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Overcoming the limitations of COVID-19 diagnostics with nanostructures, nucleic acid engineering, and additive manufacturing

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ABSTRACT

The COVID-19 pandemic revealed fundamental limitations in the current model for infectious disease diagnosis and serology, based upon complex assay workflows, laboratory-based instrumentation, and expensive materials for managing samples and reagents. The lengthy time delays required to obtain test results, the high cost of gold-standard PCR tests, and poor sensitivity of rapid point-of-care tests contributed directly to society's inability to efficiently identify COVID-19-positive individuals for quarantine, which in turn continues to impact return to normal activities throughout the economy. Over the past year, enormous resources have been invested to develop more effective rapid tests and laboratory tests with greater throughput, yet the vast majority of engineering and chemistry approaches are merely incremental improvements to existing methods for nucleic acid amplification, lateral flow test strips, and enzymatic amplification assays for protein-based biomarkers. Meanwhile, widespread commercial availability of new test kits continues to be hampered by the cost and time required to develop single-use disposable microfluidic plastic cartridges manufactured by injection molding. Through development of novel technologies for sensitive, selective, rapid, and robust viral detection and more efficient approaches for scalable manufacturing of microfluidic devices, we can be much better prepared for future management of infectious pathogen outbreaks. Here, we describe how photonic metamaterials, graphene nanomaterials, designer DNA nanostructures, and polymers amenable to scalable additive manufacturing are being applied towards overcoming the fundamental limitations of currently dominant COVID-19 diagnostic approaches. In this paper, we review how several distinct classes of nanomaterials and nanochemistry enable simple assay workflows, high sensitivity, inexpensive instrumentation, point-of-care sample-to-answer virus diagnosis, and rapidly scaled manufacturing.

1. Introduction

Since the SARS-2 virus that causes COVID-19 jumped from an animal reservoir to humans in December 2019, it has rapidly spread across the world, bringing death, illness, disruption to daily life, and economic crisis to businesses and individuals. A key failure in the health system across nearly every country has been the inability to rapidly and accurately diagnose COVID-19, attributed to factors that include a limited availability of valid test kits, a limited number of certified testing facilities, high false negative rates, and a lengthy and expensive laboratory procedure to obtain a result and provide diagnostic information to the patient [1]. The challenges underlying COVID-19 diagnosis are typical and historically evidenced by repeated tragedies during previous newly emerging epidemic and pandemic infections, especially in

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detecting etiologic RNA viruses that constitute most of the high-impact human viral diseases [2,3]. COVID-19 diagnostic testing poses difficult challenges due to the high percentage of presympomatic and asymptomatic people who are capable of transmitting the virus to others, which, for impactful testing programs, requires frequent administration of sensitive tests, rather than only testing people after they experience symptoms before arriving at a testing center [4–6].

The time and expense associated with the currently dominant gold standard for virus detection stems from the requirement of detecting unique nucleic acid sequences in a specific viral genome. In order to access genomic information, stringent and technically challenging laboratory protocols are required for lysing the viral capsid, RNA extraction from RNA viruses, RNA reverse transcription (RT), and enzymatic amplification of specific nucleic acid sequences by polymerase chain reaction (PCR) or alternatives such as loop-mediated isothermal amplification (LAMP) [7]. Although such methods can be automated and performed with high throughput using sophisticated equipment, all the nucleic acid test (NAT) methods require complex chemistries, accurate temperature control, enzymes and their conditional buffer solutions, and many sample-handling steps. PCR-based approaches can suffer from high false negative rates (e.g., COVID-19 diagnostics [8]) due to a combination of a low amount of starting material (one genome copy per viral particle), instability of the RNA during extraction and RT processes, inhibitory substances in the test sample, and quality control failure of the many reagents.

Serological antibody and antigen testing is an important diagnostic tool for combating the COVID-19 pandemic [9]. Studies have shown that measurement of SARS-CoV-2 specific antibodies may be helpful for the diagnosis of suspected patients with negative RT-PCR results and for the identification of asymptomatic infections [10]. More importantly, antibody testing can detect both recent and prior infections, while playing vital roles in epidemiology studies [11]. Measuring the immune response against SARS-CoV-2 by antibody testing is an important tool for assessing the outcomes of patients, vaccinations, and understanding global prevalence. Quantitative assessment of SARS-CoV-2 antibody titer is especially important as clinicians and researchers more fully understand the patient-to-patient variability of immune response, in terms of the onset time for post-infection antibody production, and the post-recovery time that antibodies continue to be present [12]. As vaccines become available, it will be urgent for patients to know the extent to which their immune response has been stimulated. Furthermore, quantitative measurement of SARS-CoV-2 antibody titer in donated blood is important for blood transfusion therapy, which has shown promising results for treating patients with severe symptoms [13]. Meanwhile, detection of COVID-19 antigens, such as the spike protein, in noninvasively obtained fluids has become a widely adopted alternative approach for rapid COVID-19 diagnostic testing [14–17]. While several commercially available rapid antigen tests offer low cost, simple test procedures, and rapid time to result, their rate of false negative tests has proven largely unacceptable for mitigating the spread of COVID-19, and thus their adoption has been poor.

Currently, there are three methods that represent the most widely adopted serological COVID-19 antibody and antigen tests. The traditional enzyme-linked immunosorbent assay (ELISA) performed in microplates can quantify the different isotypes and subclasses of antibodies and has been used for the detection of COVID-19 antibodies against SARS-Cov-2 spike and nucleocapsid proteins [18–20]. Chemiluminescent immunoassays (CLIA) combine the immunoassay with photon-generating chemiluminescence reporters by using a luminescent molecule-labeled antibody [21]. CLIA systems have been developed for verifying the performance of commercial COVID-19 IgG and IgM antibodies testing kits [22]. Although ELISA and CLIA offer high sensitivity and specificity, both ELISA and CLIA-based workflows are time-consuming due to the multiple sample reagent handling and washing steps, while they also require a relatively large sample volume. The lateral flow immunoassay (LFIA) is a paper-based method that has emerged as a rapid diagnostic tool for point-of-care non-quantitative detection that offers benefits, such as low cost and ease of use [23]. However, while a number of commercial LFIAs have been rapidly developed for measuring COVID-19 IgG, IgM, and spike proteins qualitatively [24,25], the sensitivity of LFIA is relatively low (ng/mL level) and it lacks the capability for quantitative analysis. Due to the limitations of ELISA, CLIA, and LFIA, there is an important unaddressed gap in the currently available technologies for quantitative and simple SARS-CoV-2 serology testing. Ideally, the diagnostic workflow would require a single step, an inexpensive/portable detection instrument, an inexpensive/disposable assay cartridge, and only a fingerstick quantity of serum.

Due to the urgency for developing and deploying solutions that could address the limitations of the existing predominant technologies for diagnosing highly infectious respiratory diseases, many of the approaches commercialized over the past year represent incremental changes to the methods that were in use before the SARS-CoV-2 pandemic. For example, injection-molded microfluidic cartridges were utilized to enable the laboratory-based protocols for PCR and LAMP to be performed in point of care settings, and modified protocols were explored that could simplify or eliminate some of the steps required for sample preparation. The purification step was bypassed for some PCR [26] and LAMP [27] protocols, and the 1-copy/μL sensitivity was achieved with LAMP-based assays for detection of SARS-CoV-2 in VTM and saliva [28]. Modified assay protocols for PCR, LAMP, and ELISA were introduced, while alternative assays that utilized enzymatic nucleic acid amplification processes were demonstrated, such as those based upon CRISPR/Cas technology as described in a recent review [29].

Despite the deployment of vaccines in 2021, the need for more effective COVID-19 diagnostic testing has not diminished, and in fact a return to a more normal environment for education, entertainment, travel, exercise, and socialization is going to require many aspects of testing to become less invasive, less costly, simpler, and faster, without compromising sensitivity and accuracy. As the underlying causes of the emergence of SARS-CoV-2 continue to be explored, it is likely that the interfaces between human civilization and nature will continue to generate pathogens with unanticipated and undesirable characteristics, and thus society would be best served by becoming better prepared for future pandemics.

Our research has been driven by the hypothesis that novel approaches for infectious pathogen diagnostic testing that meets societal and clinical needs will be uncovered by investigating material systems and technologies that will address the technological gaps in fundamentally new ways, rather than through incremental improvements of existing methods (Fig. 1). Therefore, we sought not only to perform diagnostic testing with novel biochemistry, sensing transducers, and instrumentation, but to also consider how the technology can be brought rapidly and inexpensively to mass production. In this paper, we summarize our efforts over the past year to implement rapid, simple, inexpensive, and sensitive detection of SARS-CoV-2 pathogens, as well as SARS-CoV-2 antibodies and antigens. While the research summarized here represents a variety of approaches and technologies, the common thread is the use of novel materials at the nanoscale level to enable new functional capability and manufacturability.

2. Graphene as electronic biosensors for rapid detection of DNA molecules and PCR amplification

Electrical-based devices are an attractive option for SARS-CoV-2 detection as these devices do not require fluorophores or optical components to realize the detection assay. Electrical biosensors could lead to a physically smaller and inexpensive system that can be used at the point-of-care. These electrical-based solutions can have impact on the development of antigen tests where improvement in sensitivity is required. Although antigen tests are faster than PCR techniques since they can provide results in a few minutes, antigen tests are inherently

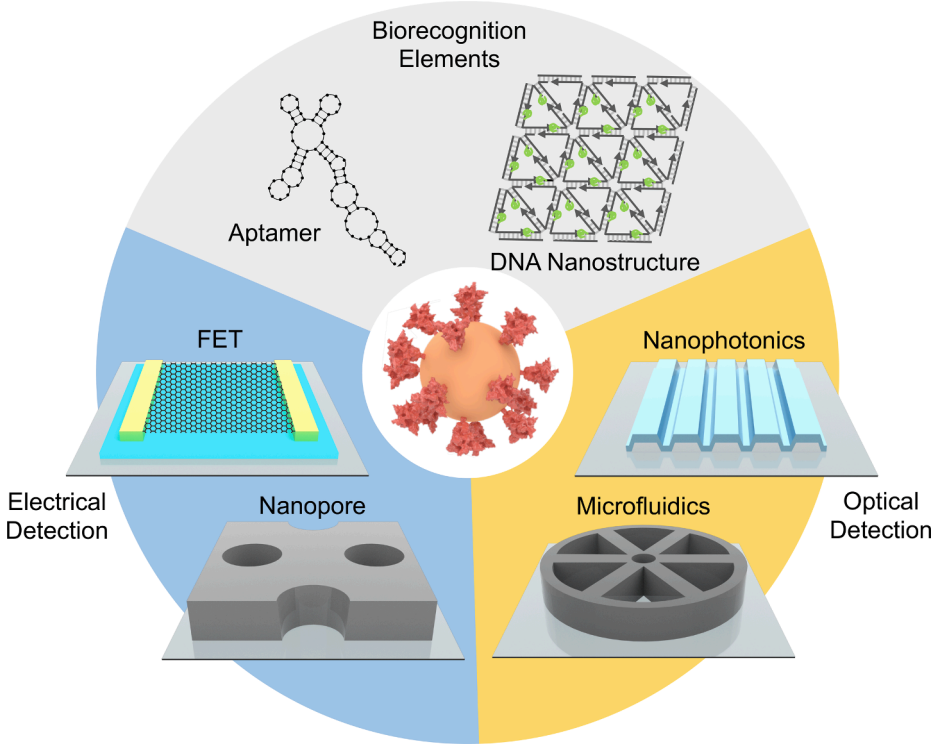

Fig. 1. Novel nanostructures and nanotechnologies for SARS-CoV-2 detection.

less sensitive as no amplification of the target is involved. Likewise, these tests provide qualitative results only (they do not quantify the viral load in the sample). Also, antigen devices have a high false-negative rate [30]. This means that a negative test result may occur if the level of antigen in a sample is below the detection limit of the test.

To improve sensitivity, several examples of new antigen detection technologies can be found in the literature as efforts towards point-of-care (POC) solutions. In this field, the use of graphene for electrical detection has been reported due to its superior properties in terms of high charge mobility and surface area [31–33]. For instance, Kim et al. reported a field-effect transistor (FET)-based biosensing device for detecting SARS-CoV-2 in nasopharyngeal swab specimens [31]. The device used an aqueous-solution-gated FET configuration using the antibody-conjugated graphene to detect the SARS-CoV-2 spike protein with a limit of detection of 242 copies/mL in clinical samples. In other example, Alatraktchi and team used a graphene working electrode, functionalized with anti-spike antibodies, to detect the signal perturbation obtained from ferri/ferrocyanide measurements after binding of the antigen with a 45-minute incubation time [32]. Likewise, graphene-based platforms have also been used for the multiplexed and portable detection of nucleocapsid protein (SARS-CoV-2), specific immunoglobulins against SARS-CoV-2 spike protein, and C-reactive protein [33]. More recently, Zhang et al. developed a rapid (2 min) and unamplified nanosensing platform for detection of SARS-CoV-2 RNA in human throat swab specimens [34]. In this approach, a gold nanoparticle (AuNP)-decorated graphene field-effect transistor (gFET) sensor was fabricated, after which a complementary phosphorodiamidate morpholino oligos probe was immobilized on the AuNP surface. This sensor allowed for testing of SARS-CoV-2 RdRp gene with limits of detection of 0.37 fM in PBS buffer (corresponding to 223 copies/μL), 2.29 fM (in throat swab), and 3.99 fM (in serum).

Graphene-based devices can also be combined with direct hybridization techniques to develop molecular POC devices for detection of SARS-CoV-2 Viral RNA without using RNA amplification methods. For instance, Pan and team developed an electrochemical [35] biosensor where the SARS-CoV-2 RNA was detected without nucleic acid amplification. In this example, a rapid (5 min) graphene-based electrochemical biosensor was developed where electrical signals amplified from AuNPs rather than molecular RNA amplification [35]. These AuNPs were capped with highly specific antisense oligonucleotides (ssDNA) targeting the viral nucleocapsid phosphoprotein (N-gene) from extracted RNA. The sensing probes of the nucleic-acid-testing device were immobilized on a paper-based electrochemical platform. In this work the total RNA was extracted and purified from the cell lysate.

2.1. *Crumpled Graphene-based biosensors*

Graphene field-effect transistors (gFETs) and their application for early detection of viruses, have significant potential due to their sensitivity and fast response [36]. However, the use of crumpled (deformed and bent) gFET-based electrical biosensors has not been explored in this field yet. These devices have recently demonstrated high sensitivity capabilities for detection of nucleic acids [37,38]. We (Hwang, Bashir, et al.) have demonstrated the ultra-sensitive detection of DNA/RNA molecules (LOD = 600 zM) on millimeter scale structures [37]. We showed how the Debye length in the ionic solution is increased at the convex region of the crumpled graphene, and therefore, results in more of the DNA being within the Debye length (Fig. 2). This makes the crumpled graphene more electrically sensitive to the negative charge of DNA. We hypothesized that although graphene does not have an intrinsic bandgap, crumpled graphene may open a bandgap, allowing an exponential change in the source-drain current from small numbers of charges. This opening of the bandgap contributes to the reported sensitivity of detection.

As an additional application of crumpled gFET devices, we have also demonstrated the indirect detection of *E. coli* DNA by detecting reduction in the specific primers in a LAMP assay [38]. The team took the advantage of the fact that graphene adsorbs single-stranded DNA due to noncovalent π–π bonds, but not double-stranded DNA. Using this functionalization-free method, we were able to electrically detect the amplification of starting concentrations down to 8×10^{-21} M when combined with crumpled gFET devices. Therefore, we consider that this

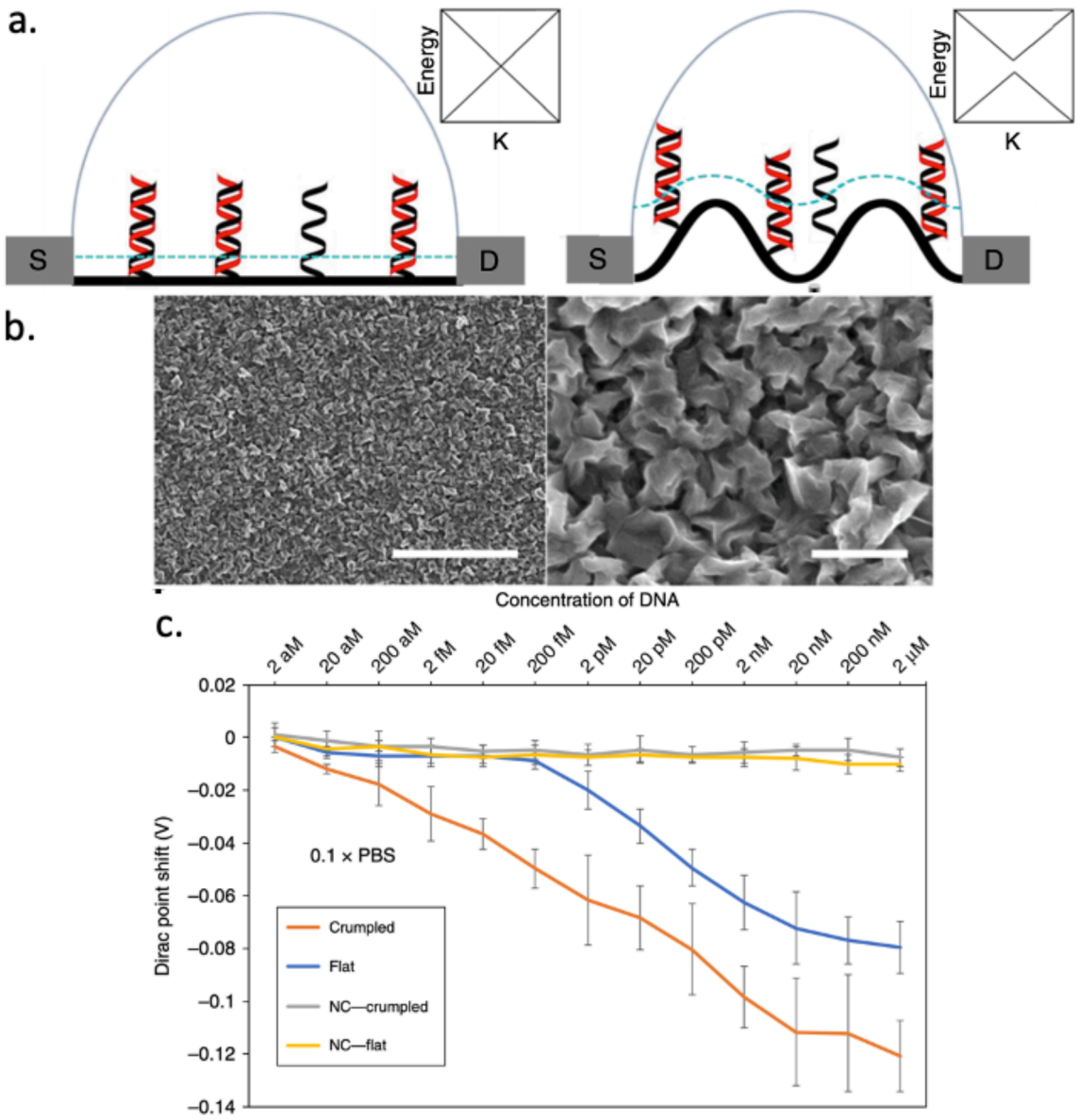

Fig. 2. Crumpled graphene FET biosensor. (a) Cross-sectional scheme of the flat (left) and crumpled (right) graphene FET DNA sensor. The blue dot lines represent Debye length in the ionic solution and the length is increased at the convex region of the crumpled graphene, thus more area DNA is inside the Debye length, which makes the crumpled graphene more electrically susceptible to the negative charge of DNA. The inset boxes represent qualitative energy diagram in K-space. Graphene does not have intrinsic bandgap. However, crumpled graphene may open bandgap. (b) SEM images of crumpled graphene. The scale bar is 5 μm (left) and 500 nm (right). (c) Dirac voltage shift of the FET sensor with detection of hybridization using DNA probe. NC is non-complementary control sequences used in the experiments. Extracted from [37] under a Creative Commons Attribution 4.0 International License http://creativecommons.org/licenses/by/4.0/.

technology has the potential for successful application to the detection of SARS-CoV-2 and other pathogens.

3. Designer DNA nanostructure-based virus particle capture probe

The current model of antigen assays continues to demonstrate fundamental limitations, rooted in workflow complexity and insufficient viral binding avidity, which in turn impacts the cost, practicality, and detection sensitivity for creating rapid and ultrasensitive antigen-based virus diagnostics technology. Many viruses, including SARS-2, present a unique spatial pattern of surface antigens. Such antigen features can be selectively captured by different antigen-targeting binders such as aptamers, antibodies, nanobodies, and peptides. We have recently designed and synthesized a star-shaped designer DNA nanostructure (DDN) to display 10 dengue virus (DENV) envelope protein-targeting aptamers in a 2D-pattern precisely mirroring the complex spatial arrangement of DENV epitopes [39]. DENV was chosen as a representative target because its epitopes represent the most complex spatial pattern among all known viruses. The DENV envelope protein domain III (ED3) is organized into a polyhedral pattern with alternating clusters of trivalent or pentavalent ED3 sites [40]. By connecting the clusters of ED3 sites linearly, we determined that a star-shape, consisting of an interior pentagon connected to 5 exterior triangles, would provide an optimal polyvalent scaffold (Fig. 3a). Based on this structural information, a 5-point, star-shaped DNA scaffold was designed to not only match the local, orthodromic distances between ED3 clusters, but also to mirror the global pattern of ED3 clusters aligning with the star's 10 vertices. Shown in Fig. 3b, each of the designer star's 10 external edges is 42-bp (base-pairs) long and connected to the internal edges through 4-arm junctions at the inner vertices. Each internal edge has a distance that fits the distance between adjacent trivalent clusters. The 5-point star was

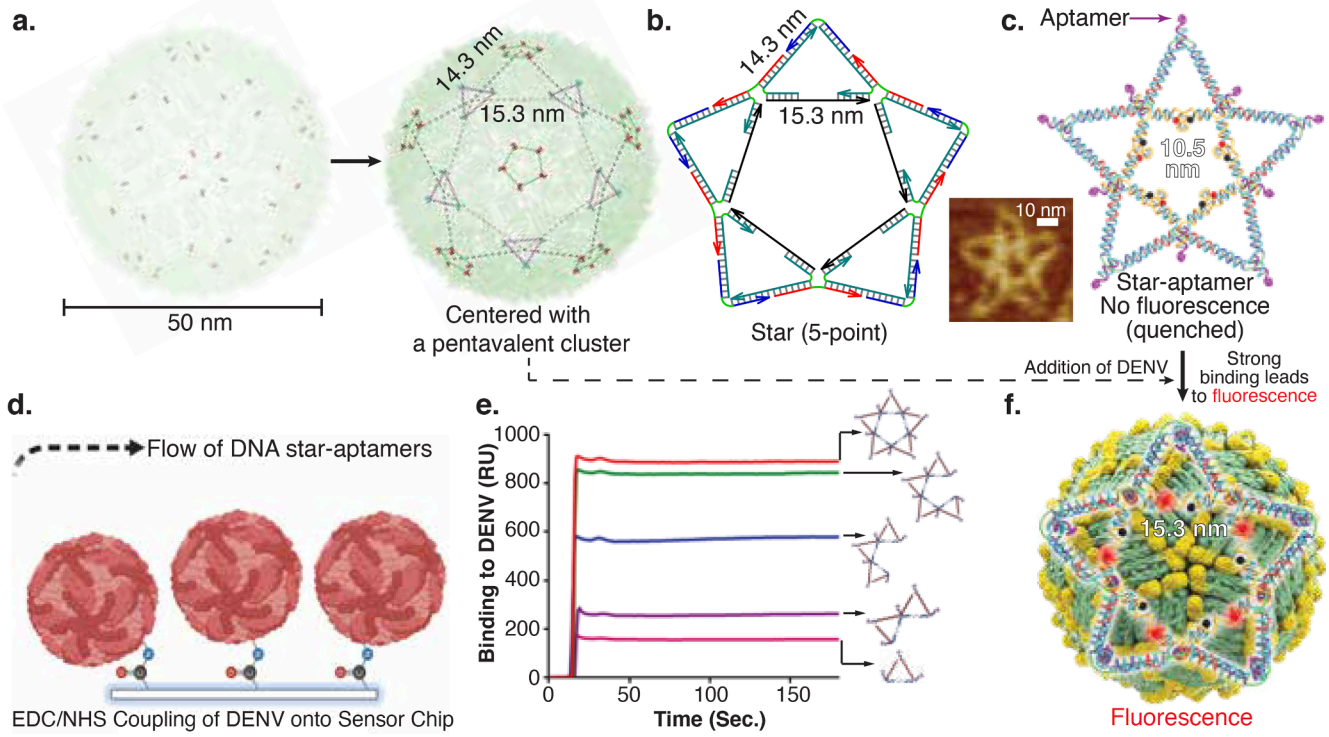

Fig. 3. Dimensional pattern analysis, scaffold design and DENV binding and sensing by DNA star strategy. (a) Distribution of DENV ED3 clusters. The diameter of a virion is 50 nm. Orthodromic distances between trivalent-trivalent and trivalent-pentavalent clusters are 15.3 nm and 14.3 nm, respectively. (b-c) The DNA star scaffold (proved by AFM imaging) is designed to incorporate 10 aptamers to match the pattern and spacing of ED3 clusters. Each DNA strand is indicated by an arrow going from the 5′ to the 3′ end. Five fluorophore–quencher pairs along the inner pentagon of the star remain in a quenching fluorescence resonance energy transfer. (d) Schematic of the SPR assay used to determine the viral binding avidity. (e) 1–4 triangle-containing and full-star were used with normalized aptamer concentrations. Better pattern matching (by adding more triangles to match the correct geometry) leads to stronger binding avidity based on relative SPR signal units. Aptamer binding avidity was used as the baseline. (f) When DENV is present, binding interactions between aptamers and ED3 domains unzip the hairpins into single-stranded (ss) DNA, enabling a fluorescent readout. a, b, d, e, extracted from [39].

functionalized for DENV binding by hybridizing a ED3-targeting aptamer [41] at each of the 10 vertices of the star to form a star-aptamer construct that geometrically matches and targets ED3 clusters (Fig. 3c). Assayed by SPR using monovalent aptamer-DENV binding signal as the baseline (Fig. 3d), binding strength increased as aptamers were placed onto scaffolds which increasingly matched the pattern of ED3 sites on DENV with the full star showing the greatest binding avidity (Fig. 3e). Viruses often escape binding by monovalent/bivalent binders such as aptamers and antibodies because the binding strength of monovalent/bivalent binders to proteins on a viral or cell surface is often weak [39,42,43]. However, the “DNA star” capture probe provides polyvalent and spatial pattern-matching interactions, affording dramatic improvement in DENV-binding avidity. Thus, polyvalent, pattern-matching interactions are critical to secure the binding of virions at very low viral loads. Carrying oligos, 6-FAM (fluorophore) and BHQ-1 (quencher) were hybridized to each inner edge, flanking the hairpin to turn the DNA star-aptamer complex into a viral sensor by forming molecular beacon like motifs (Fig. 3c). The potent polyvalent interactions with intact DENV, promoted by the matched geometric aptamer-ED3 pattern, separated the FAMs from BHQ-1s to provide a fluorescent readout (Fig. 3f). Our approach can detect intact DENV in patient samples with high sensitivity in <2 min and at a cost <$0.15, affording a limit of detection (LoD) of 100 pfu/mL which is comparable to RT-PCR sensitivity.

The above DDN-based approach can be applied to create SARS-CoV-2 virus particle capture probes by generating the requisite “binder” patterns with customized DNA architectures that afford maximum SARS-CoV-2 binding avidity *via* polyvalent, pattern-matching interactions. Importantly, instead of using a fluorophore, which is subject to photobleaching and delivers low signal-to-noise ratio, to report detection, we will utilize a newly invented form of biosensor microscopy called Photonic Resonator Interference Scattering Microscopy (PRISM) in which the photonic crystal surface amplifies laser light scattering from captured intact virions, enabling each one to be counted with high signal-to-noise ratio [44]. Such “digital” counting of intact virions offered by PRISM ensures high sensitivity, which is critical for the early detection of any virus. Moreover, directly detecting intact SARS-CoV-2 virions may also enable COVID-19-positive individuals to know when they are no longer infectious and can come out of quarantine, an added benefit over nucleic acid tests which are known to generate false positive results from the presence of nucleic acid molecules in degraded viruses [45,46].

Based on the structure and arranged pattern of the SARS-CoV-2 trimeric spike proteins on the outer surface of the virus particle (Fig. 4a), we plan to customize a DDN-based capture probe whose vertices precisely match the intra- and inter-spatial pattern of SARS-CoV-2 trimeric spike glycoprotein clusters (Fig. 4b). The DDN probe will integrate an array of SARS-CoV-2 spike-targeting binders that are designed for high affinity and specificity binding with spikes in a polyvalent, pattern-matching fashion. Like a fishing net in water, the DDN probe has mechanical flexibility to form a convex shape in solution [39,47,48]. When spherical virions are present in a patient sample, the photonic crystal (PC)-immobilized DDN probe will curve itself to fit the virus particle curvature and thus promote the interactions between structurally patterned spike-targeting binders and the viral spikes. To rapidly count each captured virus, we will use PRISM biosensor microscopy in which the PC surface, when illuminated with a laser that matches its resonant coupling condition, enhances the light scattering from DNA net-captured virus particles by up to 50x, enabling high signal-to-noise observation and rapid counting of each attached virus

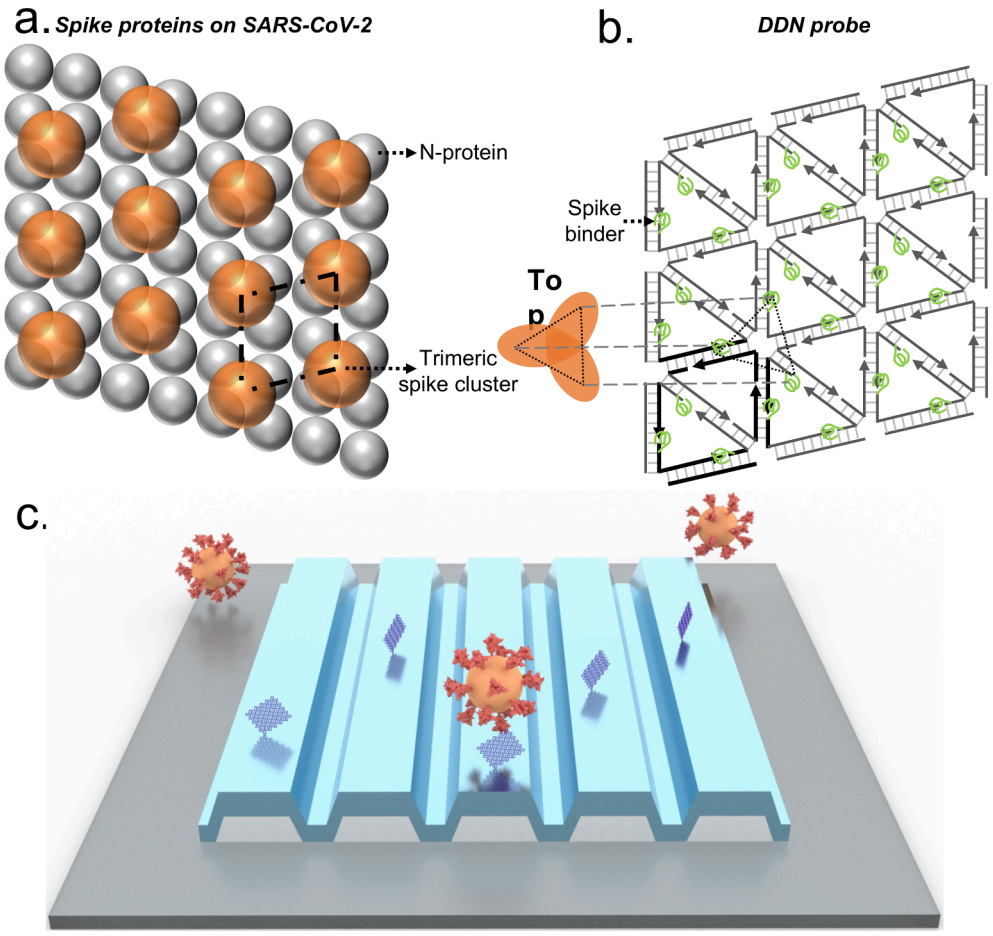

Fig. 4. SARS-CoV-2 virion sensing using PRISM. (a) Spatial arrangement of SARS trimeric spike proteins and nucleoproteins (N). (b) The DDN probe can be designed and synthesized to match the pattern and spacing of trimeric spike clusters. 3′ end of each DNA oligo is indicated by an arrow. Three spike-targeting binders wiil be placed on each vertex. (c) The PC incorporates active sensing regions with DDN probes attached to the active region for selective capture of SARS-CoV-2 virions.

(Fig. 4c).

4. Photonic crystal optical metamaterials enable new form of microscopy and digital-resolution ultrasensitive sensing of COVID-19 antibody in serum

We recently developed a single-step, wash–free, digital immunoassay for 15-minute rapid analysis of serological COVID-19 antibody (human COVID-19 IgG) by adapting the principle of "Activate Capture + Digital Counting (AC + DC)" [49]. AC + DC assay uses a PC biosensor coupled with a new form of microscopy, called photonic resonator absorption microscopy (PRAM)[50-52]. We initially developed AC + DC assay for ultrasensitive detection of cancer-specific microRNA with digital resolution and single-based selectivity without target amplification and washing steps [50]. This assay principle was further utilized to develop a PC-based microfluidic biosensing platform for quantitative HIV viral load assay with only a 35-minute process [51].

The design of the developed AC + DC assay for serological human COVID-19 IgG test is schematically illustrated in Fig. 5A. Secondary antibody-functionalized gold nanoparticles (2^{o}Ab-AuNPs) are prepared by covalently conjugating urchin-shaped AuNPs with anti-human IgG Fc secondary antibodies via EDC/NHS chemistry and heterobifunctional HS-PEG-COOH linkers. After mixing 2^{o}Ab-AuNPs with human COVID-19 IgG sample in a tube, we immediately apply the mixture on the spike protein-coated PC biosensing platform for AC + DC assay. COVID-19 IgGs will activate the system by binding either functionalized AuNPs or the spike protein-coated PC through specific antigen–antibody interaction, resulting in the subsequent capture of COVID-19 IgG-AuNP complexes or AuNPs to form sandwich immunocomplex on the PC. We then conduct PRAM imaging for quantitative measurement of human COVID-19 IgG by digitally counting the number of bound AuNPs on the PC (Fig. 5B), where each AuNP in the PRAM image represents one IgG target molecule.

We have demonstrated the capability of the method for quantitative analysis of serological human COVID-19 IgG in 15 min, with digital resolution and high sensitivity (Fig. 5C). The AC + DC immunoassay shows a high sensitivity (calculated limit of detection: 26.7 pg/mL) and a broad detection range of 100 pg/mL–100 ng/mL, which perfectly matches the clinically relevant concentration range of COVID-19 antibody. The high sensitivity of our method rivals that of ELISA or CLIA (typically 1–100 pg/mL) [53] and is >50 times more sensitive than commercial pGOLD High Accuracy IgG/IgM Assay Kit (~1.6 ng/mL). In terms of assay time, our 15-minute assay method is comparable to LFIA and substantially more rapid than ELISA and CLIA (>2 h)[19,54,55].

Our PRAM-based AC + DC immunoassay exhibits many attractive features. First, no pre-incubation, signal amplification and washing steps are required, enabling a single-step, 15-minute rapid test with a simple assay cartridge and easy operation. Second, the assay requires only a fingerstick quantity of serum (~4 μL), which is compatible with minimally invasive sample collection and well suited for point-of-care (POC) testing. Furthermore, this method can be extended to develop universal diagnostic platforms for multiplexed detection of a variety of analytes (e.g., antigens, cytokines, and other biomarkers) [56,57]. This work represents the first utilization of AC + DC assay for rapid quantitative analysis of serological COVID-19 antibodies, demonstrating a route toward point-of-care testing, using a portable detection instrument.

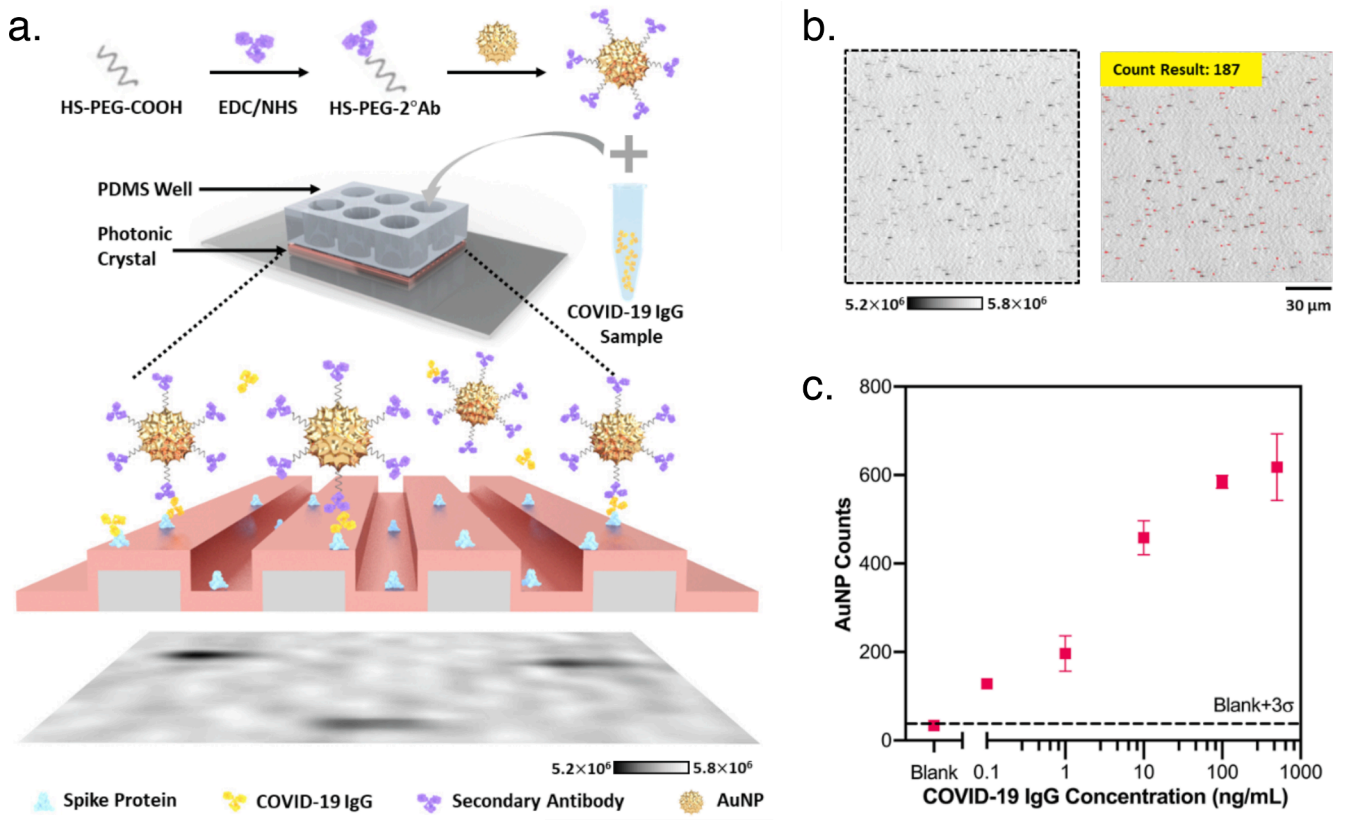

Fig. 5. AC + DC assay for rapid quantitative analysis of COVID-19 IgG in human serum. (a) Schematic illustration of PRAM-based AC + DC immunoassay. Highly cross-adsorbed secondary antibodies are conjugated with 80 nm diameter gold nanourchins via EDC/NHS chemistry and heterobifunctional HS-PEG-COOH linkers to prepare secondary antibody (2°Ab) functionalized AuNPs. Then, the COVID-19 IgG sample in a tube was added to a reagent comprised of 2°Ab-AuNPs and the mixture is immediately introduced into a PDMS well with spike proteins coating a photonic crystal (PC) surface, followed by PRAM imaging with digital resolution. (b) Digital counting of bound particles with a watershed algorithm in one representative peak intensity value (PIV) image generated by PRAM. The detected individual particles are indicated by red dots. (c) Quantification of serological COVID-19 IgG concentration by digital counting of AuNPs in AC + DC immunoassay. The assay time is 15 min. The dash line represents for the threshold (blank signal + 3 standard deviations). Reproduced from [49], with permission from Elsevier.

5. Aptamer sensors for differentiation of infectious from non-infectious SARS-CoV-2

DNA aptamers are short single-stranded DNA oligonucleotides that can fold into complex secondary and tertiary structures to bind to a target molecule with high affinity and specificity [58,59]. They can be isolated using an *in vitro* process called Systematic Evolution of Ligands by EXponential enrichment (SELEX) [60–63]. Based on their recognition capability, aptamers are often compared with their protein analogues, antibodies, however aptamers have shown to be powerful alternatives to them [64]. In addition to comparable target-binding affinities with antibodies, DNA aptamers have higher batch-to-batch consistency and stability. Moreover, DNA aptamers have shown several distinct advantages [62,65], especially for SARS-CoV-2 detection: (1) DNA aptamers are isolated in test tubes in much shorter periods (<1 month) than isolating antibodies from cell lines or animals (~6 month). This fast development cycle gives aptamers unique advantages for timely response to new emerging viruses like SARS-CoV-2 or new subtypes of viruses; (2) A major advantage of SELEX is the use of counter selection to improve the selectivity of the aptamers against competing targets, such as other viruses [66] or different subtypes of viruses [67].

Because of the advantages mentioned above, several DNA aptamers able to bind to proteins from SARS-CoV-2 have been published recently. Luo and collaborators have selected DNA aptamers that bind to different regions of the nucleocapsid (N) protein in SARS-CoV-2 [68]. Most other groups have been focused on selecting aptamers that bind to the spike (S) protein in SARS-CoV-2. Yang and collaborators have reported DNA aptamers that bind to the receptor-binding domain (RBD) of SARS-CoV-2′s S protein [69,70]. In addition, Mayer and collaborators have used trimeric S protein to select a DNA aptamer that can bind to the full S protein and inhibit pseudotyped SARS-CoV-2 infections [71]. All these efforts used purified proteins as SELEX target and the affinity of the aptamer obtained range from 0.5 to 5 nM for N protein and 3 to 100 nM for the S protein.

These aptamers can be incorporated to test for specific detection of SARS-CoV-2 proteins. This feature is shared with most COVID-19 tests where individual components of the virus such as viral nucleic acids or viral proteins are detected. However, the detection of these components does not indicate that intact infectious viruses are present, thus these tests do not inform on how contagious the patient is. The gold standard methods for direct detection of viruses that also can assess infectivity continue to be microbiology techniques involving cell culture, namely plaque assays [72]. However, culturing the virus requires growing the virus within host cells and takes several days to grow plaques, which increases the required labor, expertise, and equipment. Furthermore, performing these assays with SARS-CoV-2 is quite challenging, given the difficulties in culturing an emerging virus from clinical specimens during a pandemic. Moreover, RT-PCR and other recent tests based on detection of the viral RNA [27,73] have shown poor correlation with infectivity. For instance, SARS-CoV-2 viral RNA can remain detectable in some patients for more than one month after onset of illness, while viable virus could not be detected by culture after Week 3 in mild cases of COVID-19 [74,75]. Without a rapid and simple method that can tell if the patient is contagious, the World Health Organization has amended guidelines for releasing COVID-19 patients from isolation (i.e., 10 days after symptom onset), based on generalizing information obtained by a

few studies where cell culture assays have been performed [74]. However, some patients with mild COVID-19 have been reported to maintain viral viability for more than 10 days after the onset of symptoms, while patients with severe COVID-19 can maintain viral viability up to 32 days [76]. Despite many years of research and numerous publications, few existing methods can provide a rapid, accurate and robust information about infectivity, i.e., if a person is still contagious or not to avoid continue spreading the virus. In addition, viruses present in environmental samples, such as air, water, or different surfaces, can also be a major route for spreading infection. However, very few rapid tests are available to distinguish between the viruses that have been rendered noninfectious (inactivated) by a disinfection method from those that are still infectious (active).

Developing DNA aptamers that can differentiate infectious from non-infectious viruses opens the possibility to incorporate these versatile and highly selective recognition elements in multiple materials to develop portable and rapid tests able to assess virus infectivity. DNA aptamers can easily be attached to materials [77–79] and other molecular-signaling groups [80,81] to develop biosensors due to the plethora of DNA modifications available. Moreover, the relatively low-cost synthesis and intrinsic DNA chemical stability makes it an ideal recognition element for sensing.

While several aptamers specific to individual SARS-CoV-2 proteins have been reported [68–71], they lack the ability to identify infectivity virus status. To achieve this high level of selectivity, it is necessary to develop a recognition element that exclusively responds to the intact whole-viruses in their native state, i.e., without additional modifications on the superficial protein moieties involved in the infection process. DNA aptamers with excellent selectivity to infectious virus can be obtained based on the careful design of the selection pressures. We have demonstrated that it is possible to obtain highly specific DNA aptamers able to differentiate infectious from non-infectious viruses, such as SARS-CoV-2 [82]. First, to obtain a molecule with the capability of recognizing exclusively infectious, but not noninfectious SARS-CoV-2, we have taken advantage of DNA aptamer selection being an *in vitro* and highly versatile process. We used counter selection steps to remove sequences of DNA that bind the same virus that has been rendered noninfectious by common disinfectants such as UV-light or chemical disinfectants, as well as other viruses, to obtain a highly selective aptamer against infectious SARS-CoV-2.

While it is critical to use the whole virus as the target of the SELEX process to detect intact viruses, allowing for the selection of an aptamer that recognizes the spike protein in its native conformation, emerging viruses such as SARS-CoV-2 require a biosafety level 3 lab to handle the virus. Thus, we have used pseudotyped viruses to retain the advantage of a whole-virus selection while removing the restriction of having to use a biosafety level 3 lab. Pseudotyped viruses can be generated from a lentivirus (HIV) that displays the SARS-CoV-2 spike (S) protein within the viral envelope, and thus closely mimics the surface and entry mechanism of SARS-CoV-2 but are defective in continuous viral replication [83,84]. For intact virus detection, using pseudovirus represents two major advantages. The pseudovirus is a better mimic of real SARS-CoV-2 when compared to the purified proteins since the surface residues exposed are similar to those in SARS-CoV-2. As a result, aptamers selected using pseudovirus will bind only to the surface residues of spike protein of SARS-CoV-2, not to those that are not accessible, such as those embedded within the proteins in the envelope from the pseudovirus. In addition, since the spike protein assembles on the surface of the virus as trimmers [85], our aptamers should bind the native ensemble of conformations visited by the spike protein within the viral surface, which is not well represented by individual spike proteins in solution.

5.1. Nanopore sensors for selective and sensitive detection of COVID-19

As discussed earlier, the SELEX process enables the development of DNA aptamers with outstanding selectivity. However, these aptamers do not necessarily have enough affinity to reach the ultra-high sensitivity required for virus detection. Thus, to meet this ultrahigh sensitivity, aptamers can be easily integrated into different sensors that involve amplification steps, such as isothermal amplification steps [86–88], or nanomaterials with the capability to enhance the signal upon molecular recognition. Particularly, the incorporation of aptamers to solid-state nanopores, where the size of the pore is similar to the virus, allows a selective detection and amplification by confinement of the virus [89,90]. Amplifying the signal though confinement does not require additional steps, reactant, and time to perform the test, but does require precise control of the size and shape of the synthesized nanomaterial.

Solid state nanopores have a remarkable ability to control and manipulate the transport of chemical and biological species flowing through them. Recently, nanopores have been applied for SARS-CoV-2 detection. Using the pulse-resistive approach, nanopores have been applied for SARS-CoV-2 detection in saliva specimen, achieving a sensitivity of 90% with a rapid (5 to 15 min) measurement on a portable device [91]. In this work, to address the lack of selectivity of the nanopore itself [92], machine learning was implemented to achieve 96% selectivity. Another option is to incorporate selective recognition elements to the nanopore. For instance, synthetic peptides have been incorporated into the nanopore to gain selectivity against influenza virus A (H1N1)[93]. However, with the pulsing-resistive approach, the current signature detected still requires complex analysis.

On the other hand, single asymmetric nanochannels sensors in polymeric thick membranes can be characterized through steady-state current–voltage (I-V) measurements by sweeping the transmembrane potential at low enough frequencies (<0.1 Hz). This method significantly simplifies the signal detection and allows for direct detection of binding events that occur in sensing elements upon target recognition [94,95]. Moreover, the steady-state I-V curves contain precise information that is essential for quantification, background subtraction and identification of potential interferences. We have demonstrated that highly selective DNA aptamers can be integrated with asymmetric nanochannel sensors to selectively detect intact infectious viruses, such as SARS-CoV-2 or human adenovirus, in both biological and environmental samples with a LoD comparable with qPCR (10 copies/μL or 1×10^4 copies/mL, Fig. 6D)[82]. Single nanopore membranes were fabricated by irradiation of polyethylene teraphthalate (PET) films with single swift heavy ions and subsequent chemical etching of the generated single ion track [96,97]. During the chemical etching process, we adjusted both shape and size of the nanopore to obtain asymmetrical single nanochannels with dimensions close to virus diameter. Then, DNA aptamers are immobilized onto the inner wall of the nanopore. Next, amplification properties are obtained due to the confinement effect [89,90] and the avidity effect of having multiple aptamers in the nanopore that can bind to the same virus [39]. Fig. 6A shows a scheme on how the aptamer-nanopore system works. Virus samples are incorporated to the reservoir and incubated with the aptamer-nanopore system, followed by washing once with water to remove the excess of virus and then measuring the I–V characteristic curve. With a 2-hour incubation, the sensor detected as low as 1×10^4 copies/mL and quantified a broad range of virus concentrations, from 1×10^4 copies/mL to 1×10^8 copies/mL (Fig. 6B). Furthermore, we can differentiate SARS-CoV-2 from other coronaviruses that cause common cold, such as 229E coronaviruses, as well as pseudotyped SARS-CoV-1 and H5N1 influenza virus (Fig. 6C). Finally, our method allows the direct detection and quantification of SARS-CoV-2 pseudovirus in saliva without any pretreatment of the sample (Fig. 6D).

5.2. Other aptamer sensors for SARS-CoV-2

In addition to nanopores, other platforms have also been used to incorporate DNA aptamers to develop tests for SARS-CoV-2. Aptamers have a unique advantage, i.e., the same sensing strategy can be applied for different targets, simply by changing the sequence that determines

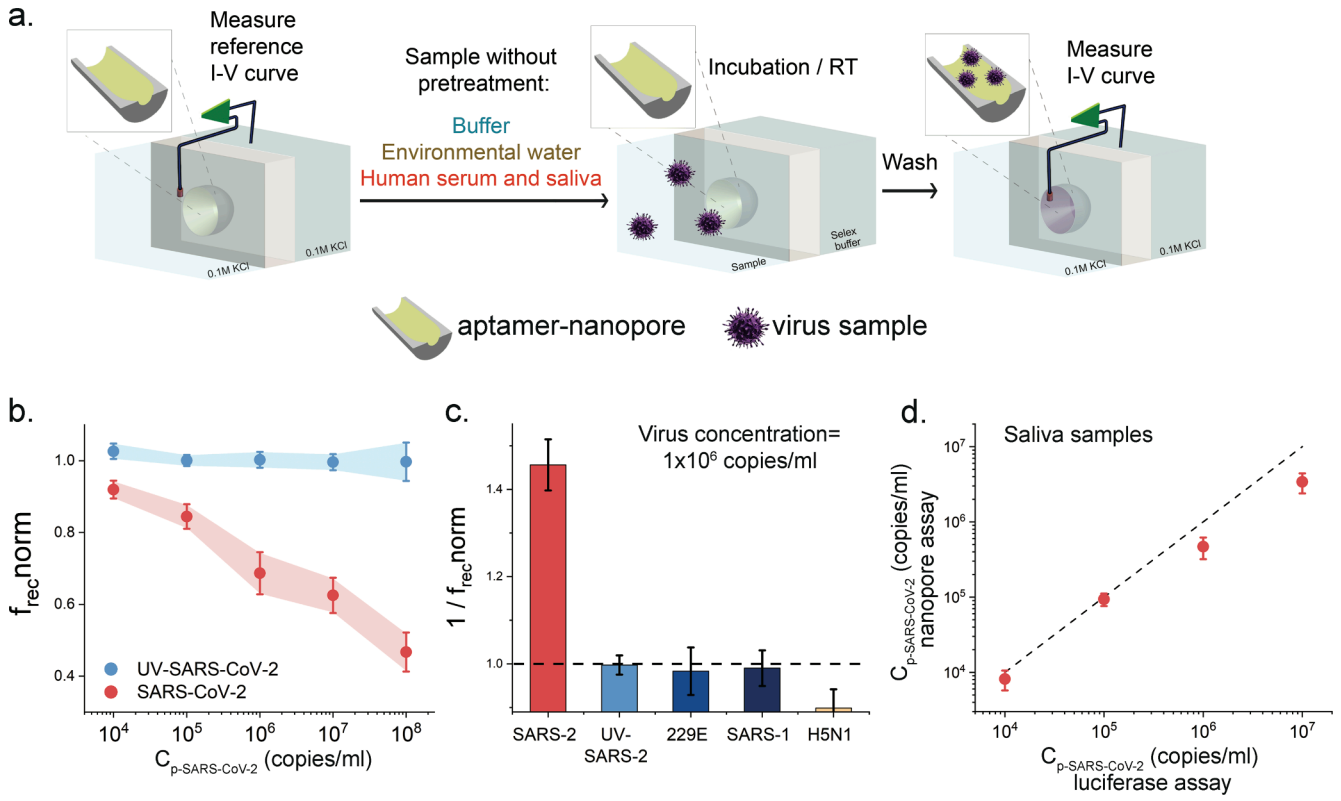

Fig. 6. Quantification of active pseudotyped SARS-CoV-2 with the aptamer-nanopore system. (a) Scheme of infectious HAdV detection by the aptamer-nanopore system. (b) Normalized rectification efficiencies versus virus concentration. n = 3, technical replicates. Colors correspond to the nanopore modified with different concentrations of UV-inactivated pseudotyped SARS-CoV-2 (blue) and active pseudotyped SARS-CoV-2 (red) (c) Selectivity assay. Inverse of the f_{rec}norm obtained for active pseudotyped SARS-CoV-2 (SARS-2), UV-inactivated pseudotyped SARS-CoV-2 samples (UV-SARS-2); another coronavirus: 229E, and two other pseudoviruses: SARS-CoV-1 (SARS-1) and influenza virus (H5N1). The concentration of each virus is 1×10^6 copies/mL. (d) Comparison of aptamer-nanopore sensor (y-axis) with luciferase assay (x-axis) to quantify active pseudotyped SARS-CoV-2 in human saliva without dilution of the biological sample. n = 3, technical replicates (mean ± SD). Each of these measurements was performed with a new membrane. Extracted from [82] under a Creative Commons Attribution-NonCommercial license.

the selectivity.

The portable glucose meter (PGM) is one of the most successful point of care devices that has already been adopted in the market at-scale. Xiang and Lu have reported a method to use a PGM to quantify non-glucose targets by linking DNA aptamers [98]. Recently, Hall and collaborators have used the same strategy to detect S and N SARS-CoV-2 proteins in saliva, able to detect COVID-19 positive clinical samples [99]. In another approach, Zhang and collaborators combined CRISPR technique with a PGM for the quantitative detection of COVID-19 related biomarkers. Using this system, a specific N gene and N protein of SARS-CoV-2 have been detected quantitatively with a simple portable device [100].

Aptamers have been incorporated into another promising point-of-care solution, lateral flow assays (LFA). LFA are fast and cheap assays but usually they have associated significant limitations, including qualitative readout and reportedly low sensitivity with high false positive rates. Aptamers can help to increase the sensitivity of LFA, and tens of pM of N SARS-CoV-2 protein has been detected using a combination of aptamers and antibody in a sandwich LFA [68].

In addition to PGMs and LFA, DNA aptamers have been integrated to a wide variety of technologies to detect different pathogens and proteins, ranging from portable fluorescence [101–103] and colorimetric assays [104,105], to rapid assays that require instrumentation such as MST [106]. Given the progress made so far, we are confident that in the near future DNA aptamers specific to SARS-CoV-2 will be interfaced with new technologies summarized in this review to make it possible for portable, on-site, and real-time detection of infectious SARS-CoV-2.

6. Additive manufacturing of microfluidic cartridges for COVID-19 assays

The COVID-19 outbreak highlighted a glaring gap in technologies for point of care testing, especially the need for an inexpensive portable diagnostic system that could provide rapid and accurate results. Several possible solutions have been proposed to address this need. For example, a smartphone can be used to optically detect the presence of specific nucleo-proteins on a molecularly imprinted polymer-based sensor [107]. In another example, a single-use microfluidic cartridge measured PH variation of samples using ion-sensitive field-effect transistors. This system was compatible with a smartphone to allow for real-time results and tracking of positive tests [108]. Other examples use optical detection in combination with simple fluidic channel geometries to measure fluorescence changes from a RT-LAMP reaction [109,110]. Another diagnostic uses nanoplasmonic sensors on a disposable chip which could be inserted into a POC device and would provide real-time data to a connected smartphone [111]. Several POC tests have been approved by the FDA, including Abbot's ID Now [112], Cepheid's Xpert Xpresss SARS-CoV-2 [113], and Mesa Biotech' AccuSarS-CoV-2 test [114].

A key challenge in the development of a POC diagnostic is manufacturing and scale up, which is traditionally the most time consuming and costly part of developing microfluidic diagnostics [115]. Additive manufacturing (AM) is a promising alternative to traditional manufacturing techniques because AM allows for rapid development. Recent advances in AM materials and equipment enable high speed production which gives AM a cost advantage over injection molding for the manufacturing of microfluidic cartridges [116]. Thus, AM can be

used for both rapid prototyping and scalable production of microfluidics with the same process. The flexible, toolless nature of AM allows for many different designs to be produced on a common platform, which is critical because rapid POC diagnostics require manufacturing flexibility and responsiveness.

We recently demonstrated the development of a POC diagnostic based on an AM microfluidic cartridge [27]. Fig. 7 shows this device and how it operates. The microfluidic cartridge performs the diagnostic as follows. The sample and reagents are loaded via dual Luer lock syringe inlets. These inlets are joined on the reverse of the chip by a Y-junction junction which connects to the serpentine mixer. The mixing region is a 3D serpentine micromixer where the fluid takes a vertical turn from one face to the other face between each horizontal U-turn. Once, the fluids pass through the mixer, the fluid is pushed into a pie shaped detection region. The microfluidic cartridges interfaces with a cradle reader system for rapid detection. The detection is based on a smartphone which images the fluorescence from the RT-LAMP amplification reaction. The cradle also includes an on-board heater to perform the amplification on chip. Using, the final cartridge design we demonstrated the detection of SARS-CoV-2 in VTM clinical samples on chip in under 40 min with a clear difference in the fluorescent levels of positive and negative samples. Although this paper highlights the application for the detection of SARS-CoV-2 in VTM, this technology can be applied to other pathogens such as *E. coli*, and to more complex matrices such as blood, if the appropriate amplification assay is used [117].

The potential for additively manufactured microfluidics produced at scale introduces compelling issues for materials engineering. Additively manufactured microfluidics in the literature, made either using prototyping processes or production processes, have channel sizes that are limited to around a few hundred micrometers in width and depth. It would be desirable to have much smaller channels, which requires advancements in materials and materials processing that can realize such geometries. a second issue that should be investigated is the long-term stability of materials when exposed to biochemical reagents. It is desirable to store cartridges that have been preloaded with reagents, and research is needed to study the chemical stability of these materials. Finally, it would be desirable to have transparent materials with very low autofluorescence. While there are some commercially available additive polymers that are transparent, only a few are compatible with production processes and all have unacceptably high autofluorescence, which prevents fluorescent detection with devices made from these materials.

We leveraged AM for rapid prototyping and were able to develop a working solution in 46 days of the start of the project. Fig. 8 shows the design evolution of the cartridge in which we tested 8 designs over 5 design cycles testing more than 10 parts with each design for fluidic and biochemical function. Given the total of 46 days for five design cycles gives an average of just over 9 days per cycle as compared to traditional manufacturing methods which could require 4 months per cycle at 20 times the cost. The parts were developed using advanced factory equipment and infrastructure, allowing for the rapid iteration. This also means that the final design is production ready without the need for any changes in materials or production processes.

7. Conclusion

This communication highlights that innovation in material science at the nanoscale is paving the way toward fundamentally new standards in the realm of pathogen diagnostics. The technological advances outlined in this work are representative of the broad and rapidly expanding fields of research in which ultrasensitive biosensing transducers, ultraselective biomolecular recognition elements, and inexpensively manufacturable,

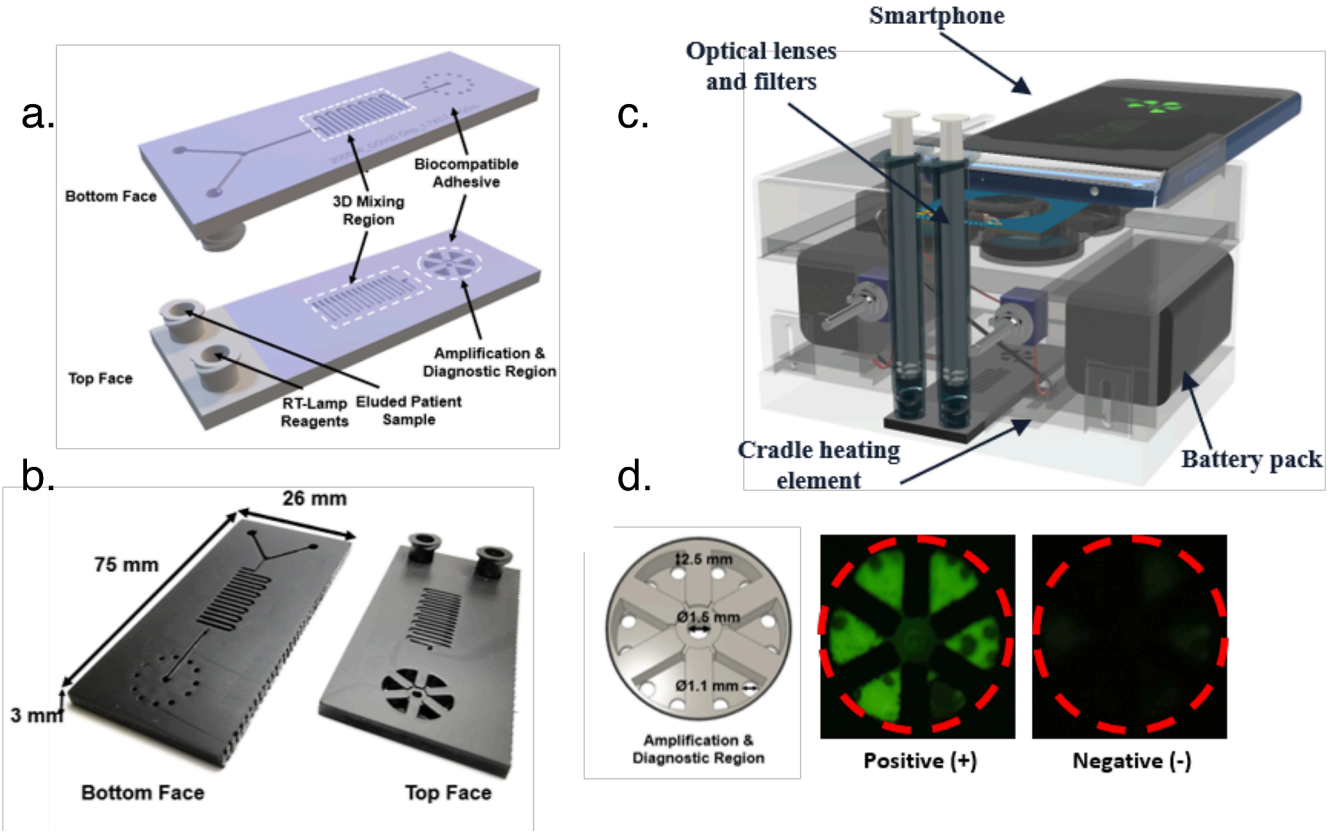

Fig. 7. AM cartridge and portable detection cradle. (a) Labeled cartridge schematic. The inlets mate with Luer lock syringes to deliver the reagents and samples to the rest of the chip. (b) Cartridge images (c) Assembled view of POC cradle with smartphone and optical components. (d) Detailed view of detection region and images of positive and negative sample tests after 40 min. a, b, extracted from [27].

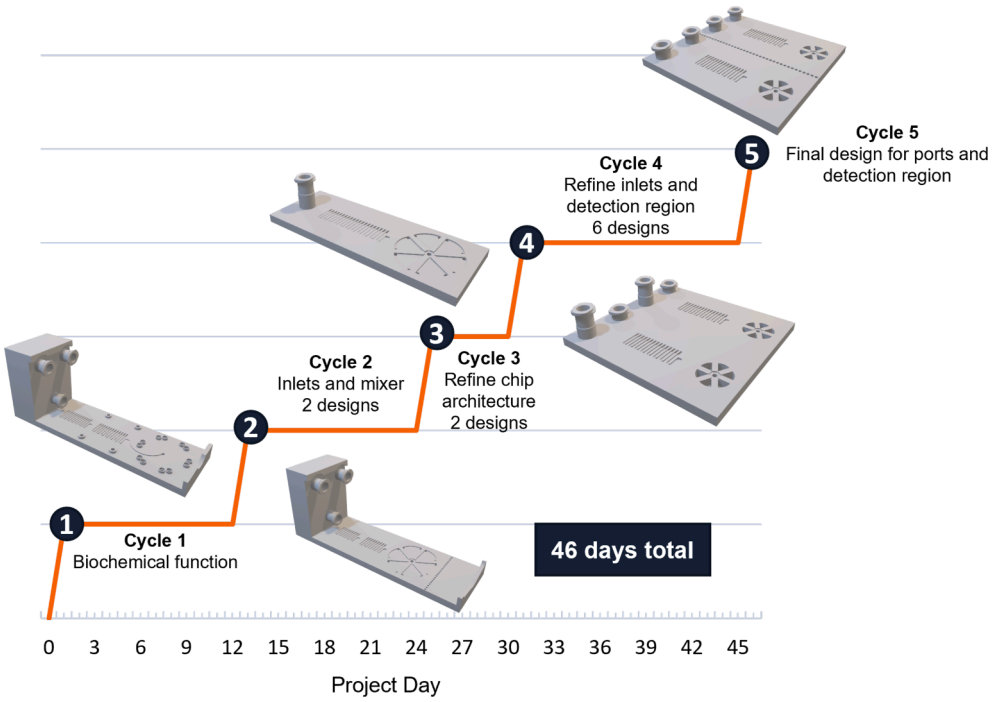

Fig. 8. Design evolution timeline of the AM SARS-CoV-2 diagnostic cartridge. Design was done in three major phases working on the inlet, mixing, and detection regions. The entire design process was completed in 46 days with 8 different designs developed and tested.

yet highly functional microfluidic cartridges come together. Here, we highlighted how both electronic and photonic biosensing technologies can take advantage of the unique properties of crumpled graphene and photonic crystal metamaterials to achieve extremely low detection limits for sensing viral nucleic acid molecules, viral antibodies, and even intact viruses. The ability to observe individual analytes with digital resolution with simple, room temperature workflows that do not require enzymatic amplification leads to inexpensive instrumentation and protocols that are more easily translated to POC settings than the current set of diagnostic tests that are performed in laboratory settings. Likewise, the capabilities afforded by high throughput selection and engineering design of nucleic acid-based nanomaterials in the form of aptamers and designer DNA nets enable highly selective recognition of unique viral features, such as spike proteins and capsid proteins, that do not suffer from the limitations of antibody-based capture molecules in terms of cost, reproducibility, and long-term stability. We shared examples of using nucleic acid capture molecules with features engineered at the sub-nanometer size scale, combined with sensing nanostructures such as nanopores and photonic crystal that are engineered with features at the 10′s of nanometer size scale, that are able to rapidly and selectively detect intact viruses, while differentiating between infectious and non-infectious versions of the same virus. Finally, the ability to apply novel polymer nanostructures that are capable of being printed into precise macro-scale structures using additive manufacturing is an enabling technology for rapid, flexible, and low-cost manufacturing of single-use disposable microfluidic cartridges that can effectively bring the biosensing transducers and biomolecular recognition elements into POC environments. We described an example of how additive manufacturing overcomes the inherent limitations of conventional 3D-printing and plastic injection molding to create structures that are compatible with storage of assay reagents, perform basic mixing sequences, and demonstrate compatibility with assay protocols that are known to be sensitive to the presence of inhibitors.

In conclusion, the COVID-19 pandemic uncovered several gaps in the capabilities of the predominant models for pathogen diagnostics, which relied predominantly upon PCR-based nucleic acid testing and ELISA-based serology testing performed in laboratory facilities using expensive equipment operated by highly trained technicians who performed complex workflows. Although the COVID-19 pandemic is still ongoing, in hindsight we can hypothesize that optimally effective COVID-19 diagnostics strategy would have incorporated testing that was more frequent, less invasive, and more easily accessible, while at the same time retaining a high degree of sensitivity while driving testing cost by at least an order of magnitude compared to current methods. The ability for the SARS-CoV-2 virus to be spread easily through exhaled breath by pre-symptomatic and asymptomatic people are features that continue to make containment of the pandemic extremely difficult, even in environments where very stringent public health measures have been strictly enforced.

The scientific community working in the fields of material science, chemistry, and sensor engineering has responded to the COVID-19 pandemic with a high degree of innovation, with a strong focus on the development of materials that derive their critical features from characteristics that are engineered at the nanoscale. As a result, we can envision new generations of diagnostic tests that can include breath analyzers, single-step saliva testers, sensing instruments that are handheld or able to use our mobile devices, and sensing that is visually observable. While widespread adoption of novel approaches will continue to require regulatory approval and the ability to meet stringent goals for accuracy, we can envision rapid POC tests serving to complement the existing infrastructure of laboratory-based testing through “screening” tests that rapidly provide only positive/negative information, while directing people toward more conventional tests for verification and quantitative assessments. We also envision the need for POC tests to become seamlessly integrated with the information infrastructure of health care systems and secure contact tracing capabilities. Importantly, the types of tests we consider in this review can serve the role of reducing costs of healthcare and reducing healthcare disparities by making testing more easily accessible to disadvantaged populations, in particular those who would not seek out testing facilities or have the ability to travel to them.

Declaration of Competing Interest

Yi Lu is a co-founder of ANDAlyze and GlucoSentient, Inc. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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