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Empowering Electrochemical Biosensing through Nanostructured or Multifunctional Nucleic Acid or Peptide Biomaterials

Susana Campuzano, María Pedrero, Rodrigo Barderas, and José M. Pingarrón**

Electrochemical biosensors continue to evolve at an astonishing pace, consolidating as competitive tools for determining a wide range of targets and relentlessly strengthening their attributes in terms of sensitivity, selectivity, simplicity, response time, and antifouling ability, making them suitable for getting a foothold in real-world applications. The design and exploitation of nanostructured or multifunctional nucleic acid or peptide biomaterials are playing a determinant role in these achievements. With the aim of highlighting the potential and opportunities of these biomaterials, this perspective article critically discusses and overviews the electrochemical biosensors reported since 2019 involving nanostructured and multifunctional DNA biomaterials, multifunctional aptamers, modern peptides, and CRISPR/Cas systems. The use of these biomaterials as recognition elements, electrode modifiers (acting as linkers or creating scaffolds with antifouling properties), enzyme substrates, and labeling/carrier agents for signal amplification is discussed through rationally and strategically selected examples, concluding with a personal perspective about the challenges to be faced and future lines of action.

1. Introduction to Electrochemical Biosensors and Their Current Empowerment

It is indisputable that electrochemical biosensors continue to establish themselves as very promising analytical tools and are at the forefront of modern detection techniques. This is due to their high selectivity and sensitivity, ease of use and low cost, fast response time, and feasibility to operate at the multiplexed and multiomics level either in centralized or field settings owing to the possibility to work both with complex instruments and with simple and portable devices.[1] The great advances demonstrated by electrochemical biosensors in recent years have gone hand by hand with the development of new electrochemical substrates, attractive surface chemistries, bioassay formats, and amplification strategies, but also with the production and application of new nanomaterials and bioreceptors, which has made possible to generate superb devices capable of facing challenging applications.

In particular, the design and exploitation of nanostructured or multifunctional nucleic acid or peptide biomaterials has proven to empower the electrochemical biosensors performance in terms of sensitivity, storage stability, response time, simplicity, and robustness, making the move of these devices from the research laboratory to the marketplace does not seem like science fiction.

As discussed in more detail in the following sections, the use of state-of-the-art nanostructured or multifunctional nucleic acid or peptide biomaterials has contributed compelling answers to important challenges conditioning the performance of electrochemical biosensors, among which it is worth introducing at this point to put the reader in context:

i) Controlling the density and orientation of recognition nucleic acid or peptide probes arranged on heterogeneous surfaces, especially in comparison with the affinity reactions in homogeneous solution, particularly those employing single-stranded DNA capture probes due to their lack of stiffness, which critically conditions the sensitivity, reproducibility, and stability of the resulting biodevices.[2–4] This goal can be attained by exploiting in a rational way DNA-, aptamer-, and peptide-based nanofabrication, enabling the preparation of highly controlled and precise programmable geometries.[4]
ii) Ensuring the reliability and robustness of the analytical results and their real-world applicability by making electrochemical biosensors less susceptible to the electrode surface state, probe packing density, environment and artificial factors,

S. Campuzano, M. Pedrero, J. M. Pingarrón
Department of Analytical Chemistry
Faculty of Chemistry
Complutense University of Madrid
Madrid 28040, Spain
E-mail: susanacr@quim.ucm.es; pingarro@quim.ucm.es
R. Barderas
Chronic Disease Programme
UFIEC
Institute of Health Carlos III
Majadahonda, Madrid 28220, Spain

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/admt.202200310.

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and the nature of the samples. All this can be approached by developing dual-signal ratiometric strategies and antifouling devices.[5,6]

iii) Improving sensitivity and reducing assay time by resorting to the clever use of nanostructured or multifunctional biomaterials of nucleic acids or peptides as recognition elements, clinically relevant enzyme substrates, electrode modifiers (such as linkers and scaffolds), and labeling/transport agents. Also noteworthy is the coupling of such biomaterials with other types of nanomaterials and their use as "dispersible electrodes" capable of bringing the sensor to the analyte instead of the analyte encountering the sensor (conventional paradigm).[7]

Enlightened by the above, the purpose of this perspective article is to offer the reader an overview of the latest advances and opportunities provided by nanostructured or multifunctional biomaterials based on nucleic acids or peptides in electrochemical biosensors, with the purpose of giving the scientific community reasons to explore them in a simple and/or combined way with artificial nanomaterials. To our knowledge, this timely and appropriate topic for the research community has not yet been covered in the literature. This is done by comprehensively presenting a selection of representative recent works (mostly since 2019), being aware of the impossibility to fully cover the immense number of reported approaches. Moreover, critical, and somewhat objective views on the challenges to be met to move the field forward and the future landscape of these biodevices is given.

2. Nanostructured and Multifunctional DNA Biomaterials

DNA is an excellent self-assembly nanomaterial due to its foreseeable base pairing, high chemical stability, and convenience of synthesis and modification. These unique properties explain its widespread use in electrochemical biosensors, both as a recognition element or multifunctional biomaterial (polyA and DNAzyme probes) and/or to generate unique nanoassemblies, including nanostructures (Y-shaped, tetrahedral, origami, and dendrimers, nanostructures and nanospheres), networks, and hydrogels.[2,8]

Table 1 summarizes the main characteristics of selected representative electrochemical (bio)sensors developed mostly since 2019 involving nanostructured and multifunctional DNA biomaterials.

2.1. DNA Nanostructures

DNA nanostructures have been employed as recognition elements, electrode modifiers, and for amplification purposes. It is important to note that, although they generate DNA nanostructures to some extent, triplex DNA nanostructures and electrochemical biosensors using proximity ligation assays (PLA)[38,39] are considered outside the main scope of this perspective article. The PLA strategy is based on DNA ligation of two adjacent probe oligonucleotides by bivalent binding. PLA has been shown to be functional with both aptamer and antibody pairs and has demonstrated potential in ÷ ÷ for detection of both genetic and protein targets.[40] This strategy has undergone several modifications to overcome its limitations, among which, the proximity extension assay should be highlighted. It being based on the extension of hybridizing oligonucleotides with DNA polymerase, has been shown to improve efficiency compared to PLA in biological samples such as plasma.[41]

2.1.1. Y-Shape, Tetrahedral, Origami, and Dendrimers Nanostructures

Y-shape DNA nanostructures, composed of three oligonucleotides, which partially hybridize to each other, have been shown to impart interesting properties to electrochemical biosensors.[17] The use of a "Y" shaped structure allows the probe to be stably fixed at the electrode surface and keeps it upright at the interface, this allowing a higher recognition efficiency compared to straight ones, particularly for long targets, also minimizing non-specific adsorptions.[5,42]

Y-shape DNA nanostructures have been exploited in developing biosensors for the determination of relevant nucleic acids (DNAs, miRNAs, lncRNAs, and mRNAs) achieving LODs at the fM level for short DNAs[19] and RNAs[5,17,16] and pM for the determination of long RNAs.[18] These biosensors were applied to the analysis of serum or total RNA samples extracted from cells, exosomes, and urine. Importantly, the high sensitivity exhibited was reached by combining these Y-shape nanostructures with amplification strategies such as mismatched catalytic hairpin assembly (CHA),[16] non-linear hybridization chain reaction (HCR),[17] assisted strand displacement reaction,[5] and CHA and terminal deoxynucleotidyl transferase (TdT) catalysis.[19] It is also noteworthy that two of these biosensors employed ratiometric readout.[5,16]

Figure 1 shows the electrochemical biosensor schemes based on the use of Y-shape DNA nanostructures as electrode modifiers for the determination of a short RNA (Figure 1a) and two types of longer RNA: lncRNA and mRNA (Figure 1b).[16,18]

The use of tetrahedral and origami DNA nanostructures in electrochemical biosensors has been proposed as efficient alternatives to improve the recognition capability of surface-confined heterogeneous DNA probes due to control of surface chemistry, conformation, and packing density of probe molecules, and surface size and geometry.[2]

The introduction of tetrahedral DNA nanostructures (TDNs, also known as tetrahedral tripods (TTs) or tetrahedral-structured probes (TSPs)) in electrochemical biosensors allows controlling the spacing and density of the immobilized probe, improving the stability of the biosensor (three anchor points instead of the single point of the single-stranded DNA capture probes) and the target accessibility, as well as imparting the biodevice with antifouling properties.[2,3,9,10] The TDNs scaffolds are easily, rapid, and reproducibly formed by a single-step self-assembly of four probes (three of them thiolated) onto gold surfaces, without requiring the use of mercaptohexanol (MCH) to lift the capture probe and prevent nonspecific adsorptions.[2,43,44] They exhibit an excellent mechanical rigidity and structural stability, imparting stability to the detection process, and have demonstrated to greatly improve the sensitivity, selectivity, and storage stability of the resultant electrochemical biosensors.[2–4] It is important to note that the TSP-modified surfaces are fully compatible with

Table 1. Representative recent electrochemical (bio)sensors exploiting the use of nanostructured and multifunctional DNA biomaterials.

Electrode	Fundamentals	Biomaterial	Detection technique	Target analytes	LR/LOD	Sample/application	Remarkable features	Ref.
DNA nanostructures: Tetrahedral, origami, dendrimers, Y-shape nanostructures, and nanospheres								
AuE	TSPs comprising a sequence complementary to the target miRNA and a G-quadruplex sequence. In the presence of the target miRNA the DSN hydrolyzed the DNA in the formed DNA-RNA heteroduplexes	TSPs + DNAzyme	DPV (Hemin/H_2O_2/L-cysteine) Signal-off	miRNA-21	0.1 fM–0.1 pM/0.04 fM	Spiked human serum	Ultrasensitive due to triple signal Amplification: TSPs + DSN + G-quadruplex–hemin conformation	[3]
AuE	TSPs in combination with proximity dependent surface hybridization and redox reporter modified probes	TSPs + PLA	DPV (Fc) Signal-on	DNA	1 fM–10 pM/0.2 fM	Spiked bovine serum and Listeria monocytogenes extraction solution	Label-free	[4]
GCE modified with AuNPs and NP-rGO	TSPs in combination with RCA	TSPs	DPV (HRP/H_2O_2/HQ) Signal-on	Thrombin	1×10^{-13}–1×10^{-7} M/3.53×10^{-14} M	Spiked human serum	–	[9]
AuNPs-SPEs	Sandwich hybridization assay in connection with AuNPs decorated with reporter probes and HRP for amplification purposes	TSPs	Amperometry (TMB)	BRCA1	1 fM–1 nM/0.1 fM	–	–	[10]
AuE	Use of TTs and MTHsA	TSPs	DPV (RuHex) Signal-on	DNA, microRNA and methylated DNA	DNA: 1 aM–100 pM/0.59 aM	Spiked human serum	Ultrasensitive	[11]
ITO	Dual aptamer-modified Fe_3O_4@SiO_2 and hairpins functionalized TSPs in combination with hyperbranched HCR	TSPs	DPV ($[Ru(NH_3)_6]^{3+}$/$[Fe(CN)_6]^{3-}$) Ru^{3+}: Signal-off Fe^{3+}: Signal-on	Exosomes	5×10^3 particles mL^{-1}	Serum samples from breast cancer patients	Ratiometric	[12]
AuE	Direct hybridization of the target miRNA at DNA origami probes immobilized on the WE via electrostatic adsorption onto a chitosan film	DNA origami nanostructures	DPV (MB) Signal-on	miRNA-21	0.1 pM–10.0 nM/79.8 fM	Spiked human serum	Label-free, amplification-free	[13]
AuE	Displacement assay based on direct hybridization of the target DNA at redox reporter modified, surface bound triangle origami receptors	DNA origami nanostructures	DPV (MB) Signal-off	Target DNA	–	–	Label-free, amplification-free	[14]
GCE	PLCHA, PtNPs-functionalized single strands and functional DNA dendrimers which remarkably facilitates the entrapping of MnTMPyP.	DNA dendrimers	SWV (H_2O_2/4-CN) Signal-on	Thrombin	1 fM–100 nM/10.7 aM	Spiked human serum	–	[8]
GCE	Sandwich immunoassay using a cDNA-labeled secondary antibody, a couple of complementary Y-shaped DNAs, and a DNA dendrimer for high loading of MB molecules	DNA dendrimers and Y-shape nanostructures	SWV (MB) Signal-on	PSA	1 pg mL^{-1}–10 ng mL^{-1}/0.26 pg mL^{-1}	Serum samples	–	[15]
AuE	Mismatched CHA amplification, Fc- and MB-tagged hairpin probes, and formation of "Y"-shaped DNA complexes	Y-shape nanostructures	DPV (MB, Fc) Fc: Signal-on; MB: Signal-off	miRNA-21	5 fM–0.1 nM/1.1 fM	RNA_t extracted from cells	Ratiometric readout, self-calibrating	[16]

Table 1. Continued.

Electrode	Fundamentals	Biomaterial	Detection technique	Target analytes	LR/LOD	Sample/application	Remarkable features	Ref.
AuE	Y-shape probes and HCR	Y-shape nanostructures	DPV $(Fe(CN)_6]^{-3/-4}$ Signal-off	miRNA-25	1 fM–10 pM/0.3334 fM	Spiked human serum	Label-free Discrimination of single base mutations	[17]
PLLy/GCE	Direct hybridization, Fc- and MB-tagged probes and assisted strand displacement reaction with a LNA-modified "Y" shape-like structure	Y-shape nanostructures	DPV (MB, Fc) Fc: Signal-off; MB: Signal-on	Exosomal miRNA-21	10–70 fM/2.3 fM	RNA_t extracted from exosomes	Ratiometric readout, regenerative	[5]
Au-SPEs	Sandwich hybridization assay using Y-shaped capture probes and multiple 6-FAM-tagged reported probes	Y-shape nanostructures	Amperometry (HRP/TMB/H_2O_2) Signal-on	PCA3 lncRNA and PSA mRNA	PCA3 lncRNA: 25 pM–10 nM/4.4 pM PSA mRNA: 25 pM–1 nM/1.5 pM	RNA_t extracted from cells and urine	Dual determination	[18]
AuE	Direct hybridization, CHA, and TdT catalysis	Y-shape nanostructures	DPV (MB) Signal-on	HPV16 oncogene	2 fM–10 pM/0.19 fM	Spiked human serum	–	[19]
TiO_2-modified GCE	The target-related ternary "Y" structure cleavage cycling reaction trigger RCA to in situ form PDA^+-decorated multifunctional DNA spheres on the TiO_2 substrate	DNA nanospheres	DPV (PDA^+) Signal-on	miRNA-141	PEC: 0.1 fM–1 nM EC: 2 fM–500 pM	–	Dual PEC and EC detection mode	[20]
DNA networks, hydrogels, and PLA assays								
AuE	MB-DNA-Au@MNPs	DNA network "dispersible electrode"	SWV (MB) Signal-on	miRNA-21	10 aM–1 nM	RNA_t extracted from cancer cells, raw serum, and 50% blood	30 min assay time. Ultrasensitive. 8 orders of magnitude linear range. Detection in unprocessed blood samples.	[21]
AuE	MB-DNA-Au@MNPs	DNA network "dispersible electrode"	SWV (MB) Signal-on	ctDNA	22 nts DNA target: 2 aM–20 nM/3.3 aM 101 nts ctDNA target indicative of NSCLC: 200 aM–20 nM/5 fM	Spiked undiluted human plasma and 50% whole human blood	Detect short- and long strand DNA targets. 20 min response time. Robustness in complex biological media.	[22]
AuE	Elongation of a specific primer and use SA-biotin-DNA-biotin networks for amplification	DNA network	EIS $(Fe(CN)_6]^{-3/-4}$ Signal-on	Telomerase	50–5000 cell mL^{-1}/2 cells	Cells	PCR-free and enzyme-free. Feasibility to perform telomerase inhibition studies.	[23]
AuE	Proximity binding-induced DNA network assembled by non-linear HCR	DNA network	DPV (MB) Signal-on	Thrombin	1.0 pM–1.0 nM/0.56 pM	Spiked human serum	Enzyme-free and ultrasensitive	[24]
AuNPs/GCE	Target-induced DNA hydrogel formation with pH-stimulated signal amplification	DNA hydrogel	EIS $(Fe(CN)_6]^{-3/-4}$ Signal-on	HPA	0.01 pg mL^{-1}–20 ng mL^{-1}/0.003 pg mL^{-1}	Spiked human serum	–	[25]
AuNPs/GCE	DNA hydrogel by coupling with DNAzyme-assisted target recycling and HCR	DNA hydrogel	EIS $(Fe(CN)_6]^{-3/-4}$ Signal-on	Hg^{2+}	0.1 pM–10 nM/0.042 pM	Spiked tap water samples	–	[26]
ITO	DNA hydrogel to enwrap enzymes (HRP, BOD)	DNA hydrogel (pure)	CV (H_2O_2/TH)	H_2O_2	30 nM–100 μM/10 nM	–	Regeneration and reusability. Feasible colorimetric and electrochemical detection	[27]

Table 1. Continued.

Electrode	Fundamentals	Biomaterial	Detection technique	Target analytes	LR/LOD	Sample/application	Remarkable features	Ref.
ITO/PET	DNA hydrogel and Fc-tagged probes	DNA hydrogel (Hybrid)	DPV (Fc) Signal-off	miRNA-21	10 пм–50 μм/5 пм	–	Good selectivity and storage stability	[28]
ITO	DNA hydrogel with TBO and G-quadruplex/hemin DNAzyme	DNA hydrogel (Pure)	CV (H_2O_2) Signal-on	H_2O_2	0.2–80 μм/0.15 μм	–	Anti-interference ability and reconstruction ability	[29]
Multifunctional DNA probes								
16-channel AuE chip	Sandwich hybridization assays using a polyA-capture probe and two biotin-labeled reporter probes	PolyA-probes	Chronoamperometry (HRP/TMB/H_2O_2) Signal-on	Five bacterial 16S rRNA (St, En, Es, Ps, and Ci)	St: 10 fм–1 пм/5 fм	*Staphylococcus aureus* genomic DNA	Reusability. Multiplexed system.	[30]
AuE	Direct hybridization at a triblock DNA capture probe (PAP)	PolyA-probes	Chronoamperometry (HRP/TMB/H_2O_2) Signal-on	DNA	10 fм–1 пм/10 fм	Asymmetric PCR amplicon from *E. coli* genomic DNA	Label-free. Discrimination of single nucleotide polymorphisms	[31]
AuE	Sandwich hybridization using a polyA-capture probe, 12 reporter probes, and 2 spacers	PolyA-probes	Chronoamperometry (HRP/TMB/H_2O_2) Signal-on	Transgene-derived long RNA (RNA_i GMO)	Long stem-loop RNA: 1 рм–300 пм/1 рм (10 ам, for a 379 bp dsDNA RT-RPA amplicon)	RNA from RNA_i-based transgenic maize leaves	Detect long RNAs	[32]
AuE	Strategy combining DNA walker (three legs DNAzyme spiders) with PLA	DNAzymes	SWV (Fc) Signal-on	Telomerase	25–2000 cells/10	Hela cells	Enzyme-free, PCR-free	[33]
GCE	Bifunctional DNAzyme nanodevice with two detection paths toward the same target	DNAzymes	DPV (MB, Fc) MB: Signal-on; Fc: Signal-off	Hg^{2+}	0.1 рм–200 пм/23 fм	Spiked tap water	Ratiometric	[34]
CNTs-SPE	Isothermal signal amplification via the polymerization/nicking reaction and DNAzyme-catalyzed cleavage of Fc-DNA from PES (prepared by assembling Fc-DNA functionalized paper and CNTs-SPE)	DNAzymes	DPV (Fc) Signal-on	miRNA-21, ALP, and CEA	miRNA-21: 1 fм–1 μм ALP: 1–10^5 mU L^{-1} CEA: 1–1500 fg mL^{-1}	miRNA-21: Spiked human serum	PES zero-background current. Universal potential for POC diagnosis in resource-limited settings	[35]
Nanostructured AuE	Electroactive RNA-cleaving DNAzymes (e-RCDs) integrated into a two-channel electrical chip with nanostructured electrodes	DNAzymes	DPV (MB) Capture channel: Signal-on; release channel: Signal-off	*E. coli*	10 CFU	Urine samples	Differential electrochemical signaling. Analysis time < 1 h. Reagent-free	[36]
SPE	Zr-MOFs-modified paper and aptamer as the recognition system and HCR and DNAzyme-mediated catalysis for signal amplification	DNAzymes	DPV (TMB) Signal-on	Exosomes	5×10^3 particles mL^{-1}	Spiked FBS	Label-free Enzyme-free Paper-based biosensor	[37]

ALP: Alkaline phosphatase; AuE: gold electrode; AuNPs: gold nanoparticles; BOD: bilirubin oxidase; CEA: carcinoembryonic antigen; CFU: colony forming units; CHA: catalytic hairpin assembly; Ci: Citrobacter f reundii; 4-CN: 4-chloro-1-naphthol; CNTs: carbon nanotubes; ctDNA: circulating tumor DNA; CV: cyclic voltammetry; DNA-Au@MNPs: network of probe DNA modified gold-coated magnetic nanoparticles; DPV: differential pulse voltammetry; DSN: duplex-specific nuclease; EC: electrochemical; EIS: electrochemical impedance spectroscopy; En: *Enterococcus faecalis*; e-RCDs: electroactive RNA-cleaving DNAzymes; Es: *Escherichia coli*; 6-FAM: 6-carboxyfluorescein; FBS: fetal bovine serum; Fc: ferrocene; GCE: glassy carbon electrode; GMO: genetically modified organism; HCR: hybridization chain reaction; HPA: heparanase; HPV16: human papillomavirus 16; HQ: hydroquinone; HRP: horseradish peroxidase; ITO: indium tin oxide; ITO/PET: indium tin oxide/polyethylene terephthalate; LNA: locked nucleic acid; LOD: limit of detection; LR: linear range; MB: methylene blue; MnTMPyP: manganese(III) meso-tetrakis (*N*-methyl-pyridinium-2-yl)-porphyrin; MTHsA: multiple tandem hairpins assembly; NP-rGO: N- and P-co-doped Graphene; NSCLC: non-small-cell lung cancer; nts: nucleotides; PAP: probe–PolyA–probe; PCA3: prostate cancer antigen 3; PCR: polymerase chain reaction; PDA^+: *N,N*-bis(2-(trimethylammonium iodide)propylene)-perylene-3,4,9,10-tetracarboxydiimide; PEC: photoelectrochemical; PES: paper-based electrochemical sensor; PLA: proximity ligation assay; PLCHA: proximity ligation-responsive catalytic hairpin assembly; PLLy: polylysine membrane; POC: point of care; Ps: *Pseudomonas aeruginosa*; PSA: prostate specific antigen; PtNPs: Pt nanoparticles; RCA: rolling circle amplification; RNA_i: RNA interference; RNA_t: total RNA; RT-RPA: reverse transcription recombinase polymerase amplification; RuHex: hexaammineruthenium(III) chloride; SA: streptavidin; SPE: screen-printed electrode; St: *Staphylococcus aureus*; SWV: square wave voltammetry; TBO: toluidine blue O; TdT: terminal deoxynucleotidyl transferase; TH: thionine; TMB: 3,3′,5,5′-tetramethylbenzidine; TSPs: DNA tetrahedron-structured probes; TTs: tetrahedral tripods; WE: working electrode.

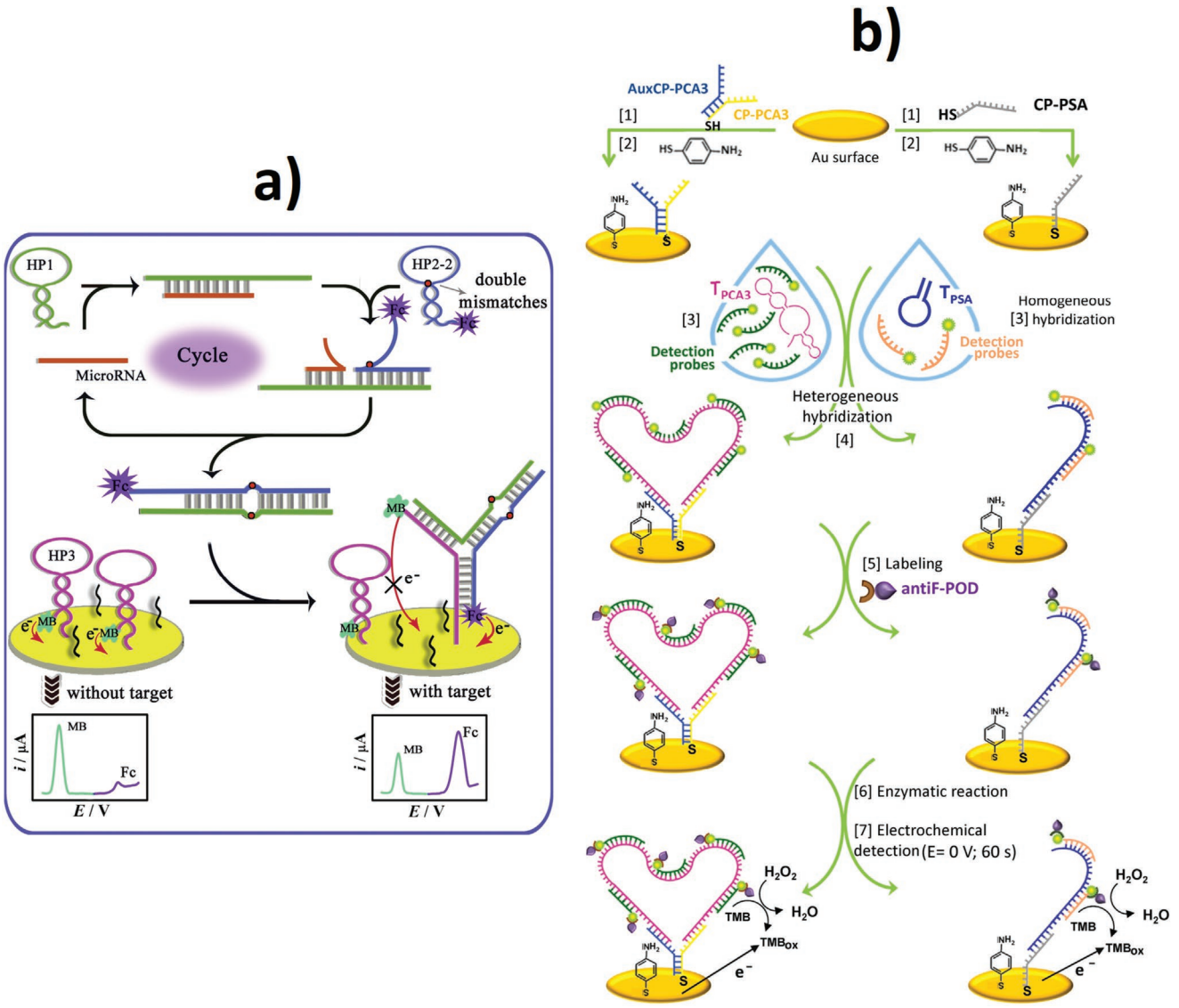

Figure 1. Y-shape DNA nanostructure-based electrochemical biosensors for the determination of a) miRNA-21 and b) PCA3 (lncRNA) and PSA mRNA. (a) Reproduced with permission.[16] Copyright 2019, Elsevier; (b) Reproduced with permission.[18] Copyright 2021, Elsevier.

electrochemical interrogation. Despite the relative thickness of the TSP layer (≈6 nm), its hollow structure allows easy diffusion of the electroactive species to the modified electrode surface.[2]

It is important to note that although they have been much less used than as electrode modifiers, TSPs have also been exploited for amplification purposes by taking advantage of the six edges they have to bind numerous electroactive molecules.[12]

Table 1 includes the characteristics of some recently reported TDNs-based electrochemical biosensors for determining a broad range of molecules (proteins, DNAs, miRNAs, methylated DNAs, and tumor exosomes). Importantly, these strategies combine the use of these DNA nanostructures with that of nanomaterials, DNAzymes, and other amplification strategies (PLA, duplex-specific nuclease (DSN), rolling circle amplification (RCA), multiple tandem hairpins assembly (MTHsA), and HCR) to achieve sensitivities at the aM–fM level of nucleic acids, fM of proteins, and 10^4 exosomes mL^{-1}.

A representative example is the electrochemical biosensor reported by Lu et al. for the determination of miRNA-21.[3] As can be seen in **Figure 2**a), the authors designed and used as electrode modifiers TDNs composed of a sequence able to recognize the target miRNA and a G-quadruplex sequence that, combined with hemin, mimicked peroxidase activity for H_2O_2 reduction and L-cysteine oxidation. A DSN hydrolyzed the DNA–RNA heterohybrids generated in the presence of the target miRNA releasing this (which can act in a next cycle) and the G-quadruplex sequence, which resulted in a decrease in the differential pulse voltammetric (DPV) response proportional to the concentration of the target miRNA. The biosensor achieved a LOD of 0.04 fM, good selectivity even for a single-base-mismatched sequence and was applied to determine the target miRNA in serum samples from breast-cancer patients providing results in line with those obtained by qRT-PCR.

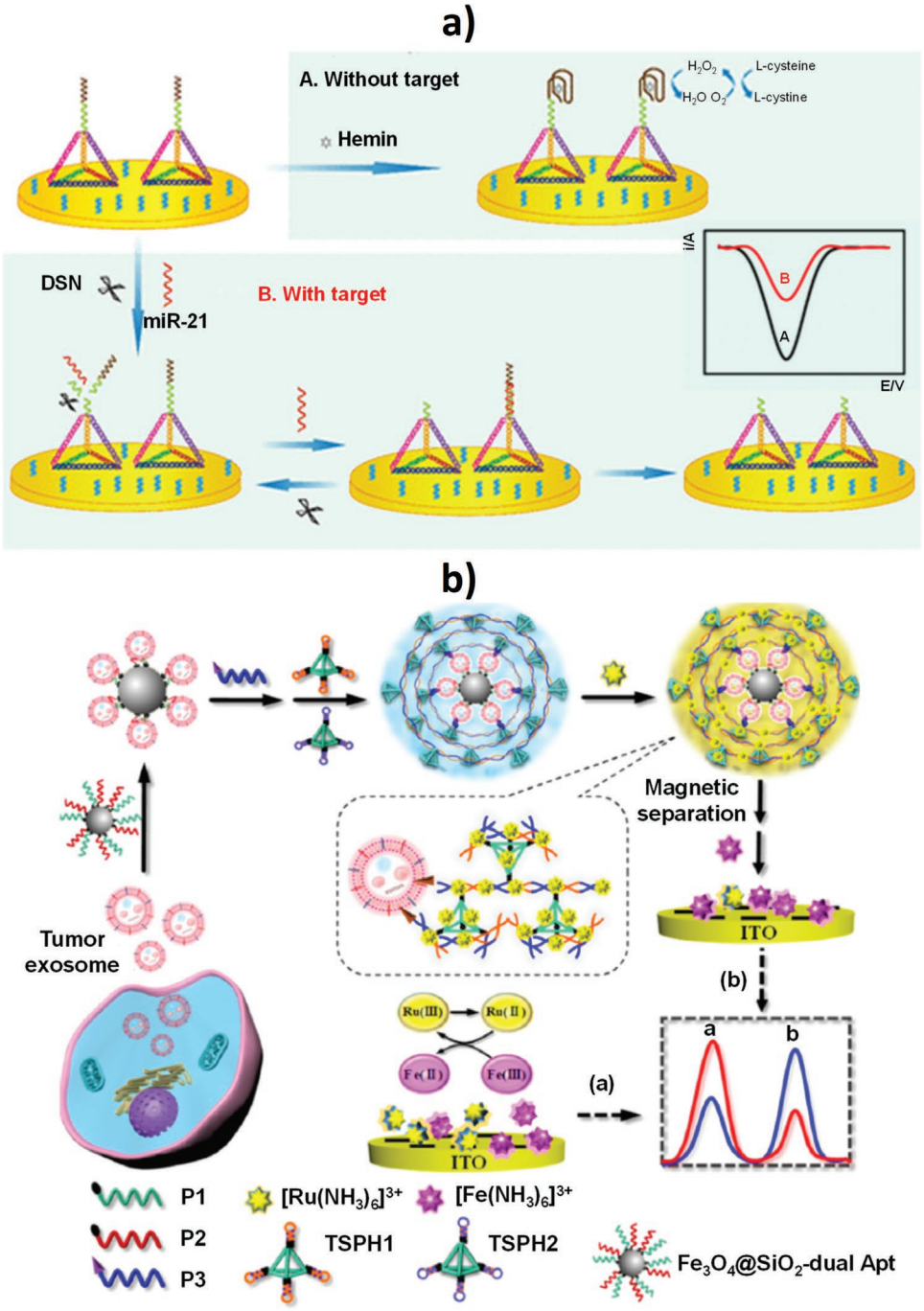

Figure 2. Electrochemical biosensors for the determination of a) miRNA-21 and b) exosomes based on the use of TDNs coupled with DSN (a) and HCR (b) as electrode modifiers and for amplification purposes, respectively. (a) Adapted with permission.[3] Copyright 2019, Elsevier; (b) Reproduced with permission.[12] Copyright 2021, ACS.

On the other hand, Figure 2b illustrates the ratiometric strategy proposed by Yang et al. for the highly sensitive determination of tumor exosomes by using dual aptamer-modified Fe_3O_4@SiO_2 and hairpins functionalized TSPs in combination with hyperbranched HCR for amplification purposes.[12] In connection with CD63 and mucin 1 (MUC1)-specific aptamers this strategy allowed the detection of as low as 3×10^4 particles mL^{-1} and differentiation of breast cancer patients from healthy individuals.

In recent years DNA origami nanostructures have aroused great interest due to their diverse structural engineering capabilities, large surface area, and unprecedented customization to precisely organizing the target binding sites at the nanoscale. Similarly to TDNs, this type of nanostructures allows the immobilization of multiple single-stranded DNA probes at desired locations thus increasing their accessibility and recognition efficiency due to the rational controlled density of DNA

probes. However, DNA origami nanostructures avoid the use of thiolated probes and, because of their larger surface area, allow the immobilization of a larger number of probes compared to TDNs.[13,14] Nevertheless, to date the use of DNA origami nanostructures in electrochemical biosensors is much smaller than that of TDNs.

An illustrative example of using DNA origami as electrode modifier is the method proposed by Han et al. reporting a label- and amplification-free electrochemical biosensor for miRNA-21 by physically adsorbing cross-shaped DNA origami nanostructures containing protruding ssDNA probes on a chitosan film and using methylene blue (MB) as a redox indicator of the hybridization process (**Figure 3**).[13] The biosensor attained a LOD as low as 79.8 fM, was able to discriminate single-base mismatched sequences, and exhibited satisfactory performance in spiked human serum samples.

DNA dendrimers (DNADS) are highly branched nanostructures formed by sequential complementary hybridization of pre-designed DNA components that can be easily employed to stably anchor a large number of signal amplifiers (small molecules, biomolecules, or metal nanoparticles) allowing amplification of the electrochemical response, improving the sensitivity and extending the linearity range of the resulting electrochemical biosensors.[8,15]

For instance, Liao et al. reported an aptasensor for the sensitive determination of thrombin where they resorted to proximity ligation CHA (PLCHA) to guide the construction of DNADS trapping Pt nanoparticles (PtNPs) and manganese(III) meso-tetrakis(*N*-methyl-pyridinium-2-yl)-porphyrin (MnTMPyP) with pseudoperoxidase activity, they acting as synergistic enhancers of the 4-chloro-1-naphthol (4-CN) oxidation in the presence of H_2O_2 (**Figure 4**a).[8]

Another DNADS self-assembled by hybridization of a pair of complementary oligonucleotides, covalently conjugated to three arms of a Y-shaped crosslinker, tris(2-maleimidoethyl)amine, was employed to amplify the electrochemical signal of an immunosensor for the determination of PSA.[15] As Figure 4b shows, this immunosensor involved a sandwich immunoassay format and the use of a complementary oligonucleotide (cDNA)-labeled detection antibody to bind the DNA dendrimer acting as carrier to load multiple electroactive MB molecules leading to the amplification of the electrochemical response. It is important to highlight the amazing sensitivity of these electrochemical biosensors with LOD values of 10.7 aM and 0.26 pg mL^{-1} for the determination of thrombin and PSA, respectively.[8,15]

2.1.2. Nanospheres

An illustrative work of these DNA nanostructures is the dual photoelectrochemical and electrochemical detection carried out with the biosensor developed by Deng et al. for miRNA-141 determination.[20] The method involved the in situ generation of cationic *N*,*N*-bis(2-(trimethylammonium iodide)

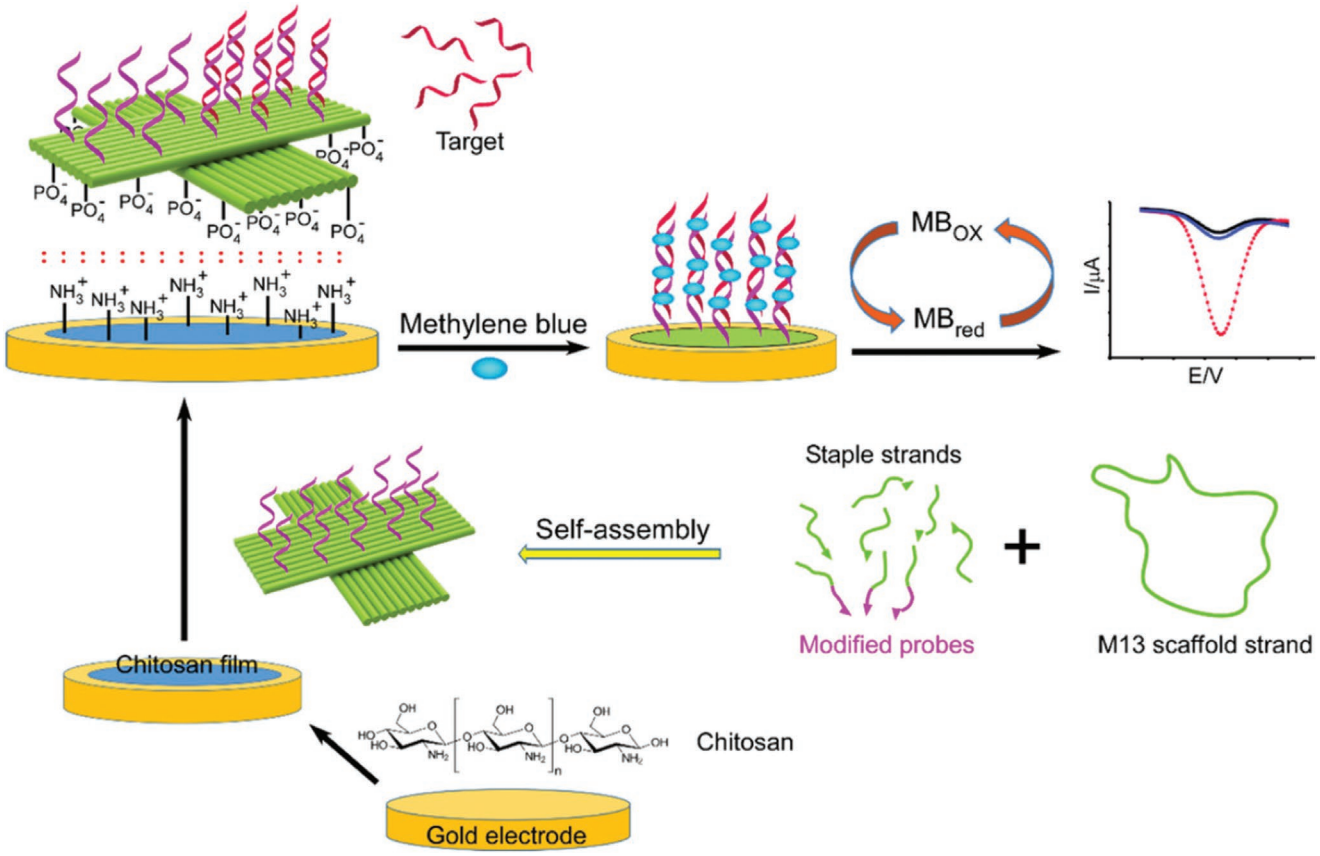

Figure 3. Electrochemical biosensor for the determination of miRNA-21 based on the use of a gold electrode modified with cross-shaped DNA origami nanostructures. Reproduced with permission.[13] Copyright 2019, ACS.

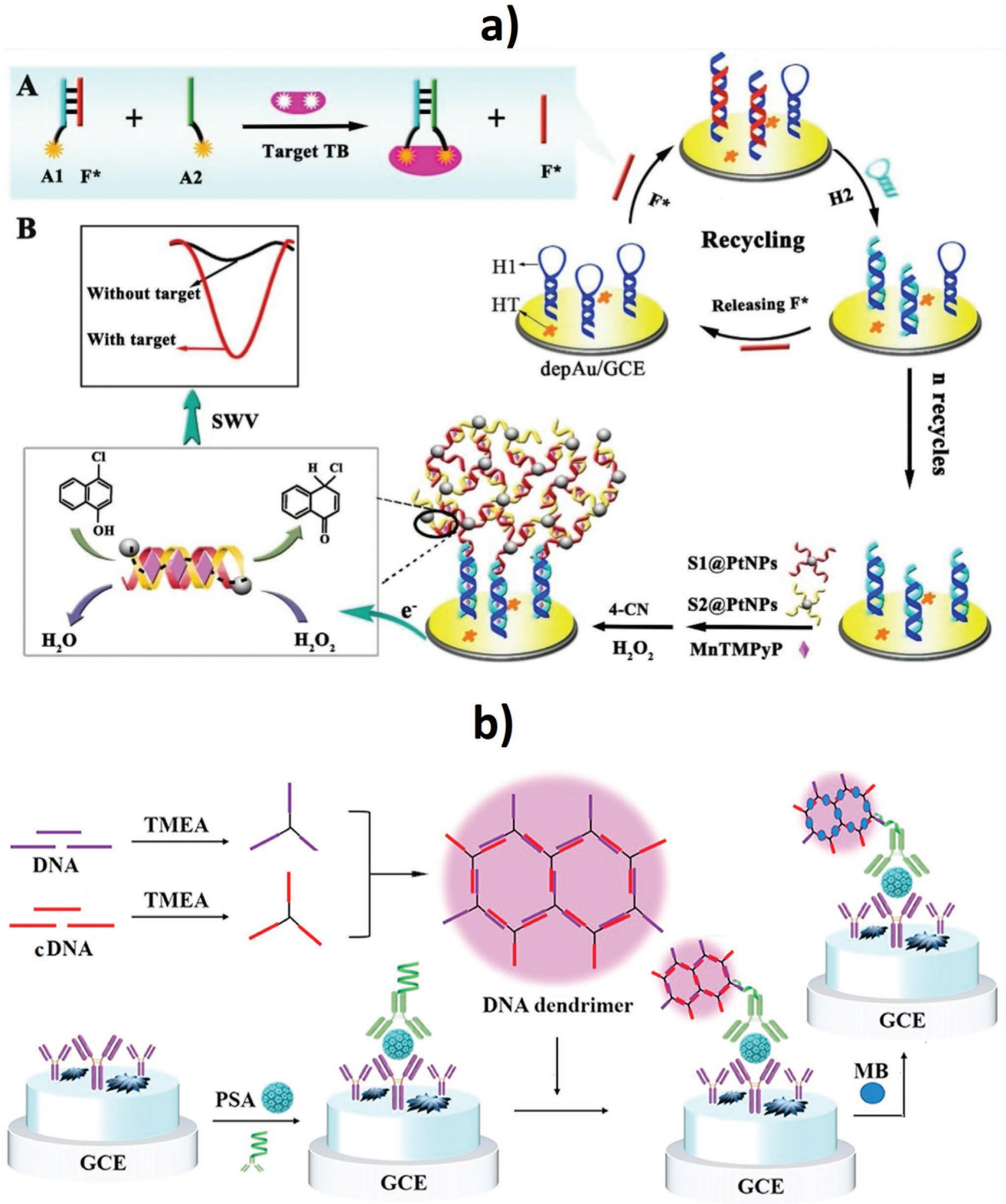

Figure 4. Electrochemical aptasensor and immunosensor for the determination of a) thrombin and b) PSA exploiting the use of DNADS to attach a large number of PtNPs + MnTMPyP (a) or MB molecules (b), respectively, for signal amplification purposes. (a) Reproduced with permission.[8] Copyright 2020, Elsevier; (b) Reproduced with permission.[15] Copyright 2021, Elsevier.

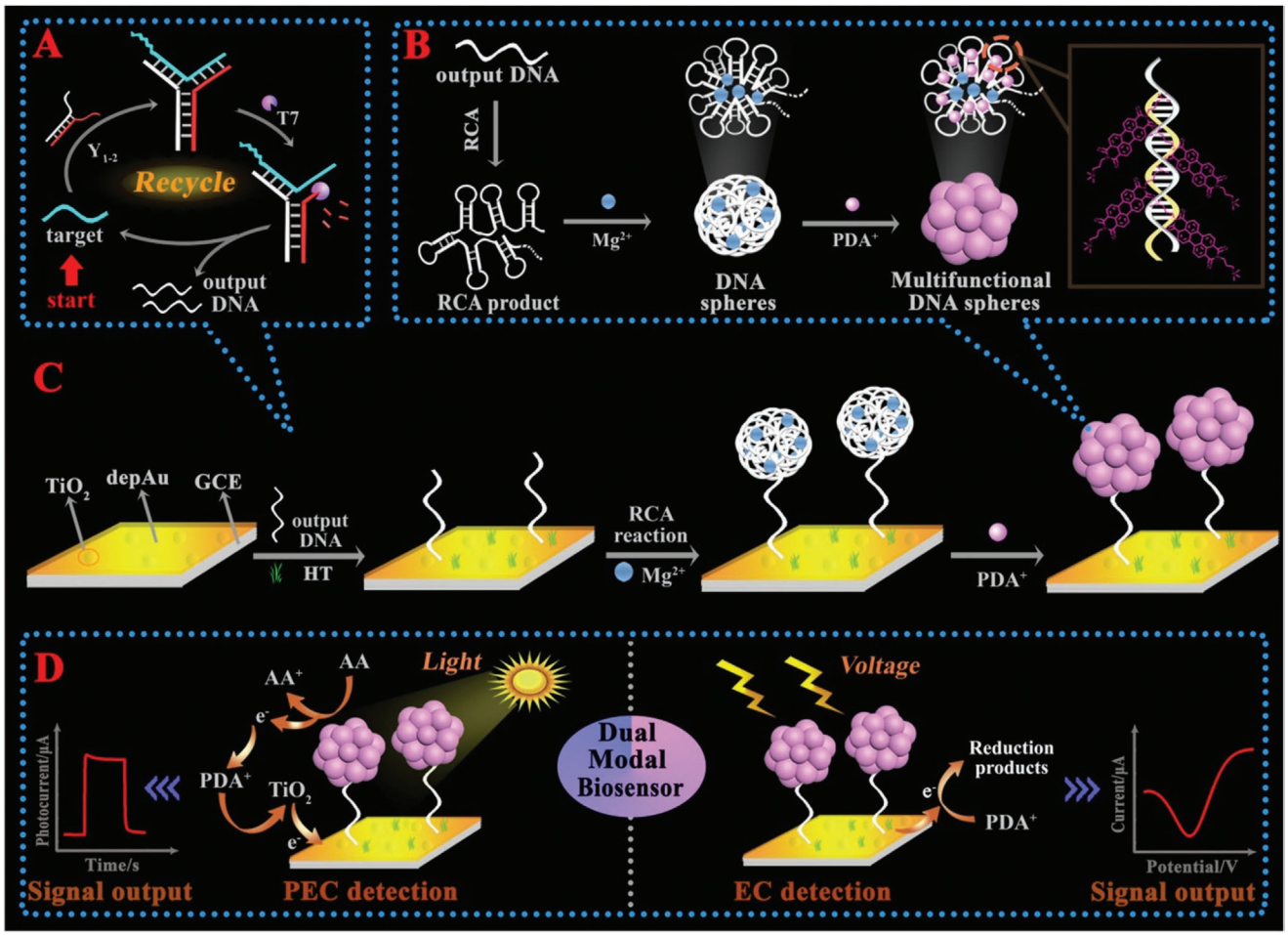

Figure 5. Electrochemical and photoelectrochemical-dual mode biosensor for the determination of miRNA-141 by utilizing multifunctional DNA nanospheres and cleavage cycling amplification and RCA strategies. Reproduced with permission.[20] Copyright 2020, Elsevier.

propylene)-perylene-3,4,9,10-tetracarboxydiimide (PDA^+)-decorated multifunctional DNA nanospheres on the electrode surface. In this bioplatform a target-related ternary "Y" structure cleavage cycling reaction converted the target DNA into massive output DNA anchored on a TiO_2 substrate, which triggers an RCA reaction. The long DNA tails of the RCA product condensed in situ in the presence of Mg^{2+} and PDA^+, forming the multifunctional DNA spheres (**Figure 5**). This biosensor displayed linearity between 2 fM and 500 pM.

2.2. DNA Networks and Hydrogels

The use of DNA networks and hydrogels has shown to be particularly interesting to empower the sensitivity of electrochemical biosensors.

DNA networks have been exploited to improve sensitivity and reduce assay time through the concept of using conductive gold-coated magnetic nanoparticles as "dispersible electrodes." as proposed by Gooding´s Group,[7,21,22,45] and for amplification using more conventional electrochemical biosensor formats.[23,24]

The key to "dispersible electrodes" is the use of Au-coated MNPs (Au@MNPs), modified with the appropriate receptor to selectively recognize the analyte, combining the ability to move the NPs with a magnetic field with electrical conductivity. This strategy showed a 1000-fold improvement in both detection limit and response time compared to the same sensing elements in macroscopic and planar electrochemical sensors.[7]

Taking advantage of this concept, electrically reconfigurable networks of DNA-Au@MNPs have been employed for ultrasensitive (in the aM range) and fast (20–30 min) detection of miRNAs and circulating tumor DNA (ctDNA).[21,22]

Figure 6 illustrates the strategy reported by Gooding's team for the determination of ctDNA that combines the use of Au@MNPs modified with a DNA probe dually labeled with thiol and MB (MB-DNA-Au@MNPs) and complementary to the target ctDNA, with the use of a gold macroelectrode modified with a binary monolayer of a single-strand DNA (also dually end-labeled with thiol and MB) and MCH to achieve signal amplification through the electroreduction of ferricyanide by MB tags from both surfaces (electrode and Au@MNPs). As Figure 6a shows, the MB-DNA-Au@MNPs were exposed to the target ctDNA and the hybridized MB-DNA-Au@MNPs were collected with the aid of a magnet onto the gold electrode surface to monitor electrochemically a redox amplification cycle in which MB is reduced to leucomethylene blue (LB) which

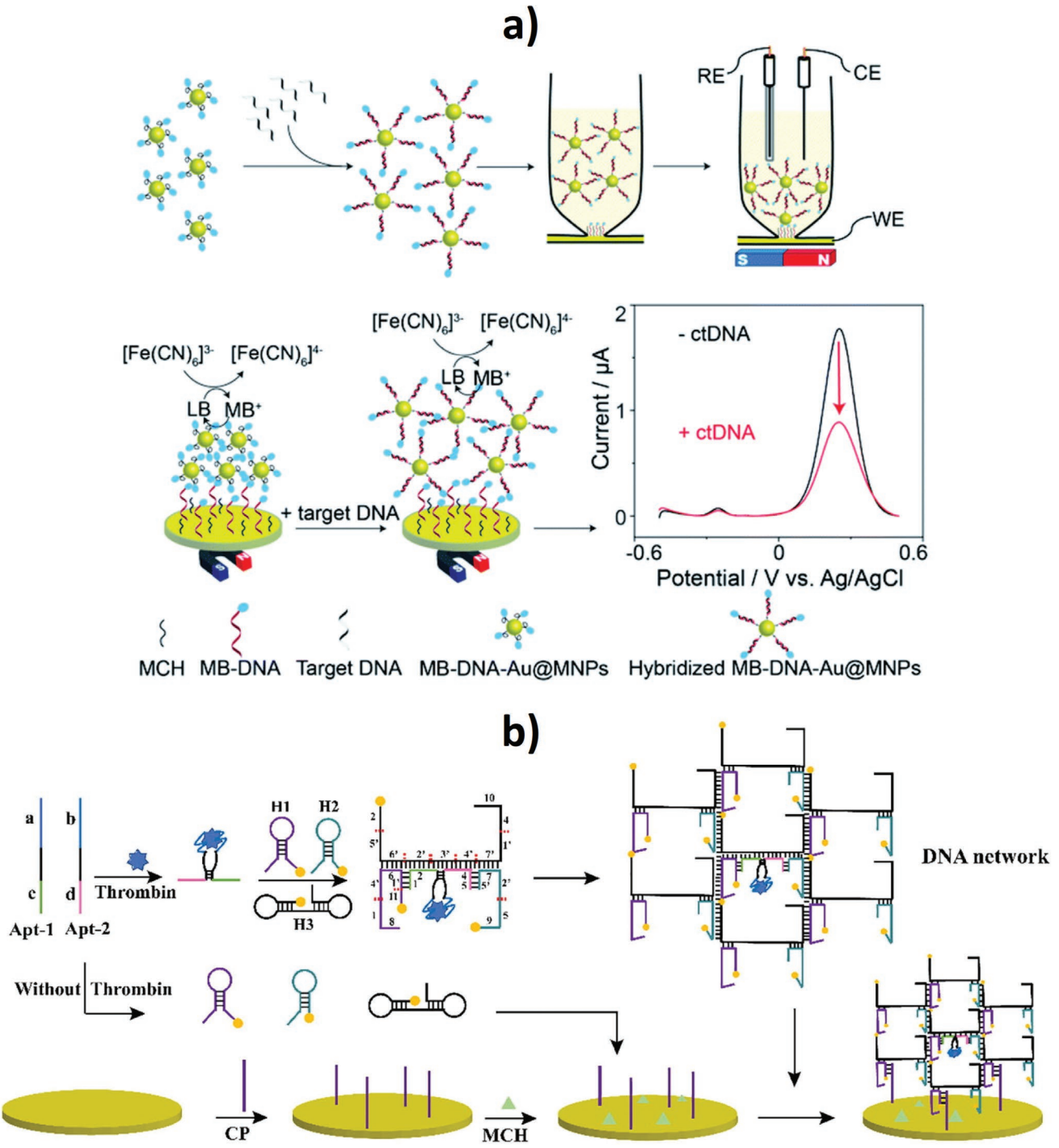

Figure 6. DNA networks used a) in "dispersible electrodes" for the ultrasensitive and rapid detection of ctDNA and b) generated by non-linear HCR for amplification purposes in the determination of thrombin. (a) Reproduced with permission.[22] Copyright 2021, RSC; (b) Reproduced with permission.[24] Copyright 2021, Elsevier.

then oxidizes back to MB (oxidation recorded by square wave voltammetry (SWV)) by $[Fe(CN)_6]^{3-}$. The decrease in the SWV signal observed in the presence of target ctDNA was attributed to the greater distance between the MB-DNA-Au@MNPs after hybridization which hindered the electron tunneling through the MB-DNA-Au@MNPs network.[21] This strategy allowed the determination of target DNAs of different length (22–101 nts) in raw human plasma and 50% whole human blood.

DNA networks generated by DNA probes functionalized with biotin at both ends[23] or by non-linear HCR amplification strategies[24,46] (Figure 6b) have been exploited to develop biosensors with improved sensitivity. Representative examples of

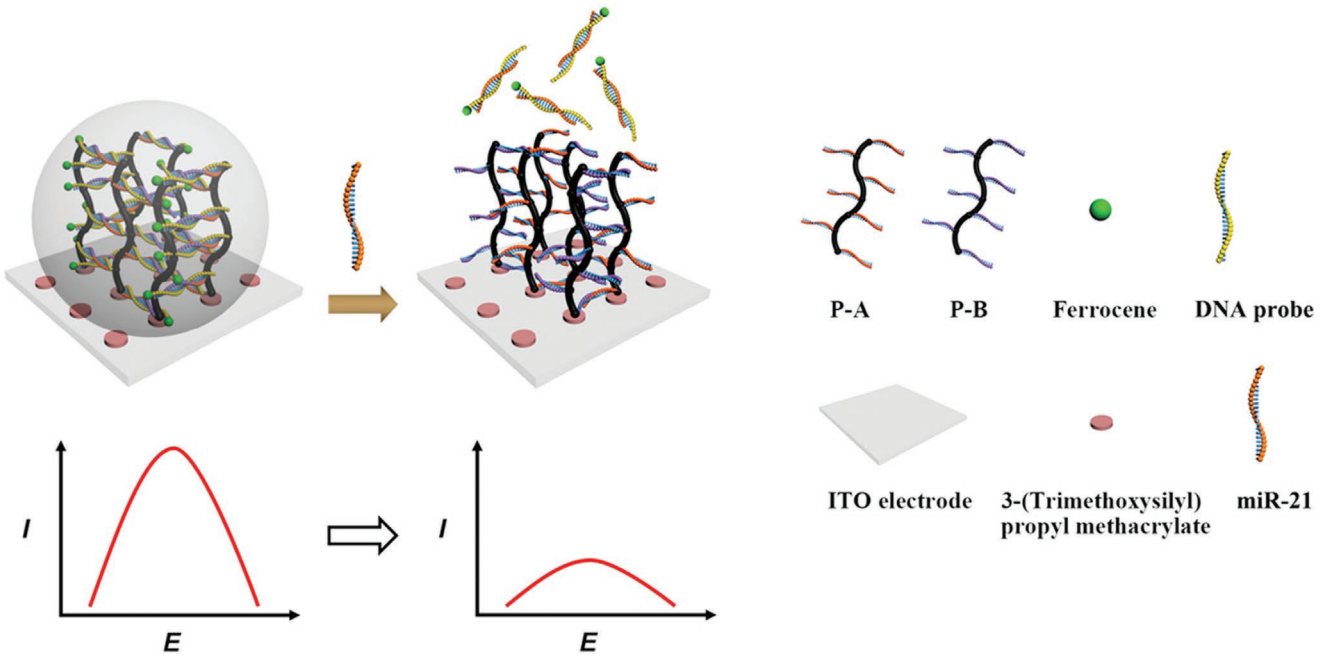

Figure 7. Hybrid DNA hydrogel-based biosensor for the determination of miRNA-21. Reproduced with permission.[28] Copyright 2018, Elsevier.

these concepts are the enzyme-free biosensors reported by Liu et al. for the determination of telomerase activity and by Li et al. for thrombin.[23,24]

DNA hydrogels are 3D porous network polymers containing a large amount of water and constructed by crosslinking only nucleic acids (pure DNA hydrogels) or grafting DNA strands on hydrophilic polymers or other materials (hybrid DNA hydrogels).[26] Pure DNA hydrogels have limited mechanical properties and high cost compared to the hybrid ones.[28]

Although they have not yet been largely explored, DNA hydrogels are considered as promising biomaterials for electrochemical biosensors because of their biocompatibility, flexibility, large specific surface area and loading capacity, high diffusivity of small molecules, mechanical stability, and ability to respond to appropriate stimuli (such as pH, light, etc.).[25] These biomaterials have been employed both as electrode modifiers, providing 3D scaffolds to enwrap catalytic or affinity receptors or 3D electron transporters and for amplification purposes.[25–29]

An interesting example is the biosensor developed by Liu et al. for the determination of miRNA-21 using an indium tin oxide/polyethylene terephthalate (ITO/PET) electrode modified with a hybrid DNA hydrogel. Ferrocene (Fc)-labeled recognition probes were cross-linked with DNAs grafted onto polyacrylamide backbones. The hybridization of the target miRNA with the recognition probe led to hydrogel dissolution, the loss of Fc tags, and a reduction in the Fc oxidation current monitored by DPV (**Figure 7**).[28]

Mao et al. reported the possibility of employing these biomaterials as 3D electron transporters imparting the modified electrodes better electron transfer and signal output intensity compared to the conventional non-functionalized electrodes.[29] The generation of hydrogels on the electrode surface using different strategies has also been exploited for amplification purposes in the development of impedimetric biosensors for the determination of heparanase and Hg^{2+}.[26,25] It is worth noting that the latter method also exploited pH-stimulation of the hydrogel density to achieve signal amplification.

2.3. Multifunctional DNA Probes

This section discusses the use of DNA probes capable of performing additional functions other than recognition of the target, such as polyA-probes and DNAzymes.

2.3.1. PolyA Probes

PolyA-type probes comprising a polyA tail and a recognition part possess interesting capabilities for developing electrochemical nucleic acid biosensors.[30] This type of probes can strongly adsorb on the gold electrode surface with an affinity comparable to that of the Au—S chemical bond.[47] Similarly to the attractive ternary monolayers of thiolated probes proposed already a decade ago,[48] PolyA probes allow the control of the capture probe density and spacing to achieve optimal hybridization efficiency, minimize nonspecific adsorptions, and impart antifouling properties to the modified surface.[49] Furthermore, unlike thiol monolayers, PolyA probes are monocomponent monolayers with no thiol chemistry involved, exhibiting enhanced storage stability due to the increased number of the probe anchor points to the gold surface. As can be seen in Table 1, so far, single polyA capture probes (**Figure 8**a)[30] or multiblock type (two recognition sequences connected through a polyA fragment (Figure 8b)[31] have been employed. It is also important to remark that the use of this type of probes, cheaper than the thiolated ones,[50] allows modulation at will of the size

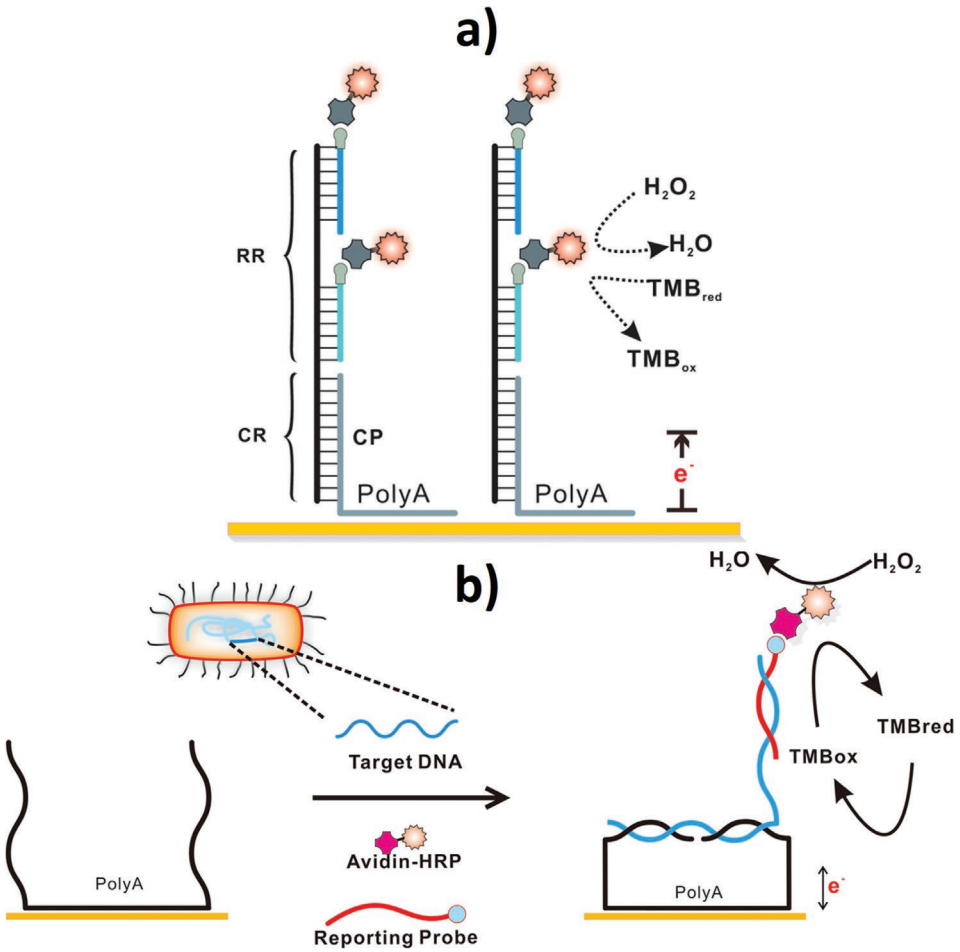

Figure 8. a) Single or b) triblock polyA-probes involved in the preparation of electrochemical biosensors for interrogating bacterial 16S rRNA or genomic DNA, respectively. In (a) RR and CR are reporting and capturing region, respectively. (a) Reproduced with permission.[30] Copyright 2019, ACS; (b) Reproduced with permission.[31] Copyright 2019, ACS.

of the polyA fragments and the evaluation of their influence on the hybridization efficiency and on other properties of the bioplatform such as the storage stability and the antifouling ability.

PolyA probes-based electrochemical bioplatforms have been reported to analyze bacterial 16S rRNA,[30] asymmetric PCR amplicons from bacterial genomic DNA,[31] and transgene-derived long RNA.[32] These three bioplatforms involved sandwich hybridization formats assisted by the use of one or multiple reporter probes, enzymatic labeling with HRP and chronoamperometric detection with the 3,3′,5,5′-tetramethylbenzidine (TMB)/H_2O_2 system.[30–32]

2.3.2. DNAzymes

Signal amplification catalyzed by nanozymes, single-stranded nucleic acids able to catalyze a specific chemical reaction, has been used in the development of electrochemical bioplaforms to achieve the highly sensitive detection of different target analytes: telomerase, Hg^{2+}, miRNA-21, alkaline phosphatase (ALP), carcinoembryonic antigen (CEA), Escherichia coli, and cancer derived exosomes.[3,33–37]

The selected strategies are summarized in Table 1. Among them, it is worth mentioning the ratiometric approach reported by Li et al. to detect Hg^{2+} involving a bifunctional DNAzyme nanodevice with two detection paths toward the same target.[34] A universal paper-based electrochemical sensor (PES) has been proposed relying on the target-induced synthesis of Mg^{2+}-dependent DNAzyme to catalyze the cleavage of a Fc-labeled probe from paper, thus leading to the release of signal molecules which generated an increased DPV electrochemical signal on CNTs-SPEs (**Figure 9**a).[35] This strategy claimed zero-background current and allowed the highly sensitive detection of miRNA-21, ALP, and CEA in connection with a miRNA recognition probe, a phosphorylated hairpin probe and a DNA aptamer, respectively. Pandey et al. reported recently a handheld platform involving the use of electroactive RNA-cleaving DNAzymes (e-RCDs) dually functionalized with biotin and MB and integrated into a two-channel electrical chip with nanostructured electrodes for detecting urinary tract infections in less than 1 h.[36] Figure 9b shows as this strategy implied a differential electrochemical signal readout between two channels, a capture channel where the DPV response of MB increased in the presence of the target bacterium (*E. coli*) and a release channel where it decreased.

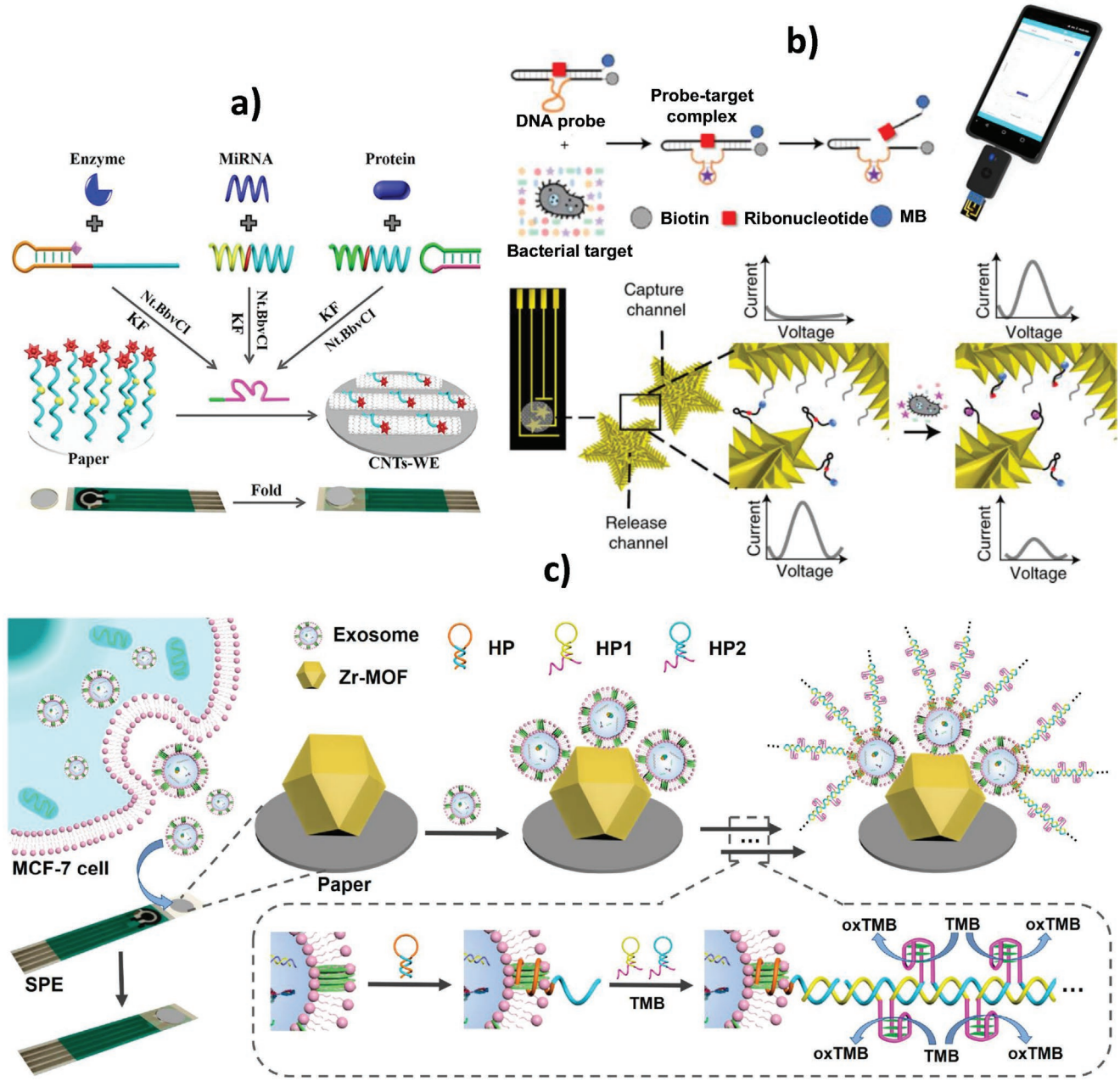

Figure 9. a) Universal PES assisted by sequence-specific Mg^{2+}-dependent DNAzymes for determination of different target analytes. b) Portable platform based on the use of e-RCDs and a differential electrochemical signal readout for the determination of *Escherichia coli*. c) MOF-functionalized paper-based electrochemical biosensor for the determination of exosomes. (a) Reproduced with permission.[35] Copyright 2019, ACS; (b) Reproduced with permission.[36] Copyright 2020, NPG; (c) Reproduced with permission.[37] Copyright 2021, ACS.

Li´s group has recently reported a paper-based electrochemical biosensor to detect cancer-related exosomes that combines the use of a Zr-MOF and an aptamer as recognition elements and HCR and the formation of a DNAzyme for amplification purposes.[37] In this method, the exosomes were absorbed on the surface of Zr-MOFs through the formation of Zr—O—P bonds. An aptamer capable of specifically recognizing a cancer-derived exosome membrane protein (CD63) initiated the HCR reaction generating double-strands DNA nanowires designed to contain numerous hemin/G-quadruples DNAzymes, which can catalyze the oxidation of TMB, reducing the TMB DPV signal response on the SPE (Figure 9c).

3. Multifunctional Aptamers

Aptamers are defined sequences of single-stranded RNA or DNA selected in vitro (systematic evolution of ligands by exponential enrichment) that interact in a tailored 3D design with the target molecule, mirroring antigen–antibody natural

Table 2. Selected recently reported multifunctional aptamer-based electrochemical (bio)sensors.

Electrode	Fundamentals	Type of aptamer	Detection technique	Target analyte/s	LR/LOD	Sample/ application	Remarkable features	Ref.
SPE	Direct affinity reaction	PolyA-aptamer	DPV $(Fe(CN)_6]^{-3/-4}$) Signal-off	Pb^{2+}	0.1–1000 ng mL^{-1}/0.03 ng mL^{-1}	Spiked river and tap water and fish samples	Label-free. Simple and one-step fabrication and 2 min response time	[50]
AuE	Dual-recognition aptazyme beacon and MB-methylene tagged signal probe	Aptazyme	DPV (MB) Signal-off	Chlorpyrifos and Pb^{2+}	Chlorpyrifos: 0.5 пм–0.5 μM/0.178 пм Pb^{2+}: 0.1 пм–0.5 μM/0.034 пм	Complex food samples	Simultaneous determination of targets with different chemical properties	[55]
AuE	Direct affinity reaction	Dimeric aptamer	EIS $(Fe(CN)_6]^{-3/-4}$) Signal-off	Spike proteins of the wildtype, alpha and delta variants of SARS-CoV-2	–/1000 viral particles mL^{-1}	Saliva	10 min assay time. Validation with saliva samples from 73 patients clinical sensitivity of 80.5% and specificity of 100%	[56]

–: not indicated;.
DPV: differential pulse voltammetry; EIS: electrochemical impedance spectroscopy; MB: methylene blue; SPE: screen-printed electrode.

interaction. At present, they are gaining a great role in developing competitive electrochemical bioplatforms as evidenced by the recent appearance of several nice reviews and editorials.[51–54] Aptamers are advantageous over antibodies in terms of smaller size, chemical stability, rapid, easy, reversible denaturation capacity, and affordable synthesis and modification, without requiring cell or animal culture and free of batch variations. Moreover, they can be generated against a wide variety of target molecules and selected in deep eutectic solvents in case the target species were poorly soluble in water.

To enhance the capabilities they currently provide, there is a tendency to design and exploit multifunctional aptamers. **Table 2** summarizes the characteristics of selected recent examples of electrochemical bioplatforms that used polyA-aptameric probes, aptazymes, and dimeric aptamers.[50,55,56]

In this context, Wang et al. reported a simple two-in-one electrochemical biosensor for the simultaneous determination of pesticides and heavy metal ions (Chlorpyrifos and Pb^{2+} used as models) by designing a dual recognition aptazyme beacon (DRAB).[55] In this approach, the target pesticide activated the self-blocked DRAB by inducing a conformational change. In the presence of the target metal, a MB-tagged signal probe was selectively cleaved resulting in a decrease of the MB electrochemical response monitored by DPV with the concentration of both analytes. Moreover, the released DRAB-pesticides complex can bind adjacent signal probes and start another cyclic cleavage.

To improve the sensitivity, multivalent aptamers have been designed by binding an appropriate number of monomers. Due to the particular relevance in these days, it should be mentioned the use of dimeric aptamers for the determination of the homotrimeric SARS-CoV-2 spike protein (**Figure 10**).[56] The resulting aptasensor selectively bound with high affinity the wild-type spike protein and those of variants of concern (Delta first identified in India and Alpha in UK) and was applied to the direct analysis in 1:1 diluted saliva samples from 36 positive and 37 negative patients within 10 min with clinical sensitivity and specificity values of 80.5 and 100%, respectively.

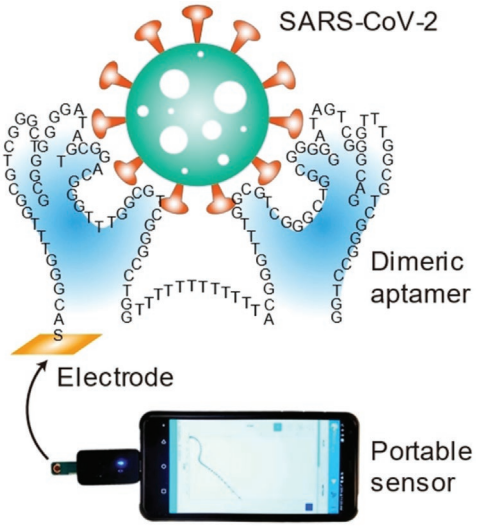

Figure 10. Electrochemical biosensor based on a dimeric aptamer for the simple and rapid detection of spike proteins of the wildtype SARS-CoV-2 virus and Alpha and Delta variants. Reproduced with permission.[56] Copyright 2021, Wiley.

4. Modern Peptides

Similarly to aptamers, peptides (amino acid sequences of different length and weight comprising synthetic or natural amino acids connected by peptide bonds), are experiencing an unstoppable rise in developing high performance electrochemical biosensors.[1,6] Peptides are promising affinity biorecognition elements that, in connection with electrochemical transducers, provide advantages primarily in terms of stability, selectivity, structural diversity, possibility of tuning the amino acid sequence, and biocompatibility. These recognition elements of synthetic nature can be easily obtained with high yield and functionalized with specific groups (affinity or electroactive tags) for binding (thiol, biotin, etc.) or signaling (Fc, MB, and enzymes) through chemical automated synthesis, thus avoiding the need for laborious in vivo protocols, to enhance binding of

peptide in a particular way while retaining its high affinity to the targets.[1,6] Although the advantages of peptides over antibodies in the development of electrochemical biosensors are evident as to their smaller size, cost-effectiveness, high yield, and ease of chemical synthesis, their comparison with nucleic acids is not straightforward. Peptides have a different acid–base behavior than nucleic acids and different functional groups that can enhance interactions, and thus affinity, with the target analyte.

Peptides have been employed as i) recognition elements; ii) enzyme substrates; iii) electrode modifiers (linker and scaffold), by harnessing their ability to interact and self-assemble to create superior highly ordered nanoscaffolds with good inherent electronic properties and biocompatibility, allowing the immobilization of other bioreceptors in a given arrangement or imparting antifouling properties to the surface;[57] and iv) labeling/carrier agents for signal amplification.[1] As biorecognition elements they can be exploited to interrogate a wide variety of analytes (nucleic acids, proteins, antibodies, cells, metal ions, etc.) and, as enzyme substrates, play an irreplaceable role to determine the activity (or inhibiting) of clinically relevant enzymes such as proteases (the peptide serves as cleavage-sensing element) and kinases (catalyzed phosphorylation reactions).[1,6] Because of their distinguished properties and the opportunities they impart to the final devices, this section discusses also the use of antifouling, multifunctional, phage-displayed, and aberrant peptides. The characteristics and performance of the selected biosensors are summarized in **Table 3**.

With the aim of developing biosensing devices suitable for real-world applications, peptides that impart antifouling properties to modify platforms, mostly zwitterionic peptides, have been increasingly employed.[57–59,61,63,64,67,68,70,71] In these biosensors, the peptide, comprising both a binding domain and an antifouling domain, was used as a bioreceptor[59,61,67,68,71] or as a surface modifier coupled with another bioreceptor (specific aptamers[58,63,64] or DNA probes[57,70]).

Another current trend is the use of multifunctional peptides. Among them, one can distinguish between those performing different functions (recognition and amplification)[62] and those more common that have different domains (anchoring, doping, linking, and antifouling), also known as "all-in-one" peptides.[59,63,67,68,71] Importantly, some have been designed with multiple binding domains (multimeric peptides) (**Figure 11**a)[68] to improve the sensitivity of the determination. In this context, it is noteworthy to mention the strategy developed by Tang et al. to identify stem-like cells in breast tumor using multifunctional peptide-based self-assembled nanofibers that, in addition to the selective analyte recognition, can amplify the response by acting as nanotransporters of multiple AgNPs to generate the electrochemical signal (Figure 11b).[62]

It is important to emphasize that some of these multifunctional peptides have been designed with characteristics shapes: Y-shaped[57,65] or branched,[68,60] providing advantages for the simplification of the biosensor manufacturing process and outperforming the properties imparted to the resulting biosensor compared to linear peptides.[65,60] Interestingly, the shape of some of these peptides (inverted Y-shaped) has been exploited to prepare scaffolds that, in addition to endowing antifouling properties, improve the sensitivity of the resulting biosensor by ensuring optimal spacing between the immobilized bioreceptors allowing a more efficient biorecognition (Figure 11c).[57]

Phage display technology, which involves the expression of foreign peptides or proteins outside the virion of a phage (a virus that only infects bacteria), has shown to be extremely useful for the production of novel receptors, such as peptides, beyond known biomolecular interactions, or against targets which are toxic or non-immunogenic.[72] Recently, Barderas and Campuzano/Pingarrón research groups reported a bioplatform that used phage display and aberrant peptides for interrogating the potential of their corresponding autoantibodies as reliable biomarkers for diagnosing Alzheimer's disease (AD) patients. The bioplatform involved the use of MBs modified with the identified four phage-derived and two aberrant HaloTag monomeric peptides to capture the corresponding serum autoantibodies efficiently and selectively.[69] The diagnostic capability of the bioplatforms was assessed with a cohort of 20 serum samples (10 from healthy subjects and 10 from AD patients). The excellent values of area under curve, sensitivity, and specificity, all above 90%, and 100% combining all peptides, obtained by receiver operating characteristic curve analysis, proved the high diagnostic ability of the developed bioplatforms for discriminating AD patients in ≈2 h, in a simple, affordable, and point-of-care manner.

5. CRISPR/Cas Systems

CRISPR/Cas systems (clustered regularly interspaced short palindromic repeats (CRISPR) and associated proteins (Cas)) are surveillance ribonucleoprotein complexes consisting of a single guide RNA with a Cas system such as Cas9, deactivated or mutated Cas9 (dCas9), Cas12a (CPF1), and Cas13a (CPF2), to bind a target sequence (dCas9) or cleave a target DNA/RNA (Cas9, Cas12a, and Cas13a) and generate a signal readout.[73]

CRISPR/Cas systems constitute powerful multifunctional biomaterials in contemporary biosensing[74–79] and, in particular, in electrochemical biosensing, improving the detection limits and accuracy for sensing both nucleic acids targets (Cas9: dsDNA; Cas12: ssDNA, and Cas13: ssRNA) or non-nucleic acid targets with high efficiency and simplified designs.[80,81]

Due to their selectivity, programmability, and double functionality, these systems have been exploited in electrochemical biosensors as: i) biorecognition elements for individual or multiplexed interrogation of nucleic acids (DNAs,[82] miRNAs,[80,83] mRNAs,[84] and viral RNA (**Figure 12**a)[85]); and ii) signal amplifiers for determining non-nucleic acid targets, such as proteins (Figure 12b), metals and bacteria.[86,87]

As can be seen in Figure 12a, the strategy developed by Liu et al. is based on the coupling of CRISPR/Cas technology with electrochemical detection using a personal glucometer (PGM) for COVID-19 screening.[85] In this strategy, a specific gene region of interest (*N* gene) of SARS-CoV-2 RNA was amplified by RCA. The RCA products bound to Cas12a/crRNA, thus activating Cas12a which cleaved the polyinvertase-DNA sequences immobilized on magnetic nanoparticles. The released invertase catalyzed the hydrolysis of sucrose to glucose, which was detected by the PGM. On the other hand, Chen et al. (Figure 12b) employed the CRISPR/Cas13a system as an

Table 3. Selected recent electrochemical biosensors involving multifunctional peptides.

Electrode	Fundamentals	Type of peptide	Detection technique	Target analyte/s	LR/LOD	Sample/ application	Remarkable features	Ref.
Macroporous Au substrate (multilayer polystyrene nanospheres self-assembled on GCE)	Direct affinity reaction at an aptamer self-assembled with a zwitterionic peptide onto a macroporous Au substrate	Antifouling peptide	DPV $(Fe(CN)_6]^{-3/-4}$) Signal-off	IgE	0.1–10 pg mL^{-1}/ 42 fg mL^{-1}	Spiked bovine serum solutions	Antifouling High sensitivity	[58]
GCE modified with electropolymerized PANI nanowires arrays	Peptide containing anchoring, antifouling, and recognition domains	Multifunctional peptide ("all-in-one")	DPV (–) Signal-off	IgG	1.0 ng mL^{-1} –10 µg mL^{-1}/ 0.26 ng mL^{-1}	Human serum	Antifouling	[59]
GCE with electrodeposited PANI films	Direct affinity reaction at branched zwitterionic peptides	Branched peptides	DPV (–) Signal-off	MUC1	50–10^6 cells mL^{-1}/20 cells mL^{-1}	MCF-7 cells in human serum	Antifouling. 6 order dynamic range.	[60]
AuNPs/ITO	Two antifouling zwitterionic peptides: a longer peptide functionalized with a GO-Fe_3O_4-Thi probe, which contain the PSA recognition sequence and a shorter one tagged with Fc and used as internal reference	Antifouling peptides	DPV (Thi) CA (GO-Fe_3O_4 as peroxidase mimic) + Thi) Signal-off (DPV) Signal-on (CA)	PSA	5 pg mL^{-1}–10 ng mL^{-1}/0.76 pg mL^{-1} (DPV) and 0.42 pg mL^{-1} (CA)	Human serum	Dual-mode PSA sensor. Antifouling properties.	[61]
AuE	Capture of BCSCs by a specific aptamer and recognition by MNFs able to recruit AgNPs	Multifunctional peptide probes (peptide-based MNFs as nanocarriers of signaling elements)	LSV ($AgNO_3$)	BCSCs	10–5×10^5 cells mL^{-1}/6 cells mL^{-1}	Human serum	–	[62]
AuNPs-GCE	Direct affinity reaction at an aptamer covalently immobilized onto multifunctional peptides (anchoring, doping, linking, and antifouling sequences) attached to AuNPs-GCE with electropolymerized PEDOT	Multifunctional peptides ("all-in-one")	DPV $(Fe(CN)_6]^{-3/-4}$) Signal-off	miRNA-21	10.0 fM–1.0 nM/2.3 fM	Human serum	Long-term antifouling performances	[63]
PABA/GCE	Direct affinity reaction at an hairpin aptamer immobilized together with an antifouling peptide onto a PABA/GCE	Antifouling peptide	DPV $(Fe(CN)_6]^{-3/-4}$) Signal-off	IgE	0.001 ng mL^{-1}– 50.0 ng mL^{-1}/0.52 pg mL^{-1}	FBS	Antifouling	[64]
PEDOT-citrate/ AuNPs-GCE	Y-shaped peptide with both recognizing and antifouling branches	Y-shaped peptides	DPV $(Fe(CN)_6]^{-3/-4}$) Signal-off	IgG	100 pg mL^{-1}–10 µg mL^{-1}/32 pg mL^{-1}	Human serum	Simple fabrication Label-free Antifouling properties	[65]
AuE	Poly adenine coating combined with highly specific CD20 epitope mimetic peptide	CD20 epitope mimetic peptide	EIS $(Fe(CN)_6]^{-3/-4}$) Signal-on	Rituximab	0.1–50 µg mL^{-1}/35.26 ng mL^{-1}	Plasma	Antifouling	[66]
PEDOT-citrate film-GCE	Direct affinity reaction at a three-in-one peptides	Multifunctional peptides (all-in-one)	DPV $(Fe(CN)_6]^{-3/-4}$) Signal-off	APN and human HepG2 cells	1 ng mL^{-1}–15 µg mL^{-1}; 50 to 5×10^5 cells mL^{-1}/0.4 ng mL^{-1}; 20 HepG2 cells mL^{-1}	Human urine	Antifouling	[67]
PANI nanowires-GCE	Direct hybridization of the target DNA with biotinylated specific probes immobilized onto inverted Y-shaped peptides-coated PANI nanowires	Inverted Y-shaped peptides	DPV $(Fe(CN)_6]^{-3/-4}$) Signal-off	N-gene (nucleocapsid phosphoprotein) of SARS-CoV-2	10^{-14}–10^{-9} M/3.5 fM	Human serum	Antifouling High sensitivity	[57]

Table 3. Continued.

Electrode	Fundamentals	Type of peptide	Detection technique	Target analyte/s	LR/LOD	Sample/ application	Remarkable features	Ref.
PEDOT-AuNPs/GCE	Direct affinity reaction at a "all-in-one" branched zwitterionic peptide	Multifunctional branched peptide ("all-in-one")	DPV (–) Signal-off	IgG	0.1 ng mL^{-1}–10 μg mL^{-1}/45 pg mL^{-1}	Human serum	Antifouling Five orders of magnitude	[68]
SPCE	Direct affinity reaction onto MBs modified with the HaloTag peptides	Phage-derived and aberrant HaloTag peptides	Amperometry	Autoantibodies	–	Human serum	Identify and validate a new signature of autoantibodies against the identified peptides to diagnose Alzheimer disease patients	[69]
PANI/GCE	Covalent immobilization of a specific DNA probe and an antifouling zwitterionic peptide onto the PANI/GCE	Antifouling peptide	DPV (–) Signal-off	miRNA-24	10 fM–1.0 nMnM/3.1 fM	Human serum	Antifouling	[70]
MXene-Au-MB composite-GCE	Direct affinity reaction at a multifunctional peptide (anchoring, antifouling, and recognizing sequences) modified with the signal probe HOOC-MBs anchored to a MXene-Au-MB-modified GCE	Multifunctional peptide (all-in-one)	DPV (Fc and MB) Signal-off (Fc)	PSA, TB	5 pg mL^{-1}–10 ng mL^{-1}/0.83 pg mL^{-1}	Human serum	Ratiometric Antifouling	[71]

–: not indicated;.
AgNPs: silver nanoparticles; APN: aminopeptidase N; AuE: gold electrode; AuNPs: gold nanoparticles; BCSCs: breast cancer stem cells; CA: chronoamperometry; DPV: differential pulse voltammetry; EIS: electrochemical impedance spectroscopy; FBS: fetal bovine serum; Fc: ferrocene; GCE: glassy carbon electrode; GO: graphene oxide; ITO: indium tin oxide; MB: methylene blue; MBs: magnetic microbeads; MNFs: multifunctional nanofibers; LR: linear range; LOD: limit of detection; LSV: linear square voltammetry; MUC1: mucin 1 protein; PABA: poly (m-aminobenzoic acid); PANI: polyaniline; PEDOT: poly (3,4- ethylenedioxythiophene); PSA: prostate-specific antigen; SPCE: screen-printed carbon electrode; TB: thrombin; Thi: thionine.

amplification strategy in a sandwich immunoassay.[86] They used a biotinylated double-stranded DNA containing a T7 promoter sequence that can be recognized by T7 polymerase to perform transcription producing many copies of single-stranded RNA molecules. The transcribed RNA molecules activated the CRISPR/Cas13 system which cleaved the reporter DNA.

The unprecedented possibilities of these systems, still in their infancy, are encouraging the development of innovative electrochemical biosensor devices achieving extremely high sensitivity (zeptomolar concentrations and single nucleotide resolution of nucleic acids and femtomolar concentrations of non-nucleic acid targets),[87] as well as specificity with simple, fast, and robust performance even on microfluidic devices.[80,83]

6. Opportunities, Challenges, and Perspectives

The rational application of nanostructured and/or multifunctional nucleic acid or peptide biomaterials (including the CRISPR/Cas systems) has made possible the birth of a new generation of electrochemical biosensors with unique attributes and capabilities for application.

In the last years, nanostructured and multifunctional biomaterials have demonstrated similar potential to artificial nanostructures for using as electrode modifiers and carriers but lag far behind them in development and application. Simply put, the works critically discussed in this perspective article show that nucleic acid and peptide biomaterials have the Janus side of bioreceptor and nanomaterial. As bioreceptors, they have relevant features with respect to other long adopted bioreceptors such as the antibodies in terms of smaller size, chemical stability, rapid, easy, inexpensive and cell and animal-free synthesis and modification, and free of batch variations. As nanomaterials, they are competitive versus the artificial ones in terms of selectivity, easy tailored structure and shape, lack of variability between batches, lower toxicity compared with carbon nanomaterials, and not to be bioinert or stiff like metal oxides. For all these reasons, it is impossible not to be convinced that we are at the beginning of a research area on the verge of exponential growth.

Although the potential of electrochemical biosensors based on nanostructured and multifunctional nucleic acid and peptide biomaterials is immense, it is important to highlight that these devices should continue collecting medals to earn the recognition of the scientific community and the interdisciplinary support necessary to progress in their advances and developments.

Fundamental studies aimed at getting an accurate prediction of the supramolecular organization from the biomaterial sequence and self-assembly mechanisms are required to select the best conditions. It is also imperative to advance in the identification and design of new peptide sequences, so far significantly smaller in number than other receptors such as antibodies, which limits the number of molecular targets that can be analyzed. In addition, novel technologies such as phage

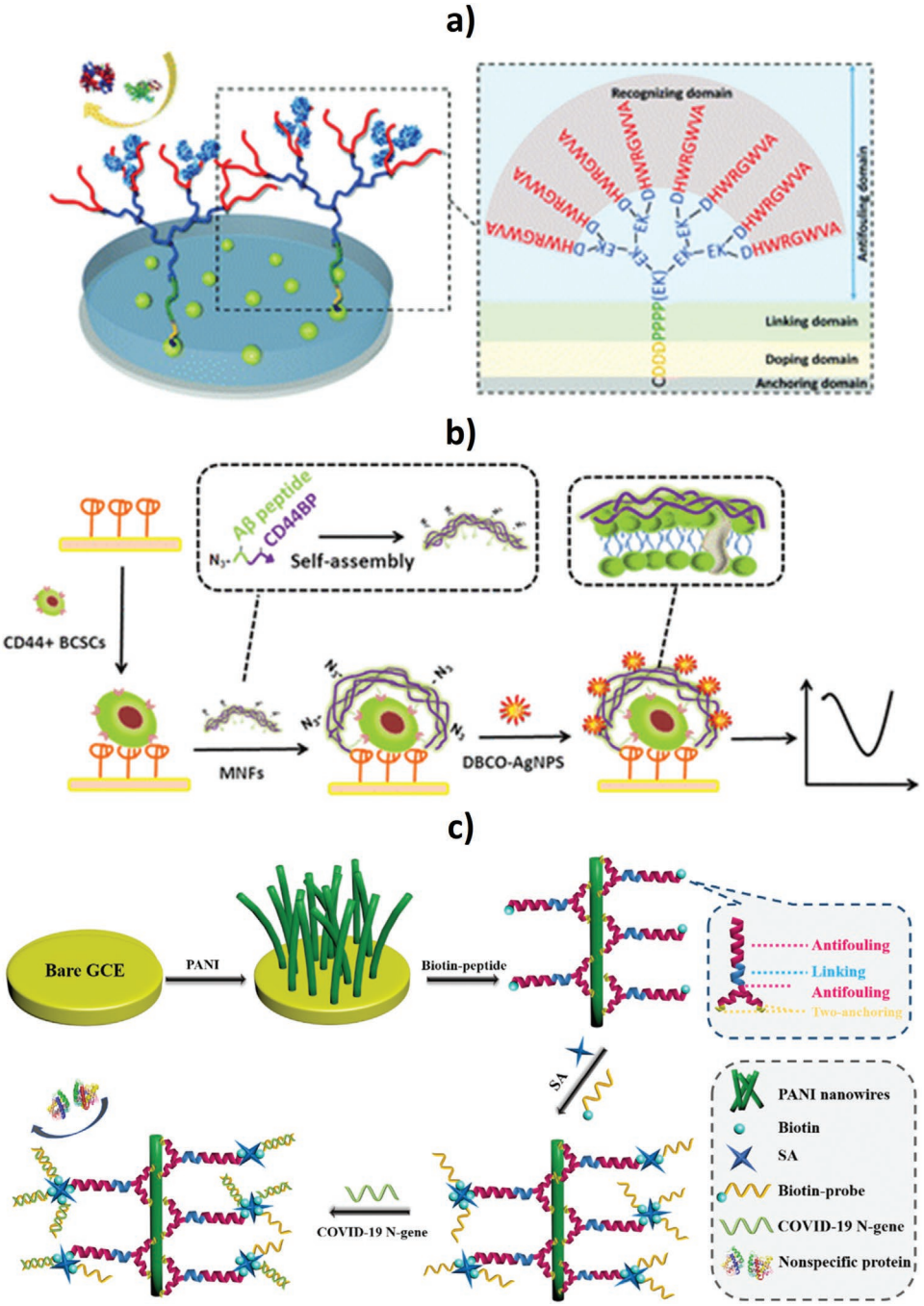

Figure 11. Schematic illustration of biosensors prepared using multifunctional peptides a) with a branched shape, b) acting both as detector bioreceptor and nanocarrier of signaling elements, and c) to construct an antifouling scaffold to immobilize bioreceptors with the appropriate spacing. (a) Reproduced with permission.[68] Copyright 2021, RSC; (b) Reproduced with permission.[62] Copyright 2019, ACS; (c) Reproduced with permission.[57] Copyright 2021, ACS.

display and HaloTag should be used in the peptide production and/or from their expression as fusion proteins, respectively.

General requirements and basic performance characteristics of the resulting biosensors, such as robustness, precision, specificity, selectivity, stability, etc. must be thoroughly evaluated and their usefulness tested in challenging real-world samples to assure that the capabilities of these biotools are not being overestimated and that it is indeed worth embarking on their further development. In fact, their ability to determine molecular targets without complicated sample processing steps, a subject always pending in biosensors, and the antifouling properties imparted by biomaterials, make these biosensors more appropriate than others to address successfully the challenging goals faced. However, it is also important to be aware that the early development stage of these devices and the applications with which they have been confronted may be responsible that

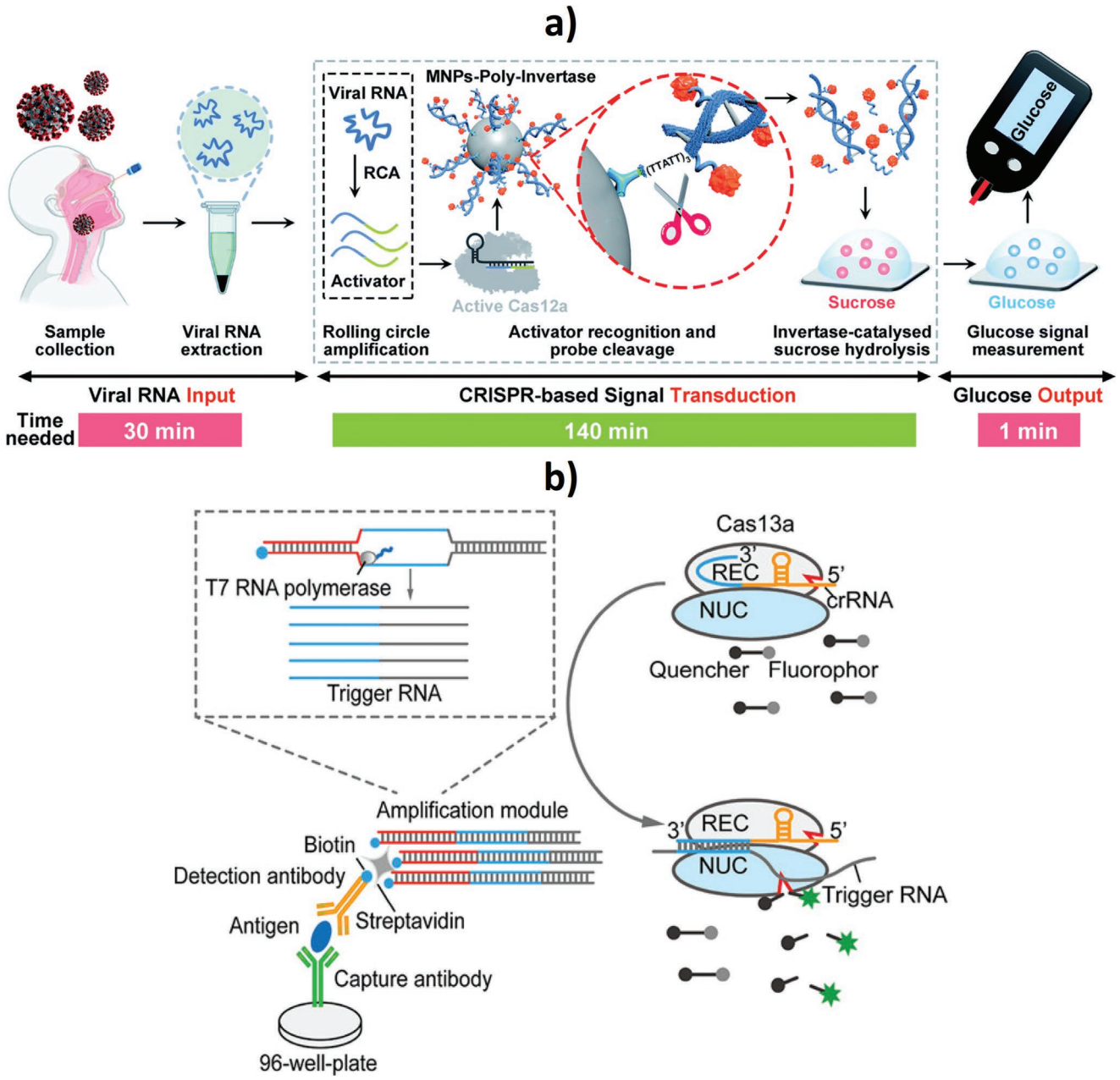

Figure 12. Strategies based on the use of CRISPR/Cas systems a) as bioreceptors or b) for amplification in electrochemical biosensing to interrogate viral RNA and protein targets, respectively. (a) Reproduced with permission.[85] Copyright 2021, RSC; (b) Reproduced with permission.[86] Copyright 2020, ACS.

the true limitations of these biomaterials have not yet become apparent. Nevertheless, some drawbacks can already be anticipated. For instance, tetrahedral and Y-shaped nanostructures require the use of multiple probes, are subjected to thiol chemistry, and restricted to the use of gold surfaces. On the other hand, although the assembly of DNA otrigami is straightforward, the tools to design new structures are not advanced and cannot be truly formalized until the relationships between design and properties are fully understood. As for dendrimers, their stability in physiological matrices needs to be further investigated.

It is also expected that the demand for improved capabilities of this biodevices will only increase as they move forward, as more is always demanded to the best, like the urgently needed simultaneous determination of multiple targets, which provides notable advantages such as increased precision, low sample consumption, high sample throughput, and reduced cost and time per assay to avoid misdiagnoses. A great effort is also foreseen on the development of new amplification strategies and in the design and application of multimeric biomaterials to meet the demand for increasingly sensitive tools to detect analytes at ultra-low levels.

Moreover, although these devices have promising potential usages, we must be aware that they are still at the conceptual level. So, their natural development must first go through

their translation from these proof-of-concepts to real practical devices and then from bench to POC. It is unquestionable that during these challenging transitions many complex obstacles must be overcome which go through simplifying and minimizing the fabrication cost and assay time, further improving the sensitivity, selectivity, storage stability, and anti-interference properties of these biodevices, demonstrating their robustness and reliability with a large number of samples and with different users in different environments, and coupling them with simple and portable electrochemical detection devices.

Further applications will require fresh breakthroughs which skillfully combine the expertise of multidisciplinary researchers. This interdisciplinary research with biomolecular nanomaterials poses great challenges and opportunities to drive the development of high-performance electrochemical biosensors, which can only be achieved with the approval and momentum of the intersecting areas of nanoscience, materials science, and molecular biotechnology.

It is important to keep in mind that although both bio and artificial nanomaterials can be used as electrode modifiers, carriers, or labels, the possibilities they provide are different but not incompatible. We personally believe that the combination of both types of nanomaterials, scarcely exploited to date, can open a new avenue where both the natural and the artificial nanomaterials will be winning horses. A good example of this is the use of DNA networks involved in the "dispersible electrodes." It is clear, for example, that biomaterials can impart to the artificial ones with selectivity which is always highlighted as the weak point of the latter. The same can be said about the use of nanostructured biomaterials combined with other types of more conventional bioreceptors.

We think that the delay in the appearance on the scene of nanostructured and multifunctional biomaterials with respect to artificial ones is partly due to a lack of knowledge or to the mistaken thoughts that they are less stable or that their manipulation requires much more advanced knowledge than for artificial nanomaterials. Hopefully, this perspective article will fulfill our purpose of reflecting the simplicity and tremendous opportunities of these biomaterials and their competitive advantages over other commonly used bioreceptors (such as the tremendously popular antibodies these days for antigen tests to detect COVID-19) or artificial nanomaterials, giving the scientific community the confidence and driving to join efforts and enthusiasm in their exploration.

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Conflict of Interest

The authors declare no conflict of interest.

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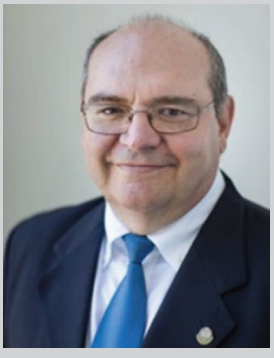
Susana Campuzano works as assistant professor at the Analytical Chemistry Department of the Chemistry Faculty of the Universidad Complutense de Madrid (Spain) where she is currently Head of "Electroanalysis and Electrochemical (Bio) Sensors" (GEBE) research group. Her areas of interest include the development of affinity-based electrochemical bioplatforms with potential for multiplexed and/or multi-omics determinations in clinical and food safety. She is associate editor of the journal Electroanalysis (Wiley-VCH).

María Pedrero is associate professor in the Analytical Chemistry Department of the Chemistry Faculty at the Universidad Complutense de Madrid (Spain) since 2002. She collaborates in the "Electroanalysis and Electrochemical (Bio)sensors" (GEBE) research group. Her main areas of interest include the development of enzymatic, immune, and DNA electrochemical sensors, at present for the detection of proteins and oligonucleotides as markers of cancer, cardiovascular, and neurological diseases.

Rodrigo Barderas received his Ph.D. in Chemistry (Biochemistry and Molecular Biology) from the Complutense University of Madrid (Spain) in 2004. Since 2004 he has been working in the field of proteomics and high-throughput screening techniques. In 2017 got a Tenured Scientist position at the Instituto de Salud Carlos III. He is currently the Head of the Functional Proteomics Unit of the Chronic Disease Programme. His areas of interest include the identification of diagnostic, and prognostic markers and new targets of intervention in chronic diseases of high prevalence by using proteomics and phage display, particularly in colorectal cancer.



José M. Pingarrón is professor of Analytical Chemistry and member of the group "Electroanalysis and electrochemical(bio)sensors" at Complutense University of Madrid. His research lines include the development of nanostructured electrochemical platforms (enzyme, immuno-, and genosensors) for single or multiplexed determination of relevant biomarkers. He is Senior Honorary Advisor of the journal Electroanalysis and since 2017 Fellow of the International Society of Electrochemistry. Currently he holds the position of Secretary General of Universities in the Spanish Government.