Supporting Information

Highly Sensitive Detection of Antibodies in a Soft Bioactive Three-Dimensional Bioorthogonal Hydrogel

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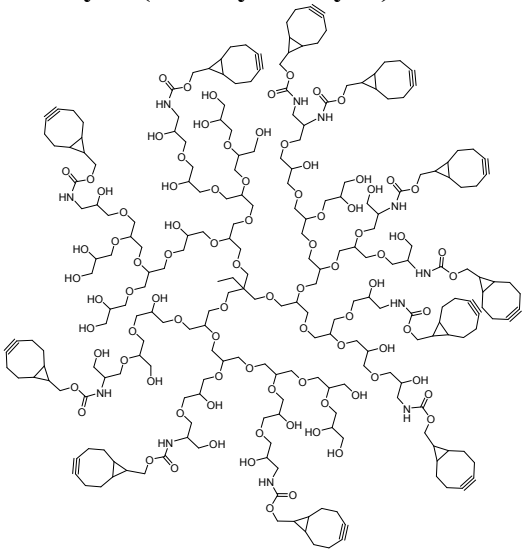
Materials

All chemicals were purchased from Sigma (Germany) and used without further purification, unless otherwise noted. α-Amino-ω-azido PEG, PEG-MW. 3000 Dalton, 5000 Dalton, 10000 Dalton, and 20000 Dalton were from RAPP Polymer (Germany). Glutathione (GSH), DYKDDDDK epitope tag antibody (Anti-FLAG®) DyLight® 650 conjugate (FG4R), DYKDDDDK epitope tag antibody, and DYKDDDDK epitope tag antibody DyLight 550 conjugate (FG4R) were from ThermoFischer. AbX™ DyLight® 549-labeled glutathione mouse monoclonal antibody, glutathione (GSH) monoclonal antibody were from Arbor Assays. Goat $F(ab')_2$ anti-mouse IgG (Fc)-Cy3 was from Dianova GmbH (Germany). 2D maleimide-functionalized slides were purchased from PolyAn GmbH. ^{1}H NMR analysis was performed at 500 MHz and 700 MHz on a Jeol™ ECX spectrometer. The chemical shifts were reported in δ (ppm) values and referenced to the indicated deuterated solvents. The samples were measured at a concentration of 15 mg/mL in $CDCl_3$, MeOD or D_2O depending on the solubility of the substance in the solvent. The solvent used in the ^{1}H NMR analysis is mentioned in the data section, which is included in the experimental section. Gel permeation chromatography (GPC) measurements were performed on an Agilent™ 1100 series instrument equipped with a refractive index detector and an UV detector (254 nm). For calibrations, PS standards were used and calculations were carried out with PSS™ Win-GPC software. Static contact angles were measured using an automated contact angle goniometer (contact angle system OCA by Dataphysics™), and the software by Northern Eclipse™ was used to determine the contact angles using a Laplace-Young fitting. A droplet with a volume of approximately 3 µL of ultrapure MilliQ™ water was used for the experiments. Average contact angles were calculated from 3 measurements. Dialysis was performed in spectra por dialysis tubing with variable molecular cut-off sizes (Carl Roth GmbH, Karlsruhe, Germany). IR spectra were recorded with a Nicolet AVATAR 320 FT-IR 5 SXC (Thermo Fisher Scientific, Waltham, MA, USA) with a DTGS detector from 4000 to 650 $cm^{-}1$. Dendritic polyglycerol (dPG) with a number average molecular weight (Mn) of 5.1 kDa was synthesized as reported previously.[1]

Synthesis experiments

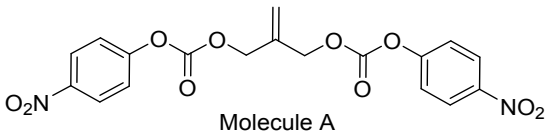
Dendritic polyglycerol-cyclooctyne (dPG-cyclooctyne)



Bicyclo[6.1.0]non-4-yn-9-yl (BCN) (0.64 g, 2.02 mmol, 1.5 eq per amino group) was added to a solution of dendritic polyglycerol amine (dPG5kDa-10%amine) (1.00 g, 1.35 mmol of amino group) and triethylamine (0.29 mL, 2.02 mmol) in dry DMF (20 mL). After stirring for overnight at room temperature (rt), the solution was concentrated. BCN was synthesized following the procedure reported in the literature by Dommerholt et al.[2] The product was purified by dialysis in MeOH/H_2O mixture 1:1 (MWCO = 2000 Dalton) followed by the removal of the solvent by freeze-drying. The product was obtained (1.1 g) and subsequently analyzed by ^{1}H NMR spectroscopy and gel permeation chromatography.

^{1}H NMR (500 MHz, δ(ppm), D_2O): 3.88-3.43 (m, dPG), 2.29-2.06 (m, 6H), 1.47-1.26 (m, 4H), 0.85-0.61 (m, 3H)

Molecule A



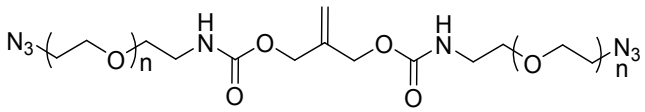
Molecule A

To a stirred solution of 2-methylene-1,3-propanediol (1.00 g, 0.011 mmol) in pyridine (4 mL, 55 mmol) 4-nitrophenyl chloroformate (5.54 g, 27 mmol) was added. The resulting reaction mixture was stirred at rt for 30 minutes. The mixture was quenched with NH_4Cl-saturated solution, extracted in DCM, dried with Na_2SO_4, and filtered and concentrated *in vacuo*. The

product was purified by column chromatography (Hexane/Ethylacetate, 10/1). A yellow powder was obtained (2.55 g, 6,54 mmol) and subsequently analyzed by ^{1}H NMR, ^{13}C NMR spectroscopy and mass spectrometry.

^{1}H NMR (500 MHz, δ (ppm), $CDCl_3$): 8.34-8.13 (m, 4H), 7.50-7.25 (m, 4H), 6.89 (d, 1H, J=9.2 Hz), 5.54 (s, 1H), 4.88 (s, 4H). **^{13}C-NMR** (500 MHz, δ (ppm), $CDCl_3$): 155.44, 152.36, 136.21, 126.30, 125.63, 125.44, 121.85, 121.74, 121.05, 120, 115.63, 68.96. **ESI-MS:** $[M+Na]^+$ – 441.3 (calculated), 441.0553 (observed), $[M+K]^+$ – 457,4083 (calculated), 457.0288 (observed).

PEG alkene di-azide (PEG-alkene-diN_3)



α-Amino-ω-azido PEG, MW. 3000 Da (0.234 g, 0.078 mmol) was dissolved in DMF (4 mL) and triethylamine (0.010 mL, 0.08 mmol) was added. Subsequently, **molecule A** was added, and the reaction mixture was stirred for 2 hours at rt. The product was purified by dialysis (MWCO = 5000 Da) in methanol then in H_2O followed by the removal of the solvent by freeze-drying and subsequently analyzed by 1H NMR, ^{13}C NMR spectroscopy and gel permeation chromatography.

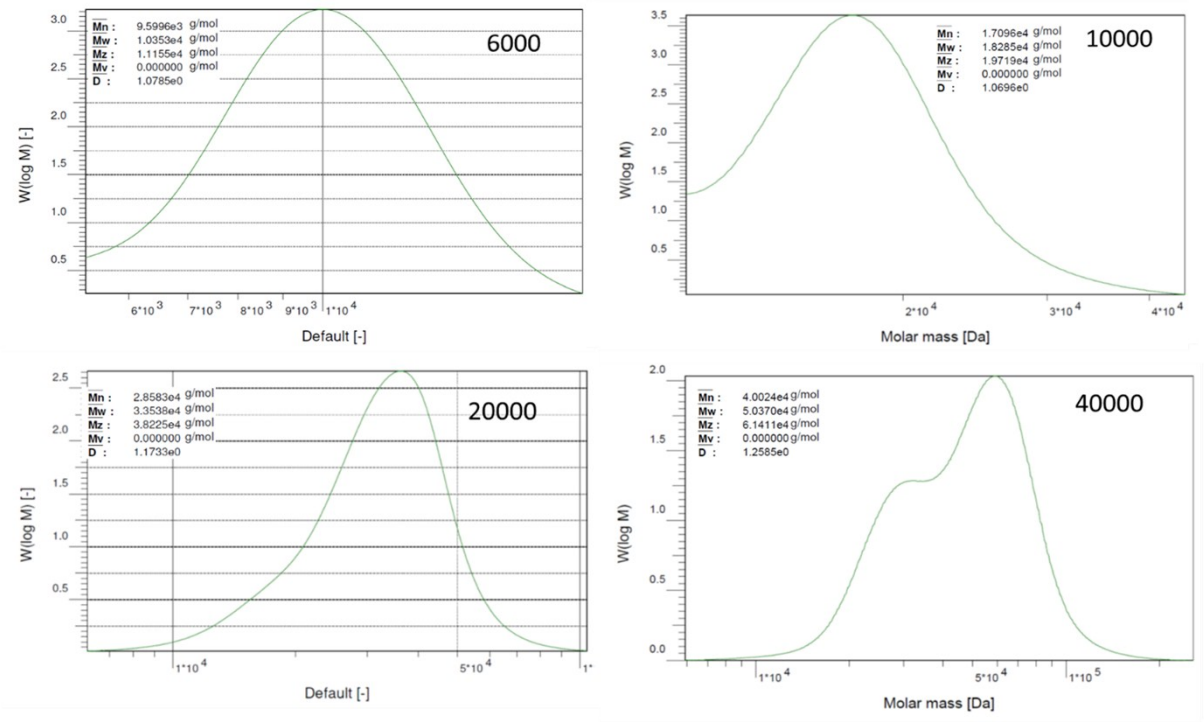
α-Amino-ω-azido PEG, MW. 5000 Da (0.234 g, 0.047 mmol) was dissolved in DMF (4 mL) and triethylamine (0.010 mL, 0.08 mmol) was added. Subsequently, **molecule A** was added, and the reaction mixture was stirred for 2 hours at rt. The product was purified by dialysis (MWCO = 8000 Da) in methanol, then in H_2O followed by the removal of the solvent by freeze-drying and subsequently analyzed by ^{1}H NMR, ^{13}C NMR spectroscopy and gel permeation chromatography.

α-Amino-ω-azido PEG, MW. 10000 Da (0.234 g, 0.023 mmol) was dissolved in DMF/DCM mixture (4 : 1) and triethylamine (0.010 mL, 0.08 mmol) was added. Subsequently, **molecule A** was added, and the reaction mixture was stirred for 2 hours at rt. The product was purified by dialysis (MWCO = 12,000-14,000 Da) in methanol then in H_2O followed by the removal of the solvent by freeze-drying and subsequently analyzed by 1H NMR, ^{13}C NMR spectroscopy and gel permeation chromatography.

α-Amino-ω-azido PEG, MW. 20000 Da (0.234 g, 0.012 mmol) was dissolved in a mixture of DMF: DCM (4 : 1) and triethylamine (0.010 mL, 0.08 mmol) was added.

Subsequently, **molecule A** was added, and the reaction mixture was stirred for 2 hours at rt. The product was purified by dialysis (MWCO = 20,000 Da) in methanol and then in H_2O, followed by the removal of the solvent by freeze-drying, and subsequently analyzed by ^1H NMR, ^{13}C NMR spectroscopy and gel permeation chromatography.

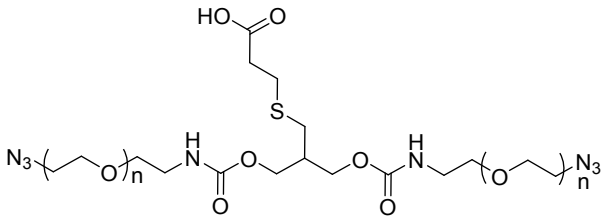
^{1}H-NMR (500 MHz, δ (ppm), $CDCl_3$): 5.23 (s, 2H), 4.59 (s, 4H), 4.06-3.46 (m, PEG), 3.39-3.35 (m, 4H). **^{13}C-NMR** (500 MHz, δ (ppm), $CDCl_3$): 70.58 (PEG).ν_{N3} (FTIR) = 2099.14 cm^{-1}.



GPC results (PEG linkers)

Figure S1. GPC results of the PEG linkers of different molecular weights (6 kDa, 10 kDa, 20 kDa, and 40 kDa)

PEG acid di-azide (PEG-COOH-diN3)



PEG-alkene-diN_3 (0.475 g, 0.047 mmol) was dissolved in distilled water (minimal amount, 8 mL) and 3-mercaptopropanoic acid (0.01 mL, 1.12 mmol) and the initiator VA-044 were added.

The reaction mixture was degassed for 1 hour under argon atmosphere then heated to 44 °C overnight. The product was purified by dialysis in H_2O followed by the removal of the solvent by freeze-drying. The product was obtained and subsequently analyzed by ^{1}H NMR, ^{13}C NMR spectroscopy and gel permeation chromatography. The conversion was determined by the disappearance of alkene groups following the disappearance of CH_2-C=CH_2-CH_2 H_2 signal in the ^{1}H NMR spectrum.

^{1}H-NMR (500 MHz, δ (ppm), $CDCl_3$): 3.88-3.38 (m, PEG), 3.30-3.20 (m, 4H), 2.90-2.87 (t, 2H). **^{13}C-NMR** (500 MHz, δ (ppm), $CDCl_3$): 70.402 (PEG), 50.754 (PEG), 29.773 (PEG)

Glutathione (GSH) peptide coupling

PEG-alkene-diN_3 (0.050 g) was dissolved in distilled water (minimal amount, 4 mL) and glutathione and the initiator VA-044 were added. The reaction mixture was degassed for 1 hour under argon atmosphere and then heated to 44 °C overnight. The product was purified by dialysis in H_2O followed by the removal of the solvent by freeze-drying. The product was obtained and subsequently analyzed by ^{1}H NMR, ^{13}C NMR spectroscopy and gel permeation chromatography. The conversion was determined by the disappearance of alkene groups following the disappearance of CH_2-C=CH_2-CH_2 H_2 signal in the ^{1}H NMR spectrum.

^{1}H-NMR (500 MHz, δ (ppm), $CDCl_3$): 3.79-3.40 (m, PEG), 3.40-3.29 (m, 4H). **^{13}C-NMR** (500 MHz, δ (ppm), $CDCl_3$): 70.66 (PEG)

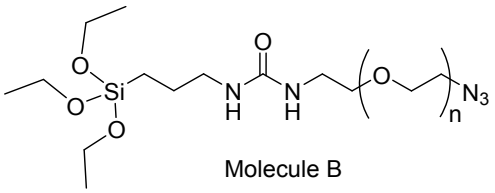
DYKDDDDK (FLAG®) peptide coupling

PEG-COOH-diN_3 (0.020 g, 0.0001 mmol) was dissolved in distilled water (minimal amount, 4 mL) and EDC, HCl (0.0425 g, 0.22 mmol) was added followed by the addition of FLAG® peptide (0.002 g, 0.002 mmol). The reaction mixture was stirred at rt overnight. The product was purified by dialysis in H_2O followed by the removal of the solvent by freeze-drying. The product was obtained and subsequently analyzed by ^{1}H, ^{13}C NMR spectroscopy and gel permeation chromatography. The conversion was determined by the presence of phenyl groups in FLAG in ^{13}C NMR spectra.

^{1}H-NMR (500 MHz, δ (ppm), $CDCl_3$): 3.79-3.40 (m, PEG), 3.40-3.29 (m, 4H).

^{13}C-NMR (500 MHz, δ (ppm), $CDCl_3$): 150.88 (benzene), 127.89 (benzene), 70.64 (PEG), 50.76 (PEG), 29.29 (PEG)

Molecule B



Molecule B

α-Amino-ω-azido PEG, MW. 3000 Da (0.600 g, 0.2 mmol) was dissolved in DMF (12 mL) and 3-(triethoxysilyl) propyl isocyanate (0.010 mL, 0.04 mmol) was added. The reaction mixture was stirred at rt overnight followed by the removal of the solvent. The product was purified twice by precipitation in DCM (10 mL) and diethylether (100 mL) at 0 °C then filtrated. The product was obtained (0.605 g) and subsequently analyzed by ^{1}H NMR spectroscopy.

^{1}H-NMR (500 MHz, δ (ppm), $CDCl_3$): 3.81 (m, 6H), 3.75-3.08 (m, PEG), 1.92 (bs, 2H, NH), 1.80-1.50 (m, 4H), 1.29-1.13 (m, 3H), 0.74-0.57 (m, 2H).

Methods

Substrate preparation

Preparation of functionalized slides. 26 mm × 76 mm glass slides (Th. Geyer GmbH & Co. KG) were cleaned in piranha solution (sulfuric acid : hydrogen peroxide, 3:1) for one hour. The slides were functionalized by immersing the substrate into a solution of **molecule B** in 30% v/v 1 M acetic acid and 70% v/v ethanol. After overnight incubation at 45 °C, the slides were washed with ethanol and dried under vacuum for one hour. The resulting azido-silanized slides were checked by contact angle measurements (CA = 39° +/- 4°) and then stored in a sealed flask until use.

Preparation of functionalized cover slips. 12-mm diameter microscope cover slips were cleaned in piranha solution (sulfuric acid : hydrogen peroxide, 3:1) for five minutes. The cover slips were functionalized by immersing the substrate into a solution of **molecule B** in 30% v/v 1 M acetic acid and 70% v/v ethanol. After overnight incubation at 45 °C, the cover slips were washed with ethanol and dried under vacuum for one hour. The resulting azido-silanized slips were checked by contact angle measurements (CA = 36° +/- 1°) and then were stored in a sealed container until use.

Rheology set-up

Rheological data were measured with a Malvern Instruments Kinexus equipped with a parallel plate geometry, with an 8-mm plate-plate. The temperature was kept at 25 °C for all experiments and an average normal force of ~ 0.07 N. A solvent trap was used to prevent evaporation. All rheological experiments were repeated three times.

Mesh sizes of the hydrogels, which are the spacing of the effective elastic units, have been investigated using oscillatory rheology and frequency sweeps with a shear stress of 1 Pa in a range of frequencies from 0.1 to 100 Hz. The mesh size ξ of the network was estimated from elastic moduli at high frequencies in the linear viscoelastic (LVE) region with the above **Equation 1**.

$$\xi = \left(\frac{G_\infty N_A}{RT}\right)^{-\frac{1}{3}} \quad (1)$$

with N_A being Avogadro constant, R the ideal gas constant, and T temperature.

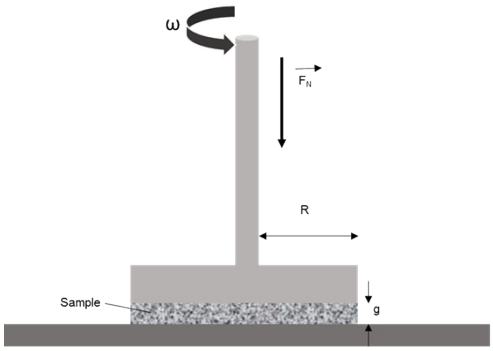


Figure S2. Schematic representation of oscillatory rheology. Which represents the general rheology set-up showing a hydrogel sample's behavior under an oscillation at a given force F_N, and a frequency ω.

Determination of Swelling Ratio and Height Expansion

For swelling tests, hydrogels (55 µL) were prepared as following. 2 mL of water was applied to the hydrogels, which were then incubated for 72 h to reach the swelling equilibrium. The surface water was then removed from the samples by using a blotting paper and the hydrogels were weighed (ms). Then, the swollen hydrogels were freeze-dried and weighed again to get the dried mass (md). The experiments were performed in triplicate and the mass swelling (q) was calculated with the **Equation 2** following the literature: [3]

$$q = \frac{ms - md}{md} \quad (2)$$

For height expansion tests, microscope cover slips were coated by spin coating 60 µL of hydrogel solution prepared as following. The samples were prepared by spin coating a 2% wt solution mixture of PEG-diazide and dPG-cyclooctyne on a functionalized cover slip, with a speed of 12,000 rpm for 30 seconds. The resulting surface was stored in a sealed container before measurement. Subsequently, the height and morphology of the coating on the cover slips were measured by atomic force spectroscopy in dry (hd) and then in swollen state (hs) (in the presence of Milli-Q water). To quantify changes in hydrogel thickness from dry to hydrated state, a needle was used to induce and scratch on the soft hydrogel surface and produce a reference for thickness determination from the substrate. Therefore, hydrogel swelling was determined by imaging the same scanned (70x70µm) region first in dry state and then in MilliQ. The height expansion e was obtained with the following **Equation 3**:

$$e = \frac{hs - hd}{hd} \tag{3}$$

Study of Hydrogel Morphology by AFM and SEM

Morphologies of different hydrogels thin films were studied by AFM imaging in dry and liquid conditions in PeakForce mode with a maximum applied force of 500 pN. In MilliQ water, images were taken at 10, 5 and 1 µm to observe changes in hydrogel surface morphology from the micro to nanoscale. Imaging in dry state lead to a poor resolution of the surface structure as expected from strong effects arising between tip and sample related to meniscus and adhesion forces which tend to obscure the surface topography of objects with high water retention capacity.

All AFM imaging of hydrogels were conducted with a Multimode 8 from Bruker with a Nanoscope V controller inside an assembled fluid chamber at room temperature. Scanning of large surface areas with a scratch in the middle for thickness determination was performed in contact mode using an optimal setpoint value. This setpoint value reflected the minimal force applied to the hydrogel surface without causing any structural damage but still allowing to obtain images with reliable surface profiles. Imaging of these large scanned areas were made with 512 points per line and at 0.3 Hz scan rate. Imaging morphological features on the hydrogel surface at higher resolution was carried out in PeakForce with a minimal tip loading force of 500 pN, resolution of 512 points per line and 0.7 Hz scan rate. In all imaging experiments, AFM tips A from Bruker, model SNL-10 with nominal tip radius of 2 nm were used.

Morphologies of bulk hydrogels were studied by scanning electron microscopy (SEM, SU8030, Hitachi, Germany). Before measurement, hydrogels were frozen in liquid nitrogen

and lyophilized. Samples were mounted on an aluminum stub with double-sided conductive carbon tape. An accelerating voltage of 20 kV was used to obtain images.

Determination of Young's Modulus: Colloidal Force Spectroscopy

Colloidal force spectroscopy (CFS) was used to study the material properties of hydrogels as a function of total molecular weights of the PEG linkers (6 kDa, 10 kDa, 20 kDa, and 40 kDa). In CFS a hard silica colloid is firmly attached at the end of a flexible tipless cantilever and used as an indenter to induce deformations on the soft hydrogel surface. Repetitive approach-retraction cycles allow to obtain force-separation curves which can be used together with contact models to extract elasticity parameters like the hydrogel´s Young´s modulus E. In this study, an AFM Nanowizzard 4 from JPK (Berlin, Germany) was used in force spectroscopy mode to map the hydrogel surface when in MilliQ, using a 16x16µm grid with a single approach-retraction cycle at each point. Silica colloids 23 µm in diameter from Microparticles GmbH (Berlin, Germany) where glued with UHU plus Endfest epoxy to the apex of tipless cantilevers D from Bruker model MLCT-010 (nominal spring constant k = 0.03N/m). Prior to an experiment the system (cantilever+colloid) was calibrated by compressing a hard surface (i.e. mica) to obtain the cantilever sensitivity. Then, an available thermal noise method was used to extract the spring constant of the system cantilever+colloid.

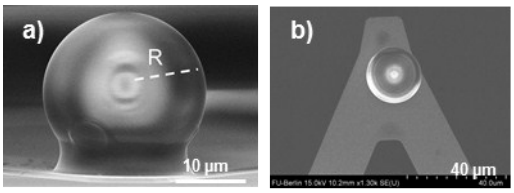


Figure S3. Features of the colloidal force spectroscopy (CFS) probe used in this study. (a) SEM image of a colloid probe attached to the cantilever used to induce deformations on the hydrogel surface. b) Lateral view optical microscopy photograph of colloidal probe.

The JPKSPM data processing software was used to process all obtained force distance curves and fit the experimental data with the contact model of Hertz. All curves were base-line corrected and all curves were transformed from force-piezo displacement curves to force-separation curves, to account for the determination of tip-sample separation distance or point of contact. A Poisson ratio of 0.5 characteristic of soft rubber-like materials was used, and only experimental deformation data below 35 nm was considered within the fit.

ELISA

A rat-reduced glutathione ELISA kit (lot no. MBS724319) was purchased from Mybiosource.com and used as received. The content of each kit was: a sealed microtiter plate with coated 48 wells, an enzyme conjugate, six standard solutions with a range of concentrations from 0 ng/mL to 10 ng/mL, two substrate solutions, a stop solution, a wash solution, and a balance solution.

Procedure: each well was filled with a constant volume of each standard solution and a blank control well was filled with PBS buffer (10 mM, pH 7.4). The conjugate solution was then added to each filled well except the blank control. The strip plate was then covered and subsequently incubated at 37 °C for 1 h. After incubation, the wells were completely emptied by inverting the plate, and blot dried by hitting the plate onto absorbent paper or paper towels until no moisture appeared. Each well was filled with washing solutions. The procedure was repeated five times for a total of five washes. After that, substrate solutions A then B was added to each well including blank control, covered avoiding sunlight, and incubated at 37 °C for 10 to 15 min. Finally, stop solution was added on each well including blank control and the optical density (O.D.) was determined at 450 nm using a microplate reader.

Two-dimensional (2D) Slides

Array preparation. The peptide coupling to maleimide slides was performed by following the protocol by Zimmermann et al.[4] All serum antibodies stocks were prepared in a spotting buffer (phosphate buffer 0.2 M pH 8, 0.1 M NaCl, 1% w/v BSA, 0.02% w/v Tween 20) and stored at 4 °C before use. Antibodies were fluorescent-labeled with DyLight® 549. Slides were spotted by hand with a constant volume solution of 1 μL of antibody serum with the same antibody concentration.

Three-dimensional Hydrogel Slides

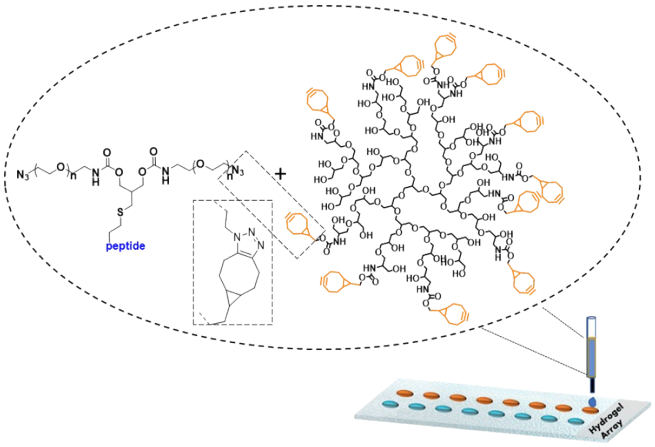


Figure S4. Hydrogel formation on a glass slide by SPAAC

Formulation of hydrogels

10% wt PEG alkene di-azide and 10% wt dPG-cyclooctyne were prepared and the hydrogel was formed by mixing both components with a 1 : 10 ratio dPG-cyclooctyne : PEG-diazide.

Array preparation. Functionalized slides were spotted by hand with a constant volume solution of 1 µL. Hydrogel spots generated an array of hydrogels of approximately 2 mm diameter and were printed with a spot-to-spot spacing of approximately 1 mm. During the process, the slides always lay in a humid chamber to keep the hydrogel swollen. After the gel formation on the slides, two different types of assays were performed on the array, a direct assay and an indirect assay. In the direct assay, a constant volume of a corresponding fluorescently labeled target antibody to the peptide was dropped on the hydrogel spot, the slide was then incubated for 1 h, then washed three times with 10 mM PBS at pH 7.4 for 15 min, and thoroughly rinsed with distilled water. The resulting printed slides were dried with pressured N_2, followed by fluorescence detection. In the indirect assay, a constant volume of a corresponding unlabeled target (primary) antibody was dropped on the hydrogel spot. The array was subsequently washed before adding a secondary fluorescently labeled F'$(ab)_2$ fragment antibody, then incubated for 1 h and washed once again. The resulting printed slides were dried with pressured N_2, followed by fluorescence detection. All serum antibodies stocks were prepared in a spotting buffer (phosphate buffer 0.2 M pH 8, 0.1 M NaCl, 1% w/v BSA, 0.02% w/v Tween 20) and stored at 4 °C before use.

Array spotting formats

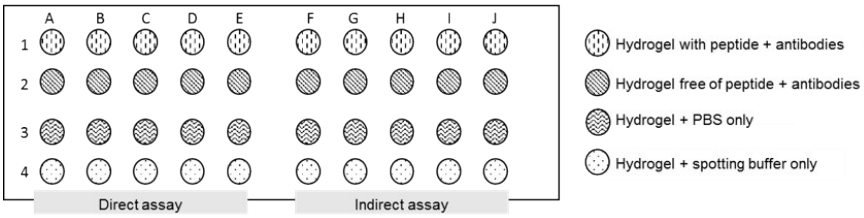


Figure S5. On each slide, four rows were present. (1) A row of hydrogels containing peptides and supplied with a constant volume of serum antibody, (2) a row of hydrogel spots free of peptide and supplied with a constant volume of serum antibody (control), (3) a row of hydrogel spots supplied of a constant volume of only PBS instead of an antibody serum (control), and (4) a row of hydrogel spots supplied with a constant volume of spotting buffer instead of an antibody serum (control).

Concentration-dependent Experiment

Direct assay. Different concentrations of fluorescent-labeled antibodies were applied onto the different spots of the same slide and incubated in a humid chamber for 1 hour at rt followed by fluorescence detection.

Indirect assay. Different concentrations of unlabeled antibodies were applied onto the different spots of the same slide and incubated in a humid chamber for 1 hour at rt. The slide was then washed three times with 10 mM PBS at pH 7.4 for 15 min and thoroughly rinsed with distilled water. Subsequently, the same concentration of DyLight 549-labeled secondary antibodies was applied onto the different spots of the same slide and incubated in a humid chamber for another 1 hour at rt. Following that, the fluorescence detection was performed.

Incubation Time-dependent Experiment

Slides were incubated with the same concentration of antibodies for different periods of time (0 min, 5 min, 15 min, 30 min, 1 h, 2 h, overnight, one week) with the slides laid out in a humid chamber.

Washing Time-dependent Experiment

Slides were immersed in a stirring staining rack containing a washing buffer (10 mM PBS solution, pH 7.4) for different periods of time (15 min, 30 min, 45 min, overnight, 3 days) and

rinsed in distilled water then dried with a nitrogen flow before fluorescence measurements. This washing procedure remained the same for the different assays performed.

Specificity of the System and Effect of the Peptides

Direct and indirect assays were performed by using the specific antibody to the peptides *or* the unspecific antibody to the peptides following the same protocol as reported above.

Selectivity of the System

Direct and indirect assays are performed by using specific antibodies to the peptides *and* the unspecific antibody to the peptides following the same protocol as reported above.

Fluorescence Detection, Data Extraction and Analysis

Fluorescence measurements and imaging were performed on a ChemiDoc MP™ imaging system equipped with a 16-bit charge-coupled device (CCD) camera, a built-in UV and white light illumination, as well as red, green, and blue epi LED light sources. Imaging and quantification of the fluorescence data were performed using Image Lab™ software. Values from quintuplicate and quadruplicate points were background subtracted and averaged. Points that were inconsistent across different slides were removed. The dataset showed a good consistency across triplicated experiments.

Limit of detection (LOD). The limit of detection is the lowest amount of a substance that is distinguishable from the background noise, which means the signal corresponding to k times the standard deviation, s, of the blank above the mean blank value. Typically, k = 3, i.e., the probability of a signal higher than 3s above the blank originating from the blank is less than 5%.

Limit of quantitation (LOQ). "The limit of quantitation is the point at which the [method] can distinguish between two different amounts of analyte. Ideally, measurements would be acquired along the linear dynamic range, the portion of the curve in which the intensity increases linearly with analyte concentration. The boundaries of the linear range are defined as the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ)."[5]

Sensitivity. The sensitivity of a biosensor measures how a small change in concentration or quantity of the analyte can make an important response. The linear regime in the calibration curve defines well this characteristic. Consequently, the slope of the analytical calibration curve represents the sensitivity of a given analyte.[6]

Results

AFM characterization of thin hydrogel films

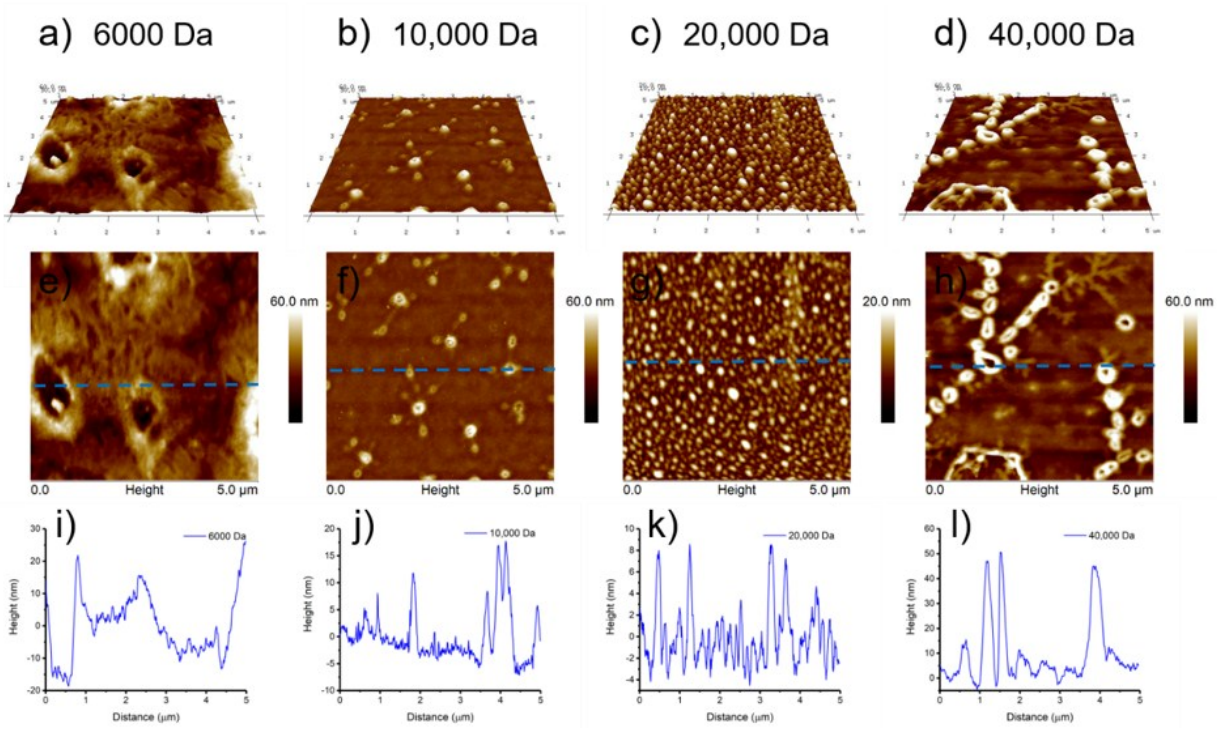


Figure S6. 3D (a-d) and 2D (e-h) AFM images of the surface topography of hydrogels in swollen state as imaged in Milli-Q water. For these 5 x 5 µm surface areas, the presence of bifurcations and pores on the hydrogel surface was evident. Although the AFM tip could not penetrate inside the hydrogel, their soft nature allowed to reveal protrusions and connections directly underneath the surface. Cross-sections provided in (i-l) give a glimpse into the surface profiles and the dimensions of height and depth of the imaged pores.

Table S1. Main 3D surface roughness parameters calculated with Nanoscope analysis software over the 10 x 10 µm region shown in **Figure 3** in the main text. R_a stands for average roughness or mean value of vertical deflections at the surface. R_q is the root mean square roughness, which considers smaller variations of the surface roughness. R_{max} describes the maximal peak to peak variations in differences of height and depth along the scanned region. In the present case, large deviations in R_{max} reflected the dimensions of pore protrusions or pore depth.

Surface roughness	**R_a (nm)**	**R_q (nm)**	**R_{max} (nm)**
6000 Da	9.6	12.6	104
10000 Da	2.8	3.9	61.4
20000 Da	2.8	4.3	70.4
40000 Da	6.2	9.1	81.6

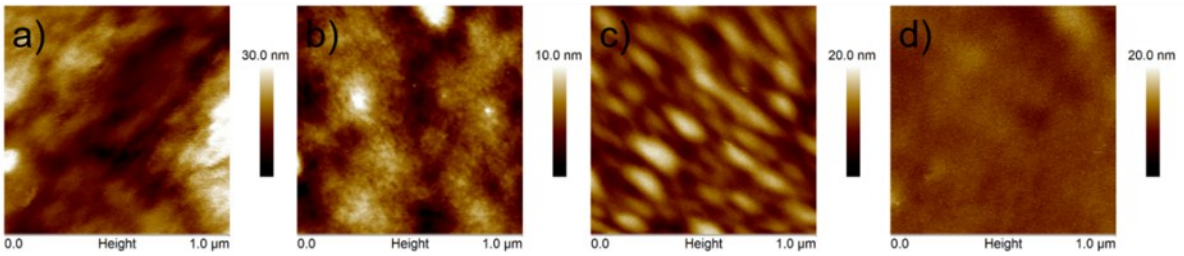


Figure S7. High resolution AFM images for 6000, 10000, 20000, and 40000 Da are respectively shown in (a-d). Despite the use of very sharp AFM tips (SNL-10 from Bruker) with nominal tip radius of 2 nm, the nanoscopic nature of the hydrogels was only revealed to some extent. Patterns showing junctions and packing domains were common but imaging a lower scan sizes than 1 µm usually led to nebulous features.

SEM images

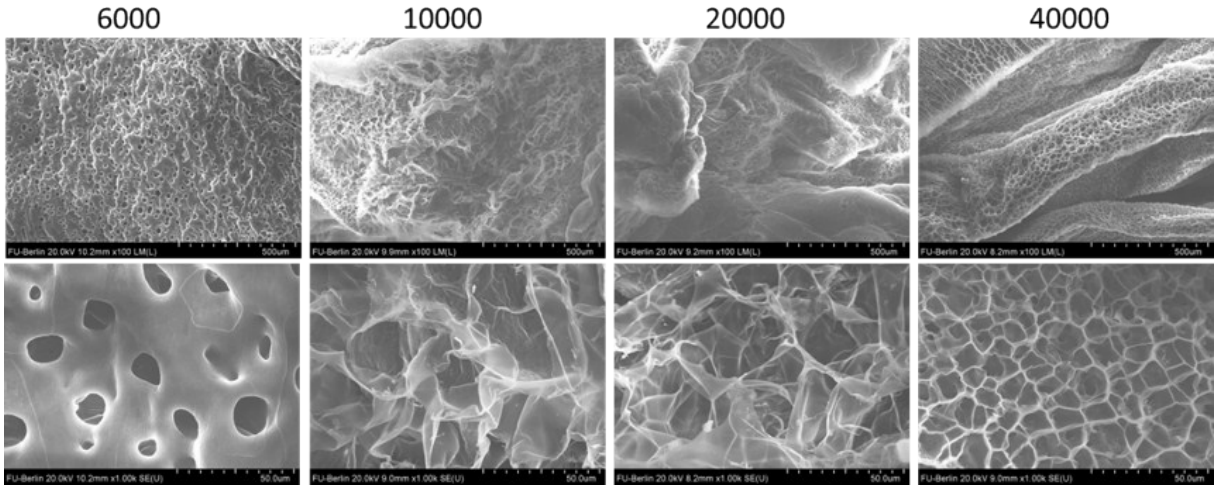


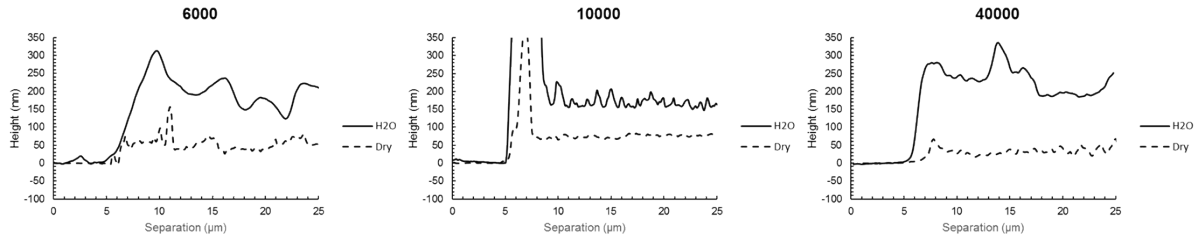
Figure S8. SEM images acquired for the bulk hydrogels with different molecular weights of PEG linkers, at two different magnifications, suggesting a structured pore distribution for 10 kDa, 20 kDa, 40 kDa.

Expansion of Thin Hydrogel Films and Swelling of Bulk Hydrogel

Table S2. Height expansion summary of thin hydrogel films in dry and swollen states with PEG 6 kDa, 10 kDa, 20 kDa, and 40 kDa and summary of the swelling in dry and swollen states for dPG-PEG bulk hydrogels with PEG 6 kDa, 10 kDa, 20 kDa, and 40 kDa.

Thin hydrogel film			
PEG MW (Da)	h_{dry} (nm)	h_{H2O} (nm)	Height expansion ratio He
6000	52.19 ± 2.72	191.75 ± 6.01	3.67
10000	74.61 ± 4.40	166.67 ± 10.56	2.23
20000	31.49 ± 6.21	273.64 ± 8.91	8.68
40000	31.29 ± 4.34	224.93 ± 6.38	7.18

Bulk hydrogel			
PEG MW (Da)	W_{dry} (nm)	W_{H2O} (nm)	Swelling ratio
6000	1.27 ± 0.90	35.93 ± 23.97	27.85
10000	1.20 ± 0.35	51.00 ± 7.50	51.7
20000	0.90 ± 0.17	65.33 ± 22.12	76
40000	3.33 ± 0.58	337.03 ± 24.50	101



Rheology

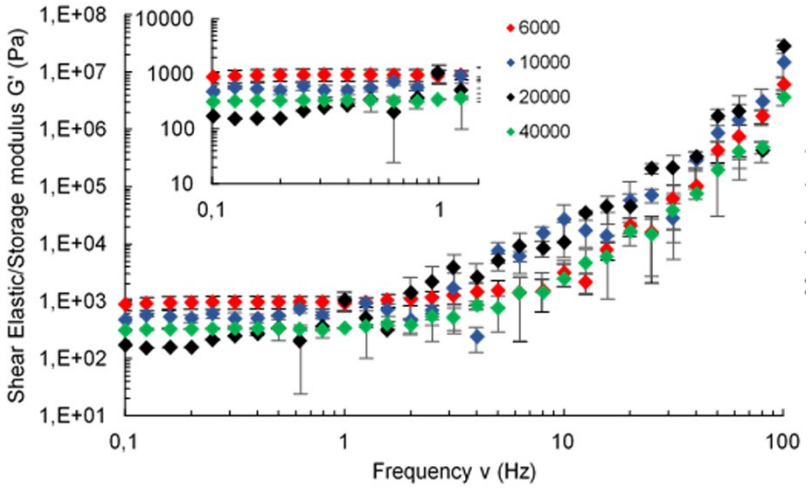


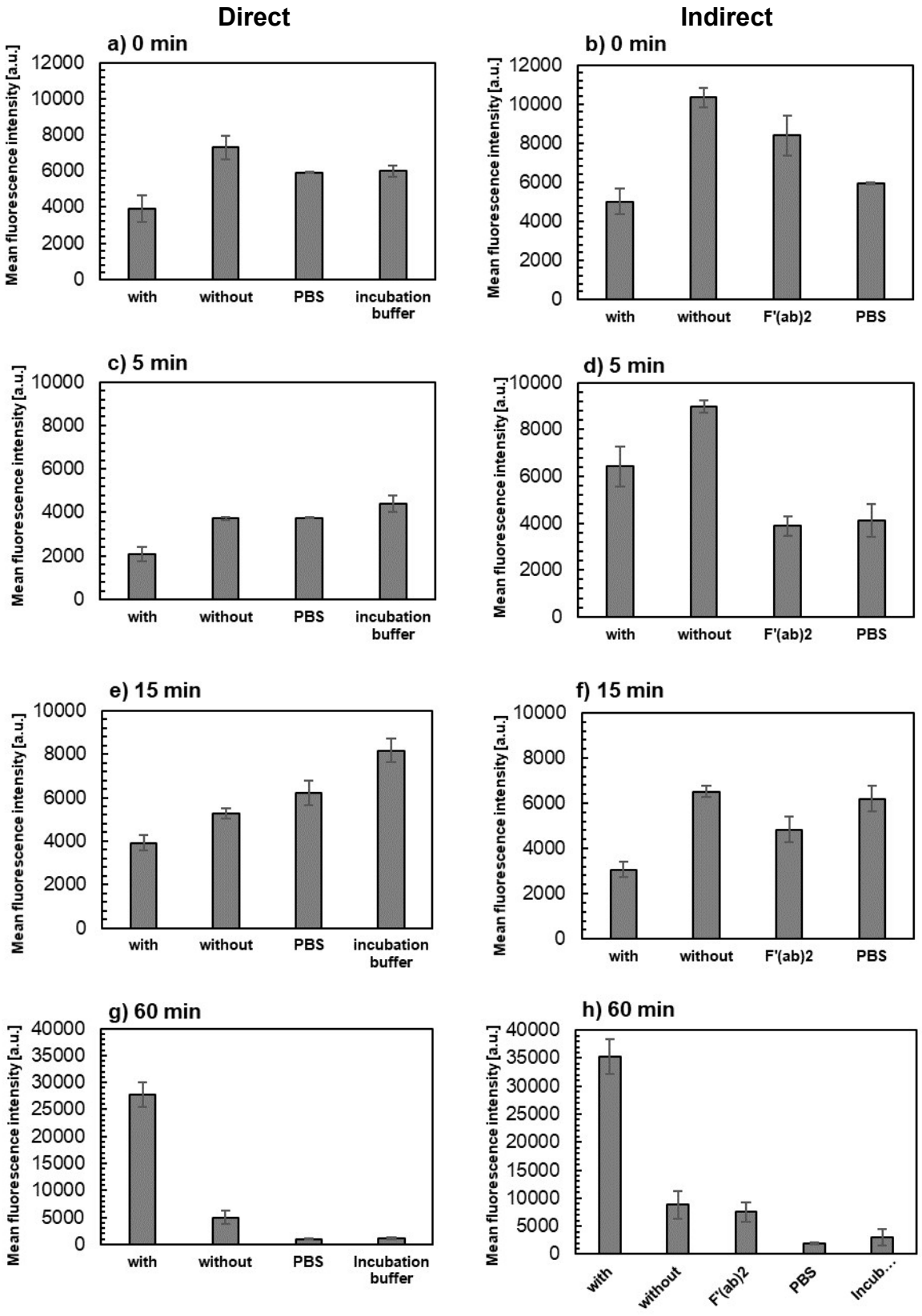
Figure S9. Mechanical characterization of dPG-PEG-based hydrogels at different scales of the material. A representation of shear elastic modulus as a function of frequency for different molecular weights of PEG linker. 6000, 10,000, 20,000, and 40,000 Da correspond to total molecular weights of the different PEG linkers studied. Hydrogels exhibited linear viscoelastic (LVE) behavior up to 2 Hz. At low frequencies, G' approaches a plateau. The increase after 2 Hz, typical of a crosslinked gel, characterizes the glass transition of the sample, until reaching a second plateau at frequencies above 100 Hz where the glassy region starts. [7]

Table S3. Summary of hydrogels macro and nano-characteristics as a function of the PEG linker's total molecular weight by oscillatory rheology and colloidal force spectroscopy. Bulk hydrogel's stiffness decreases by increasing PEG linkers Mw, quantified by shear elastic modulus at high frequency, and mesh size increases by increasing PEG linker's MW. Elastic modulus obtained by both CFS and rheology, without significant difference between nano and macro in terms of modulus of elasticity. Diffusion rates increase with PEG MW due to larger mesh size because of the longer PEG chains. Diffusion rate is calculated with Peppa's equation: $D = D0 - \frac{(Rh * D0)}{\xi}$, D0 being the diffusion rate in water, Rh the hydrodynamic radius of IgG antibodies. [35]

PEG linker Mw (Da)	Shear elastic modulus G'∞ (kPa) by rheology	Young's Modulus E (kPa) by AFM	Mesh size ξ (nm)	Diffusion rate D ($cm^2.s^{-1}$)
6000	0.962 +/- 0.169	0.86 ± 0.43	16	$2.49*10^{-7}$
10000	0.567 +/- 0.241	0.41 ± 0.09	19	$2.71*10^{-7}$
20000	0.512 +/- 0.413	0.32 ± 0.07	22	$2.87*10^{-7}$
40000	0.317 +/- 0.210	0.19 ± 0.03	24	$2.96*10^{-7}$

Peptide immobilized in 3D hydrogel-based immunosensor

Finding the optimal incubation time



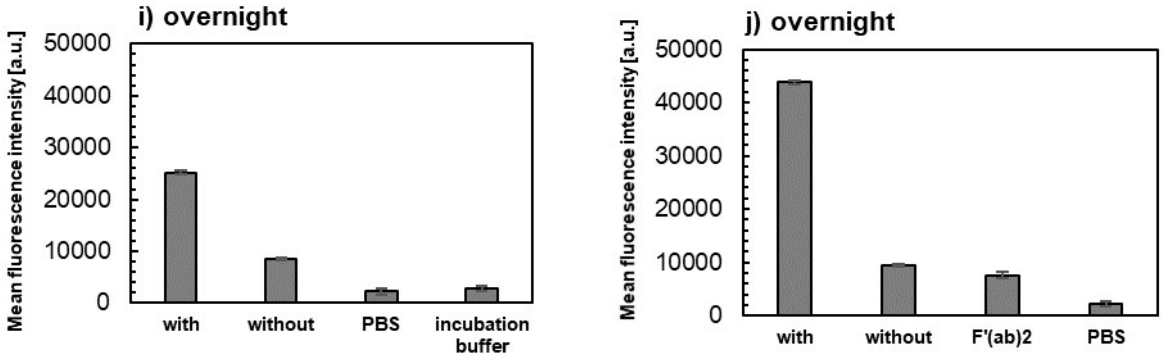


Figure S10. Relative mean fluorescence intensity (emission field at λ=549 nm) obtained for the study of the incubation procedure revealed no significant difference in fluorescence of a hydrogel matrix with FLAG peptides and without peptide for an incubation of the DyLight550 labelled Anti-FLAG antibodies in hydrogel spots for 0 to 15 min in graphs (a), (b), (c), (d), (e), (f). Graphs on the left are the results for direct assay and graphs on the right the results for indirect assays. The system did not reach the equilibrium before 1h. Relative mean fluorescence intensity obtained for the study of the incubation procedure revealed significant differences in fluorescence of a hydrogel matrix with peptides and without peptide occurring after 1 h incubation of antibodies in hydrogel spots in graphs (g) and (h). After 1 h incubation, this significant difference remained for both direct and indirect assay after overnight incubation in graphs (i) and (j) ensuring a stability and reproducibility of the array. A minimal time of 1 h incubation of antibodies was thus the optimal incubation time of antibodies in hydrogels. The same optimal incubation time was observed in both direct and indirect assays.

Finding the optimal washing time

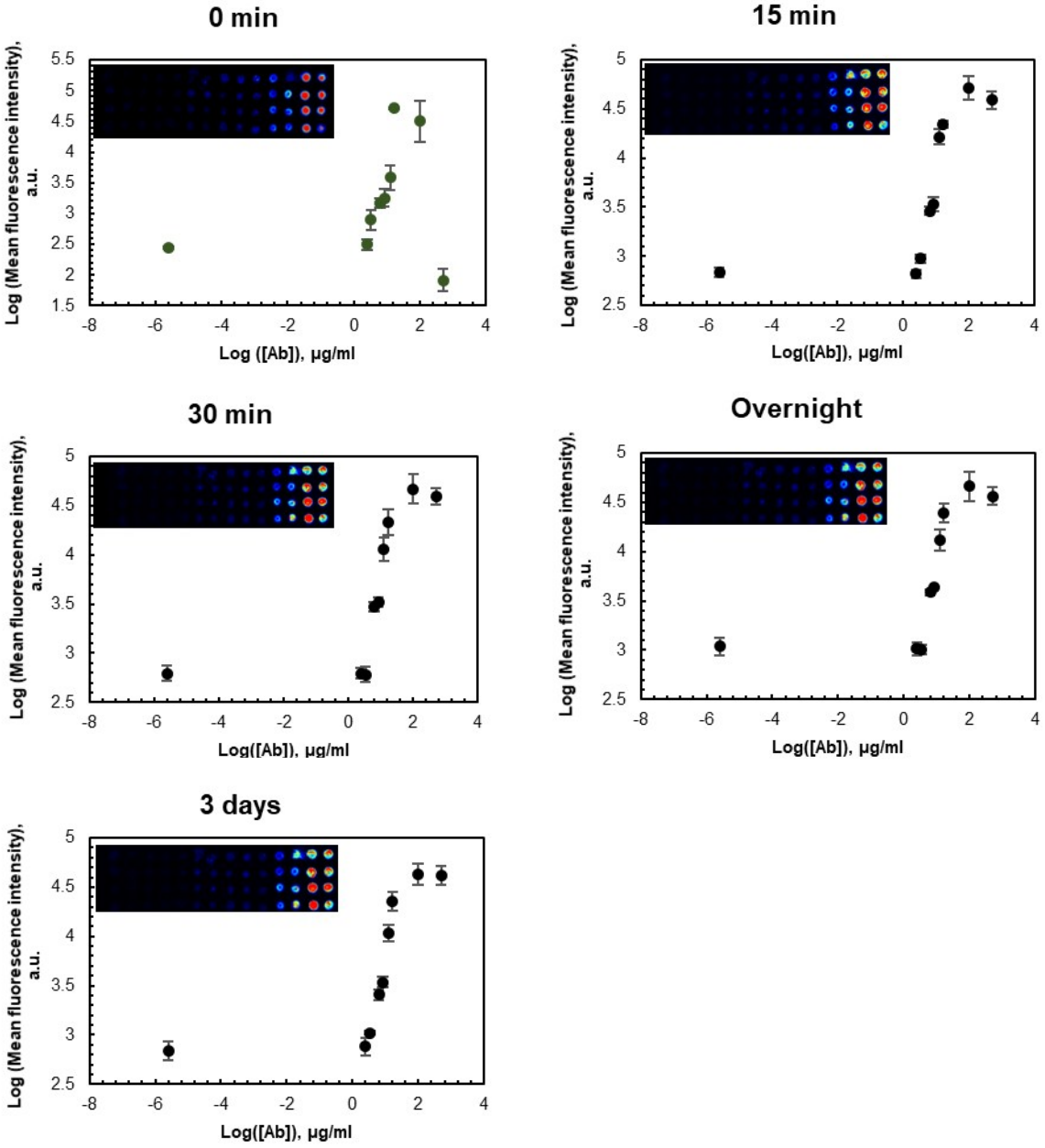


Figure S11. Mean fluorescence intensity (emission field at λ=549 nm) obtained for the study of the washing time of the hydrogel matrix with FLAG peptides and without peptide for an incubation of the DyLight550 labelled Anti-FLAG antibodies in hydrogel spots revealing a constant number of fluorescence after first wash requiring only 15 min in stirring PBS buffer. After 30 min to 3 days, the fluorescence signals remained stable. The non-significant difference in mean fluorescent intensity between 15 min incubation to 3-day incubation reflected the

stability and strong antibody-peptide interaction, which ensured a minimum loss of material during the array process. The color indicates a high to low fluorescent signal from red to blue.

Specificity and effect of peptides

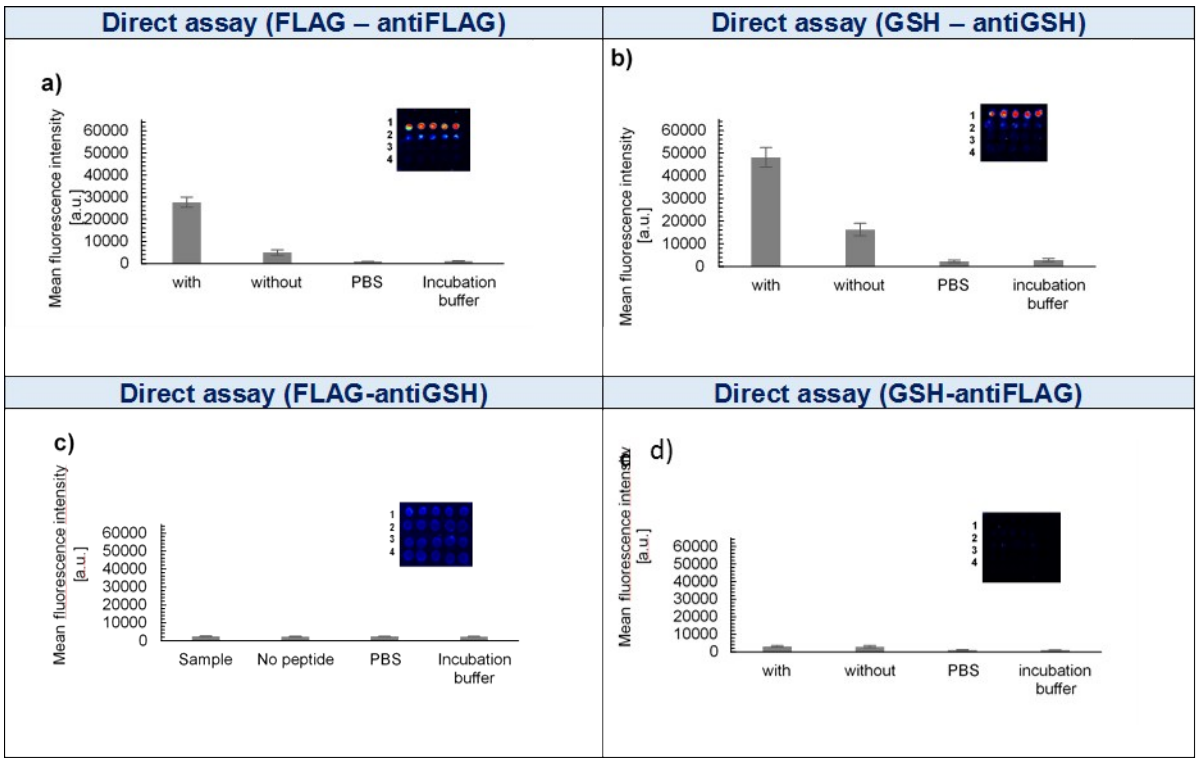
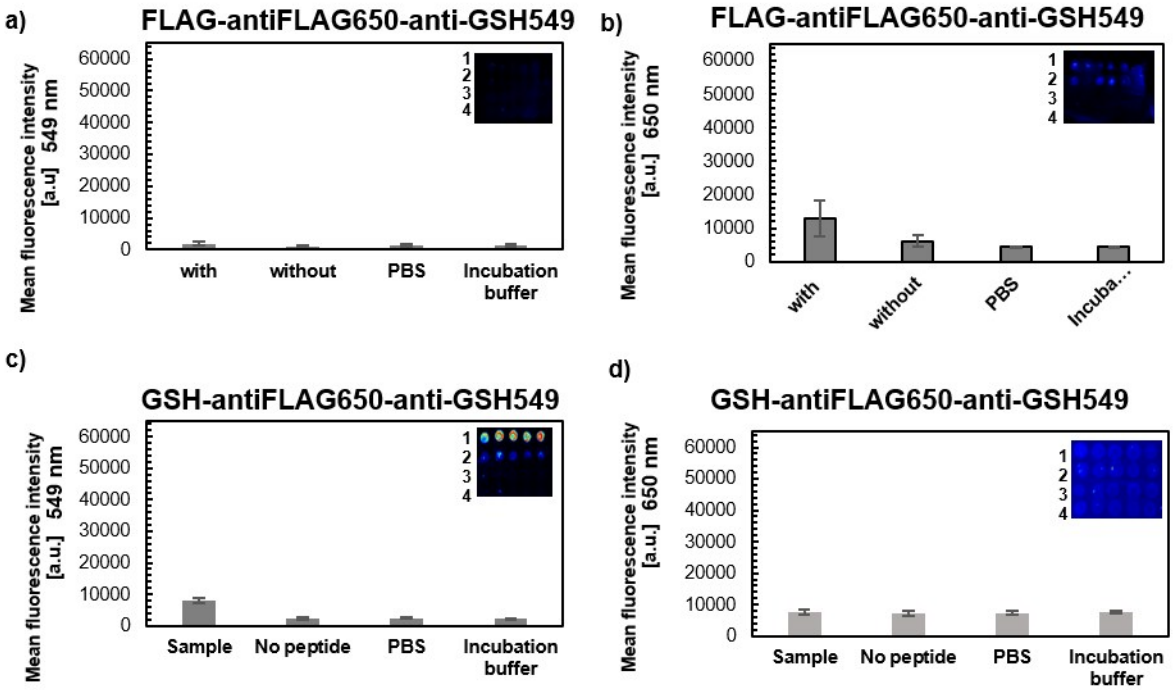


Figure S12. Mean fluorescence intensity (emission field at λ=549 nm) obtained for the study of the specificity of the detection method with FLAG and GSH peptides against DyLight550 labelled Anti-FLAG and DyLight549 labelled Anti-GSH antibodies in hydrogel spots. (a) and (c) depict the assays with FLAG peptides and (b) and (d) are with GSH peptides. In (a), (b) the specific antibody to the peptides were used and in (c), (d), the unspecific antibody to the peptides were used. In the row numbering in the fluorescence images: 1 to 4 are the numbering for direct assays. (1) is with peptide covalently immobilized in the hydrogel, (2) without peptide in the hydrogel, (3) PBS only, no antibodies, and (4) with only the incubation buffer, no antibodies.

Selectivity of the 3D hydrogel sensor

The system is selective to a given antibody if only the specific antibody to the peptide is recognized by the sensing system in presence of other interferences, i.e., an unspecific antibody. In our case, the selectivity of the hydrogel peptide system was studied by diffusing two types of fluorescently labeled antibodies, one is specific to GSH peptide (anti-GSH labeled with

DyLight 549) and the other is specific to FLAG peptide (anti-FLAG labeled with DyLight 650). As a result, the unspecific peptide to antiFLAG-Dylight650 would be GSH peptide and the unspecific peptide to antiGSH-DyLight 549 would be FLAG peptide. If only a fluorescent signal on the channel of emission of the specific antibody to the peptide is recorded, then the system in selective. The results are presented in **Figure S14**. We observed that a very low fluorescence intensity was recorded in **Figure S14a and d**, which meant that no antibody of the unspecific species remained in the hydrogel matrix. The specific antibodies (**Figure S14b and c**) fluorescence signal was slightly significant for the row with peptides and controls present no fluorescence. The sensing system did not exhibit the same fluorescence signal as without interference though. These observations have demonstrated that the sensing system could discriminate between unspecific antibodies and the specific ones but only at a ratio 1:3



(with interference: without interference).

Figure S13. Hydrogel arrays fluorescence spectra and imaging recorded in two emission fields (549 nm and 650 nm) were plotted using the arithmetic means of each quintuplicate. (a) FLAG peptide containing hydrogel and diffusion of anti-GSH DyLight 549 and anti-FLAG DyLight 650, recorded on 549 nm, (b) FLAG peptide containing hydrogel and diffusion of anti-GSH DyLight 549 and anti-FLAG DyLight 650, recorded on 650 nm, (c) GSH peptide containing hydrogel and diffusion of anti-GSH DyLight 549 and anti-FLAG DyLight 650, recorded on 549 nm, (d) GSH peptide containing hydrogel and diffusion of anti-GSH DyLight 549 and anti-

FLAG DyLight 650, recorded on 650 nm. The numbering corresponds to the following: (1) with peptide covalently immobilized in the hydrogel, (2) without peptide in the hydrogel, (3) PBS only, no antibodies, and (4) incubation buffer only, no antibodies.

Limit of detection, lower limit of quantitation, upper limit of quantitation (3D system)

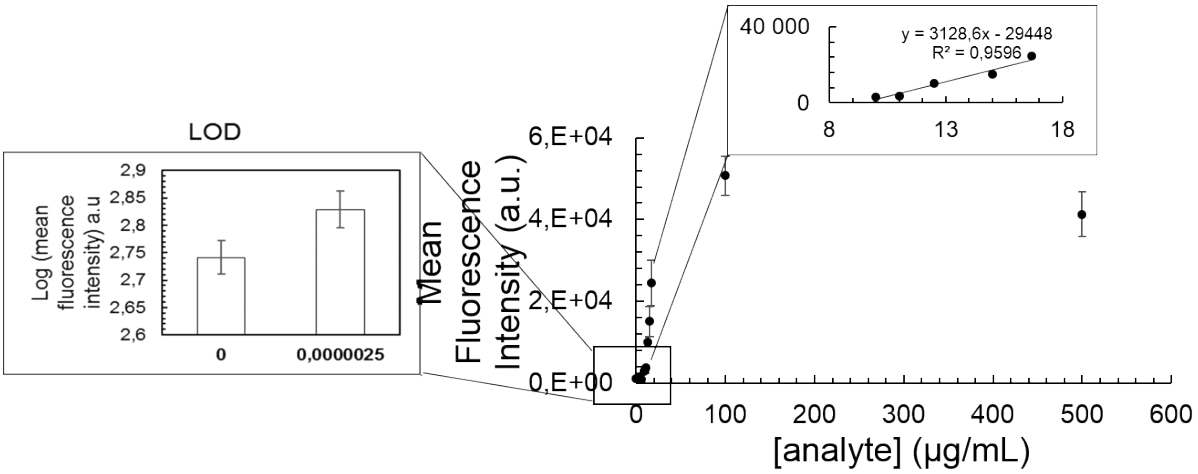


Figure S14. Mean fluorescence intensity (emission field at 549 nm) in a system FLAG-DyLight550 labelled antiFLAG, for the study of the concentration dependence experiment revealed a calculated LOD of 27 pg/mL. Comparison of the lowest detectable concentration of antiFLAG antibodies to blank "0 µg/mL" (PBS). LLOQ = 2.5 µg/mL. ULOQ = 17 µg/mL.

LOD = (concentration corresponding to the lowest detectable fluorescence signal different from blank) * 3

LLOQ = (concentration corresponding to the lowest detectable fluorescence signal that can distinguish between two amounts of analyte)

ULOQ = (concentration corresponding to the highest detectable fluorescence signal that can distinguish between two amount of analyte)

Limit of detection (2D system)

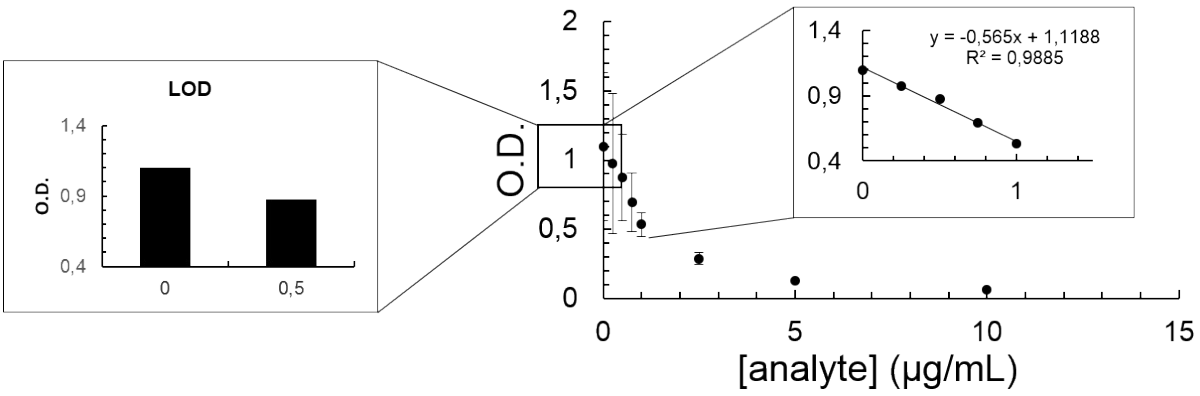


Figure S15. O.D vs concentration of analyte for the study of the concentration dependence experiment revealed a calculated LOD of 3 ng/mL in a commercial ELISA kit.

Glass surface functionalization

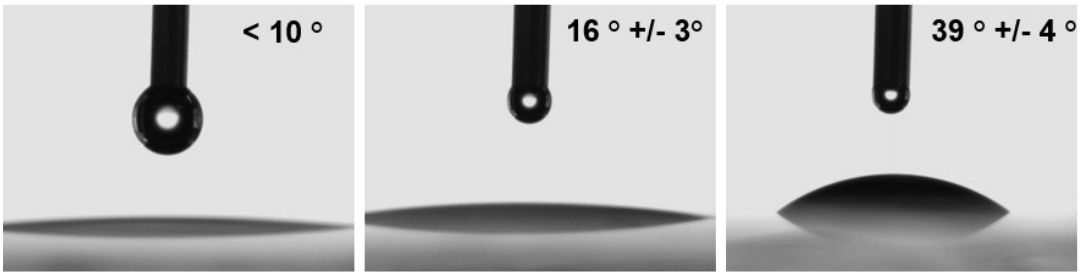


Figure S16. Glass substrates before (left) and after treatment with Piranha solution (middle) and after silanization (right).

References

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