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DNA -based hydrogels for high-performance optical biosensing application

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ABSTRACT

Analyte-sensitive DNA-based hydrogels find multiple applications in the field of biosensors due to their adaptable nature. Here, the design of DNA-based hydrogel and its application as sensing platform for the detection of a specific target sequence are presented. DNA-functionalized hydrogel structures were formed via a free radical co-polymerization process. A simple one-step probe immobilization procedure is reported: DNA probe molecules are added to the photoactive polymer mixture, dispensed onto a solid support, or a mold, and covalently attached while the hydrogel is formed through UV light exposure. Such hydrogels can be synthesized with desired recognition ability through the selection of a certain nucleotide sequence. Here we show the application of DNA-based hydrogel to detect the target with high performance in fluorescence microarray format and, additionally, to fabricate holographic surface relief gratings for label-free sensing assays.

1. Introduction

Hydrogels have found wide application in the biomedical field due to their versatile nature. They are three dimensional networks of polymers, characterized by the ability to retain a large amount of water being permeable. This feature makes them ideal for a variety of applications, such as biosensing [1], drug delivery [2], immunotherapy [3] and tissue engineering [4]. Furthermore, they are suitable for multiplexing, miniaturization and label-free systems. Hence, they are being increasingly used, thanks to their suitable properties both mechanical (porosity, sorption capacity, elasticity, strength) as well as optical (transparency), chemical (easy fabrication process, ability to be chemically derivatized) and biochemical (low nonspecific signals, biocompatibility, bio-specificity) [5–7]. The immobilization of nucleic acids on solid supports has been broadly employed in sensor technology for the detection of DNA and other biomolecules [8]. Microarrays are versatile tools in biomedical research, as they permit highly parallel analysis of various samples [9]. Immobilization strategies of biomolecules at the surfaces of the chips plays a decisive factor for the assay performance. Three-dimensional (3-D) hydrogel matrixes offer significant advantages for catching probes over more conventional two dimensional (2-D) rigid substrates and, additionally, they provide a solution-mimicking environment that makes them attractive supports for bio-analysis [10–12]. Moreover, DNA can be modified with several functional groups including amino, biotin, acrydite, azide, and thiol to promote the covalent binding to the substrate [13]. DNA-based hydrogel chips were prepared by simple and fast procedure; in comparison to other multiple steps and thus time consuming procedures presented in literature [14]. Initially, in this work, the functionalization approach of acrylamide hydrogel was based on the polymerization with glycidyl methacrylate (GMA), as a co-monomer functional group, and the immobilization of thiol-modified DNA by thiol–epoxy coupling reaction. This immobilization procedure was performed by click photochemistry, during or after the polymerization. Secondly, acrydite-modified DNA were used to direct co-polymerize with acrylamide monomers during gel formation, as already reported in literature [15]. Compared to the already published method, our strategy for the immobilization in the 3D-hydrogel is based on the same chemical reaction but activated photochemically and/or thermally, thus avoiding the use of toxic catalyst for future biomedical applications and, moreover, obtaining better porosity. This last approach was used for the microarray fabrication in which acrylamide-based hydrogel were attached on a glass substrate, by UV irradiation, and simultaneously functionalized by co-polymerization with acrydite-modified DNA probes. In this method, the UV light plays a double role as it catalyzes both the co-polymerization of acrylamide monomers with DNA probes and the covalent attachment to the

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microarray substrate. Basically, a simple one-step immobilization process for hydrogel 3D-microarray fabrication is shown and its transfer in label-free biosensing was explored [16]. Towards future applications as label-free system, a diffraction grating has been produced on the hydrogel surface by replica molding (REM) and employed as optical transducer [17–20]. So far, very few hydrogel-based diffractive gratings have been developed for label-free biosensing applications, and none of them is for nucleic acid recognition [21–24]. Sensitive DNA-based hydrogels as label-free sensors are highly promising in disease diagnosis and healthcare monitoring applications.

2. Experimental

2.1. Material and methods

Acrylamide (AA), N,N′-methylenebis (acrylamide) (MBA), Potassium persulfate (KPS), glycidyl methacrylate (GMA), 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959), toluene ≥99.5% and 3 (trimethoxysilyl)propylmethacrylate (TMSPMA) ≥98% were purchased from Sigma–Aldrich (Madrid, Spain). The employed oligonucleotides were supplied by Sumilab (Valencia, Spain), sequences used are listed in Table 1. Phosphate buffered saline solution with Tween 20 (PBS1x: 0.008 M disodium phosphate, 0.002 M monosodium phosphate, 0.137 M sodium chloride, 2.7 M potassium chloride, pH 7.5 with 0.05%Tween 20) and Saline-sodium citrate (SSC1x: 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7) were prepared following the preparation procedures. Polydimethylsiloxane (PDMS) Sylgard 184 was purchased from Dow Corning (Wiesbaden, Germany). Glass microscope slides were provided from Labbox (Barcelona, Spain). Microarray printing was carried out with a low-volume noncontact dispensing system from Biodot (Irvine, CA, USA), model AD1500. Irradiation was carried out with a UV-ozone cleaning system (FHR, Ottendorf, Germany). Hydrogel fluorescence measurements are registered with a homemade surface fluorescence reader (SFR) having a high-sensitivity charge-coupled device camera [25]. Microarray fluorescence measurements were carried out with a fluorescence microarray analyzer SensoSpot (Radolfzell, Germany). For fluorescence image analysis and quantification, GenePix Pro 4.0 software from Molecular Devices, Inc. (Sunnyvale, CA, USA) was employed. Images were also analyzed with ImageJ software. Morphological characterization of hydrogel was carried out using scanning electron microscopy (SEM, Gemini SEM 500 system, Zeiss), and optical microscopy (OM, Leica microsystems, MZ APO). For SEM characterization hydrogels were completely swollen in distilled water and frozen at −20 °C. Then, they were lyophilized overnight in a Telstar Lyoquest freeze-drier to yield completely dry aerogel samples. Finally, dry samples were prepared by sputter coating with an Au layer of about 15 nm (BAL-TEC SCD 005 sputter coater, Leica microsystems). Moreover, swelling behavior study was carried out with lyophilized hydrogel samples. Samples with a size of approximately 1 cm were immersed in PBS-T (10 mL) at room temperature. The weight of the swollen hydrogels was recorded at different times until they were totally swollen (reaching of a constant weight). Water excess on the surface of the hydrogel was removed with a filter paper before weighing. The swelling degree was calculated from equation (1), where Wt is the weight of the hydrogel after being immersed in water during time "t" and W_0 is the weight of the lyophilized hydrogel before the immersion.

Table 1
Nucleotide sequence of probes and target used.

Name	Sequence (5′–3′)	5′ end	3′end
Probe 1	$(T)_{15}$ – CCCGATTGACCAGCTAGCATT	SH	Cy5
Probe 2	CCCGATTGACCAGCTAGCATT	acrydite	Cy5
Probe 3	CCCGATTGACCAGCTAGCATT	acrydite	none
Target	AATGCTAGCTGGTCAATCGGG	Cy5	none
control probe	CCCGATTGACCAGCTAGCATT	none	none

Hydrogel diffraction gratings, as surface relief gratings, have been fabricated through replica molding technique (REM) and characterized by a simple home-made optical set-up.

$$\%\ Swelling = \frac{Wt - W0}{Wo}\ 100 \quad (1)$$

2.2. Optimization of hydrogel synthesis

AA/GMA hydrogels were prepared by free radical polymerization (FRP) thermally initiated at 60 °C. Synthesis optimization was reached varying the % of the monomers (AA and GMA) and the crosslinker (MBA) in 1 mL of distilled water, while the thermal initiator (KPS) was always kept at 1% *w*/v (supporting information). The prepared hydrogels were stored at 4 °C.

2.3. DNA-based hydrogels preparation and hybridization assay

Hyd10 and hyd11 compositions were finally used for DNA-based hydrogels preparation in milli-Q water. Firstly, DNA-based hydrogels solutions were prepared in 1 mL of milli-Q ultrapure water with 25% *w*/*v* AA, 0.5 μM of 5′-thiol oligonucleotide (Probe 1), 0.6% *w*/*v* MBA, 0.01% *w*/*v* GMA and 1% *w*/*v* of KPS. The free radical polymerization of AA/GMA hydrogel (hyd11) was initiated thermally at 60 °C. Secondly, for hyd10 preparation, DNA-based hydrogels solutions were prepared in 1 mL of milli-Q ultrapure water with 25% *w*/*v* acrylamide, 0.6% *w*/*v* MBA and 0.5 μM of 5′-acrydite modified DNA (Probe 3). Hydrogel pregel solutions (hyd10) with DNA probes were stirred during 1 h at RT and, after that, 1% *w*/*v* of the photo initiator (Irgacure 2959) was added in order to trigger the free-radical polymerization photochemically at 254 nm. Alternatively, the AA-hydrogel (hyd10) polymerization was thermally activated with KPS. To study the biosensing performance, hybridization of acrydite-functionalized hydrogel (hyd10-DNA) with the labeled anti-probe (Target) was carried out and compared with two reference systems: hyd10,without any probe, and hyd10-control probe (the same oligonucleotide sequence of probe 3 but without any modification). Labeled anti-probe solutions were prepared at growing concentrations (0.5, 0.1, 0.2, 0.3, 0.4, 0.5 μM) in SCC1 × . Hybridization was performed in triplicate by incubating hydrogels (0.5 × 0.5 cm) with the prepared anti-probe solutions for a duration of 2 h at 37 °C. Subsequently, fabricated hydrogels were washed three times with PBS-T in order to eliminate the unbinding oligonucleotides probes. Fluorescent detection was performed before washing, after 2 h washing and after overnight washing with PBS-T.

2.4. Glass surface modification

In order to modify the glass slides surface with acrylate groups, firstly, they were washed with ethanol and dried. Next, surfaces were activated with a UV-ozone cleaner for 3 min and, immediately after, immersed in a toluene solution containing 3-(trimethoxysilyl)propylmethacrylate (TMSPMA) organosilane (2% *w*/*v*). After 2 h at room temperature, glass slides were washed with toluene and placed in the oven at 120 °C for 20 min.

2.5. Microarrays fabrication and hybridization assay

DNA-based hydrogel microarray was obtained by free radical polymerization (FRP) initiated by UV light. Hence, pre-polymeric solutions containing the DNA-probe (Probe 3) were prepared at different concentrations (5, 1, 0.5, 0.25 μM) in PBS1 × . As comparing system, conventional DNA-microarray were used. For that, DNA-probe (Probe 3) was solved in PBS1 × at different concentration (25, 10, 5, 1 μM) with 1% *w*/*v* of the photo initiator and directly immobilized on a glass slide. The prepared solutions were spoted on the microscope glass slides with a template of 5 rows, 3 spots per row (10 nL/spot), and 12 microarray

replicas per glass slide. DNA-microarrays were irradiated by UV light at 254 nm (50 mW cm^{-2}) for 10 min, while 25 min were necessary in the case of DNA-based hydrogel microarrays. An extra row with the labeled DNA-probe (Probe 2) was included as a positive immobilization control (at 10 μM and 1 μM for simple DNA and DNA-based hydrogel microarrays, respectively). The 3 × 5 microarrays were spotted at 24 °C and with 80% of relative humidity. After UV irradiation, they were washed with PBS-T 10 × , in order to eliminate the non-bonded oligonucleotides probes, and dried. Furthermore, 20 μL of the complementary oligonucleotide 5′ Cy5-labeled (Target) in SSC 1 × was dispensed and spread out with a coverslip and incubated in a dark and humidified chamber for 1 h at 37 °C. The microarrays were then washed with PBS-T and dried. To study the immobilization density for the two microarray platforms, fluorescence images were measured at λ 650 nm with the fluorescence microarray analyzer SensoSpot and quantified by the GenePix 4.0 software.

2.6. Label-free format of DNA-based hydrogel

Holographic grating masters, employed as optical transducers, were fabricated using the Direct Laser Interference Patterning (DLIP) technology [26]. PET grating masters were supplied by Fraunhofer Institute (structuring parameters indicated in Table 2). Afterwards, hydrogel diffraction gratings were made by reproducing the grating of the master via replica molding (REM). REM is a soft lithography technique which consists of 3 steps: i) a line-like diffractive pattern was recorded by DLIP on a PET master; then ii) transferring the pattern on the master into PDMS, thus a negative copy of the pattern was obtained using PDMS as "ink"; iii) the original pattern is replicated on the hydrogel surface, during the curing process, by solidifying the prepolymer solution against the PDMS mold. In the last step, the prepolymer solution was poured into vials with the PDMS molder stuck to the base. In this case, DNA-based hydrogel polymerization had been triggered thermally at 60 °C for 2 h, adding to the prepolymer mixture only KPS as thermal initiator. Before solidifying the hydrogel with the PDMS mold in oven, vacuum was applied for about 15 min to remove any trapped air bubbles and to allow a greater adhesion to the mold. Afterwards, the diffraction of the grating fabricated within the hydrogel was observed through samples irradiation, with a laser beam (650 nm) incident from the bottom side of the hydrogel. The diffraction was projected on a white screen, placed at fixed distance where a digital camera captures the images.

3. Results and discussion

3.1. Poly(AA) hydrogels preparation

Polyacrylamide (PAA) hydrogels are biocompatible, low cost and ease to prepare. Usually, the preparation of PAA hydrogels by free radical polymerization (FRP) involves mixtures of acrylamide, bisacrylamide and catalysts for the polymerization such as tetramethylenediamine (TEMED) and ammonium persulfate (APS). In this work, PAA hydrogels were made both using ultraviolet (UV) photocrosslinking with Irgacure 2959 or through thermal crosslinking with KPS in the oven at 60 °C, thus avoiding the use of toxic catalysts. PAA hydrogel composition has been optimized varying the ratio of monomer (acrylamide) to crosslinker (bisacrylamide) in order to obtain the desired hydrogel properties. Moreover, hydrogel properties such as transparency, porosity, mechanical stability, and malleability have been optimized for applying them as label-free optical sensors. The optical and mouldability properties sought were observed for hyd10 and hyd11 compositions (Table S1, supporting information). From the swelling and morphology characterizations, hyd10 composition (Fig. 1.) was chosen for DNA-based hydrogel preparation both for microarray format and for surface relief gratings fabrication.

Table 2
Structuring parameters of DILP fabrication process for PET masters.

Master by DILP	Period (μm)	P_{laser} (J/cm^2)	Spot overlap[a] p (μm)	hatch distance h (μm)
P6H–H	5.90	1.8	8	23.6
P4H–H	4.00	1.8	8	19.5
P3H–H	3.00	1.8	5	20.9

[a] During the holographic pattern registration, the spot overlap is the pulse separation distance that measures the overlap of two successive laser spots, when the pulsed laser moves vertically [27].

3.2. Hydrogel bio-functionalization

In order to provide the sensitivity and selectivity for the hydrogel-based sensors designed, they were functionalized with a DNA probe, as biorecognition group. Firstly, thiol-modified DNA were used to introduce the DNA functionality in AA/GMA hydrogels by thiol-epoxy covalent binding, triggered by UV light. Secondly, another approach for DNA-based hydrogel preparation, was to use an acrydite-modified DNA to directly co-polymerize with AAM monomers. Commercially available thiol and acrydite-modified oligonucleotides were used for the proposed approaches. Initially, several AA/GMA hydrogel compositions were tested for the immobilization of thiolate probes (highlighted in red in Table S1, supporting information) which was carried out both before and after the curing process. Fluorescence was measured after irradiation and after washing with a homemade surface fluorescence reader (SFR). It was not possible to directly monitor the immobilization using the fluorophore-labeled probes, because both KPS initiation and prolonged UV irradiation cause fluorophore quenching. Therefore, in order to demonstrate that the immobilization occurs and how effective it is for the hydrogel, unlabeled probes were used and the hybridization assay performed with the targets marked with the fluorophore. Following various immobilization strategies being investigated, polyAA/GMA hydrogel with thiol-modified DNA did not provide a higher fluorescent signal compared to the negative control which did not bear the thiol group. Hence, it was possible to conclude that thiol-probes (Probe 1) were physically trapped in the 3D hydrogel network. Subsequent hybridization assays with the target did not show good selectivity. Thus, the hyd10 composition, without GMA co-monomer, was employed for the second approach. Hydrogels (hyd10) with 0.5 μM of the acrydite-modified DNA were prepared by both thermal and photochemical activations. After the hybridization assay, in both cases, it was demonstrated that a chemical immobilization of the acrydite-modified DNA occurs. Acrydite modified oligonucleotides had a similar reactivity as free acrylamide monomers allowing high incorporation efficiency.

The swelling of hyd10 with and without probe was measured and compared, hyd10 without probe had higher swelling capacity (550%) than hyd10 with the probe incorporated (200%), which supports the effective inclusion of the probe in the polymer (Fig. 2.). Indeed, after hybridization with the labeled target and overnight washing with SCC1x buffer, a significant difference was observed in the fluorescence signals between hyd10-DNA and its controls systems not bearing the acrydite termination (Fig. 3.). This result indicated that the covalent binding of acrydite modified oligonucleotides to the acrylamide chains occurs and, in addition, the DNA-based hydrogel specifically recognized its complementary DNA strand.

The optimized DNA-hydrogel composition was applied to create fluorescence microarrays as well as for the fabrication of diffractive surface relief gratings, as examples of its versatile possibilities and applications.

3.3. DNA-hydrogels chips fabrication and hybridization assay

For the microarray fabrication, glass surfaces were functionalized with 3-(trimethoxysilyl)propylmethacrylate (2% in toluene). Hence,

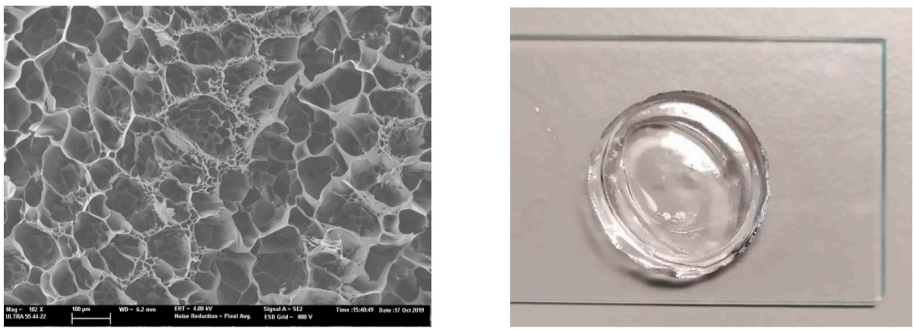


Fig. 1. On the left: SEM micrograph of lyophilized acrylamide hydrogel (hyd10) cross-section; on the right its digital photo.

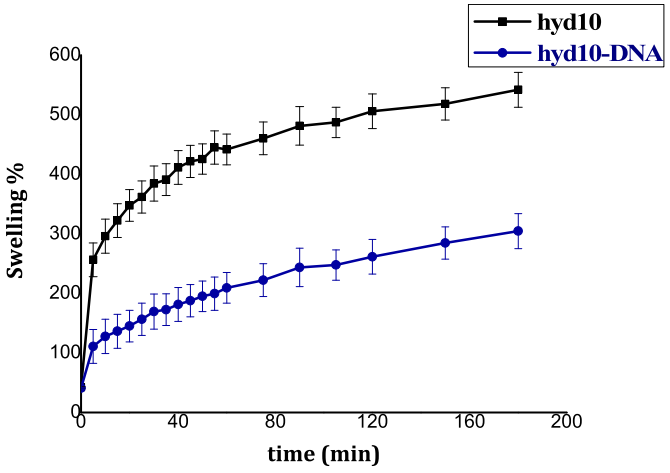


Fig. 2. Swelling kinetics of hyd10 and hyd10-DNA after immersion in PBS-T.

DNA-hydrogels chips were prepared by spotting the prepolymer solution with acrydite oligonucleotides, as well as with the crosslinker and the photo initiator, onto a modified glass slide. Whereas, to prepare DNA-chips comparing system, dilutions of acrydite-modified probes with the photo initiator were directly spotted on modified glass slides. Subsequently, the anchoring of the spots to the surface was induced by irradiation at 254 nm. As mentioned above, it was not possible to quantify the amount of probe immobilized using the labeled probe due to the quenching of the fluorophore caused by the irradiation. Thus, after exposure and washing, the applicability of the proposed strategy to attach DNA probes onto the hydrogel network was assessed through hybridization assays with the target, establishing its sensitivity (Scheme 1). For that, Cy5-labeled complementary oligonucleotide in the hybridization buffer was dispensed, incubated for 1 h at 37 °C, and, after rinsing and drying, the fluorescence intensity was registered with a microarray surface reader SensoSpot.

Results obtained demonstrated that the acrydite-probe was covalently immobilized into the 3D hydrogel network and, moreover, it specifically recognized the target. Further, an increased binding capacity of DNA in hydrogel spots compared to planar spots was observed. Thus, a calibration curve for Target (Fig. 4.) was measured by spotting increasing concentrations of Target (from 0 to 5 μM) and fluorescence obtained after hybridization was interpolated in the curve to determine the amount of target retained on the spot. For a probe concentration of 5 μM in the case of hyd-DNA 2.36 pmol cm^{-2} of target were quantified, while in the case of direct immobilization without hydrogel, for a probe concentration of 10 μM only 0.08 pmol cm^{-2} of Target resulted hybridized. Another advantage of these hydrogels was their transparency and consequently a very low signal background.

It is well-known that three-dimensional (3D) volume immobilization in porous gels provides increased loading capacity, compared to two-

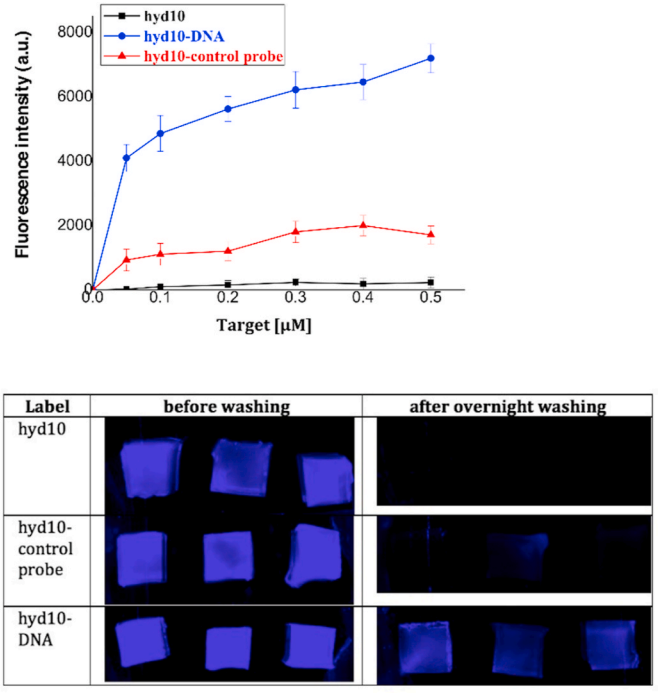
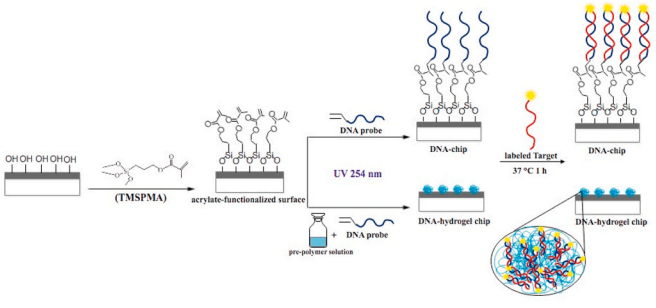


Fig. 3. On the left, hybridization assay curve of thermally obtained hyd10-DNA and its reference systems (hyd-without probe and hyd-control probe not bearing the acrydite tag) after washing overnight with SCC1x. On the right, images of hyd10-DNA and its controls obtained with a homemade surface fluorescence reader (SFR) after the hybridization with 0.2 μM of labeled Target (Table 1), before and after overnight washing.



Scheme 1. Immobilization process of oligonucleotides for both 2D and 3D microarrays.

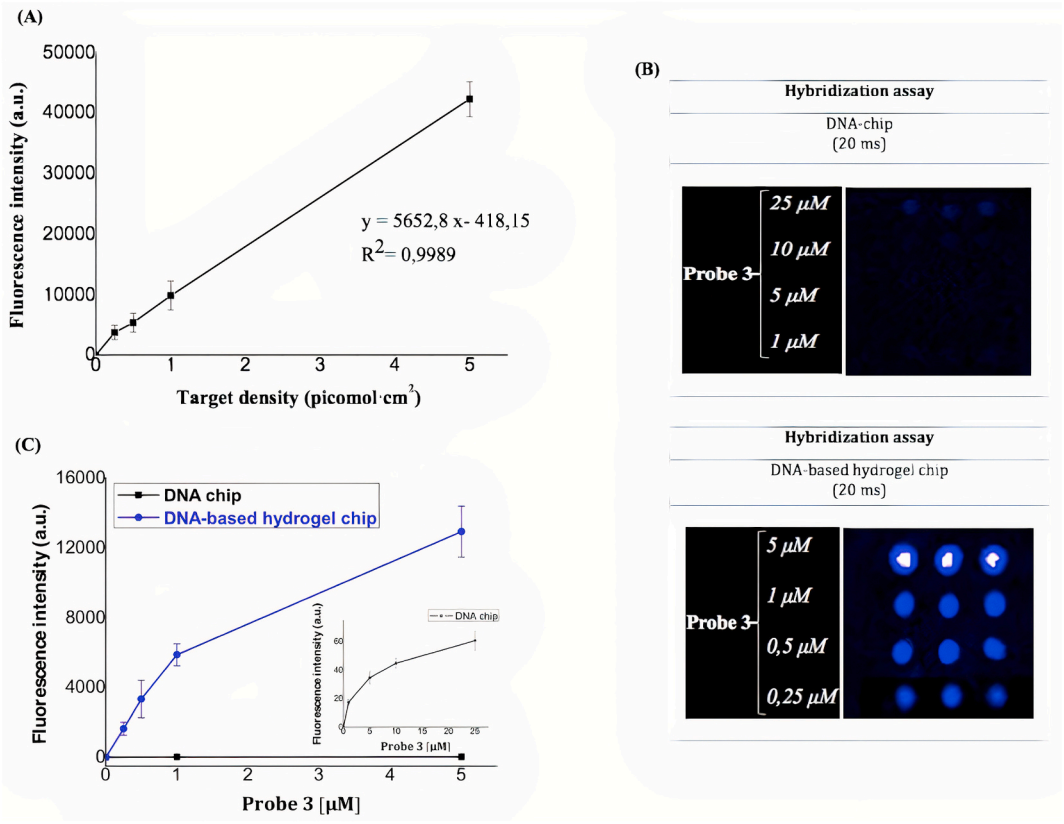


Fig. 4. (A) Calibration curve of the Target ranging from 0 to 5 mM. Spotted concentrations were transformed into picomol·cm^{-2} considering the spotted volume (10 nL) and the spot diameter (1000 mm). The regression equation and regression coefficient are shown in the plot. (B) Fluorescence images of DNA-based hydrogel chip and DNA-chip, after hybridization with the complementary labeled probe (Target at 5 μM), measurements at 20 ms are shown to appreciate the signal in the DNA-chip microarray. (C) Hybridization assay curve (5 μM of the labeled target) obtained measuring at λ 650 nm, 5 ms for different probe concentrations. The inset in the plot is a close up of the hybridization curve for the DNA chip.

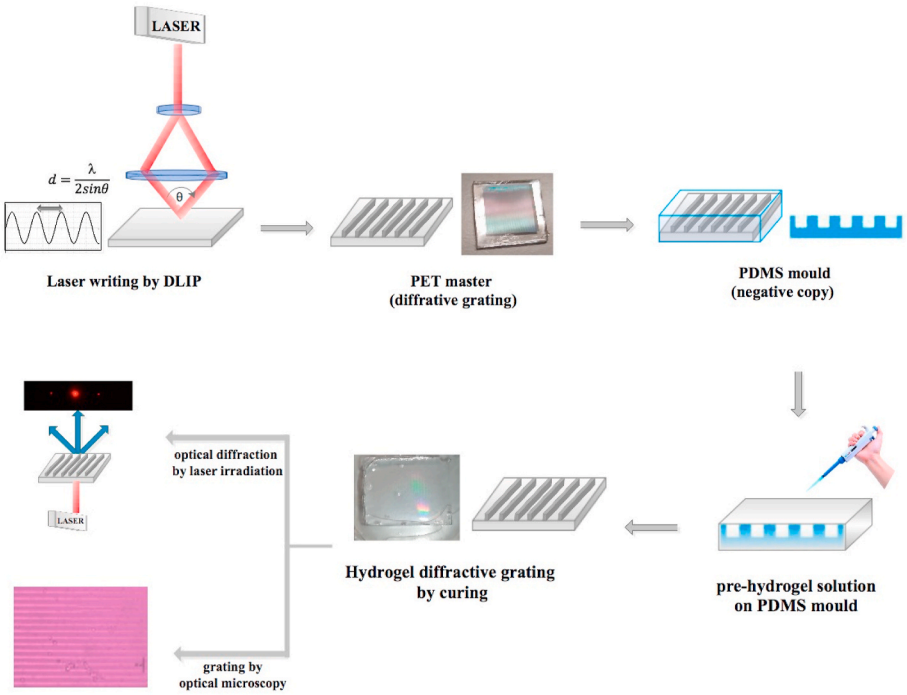


Fig. 5. Schematic representation of the sensor design process, obtained by direct laser writing (DLIP) and replica molding technique (REM). REM consist of transferring the pattern on the master into PDMS stamps and finally transferring the pattern on the PDMS back into a replica of the original master by solidifying the prepolymer solution against the PDMS mold. After the fabrication, hydrogel diffractive gratings (rainbow diffraction when illuminated by white light), when hydrated, were optically characterized under laser illumination and by optical microscope (OM).

dimensional (2D) surfaces and, as a result, the sensitivity of the microarray was highly improved.

3.4. Towards label-free biosensing

Diffraction gratings can be employed to transduce biorecognition processes into measurable signals, with the aim of label-free detection of specific molecules. Thus, hydrogels (hyd10) diffraction gratings were fabricated by replica molding from holographic PET molds (see Materials Section). The tuned properties of the optimized material allowed the grating replication and the diffraction of the gratings was observed through a laser beam (660 nm) incident from the bottom side of the hydrogel (Fig. 5.). The diffraction pattern obtained on the hydrogel surface showed equally spaced diffraction spots, which indicated that a regular, periodic hydrogel grating was formed as it was possible to observe by laser irradiation and optical microscopy OM (Fig. S5, supporting information).

Notably, the distance between the diffraction spots changed when the water content in the hydrogel raised. The grating period increased when the hydrogel swells owing to the absorption of water, but the straight shapes of the line structures were preserved, which demonstrated the optical tunability of the fabricated structure. Surface relief grating with good optical and physical properties were obtained, as resulted from the optical characterization. Diffraction efficiencies of PET masters, and DNA-hydrogels dry, fully hydrated, and after incubation with the complementary strand were measured, they varied between 2% and 11%. Distances in the diffraction first order were also measured. The data are provided in Table S2 in the supporting information. Typically, the binding of the analyte triggers a response of the hydrogel, such as changes in its swelling volume, mass, optical or mechanical properties. Our next step is the continuous monitoring of these changes for future application in label-free detection.

4. Conclusions

A rapid strategy for the covalent immobilization of DNA onto hydrogel network using the UV-initiated free radical polymerization has been developed to efficiently detect DNA. The DNA-hydrogel microarrays fabricated, thereby, allowed high and reproducible yields, increased stability of the attachment and low non-specific binding. The fabrication process was simple, consisting of one step and was very rapid. Overall, it was possible to covalently incorporate the bioreceptor group in the hydrogel network and demonstrate the bio-sensing properties by DNA hybridization assays. Indeed, the resulting DNA-hydrogel microarrays exhibited a high density of bioavailable oligonucleotide probes. Furthermore, the hydrogel containing the DNA probe was easily fabricated into a diffractive grating and shown optimal diffractive properties thanks to its high optical transparency and mouldability. Accordingly, our future objective will be the combination of this high-performance biosensor with transductors that allow working in label-free format. Hence, the developed strategy could be applied to the fabrication of point-of-care devices for biomolecular detection in medical diagnostics.

CRediT authorship contribution statement

Paola Zezza: Investigation, Writing – original draft. **María Isabel Lucío:** Methodology, Writing – review & editing. **Ángel Maquieira:** Writing – review & editing, Supervision, Funding acquisition. **María-José Bañuls:** Conceptualization, Methodology, Writing – original draft, Supervision, Funding acquisition.

Declaration of competing interest

The 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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Appendix A. Supplementary data

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