In environmental monitoring, FET-biosensors can be used to detect toxins, pollutants, and heavy metals in air, water, and soil.[20,21] Others fascinating examples of FET-based biosensors application are CRISPR-Cas9-gFET for the detection of mutations related to Duchenne muscular dystrophy[22] and a graphene FET (gFET)-biosensor for the detection of interactions between proteins and small-molecule drugs (**Table 1**).[23]

As such, they are an exciting and promising technology that has the potential to impact many aspects of modern life. Among others, graphene and its derivatives have emerged as one of the most attractive nanomaterials for biosensing applications. Since its discovery almost 20 years ago its potential has been immediately clear.[24]

In particular, after the COVID-19 emergency, there has been an increased focus on the potential of graphene and FET biosensor in general.[25] Rapid diagnostic testing plays a critical role in enabling timely decision-making for the treatment and isolation of infected patients, ultimately helping to curb the spread of infectious diseases. To enable quick and easy confirmation of the presence or absence of SARS-CoV-2 in asymptomatic cases, it would be beneficial for readily available in-house biosensor devices to be accessible to individuals in all locations, but also to screen people at hospitals, airports, and other crowded areas. Numerous biosensors for detecting SARS-CoV-2 have been developed.[25–28] In this scenario, the potential of FET-biosensors has been demonstrated, in terms of their advantages but above all in the areas for improvement. In fact, there are still some challenges that need to be addressed, such as sensitivity, reproducibility, and high cost. Specifically for FET diagnostic biosensors, which rely on the selective interaction between bioreceptors attached to the surface and analytes, the majority of challenges arise from a biological perspective. For instance, graphene is commonly functionalized with bioreceptor, usually proteins, such as antibodies or membrane receptors in a liquid environment (Table 1). The difficulties that emerge include producing membrane proteins or chimeras, maintaining their stability, and

Table 1. Overview of some of FET-based biosensors and their different applications.

Application	Bioreceptor type	Target analyte	Refs.
Cancer diagnosis	Antibody	CEA	[18, 31, 32]
	Antibody	Chondroitin sulfate proteoglycan 4	
	Antibody	HER	
	Aptamer	hCG	[33]
	Aptamer	PSA	[34]
	Antibody	hCG	[35]
Emerging pollutant	Antibody	Chlorpyrifos	[36, 20]
	Aptamer	Kanamycin	
	Aptamer	17β-estradiol (E2)	[37]
	Aptamer	17β-estradiol (E2)	[38]
Food contaminants	Aptamer	Aflatoxin B1	[9, 39]
	Antibody	*E. coli*	
	Antibody	*E. coli*	[40]
Extracellular vesicles	Antibody	Extracellular vesicles	[17, 41]
	Antibody	Extracellular vesicles	
Covid-19 diagnosis	Spike S1 protein	SARS-CoV-2 antibody	[42, 43, 44]
	ACE2	SARS-CoV-2	
	ACE2	Spike	
	ACE2-Fc	SARS-CoV-2	[27]
	Y-shaped DNA dual probes	SARS-CoV-2 nucleic acid	[45]
Alzheimer disease biomarker detection	Antibody	Aβ1-42 and t-TAU	[12]
Glucose monitoring	PBA	Glucose	[46, 47]
	Enzyme	Glucose	
Diseases diagnosis	Antibody	Cardiac Troponin T	[48, 49]
	ssDNA	H5N1AIV	
	Enzyme	L-carnitine	[50]

ensuring that they acquire the correct conformation and orientation when immobilized on the biosensor device.

To overcome these issues, researchers are increasingly turning to combined computational/experimental techniques to improve biosensor design and performance. There is a limited number of examples that demonstrate the advantages of communication between the distinct approaches.[27,29,30] In this perspective, we will highlight the importance of teaming up computational design and molecular biology for the optimization of bioreceptors, with the final goal of improving FET-biosensors' performance. We will also discuss the challenges and opportunities in this rapidly evolving field, as well as the future directions for biosensor development.

2. Sensing at the Boundaries of FET

FET sensors are a ground-breaking technology that allow rapid, label-free and sensitive detection. Traditionally, FET sensors are based on the change in conductance of the 3D semiconducting channel before and after the target molecule's adsorption. However, due to the peculiar electronic characteristics of bulk materials, external stimuli only affect their upper surface, resulting in a low efficiency. To replace 3D semiconductor channels in FETs, low-dimensional alternatives were reported such as 1D nanotubes, nanoribbons, nanowires, and 2D graphene, molybdenum disulfide (MoS_2), and black phosphorus (BP).[2] 2D materials are easier to manipulate and can form a stricter contact with the metal electrodes. In the field of sensing applications, 2D thin layer materials such as graphene, phosphorene, transition metal dichalcogenides (TMDs), and transition metal oxides (TMOs) are commonly used.[51] Graphene and its derivatives possess a cloud of pi-electrons due to sp^2 hybridized carbon atoms, facilitating the binding of molecules through Van der Waals (VDW) or π–π interactions. Phosphorene, on the other hand, interacts with the target through charge transfer from the p_z-orbitals of P atoms. Similarly, charge transfer from p-orbitals of chalcogens in TMDs enables their interaction with molecules. TMOs structure, such as MoS_2-FET, includes d-orbitals of molybdenum, where four electrons from Mo fill the bonding state, and the surface layers are completed through long pairs of electrons, showing efficient attachment with biomolecules, such as DNA. The choice of material depends on the application, but the principle remains the same, namely modulation of the electrical properties due to the adsorption of analytes such as gas, ions, biomolecules, or chemicals. For chemical and physical sensing, materials such as MoS_2 and phosphorene FET are commonly employed due to their electrostatic doping. In biological sensing, graphene is the most used 2D layer due to its atomically thin honeycomb lattice and high surface-to volume ratio.[51,52]

The basic structure of a FET includes at least three electrodes: source, drain, and gate (Figure 1).[2] The density of charge in the channel (i.e., the current) is modulated by the local

electrostatic field and the resulting change in the electrical metrics is used to determine whether the molecule of interest is present or not. The channel's conductivity is modulated by regulating the gate bias voltage, which controls the charge current flowing through the source–drain electrodes. These electrical changes in FET can be evaluated with three characteristic curves: transfer curves (current vs gate bias), output curves (current vs drain–source bias), and real time response curves (current vs time) with fixed drain and gate voltages.[1] Depending on the experiment and analyte to detect, the gate electrode can be positioned differently (Figure 1). For gaseous or air sensors a "back-gate" configuration is favorited. In this architecture, the conductive bottom layer of the substrate serves as the gate electrode, which is isolated from the channel and drain–source electrodes by a dielectric layer. A "dual-gate" geometry biosensors refers to a structure that incorporates two separate gates, namely the top gate and the back gate. Both gates are used to control the flow of current through the graphene channel in the transistor and are typical of pH sensors (Figure 1).[2,53]

Whereas, when working in solution, the gate voltage can be applied either with a coplanar electrode located on the substrate, or a reference electrode immersed in the medium. These gate structures are usually referred to as "liquid gate" or "top gate."[2]

Liquid gate can effectively replicate the physiological environment where biological interactions occur naturally. Various biological molecules, including nucleic acids, proteins, drugs, and microorganisms, interact with their target bioreceptors in aqueous physiological environments (i.e., blood, serum, cerebrospinal fluid, and urine). However, FET with liquid gate configuration presents some constraints, such as bioreceptors instability and their uncontrolled orientation when attached on the surface of biosensor, which can negatively impact the accuracy and reliability of sensing results. To address these issues, we present here the combination of computational and molecular biology techniques to engineer and produce more stable and well-immobilized bioreceptors. This approach is expected to improve biosensor performance, resulting in more accurate and robust results. This could lead to the development of standardized protocols which is crucial to ensure consistent quality and reproducibility for large-scale production. Finally, considering that over the past few years FET have been commercially available (Graphenea—https://www.graphenea.com, Cardea—https://cardeabio.com), the production of easily purifiable proteins has become essential in this regard. By adopting this strategy, we open the possibility to create high-quality biosensors, paving the way for a wide range of applications in biotechnology and medical diagnostics.

3. Exploiting Computational Approaches to Tailor Bioreceptor Structure and Function

Bioreceptors usage as recognition elements in biosensors offer several advantages due to their high binding affinity, selectivity, and interfacial stability.[54] The selection, design, and application of a bioreceptor in biosensing involves a cyclical workflow (**Figure 2**). Computational design of the bioreceptor can be employed to ensure that it maintains the proper functional conformation. This step can significantly reduce the number of experiments required in later stages, helping to streamline the overall process.

The starting point in the computational design of a bioreceptor is its 3D molecular structure. The atomic coordinates of many proteins, nucleic acids, and complex assemblies are readily available in databases, such as the Protein Data Bank (PDB). However, not all bioreceptors are known. Thus, considerable computational effort has been dedicated to predict 3D proteins' architecture. After decades of efforts in developing successful homology models through classical algorithms, a revolutionary advancement was brought by the artificial intelligence (AI) system named AlphaFold2 (AF2).[55] This approach was extensively evaluated during the 14th Critical Assessment of Protein Structure Prediction (CASP14) and was the top-ranked protein structure prediction method by a large margin, producing predictions solely from the amino acid sequence, with high accuracy.[56] In addition to structures already available in databases, protein de novo design approaches can be used to create efficient bioreceptors. These type of approaches can be used to create novel and tunable protein bioreceptors from the ground up using physical principles and sequences which do not exist in nature.[57,58] Of note, a new robust deep-learning software, called ProteinMPNN (protein message-passing neural network), is broadly applicable to the design of monomers, cyclic oligomers, and protein–protein interfaces.[59] The customization capabilities of ProteinMPNN enable the generation of unique sequences that fold into protein structures with the desired function. The same group who developed ProteinMPNN, introduced RoseTTAFold (RF) Diffusion, which is based on a machine learning approaches termed denoising diffusion probabilistic models (DDPMs) and is implemented within the well-known RF software.[60] This method works by adding and removing noise, like the diffusion model used for image generation, and it is particularly well suited for protein binder and functional protein design.[61] Additionally, methods to design ligand-binding domains have been developed to devise bioreceptor for sensing small ligands. The rotamer interaction field (RIF) docking method generates billions of amino acid side chains that form hydrogen bonds with the target, and then searches for a backbone with the same geometry.[62] Other approaches leverage protein–ligand interactions that have been deposited in the PDB. These data combined with Monte-Carlo simulated annealing generate new binding sites for the ligand target, thus introducing the opportunity to design novel binders.[63]

To increase the specificity and sensitivity of the FET-biosensor, it is essential to deeply comprehend how the ligand binds to the receptor. To achieve this goal, protein–ligand docking is a valuable technique (**Figure 3**A).[64,65] Predicting the most likely way a ligand would bind to a bioreceptor, as well as the number and types of interactions established between the two agents are some of the advantages of this technique.

Recent research has demonstrated the importance of protein 3D structures in determining which region of the bioreceptor to modify for appropriate immobilization on FETs. For example, Maddalena et al.[66] mutagenized a surface-exposed glycine to a cysteine in the known structure of the sulfate binding protein (PDB ID 1SBP) (i.e., G289C) to obtain a possible anchor for chemical coupling. The alteration was carefully designed to ensure that it did not disrupt the overall protein architecture or interfere with the sulphate binding site. The modified protein was

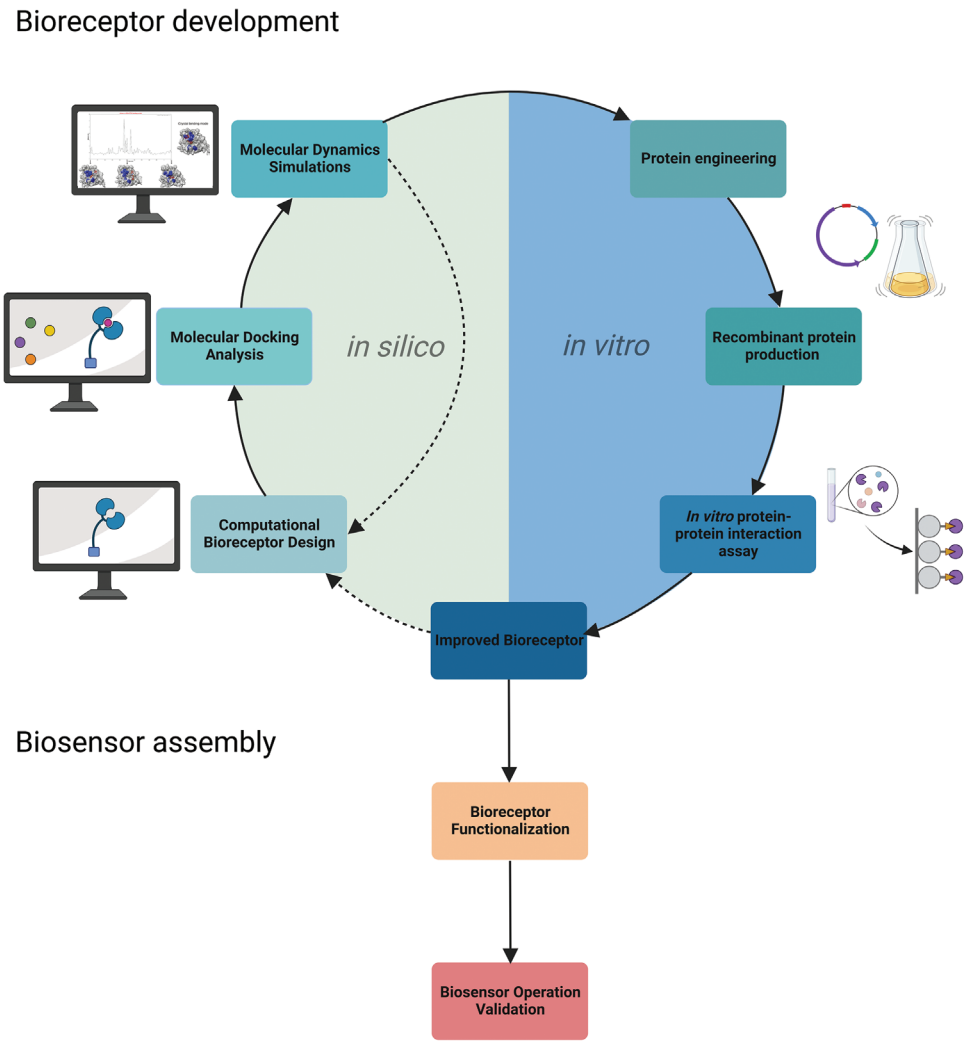

Figure 2. Schematic representation of the ideal bioreceptor development process and biosensor assembly.

then immobilized via covalent bonding of the cysteine residue to a maleimide-functionalized insulator layer of a FET to detect sulphate ions. It resulted in a well-aligned surface of properly oriented bioreceptors, each capable of effectively binding a single sulfate ion.

Even if the 3D structure of a protein receptor has been determined, it is good practice to ensure that it satisfies the environmental constraints of liquid gate FET before it can be utilized. This is a pressing issue regarding membrane receptors, which are deeply involved in signaling and consequently widely applicable in biosensing. Typically, membrane proteins do not allow for their usage in a water-soluble system without modifications. In a groundbreaking study, researchers have successfully designed soluble versions of integral membrane proteins using a deep learning pipeline called AF2seq-MPNN. The designed proteins demonstrated high thermal stability, as confirmed by biophysical analysis. Moreover, they obtained experimental structures that closely matched the predicted structures, showcasing remarkable accuracy.

This achievement opens possibilities for utilizing these proteins as novel bioreceptors in gFET applications.[67] Perez-Aguilar and colleagues have developed a protocol which exploits computational tools to modify the structure of membrane proteins to make them soluble.[29] The resulting engineered receptor was then applied to a gFET for opioid biosensor development.[68,69] The possibility of manipulating the water-soluble version of the μ-opioid receptor, a G-protein coupled receptor (GPCR), outside the membrane led to the development of a gFET biosensor with a limit of detection (LOD) of 10 pg mL^{-1} for detecting naltrexone, an opioid receptor antagonist.

Despite the knowledge of the 3D structure of a protein is of paramount importance for the rational development of an efficient bioreceptor, static structures lack the description of the dynamic behavior of the system, which is fundamental to unveil its functionality. Molecular dynamics (MD) has emerged as a useful tool for describing the motion of a system, reaching nowadays timescales up to milliseconds. This method enables the simulation of protein motion over time, driven by the laws of classical

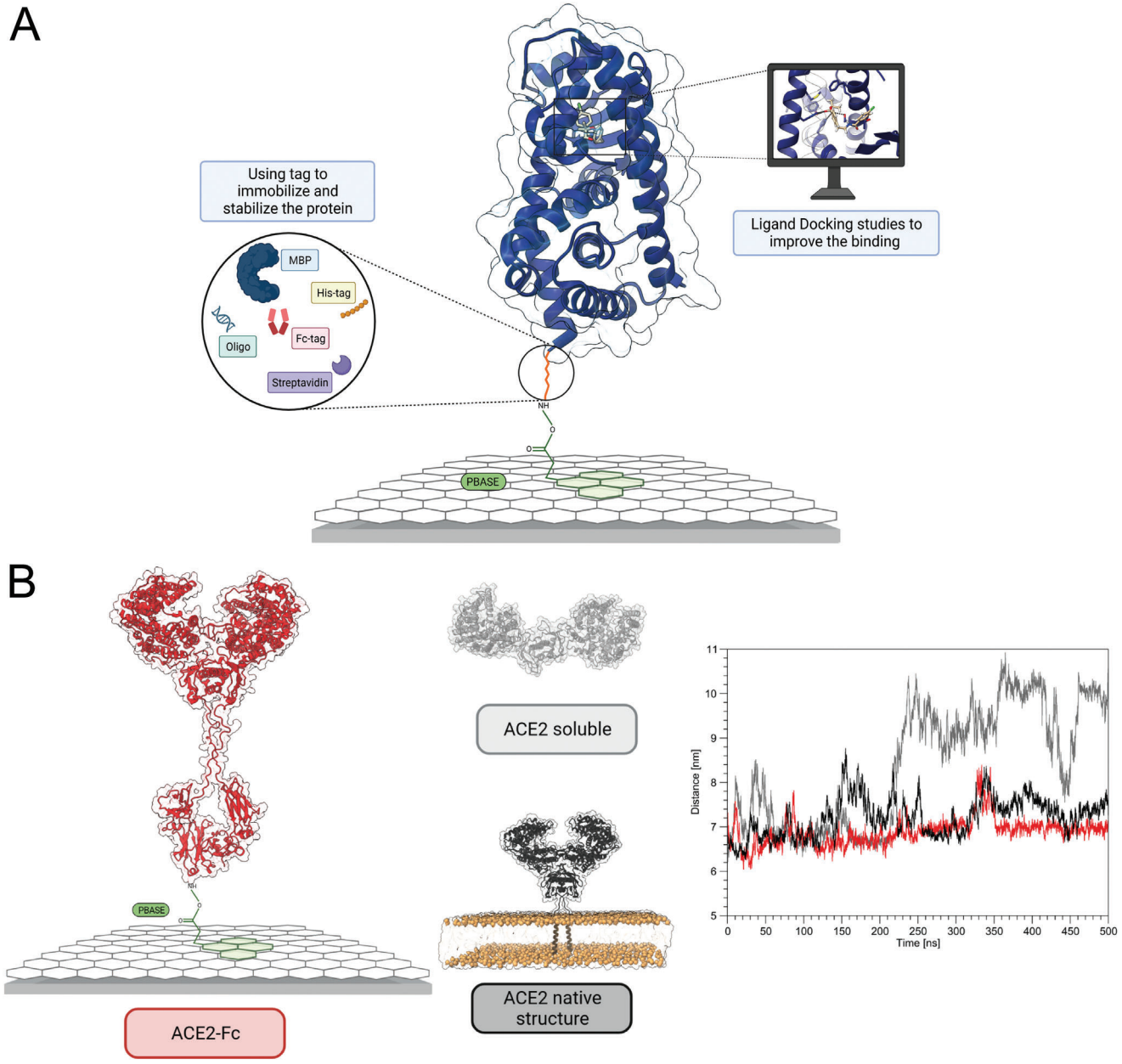

Figure 3. Illustration of the operating methodologies for improving biorecognition elements. A) Example of a generic protein immobilized on graphene sheet. X-ray structure of PPAR-γ cocrystalized with benzafibrate (PDB ID 7WGL) is represented as ribbon with transparent surface. 1-pyrenebutanoic acid succinimidyl ester (PBASE) is used as protein linker. The fusion-tags, used to increase stability and immobilization, are displayed alongside ligand docking binding studies to enhance binding proficiency. B) ACE2-Fc (red), ACE2 soluble (gray) ACE2 full length (black) oligomeric state stability was evaluated performing MD simulations. Time evolution of the intermonomer distance measured between the ACE2 peptidase domains (PD) is shown and suggest the proper form of the protein to be immobilized on the gFET. Reproduced with permission.[27] Copyright 2023, NanoToday.

mechanics. The primary benefit of MD is the potential to reveal information that lies beneath the 3D static structure. It can be employed to rank, select, identify, and assess protein designs, as well as to modulate protein stability, optimize engineered protein functional regions and predict protein folding, unfolding, and the impact of point mutations.[70–72] Furthermore, MD can be applied to multiple interacting (bio)molecules, thus allowing the simulation of the entire biosensing system (e.g., graphene, linker, protein receptor, and small ligands).[73] MD simulations can be used to improve results of docking calculations. The predominant issue with this technique is that rigid body docking restricts the conformational flexibility of the ligand and bioreceptor.[74] This is particularly problematic when the ligand binds the protein using a conformational selection model.[75] An approach to address this dilemma is ensemble docking. This method involves the generation of an "ensemble" of drug target conformations, typically generated with MD simulations.[76] Long-time MD simulations can identify unseen druggable pockets,[77,78] but enhanced sampling

methods, such as replica exchange,[79,80] parallel tempering,[81] Temperature Accelerated MD (TAMD),[82] and accelerated MD (aMD),[83,84] are more effective. Finding the most representative conformations among the ensemble can be challenging, but original clustering methods, multiensemble Markov models and machine learning techniques have been developed to address this issue.[85,86]

Another enhanced sampling method that allows to probe biomolecule's mechanical function and to simulate slow molecular processes is the steered MD (SMD).[87] By applying an external force, SMD can be employed to induce ligand unbinding and ligand conformational changes on a short timescale. In our recent paper,[27] this technique was used to assess the potential of using a selected protein as a bioreceptor. SMD was employed to estimate the force necessary to completely dissociate the Spike Protein RBD from Angiotensin Converting Enzyme 2 (ACE2) or Ab- Anti Spike. The SMD results clearly indicated that the unbinding force between the Ab-Anti Spike and the receptor was comparable, indicating that ACE2 could be successfully used as a bioreceptor in FET biosensors. Other enhanced sampling techniques, such as metadynamics[88] and umbrella sampling,[89] allow the study of such slow processes, but a detailed description of such methods have been provided elsewhere and are beyond the aim of this perspective.[90,91]

In conclusion, the use of computational structural-functional analysis has proven to be an effective approach in the development of customized bioreceptors with enhanced selectivity toward specific binding partners. This, in turn, has led to significant improvements in the performance of biosensors. Increasing use of computational tools in the study of gFETs [73,92,93] has demonstrated the predictive power of computational approaches in protein engineering for sensing applications.

4. Frontiers in Molecular Biology for Protein Engineering

The use of engineered proteins in biosensors represents a significant leap forward in the development of protein-based sensors with the ability to identify the target analyte accurately and specifically. Depending on the required changes, diverse approaches can be applied to improve the properties of the selected bioreceptor, such as mutagenesis, production of truncated proteins, addition of fusion tags, and addition of short cross-linkers (Figure 3). One technique for modifying a protein is site-direct mutagenesis, which involves introducing amino acids substitutions. Initially, this technique was mainly used to analyze and improve enzyme catalysis and stability, then it became pivotal to characterize the folding of proteins through structural–functional studies.[94] As a result, it has become increasingly widespread to apply this technique to improve all types of proteins.

Membrane receptors are essential in cellular functioning, and they are involved in many biological pathways, making these proteins intriguing in biosensing for diagnostic application.[95] However, producing membrane proteins in a soluble form is really challenging due to their complex structure and hydrophobic nature.[96] Even if they are embedded in the membrane, such as the GPCRs, some of them have an extracellular domain which is involved in the recognition of the ligand (i.e., extracellular ligand-binding domain). Perez-Aguilar and colleagues[29] demonstrated in their study that by combining computational design with protein engineering approaches, it is possible to make a previously insoluble membrane protein, such as a GPCR, soluble. The authors computationally identified and experimentally produced a water-soluble variant of MUR protein. Circular dicroism (CD), intrinsic tryptophan fluorescence, thermostability and ligand-binding studies demonstrated a native-comparable conformation of the water-soluble receptor.[29]

Recent research findings gave a concrete example of how computational protein design and mutagenesis led to the development of a nicotine-fluorescent sensor (iNicSnFR) that is nine times more sensitive compared to previously constructs. In fact, the two mutant iNicSnFR proteins have higher sensitivity to nicotine (<100 nM) in both diluted mouse and human serum, according to fluorescence assays, molecular dynamics simulations, and absorbance measurements).[30]

An alternative to amino acid substitutions is to engineer membrane proteins in order to produce only the soluble extracellular portion of the receptor, which can eventually be stabilized by the addition of a specific fusion tag. This is exactly what we did in our recent work.[27] We produced and deeply characterized an ACE2-Fc chimera by combining MD simulations and protein engineering experiments. The extracellular-soluble part of the receptor was coupled with the Fc-tag in order to promote the active oligomeric state of the protein, leading to an improved biosensor performance (Figure 3B). Through multiple measurements using the antibody anti-Spike, soluble ACE2 protein, and ACE2-Fc protein, it was consistently observed that the ACE2-Fc receptor displayed heightened sensitivity compared to other receptors. Notably, the proteins exhibited sensitivity values of 0.02, 0.2, and 20 pg mL^{-1}, respectively. Furthermore, a LOD of 65 cps mL^{-1} was successfully achieved directly from nasopharyngeal swabs.[27]

The Fc-tag consists of the constant region of the Immunoglobulin-G heavy chain, which retains a stable and well-defined structure allowing the production of a stable chimera. Fc-tag is also widely used to produce therapeutic proteins, as its use can positively affect solubility, stability, and the yield of the recombinant proteins.[97,98]

Fusion tags, including both proteins and peptides, have been utilized extensively over the years to solve various challenges related to recombinant proteins production, promoting proper protein folding.[99,100] The most commonly used protein fusion tags are maltose binding protein (MBP),[101,102] Small ubiquitin-related MOdifier (SUMO),[103] Glutathione S-Transferase (GST),[104] Thioredoxin (Trx),[105,106] N-utilization substance A (NusA),[107] and Fh8,[108] among others. In some cases, these tags facilitate the correct folding of the fusion protein, thus resulting in improved stability and solubility. However, due to their size, some of them (i.e., NusA, MBP, GST) may not be suitable for the biosensor field, as they can affect bioreceptor structure and activity. As an alternative to fusion protein tags, peptide fusion tags can be used both for enhancing protein expression and for their immobilization over specific substrates. Among them, FLAG-tag, His-tag, Strep-tag, HA-tag are the most widely employed.[107] These tags can be added to either the N- or C-terminus of a protein and, thanks to their small size (i.e., 10–15 residues), they are less likely to affect protein structure and

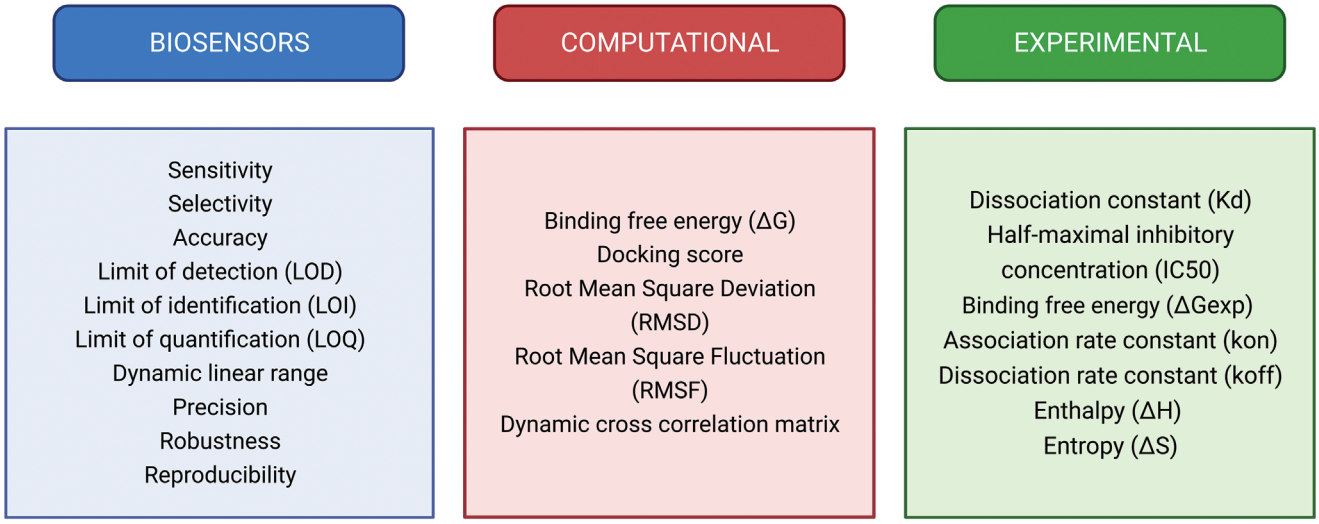

Figure 4. Figures of merit of different systems discussed. For computational and experimental approaches, we refer to the most common figures of merit used to assess protein–protein interactions from different techniques.

function. Although these tags facilitate protein production and detection, they are not as efficient as fusion protein tags in increasing protein solubility and stability.[109] However, peptide tags, are at the same time still useful. In fact, due to their intrinsic affinity for their binding partners, they can be used to immobilize the protein of interest for protein–protein or protein–peptide interaction assay in order to test their activity, and to identify potential therapeutic targets.[110,111] In vitro binding techniques, such as bio layer interferometry (BLI), surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), protein microarrays and others, through the calculation of different figures of merit, such as dissociation constant (Kd), or half-maximal inhibitory concentration (IC50) (**Figure 4**), enable the quantitative measurement, characterization, and validation of protein–protein interactions in biosensing applications.

Furthermore, in biosensors, peptide fusion-tag can be exploited to attach bioreceptors on the substrates for analyte detection (Figure 3A).

Similarly, proteins can be engineered for their proper immobilization through the addition of short cross-linker which can be performed with the novel and forefront click chemistry strategy.[112–114] It is a recently developed method which offers a reliable and robust system for protein attachment to substrate making this winsome for biosensors.[115,116] Click chemistry reactions typically involve the use of small molecules that can be easily synthesized and modified, which are able to form stable and biocompatible covalent bonds.[114] This method entails a cycloaddition reaction between an azide and an alkyne group, which generates a 1,2,3-triazole ring in presence of copper.[117] In literature, it was reported how this method can be used to get a solid binding to clickable modified substrates.[112,118] However, this procedure does not involve directly protein engineering but chemical modification techniques of molecules (i.e., addition of azide or alkyne containing groups). To do so, the site-specific insertion of non-canonical amino acids (NCAAs), which contain azide group, it might be exploited. The resulting engineered protein can be bioconjugated to an alkyne-modified oligonucleotide through click reaction.[119,120] In this way, the protein is ready to be first coupled with a complementary oligo, then immobilized over the sensing area. This leads to the production of a solid attachment of the protein of interest to the biosensor substrate using a complementary oligo, increasing its sensitivity. However, it must be considered that the resulting NCAA-modified protein needs a specific cell line with the modified translational machinery which involves a particular aminoacyl-tRNA synthetase. Hence, this can result in low expression yield.[119]

An easier alternative to the use of short cross-linker could be its direct coupling to a lysine or a cysteine residue of the protein of interest, which can be either endogenous or introduced via mutagenesis.[120] Because it contains a nucleophilic primary amine group, lysine can easily react with electrophilic reagents such as N-hydroxysuccinimidyl (NHS) esters which is largely used in the biosensing field.[121] On the other hand, lysine is very abundant in proteins, consequently exploiting this amino acid could lead to the formation of nonspecific bonds. The other choice is cysteine, a scarcely abundant amino acid, containing a thiol (SH) reactive group.[120,121] This characteristic makes cysteine an appealing tag for protein immobilization via thiol-maleimide coupling.[119] If the protein of interest does not contain cysteines by itself, it can be easily inserted via site-directed mutagenesis. The resulting cysteine-engineered protein can be coupled with a maleimide-modified DNA to form a disulfide bond.[106,122] In this way, the conjugated protein can be firmly immobilized at the specific substrate as stated above.[120] Even in this case, the ability of cysteine to form both disulfide bonds as well as noncovalent interactions must be considered as it can affect protein folding and structure. Overall, advances in molecular biology have made it possible to engineer proteins for biosensors purposes. Since each protein has unique properties, there is no universal protocol for improving bioreceptors. Therefore, the combination of computational and experimental techniques can significantly accelerate the development of effective bioreceptors for biosensing applications (Figure 2).

5. Final Considerations

The growing interest in biosensors is pushing the scientific community to increasingly optimize their performance in terms of specificity and sensitivity. With this perspective, we aim to illustrate the pivotal role of both computational and molecular biology in protein design, making it a valuable tool for the development of next-generation biosensors. Currently, protein engineering has made it feasible to modify native proteins structure into engineered ones. Several approaches have been explored, including mutagenesis, fusion tags, and the addition of short cross-linkers, all of which have been shown to enhance protein stability, solubility, orientation, and immobilization. These techniques require key information concerning structural, physicochemical, and functional properties of the protein which may be not available or completely exhaustive. Computational approaches aim to fill this lack of knowledge. Structure prediction and de novo software ensure a global description of the bioreceptor allowing its structural study. On the other hand, methods such as MD and ensemble docking can be used to deeply investigate the dynamic properties and, consequently, the functional aspects of the specific engineered proteins. Therefore, this type of analysis allows identifying the target modification thus establishing the technique to apply for bioreceptors' improvement. The choice of the bioreceptor is fundamental when it comes to evaluate sensitivity and selectivity which are two of the most important biosensing figures of merit (Figure 4). The sensitivity is the ability to sense the lowest concentration of analyte, which in the last years has been remarkably associated with the LOD.[123] The selectivity instead refers to the probe bioreceptor (e.g., antibody, aptamer, receptors) used to detect the target analyte. The probe bioreceptors span from aptamer to antibodies and receptor proteins among others (Table 1). Aptamers are small single stranded oligonucleotides designed through systemic evolution of ligands by exponential enrichment (SELEX) which can target several molecules. In recent years, they have gained a lot of attention due to their small size and stable chemical structure, but at the same time they can make nonspecific reactions because of electrostatic interactions.[123,124] Antibodies, with their Y-shaped structure, can specifically bind to their target bio-analyte, while receptors function by binding to a specific ligand molecule. Although antibodies form stable bonds with antigens, they can only recognize one epitope. The COVID-19 pandemic has highlighted the importance of utilizing receptors rather than antibodies for sensing purposes. As a matter of fact, the multiple mutations of the SARS-CoV-2 affected mainly the antibody binding, thus making antibody-based sensors less performing. In the SARS-CoV-2 detection there was a radical shift from an immuno-based biosensor to a bioreceptor one due to the constantly emerging virus variants escape antibody targeting (Table 1). As such, the combination of computational approaches, and molecular biology protocols will be essential for the design and optimization of biosensors. However, this multidisciplinary approach is not yet widely used. We assume that the concerted combination of in vitro and in silico techniques will overcome the existing difficulties in designing and producing stable and specific bioreceptors when working with liquid-gate FET.

Over the years, FETs have been characterized by an "in house" production, but recently they have also turned into commercially available products that can be purchased by companies. This development has resulted in a pressing need for the largescale production of stable proteins. As such, it is now imperative to establish standardized protocols for the production of improved recombinant proteins, first ensuring consistent quality and reproducibility, then simplifying the commercialization process.

In the not-too-distant future point-of-care devices will enable personalized medicine by identifying different markers from small volumes of biologic fluids. The advent of liquid biopsies will lead to a new era of diagnostic, and thus, novel sensing approaches are needed. We speculate that all the above-mentioned approaches will lead to an accurate real-time identification of the target, saving costs, reagents, precious time and finally, giving minimal to zero false positive or false negative. Having the potential to be compared with the existing gold standard methods, these new POC can revolutionize the diagnostic field.

Acknowledgements

The authors acknowledged funding from the Italian Ministry of Research (Project No. FISR2020IP 03475) and the Italian Foundation for Cancer Research AIRC (Project No. IG 2022 ID 27534).

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

J.R. and N.P. contributed equally to this work. D.D.M. conceived the idea. J.R., N.P., A.Ros. and A.Rom. wrote the original draft. D.D.M., A.Rom., A.L.T., S.M., and L.M. have read and corrected the manuscript. D.D.M. and A.Rom. have coordinated the work. All authors reviewed the manuscript.

Keywords

biosensors, computational biology, field-effect transistor, molecular biology, protein engineering

Received: March 23, 2023
Revised: June 20, 2023
Published online: July 19, 2023

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