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Designer Extracellular Matrix Based on DNA–Peptide Networks Generated by Polymerase Chain Reaction

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Abstract: *Cell proliferation and differentiation in multicellular organisms are partially regulated by signaling from the extracellular matrix. The ability to mimic an extracellular matrix would allow particular cell types to be specifically recognized, which is central to tissue engineering. We present a new functional DNA-based material with cell-adhesion properties. It is generated by using covalently branched DNA as primers in PCR. These primers were functionalized by click chemistry with the cyclic peptide c(RGDfK), a peptide that is known to predominantly bind to αvβ3 integrins, which are found on endothelial cells and fibroblasts, for example. As a covalent coating of surfaces, this DNA-based material shows cell-repellent properties in its unfunctionalized state and gains adhesiveness towards specific target cells when functionalized with c(RGDfK). These cells remain viable and can be released under mild conditions by DNase I treatment.*

Multicellular organisms have to orchestrate a large variety of cells with different functions. The positioning of specialized cells and information exchange between cells through signaling are important to fine-tune cellular functions.[1] Most mammalian cells adhere to a biological matrix, the extracellular matrix (ECM), which consists of a dense network of a multitude of different structural and functional proteins.[2] The ECM provides survival signals[3] and is also necessary for mechanical stability.[4] For cultivation in vitro, artificial substrates are needed, to which the adherent cells stick.[5] Without attachment to a matrix, most mammalian cells undergo apoptosis.[2b] Most commonly, essentially rigid materials like glass or polystyrene with adsorbed adhesion proteins or peptides are used to induce adhesion to a wide variety of cells.[6] The peptide motif arginine-glycine-aspartate (RGD) is one of the best characterized and most used of these.[7] This sequence can be found in several proteins, such as fibronectin and laminin, and plays an important role in mediating cell adhesion.[3] About half of all integrins, a large family of heterodimeric transmembrane cell receptors, recognize this peptide motif and thereby establish a connection to the ECM.[8] Selectivity for certain integrins can be strongly improved by using cyclic forms of RGD due to their restricted conformation.[9] For instance, the cyclic peptide c(RGDfK) is used to address αvβ3 integrins, which are overexpressed in cancerous cells, for example, HeLa cells.[10] Moreover, the incorporation of additional amino acids beside RGD enables modification of the exterior peptide scaffold, for example, the attachment of dyes or solid supports.

To be able to meet the requirements for the attachment of different cell types, a programmable material would be beneficial. The possibility to program DNA-based materials through Watson–Crick base-pairing rules,[11] the ability to easily synthesize modified nucleic acids by chemical means, and the toolbox of available enzymes that manipulate DNA at single-base resolution, render DNA a very interesting candidate for such a programmable material. Examples along these lines include approaches in which cells were bound to immobilized DNA nanostructures via oligonucleotide-decorated cell surfaces,[12] protein-conjugated DNA microarrays,[13] or structurally tunable DNA/protein-based matrices.[14] Furthermore, aptamers, which are oligonucleotides that can bind specific target molecules, have been employed to form DNA hydrogels that additionally attract cells.[15] Finally, the selective recognition of oligonucleotide-modified surfaces of defined cell types by complementary binding partners is being used to form precisely engineered tissues with programmed cell–cell or cell–surface connectivity.[16]

In this work, we developed a DNA-network-based material that is generated and amplified by the polymerase chain reaction (PCR). The concept is based on branched DNA strands that serve as primers in PCR (Figures 1 A, 2 A). We aimed at synthesizing the DNA network on glass surfaces that are functionalized with branched primers. During the enzymatic reaction, a multilayered DNA network is expected to be built up on the surface (Figure 2 A). This would fulfill the common prerequisites of a hydrogel, being a cross-linked polymeric network that can be swelled with water, with the latter being an intrinsic property of DNA that is influenced by its hydration shell.[17]

Since the branched primer molecules are modified with a bait molecule for cell adhesion, the functionalized DNA networks mimic the extracellular matrix and enable the adhesion and growth of specific cells that interact with the bait. For this purpose, branching unit **1** was used during oligonucleotide synthesis to prepare the branched primers (Figure 1 B,C). The alkyne modification on **1** allows functionalization of the networks by copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC).[18] We employed the cyclic RGD-peptide, c(RGDfK) based on its high affinity to cells expressing the αvβ3 integrin, such as HeLa and MEF cells.[19] To make it suitable for CuAAC, the peptide was

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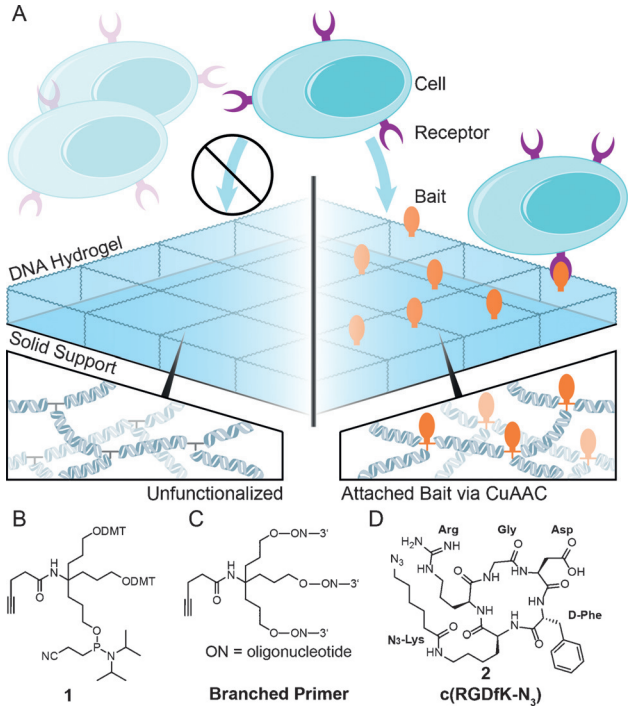

Figure 1. A) A schematic representation of surface-bound DNA hydrogels for selective cell adhesion. DNA hydrogels that are based on branched DNA and built on surfaces adhere specifically cells when they are decorated with bait molecules. Branched DNA is covalently attached to the surface. Branched primers are used in a PCR reaction and attach to the surface-bound DNA. B) The building block for the solid-phase synthesis of branched primers. C) The general structure of branched primers. D) The bait molecule, a cyclic RGD peptide modified with 6-azidohexanoic acid at the lysine residue.

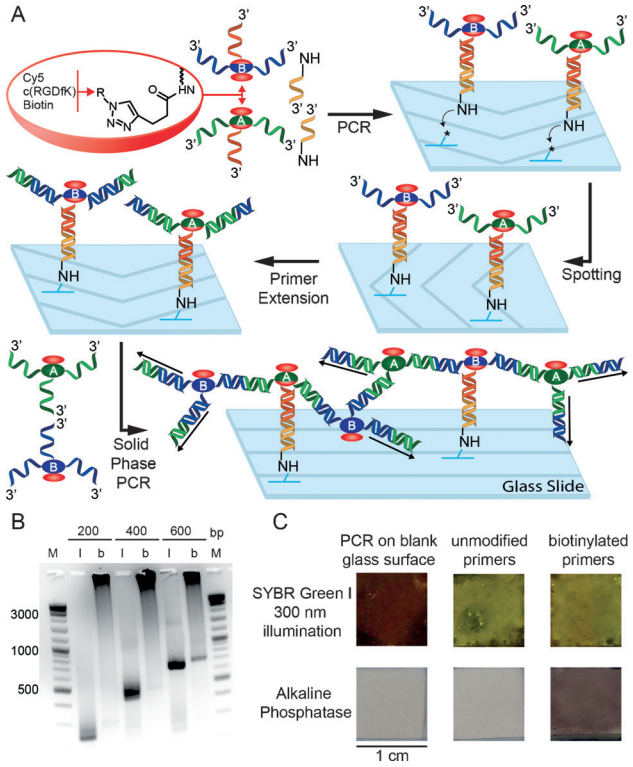

Figure 2. A) The formation of DNA networks on glass surfaces. Amine-functionalized primers and branched primers undergo PCR in solution. This PCR product is covalently attached to glass activated with *p*-phenylene diisothiocyanate (*). Primers not used in the first PCR are elongated in a primer-extension reaction. Performing branched PCR with branched primers **A** and **B** on this surface leads to the incorporation of branched primers into the surface-bound network. B) Branched PCR in solution with templates of different length. l: linear primers, b: branched primers, bp: base pairs, M: marker. C) Network formation on the surface. Staining with SYBR Green I (top) and colorimetric analysis of streptavidin-conjugated alkaline phosphatase and a chromogen (NBT/BCIP; bottom) on glass slides after solid-support PCR on a surface devoid of spotted branched DNA (left), and surfaces with spotted branched DNA and unmodified primers (middle) or biotinylated primers (right) for DNA network formation.

modified with 6-azidohexanoic acid at the lysine residue to give **2** (Figure 1D).

Initially, the DNA network was established in solution by the following approach: branched primers were synthesized using building block **1** (for details of synthesis and characterization, see the Supporting Information).[20] Next, the primers were used for PCR with *Taq* DNA polymerase, which accepts the branched primers and elongates the "DNA arms" to form a DNA network. Two primer sequences complementary to the ends of the template were used. The formation of the DNA network was indicated by retarded migration of the DNA product in agarose gel electrophoreses in comparison to the products obtained with linear, non-branched primer strands. Interestingly, the majority of the obtained products exhibited hardly any observable mobility when the branched primers were used (Figure 2B).

Next, the preparation of DNA networks was established on solid support. PCR was performed with amine-modified primers and two branched primers. One of the three original branched primer sequences was substituted to link to the amine-modified primer through a mutual template. The PCR product was covalently attached to amine-functionalized glass slides activated with *p*-phenylene diisothiocyanate. Reaction chambers were glued onto the surfaces and a first primer extension was performed in a slide thermocycler. When compared to the results obtained from unextended surface-bound branched primers, this led to an improved yield of the surface-bound DNA in the final PCR on solid support for DNA network formation. The same branched primers were used as for the DNA networks in solution. In contrast to a PCR product that is formed in solution, PCR on solid support lead to a DNA hydrogel, which was constrained to become an outstretched layer. The deposition of DNA was analyzed by staining with SYBR Green I. Using a blue-light transilluminator, green fluorescence was exclusively observed when PCR was performed with branched primers on the branched-DNA-functionalized slides (Figure 2C).

We next investigated whether the branched primers and the DNA networks are amenable to CuAAC. For this purpose, the primers were first conjugated to Cy5 azide, with the reaction being monitored by anion-exchange chromatography (Figure S3). After 4 h, the reaction proceeded to completion. To further investigate whether modified primers were incorporated into the DNA, branched primers were modified with azide-functionalized biotin.[21] In addition to

staining with SYBR Green I, the formation of biotinylated DNA networks was verified through a colorimetric reaction, in which streptavidin-conjugated alkaline phosphatase was bound to the surface, followed by incubating with a suitable chromogen. Color development indicated the presence of biotin in the DNA network, and showed that the biotin moieties are still accessible by proteins (Figure 2C).

Encouraged by these results, we envisioned the generation of DNA networks that harbor the c(RGDfK) moiety as a specific cell bait. We synthesized **2** and conjugated it to the branched DNA primers through CuAAC, based on the procedure used for Cy5 azide. Finally, these primers were used to build up DNA networks which served as cell substrates on solid support.

Next, we investigated the response of cells to the DNA-modified surfaces. HeLa cells and mouse embryonic fibroblasts (MEF) were chosen since they are known to bind to the RGD peptide motif.[19] This is accomplished by different receptors, although the αvβ3 integrin appears to play the major role.[22] In order to probe the specificity of our approach, we also investigated HEK 293T cells, which are reported not to interact with the RGD motif.[23]

To present either unmodified or **2**-functionalized DNA networks to the cells, glass slides were upgraded with walling to hold the medium, into which the cells were seeded and allowed to adhere. Loose cells were removed by washing with PBS buffer, before assessing the cell numbers by phase-contrast microscopy. Subsequent staining with SYBR green I confirmed the quality of the DNA networks on the basis of the observed green fluorescence.

In all cases, only few cells attached to the DNA-network-based hydrogels devoid of the bait peptide motif c(RGDfK). 11 ± 4 HeLa cells, 43 ± 5 MEF cells, and 45 ± 31 HEK cells were counted per mm^2 (Figure 3A,B, mean $\pm$ SEM, $n = 4$). This means that the unmodified DNA-network-based hydrogels have cell-repellent properties, as shown by the fact that cell adhesion, which readily occurs on glass supports devoid of the DNA layer (Figure S4), was prevented. This feature is advantageous since unspecific cell binding is decreased to a minimum and should thus have only a minor impact on binding through specific interactions.

On **2**-modified DNA networks, cell densities of 734 ± 162 HeLa cells per mm^2 and 915 ± 121 MEF cells per mm^2 were observed. In comparison to the unmodified DNA networks, adhesion increased 67-fold for the HeLa cells and 21-fold for the MEF cells. Additionally, sustained cell attachment and a healthy state of the cells was confirmed after 2 days. HEK cells showed a minor improvement in adhesion to the modified DNA networks when compared to the unmodified networks. However, the total cell numbers stayed comparably low, with a twofold increase to 92 cells per mm^2. This result is consistent with previously reported data indicating that cell adhesion is mediated by the binding of RGD to integrins that show a high affinity towards this peptide, such as the αvβ3 integrin. The integrins of HEK cells and their expression levels, however, lead only to weak binding to the network.[24]

In addition to capturing cells, mild release of the cells by harnessing DNA-cleaving enzymes should be feasible and was investigated. Cultures containing HeLa and MEF cells

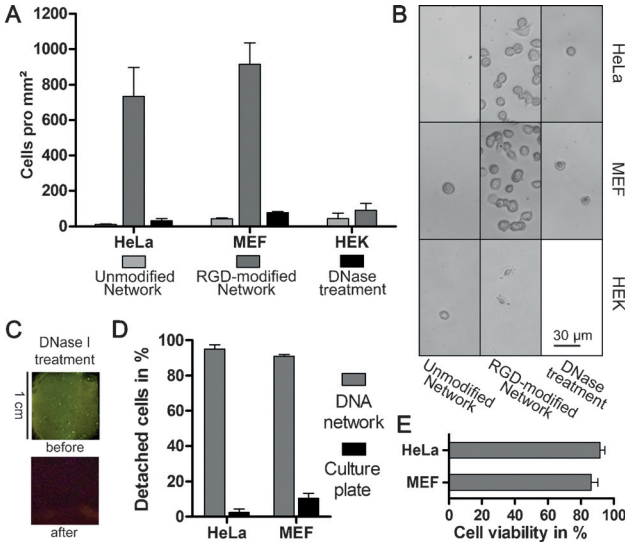

Figure 3. A) Cell attachment on the DNA networks. Hela, MEF, and HEK cells were incubated on unmodified and **2**-modified DNA networks. Unattached cells were washed with PBS buffer and the cell density was assessed by phase-contrast microscopy. On unmodified DNA, low cell density was determined for all three cell types: 11, 43, and 45 cells per mm^2 for Hela, MEF, and HEK cells, respectively. The cell density on **2**-modified DNA was 734, 915, and 92 cells per mm^2, respectively, showing a high increase for MEF and HeLa cells. DNase I treatment decreased Hela and MEF cell density to 32 and 79 cells per mm^2, respectively. $n = 4$, mean $\pm$ SEM. B) Representative images of adhered cells on DNA networks. Phase-contrast images of HeLa, MEF, and HEK cells adhered on unmodified and **2**-modified DNA networks, showing the approximate cell density. C) SYBR Green I staining of DNA before and after DNase I treatment. D) Cell detachment after DNase I treatment, $n = 4$, mean $\pm$ SEM. E) Cell viability of cells treated with DNase I, $n = 4$, mean $\pm$ SEM.

bound to the DNA networks were therefore subjected to DNase I. Cleavage of the DNA networks upon DNase I treatment was confirmed by SYBR Green I staining of the DNA-modified surfaces prior to and after DNase I treatment (Figure 3C). Cells readily detached to a high degree: after incubation for 10 min, 32 ± 13 HeLa cells per mm^2 and 79 ± 6 MEF cells per mm^2 remained, which indicates the detachment of about 95 ± 3% of the HeLa cells and 91 ± 1% of the MEF cells. In contrast, only 2.4 ± 1.9% of the HeLa and 11 ± 3% of the MEF cells detached from standard cell culture plates (Figure 3D). These findings support the idea that the cell release is mediated by DNA digestion. Furthermore, detached cells from the DNA-modified glass slides could be successfully transferred back onto cell culture plates, where they continued proliferating after initial adhesion. Trypan blue cell assays indicated cell viability of 92 ± 5% for the HeLa cells and 86 ± 7% for the MEF cells with the applied DNase I concentrations and incubation times (Figure 3E). The tolerance of cells towards DNase I renders it a suitable reagent for the mild release of cells, and it has also been employed by others for similar cases.[15,25]

The production of artificial ECM has become an attractive research field in the past few decades.[26] ECMs can be classified based on different characteristics, for example, according to their building blocks. These can be derived from

natural tissue, like collagen, or be synthetic, like polyethylene glycol (PEG).[26] The approach we developed connects the two cases. Although the building blocks (DNA) are derived from natural sources, they were assembled in a synthetic manner. Obviously, synthetic scaffolds lack standard motifs as "bait" to attract cells. We addressed this shortcoming by enabling the possibility to attach bait molecules through bioorthogonal chemistry. The nature and concentration of displayed bait molecules can be controlled to address different types of cells. Furthermore, a defined conjugation of our material with the bait gives lower batch-to-batch variation than that found in protein-mixture extracts like Matrigel.[27] Besides cell-binding motifs, the mechanical properties of the scaffold have also an impact on the cells. For example, it has been shown that a soft support favors neurogenic over osteogenic differentiation.[28] In other cases, porous materials can be used for controlled drug release or 3D cell culture systems for improved cell interactions.[29]

Unfortunately, the porosity and material preparation do not allow any cell encapsulation yet, although the mesh size and character of the DNA networks lead to a soft and flexible material. In contrast to DNA-based approaches that are based on specific DNA–DNA interactions,[12–16] the herein descripted approach does not require sophisticated sequence design in order to avoid unwanted interactions with the DNA scaffold through hybridization.

In summary, we report on a new DNA-network-based hydrogel that is immobilized on a solid support by enzymatically elongating and linking branched primer molecules. The results of cell culture experiments show on the one hand that the DNA networks have cell-repellent properties and thus show promise as a viable coating for solid supports to reduce unspecific cell attachment. On the other hand, we demonstrate that by modifying the DNA-network-based hydrogels with bait molecules, specific cell attachment through specific interactions with desired cell-surface markers is enabled. Furthermore, these cells can be efficiently detached afterwards by DNase I treatment for further use since they remain viable.

Although at the current stage, our approach is less attractive for bulk production as followed for PEG-based materials,[30] it might well be suited for smaller, target-oriented applications. A wide range of issues in cellular studies might be addressed by employing this DNA-based material, which can be equipped with different kinds of bioactive molecules. In addition to bait molecules for selective cell adhesion, the material could, for example, be equipped with covalently linked growth factors in order to improve proliferation or induce the differentiation of attached cells.[31]

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