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A Double-Imprinted Diffraction-Grating Sensor Based on a Virus-Responsive Super-Aptamer Hydrogel Derived from an Impure Extract**

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Abstract: *The detection of viruses is of interest for a number of fields including biomedicine, environmental science, and biosecurity. Of particular interest are methods that do not require expensive equipment or trained personnel, especially if the results can be read by the naked eye. A new "double imprinting" method was developed whereby a virus-bioimprinted hydrogel is further micromolded into a diffraction grating sensor by using imprint-lithography techniques to give a "Molecularly Imprinted Polymer Gel Laser Diffraction Sensor" (MIP-GLaDiS). A simple laser transmission apparatus was used to measure diffraction, and the system can read by the naked eye to detect the Apple Stem Pitting Virus (ASPV) at concentrations as low as 10 ng mL^{-1}, thus setting the limit of detection of these hydrogels as low as other antigen-binding methods such as ELISA or fluorescence-tag systems.*

The detection of viruses is important for saving lives through biomedical applications, management of agriculture and the environment, and safeguarding national security.[1] Virus detection further enhanced by methods that do not require expensive equipment or trained personnel, especially if the results can be read by the naked eye. Hydrogels that are responsive to specific molecular recognition stimuli can satisfy these requirements;[2] however, current strategies for detecting the volume change of hydrogels that shrink in response to molecular stimuli, for example, gel length- or weight-change measurements, are prone to error. Many reports on bioresponsive hydrogels triggered by molecular recognition employ bioimprinting methods to impart selective recognition. For example, Miyata et al. bioimprinted the protein APF through precomplexation to a polymerizable lectin and antibody, which led to a shrinking response when both the lectin and antibody simultaneously bound AFP.[3] Recently a virus displacement assay based on an aptamer hydrogel was demonstrated, however, signal detection required the use of expensive and sensitive quartz crystal microbalance (QCM) equipment.[4] For a more comprehensive overview of molecular recognition responsive hydrogels that use DNA, protein, and other molecular recognition elements, several excellent reviews are available.[2,5]

Using an aptamer-based bioimprinting strategy recently introduced by our group for protein recognition,[6] Apple stem pitting virus (ASPV) was targeted for the MIP-GLaDiS technology in part as a result of its destruction of approximately 30% of the apple and pear crops in the United States and Europe.[7] ASPV is a filamentous virus with a capsid comprised of MT32 proteins that is approximately 15 nm wide and 800 nm long[8] before degradation to myriad smaller fragments, as reported for general extraction methods[9] and in previous virus imprinting studies,[10] which facilitates mass transfer in hydrogels. Polymerizable aptamers elicited for specific binding to the MT32 protein[11] are assembled in the presence of ASPV and copolymerized with additional monomers to create a multi-aptamer binding site that shrinks in the presence of ASPV (Scheme 1). These virus-responsive super-aptamer hydrogels are envisioned to provide not only a useful assay for the low-cost, easy to use, and portable detection of ASPV; but as a proof-of-principle that can be applied to the detection of any virus for which a polymerizable bioreceptor can be made.

Polymerization of the virus-responsive hydrogel within a lithographic mold (Scheme 2a) creates a "double-imprinted" hydrogel, with the virus imprinted at the molecular scale and the diffraction grating imprinted at the macromolecular scale. The key aspect of this methodology comes in the measurement of the volume-shrinking event, which is accomplished by passing laser light through the grating-patterned hydrogel and measuring the distance between spots from the 1D diffraction pattern as shown in Scheme 2b. The only detector used to measure the diffraction distance projected on the ruler is the naked eye, thus preserving the naked-eye-detection classification of this methodology. All that is needed is a simple and inexpensive laser pointer, hand-held if necessary, placed at an arbitrary distance (h in Scheme 2b) from the hydrogel and pointed normal to the plane of the hydrogel. While the distance h is arbitrary, larger h values can amplify small signals by increasing the diffraction distance (D in Scheme 2b), thus making accurate measurements easier.

For the molecular-scale bioimprinting of the virus (Scheme 2a; details provided in the Supporting Information), the prehydrogel solution was prepared by incubation of the polymerizable ASPV-specific aptamers with an impure ASPV extract (Bioreba AG, Switzerland) that was reported to contain 10 μg mL^{-1} of the ASPV contagium. Prepolymer complexation of the aptamers to the virus was unaffected by other impurities in the extract owing to the specificity of the

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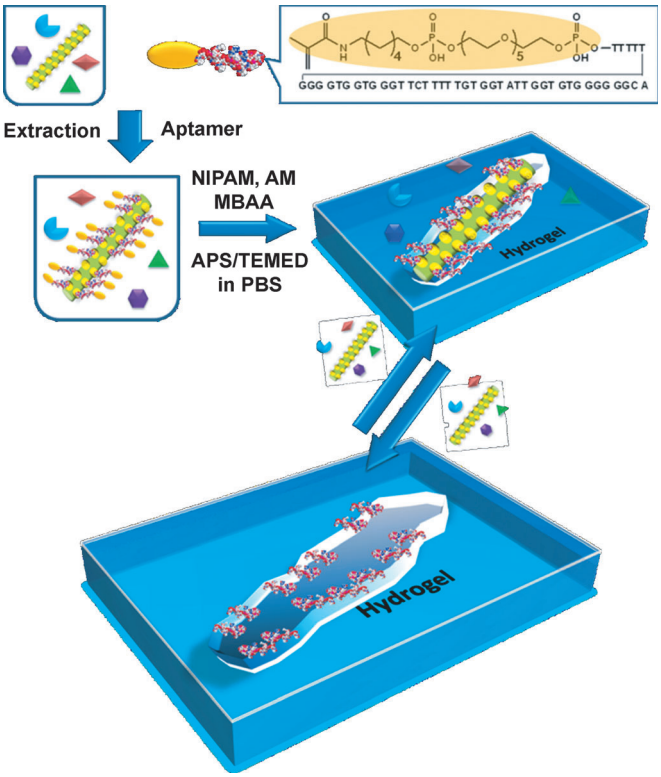
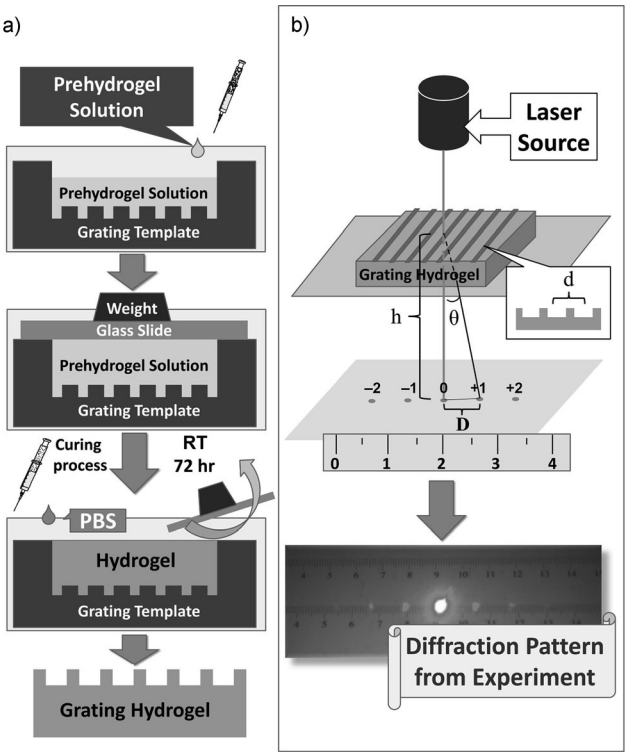


Scheme 1. Outline of the bioimprinting process used to create virus responsive super-aptamer hydrogels. NIPAM = *N*-isopropylacrylamide, AM = acrylamide, MBAA = *N*,*N*′-methylene bisacrylamide, APS = ammonium persulfate, TEMED = *N*,*N*,*N*′,*N*′-tetramethylethylendiamine, PBS = phosphate-buffered saline.



Scheme 2. a) Preparation of "double imprinted" diffraction grating bioimprinted hydrogels for MIP-GLaDiS materials. b) Schematic of the laser diffraction apparatus used for the measurement of the laser diffraction pattern projected onto a desk ruler (full photograph in the Supporting Information).

aptamer. The remaining components (Table S1 in the Supporting Information) were added to the prehydrogel solution, which was then cast onto the elastomer grating micromolds and allowed to polymerize. Once the hydrogel is formed, the film is delaminated from the elastomeric grating template and the virus template removed by immersing the hydrogel film several times in an ethanol/NaOH regenerating solution. Spectroscopic studies on the eluent revealed that approximately 90% of the aptamer monomers remained incorporated in the MIP-GLaDiS material (see the Supporting Information).

Rebinding of the virus was first evaluated by incubation of the MIP-GLaDiS with ASPV, and the shrinking response at equilibrium was evaluated by microscopy and laser diffraction (Figure 1). The microscopy image in Figure 1c shows that the grating period (d; the distance from the middle of one trough to the middle of the adjacent trough) of the ASPV-exposed hydrogel shrinks versus the original hydrogel without ASPV (Figure 1a). The grating period (d) and the wavelength of the incident laser (λ) give the angle of diffraction (θ) for the laser light according to Equation (1):

$$\theta = \sin^{-1}(\lambda/d) \quad (1)$$

θ thus becomes larger as d becomes smaller. The angle of diffraction determines the distance (D) between two different projected laser points, which is determined by the angle of

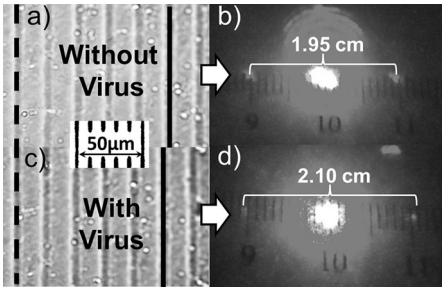


Figure 1. Optical microscopy images of ASPV-responsive MIP-GLaDiS and the corresponding laser diffraction patterns in 2.0 mM phosphate buffer without virus (a and b), and in the presence of the ASPV extract (c and d).

diffraction and the geometrical arrangement of the measuring apparatus shown in Scheme 2b (and Figure S4) according to Equation (2):

$$\theta = \tan^{-1}(D/h) \quad (2)$$

Combining the two equations gives the distance between projected laser points (D) as a function of the hydrogel grating period [d; Eq. (3)]:

$$D = h\tan[\sin^{-1}(\lambda/d)] \quad (3)$$

Equation (3) shows how the decrease in d as a result of MIP-GLaDiS shrinking expands the distance (D) between the two projected laser points that are used to quantify the hydrogel volume change. The two laser points used to measure twice distance D are shown in Figure 1b for MIP-GLaDiS without ASPV and Figure 1d in the presence of ASPV. It can be clearly seen that this type of measurement can be carried out with greater ease and accuracy compared to length measurements of the entire MIP-GLaDiS (top of Figure 2) or the corresponding hydrogels made in capillaries (top of Figure 3). Quantification of the MIP-GLaDiS shrinking response as a function of the amount of ASPV bound to the hydrogel is given by the isotherm in Figure S5, which shows a dynamic range for virus detection of 1.0 to 1.0×10^{-2} μg mL^{-1}. The ASPV-specific response can be seen at concentrations as low as 10 ng mL^{-1}, thus setting the limit of detection for these hydrogels as low as other antigen-binding methods.[12]

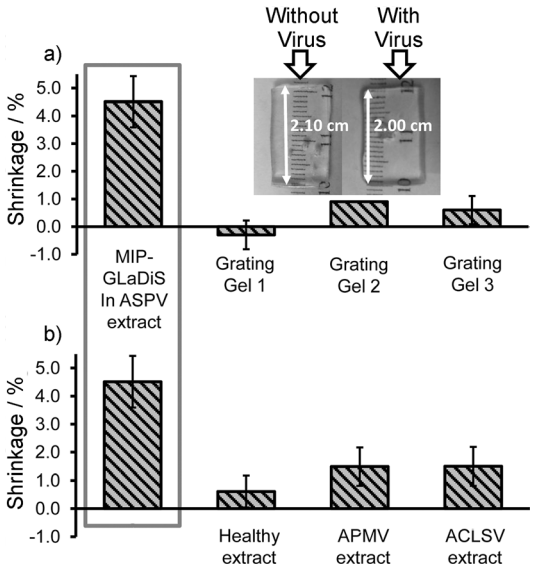


Figure 2. The volume-shrinking response for the MIP-GLaDiS in ASPV extract is shown in both parts a and b (boxed) for comparison to nonimprinted control hydrogels (a) and cross-reactivity responses to extracts from healthy leaves not infected with ASPV or leaves infected with different apple viruses (b).

To verify that the MIP-GLaDiS shrinking response is due to the bioimprinted virus, three similarly formulated control gels were also synthesized (Figure 2a); one without the ASPV extract or the aptamer monomer (Grating Gel 1), a second without the aptamer monomer (Grating Gel 2), and a third without the virus extract (Grating Gel 3). All control hydrogels show low background responses compared to the MIP-GLaDiS when exposed to a saturating virus solution. Especially important is the low response of Grating Gel 3, in which the aptamers are randomly polymerized within the gel. This clearly demonstrates the need for aptamer preorganization by using the ASPV template to give an appreciable response, a result that supports the importance of the bioimprinting mechanism for creating ASPV-specific sites.

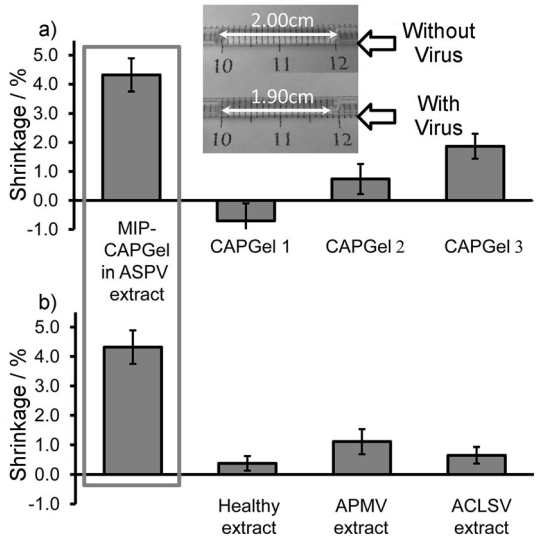


Figure 3. The volume-shrinking response for the MIP-CAPGel in ASPV extract is shown in both parts (a) and (b; boxed) for comparison to nonimprinted control hydrogels (a) and cross-reactivity responses to extracts from healthy leaves not infected with ASPV or leaves infected with different apple viruses (b).

Equally important, the response of Grating Gel 3 also shows that the aptamer alone in the MIP-GLaDiS does not result in an appreciable number of nonselective binding sites that could interfere with the bioimprinted response to the ASPV template. The slight swelling of Grating Gel 1, which has neither virus nor aptamer monomers, may be due to nonspecific disruption of the hydrogen bonding in the hydrogel polymer network by components of the extract.

For any sensor, it is important to minimize any false-positive responses; therefore, the selectivity of the MIP-GLaDiS class of materials was investigated. Possible interference from components of the leaf extract was investigated first by incubation of the MIP-GLaDiS in a negative-control solution extracted from healthy, noninfected apple leaves. As shown by the second column in Figure 2b, there is very little volume change of the bioimprinted hydrogel in response to healthy-leaf extract supplied by Bioreba AG (Switzerland) as a negative control for assays involving the ASPV extract. Next, selectivity was more rigorously tested against two different related viruses; apple mosaic pitting virus (AMPV) and apple chlorotic leafspot virus (ACLSV), which is often also present in ASPV-infected apple trees. The responses plotted in Figure 2b show that both AMPV and ACLSV exhibit approximately 30% cross-reactivity that is due to the aptamer itself, rather than the bioimprinting process.[11a] Overall, good selectivity by the MIP-GLaDiS is found for the target ASPV, a result which confirms that the mechanism of the hydrogel volume change involves specific binding interactions between the ASPV virus and the hydrogel-bound aptamers.

The performance of the "double-imprinted" hydrogels was compared to capillary-encapsulated hydrogels to determine whether differences in binding or selectivity arise from the different morphologies. Fabrication of the virus-sensor hydrogels in the capillary format is procedurally easy (Ref. [3]

and the Supporting Information); however, the equilibrium time for rebinding was in the order of 3 days versus 12 h for the MIP-GLaDiS materials. Naked-eye measurements of the meniscus to meniscus distance changes in the capillary gel were determined as "percent shrinkage" according to published procedures,[6] and the resulting isotherm closely resembled that found for the MIP-GLaDiS with good reproducibility (see the Supporting Information).

The uptake of ASPV and two different control viruses by the bioimprinted capillary hydrogel versus the nonimprinted control capillary hydrogels is graphically shown in Figure 3a. The first column shows a 4.3% shrinking of the imprinted hydrogel (referred to as the MIP-CAPGel) after incubation with the ASPV-infected leaf extract; a close match to the results found for the MIP-GLaDiS in Figure 2a. The control hydrogels CAPGel 1, CAPGel 2, and CAPGel 3 are formulated identically to the previous control polymers, Grating Gel 1, Grating Gel 2, and Grating Gel 3, respectively. The results for all of the capillary-based control gels parallel the results for the MIP-GLaDiS materials, and it can be concluded that there is no difference in terms of the bioimprinting process between the capillary and MIP-GLaDiS formats. The selectivity of the ASPV bioimprinted hydrogel (Figure 3b) was evaluated as before and showed similar cross-reactivity to that seen for the MIP-GlaDiS materials. Finally, the long term stability and ease of transport of the bioimprinted hydrogels can be enhanced by dehydration, and investigations into the effects of dehydration of the MIP-CAPGel in the presence or absence of ASPV showed that the latter treatment lead to good responses by the rehydrated material (see the Supporting Information).

In conclusion, a new "double imprinting" method has been developed whereby a virus-bioimprinted hydrogel is further micromolded into a diffraction-grating sensor to give a "Molecularly Imprinted Polymer Gel Laser Diffraction Sensor" (MIP-GLaDiS). The simple laser transmission apparatus used to measure the change in the diffraction of the MIP-GLaDiS in response to virus samples demonstrates the ease of acquiring data while maintaining high accuracy, compared to the less sophisticated capillary gel formats. Furthermore, the laser-diffraction format could be automated for fast and easy measurements, or to allow incorporation into a multi-array format.[13] An additional novel aspect of this bioimprinting study is that it is the first example of the use of an impure virus extract as the source of the template, an approach that was facilitated by the use of virus-specific aptamers that excluded interference from the other extract components in the prepolymerization complex. Multivalent interactions of polymer-bound aptamers with ASPV led to a clearly visible volume-shrinking response upon reintroduction of the virus. Control hydrogels polymerized with random placement of the aptamers (i.e. in the absence of the ASPV template) did not show an appreciable response to the ASPV samples, thus emphasizing that random incorporation of aptamers cannot compete with the preorganized aptamers in the bioimprinted hydrogel. Overall, the methods described herein are anticipated to advance the development of assays and sensors for a wide array of biopathogens.

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[1] a) C. Qi, G. F. Gao, G. Jin, Biosensors for Health, Environment and Biosecurity **2011**, 165–182; b) D. R. Roach, A. M. Svircev, *Princ. Pract. Adv. Technol. Plant Virol.* **2010**, 39–58; c) L. W. Stobbs, *Princ. Pract. Adv. Technol. Plant Virol.* **2010**, 1–38.
[2] a) J. Liu, H. Liu, H. Kang, M. Donovan, Z. Zhu, W. Tan, *Anal. Bioanal. Chem.* **2012**, *402*, 187–194; b) X. Xiong, C. Wu, C. Zhou, G. Zhu, Z. Chen, W. Tan, *Macromol. Rapid Commun.* **2013**, *34*, 1271–1283.
[3] T. Miyata, M. Jige, T. Nakaminami, T. Uragami, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 1190–1193.
[4] R. Wang, Y. Li, *Biosens. Bioelectron.* **2013**, *42*, 148–155.
[5] a) T. Miyata, *Polym. J.* **2010**, *42*, 277–289; b) R. V. Ulijn, N. Bibi, V. Jayawarna, P. D. Thornton, S. J. Todd, R. J. Mart, A. M. Smith, J. E. Gough, *Mater. Today* **2007**, *10*, 40–48; c) see Ref. [2a].
[6] W. Bai, N. A. Gariano, D. A. Spivak, *J. Am. Chem. Soc.* **2013**, *135*, 6977–6984.
[7] a) Z. B. Wu, H. M. Ku, C. C. Su, I. Z. Chen, F. J. Jan, *J. Plant Pathol.* **2010**, *92*, 721–728; b) A. Negi, T. Rana, Y. Kumar, R. Ram, V. Hallan, A. A. Zaidi, *J. Plant Biochem. Biotechnol.* **2010**, *19*, 91–94; c) N. Pūpola, I. Moročko-Bičevska, A. Kāle, A. Zeltiņš, *J. Phytopathol.* **2011**, *159*, 597–605.
[8] L. Li, H. Yang, *J. Phytopathol.* **2011**, *159*, 676–681.
[9] M. H. V. Van Regenmortel, Serology and Immunochemistry of Plant Viruses, **1982**.
[10] L. D. Bolisay, P. Kofinas, *Macromol. Symp.* **2010**, *291–292*, 302–306.
[11] a) Z. Balogh, G. Lautner, V. Bardoczy, B. Komorowska, R. E. Gyurcsanyi, T. Meszaros, *FASEB J.* **2010**, *24*, 4187–4195, 4110 1096/fj 4109-144246; b) G. Lautner, Z. Balogh, A. Gyurkovics, R. E. Gyurcsanyi, T. Meszaros, *Analyst* **2012**, *137*, 3929–3931.
[12] a) T. Q. Huy, T. H. H. Nguyen, T. T. Nguyen, P. V. Chung, P. T. Nga, M. A. Tuan, *Talanta* **2011**, *86*, 271–277; b) A. C. M. S. Dias, S. L. R. Gomes-Filho, M. M. S. Silva, R. F. Dutra, *Biosens. Bioelectron.* **2013**, *44*, 216–221.
[13] T. M. Straub, *Environ. Microbiol.* **2011**, *13*, 127–142.

