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Research Progress of Functionalized DNA Hydrogels in Biosensing

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Abstract. DNA hydrogels are programmable 3D networks that integrate molecular recognition modules (aptamers, DNAzymes, CRISPR) to translate binding events into macroscopic material responses. The central challenge is reliably amplifying these events into quantifiable changes within complex biological matrices. Sensor behavior is governed by coupled factors: material thresholding (gel–sol transition boundaries), pore architecture and mass transport, matrix interference (nucleases, proteins, ionic strength), and readout modality constraints. This review analyzes key scientific questions connecting molecular recognition to signal transduction. We summarize progress in construction routes, functionalization strategies, biosensing applications, and principal bottlenecks in complex samples. Priority directions for clinical translation include thin-film CRISPR geometries with distance readout, wireless integration for longitudinal monitoring, and standardized matrix validation frameworks. Ultimately, translation requires not only low detection limits but also reproducible manufacturing, robust real-matrix operation, and explicit mapping between hydrogel responses and clinically relevant biomarker ranges.

Keywords: DNA hydrogels, biosensing, functionalization strategies, signal transduction, clinical translation

1. Introduction

DNA hydrogel is a 3D hydrophilic mesh that is a cross-linked network of DNA strands, integrating the programmable recognition property of nucleic acids with the versatile properties of hydrogels to do biosensing. The essence of their design is that they incorporate recognition elements (aptamers, DNAzymes, CRISPR) to attach binding events to observable material changes.

Although improvement has been made, there still remains an inherent problem, namely, the ability to unquestionably translate molecular recognition into measurable changes in hydrogel states, which are still interpretable in more complex biological environments. This dilemma is multidimensional by its nature.

A network structure defines the recognition thresholds, too large thresholds insufficiently disrupt the system, and too small thresholds make it vulnerable and tend to raise false alarms. The mass transport is controlled by pore architecture wherein not only mass transport but also diffusion restrictions can dominate the performance of large biomolecules. The presence of complex matrices leads to interference: the nucleases degenerate the DNA networks, adsorbing proteins and ionic

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variations are changed by hybridization and enzyme activity respectively. The modalities of readout also diverge with respect to environmental sensitivity, with colorimetric and fluorescence readouts experiencing optical interference, electrochemical kinds of readout relying on electrode interfaces, and distance based format all minimizing instrument reliance but needing geometric regulation.

Scientists have studied aptamer-crosslinked networks, nucleic-acid amplification loops, CRISPR insertion, and nanomaterial in order to increase output. Platforms have now recognized small molecules, proteins and nucleic acids with a variety of readouts. But multi-stage amplification is often required in the highly sensitive systems itself, and in real samples they are vulnerable to the leakage reactions and to the inhibition of the system by the matrix. This means that sensitivity, stability and specificity are always held in trade-off, and there is no broad-based solution to this issue. Clinical translation Standardized financial schemes of evaluation such as reproducibility, interference examination, gold-standard comparison have not been well developed.

This is a systematic review of advancements in functionalized DNA hydrogel-based biosensing, critically evaluating methods of construction, implementation and signal readings, highlighting bottlenecks of complex-sample biosensing and future areas of priority clinical application.

2. Fundamental principles and characteristics of DNA hydrogels

2.1. Construction of DNA hydrogels

These two types of DNA hydrogel building are usually built through self-assembling of the DNA or by creating composites using traditional polymers and/or nanomaterials. DNA-only networks Y-shaped or X-shaped DNA building blocks may be self-assembled by complementary sticky-end hybridization. Alternatively, long strands of DNA could be made through the rolling circle amplification (RCA) technique and crosslinked into networks. One of the representative mode of construction entails polymerization of DNA gels on indium tin oxide (ITO) electrodes to come up with a fixed-shape form of a 3D network construction that could be used to biosense multichannel.

These construction paths involve obvious trade-offs. High programmability and flexible design, Pure DNA networks have limitations, due to the cost of synthesis and stability in biological systems. Hybrid/composite hydrogels offer better mechanical behavior and can allow more useful fabrication and manipulation, however, they generally compromise the ability to program network behavior solely by nucleic-acid sequence design and hybridization design.

Construction has direct control on the material threshold and the mass-transport window. The material threshold explains the nature of the network that is continuous in the hydrogel and in real sense, when there is gel-sol transition. This threshold is controlled by effective connectivity probability amongst crosslinking points in DNA hydrogels. In the case of biosensing, the threshold is the extent to which network disruption (e.g. crosslink cleavage or dehybridization) must be caused by a recognition event to produce a visible or otherwise measurable signal. Methods in practice that can be used to tune thresholds in the form of DNA fragment length variation, flexibility variation, and crosslink density variation.

Simultaneously, pore size and swelling behavior determines diffusion and release rates, and thus kinetics and time-to-result. Hydrogel based on thin films or paper reduce diffusion distances, and can further reduce response time. Porosity and water absorption affect infiltration, reaction limitations and reporters liberation in bulk gels, especially with large targets like proteins and nucleic acids. The geometric uncertainty can be minimized by immobilizing the hydrogel on specific interfaces (e.g., electrodes or silicon substrates), which constitute a method of enhancing reproducibility. As a whole, the design of DNA hydrogel biosensing systems necessitate an active

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balancing of stability of the network (threshold) against the performance of the network transport to ensure that the network will be stable under operation without necessarily diminishing the sensitivity to alteration of recognition to a measurable state change.

2.2. Physicochemical and biological properties

In addition to programmable architecture, DNA hydrogels may also give reversible responsitivity and manageable degradability, which can be used to create sensing opportunities. The mechanical characteristics of them mainly rely on the rigidity and density of DNA crosslinks. Microrheology measurements by Xing et al. indicate that with constant network topology, crosslink rigidity and decrease in the length of flexible chain segments have a significant positive effect on the elastic modulus and shear resistance [1]. This finding offers a design suggestion to biosensing: when robustness in operation (e.g. shear resistance during manipulation) is needed, stability can be enhanced by strive based molecular design (e.g. to enhance the crosslink rigidity).

Two significant sources of failure have been limiting with regards to biostability in vitro. The former is the nonspecific degradation by nuclease. Nucleic acids can be degraded randomly in biological samples e.g. serum and cell lysates by deoxyribonucleases (DNases) and ribonucleases (RNases). Depending on the extent of degradation, such degradation may pseudo-trigger gel collapse and give a false positive, or damage embedded amplification circuits and give a false negative. Notably, this instability too can be re-purposed as the sensing means. Xiong et al. presented infection-associated DNase activity as a source of signal and quantified early infection as change in dielectric-constant changes caused by DNase-mediated cleavage of DNA hydrogel (as shown in Figure 1) [2]. Although this design represents the method of transforming intrinsic instability into particular responsiveness, it is also necessary to note that the effects of endogenous nucleases should be strictly evaluated each time the target is non-nuclease-related.

Nonspecific interaction and signal drift caused by ionic strength and protein adsorption is the second substantial source of failure. The negative charge of DNA is very strong, and it is what facilitates the binding with the plasma protein, multivalent cations and nanoparticles that can modify the structure of hydrogel and ultimately the performance. Nonspecific binding of proteins can change the distributions of pore sizes and block mass transport, and salt differences can directly affect a hybridization stability and enzyme module activity (including CRISPR/Cas systems). These background effects have generally been known as some of the major impediments that need to be circumvented before DNA-hydrogel biosensors can find favorable application in a real sample. Consequently, antifouling design techniques, chemical modification, nuclease-resistance design techniques and matrix-matched calibration systems constitute key elements in realistic DNA-hydrogel sensor design.

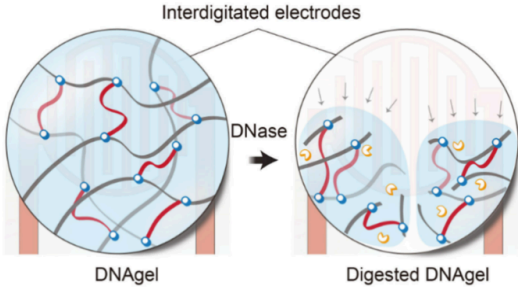

Figure 1. DNase-triggered dielectric sensing via DNA hydrogel degradation [2]

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3. Construction strategies of functionalized DNA hydrogels

3.1. The nucleic-acid-based functionalization is used to functionalize nanopolymers

A strategy directly based on aptamers as crosslinked hydrogel is Aptamer-crosslinked hydrogels: Binding of the target destabilizes the crosslinks, leading to the disassembly of the hydrogel. Critical design Threshold tuning is a critical design issue, ranging into becoming too thin to be connected to network connectivity. In cases where the binding energy of small molecules is not very high, high crosslink density will not allow any measurable output to be observed and low crosslink density will allow the system to be sensitive but unstable and inhibit false signals in complex samples.

The assay of nucleic-acid circuits (RCA, HCR, CHA) showed a significant increase in sensitivity due to cascade amplification. Nevertheless, leakage reactions of the circuit, without a target, are a significant problem. Also, small leakage may give positive threshold-based readouts. Sequence optimization, temperature handling, and distance-based readouts are mitigation measures that cannot fully remove them in biological matrices.

CRISPR/Cas systems can be used to gain sequence-specific recognition in combination with collateral cleavage to interrupt hydrogel crosslinks and generate measurable outputs. Although the use of upstream amplification (e.g. RPA) has been applied to many platforms in an attempt to increase sensitivity, optimised thin-film or paper-based designs have been shown to achieve amplification-free detection under specified conditions. The quantitative calibration and matrix inhibition are some of the challenges.

There are still challenges: numerous CRISPR systems need upstream amplification (RPA/RT-RPA) which means that they get more complex and miscellaneous; sample matrix inhibitors suppress the Cas activity; and quantitative models that would connect cleavage kinetics studies to hydrogel mechanics are still lacking. This difference makes the calibration challenging, since the indication of the threshold increases signal and uncertainty. Reproducibility of the outputs depends on systematic characterization of operating windows, matrix effects, and material mechanics, which are necessary to obtain clinically interpretable outcomes.

There is no doubt about the importance of emphasizing the functionalization of nanomaterials, which has achieved significant success in biology.

3.2. Nanomaterial-based functionalization

There is no doubt that the focus on functionalization of nanomaterials has been quite successful in biology.

The functionalization of nanomaterials is intended to amplify signal and/or support multimodal detection forms. To enhance readouts of output, classic methods entrap auxiliaries of gold nanoparticle (AuNPs), quantum dots (QDs) or catalysts (catalytic nanozyme).

An example of this is the AuNP-based colorimetry, in which disassembly of hydrogel releases AuNPs, and results in a visible change in color. It is an intuitively sensitive format. Nevertheless, the colloidal stability of AuNP is very much reliant on ionic strength, pH and protein adsorption; the non-optimal conditions may result in nonspecific aggregation, which causes spurious color changes. In order to enhance robustness, AuNP surfaces could be functionalized with polymers including polyethylene glycol (PEG) or with layers of nucleic-acid. However, the selection of buffers and sample pre-treatment should still be pertinent to minimize non-specific aggregation in intricate matrices.

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QDs or silver nanoclusters attached to the hydrogel can be used in order to enable fluorescence enhancement. Figure 2 illustrates that Xu et al. designed a target-responsive aptamer-Crosslinked DNA hydrogel with quantum dots (QDs) into the hydrogel to create a target-responsive avian influenza virus H5N1 [3] detection system. When the hydrogel was crosslinked in the absence of target, it was held in a shrunken form and QD fluorescence was suppressed. When bound by the virus, the crosslinks breaks off and hydrogel swelling and release of QD-labeled strands happened, therefore, regaining fluorescence. The system reached a detection limit of 0.4 HAU in the 30 minutes time depicting quick and field reproducible viral sensing. Fluorescence-based systems may also be highly sensitive, but their stability is also susceptible to quenchers, energy-transfer effects, and drift in the signal caused by the matrix, potentially necessitating calibration to the natural environment conditions to be reliable in its quantitative aspect.

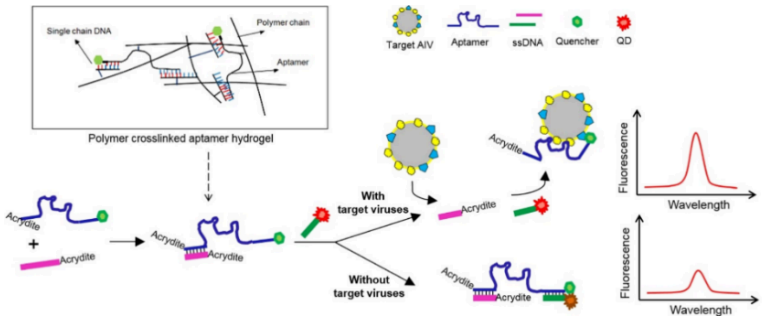

Figure 2. Working principle of a QD-based aptamer hydrogel sensor for AIV H5N1 detection [3]

Another way of amplification is through nanozyme catalysis. The hydrogel may be used in cases of detection of the environmental toxin microcystin-LR (MC-LR): the hydrogel can serve as a gate through which the release of the catalysts, the Cu/Au/Pt trimetallic nanozymes, can take place; and once the substrate is added, catalytic color formation can be observed with high sensitivity. Nevertheless, the activity of nanozymes may change significantly under varying pH, ionic-strength conditions and organic-matter backgrounds, so the activity of nanozymes has to be calibrated when used with each type of sample. On the whole, amplification of signals with nanomaterials may be extremely useful, although the degree of reliability in real matrices requires the accuracy of control over transitions between material states and the systematization of the influence of factors of an external environment.

3.3. Visible stimulus-responsive functionalization

Stimulus-responsive approaches are based on the use of certain environmental factors to produce a change in hydrogel to convert detection into visual, morphological or wireless signals. One well known class is comprised of enzyme-responsive designs, where the source of stimulus is the enzyme itself. Xiong et al. wound-infection sensor is based on infection-related DNase which cuts a DNA hydrogel and detects wound-infection with the help of dielectric-constant variations [2]. This is an explanatory reason: informative readouts can be produced through the utilization of endogenous sample characteristics even prior to the onset of overt clinical symptoms. The sensor is however intended to be applied to other targets, background DNase is an interferent, logic designs that are higher specificity or enzyme inhibition might be required to silence nonspecific activation.

In addition to nuclease responsiveness, hydrogel changes can also be caused by stimuli like pH, temperature and light. Examples are the incorporation of ultraviolet (UV)-sensitive bonds into the strands of the hydrogel structure so that the crosslinks can be broken by UV exposure to allow

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pollutant-oriented sensing, and the creation of a phase-change which is triggered by magnesium-ion exposure to detect phosphatase activity. Another common design idea in these strategies is to convert output on the basis of intensity signals to physical measures (distance or morphology) removing background noise [4]. Readouts based on distance to which the fixed length variations are applied tend to be mostly unresponsive to ambient light as well as to sample colorations.

In case of clinical translation, stimulus-responsive systems have to be sensitive to environmental conditions, consumable batch variance and long-term stability. Readout magnitude and time scale may be dependent on temperature and humidity variation and a difference in batch-to-batch variation of substrates or devices. In this regard, internal standards, standard operating procedures and high quality control are required to generate consistent strong comparable readouts.

4. Uses of functionalized DNA hydrogels in biosensing

4.1. Small-molecule biosensing

Small-molecule targets (e.g. toxins and drug residues) are characterized by bottlenecks such as small binding affinity, and by complex matrices. Apptamers or DNAzymes are also frequently used as recognition modules in functionalized DNA hydrogels, which are used to combine recognition and signal amplification.

Wang et al. as indicated in Figure 3 were able to come up with an aptamer-crosslinked polyacrylamide hydrogel with embedded AuNPs to detect melamine. Melamine interfered with the network and the hybrid release of AuNPs triggering a capillary based colorimetric detection limit to about 0.04 μM of melamine [5]. The platform combined a microfluidic chip, which streamlined assay geometry and readout conditions allowing better comparability through minimizing geometrical uncertainties. However, cross-calibration to a variety of optical instruments still needs to be performed, as differences in background can still affect reported sensitivity, and hence the practical use of LOD in any other environment.

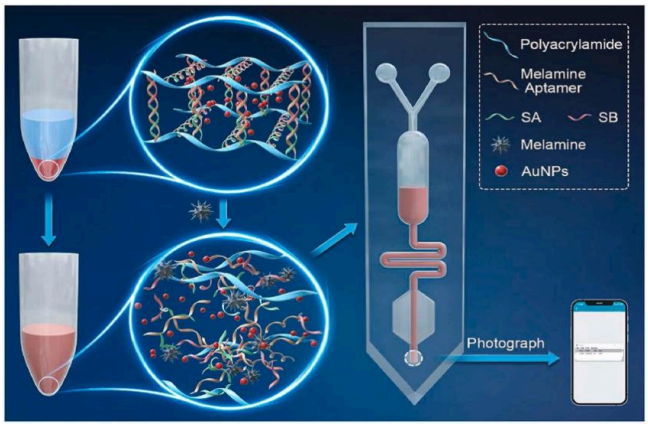

Figure 3. Schematic illustration of the aptamer-crosslinked AuNP-embedded hydrogel for capillary-driven colorimetric detection of melamine [5]

Another representative avenue is the nanozyme-based amplification. Wu et al. implemented a hydrogel-gated catalytic system of trimetallic nanoparticles made of Cu/Au/Pt and attained ng/L-level LOD of MC-LR [6]. Whereas performance is good in purified water samples, complex water bodies that harbor organic matter or metal ion may effectively compromise nanozyme performance. Foremost therefore, the quantitative precision relies on the environmental calibration of matrices and

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a practical application must take care to consider across conditions representative matrix conditions and not just on the tests performed in buffers.

In general, the representative small-molecule experiments indicate that DNA-hydrogel systems can be made to have a high sensitivity of analysis. The major practical concern is consistency in cross-samples and interference control in a variety of samples. It would only be possible to make high-sensitivity platforms practically useful once calibration schemes where the target matrix is explicitly considered are established.

4.2. Enzyme and protein biosensing

Additional constraints are found with protein targets (e.g. cancer biomarkers) and enzyme activities due to their larger size and a tendency to nonspecific adsorb. To this end, interfacial enrichment, magnetic separation, or multi-stage amplification are sensing strategies that are usually employed to enhance specificity as well as counteract matrix interference.

One method in alpha-fetoprotein (AFP) detection involves surface-enhanced Raman scattering (SERS) reporter hydrogel-mediated release in combination with magnetic-bead enrichment and quality LOD of about 50 pg/mL. The background can be economically supressed in the serum-derived background by the design, and the workflow presents numerous stages. Consequently, controlling the operational complexity and retaining the robustness should continue to be a serious direction of further optimization.

In a system applying PSA detection, Xu et al. integrated a DNAzyme in the hydrogel, in the occurrence of PSA, the gel disassembly liberates silver nanoclusters of aggregation-induced luminescence attributes with an LOD of 4.4 pg/mL. The design is not based on exogenous fluorescent enzymes or any form of added substrates, and the effectiveness of the design is affected by the stability of the silver clusters in the sample. Luminescence behaviour can be disturbed by serum proteins and salts, necessitating stabilisation measures and/or environment-specific calibration, to be confident about measurement. Procedures The detection of enzymes can also be mode to minimize optical background by means of readout distance. Jiang et al. suggested a uracil-DNA glycosylase (UDG) distance sensor, which operates under membrane filtration and a distance-output format and showed that they were feasible with cellular samples [7]. Furthermore, wireless infection sensor involves DNA hydrogels to control the dielectric characteristics of radio frequency identification (RFID) antenna, and allows passive, nonstop monitoring without power supply on board.

Taken together, such examples suggest that the key issues in protein/enzyme biosensing are an effective interference control and creation of biologically relevant thresholds. Clinically relevant ranges of concentrations and biologically interpretable endpoints should be matched with detection systems lest a low LOD cannot remotely be converted to clinical quantitative inference in real samples.

4.3. Nucleic acid and exosome biosensing

Ample application DNA hydrogels have been clinically developed in detecting nucleic-acids especially in infectious diseases and cancer diagnostics. The most interesting feature of CRISPR-responsive systems is the combination of sequence specificity with catalytic amplification to cause plain material responses.

Figure 4 reflects the Cas12aRNA-based detection of mecA gene in clinical samples, where Zhang et al. have determined that the concentration of Cas12aRNA-RCA is insufficient to detect fewer than

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10 copies/ uL of the gene [8]. The μReaCH-PAD system incorporates Cas12a into paper chips in order to detect fungal DNA, which is consistent with culture techniques. Thin-film geometries provide lower diffusion length and also provide distance based readouts with less instrument dependence.

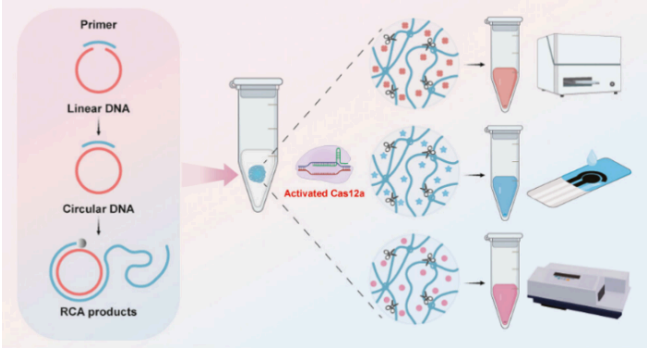

Figure 4. Schematic illustration of the Cas12a–RCA hydrogel-based nucleic acid detection system [8]

The amplification-free RNA amperometric detection of 100 aM of SARS-CoV-2 in cas13a-responsive RNA hydrogel, is comparable to RT-PCR SARS-CoV-2 detection in saliva and swab samples. These show ultra-low LODs but matrix inhibition and defined operating windows are key factors of concern.

Along with CRISPR-based approaches, nucleic-acid amplification circuit-based hydrogels regarding exosomal miRNA detection have been created [9]. Tang et al. introduced DNA-hydrogel columns in order to separate and subtype the exosomes [10]. Zou et al. performed exosomal miRNA detection based on hydrogelation-enhanced imaging ellipsometry with tunable range and enhanced sensitivity demonstrating that hydrogels can be used as both detecting and enriching medium [11].

One of the common needs across the applications is a standardization of sample handling and time of readout. Readouts based on the threshold (gel rupture vs. intact) are time dependent particularly when using leakage or inhibition. Inter-laboratory comparability and clinical validation require defined reaction windows, internal controls and matrix-matched calibration.

In general, the sensitivity of nucleic acids and exosomes using DNA-hydrogel strategies is suitable to high requirements. The challenges still remain on how to create a better batch-to-batch reproducibility, manage circuit leakage and over-amplification, and conduct more clinical validation using standardized protocols and gold-standard comparison.

4.4. Other targets and higher-order biological events

Higher-order biological events other than individual analytes have also been detected using DNA hydrogels and capitalizes on in situ enrichment and localized signal amplification. Indicatively, DNA hydrogels functionalized with antibodies have been utilized to entrap the immune cells in integrated, early-diagnosis approaches in the disease models [12]. Other studies also utilize hydrogels with tumor associated antibodies to trap recurrent tumor cells and provide recurrence warning on the basis of local adenosine triphosphate (ATP) metabolism variation. Such applications indicate that hydrogels also play a role as local enrichment and local signal amplification platforms, and not just as solution-phase sensors.

In higher-order targets, crucial performance indicators cannot be confined to solution-phase LOD, and encompass capture efficiency, temporal resolution, and specification of biologically

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meaningful thresholds [13]. In in vivo sensing applications, the calibration of dynamic-range to disease progression is necessary; otherwise, material readings will not be converted to diagnostic inference even when indicative of actual biochemical variation.

5. Conclusions and outlook

The main one is that functionalized DNA hydrogels in biosensing utilizing the principles of linking molecular recognition to material-state changes to allow bio-relevant diagnostic and monitoring capabilities [14]. In present literature, the study of hydrogel products is based on the interactions between strongly coupled molecules and materials; therefore, rational design must be clear in the behavior of network structure, diffusion routes, and matrix crossbreeding to transform the threshold-transport-interference phase diagram. Such mechanistic linkages can be clarified to enhance cross-platform comparison as well as translational robustness.

CRISPR recognition and distance-based readout thin-film hydrogels also appear to be a highly promising translational route [15]. Thin-film geometries help achieve operational stability by decreasing the transport ranges and minimizing matrix inhibition, whereas programmable CRISPR systems can be more specific. Nonetheless, quantitative mapping of hydrogel readouts to clinical endpoints, which are not only meaningful in clinical terms but also essential to improve, (like infection burden or tumor recurrence) is mandatory to leave qualitative detection behind.

The possibilities of longitudinal and dynamic monitoring are also extended by wireless sensing and implantable hydrogel interfaces. However, biocompatibility on the long-run, stability of signals and diagnostic thresholds that are clinically verified and valid should be strictly determined in order to guarantee its applicability in the real-world.

By contrast, strategies that have a high instrumental dependence, e.g. SERS multiplex assays and multi-layer amplification architectures, are more unfavorable to translational implementation because they are expensive, difficult to reproducibly scale, and have a strong chance of false-positive bias in complex matrices.

Future directions should focus on: (i) quantitative correlations between sensing results and clinical responses; (ii) physiologically compatible, degradable and enzyme-responsive designs in vivo; and (iii) uniform validation systems in complicated biological systems to ascertain reproducible and clinical interpretable functionality.

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