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Investigation of Methacryloyl-Modified Gelatin-Based Hydrogels for Inkjet-Printed Biosensors and Their Adherence to Polyethylene Terephthalate and Oriented Polypropylene Substrates

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ABSTRACT

The COVID-19 pandemic has highlighted the need for rapid, simple, and cost-effective point-of-care testing (POCT) methods for pathogen detection. Hydrogel-based biosensing has emerged as an increasingly popular approach, offering advantages such as reagent storage and multiplexing capabilities. In this study, we have functionalized different polymer substrates to ensure an adequate adherence of methacryloyl-modified gelatin-based hydrogel spots that function as biosensors. Different hydrogel formulations were tested for their suitability in a point-of-care testchip. Our findings demonstrate the good adherence properties of amino-functionalized and subsequent methacrylated polymer materials, specifically polyethylene terephthalate (PET) and oriented polypropylene (OPP), when used as substrates for hydrogel biosensors. Moreover, we successfully identified an optimal formulation for the hydrogel ink, consisting of amino-functionalized methacryloyl-modified gelatin with a biopolymer content of 3.5% (w/w) and a photoinitiator content of 0.0875%. This formulation not only enables printing with a piezoelectric 2D printer but also exhibits excellent hydrogel stability and adherence to the functionalized substrates. These results contribute to the development of reliable and efficient POCT methods for pathogen detection, addressing the limitations of current diagnostic capabilities. The study emphasizes inkjet-based functionalization, with comprehensive characterization of printability including viscosity, surface tension, and density, and provides expanded methodological details to ensure reproducibility.

Practical application: This study demonstrates the successful functionalization of PET and OPP polymers as substrates for point-of-care test chips, paving the way for advanced diagnostic solutions. By amino-functionalization and methacryloylation of the surfaces, covalent bonding with biosensors was achieved, ensuring stability and adherence. A methacryloyl-modified gelatin-based hydrogel ink, optimized for piezoelectric printing, was identified for biosensor fabrication. The selected ink minimizes fluorophore quenching, preserving biosensor sensitivity. Among the tested materials, OPP showed superior adherence due to its non-polar characteristics. These findings enable the creation of multiplexed test chips capable of detecting multiple pathogens simultaneously, addressing a crucial gap in rapid and reliable diagnostics. Although the hydrogel-based biosensors have not yet been tested with encapsulated LAMP, this integration marks the next step toward fully functional point-of-care testing. Ultimately,

this research advances the development of robust diagnostic platforms, with applications in healthcare settings for timely pathogen identification and disease management.

1 | Introduction

The COVID-19 pandemic has highlighted the increasing need for rapid, simple, and cost-effective point-of-care testing (POCT) methods [1–4]. The availability of such testing methods can help ease the burden on healthcare systems, reducing the need for expensive and time-consuming laboratory testing and skilled personnel [1, 5]. Hydrogel-based biosensing for POCT applications have become increasingly popular [6–8]. Hydrogels are hydrophilic crosslinked polymer networks that can absorb a large amount of water and allow diffusion of analytes through the hydrogel matrix [9]. Du et al. [6] demonstrated the use of a hydrogel as part of a simple optical POCT sensor platform that is capable of detecting metal ions based on an analyte-induced dye release from the hydrogel. However, hydrogels can also be used to detect more than just specific ions. The large water content of hydrogels and its crosslinked nature make them a suitable material for encapsulating biological materials, as it can protect from degradation [10]. This advantage can be used for detecting important biomolecules such as enzymes, hormones, and DNA [11]. Additionally, the hydrogel's storage capability enables it to store reagents needed for the detection of pathogens and other analytes [12]. Yang et al. developed a hydrogel-based assay for detecting the SARS CoV 2 virus on food samples [13]. The authors used a hydrogel-based RT LAMP (reverse transcription loop-mediated isothermal amplification) technique that can detect virus RNA by doing a fluorescence readout. The LAMP is a special polymerase chain reaction (PCR) method, which is very promising for POCT procedures due to its simple, fast, specific, sensitive, and cost-effective properties [1, 14, 15]. However, the method proposed by Yang et al. has the limitation that it does not allow parallel detection of multiple pathogens within a single reaction. To make this possible, individual hydrogel spots encapsulating the LAMP assay will be applied to the surface of a point-of-care test using a piezoelectric 2D printer. These hydrogel spots with the encapsulated LAMP assay will act as biosensors. Applying the hydrogel as individual spots on the surface of a point-of-care test chip makes it possible to divide an entire LAMP reaction into many individual reactions. This allows false-positive or false-negative results to be clearly detected. However, it also enables multiplexing, which allows the detection of multiple pathogens using one test chip. The challenge in producing the point-of-care test chip is that the biosensors must adhere to the test chip and should not detach through the introduction of liquid. Therefore, a suitable substrate is needed as a base for the biosensors to which the hydrogel adheres. Promising materials for POCT are polymer films such as Polyethylene terephthalate (PET) and Oriented Polypropylene (OPP) due to their low cost and simple manufacturing processes [16, 17]. Moreover, polymer films are flexible and durable materials that have been widely used in microfluidic devices [18, 19]. Polymer films also have the advantage of possessing heat-resistant properties [19, 20], making them promising for the test chip, as the LAMP assay takes place at a temperature of around 65°C and the substrate must withstand this temperature. Additionally, the surface properties of polymeric foils, such as wettability, can easily be modified [19]. This property can be used to allow sufficient adhesion of the hydrogel to the substrate. Thus, a suitable hydrogel is necessary for good adhesion. Furthermore, the hydrogel is required to exhibit good hydrogel stability, so that the hydrogel spots do not dissolve on contact with liquids. Moreover, it is important for the hydrogel to be printable in order to allow scalability, reproducibility and precision [21, 22]. Using printing also reduces the risk of contamination [23], which is an important aspect for POCT that will detect pathogens. Since hydrogels often exhibit a high viscosity, they tend to clog the printer nozzles [24]. To prevent clogging a hydrogel precursor solution with a lower viscosity that can be used as an ink is beneficial [24]. This hydrogel precursor should have the ability to form a stable hydrogel after printing [24]. Hydrogels with such prospects are hydrogels from modified gelatin derivatives [24]. Via modifications like methacryloylation it is possible to influence physico chemical properties of such hydrogels [25]. The modification of crosslinkable gelatin by methacryloylation was first introduced by Van Den Bulcke et al. [26]. They derivatized gelatin with methacrylamide side groups and subsequently crosslinked the hydrogel by radical polymerization using photoinitiation. Photo-crosslinkable hydrogel precursor solutions form only after crosslinking stable hydrogels [27]. The degree of crosslinking of such hydrogels can be modulated by adjusting various parameters, including the concentrations of biopolymer and photoinitiator. Over the years additional modifications for gelatin-based hydrogels were introduced [24, 25, 28], such as the modification of methacrylated gelatin with ethylenediamine (EDA) which yields at neutral pH a more positively charged crosslinkable gelatin hydrogel [25]. This effect can be useful to ensure a better encapsulation of the negatively charged detection method components inside the hydrogel. Additionally, the crosslinkable methacryloyl groups can be helpful to promote sufficient hydrogel adherence to the substrates through the establishment of covalent bonds. This can be achieved by introducing the crosslinkable methacryloyl groups through methacryloylation of the substrate surfaces. Comeau et al. [29] presented such a method for treating nanohydroxyapatite (nHA) particles, where methacrylic anhydride was utilized in the process. However, it is also possible to apply this approach to polymer substrates. Prior to that, amino-groups, to which the methacryloyl groups can bind, need to be introduced onto the surface through amino-functionalization. A well-known method for this is the use of ammonia plasma [30]. With promising substrates and a hydrogel, it is needed to investigate the substrates PET and OPP for their suitability as materials for POCT, but also to find the ideal hydrogel and its respective hydrogel precursor formulation. With all these aspects in mind, we have functionalized the polymer substrates PET and OPP and investigated their suitability with characterization of the surfaces and investigating their hydrogel adherence in this paper. In addition, we synthesized methacryloyl-modified gelatin derivatives containing crosslinkable methacryloyl groups. The

chosen modified gelatin derivatives were a GM10 and a GM10 that was additionally amino-functionalized with EDA (GM10E10) to provide a more positive charge. Different gelatin hydrogel formulations were investigated with the aim of finding a hydrogel that is both printable and exhibits good hydrogel stability and adhesion. Additionally, we analyzed the data obtained from our experiments and drew conclusions regarding the suitability of the hydrogel and substrates for use in a point-of-care test.

2 | Materials and Methods

2.1 | Materials

Methacrylic anhydride (MAAnh), dimethylformamide (DMF), perchloric acid, ethylene diamine (EDA), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), hydrochloric acid (HCl), lithiumphenyl-2,4,6-trimethylbenzolphosphinate (LAP), glycerol and rhodamine 6G were purchased from Sigma–Aldrich (Germany). Other reagents were purchased from the following sources (given in the parenthesis): ethanol absolute (EtOH; VWR, Germany), sulfosuccinimidyl-4-o-(4,4-dimethoxytrityl) butyrate (Sulfo-SDTB; Bioworld, USA), sodium hydroxide (NaOH; Merck, Germany), 41-mer DNA oligo with Cy5 conjugated to the 5' end (Cy5-oligo; IDTDNA, USA). Sodium bicarbonate and eosin B were purchased from Carl Roth, Germany. Gelatin type B (limed, bovine bone) and type A (Medella Pro, pig skin) were donated by Gelita (Germany). The substrate samples were purchased from the following sources (given in the parenthesis): Oriented Polypropylene (OPP) films with a thickness of 11 µm (Franz Mensch, Germany), 40 µm thick OPP (Fibrolux, Germany), Polyethylene terephthalate (PET) films with a thickness of 46 µm (Schmedt, Germany). The three polymeric materials are abbreviated according to their thickness as OPP-11, OPP-40, and PET-46.

2.2 | Amino-Functionalization of Substrates (Ammonia Plasma Functionalization)

The samples were cut out into square shapes with approximate dimensions of 10 cm × 10 cm. To enhance the adherence properties of the polymer foil surfaces, they were treated with ammonia plasma, which generated free amino-groups. Amino-functionalization of polymer substrates was performed using a low-pressure ammonia plasma process. The plasma reactor and process parameters were chosen in accordance with previously established protocols for the surface modification of polymeric biomaterials, as described by Kleinhans et al. [31, 32]. Specifically, plasma activation was conducted in a capacitively coupled RF plasma reactor (as detailed in [33]), operating at a pressure of 13 Pa, with an ammonia (NH_3) gas flow rate of 20 sccm, a power setting of 20 W, and a treatment duration of 30 s. The distance between the plasma source and the substrate was approximately 5 cm. This approach has been shown to reliably introduce primary amine groups onto polymer surfaces, thereby enhancing subsequent chemical functionalization and cell adhesion [31, 32]. The plasma reactor design and deposition uniformity are further described in [33]. The amino-functionalization specifically targeted one side of the polymer films. To maintain their stability, the functionalized polymer films were stored in a desiccator filled with argon. The films were further processed within 7 days due to the loss of stability of the amino-groups.

2.3 | Methacryloylation of Substrates

After amino-functionalization, methacryloylation was performed on polymer films. The methacryloylation of OPP and PET with methacrylic anhydride was developed using the approach of Comeau et al. [29]. A 0.1 M phosphate buffered saline (PBS) with a pH of 7.4 was prepared. The amino-functionalized films were punched into 44 mm circles for easier handling. An emulsion of 0.425 M MAAnh in PBS was prepared and swirled in an ultrasonic bath. Each foil was individually placed in the MAAnh emulsion, with the amino-functionalized side facing the emulsion and shaken on a shaker at 50 rpm for 2 h. Subsequently, the films were thoroughly rinsed with water and absolute ethanol to remove any excess MAAnh. Finally, the films were air-dried and stored in a desiccator under argon.

2.4 | Characterization of Substrates by Sulfo-SDTB Assay

To determine the concentration of primary amines on amino-functionalized polymer films, a sulfo-SDTB assay was conducted. A fresh sodium bicarbonate buffer solution (2.25 g/L, pH 8.5) was prepared. A staining solution was prepared by weighing 0.5 mg sulfo-SDTB per sample into a sample vial. The reagent was pre-dissolved in DMF. Fifty microliters of DMF is used for 4 mg sulfo-SDTB. Then, for each 0.5 mg of SDTB, 1.0 mL of the buffer solution was added, gently spun, and used within 1 h. Before the sulfo-SDTB assay, amino-functionalized polymer films were punched into 3 cm circles. For each polymer foil, 1 mL of buffer solution was added into a Petri dish, followed by 1 mL of staining solution. The covered Petri dishes were shaken at room temperature for 30 min on a shaker at approximately 55 rpm. The films were then washed with water and ethanol. Subsequently, the films were air-dried until no ethanol was visible. To each foil, 2 mL developer solution (35% perchloric acid) was added and shaken for 20 min on a shaker at 55 rpm. To detect the 4,4'-dimethoxytrityl cation (DMTr Cation), absorbance readings were performed at a wavelength of 498 nm. The concentration of primary amine groups on the surface was quantified using a calibration line. A total of three films per material were examined, from which the mean value and the standard deviation were then determined.

2.5 | X-Ray Photoelectron Spectroscopy of Substrates

X-ray photoelectron spectroscopy (XPS) measurements were performed for amino-functionalized PET-46, OPP-11 and OPP-40. For comparison the unfunctionalized substrates were measured as references. XPS spectra were acquired on a KRATOS Axis Supra spectrometer using monochromatic Al Kα radiation (1486.6 eV). Charge neutralization was used for all samples. The spectra were calibrated to the center of the C–C/C–H peak at 285.0 eV. Survey scans were acquired at PE = 160 eV PE, detail spectra at PE = 20 eV.

2.6 | Contact Angle Measurement of Substrates

Contact angle measurements to characterize the substrates and verify the modification quality were performed before and after each functionalization using an optical contact angle goniometer (DataPhysics OCA40, Germany). Static contact angles were determined for each foil using the sessile drop method with deionized water (drop size of 2 µL). For the amino-functionalized films, dynamic contact angles were also measured using deionized water (drop size of 2 µL ± 10 µL). At least five measurements from different places on the substrate were taken for each polymer foil and a mean value was calculated. The contact angle measuring device was operated in a climate-controlled laboratory with a humidity between 45% and 55% and an ambient temperature of 21–24°C. The samples were stored in the same laboratory for at least 30 min before measurement.

2.7 | Synthesis of Methacryloyl-Modified Gelatin Derivate GM10

Methacryloyl-modified gelatin derivates GM10 (type B) with a ten-fold excess were prepared according to a previously described procedure by Claaßen et al. [28]. The method was initially described by Van Den Bulcke et al. [26]. The ten-fold excess of MAAnh refers to a nominal concentration of free amino-groups in gelatin of 0.35 mmol/g [26]. The degree of methacryloylation was 1.1 mmol/g (mmol methacryloyl groups per gram gelatin).

2.8 | Cationization of Methacrylated Gelatin

EDA modification was performed using a previously published procedure by Claaßen et al. [25]. Five grams GM10 (type A) was briefly dissolved in deionized water at 37°C while stirring. After adding 2.27 g EDA, the pH of the solution was adjusted to 4.5 using 2 M HCl. 0.725 g EDC was weighed in and dissolved in 2.5 mL water, and added to the reaction mixture. After stirring the solution for 18 h at 37°C, the solution was filtrated, and pH was adjusted to 7 using NaOH. Afterward, the solution was dialyzed for 7 days against deionized water at room temperature and afterward freeze-dried. The degree of methacryloylation is 0.59 mmol/g (mmol methacryloyl groups per gram gelatin).

2.9 | Synthesis of Methacryloyl- and Acetyl-Modified Gelatin Derivate GM2A8

For preliminary testing only, the methacryloyl- and acetyl-modified gelatin derivate GM2A8 was prepared as previously described by Claaßen et al. [28]. The method was first introduced by Hoch et al. [24]. The degree of methacryloylation is 0.372 mmol/g (mmol methacryloyl groups per gram gelatin).

2.10 | Preparation of Hydrogel Precursor Solution

Two different modified gelatin derivatives were used, more precisely, GM10 and GM10E10. For preliminary experiments where the hydrogel spots were pipetted manually on the substrates, a 10% (w/w) GM10 and GM10E10 solution was prepared. The 10% (w/w) GM10 hydrogel precursor solution was prepared according to Table 1.

TABLE 1 | Components (in weight [mg] and percentage in relation to the total mass [%]) of 10% (w/w) GM10 hydrogel precursor solution.

Components	Weight (mg)	Percentage (%)
GM10	100	10.00
LAP	0.3	0.03
Glycerol	50	5.00
Rhodamine 6G	0.5	0.05
Deionized Water	849.2	84.92
Total	1000	100

2.11 | Pipetting Hydrogel Spots Manually on Functionalized Substrates

In preliminary tests, the 10% (w/w) GM10 and GM10E10 hydrogel precursor solutions were pipetted by hand onto the respective substrate material to generate spots. For each substrate, 16 spots with a volume of 0.6 µL were pipetted. To avoid air bubbles in the spots, reverse pipetting was used. The pre-tests were performed on all substrates with the different modifications (amino-functionalized and amino-functionalized followed by methacryloylation) and one control (an untreated sample). Immediately after pipetting, each sample was crosslinked separately.

2.12 | Crosslinking Hydrogel Precursor Solutions Onto Modified Substrates

A UV chamber with a built-in-argon supply was used to crosslink the pipetted or printed hydrogel spots. For this purpose, the samples were placed in a specially made aluminum mold, which was provided by University of Stuttgart, Institute of Interfacial Process Engineering and Plasma Technology (IGVP). Humidified argon was passed through the mold during crosslinking. This prevents the hydrogel spots from drying out, and at the same time, the premature reaction of the radicals produced during UV crosslinking should be avoided, as these can react with oxygen. To prevent the samples from being forced upward by the argon flow, they were secured on a glass slide with adhesive tape. The aluminum mold was placed in a UV chamber of Paul Hartmann GmbH (Hainichen, Germany), which is combined with a UV handlamp at 365 nm (max.), irradiance of 4.4 mW/cm^2, exposure time of 10 s, and a sample-to-lamp distance on the lowest rail (22 cm). After crosslinking, the samples were dried for at least 24 h and protected from light at room temperature.

2.13 | Effects of Photoinitiator on Fluorescence of Hydrogel Spots

To evaluate the potential effects of the photoinitiator on the fluorescence of the ultimate loop-mediated isothermal amplification (LAMP) reaction specific to Dengue virus, Cy5 was conjugated to the 5' end of a 41-mer DNA oligo. To study the effects 1.8 µM Cy5-

oligo, 5% (w/w) GM2A8 hydrogel, and either 3% (w/w) LAP or 0.3% (w/w) LAP was crosslinked with UV for 30 s ($\lambda = 365$ nm, 1 mW/cm) in 50 µL total reaction volume. Reactions were imaged in-tube at Ex/Em maxima, 649/666 and intensity values were measured using ImageJ (NIH, USA). Data are representative of $n = 5$ replicates and 2–3 independent experiments.

2.14 | Adherence Tests

Adherence tests of the hydrogel spots on each substrate were performed under LAMP conditions, that is, temperature and duration. Therefore, after crosslinking and drying, each sample was placed in a beaker with 60 mL pre-tempered deionized water and placed on a thermal shaker at 80 rpm for up to 3 h at 65°C. During the 3 h incubation time, the samples were removed and visually assessed after 5 and 40 min, corresponding to the actual LAMP reaction time. In addition, pictures of the hydrogel spots were taken with a camera to conclude about the adherence by counting the still adhering spots.

2.15 | Printing of Hydrogel Precursor Solution

For printing, the NanoplotterTM2.1 (GeSiM, Germany), a drop-on-demand piezoelectric inkjet printer with the possibility of dispensing volumes in a nanoliter range, was used. A Nano-Tip micropipette, delivering drops of approximately 400 pL (manufacturer's specification) was used. Printing was carried out in a climate-controlled laboratory at constant room temperature in the range of 21–23°C. The print pattern was designed using the software SpotFrontEnd (GeSiM, Germany). 4 × 4 spots of the hydrogel precursor solution, each spaced 2.5 mm apart, were spotted on the substrate samples. Each spot consisted of 40 drops with a total volume of 25–32 nL. The spot size was chosen due to visibility since smaller spots are challenging to detect macroscopically. For printing, the substrates were attached to glass slides with adhesive tape to prevent them from shifting. At least three printing tests were performed per hydrogel precursor solution. The printed substrates were dried for 5 min and then incubated in a humid chamber for 10 min to provide the same conditions before UV crosslinking. After the crosslinking, the samples were stored at room temperature in a dry, light-protected environment until they underwent further processing. Printing was performed using a GeSiM Nanoplotter 2.1 drop-on-demand piezoelectric inkjet system under standard operating conditions. The actuation parameters applied were those provided by the manufacturer's default settings, including the standard waveform configuration, dwell time, and amplitude. The following parameters were selected for the printing process of GM10 and GM10E10: pulse width: 90 µs, frequency: 100 Hz and for the voltage, the value was varied between 75 V (2.5% (w/w)) and 80 V (3.5% (w/w)) depending on the gelatin concentration. The printing was conducted at ambient laboratory temperature (21–23°C and 50%–60% r.h.). No optimization of waveform or pressure beyond the default settings was implemented. Jetting performance was therefore evaluated indirectly based on the reproducibility of printing runs and the morphological integrity of the deposited hydrogel spots. Arrays were inspected for uniformity, absence of satellite droplets, and consistent spot size as indicators of stable jetting behavior.

2.16 | Printability of Hydrogel Precursor Solution

After preliminary tests, the printability of both GM10 and GM10E10 hydrogel precursor solution was investigated. In the context of printing, the hydrogel precursor solution will also be referred to as ink. For this reason, the GM10 and GM10E10 inks were prepared as previously described. In addition to the 10% (w/w) hydrogel precursor solutions, 2.5% (w/w), 3.5% (w/w), and 5% (w/w) GM10 and GM10E10 hydrogel precursor solutions were tested. The respective compositions are shown in Tables 2 and 3.

2.17 | Rheological Measurements of Hydrogel Precursor Solution

Rheological measurements were performed to characterize the hydrogel precursor solutions. All hydrogel precursor solution concentrations used for printing (2.5% (w/w), 3.5% (w/w), 5% (w/w)) were measured, as well as the 10% (w/w) hydrogel precursor solution and a pure 10% (w/w) GM10 or GM10E10 solution in deionized water. The viscosity was measured at room temperature (22°C) in a shear rate range from 1 to 1000/s on the rheometer Physica MCR 301 (Anton Paar, Germany). The measuring point duration was logarithmic, starting at 10 s up to 1 s. The hydrogel precursor solutions were prepared the same as for the printing process. Only the LAP and dye content were replaced by deionized water. LAP could have falsified the measurement result if crosslinking had occurred. Due to the low concentration of LAP, no effect on the viscosity has to be assumed. At least three measurements per sample were performed.

2.18 | Adherence Tests With Printed Hydrogel Spots

The procedure for testing the adherence of printed hydrogel spots was identical to manually applied hydrogel spots. The printed spots were examined and evaluated with a light microscope (Keyence, Germany), due to the much smaller spot size. The spots were photographed under 20-fold magnification.

2.19 | Adjustment of LAP Content in the GM10E10 Ink

In order to influence the stability and adhesion of the printed GM10E10 the content of the photoinitiator LAP was varied. This was done exemplary with the 3.5% (w/w) GM10E10 hydrogel precursor solution. The hydrogel precursor solutions were prepared following the previously described method, with a modification in the LAP content. Initially, the original LAP concentration of 0.03% and a much higher concentration of 0.5% were tested to set a framework for the LAP concentration. Subsequently, LAP contents of 0.0350%, 0.0875%, and 0.1225% in relation to the total mass of the hydrogel precursor solutions were used. The adapted GM10E10 ink was printed on each substrate, crosslinked and afterward adherence tests were performed as previously described.

TABLE 2 | Components ([%] in relation to the total mass) for preparation of 2.5% (w/w), 3.5% (w/w), and 5% (w/w) GM10 hydrogel precursor solution.

Components	2.5% (w/w) [%]	3.5% (w/w) [%]	5% (w/w) [%]
GM10	2.50	3.50	5.00
LAP	0.0075	0.0105	0.0150
Glycerol	5.00	5.00	5.00
Rhodamine 6G	0.05	0.05	0.05
Deionized Water	92.4425	91.4395	89.9350
Total	100.00	100.00	100.00

TABLE 3 | Components ([%] in relation to the total mass) for preparation of 2.5% (w/w), 3.5% (w/w), and 5% (w/w) GM10E10 hydrogel precursor solution.

Components	2.5% (w/w) [%]	3.5% (w/w) [%]	5% (w/w) [%]
GM10	2.50	3.50	5.00
LAP	0.125	0.175	0.250
Glycerol	5.00	5.00	5.00
Rhodamine 6G	0.05	0.05	0.05
Deionized Water	92.3250	91.2750	89.7000
Total	100.00	100.00	100.00

3 | Results and Discussion

3.1 | Surface Characterization of Functionalized Substrates by Sulfo-SDTB Assay

Amino-functionalization is intended to produce amino-groups on the surfaces of the substrates. To assess whether the amino-functionalization of the polymeric substrates was successful sulfo-SDTB assays were performed. The sulfo-SDTB assay measures the concentration of primary amines on the polymer films. The respective untreated polymer foil was examined as a reference. Unfunctionalized PET-46 had an amino-group concentration of 0.0198 ± 0.0036 nmol/cm^2. Amino-functionalized PET-46, on the other hand, had a significantly higher amino-group content of 0.0918 ± 0.0078 nmol/cm^2. The reference of OPP-11 was only minimally different from the PET-46 reference at 0.0264 ± 0.00453 nmol/cm^2. There is also a significant increase in amino-group content in OPP-11 due to amino-functionalization, as shown by the value 0.106 ± 0.0136 nmol/cm^2. The reference for OPP-40 also has with a concentration of 0.0168 ± 0.00453 nmo/cm^2 similar value to the other references. There was also an increase in amino-group content due to amino-functionalization, but the value of 0.0678 ± 0.0923 nmol/cm^2 is slightly lower than the values of OPP-11 and PET-46. Nevertheless, for all three materials it is evident that the amino-functionalization was successful.

3.2 | X-Ray Photoelectron Spectroscopy

To further validate amino-functionalization of the polymer substrates x-ray photoelectron spectroscopy (XPS) measurements were performed to detect the amount of nitrogen on the surface. There was no nitrogen detected for the untreated reference of PET-46, whereas the amino-functionalized PET-46 had a nitrogen content of 7.1 at%. For the reference of OPP-11 no nitrogen was detected, while the amino-functionalized OPP-11 had a nitrogen content of 2.6 at%. In the case of untreated OPP-40, a nitrogen content of 0.3 at% was already present, which increased to 3.5 at% because of the amino-functionalization. These results confirm that nitrogen is present on the substrates after amino-functionalization, which additionally confirms the success of the functionalization.

3.3 | Contact Angle Measurement

The surface properties, such as hydrophobicity and hydrophilicity, can be changed by functionalizing the substrates. This property can be investigated by assessing the wettability of surfaces using contact angle measurements. Therefore, contact angle measurements were used to evaluate the success of the amino-functionalization and the subsequent methacryloylation. The contact angles were measured on untreated PET-46, OPP-11, and OPP-40 as a reference, along with amino-functionalized, and amino-functionalized and methacrylated polymer films. The results are shown in Table 4.

Untreated OPP has, by default, a lower polarity than PET, regardless of thickness. Thus, both OPP films have a 30° higher contact angle than PET. The thickness of the untreated OPP foil did not affect the contact angle, as shown by the similar values.

After amino-functionalization, PET-46, OPP-11, and OPP-40 showed a decrease in contact angle and thus increased polarity. The polar amino-groups on the surface can explain the

TABLE 4 | Average static water contact angles of PET-46, OPP-11, and OPP-40 with different functionalization's.

Samples	PET-46	OPP-11	OPP-40
Ref	71 ± 3	102 ± 2	100 ± 5
A	40 ± 2	80 ± 6	85 ± 3
a+m	62 ± 3	82 ± 8	83 ± 4

Note: The contact angle measurements were performed on the untreated reference substrates (ref), amino-functionalized substrates (a), as well as amino-functionalized and methacrylated substrates (a + m). Contact angle values for each sample from several batches were averaged (±SD).

TABLE 5 | Adherence of 10% (w/w) GM10 hydrogel spots after 40 min on different substrates in percentage ($n = 3$).

Samples	PET-46	OPP-11
Ref	2 ± 4	0 ± 0
A	58 ± 52	25 ± 43
a + m	77 ± 22	79 ± 19

Note: Spot adherence test of crosslinked 10% (w/w) GM10 hydrogel was on untreated reference substrates (ref), amino-functionalized substrates (a), as well as amino-functionalized and methacrylated substrates (a + m). The substrates with hydrogel spots were incubated for 40 min in 65°C water to investigate spot adherence.

increased polarity due to amino-functionalization. These results are consistent with the sulfo-SDTB assays and XPS measurements, where the amino-groups and nitrogen was detected on the substrates after amino-functionalization. In conclusion, the contact angle measurements could also demonstrate a successful amino-functionalization of all three materials. For the subsequent methacryloylation, a decrease in polarity and enhanced hydrophobicity was expected. Therefore, the attachment of non-polar methacryloyl groups on the surface would result in higher contact angles. This expectation was validated only for PET, where the contact angle for amino-functionalized and methacrylated PET films showed, on average, a difference of 20° compared to amino-functionalized PET films. Therefore, it can be concluded that the methacryloylation process was successful for PET. In the case of OPP, however, there were no significant differences in contact angles between amino-functionalized and amino-functionalized and methacrylated OPP of both thicknesses, which means that no statements can be made about the success of methacryloylation. The similar values of OPP-11 and OPP-40 are indicated that thickness does not play a role in functionalization.

3.4 | Adherence of Modified Gelatin to Functionalized Polymeric Surfaces

For the development of the point-of-care test, it is crucial that the hydrogel spots, which act as nanosensors, withstand subsequent test conditions such as exposure to liquid (patient sample) and heat (conditions of the detection method). Therefore, the hydrogel spots must have sufficient stability and adhere to the surface of the selected substrate. In order to optimize adherence, a functionalization of polymeric substrates was developed within this project's scope, to which the hydrogel spots should adhere through covalent bonds of the methacryloyl groups through crosslinking. At first, the adherence of 10% (w/w) GM10 hydrogel and 10% (w/w) GM10E10 hydrogel was tested on functionalized PET-46 and OPP-11, as well as their respective references (untreated materials). For this purpose, the hydrogel precursor solutions were hand pipetted onto the substrates to generate spots, which were subsequently crosslinked at 4.4 mW/cm^2 for 10 s using UV radiation and then tested using adherence tests. The results for the adherence tests with 10% (w/w) GM10 are shown in Table 5.

For PET-46 and OPP-11, the different functionalization's significantly improved the adherence of the hydrogel spots to the materials. PET-46 and OPP-11, which were amino-functionalized and methacrylated, demonstrated the best adherence, presumably due to the ability to establish covalent bonds by the presence of crosslinkable groups on the surface and within the hydrogel. The significantly better adherence after methacryloylation, which is also evident for OPP-11, suggests that methacryloylation was successful, even if it was not evident from the contact angle measurements for OPP. Higher standard deviations in spot adherence for a + m films may be caused by non-uniform functionalization or air bubbles generated during manual pipetting. The latter will be avoided by using a printer at a later stage. The amino-functionalized films exhibit notably higher standard deviations, potentially attributable to the lack of crosslinkable groups on the material surface. Likely, the adherence is predominantly facilitated by electrostatic forces, which are susceptible to external influences. The adherence properties of GM10E10 hydrogel precursor solution were significantly different from those of GM10, as indicated by the detachment or complete dissolving of almost all GM10E10 spots from surfaces within 5 min of exposure to water. This occurred regardless of the functionalization of the substrates. Based on the poor adherence and dissolving of the hydrogel, it can be inferred that the precursor solution of GM10E10 hydrogel was not crosslinked adequately. This insufficient crosslinking may be attributed to the presence of fewer crosslinkable groups in GM10E10 as compared to GM10 [25]. The degree of methacryloylation of the GM10E10 biopolymer was significantly lower (0.589 mmol/g) than that of GM10 (1.1 mmol/g), indicating fewer methacryloyl groups. The GM10E10 biopolymer was prepared from GM10 biopolymer with a higher degree of methacryloylation (0.917 mmol/g). The treatment with EDA has probably resulted in the cleavage of methacryloyl groups and, therefore, fewer crosslinkable groups and a lower degree of methacryloylation. To enhance the crosslinking of GM10E10, the concentration of the photoinitiator LAP was increased from 0.03% to 0.5%, and subsequent adhesion tests were conducted. The results are shown in Table 6.

After adjusting the LAP content in the hydrogel, the GM10E10 exhibited excellent spot adherence. Due to the higher LAP content, the spots no longer dissolved in the water during the adherence test, indicating sufficient crosslinking. Both materials amino-functionalized and methacrylated, as well as their amino-functionalized films, showed 100% spot adherence after 40 min and even after 3 h in water. The spot adherence of the references is also comparatively good.

TABLE 6 | Adherence of 10% (w/w) GM10E10 hydrogel spots with 0.5% LAP after 40 min on different substrates in percentage ($n = 3$).

Samples	PET-46	OPP-11
Ref	94 ± 11	67 ± 30
A	100 ± 0	100 ± 0
a + m	100 ± 0	100 ± 0

Note: Spot adherence test of crosslinked 10% (w/w) GM10 hydrogel was tested on untreated reference substrates (ref), amino-functionalized substrates (a), as well as amino-functionalized and methacrylated substrates (a + m). The substrates with hydrogel spots were incubated for 40 min in 65°C water to investigate spot adherence.

The results of the two modified gelatins cannot be compared one-to-one since different LAP concentrations were used. However, it is reasonable to assume that increasing the LAP content in the GM10 ink would exhibit a similar adherence result as the GM10E10 spots. Since the degree of spot adherence for GM10 was determined to be sufficient for preliminary tests, the LAP content was never adjusted in GM10. In addition, it was intended to keep the LAP concentration low, as the reduction of LAP content lowers the costs, which is desirable for developing a point-of-care test. The excellent adhesion of GM10E10 with 0.5% LAP suggests that it is possible to reduce the LAP content of this ink to a certain extent and still achieve good adhesion.

3.5 | Effects of Photoinitiator on Fluorescence of Hydrogel Spots

The reduction of the LAP content reduces the cost factor as mentioned, but it is also necessary since the LAP concentration can have a negative effect on the detection method. Here, we compared the effect of 3% (w/w) LAP and 0.3% (w/w) LAP on Cy5 fluorescence after UV crosslinking. Cy5 is a far-red fluorescent dye that can be conjugated to oligonucleotides for detection in nucleic acid amplification tests. As shown in Figure 1, Cy5 fluorescence was largely quenched at 3% (w/w) but not 0.3% (w/w) LAP to hydrogel concentration. Such an inhibitory effect on fluorescence was also noted for the photoinitiator APS on fluorescent dyes SYBR Green X and LC Green Plus by Atrazhev and Acker [34]. As 0.3% (w/w) saturated the detector, it is likely that greater concentrations of LAP such as 0.5% (w/w) used for GM10E10 will exhibit some level of measurable fluorescence. We are investigating the tradeoffs between attachment and fluorescence further.

3.6 | Rheological Measurements of Hydrogel Precursor Solution

After the preliminary tests with the 10% (w/w) GM10 and GM10E10 had led to first conclusions about the adhesion of the hydrogel spots and its influencing factors on the different functionalized substrates, a printer was to replace the manual pipetting. By using additive manufacturing, it is possible to increase scalability, reproducibility, and precision [21, 22]. In addition, influencing factors that occur through manual pipetting, such as air bubbles in the hydrogel, which can negatively influence adhesion, will be avoided. Before the spots could be spotted with the piezoelectric printer, the differently modified gelatin inks had to be tested for printability. An essential factor for the printability of the hydrogel precursor solution is the viscosity [24, 35]. With increasing viscosity, the ability to dispense decreases, and the risk of print nozzle clogging increases. The manufacturer specifies a viscosity limit of 5 mPa·s for the GeSiM Nanoplotter 2.1 printer. To assess printability, hydrogel precursor solutions of GM10 and GM10E10 with varying biopolymer contents (2.5% (w/w), 3.5% (w/w), 5% (w/w), and 10% (w/w)) were prepared. Rheological measurements were used to determine the viscosities of the hydrogel precursor solutions that serve as ink for the printer. As shown in Figure 2, there was no significant difference in viscosity between the GM10 (A) and GM10E10 (B) hydrogel precursor solutions. Only at the biopolymer content concentrations below 5% (w/w) the differences increased slightly.

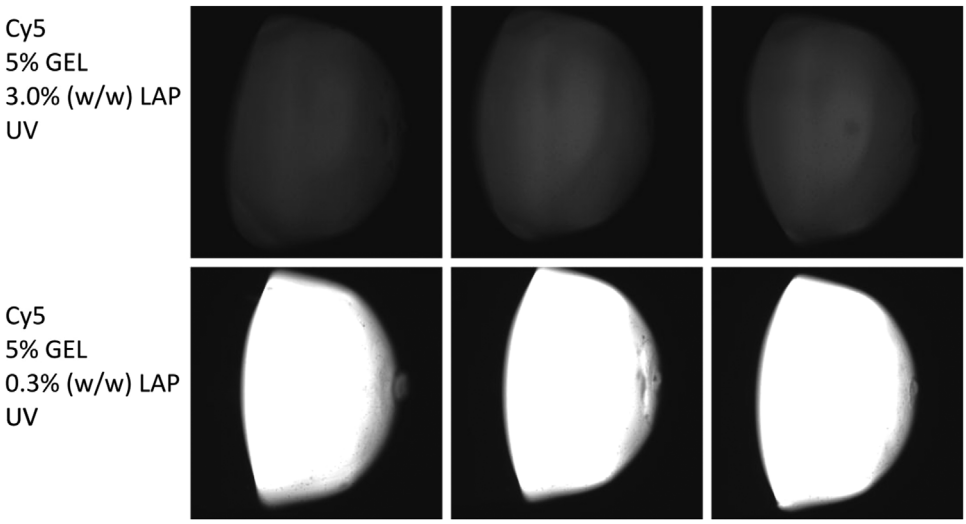

FIGURE 1 | Reducing LAP concentration restores fluorescence. Incubation of 3.0% (w/w) LAP and hydrogel and UV crosslinking resulted in Cy5 fluorescent intensities of 10731 ± 947 RFU. Cy5 fluorescence with 0.3% (w/w) LAP to hydrogel under the same UV crosslinking conditions saturated the detector (>65,000 RFU) similar to Cy5 alone (data not shown).

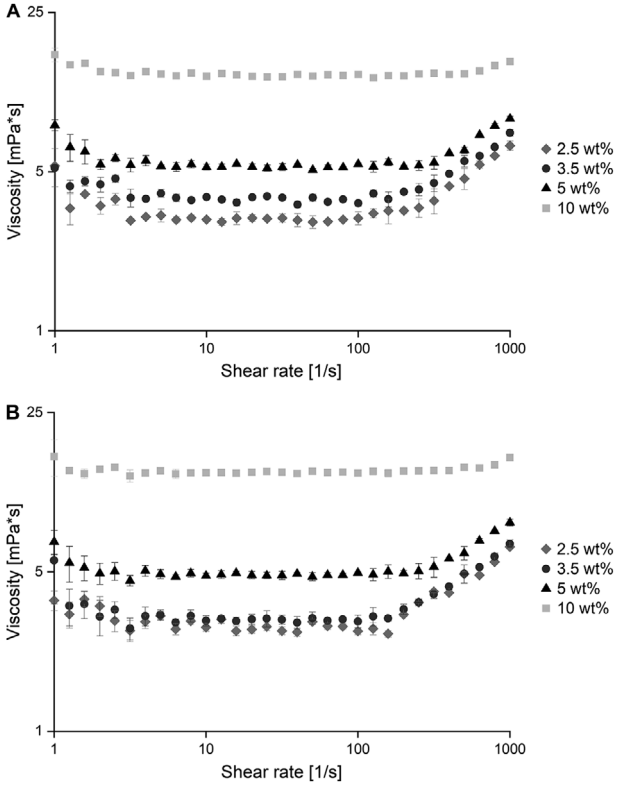

FIGURE 2 | Logarithmically represented viscosity measurements of different modified Gelatins in a shear rate range from 1/s to 1000/s at 22°C. (A) GM10 and (B) GM10E10 hydrogel precursor solutions with different biopolymer content of 2.5% (w/w), 3.5% (w/w), 5% (w/w), and 10% (w/w). Rheometer Physica MCR 301 from Anton Paar was used for the measurements ($n = 3$).

Only hydrogel precursor solutions with a biopolymer content of 2.5% (w/w) and 3.5% (w/w) for both GM10 and GM10E10 were found to be below the viscosity limit specified by the printer manufacturer. The viscosity of the 5% (w/w) precursor solution was measured to be 4.95 mPa·s ± 0.05 mPa·s at a shear rate of 100/s, which falls within the range of the viscosity limit. Although rheological characterization of the hydrogel precursor solutions was performed across a shear rate range up to 1000/s, it is important to note that the actual shear rates encountered within inkjet nozzles can be substantially higher, often exceeding 10^4/s. Consequently, the viscosity values reported in this study should be regarded as an approximation for printability rather than an absolute predictor of jetting behavior. In addition to shear viscosity, other factors such as extensional viscosity and transient shear effects during droplet formation may significantly influence jetting performance. These parameters were not assessed in the present work and represent an important direction for future studies aimed at achieving a more comprehensive understanding of inkjet printing dynamics for hydrogel-based inks. Based on these viscosity measurements, we anticipated that only hydrogel precursor solutions with lower biopolymer concentrations (2.5% (w/w), 3.5% (w/w), and possibly 5% (w/w)) would be printable. To evaluate the results of rheological measurements, initial printing tests were conducted using different inks. An ink was considered printable if it could complete at least three printing runs with six arrays each without any issues, and this process had to be repeatable for three hydrogel precursor solution approaches. As expected, the 10% (w/w) GM10 and GM10E10 were too viscous to be printed. The 2.5% (w/w) and 3.5% (w/w) GM10 and GM10E10 could be printed without difficulty, as per the viscosity measurements. However, printing the 5% (w/w) inks was less successful than lower concentrations. Although the 5% (w/w) GM10E10 showed slightly better printability than the GM10 due to slightly lower viscosity, neither could meet the required standards and were therefore not printable.

In summary, GM10 and GM10E10 hydrogels with a biopolymer content greater than or equal to 5% (w/w) are not suitable for use in the POCT. Hydrogel precursor solutions with a biopolymer content of 3.5% (w/w) have the highest concentration of biopolymer that can be reliably printed while having the most crosslinkable groups. Therefore, this concentration was deemed promising and was further studied. Although this study provides a comprehensive evaluation of hydrogel printability and substrate functionalization, certain aspects were beyond its scope. Printability was primarily assessed through viscosity measurements, as this parameter is most relevant for the employed inkjet system. Other factors such as surface tension and density, which would allow calculation of dimensionless numbers (e.g., Z number), were not included and represent an opportunity for future work. Similarly, rheological measurements were performed up to 1000/s, which offers a reasonable approximation, although actual shear rates in inkjet nozzles can be higher. High-speed imaging of droplet formation was not conducted; instead, jetting performance was evaluated based on reproducibility and spot morphology. Finally, integration of the LAMP assay and further optimization of photoinitiator concentration will be addressed in subsequent studies. These considerations do not affect the main conclusions but highlight areas for continued development.

3.7 | Adherence of Printed Hydrogel Spots

The lower biopolymer content and smaller ink volume (25–32 nL) used in the printing process compared to manual pipetting can result in reduced crosslinkable groups and altered hydrogel adhesion to substrates. Adhesion tests were therefore conducted on printed substrates, including OPP-40 with untreated, amino-functionalized, and amino-functionalized and methacrylated films, in addition to previously tested PET-46 and OPP-11 materials. The first adherence tests of the substrates printed with 3.5% (w/w) hydrogel precursor solution showed similar results to manually spotted hydrogel spots. In Figure 3, a microscopic image shows a 4 × 4 array of printed hydrogel spots on amino-functionalized and methacrylated OPP-40. Variations in droplet diameter observed in Figure 3 were analyzed with respect to jetting conditions and substrate wetting dynamics. Factors such as drop velocity, satellite formation, contact angle dynamics, and substrate heterogeneity were considered. Again, 100% of the GM10E10 spots still adhered to all functionalized substrates after exposure to water for 3 h, and the adhesion was also better on the non-functionalized substrates. These findings were unexpected given the lower biopolymer content and fewer crosslinkable groups in the ink. However, the high LAP content (0.175% LAP) in the hydrogel is believed to contribute to good adhesion. In contrast, GM10 with lower LAP content (0.105% LAP) exhibited

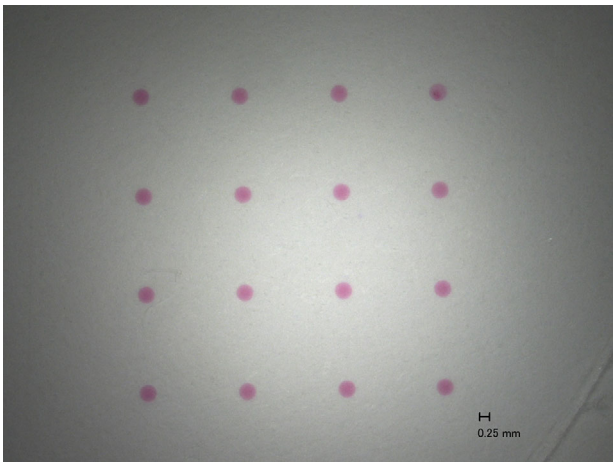

FIGURE 3 | Microscopic image of a 4 × 4 array of printed hydrogel spots on amino-functionalized and methacrylated OPP-40. The image was captured at 20× magnification and depicts the hydrogel spots fabricated using a 3.5% (w/w) GM10E10 solution with 3.5% LAP photoinitiator.

poorer adherence, with some spots detaching after 40 min in water and complete detachment from all substrates except OPP-11 (30% ± 50% still adhered) within 3 h. Adjusting the LAP content could improve spot adherence for GM10, but the positively charged GM10E10 is promising for the later detection application. The EDA modification in GM10E10 imparts a positive charge [25] and allows trapping of negatively charged components of the detection method and presumably enables the transport of the negatively DNA, which is desirable for the application.

3.8 | Adjustment of the LAP Content in the GM10E10 Ink

To further adapt the 3.5% (w/w) GM10E10 ink to its subsequent application, the LAP content was further reduced. This is possible, as could be seen from the 100% hydrogel spot adherence on all substrates. The reduction of the LAP content reduces the cost factor and the risk of fluorophore quenching, as already mentioned. In addition, there is the risk that at higher photoinitiator levels, the hydrogel precursor solution will react before printing due to ambient light. To reduce the LAP content, 3.5% (w/w) GM10E10 was prepared with different photoinitiator levels. LAP concentrations between 0.0105% (LAP concentration of 3.5% (w/w) GM10) and 0.175% were chosen. Since it was known from the preliminary tests that a higher LAP content is required for GM10E10 than for GM10, and more than sufficient spot adherence was already observed at 0.175% LAP, LAP levels of 0.035%, 0.0875%, and 0.1225% (relative to the total mass of hydrogel precursor solution) were investigated. The evaluation of the adherence tests of the ink with different LAP concentrations ($n = 2$) is summarized in Figure 4.

The hydrogel with 0.035% LAP adhered to the different substrates. However, a few of the spots detached from some substrates, whereas the hydrogels with 0.0875% and 0.1225% LAP showed excellent adhesion. Since 0.0875% already showed sufficient spot adherence and should not limit the detection method, this LAP level was selected as the best choice for the 3.5% (w/w) GM10E10.

3.9 | Comparison of the Different Polymeric Substrates

After it was possible to select a suitable composition of the hydrogel that is printable and exhibits good hydrogel adherence, it is necessary to determine the most suitable material. For the later application in the point-of-care test chip, spot adherence is essential for selecting the material. For the selected 3.5% (w/w) hydrogel of GM10E10 with 0.0875% LAP, there were no significant differences in spot adherence between PET-46, OPP-11, and OPP-40. For PET-46 and OPP-11, the amino-functionalized and methacrylated, amino-functionalized, and untreated reference films had spot adherence of 100% (see Figure 4). Amino-functionalized and untreated OPP-40 also showed spot adherence of 100%. Amino-functionalized and methacrylated OPP-40 had a slightly lower spot adherence of 90% ± 13%, but this difference was not significant enough to exclude the material. From the preliminary tests, it is known that the functionalization's influence spot adherence by increasing it. This is not visible in the spot adherence of the selected hydrogel due to the LAP content. Nevertheless, it is known that amino-functionalization with subsequent methacryloylation has the advantage of a covalent bond between the hydrogel and the material surface. By applying the crosslinkable methacryloyl groups to the substrate surface, as demonstrated by the change in contact angles, they can be covalently crosslinked with the methacryloyl groups in the hydrogel under the influence of UV radiation. This is advantageous for longer and stronger adhesion, as it is not only based on electrostatic forces. Since the various substrates that were amino-functionalized and methacrylated did not differ significantly in their spot adherence, further aspects can be considered in the selection of the substrate. As OPP has higher contact angles than PET and thus has more hydrophobic properties, the applied hydrogel spots are rounder and less flat. This enables more spots to be applied to the same area of the substrate material, which can result in a more accurate detection of pathogens since false positives and negatives can be better excluded. Although working with the materials, differences in the handling of the materials became apparent. OPP-11 was found to be highly statically charged and thin, making it challenging to handle. Therefore, a thicker OPP-40 was also investigated. The handling of OPP-40 was easier since it had a lower electrostatic charge. There was no difference between OPP-40 and PET-46 in regards of their handling, due to its similar thickness. In view of the later detection method, the background fluorescence is of relevance. It was found that the amino-functionalized and subsequently methacrylated OPP exhibited low background fluorescence for the used fluorescence dye, while the functionalized PET exhibited medium background fluorescence. However, it is possible to use a different fluorescent dye for the detection method. If the dye is changed, the substrates must therefore be re-evaluated in this regard. In conclusion, no final decision could be made as to which of the three substrates is the more suitable for a point-of-care test. However, it has been shown that all of them can be used for this purpose, as they exhibit good hydrogel adherence.

4 | Concluding Remarks

In this study, we successfully identified a suitable functionalization approach for the polymers PET and OPP to serve

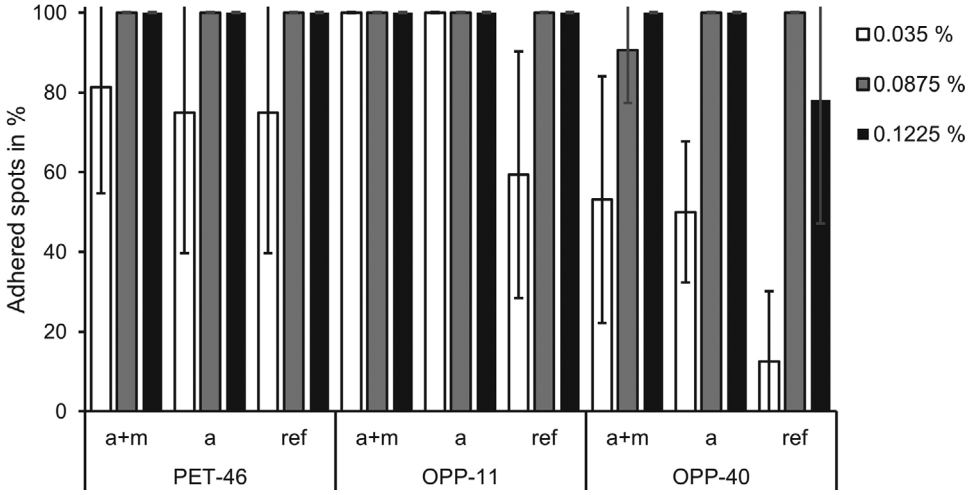

FIGURE 4 | Adhered hydrogel spots of 3.5% (w/w) GM10E10 with different LAP concentrations on different substrates in percentage. The tested LAP concentrations were 0.035%, 0.0875%, and 0.1225%. Spot adherence test of crosslinked 3.5% GM10 hydrogel was tested on amino-functionalized and methacrylated substrates (a + m), amino-functionalized substrates (a), and untreated reference substrates (ref). The graph shows the percentage of adhered spots after 40 min, including the respective standard deviation. The substrates with hydrogel spots were incubated in 65°C water for 3 h to investigate spot adherence. $n = 2$.

as substrates in a point-of-care test chip. The substrates were amino-functionalized, incorporating amino-groups on the surface, which were detectable through XPS measurements and sulfo-SDTB assay. Subsequently, methacryloylation was performed to enable covalent bonding between the substrate surface and the biosensors, allowing their adherence to the point-of-care test. Additionally, we successfully identified a suitable hydrogel ink precursor solution for fabricating the biosensors. This methacryloyl-modified gelatin-based solution is compatible with piezoelectric printing technology and exhibits good hydrogel adherence to the functionalized substrates. The ink selected as the most suitable for the hydrogel is a 3.5% (w/w) GM10E10 with a LAP content of 0.0875%. By reducing the concentration of the photoinitiator LAP, we successfully minimized the risk of fluorophore quenching. This precautionary measure is crucial as it prevents any potential adverse effects on the biosensor's detection method. For the selected ink, no significant variations in hydrogel adherence were observed across all three materials. Considering the greater non-polar characteristics of OPP in comparison to PET, OPP stands out as the more suitable material for adhesion. In summary, three suitable materials could be found for a point-of-care test, which offer good adherence for biosensors through two subsequent surface functionalization's. These biosensors consist of an equally suitable hydrogel that can be printed as individual spots on the substrates. However, the materials could not yet be thoroughly studied with the hydrogel spots already encapsulating the LAMP. Therefore, the next step is to incorporate the detection method into the hydrogel. Once this is done, the next step is to test the point-of-care chip. Here, the focus is particularly on multiplexing, where the aim for the chip is to be able to detect several pathogens at the same time. In conclusion, this study contributes to the development of reliable and efficient POCT methods for pathogen detection, addressing the limitations of current diagnostic capabilities.

Author Contributions

Caroline M. Trust, Regina M. Lehmann, and Verena Singer performed the measurements on contact angles and adherence, Dominic Baum conducted experiments on LAP-induced fluorescence quenching, Sarah Schmidt conducted the rheological measurements, Christine McBeth and Achim Weber were involved in planning and supervised the work, Caroline M. Trust and Regina M. Lehmann processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. Caroline M. Trust, Sarah Schmidt and Regina M. Lehmann manufactured and printed the samples and characterized them with rheology. Achim Weber and Christine McBeth aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

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Conflicts of Interest

The authors have declared no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

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