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Functionalized DNA Hydrogels Produced by Polymerase-Catalyzed Incorporation of Non-Natural Nucleotides as a Surface Coating for Cell Culture Applications

*Alexander Finke, Ann-Kathrin Schneider, Anna-Sophie Spreng, Marcel Leist, Christof M. Niemeyer, and Andreas Marx**

Cells from most mammalian tissues require an extracellular matrix (ECM) for attachment and proper functioning. In vitro cell cultures therefore must be supplied with an ECM that satisfies both the biological needs of cells used and the technical demands of the experimental setup. The latter include matrix functionalization for cell attachment, favorable microscopic properties, and affordable production costs. Here, modified DNA materials are therefore developed as an ECM mimic. The material is prepared by chemical cross-linking of commonly available salmon sperm DNA. To render the material cell-compatible, it is enzymatically modified by DNA polymerase I to provide versatile attachment points for peptides, proteins, or antibodies via a modular strategy. Different cells specifically attach to the material, even from mixed populations. They can be mildly released for further cell studies by DNase I-mediated digestion of the DNA material. Additionally, neural stem cells not only attach and survive on the material but also differentiate to a neural lineage when prompted. Furthermore, the DNA material can be employed to capture and retain cells under flow conditions. The simple preparation of the DNA material and its wide scope of applications open new perspectives for various cell study challenges and medical applications.

1. Introduction

Most mammalian cells attach to an extracellular matrix (ECM), formed by proteins like collagen, laminin and fibronectin.[1–3] The ECM regulates proliferation, differentiation and migration of attached cells and it modifies their propensity to undergo apoptosis.[4] The cell-ECM interaction is based mainly on cellular integrins binding to their cognate ligands on the dense ECM network. In this context, the interaction of fibronectin and integrins is particularly well studied.[5] Nearly half of the known heterodimeric integrins bind to fibronectin by recognition of its short tripeptide sequence Arg-Gly-Asp (RGD), which is present on the 10th repeat of the type 3 domain of fibronectin.[6] Aditionally, the attachment motif RGD is also found in other structural proteins like fibrinogen and vitronectin.[7] Numerous studies report on the mimicry of features of ECM by using synthetic or natural materials to obtain widely tunable, but highly controlled conditions.[8–10] However, for each application an artificial ECM with most appropriate properties has to be chosen. For instance, an extract of natural ECM, Matrigel, is often used. However, it is problematic due to its batch to batch variation and poor material definition.[11] But even well-established types like collagen-based hydrogels are not suited for all purposes, e.g., due to reduced stiffness.[12] Therefore, a wide range of hydrogels is proposed with different building blocks functioning as the backbone, often also with conjugated bait molecules like RGD to make the material susceptible to cell attachment.[8,13] While polyethylene glycol is predominantly used for these scaffolds,[14] DNA is also a viable candidate due to its hydrophilic character, the possibility to program its properties by sequence design, chemical or enzymatic incorporation of modified nucleotides and its susceptibility to modification with a large toolbox of enzymes like restriction enzymes.[15–18] To this end, various DNA-based materials were reported for different applications.[19–21]

Likewise, oligonucleotide-decorated cell surfaces have a huge potential for sophisticated studies in cell biology because they can be engineered to precisely control cell/cell and cell/substrate interactions.[22–25] Therefore, DNA/protein, DNA/peptide and DNA/aptamer-based networks on solid substrates were successfully used to bind to specific cell receptors for cell immobilization and cell culturing.[26–29] Additionally, the particular properties of DNA allow the specific release of captured cells, which was, e.g., demonstrated using enzymatic degradation, strand displacement or induced structural changes

A. Finke, A.-S. Spreng, Prof. M. Leist, Prof. A. Marx
Departments of Chemistry and Biology
Konstanz Research School Chemical Biology
University of Konstanz
Universitätsstraße 10, 78464 Konstanz, Germany
E-mail: andreas.marx@uni-kostanz.de
A.-K. Schneider, Prof. C. M. Niemeyer
Karlsruhe Institute of Technology (KIT)
Institute for Biological Interfaces (IBG 1)
Hermann-von-Helmholtz-Platz D-76344, Eggenstein-Leopoldshafen, Germany

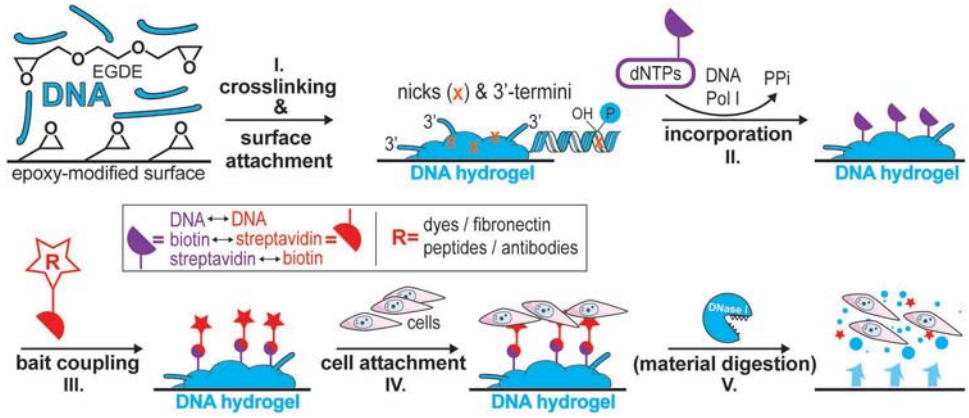

Figure 1. Working scheme toward bait-decorated DNA hydrogels. I) Salmon sperm DNA is cross-linked by EGDE and attached to epoxy-modified glass slides in alkaline conditions. II) Modified dNTPs are employed at nicks and 3′-ends by DNA polymerase I to incorporate DNA, biotin, or streptavidin into the DNA hydrogel. III) Molecules of interest, like dyes, peptides, or proteins, can be bound to the DNA hydrogel by biotin-streptavidin-mediated or complementary oligonucleotide-mediated attachment. IV) Specific bait-mediated cell attachment. V) Controlled release of cells by DNase I-mediated digestion of the material if needed. PPi: inorganic pyrophosphate; DNA Pol I: DNA polymerase I.

of the DNA scaffolds.[29–31] Such artificial ECMs may have the potential to bridge the gap between 2D cell culture and animal models by reducing the natural complexity to a more defined number of variables.[32,33] In tissue engeneering, artificial scaffolds could be designed to accommodate cells, e.g., for regenerative purposes.[14]

Here, we report on the development of a chemically cross-linked DNA hydrogel that can be readily modified by enzymatic modification to incorporate functional moieties that render the material attractive for cells (**Figure 1**). Our material is based on bulk salmon sperm DNA which is available in large quantities and much lower costs than synthetic oligonucleotides. It can be formed directly on reactive surfaces, followed by functionalization through DNA polymerase-catalyzed nick translation reaction to incorporate modified nucleotides in order to install "bait" molecules that mediate cell adhesion.

Specific cell attachment on the developed DNA hydrogels was evaluated for several cell types. Since the hydrogel can be degraded under mild conditions by specific DNA-cleaving enzymes, we also explored cell detachment by these enzymatic reactions. This approach opens the door to applications where cells of interest can specifically be captured and released from DNA hydrogel-coated surfaces or when cells need to be detached without proteolytic damage.[34]

2. Results and Discussion

The preparation of the DNA hydrogel is based on the work of Tanaka et al., using ethylene glycol diglycidyl ether (EGDE) as a diepoxide cross-linker to connect DNA strands to each other in an alkaline environment and in presence of tetramethylethylenediamine (TEMED) as a catalyst.[35] It has been shown that the epoxy-moieties of EGDE perform cross-linking via the N7-position of guanosines to covalently connect the strands to each other.[36] This procedure was modified by us to allow flexible attachment to support structures and enzymatic modification for DNA hydrogel functionalization.

To generate the cross-linked DNA hydrogel on solid support, salmon sperm DNA was dissolved under heating to promote solubility, denaturation of the double strands, and facilitated liquid handling. After addition of EGDE, TEMED, and other reaction supplements (experimental part in the Supporting Information), the hot solution was cast on epoxy-modified glass slides within sealable frames which were afterward cooled to solidify the reaction mixture. The solution was slowly dried, soaked with high salt buffer, and sealed for final polymerization overnight. Our method differs from others,[35,37] as the initial DNA concentration of 3% used by us was relatively low, leading to low viscosity and easy handling of the casting process, while the drying step still allowed the polymerization with only minor cross-linker concentrations (0.3%). Verification of the hydrogel homogeneity was performed via staining with SYBR Green I that binds double-stranded DNA resulting in an increase of fluorescence intensity. The equal distribution of fluorescence indicates a high degree of homogeneity of the DNA hydrogel (**Figure 2**A). Further analysis by scanning electron microscopy shows that the casting process of the DNA hydrogel using a flat foil-cover yields a DNA material with a flat surface and minor wrinkles (Figure 2B I). Analysis of gel cross-sections (Figure 2B II) revealed honeycombed structures which form the bulk of the material and show similar morphologies of other hydrogels.[38,39]

Next, the incorporation of modifications into the DNA hydrogel was explored by using DNA nick translation reactions, harnessing the ability of DNA polymerase I to incorporate modified nucleotides even with bulky substituents.[40,41] Abundant 3′-ends and nicks, which arise during handling of salmon sperm DNA were envisioned to be recognized by DNA polymerase I and to guide the incorporation of the modified nucleotides. As modified dNTPs, we used commercially available biotin-dUTP. We also prepared modified dUTPs bearing streptavidin moieties (Stv-dUTP) or oligonucleotides (ON-dUTP), following recently reported protocols.[42,43] The incorporation of the three types of modified nucleotides by the DNA polymerase was validated by primer extension reactions (Figure S1, Supporting Information), and we went on to

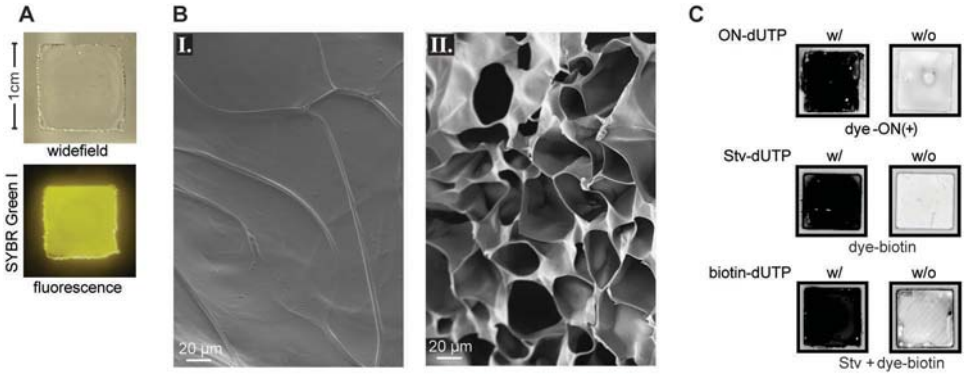

Figure 2. Preparation and modification of DNA hydrogels. A) Widefield image of DNA hydrogel untreated (top) and incubated w/ SYBR Green I on a blue light transilluminator (bottom). B) Scanning electron microscopy images from I) the DNA hydrogel surface and II) the cross-section. C) DNA hydrogel and nick translation w/ or w/o modified dUTPs, incubation w/ dye-modified molecules and fluorescence readout. Oligonucleotide-dUTP (ON-dUTP) and complementary dye-modified ON (top). Streptavidin-dUTP (Stv-dUTP) and dye-biotin (middle). Biotin-dUTP and 2-step incubation w/ streptavidin (Stv) and dye-biotin (bottom).

investigate modification of the chemically cross-linked DNA hydrogel.

First, nick translation was performed in the presence of DNA polymerase I and either ON-dNTP, Stv-dUTP, or biotin-dUTP, respectively. Incubation was performed in a humidity chamber at 37 °C by overlaying the DNA hydrogel with the reaction solutions followed by thorough washing of the modified surfaces (see the Supporting Information for details). In order to verify incorporation of the functionalities, the surfaces were incubated with dye-modified binding partners, i.e., dye-modified complementary oligonucleotide when ON-dNTP was used, dye-modified biotin for Stv-dUTP, and streptavidin/dye-modified biotin in case of biotin-dUTP, respectively. A modification-specific staining of the DNA material was observed via fluorescence read-out in all cases. Using different cross-linker concentrations (0.1–0.6%) during DNA hydrogel preparation had no significant impact on functionalization and was therefore left at 0.3%. After demonstrating the feasibility of our approach to modify the surface the stage was set for the immobilization of baits that mediate selective cell adhesion.

First, we investigated HeLa cell attachment on the DNA hydrogel that was modified with biotin as described above (Figure 2C). As bait molecule, a fibronectin streptavidin fusion protein was prepared (Fn-Stv, Figure S2, Supporting Information) to bind on the one side to integrins like the $\alpha v\beta 3$ variant which is the predominantly expressed integrin in HeLa cells and on the other side, to the biotin-modified surface.[26,44] Thus, the biotin-modified DNA hydrogels were further functionalized with Fn-Stv and HeLa cells were incubated on the DNA hydrogel. After washing, the cell density was assessed by phase contrast microscopy. We found cell densities in the range of about 800 cells mm^{-2}. This is significantly higher in comparison to control experiments conducted with streptavidin-modified (Stv) or unmodified DNA hydrogel surfaces where only up to 40 cells mm^{-2} were detected under identical conditions (**Figure 3**A).

Next, we investigated ON-modified immobilized DNA hydrogels. As ligands, we synthesized azide-modified cyclic c(RGDfK) and as control c(RADfK),[45] and coupled them to a DBCO-modified oligonucleotide which is complementary to the oligonucleotide incorporated into the DNA hydrogel. We found elevated cell adhesion when the c(RGDfK) was used and suppressed cell adhesion in case of the nonfunctional c(RADfK) (Figure 3A).

Finally, we investigated a biotin-modified antibody (α-CD81) which was expected to mediate cell adhesion via its specific

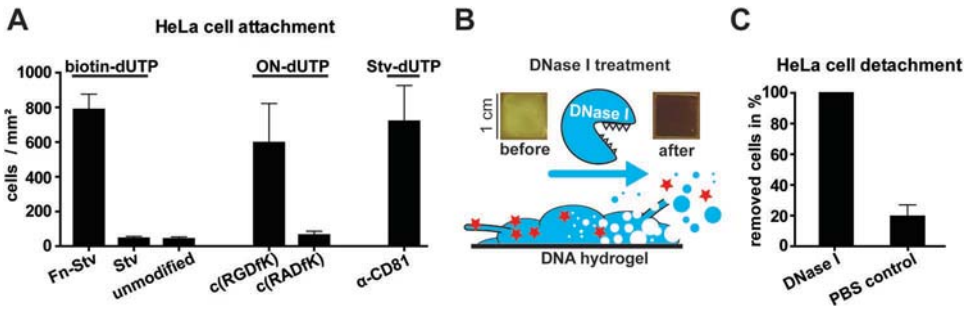

Figure 3. Evaluation of cell attachment and detachment methods. A) HeLa cells incubated on differently modified DNA hydrogels and washed w/ PBS buffer. Cell density assessed by phase contrast microscopy. B) Schematic overview of DNase I-mediated digestion of the DNA hydrogel. C) HeLa cell detachment by DNase I-mediated DNA hydrogel digestion, assessed by the difference in cell densities after initial cell attachment and after DNase I treatment. Control experiment was performed with PBS buffer w/o DNase I. α-CD81: biotinylated antibody against CD81; Fn-Stv: fibronectin streptavidin fusion protein; Stv: streptavidin, mean ± SEM.

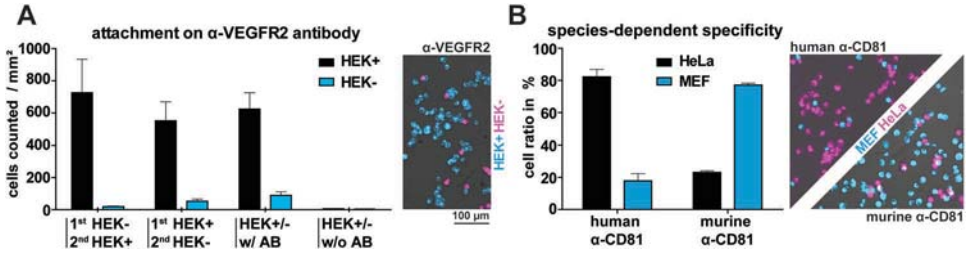

Figure 4. Evaluation of specificity by antibody-mediated cell capture. A) Incubation of a cell mixture containing HEK+ and HEK– cells or their alternating incubation on α-VEGFR2 antibody-modified DNA hydrogel. Assessed HEK+ and HEK– cell density of the imaging data. Representative image of experiment (HEK ± w/ AB) showing approximate cell density and population ratio. B) Incubation of a cell mixture containing Mouse Embryonic Fibroblast (MEF) and HeLa cells on DNA hydrogel modified w/ human or murine α-CD81 antibody. Cell ratios assessed by epifluorescence imaging. Representative images of experiments showing approximate cell densities and population ratios. AB: α-VEGFR2 antibody, mean ± SEM.

interaction with CD81 on HeLa cells. Stv-modified immobilized DNA hydrogels were incubated with biotinylated α-CD81, washed, and incubated with a HeLa cell suspension. After subsequent washing, we found again an elevated cell adhesion also in this case. Thus, a platform for cell culturing can be established in a straightforward and modular manner by the functionalization of chemically cross-linked DNA hydrogels. While all cell attachment experiments were performed with optimized conditions, cell densities can be influenced, e.g., by the amount of used bait molecules (Figure S3, Supporting Information).

We next established a method to mildly detach the cells from the immobilized DNA hydrogel. This approach would open the door to downstream applications with cells (vide infra). We expected that digestion of the DNA hydrogel by DNase I would liberate the attached cells. Preliminary results indicate that the DNA hydrogels are degraded upon treatment with DNase I since no remaining fluorescence signal was detected after staining with SYBR Green I (Figure 3B). Therefore, we investigated the detachment of HeLa cells that were immobilized on a biotin-modified surface which was incubated with Fn-Stv. Medium was exchanged with DNase-I in modified PBS buffer. After only short incubation (10 min) and gentle washing, only a few cells remained in the DNase I-treated samples (Figure 3C). Moreover, the detached cells could be aspirated and placed on a new cell culture plate with no visible decrease of viability or impaired proliferation, as previously shown.[28,39]

We then investigated the specificity of our system to address particular receptors on the cell membrane. The adhesion of VEGFR2-overexpressing HEK (HEK+) cells on an Stv-modified DNA hydrogel decorated with biotinylated α-VEGFR2 antibody was assessed in comparison to wt HEK cells (not expressing VEGFR2; HEK–) (**Figure 4**A). This attachment was performed either in simultaneous competition of both cell types (HEK+ and HEK–) or in separate incubation cycles of each respective cell population with washing steps in between (i.e., first HEK+ followed by HEK–, and vice versa). For the assessment of cell numbers, the respective cell lines were prestained with CF555 or CF680-modified wheat germ agglutinin (WGA). Best discrimination of adhesion (97% HEK+ cells) was observed when HEK+ cells were incubated on the DNA hydrogel after the addition and brief washing of HEK– cells. Still, the attachment and the discrimination were satisfactory when the order was changed and HEK+ cells were added first (92% HEK+ cells) or when both cell lines were placed on the DNA hydrogel at the same time (87% HEK+ cells). Upon closer scrutinizing, we observed that most of the attached HEK– cells were in direct contact with HEK+ cells, which presumably explains the observed minor increase of nonspecifically bound cells. A control experiment with DNA hydrogel without α-VEGFR2 antibody showed only little attachment of either cell line, and no specificity for HEK+ cells was observed as expected.

Additionally to the manual cell counting, the established cell detachment method using DNase I-assisted DNA hydrogel degradation was successfully employed to measure flow cytometry with all hydrogel-bound cells in order to validate the results. Results of both assessments were comparable, but flow cytometry showed a slightly decreased specificity (90%, 81%, 72% HEK+ specificity, respectively, compared to the results stated above).

We probed the specificity even further and investigated species-specific baits. HeLa cells and mouse embryonic fibroblasts (MEF) possess a species-dependent binding prevalence to human or murine α-CD81 antibodies in life cell imaging as shown in Figure S4 (Supporting Information). We also investigated if this can be applied to antibody-modified DNA hydrogels. Thus, Stv-modified DNA hydrogel samples functionalized with biotinylated human or murine α-CD81 antibody were presented to a cell mixture of HeLa and MEF cells (Figure 4B). Hela cells preferred human α-CD81 and MEF cells preferred murine α-CD81 antibodies with a specificity of 82% and 77%, respectively.

Next, we went on to investigate more complex systems and established the DNA hydrogels as a platform for culture and differentiation of neural stem cells (NSCs). NSCs easily attached to fibronectin- or c(RGDfK)-modified DNA hydrogels and as expected did not adhere to DNA hydrogels which were unmodified or modified with either streptavidin or c(RADfK) peptide (Figure S5, Supporting Information).

Differentiation of the attached NSCs was explored next (**Figure 5**A). After attachment and proliferation of NSCs on the DNA material, growth factors were removed by medium exchange which triggers neuronal differentiation. After the differentiation phase of 4 d, cells were stained with Neurofluor NeuO, a life-cell imaging dye, described to bind specifically to neuronal cells.[46] Additionally, the dye Hoechst H-33342 (Hoechst) was used to stain cell nuclei, as well as the surrounding DNA hydrogel. We detected characteristic neuronal networks on fibronectin- and c(RGDfK)-modified DNA hydrogels. The soft properties of the material might beneficially influence differentiation as previously shown for other systems by others.[47,48]

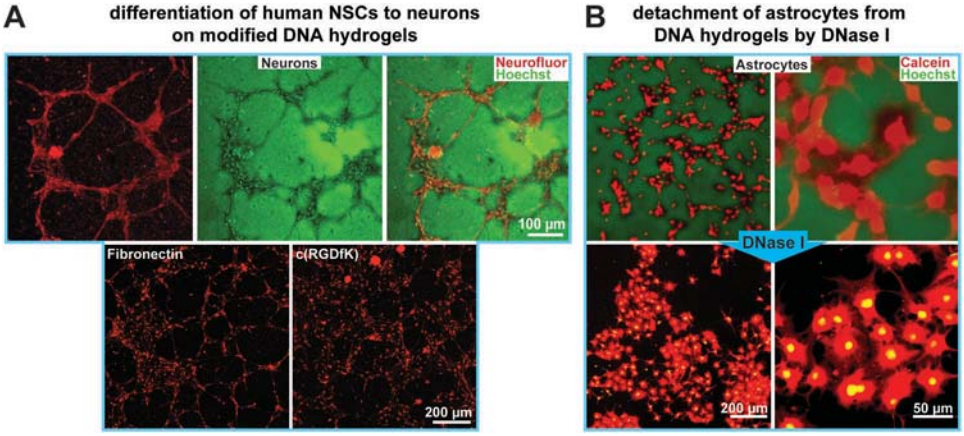

Figure 5. Neural stem cell (NSC) attachment and differentiation on modified DNA hydrogel. Hoechst was used for staining cell nuclei and DNA hydrogels (green). A) Differentiation of NSCs to neurons, split and merged views of cultivated neurons on fibronectin-modified DNA hydrogel (top). Zoomed-out view of differentiated neurons cultivated on DNA hydrogel w/ fibronectin or c(RGDfK) (bottom). Neurofluor was used for staining neurons (red). B) Differentiation of murine NSCs to astrocytes on fibronectin-modified DNA hydrogels. Wide (left) and close-up views (right) of cells before DNase I treatment on DNA hydrogel (top) and after DNase I treatment on poly-L-ornithine/laminin-coated cell culture plates on the next day (bottom). Calcein was used for staining live cells (red).

After having shown that the differentiation of human NSCs toward neurons on DNA hydrogels is possible, we also examined murine NSC which are well established to differentiate to an astrocyte phenotype within 3 d but show resistance to detachment by trypsin (Figure S6, Supporting Information).[49–51] We found that these cells tightly bind to their substrate and thus need harsher conditions like prolonged incubation times followed by mechanical resuspension, affecting cellular signaling and cell viability. To solve this problem, we implemented the herein developed detachment protocol by DNA digestion through DNase I treatment. Therefore, we seeded murine NSC onto fibronectin-modified DNA hydrogels and replaced the growth factors in the medium with BMP4 to trigger astrocyte differentiation (Figure 5B). Hoechst and Calcein-AM staining verified the characteristic morphology of astrocytes after 3 d. After treatment with DNase I, cells were easily detached from the material and transferred onto a poly-L-ornithine/laminin-coated slide for reattachment. The detachment efficiency was similarly quantitative as previously performed with HeLa and HEK cells. Imaging of stained cells on the next day showed that they are healthy and in a nonactivated state. This example effectively shows how the herein developed DNA-based material can be employed for the initial attachment phase, for cell differentiation experiments, and up to the final detachment of the cells for other applications.

While all the previous experiments were carried out under static conditions on flat surfaces, we also wanted to investigate the potential of the new DNA hydrogels under fluidic culture conditions. To this end, we used a cylindrical flow channel (2 mm diameter) prepared by microthermoforming, as previously described (**Figure 6**A).[52] To immobilize the DNA hydrogel, cyclic olefin polymer foils were first chemically activated with epoxy groups and subsequently formed into half-channels by microthermoforming. The prepared DNA solution was filled into the half-channel, into which a negative PDMS cast of the half-channel was then carefully installed to cover the entire channel surface and to ensure a curved shape of the hydrogel. Subsequent to polymerization, two half-channels were assembled by self-adhesive foil to form a complete channel in which all further modification steps were performed. DNA hydrogel deposition and shape were verified by SYBR Safe staining and confocal microscopy 3D Z-stacks (Figure 6B). Channels equipped with unmodified or α-VEGFR2-modified DNA hydrogels were tested for cell adhesion. To this end, the channels were mounted in a chip-to-world interface that enables microscopy and connection to the flow system (Figure S7, Supporting Information). Medium containing Syto16 pre-stained α-VEGFR2-overexpressing HEK (HEK+) cells was perfused through the channel for 30 min. To balance the cell migration speed in the fluidic system against the interaction time for the cells to attach to the material, a flow rate of 200 µL min^{-1} was chosen. We found that HEK+ cells were detected only on the modified DNA hydrogel, whereas no cell adhesion was observed in the case of control channels containing DNA hydrogel that lacked the antibody (Figure 6C,D). While cell attachment was reduced in comparison to HEK cell attachment under static conditions and thus needs further optimization, these results confirmed the utility of the novel surface coating for cell culture under dynamic conditions, which is important, for instance, in prosthetics.

3. Conclusion

In this work, we established a DNA-based hydrogel which is easy to prepare and due to its modular modification very versatile. The modification by DNA polymerase I-mediated nick translation renders the material to be specifically recognized by desired cells. A broad range of available biotinylated antibodies and the use of common components allow binding of even rare cell lines without major effort. Cells can be processed on the material or transferred to other plates using the mild detachment by DNase I treatment. This can be especially beneficial when cells, for example astrocytes, are rather resistant to detachment by trypsin treatment. It can be also a potent approach for co-culturing cells. For the orthogonal release of different cell

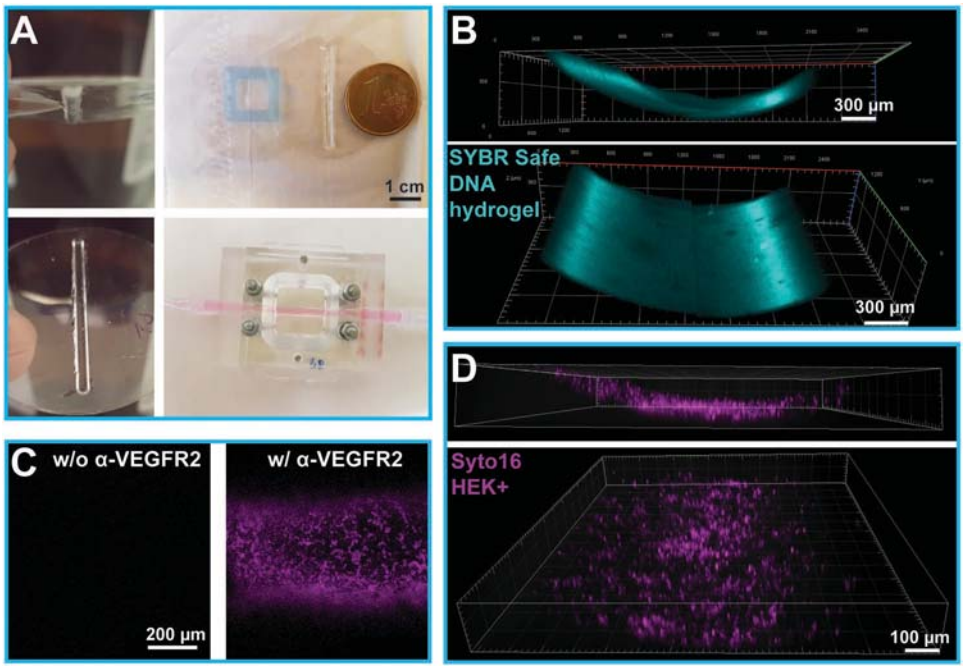

Figure 6. System setup for cell attachment in a flow cell. A) DNA hydrogel preparation on thermoformed cyclic olefin polymer half-channels (left). Size-comparison with DNA hydrogel used in previous results (blue frame, inner surface 1 cm^2) and with a 1-Euro coin (top right). Channel assembly with a chip-to-world mounting suitable for flow experiments and microscopy (bottom right). B) 3D fluorescence imaging of SYBR safe-stained DNA hydrogel prepared in a half-channel. C) Top view of Syto16-stained, VEGFR2+ HEK (HEK+) cells which attached under flow on DNA hydrogels that were unmodified or modified w/ α-VEGFR2 antibody. D) 3D fluorescence imaging of Syto16-stained HEK+ cells in the flow channel on α-VEGFR2-modified DNA hydrogel.

lines, one can be placed on the DNA hydrogel and the other one on the regular cell culture plastic. In contrast to standard proteolytic detachment, cell surface proteins are furthermore not degraded. Besides HeLa, MEF and HEK cells, more delicate cell lines were tested. Here, neuronal stem cells could attach and proliferate on the material. After differentiation, they easily formed neurite outgrowths to connect to each other. So far, differentiation was initialized by removal of growth factors or supplementing with differentiation factors. For differentiation signals which are initialized by cell surface receptors, one could think of decorating the DNA hydrogel not only with bait molecules but also with differentiation factors. Furthermore, the influence of the mechanical properties of the DNA hydrogel on cells and their behavior can be investigated. Here, DNA hydrogel stiffness could be altered by changes in the preparation like varying cross-linking concentrations. The cell attachment in DNA-coated channels under flow shows the applicability in more complex settings and opens possibilities in the medical area when further optimized. A possible application could be DNA hydrogel-coated stents which are functionalized to attract endothelial progenitor cells which built up an epithelium and reduce adverse immune responses in the human body. In summary, our cell-viable DNA material should be considered, when scientific questions are addressed which demand elaborated cell culture settings.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

cell adhesion, chemical cross-linking, DNA hydrogels, nick translation, tissue engineering

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