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Lab in a Tube: Purification, Amplification, and Detection of DNA Using Poly(2-oxazoline) Multilayers

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Fast and easy purification and amplification of DNA are prerequisites for the development of point-of-care diagnostics. For this reason covalent coatings of amine containing poly(2-oxazoline)s (POx) on glass and poly(propylene) surfaces are prepared, to reversibly bind genetic material directly from biological samples. The polymer is deposited in a layer-by-layer process, whereas initial immobilization of macromolecules on the surface is accomplished by the use of an epoxy functionalized siloxane monolayer. Alternating treatment with polymer and cross-linker leads to the construction of amine containing POx multilayers on the substrates. Successful deposition is investigated by confocal laser scanning microscopy (using labeled polymers), contact angle measurements, as well as reflectometric interference spectroscopy. The interaction of these layer systems with DNA regarding binding and temperature dependent release is studied using labeled genetic material. Finally, polymerase chain reaction (PCR) vessels are coated with POx layers on the inside, and used for quantitative real-time PCR (qPCR) experiments. It is possible to bind genetic material directly from cell lysates to perform qPCR assays from surface adsorbed DNA within the same tube including amplification, as well as detection. The presented system displays an easy to use device for a point of care diagnostic.

1. Introduction

A reliable and time saving point-of-care diagnostic is one of the most important targets in bioanalytical science.[1] The optimal method would comprise an analysis of sample material directly on the spot, however, operating with the same accuracy as laboratory tests.[2] Moreover, cost efficiency is a major requirement in this context, rendering the design of new bioanalytical devices a highly challenging task.[3] The detection of diseases based on bacterial, fungal, or viral infections can be accomplished by the investigation of the pathogens nucleic acid profiles,[4] providing the opportunity of a fast determination and a targeted medication.[5] The recognition of the genetic material can be accomplished via specialized DNA assays,[6] e.g., based on microchip systems.[7] However, this presents only the last step in bioanalytics. The isolation of nucleic acids from whole tissue and cell samples and, in particular, the separation from proteins is still a prerequisite for downstream analytical processes. The general steps of nucleic acid purification include 1) tissue and/or cell lysis, 2) inactivation of cellular nucleases, and 3) separation of the desired nucleic acids from the cell debris.[8] To separate proteins from genetic material, phenol-chloroform extraction and density gradient centrifugation displayed the method of choice for many years.[8a,9] Besides the use of toxic substances,[9] these liquid phase based methods had the drawback of very time consuming and laborious working steps.[10] An enormous improvement was achieved in 1989, when the first solid phase extraction (SPE) system for DNA purification was described by McCormick et al.[8b] It has the advantage to separate nucleic acids from proteins more effectively, and without the generation of toxic by-products. Since its introduction, the SPE system evolved to the most common and easily adaptable purification method for genetic material.[11] Still, there are remaining disadvantages, such as the labor-intensive multiple washing procedures, the limited loading capacity of the silica matrix, as well as limitations in the extraction efficiency.[8a]

To overcome these restrictions, a challenging alternative approach is the binding of DNA within polymer matrices. Such scaffolds can be considered as a potential alternative for DNA immobilization, based on the fact that they have the potential of low cost production at large scales and mostly exhibit a biocompatible character.[12] Furthermore, they can be functionalized easily,[13] e.g., with positively charged groups, able to interact with the negatively charged phosphate backbone of polynucleotides.[14]

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However, these polymers are mostly water soluble and, therefore, an assignment in biological, water based systems is not possible, since a separation from solution is difficult. For this reason, polymer-based hydrogels offer a great opportunity for a reversible DNA binding and release.[15] Because of their high water content and the resulting permeability of the 3D matrix, as well as the adjustable functionalities and a high surface to volume ratio, hydrogels represent attractive candidates for DNA immobilization. The suitability of poly(2-oxazoline) (POx) based hydrogels[16] and networks composed of POx–poly(ethylenimine) copolymers[17] was already investigated. The amine content and, therefore, the affinity to DNA are adjustable in both systems, resulting in a tunable DNA binding behavior. The combination of charged moieties with hydrophilic monomers such as 2-ethyl-2-oxazoline (EtOx) results in water swellable networks able to interact with genetic material. Furthermore, a release can be induced by the addition of heparin which replaces the DNA, generating eluates compatible with subsequent polymerase chain reaction (PCR) based DNA amplification.[16] However, the diffusion of genetic material within the gel is the limiting factor preventing fast purification procedures. Additionally, also non- or low-charged residues from cell lysis could be entrapped within the pores of the gel and, hence, interfere with downstream processes.

Surface bound polymer coatings are a potential way to overcome the drawbacks discussed above and combine the advantages of hydrogels with diffusion independent, surface mediated processes. The covalent immobilization of POx on glass substrates was already demonstrated in the context of low-fouling coatings;[18] however, to the best of our knowledge, no investigations regarding their bioanalytical potential are reported. A major requirement for DNA purification systems is their compatibility with subsequent PCR processes which are indispensable for a later detection. The PCR was invented by Mullis in 1983 and enveloped to one of the most important methods in bioanalytical science.[19] Via PCR, a few copies of a specific sequence of genetic material can be amplified by the naturally thermostable enzyme polymerase,[20] using specifically, synthesized oligonucleotides (primers) for the gene-of-interest.[19] The process itself consists of three steps:[21] 1) The denaturation (melting), 2) the primer annealing to the specific gene sequences, and 3) the elongation at a polymerase-specific temperature. Without PCR, DNA detection would be impossible due the low amounts of genetic material in biological samples.[22] However, the PCR process is limited by the purity of the bioanalytical samples, as well as by the template DNA amount.[23] Cellular components, remaining from cell lysis or other impurities, can interfere with the process and lead to nonexponential amplification or even a complete inhibition of the process. Therefore, pure polynucleotide solutions with a high concentration are preferable.

In this contribution we describe the synthesis of POx based surface coatings, containing a defined amount of amine groups for DNA binding and release. The polymers are covalently immobilized in a layer-by-layer (LbL) approach, generating films of defined thicknesses, and DNA binding/release profiles. The coating procedure is established on glass and, subsequently, transferred to poly(propylene) (PP), being one of the most common and important materials in bioanalytics. After investigation of the DNA interaction on plain substrates, PCR tubes are coated on the inside, creating a system able to separate DNA from impurities and, moreover, amplify and detect specific sequences by quantitative real-time PCR (qPCR). Exploiting the temperature dependent DNA binding behavior of POx multilayers it is possible to extract and detect DNA directly from cell lysate rendering this "lab in a tube" system a highly versatile and likewise easy to use tool for pathogen detection.

2. Results and Discussion

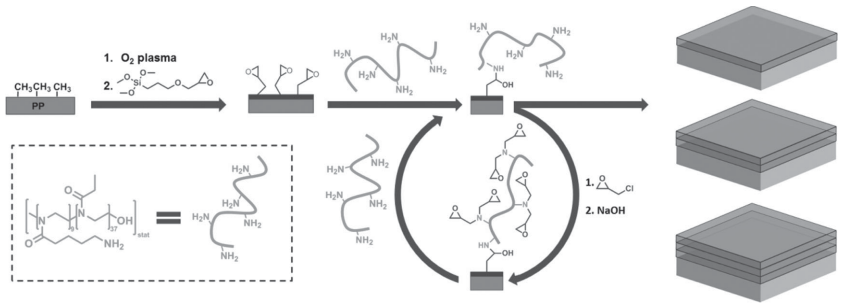
2.1. Polymerization and Fluorescent Labeling

The synthesis of amine containing POx was reported previously by our group.[15] Briefly, a 2-oxazoline bearing a Boc protected amine group (BocOx) was used in a copolymerization with EtOx to yield a statistical copolymer having 24% of functional comonomer incorporated (P(EtOx$_{35}$-*stat*-BocOx$_{11}$, **1**). Deprotection using trifluoroacetic acid (TFA) was applied to obtain amine containing POx (P(EtOx$_{37}$-*stat*-AmOx$_9$, **2**) with a molar mass (M_n) of 4600 and a narrow size distribution. Recalculation of the composition by NMR measurements after deprotection resulted in a final AmOx content of 20% (**Table 1**). Since this polymer should be used as a surface coating material, labeling was conducted using fluorescent dyes (fluorescein-5-*N*-hydroxysuccinimide ester for polymer **3** or rhodamine B *iso*-thiocyanate for polymer **4**) which are able to react with amine groups to enable a later detection of deposits. The coupling reaction was performed using one equivalent of dye per polymer chain, leaving a sufficient amount of free amine groups for surface attachment and DNA interaction. The success of the dye functionalization was verified by size-exclusion chromatography (SEC) measurements, comparing the signal from the refractive index (RI)

Table 1. Selected characteristics of the synthesized polymers.

Polymer	Compound	Dye	M_n [gM^{-1}]	$Đ$	Degree of func. [%]	Absorption maximum [nm]	Emission maximum [nm]
1	P(EtOx$_{35}$-*stat*-BocOx$_{11}$)	–	4600[a)]	1.30	–	–	–
2	P(EtOx$_{37}$-*stat*-AmOx$_9$)	–	9300[b)]	1.13	–	–	–
3	P(EtOx$_{37}$-*stat*-AmOx$_8$-*stat*-FAmOx$_1$)	Fluorescein	11 000[b)]	1.16	78	485	530
4	P(EtOx$_{37}$-*stat*-AmOx$_8$-*stat*-RAmOx$_1$)	Rhodamine B	12 000[b)]	1.19	5	555	590

[a)]SEC measurements in chloroform; [b)]SEC measurements in DMAc.



Scheme 1. Schematic representation of the deposition of P(EtOx$_{37}$-*stat*-AmOx$_9$) on substrates by LbL immobilization.

detector with the UV–vis signals. Purification (precipitation, and in the case of polymer **4** preparative size exclusion chromatography) was conducted until no trace of unbound dye was detected in the SEC traces. Overlapping RI and UV–vis traces prove a homogeneous functionalization of the polymeric material (Figure S1, Supporting Information). The efficiency of the labeling reaction was quantified by NMR spectroscopy (Table 1).

2.2. Surface Functionalization

The covalent attachment of amine functionalized POx to (activated) glass substrates was already reported by our group.[24] The method comprises the cleaning and activation by plasma and the subsequent deposition of a siloxane monolayer using 3-glycidyloxypropyl trimethoxysilane (GOPTMS). The epoxide groups of the siloxane layer react readily with the amine groups of the POx material to form a polymeric monolayer on the surface. While glass is a substrate frequently used for functionalization by silanization, also PP was utilized as a support material in this study since it is cheap, flexible, and relatively inert regarding biological processes. For this reason, the surface chemistry described before was transferred to PP. An overview over the performed coating procedures is depicted in **Scheme 1**.

To generate oxygen based functionalities on the highly inert substrate, O_2-plasma was applied. The oxidative conditions lead to the formation of OH groups and other oxygen based functionalities on the surface.[25] These functional groups can be used to attach a siloxane monolayer of GOPTMS. The present epoxide groups were subsequently quenched by the reaction with the amine groups of polymers **2**, **3**, or **4**. The success of the reaction could be verified by contact angle (CA) measurements determining the surface energy, as well as by fluorescence using confocal laser scanning microscopy (CLSM).

To optimize the amount and homogeneity of the deposited POx, the O_2-plasma treatment time, as well as the GOPTMS incubation temperature was altered since coating experiments with conditions described for glass resulted in heterogeneous coated surfaces. Additionally, the incubation time of the polymer was varied between 1, 2, and 24 h. The success of coating experiments was analyzed via CA and CLSM measurements (**Figure 1**). The CA measurements revealed that the hydrophobic character of untreated PP (CA = 88°) decreases after activation with O_2 plasma (CA = 36°), most probably due to the formation of hydroxyl groups. These oxygen functionalities react with GOPTMS to form a monolayer on the surface (CA = 25). After coating with polymer **4**, the hydrophilicity increases (CA < 20°) as a result of the covalently bound macromolecules. According to the CA results, there are no differences in the coating efficiencies for varying incubation times. CLSM measurements at λ_{ex} = 543 nm (λ_{em}: 560–615 nm) were performed for all coated slides, as well as for the blank substrates measuring an increase of fluorescence after coating with fluorescently labeled POx (**4**). The fluorescence intensity does not change significantly by varying either coating conditions or POx incubation time; however, the film homogeneity does, which is indicated by the standard deviation of the signal. An O_2 plasma treatment of 30 min and 1 h GOPTMS incubation at

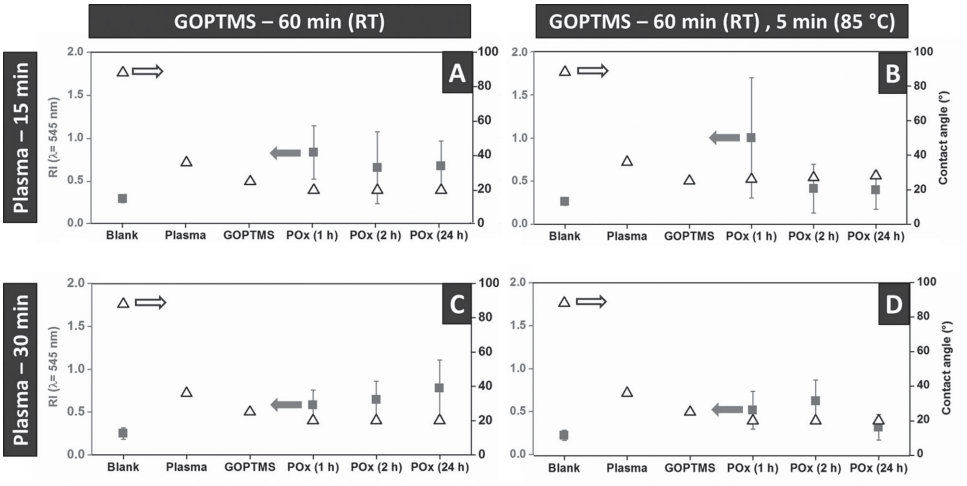


Figure 1. Optimization of the coating conditions for POx (**3**) on PP. The plasma incubation time was investigated using A,B) 15 min or C,D) 30 min of treatment. GOPTMS incubation was performed for A,C) 60 min at RT, or B,D) with an additional heat treatment for 5 min at 85 °C. POx coating was performed for 1, 2, or 24 h at 50 °C. Success and homogeneity of the coating procedure was verified by CA and CLSM.

Table 2. Analytical data of the multilayer POx coatings.

Coating step	CA [°]	POx amount by TGA [wt%]	Layer thickness (on glass) [nm]
Blank	100	–	0.0
O_2 plasma	80	–	0.6
GOPTMS	48	–	0.8
1st POx layer	<20	0.7	3.5
ECH	57	n. d.	n. d.
2nd POx layer	<20	1.3	28.4
ECH	72	n.d.	n.d.
3rd POx layer	<20	1.8	109.4

room temperature (RT) were chosen as the best activation and functionalization conditions because of a high mean fluorescence in combination with the most homogenous distribution of polymer on the surface within a reasonable time (Figure 1C).

2.3. LbL Assembly

The aim of this work is the production of POx coatings having a defined thickness to tailor the strength of interaction with the genetic material. To this end, an LbL deposition method was conducted to produce multilayers of amine containing POx on PP. The general procedure is shown in Scheme 1, starting with the already described deposition of a POx coating on a GOPTMS monolayer. Subsequently, the remaining amine groups were activated by the use of an excess of epichlorohydrin (ECH). The epoxide groups of this cross-linker molecule react with primary and secondary amines present in the first POx layer resulting in tertiary amine groups. In a second step, alkaline conditions are applied, leading to a reformation of an epoxide ring, based on the elimination of hydrochloric acid. The generated epoxide groups can be used to attach a second layer of POx as described before. This cycle can be repeated to immobilize a defined amount of POx layers on the substrate. Within this study films composed of up to three layers were produced. A first indication of a successful layer deposition was generated by CA measurements (**Table 2**). The blank PP substrate produced high CA values due to the hydrophobicity of the material.

Plasma activation, as well as GOPTMS deposition reduces the CA significantly, indicating an effective functionalization. Upon the attachment of POx, the CA decreases below 20° which displays the minimum measurable CA. The activation using ECH cross-linker yields CA values above 50° which is decreased again below 20° upon the addition of another POx layer. This behavior of surface energy changes is in perfect agreement with the successful deposition of POx in an LbL approach.

To gain further insights into the system, thermogravimetric analysis (TGA) experiments were conducted. Pure PP substrates, as well as POx (**2**) were subjected to thermal combustion. The weight loss as a function of temperature was recorded revealing different decomposition temperatures (Figure S2, Supporting Information).

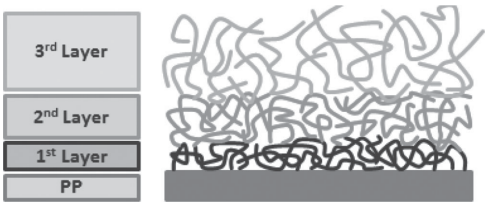


Figure 2. Schematically representation of water-swollen POx multilayers.

Using this information it is possible to calculate the amount of deposited POx on PP from the TGA measurements of the coated samples (Table 2). The relatively linear mass increase per POx layer indicates a homogeneous LbL deposition.

Further analysis of the POx coating was conducted using reflectometric interference spectroscopy (RIFS) measurements. The method determines the thickness of layers on glass substrates having a reflective Ta_2O_5 layer on the opposite site of the coating by measuring the peak shift of light reflected by the polymer layers.[26] For this purpose, glass slides were coated applying the conditions used for PP and analyzed accordingly (Table S1, Supporting Information). During the measurements, a water flow was channeled over the POx surface continuously to generate information about the swollen layers. The exponential increase of the layer thickness is, at first sight, inconsistent with the information obtained from TGA. A varying cross-linking density of individual layers could be an explanation for this discrepancy. The first layer is attached to the substrate via the reaction with epoxide groups directly on the surface, generating a flat film of polymer chains. With every additional layer the mobility of the epoxide binding sites increases generating more loosely bound and mobile POx layers able to incorporate more water into the polymeric matrix, describing the transition from a surface coating to an immobilized hydrogel (**Figure 2**).

As a final proof of the LbL deposition, fluorescence analyses were performed using polymer **3**. All investigated PP slides were coated with three POx layers; however, fluorescently labeled POx was only used in the first, second, or third coating step, respectively. For the other two layers, polymer **2** was used. In this way samples with one fluorescently labeled POx layer and two unlabeled layers are obtained. Every coating step was investigated using CLSM measurements (**Figure 3**). The pictures illustrate the control over the layer deposition by the applied method. A significant increase in fluorescence upon coating with polymer **3** shows the successful immobilization of the material (e.g., A1). The fluorescence signal remains relatively constant upon the addition of further layers (e.g., A2 and A4) proving the existence of LbL structures on the surfaces.

2.4. DNA Binding and Release

The ability of the presented POx films to adsorb genetic material in a reversible manner is a prerequisite for the aimed application. First tests were conducted using the RIFS method (BIAffinity) as described before, however, using DNA solutions instead of pure water to swell the POx layers. Differences at the peak minimum (500 nm) enable information about the layer

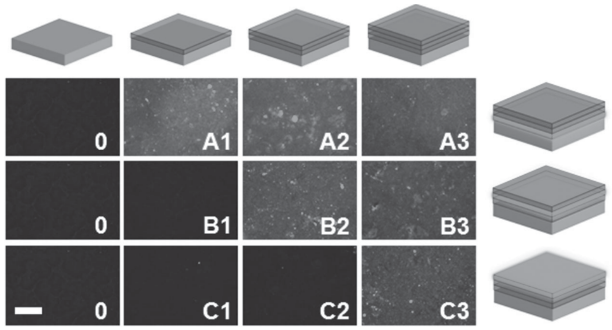


Figure 3. CLSM pictures of fluorescently labeled POx multilayers. Polymer **3** was used for the A) first, B) second, or C) third polymer layer. Pictures were captured of blank PP (0), and after the first (1), second (2), as well as third (3) coating step at a wavelength between 505 and 530 nm. Scale bar: 200 µm.

thickness which, in the end, allows a conclusion about the relative amount of bound DNA (**Figure 4**).

Slides containing a POx monolayer do not show a significant swelling upon addition of DNA while the thickness of coatings with two as well as three polymer layers increases. This trend correlates well with the increase of the hydrogel character per deposited layer. A weaker interconnection between the polymer chains of a film leads to a higher DNA adsorption due to the incorporation of DNA molecules within the layer and an increased interaction between amine groups and the DNA backbone. However, the presence of DNA as a polyanion could also lead to an alteration of the swelling behavior without a distinct binding.

To track the fate of the genetic material in the presence of POx multilayers directly by CLSM investigations, Cy5-labeled DNA strands were used. These measurements allow a quantification of the amount of bound DNA on the substrate assuming that the fluorescence emission increases proportional to the amount of immobilized genetic material. Cy5-labeled DNA lacZ segments were synthesized via PCR using Cy5-labeled primers. After amplification, the PCR products were purified and analyzed via gel electrophoresis. The DNA amount was determined using UV–vis spectroscopy (ScanDrop250).

The time required to establish an equilibrium between bound and unbound DNA is crucial for a later application since

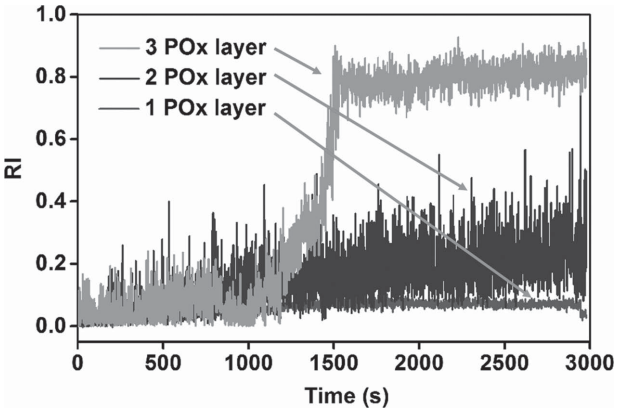


Figure 4. RIFS measurements using a DNA solution of 100 ng µL^{-1} to swell POx multilayers.

it determines the velocity of the first step of, e.g., pathogen detection. To this end, the immobilization of labeled DNA on POx multilayers was investigated (**Figure 5**A). PP chips with a defined surface (78 mm^2) were covered with a water droplet (15 µL) containing 1.5 µg of DNA for a defined time. To exclude an unspecific interaction, the chips were subsequently washed with water at RT.

The CLSM measurements show an increase of fluorescence emission that is proportional to the incubation time of Cy5-labeled DNA strands. Chips containing one POx layer do not show a pronounced uptake behavior which is assumedly a result of the diminished swelling of this monolayer preventing an efficient uptake of the oppositely charged species. In contrast, PP chips with two POx layers reveal an increase in fluorescence up to 5 min incubation time. PP chips with three POx layers show a constant increase in emission up to 2 h. Those results indicate a correlation between the amount of polymer layers and the total DNA adsorption. It is assumable that the hydrogel-like character of chips coated with three layers of POx results in an increasing impact of diffusion processes on the DNA adsorption which is not pronounced for the two-layered systems.

As the polymer associated DNA will be used for surface mediated PCR experiments, the ability of a controllable DNA release behavior is likewise important. Since the genetic material must be available for the polymerase to be amplified, a quantitative release is preferable.

For these experiments, chips loaded for 1 h with the above described procedure were subjected to a temperature dependent washing process (Figure 5B). DNA release studies were not performed for chips containing one polymer layer, due to the fact that the long incubation times necessary for binding makes them unfavorable for further studies. All samples were investigated regarding Cy5 fluorescence before and after the release was accomplished resulting in a percentage amount of released genetic material. The measurements show a release of around 40% of the initially bound DNA at a temperature of 65 °C or above representing ideal conditions for PCR experiments, since the temperature in the amplification cycles varies between 57 and 95 °C. Since no significant improvement regarding DNA binding and release is achieved by the deposition of three instead of two POx layers, further experiments were conducted using a POx double layer.

2.5. PCR Experiments

One major advantage of the presented method is the possibility of coating PP substrates enabling a larger variety in the design of analytical systems. Up to now, only 2D PP slides were used for polymer immobilization. For detection applications, however, it would be favorable to apply POx layers on the inside of PCR reaction vessels. For this reason, PP-tubes (RoboStrip PP white 8-well strips low profile) were coated using the already described procedure. The success of the coating was verified using CLSM measurements of layers deposited using polymer **3** (Figure S3, Supporting Information). However, for qPCR experiments, unlabeled polymers (**2**) were used to avoid interference with fluorescence based detection process. To demonstrate the

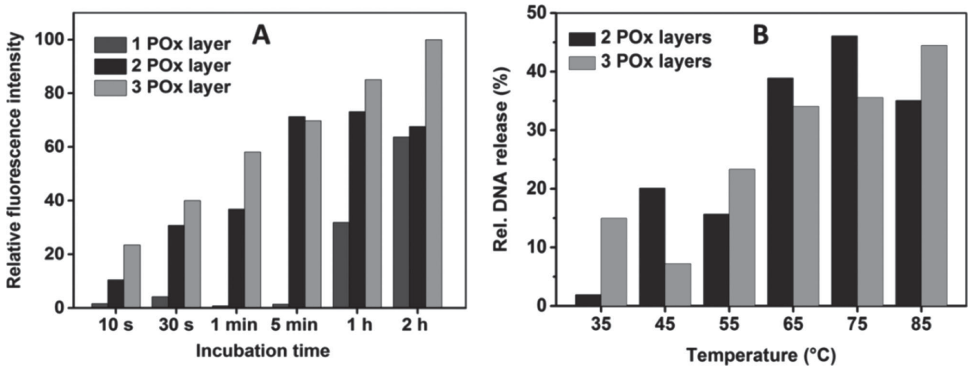


Figure 5. Adsorption and release of Cy5 labeled DNA by POx multilayers. A) Time dependent uptake of labeled genetic material determined by mean fluorescence of POx multilayers after treatment. B) Temperature dependent release of POx multilayers loaded with DNA for 1 h displayed as percentage of the initially bound DNA amount.

convenience of the "lab in a tube"-approach, qPCR assays were performed using coated PCR tubes.

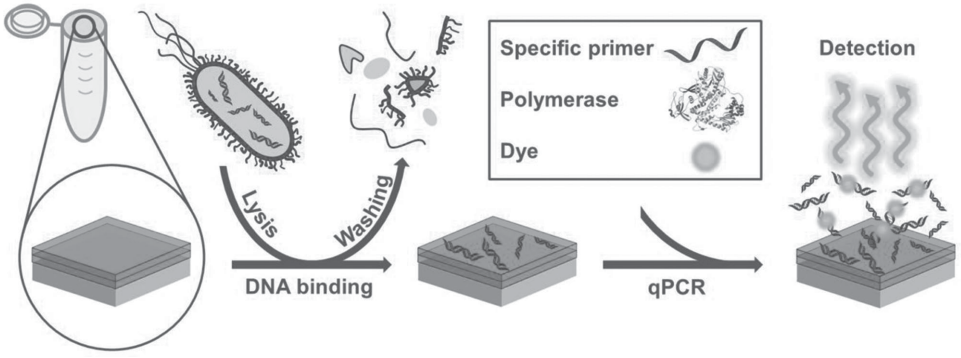
In first experiments, DNA from *Escherichia coli* BL21 (*E. coli*) was extracted with innuPREP DNA Mini Kit (AJ Innuscreen GmbH, Berlin), bound within POx coatings for 1 h at RT, and washed thoroughly. The DNA loaded tubes were filled with 10 µL of qPCR master mix and subjected to qPCR. The assay detects specific gene sequences, like the LacZ-Gene as *E. coli* specific target, by the use of specific primer sequences which only bind to the desired gene. A schematic representation of the whole process is displayed in **Scheme 2**.

The cycle threshold (c_t) value of the qPCR assay shows at which cycle the DNA has reached a defined concentration providing an indication about the efficiency of the process and the amount of available template. After amplification, a positive result is reported by the melting peak of the PCR product, as detected by the use of intercalating dyes. In the case of LacZ, the melting curve of the product should exhibit a peak around 87.5 °C. This melting-point is equivalent to the expected product of 224 base pairs (GenBank sequence accession number: AM946981.2). The results of the detection of extracted *E. coli* samples are shown in **Table 3** and the amplification curves as well as melting points are depicted in Figure S4, Supporting Information. Uncoated tubes with additional template served as a positive control, whereas, a PCR experiment without sample DNA was used as a negative control.

The qPCR result of surface bound DNA is positive for the specific *E. coli*-target and the amplicon shows the same characteristic melting point as the positive control. Also the assay is able to detect *E. coli* DNA in the presence of a background of Herring testes DNA, proving the working principle in the presence of undesired genetic material.

However, for an easy and cost-effective sample analysis it is necessary to bind DNA directly from a cell lysate instead of using already purified genetic material. Consequently, the presence of other (charged) cellular components has to be tolerated. Therefore, an *E. coli* suspension (1.36×10^9 cfu mL^{-1}) was incubated for 10 min at 95 °C to lyse the cells by heat induction. Each 50 µL of this cell lysate were filled in POx modified tubes and incubated for 1 h. To investigate the influence of temperature to the binding behavior during the incubation, the tubes were incubated at 25, 35, 45, 55, and 65 °C (incubation performed on BioShake iQ with adapter for 96×0.2 mL tubes) and the supernatant solution was removed at the specific temperature. Every incubation temperature step was tested positive for *E. coli* DNA with a melting point around 87.3 °C with no detectable melting point on negative control (**Figure 6**).

As shown in the box-plot the c_t values of the qPCR decrease with increasing temperature which indicates a faster amplification process. Generally, the c_t value is dependent on the amount of accessible template (a higher initial DNA concentrations leads to lower values) and the presence of inhibitors



Scheme 2. Working principle of the "lab in a tube" approach. Cells are lysed by heat treatment and genetic material is adsorbed in POx surface coatings. After addition of a specific primer and a PCR master mix, amplification and detection is accomplished in a qPCR process.

Table 3. Results of *E. coli* detection from extracted DNA using the LacZ-gene as target. The amount of bound DNA within the POx coatings could not be determined.

Template	Sample	Amount of DNA [ng]	Melting point [°C]	Mean c_t value	Sample count
E. coli	Positive control	0.6	87.3	16.4	3
	Negative control	0	–	–	3
	Surface bound	n. d.	87.5	20.5	4
E. coli + Herring testes	Positive control	1	87.5	13.7	3
	Negative control	0	–	–	4
	Surface bound	n. d.	87.2	20	4

which increase the c_t value by lowering the efficiency of the reaction. Since the amount of bound DNA is not increasing at higher temperatures (Figure 5B) the latter reason is presumably responsible for the faster amplification. A further indication is given by a narrowing of the value distribution at higher temperatures.

To explain this behavior, the complex composition of the cell lysate can be accounted. While DNA has a high density of negative charge, also other former cell components have potentially negative charges and can interact with the POx layers. The presence of these compounds leads to a disturbance or inhibition of the PCR process, resulting in high c_t values. However, the binding affinity of negatively charged material decreases with increasing temperature, as already demonstrated by temperature dependent release studies (Figure 5B). It is likely that these compounds are released from the POx layers at lower temperatures than the DNA with its multiple negative charges. At 65 °C, the majority of contaminants are present unbound in solution and can be washed away while a considerable amount of genetic material is still adsorbed to the polymer layers, accessible for a subsequent amplification. The performed experiments demonstrate the suitability of POx modified surfaces for a qPCR detection of DNA. Due to the compatibility of the modified PP surface with the qPCR process a combination of sample purification and detection in a single tube or cavity is possible.

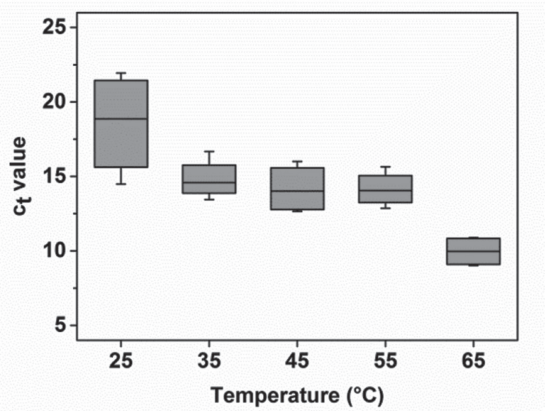


Figure 6. Box-plot of qPCR experiments, performed in POx coated reaction vessels. The tubes were loaded with genetic material from cell lysates at different temperatures and the c_t value of the amplification was recorded.

3. Conclusion

We demonstrated the production of a poly(2-oxazoline) (POx)-based separation and detection system for pathogen analysis without prior purification steps. Covalent amine containing POx multilayers was deposited on PP substrates in a layer-by-layer process. The affinity of DNA to the surface coatings was studied in detail and the binding and release was found to be temperature dependent. PCR tubes, coated with POx were used to bind and purify DNA directly from cell lysates and the amplification of genetic material can be accomplished directly from the adsorbed DNA templates. Moreover, detection can be performed using a qPCR assay. The presented system displays an easy and elegant way to collect sample DNA from biological materials, as well as to perform purification, amplification, and detection within just one coated PCR tube. The method is likewise effective and easy-to-use while requiring only low amounts of functional polymer coating. The straight forward preparation of the layer system and the cost efficiency of the support material should enable for large scale production and automation of detection processes. For this reason, this "lab-in-a-tube" displays a promising tool for bioanalytics. Further studies have to show how reliable the method is concerning varying samples sources, primers and in which concentration ranges a successful detection is possible.

4. Experimental Section

Material and Instrumentation: All chemicals were purchased from Sigma-Aldrich and Merck. 2-Ethyl-2-oxazoline (EtOx) and methyl tosylate (MeTos) were distilled to dryness under argon atmosphere prior to usage. PCR ingredients as well as polypropylene (PP) substrates were provided by Analytik Jena. PP slides (1 × 3 cm) were used for optimization of the coating procedure and verification of the LbL deposition (CA and CLSM measurements). Round PP-chips with a diameter of 0.5 cm were utilized for TGA investigations as well as DNA binding and release studies. For PCR experiments, PCR-tubes (RoboStrip PP white 8–well strips low profile) were coated on the inside. 2-(4-((*tert*-Butoxycarbonyl)amino)butyl)-2-oxazoline (BocOx) was synthesized according to a published procedure.[15]

The polymerization was performed under microwave irradiation, using an Initiator Sixty single-mode microwave synthesizer from Biotage, equipped with a noninvasive IR sensor (accuracy: 2%). Microwave vials were heated overnight to 100 °C under vacuum and allowed to cool to RT under argon before usage. Polymerizations were performed under temperature control.

SEC of the protected statistic copolymers was performed on a Shimadzu system equipped with a SCL-10A system controller, a

LC-10AD pump, a RID-10A RI detector, and a PSS SDV column with chloroform-triethylamine (TEA)-2-propanol (94:4:2) as eluent. The column oven was set to 50 °C.

SEC measurements of the deprotected statistic copolymers and the fluorescently labeled macromolecules were accomplished an a Shimadzu system equipped with a LC-10AD pump, a RID-10A RI detector, a degasser DGU-14A, and a CTO-10A column oven utilizing *N,N*-dimethylacetamide with 2.1 g L^{-1} LiCl as eluent. The column oven was set to 50 °C. Poly(styrene) (PS) samples were used as calibration standards for both solvent systems.

Proton NMR spectroscopy (^{1}H-NMR) measurements were performed at RT on a Bruker AC 300 MHz spectrometer, using $CDCl_3$ or D_2O as solvents. The chemical shifts are given in ppm relative to the signal from the residual nondeuterated solvent.

Absorption and emission spectra of the fluorescently labeled POxs were recorded using the Tecan M200 Pro fluorescence microplate reader (Crailsheim, Germany) at wavelengths from $\lambda = 350$ to 800 nm.

Fluorescence images were obtained using a confocal laser-scanning microscope (CLSM 510 Meta, Zeiss, Jena, Germany), equipped with a 10× objective. Fluorescein-labeled polymers were excited with the 488 nm line of the argon laser. The emitted fluorescence was collected using a 505–530 nm band-pass filter. Scans for the rhodamine-labeled polymers were accomplished using the 543 nm line of the He/Ne laser. The fluorescent emission was recorded with a 560–615 nm band-pass filter. To excite the Cy5-labeled DNA, the He/Ne 633 nm laser was used. Fluorescence was recorded using a 650 nm long-pass filter. To allow a comparison, all images were captured under identical conditions and instrument settings (laser power, pinhole diameter and detector gain). Quantitative image analysis was performed on grayscale converted images using the ImageJ software.

The hydrophilicity of the substrate surfaces was determined using a CA measuring system (OCA 30, Dataphysics, Germany) with droplets of distilled water having a volume of about 10 µL. The measurements were performed in triplicates on different positions of the surface area of the substrates at RT.

TGA was performed under a nitrogen atmosphere on a Netzsch TG 209 F1 Iris in the range from RT to 800 °C with a heating rate of 10 K min^{-1}. The amount of surface bound POx was calculated at a temperature of 280 °C comparing uncoated and coated samples.

The RIFS (BIAffinity) the swelling and DNA binding measurements were accomplished in a new set up, the parallel flow device (Analytik Jena AG), using a flow rate for polymer swelling of 20 µL min^{-1} and for the gDNA binding a flow rate of 5 µL min^{-1} at RT. During DNA binding experiments 100 µL of a gDNA *E. coli* BL21 solution (100 ng μL^{-1}) was injected and channeled in Channel 1 over the coated chip surface and in Channel 2 over the coated chip surface parallel with pure water. The residual DNA content was determined by UV–vis spectroscopy (ScanDrop250).

*Synthesis of P(EtOx$_{35}$-stat-BocOx$_{11}$) (**1**)*: In a microwave vial EtOx (35.3 µL, 0.35×10^{-3} M), MeTos (17.7 µL, 0.12×10^{-3} M), and acetonitrile (2.6 mL) were mixed under inert conditions. After heating in the microwave synthesizer at 140 °C for 19 min EtOx (530 µL, 5.95×10^{-3} M) and BocOx (350 µL, 0.70×10^{-3} M) were added under inert conditions and the reaction mixture was heated to 140 °C for another 13 min. The solution was diluted in CH_2Cl_2 (10 mL) and precipitated in cold diethyl ether (300 mL). The white solid was filtered off and dried in high vacuum to obtain the product as a white powder (810 mg, 88%). ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 4.99$ (s, 0.2 H, NH), 3.45 (s, 4 H, backbone), 3.11 (s, 0.3 H, CH_2-CH_2-NH (BocOx)), 2.40 (s, 1.9 H, CH_2 (EtOx)), 1.92 (s, 0.3 H, CH_2-CH_2-CO (BocOx)), 1.64 (s, 0.3 H, CH_2-CH_2-CH_2 (BocOx)), 1.53 (s, 0.3 H, CH_2-CH_2-CH_2 (BocOx)), 1.42 (s, CH_3 (BocOx)), 1.21 (s, 1.3 H, CH_3 (EtOx)) ppm. SEC (eluent: $CHCl_3$-i-propanol-TEA, PS-standard): $M_n = 4600$ g M^{-1}, $M_w = 6000$ g M^{-1}, $Đ = 1.30$.

*Synthesis of P(EtOx$_{37}$-stat-AmOx$_9$) (**2**)*: TFA (10 mL) was added to solid P(EtOx$_{37}$-*stat*-BocOx$_{11}$) (810 mg), heated to 60 °C, and stirred for 1 h. Subsequently, the solution was cooled to RT, stirred for another 12 h, and was diluted by the addition of 10 mL methanol (MeOH) followed by precipitation in cold diethyl ether (500 mL). The white solid was filtered and redissolved in MeOH (50 mL). Amberlyst A21 free base was added and the mixture was stirred at RT for 24 h. The solid phase was filtered off and the solvent was evaporated under reduced pressure. The product was obtained as a slightly yellow powder (450 mg, 60%). ^{1}H-NMR (D_2O, 300 MHz): $\delta = 3.48$ (s, 4 H, backbone), 2.98 (0.23 H, s, CH_2-CH_2-NH_2), 2.23 (2 H, s, CH_2 (EtOx) + CH_2-CH_2-CO (AmOx)), 1.53 (0.41 H, s, CH_2-CH_2-CH_2-CH_2 (AmOx)) 1.11 (2.8 H, s, CH_3 (EtOx)) ppm. SEC (eluent: DMAc-LiCl, PS-standard): $M_n = 9300$ g M^{-1}, $M_w = 12\,000$ g M^{-1}, $Đ = 1.13$.

*Fluorescent labeling of P(EtOx$_{37}$-stat-AmOx$_9$) (**2**) with fluorescein-5-N-hydroxysuccinimide ester (Fluorescein-NHS-ester) (**3**)*: P(EtOx$_{37}$-*stat*-AmOx$_9$) (**2**, 1.35 g, 0.24×10^{-3} M) was dissolved in dimethylformamide (DMF, 50 mL) followed by the addition of the fluorescein-NHS-ester (112 mg, 0.24×10^{-3} M) and TEA (2.5 mL, 18×10^{-3} M). The reaction mixture was stirred at RT for 3 h. Subsequently, the solvent was evaporated and the crude product was redissolved in MeOH (10 mL). The polymer was precipitated in cold diethyl ether (800 mL), filtered and redissolved in MeOH (50 mL). The solvent was evaporated and the product was obtained as yellow powder (1.17 g, 80%). ^{1}H-NMR (D_2O, 300 MHz): $\delta = 6.60$–6.78 (m, 4.7 H, dye), 3.48 (s, 4 H, backbone), 2.98 (0.23 H, s, CH_2-CH_2-NH_2), 2.23 (2 H, s, CH_2 (EtOx) + CH_2-CH_2-CO (AmOx)), 1.53 (0.41 H, s, CH_2-CH_2-CH_2-CH_2 (AmOx)) 1.11 (2.8 H, s, CH_3 (EtOx)) ppm. SEC (eluent: DMAc-LiCl, PS-standard): $M_n = 11\,000$ g M^{-1}, $M_w = 13\,000$ g M^{-1}, $Đ = 1.16$

*Fluorescent Labeling of P(EtOx$_{37}$-stat-AmOx$_9$) (**2**) with Rhodamine B iso-thiocyanate (**4**)*: P(EtOx$_{37}$-*stat*-AmOx$_9$) (**2**, 300 mg, 0.05×10^{-3} M) was dissolved in DMF (20 mL). After addition of rhodamine B *iso*-thiocyanate (26.1 mg, 0.05×10^{-3} M), the reaction was stirred at RT for 48 h. Subsequently, the solvent was evaporated and the crude product was redissolved in MeOH (10 mL). The polymer was precipitated in cold diethyl ether (800 mL), filtered and redissolved in MeOH (50 mL). After evaporation of the solvent, the crude product was purified via preparative size exclusion chromatography (SEC) using bio beads SX-1 and DMF used as eluent. The product was obtained as a pink powder (280 mg, 86%). ^{1}H-NMR (D_2O, 300 MHz): $\delta = 6.70$–6.88 (m, 0.3 H, aromatic peaks), 3.48 (s, 133.2 H, backbone), 1.56 (s, 37.7 H, CH_2 (AmOx)), 1.11 (s, 111.0 H, CH_3 (EtOx)) ppm. SEC (eluent: DMAc-LiCl, PS-standard): $M_n = 12\,000$ g M^{-1}, $M_w = 14\,000$ g M^{-1}, $Đ = 1.19$.

Surface Coating: PP slides (3 × 1 cm), PP Chips (diameter = 0.5 cm), and PCR tubes were washed in a water/ethanol (EtOH) mixture (1:1) for 24 h. The dried material was treated with O_2 plasma for 30 min. Subsequently, the slides were incubated in an excess of GOPTMS molten on the substrates at RT for 1 h and cleaned by washing with DMF repeatedly.

The epoxide functionalized PP was coated in a layer-by-layer (LbL) approach. First, the substrates were incubated in a POx solution (10 wt%, DMF) at 50 °C for 1 h and washed with DMF to create the first polymer. Subsequently, the material was incubated in an ECH solution (1 wt%, DMF) at 50 °C for 1 h and washed with DMF as well as water. Afterwards, the slides were incubated in an aqueous sodium hydroxide solution (5 wt%) at 50 °C for 1 h and washed with water and DMF. This cycle was repeated, until the desired amount of POx layers were deposited on the glass slides. The same procedure was used for BIAffinity glass slides (10 mm × 10 mm; thickness: 1.1 mm; single-sided coated with Ta_2O_5).

DNA Binding and Release: DNA binding and release experiments were performed using Cy5-labeled lacZ amplificates that were synthesized by standardized PCR experiments from lacZ transfected *E. coli* BL21 using Cy5-labeled primers (SI). All experiments were conducted using PP chips coated with one, two, or three POx layers. In each case the first polymer layer was fluorescein labeled (polymer **3**). DNA solution (15 µL) containing 100 ng μL^{-1} Cy5 labeled lacZ DNA was added to the POx coated PP chips and incubated at RT for different times (10, 30 s, 2, 5 min, 1, 2 h). Afterwards, the chips were washed with water (5 × 500 µL) and dried using compressed air. Subsequently, the mean fluorescence was determined by LSM measurements ($\lambda_{ex} = 633$ nm, $\lambda_{em} \geq 650$ nm).

For release, the PP chips were loaded with DNA as described above for 1 h and, subsequently, incubated in water for 1 h at different temperatures. After separation from the solution and drying using compressed air, the mean fluorescence was determined via CLSM

Table 4. Used primer sequences.

Label	Target	Sequence	Nucleotides	Guanin and Cytosin content [%]	T_m [°C]
SekuLZF	LacZ	5′-cgcagcctgaatggc-gaatg-3′	20	60	61.4
SekuLZR	LacZ	5′-gtgagcgagtaacaaccc-gtcg-3′	22	59.1	64.0

(λ_{ex} = 633 nm, $\lambda_{em} \geq$ 650 nm) before and after treatment and the relative DNA release was determined by comparison of the two values.

PCR Experiments: The qPCR assay was designed to detect DNA of *E. coli* in a POx coated and uncoated cavities. Therefore, every qPCR experiment had a minimum three PP POx modified tubes with qPCR master mix and no template as negative control and 1 µL of DNA extract out of 10^7 cfu mL^{-1} *E. coli* as positive control. The qPCR master mix contains: 3×10^{-3} M of $MgCl_2$, 1 × Hot Start Buffer w/o $MgCl_2$ pH 8.5, 0.1×10^{-3} M dNTPs each, 0.025 U μL^{-1} InnuTaq HOT-A DNA polymerase (provided by AJ Innuscreen GmbH, Berlin), one EvaGreen (Jena BioScience GmbH, Jena), 1×10^{-6} M Primer (**Table 4**) each (Eurofins Genomics, Ebersberg), 3% dimethyl sulfoxide (DMSO) (Carl Roth GmbH + Co. KG, Karlsruhe). The qPCR protocol was optimized for sensitivity and specificity up to $<10^1$ cfu (data not shown). For amplification an initial denaturation for 2 min at 95 °C was followed by 10 cycles 95 °C for 5 s, 72 °C for 15 s and 30 cycles 95 °C for 5 s, 67 °C for 5 s, 72 °C for 10 s with one final amplification of 2 min at 72 °C. The qPCR products were analyzed by melting curve measurements with a temperature range from 60 to 95 °C. The whole qPCR was performed using a TOptical Gradient Thermocycler (Biometra GmbH, Göttingen)

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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