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Quantitative Analysis of EDC-Condensed DNA on Vertically Aligned Carbon Nanofiber Gene Delivery Arrays

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ABSTRACT: Vertically aligned carbon nanofibers (VACNFs) with immobilized DNA have been developed as a novel tool for direct physical introduction and expression of exogenous genes in mammalian cells. Immobilization of DNA base amines to the carboxylic acids on nanofibers can influence the accessibility and transcriptional activity of the DNA template, making it necessary to determine the number of accessible gene copies on nanofiber arrays. Polymerase chain reaction (PCR) and in vitro transcription (IVT) were used to investigate the transcriptional accessibility of DNA tethered to VACNFs by correlating the yields of both IVT and PCR to that of non-tethered, free DNA. Yields of the promoter region and promoter/gene region of bound DNA plasmid were high. Amplification using primers designed to cover 80% of the plasmid failed to yield any product. These results are consistent with tethered, longer DNA sequences having a higher probability of interfering with the activity of DNA and RNA polymerases. Quantitative PCR (qPCR) was used to quantify the number of accessible gene copies tethered to nanofiber arrays. Copy numbers of promoters and reporter genes were quantified and compared to non-tethered DNA controls. In subsequent reactions of the same nanofiber arrays, DNA yields decreased dramatically in the non-tethered control, while the majority of tethered DNA was retained on the arrays. This decrease could be explained by the presence of DNA which is non-tethered to all samples and released during the assay. This investigation shows the applicability of these methods for monitoring DNA immobilization techniques.

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KEYWORDS: nanofibers; DNA; quantitative PCR; EDC; nanotechnology; impalefection

Introduction

There has recently been a heightened interest in the integration of synthetic, nanostructured materials with biological systems. Use of such materials to deliver tethered, transcriptionally active DNA into mammalian cells has enabled novel approaches to genetic manipulation. This includes nuclear targeting of delivered DNA via heterofunctional metallic nanorods (Salem et al., 2003) and controlled, non-segregated transgene expression from arrays of nuclear-penetrant, vertically aligned carbon nanofiber (VACNF) arrays (McKnight et al., 2003, 2004). This latter method, termed *parallel impalefection*, is a technique similar to microinjection, differing in that it can be conducted on a highly parallel basis to manipulate many cells simultaneously. VACNFs with micron lengths and less than 100 nm diameters are arrayed in parallel normal to the surface of a solid substrate. Pressing this array into a cell or tissue matrix results in cellular penetration and "microinjection" of surface-bound material into a large number of target cells. Due to the nanoscale diameter of VACNFs, the plasma

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membranes of mammalian cells can recover following fiber penetration which enables the proliferation of the interfaced cell (McKnight et al., 2003). Often, impalefection appears to result in direct nuclear delivery, particularly for suspended or unattached mammalian cells with large nuclear cross-sections as compared to the overall cross sectional area of the cell.

Although DNA has been successfully delivered to mammalian cells using VACNFs, the details of this process are still being investigated. One element of this process that has not been previously addressed is a quantitative determination of the amount of DNA immobilized on the nanofiber platform. In previous work, plasmid DNA was tethered to impalefection arrays using a carbodiimide mediated condensation reaction between DNA base amines and carboxylic acid sites on nanofibers (McKnight et al., 2003, 2004). This approach has been effectively used for DNA immobilization onto a variety of substrates, including polymers (Taira and Yokoyama, 2004), microwells (Rasmussen et al., 1991), glass slides (Zammatteo et al., 2000), glass beads (Walsh et al., 2001), gold electrodes (Ge et al., 2003), carbon electrodes (Millan et al., 1992), carbon nanotubes (Dwyer et al., 2002; Nguyen et al., 2002), and carbon nanofibers (McKnight et al., 2003). Millan demonstrated that when using 1,1-ethyldiaminopropylcarbodiimide (EDC) for DNA immobilization to carbon, the amide bond is formed specifically at exposed guanine and cytosine amines (Millan et al., 1992). Since DNA bound to carboxyl groups at these binding sites cannot be effectively directed, binding may occur at undesirable locations and might render the tethered DNA template transcriptionally inactive.

Covalent binding within or near the promoter region may also limit or eliminate transcription initiation due to steric hindrance of polymerase and/or transcription factor binding. In the eukaryotic cell, transcription initiation is similarly impacted by steric hindrances imposed by ionic interactions between histones and DNA template, with, for example, acetylation of lysine residues of these chromatin proteins being a significant regulatory mechanism of gene expression (Latchman, 2004). Binding within the coding sequence may also interfere with reading and complementation of the base during mRNA elongation. Schaffer et al. (2000) found that the disassociation of non-covalent interactions between polycations and DNA was required for efficient expression of the DNA by transcriptional machinery. In either case, the transcriptional activity of template can be significantly reduced, if not completely hindered by the interaction of the template with large molecules. Therefore, tethered gene strategies, where solid scaffolding can present even larger hindrances, may present an additional variable with respect to evaluation of efficiency (i.e., transgene expression per unit DNA). Having knowledge of the amount of *transcriptionally active* DNA that is successfully being delivered to cells will be helpful for future studies where the number of gene copies per cell will play a significant role. In this manuscript, using impalefection-based nanofiber arrays as our substrate, we explore cell-free methods to obtain a first order approximation of the accessibility and transcriptional activity of immobilized DNA template by correlating the yields of PCR, quantitative PCR, and in vitro transcription (IVT) against that of unbound, free DNA.

Materials and Methods

Synthesis of VACNF Arrays

Arrays of VACNFs were fabricated as previously described (Melechko et al., 2003). In brief, 100 mm silicon wafers (n-type, <100>) were spun with photoresist (SPR 955 CM 0.7) and patterned with 500 nm diameter holes on a 2.5 μm pitch using projection photolithography (GCA Autostep 200) and development (Microposit, CD26). A 30 s reactive ion etch (RIE) (Trion Oracle, 150 torr, 50 sccm oxygen, and 150 W of power) in oxygen plasma was used to remove residual resist from the developed regions. A Ni layer (500 Å) was deposited onto a wafer using electron-gun evaporation at 10^{-6} torr. The excess metallization and photoresist was lifted off using a 1 h soak in acetone, followed by rinsing in a spray of acetone, followed by 2-propanol. VACNFs were then grown from the patterned Ni dots using dc catalytic plasma enhanced chemical vapor deposition (C-PECVD) (Melechko et al., 2003). Typically, nanofibers were grown to a length of approximately 7 μm with a conical shape featuring a tip diameter of <100 nm and a base diameter of 200–300 nm. Following synthesis, wafers were coated with a protective layer of photoresist (SPR 220 CM 7.0) and cut with a dicing saw into sized pieces. Prior to use, each piece was cleaned of the protective photoresist with a 30 min soak in acetone, followed by rinse in acetone, 2-propanol, and water.

Covalent Attachment of DNA to Carboxylated Microspheres and VACNF Substrate

The DNA vector pd2EYFP-N1 (BD Biosciences, San Jose, CA) was transformed into DH5α *E. coli* cells and isolated using the Wizard Plus Miniprep Kit (Promega, Madison, WI). DNA was covalently attached to carboxylated beads using 100 ng of pd2EYFP-N1 vector (BD Biosciences) placed in 200 uL of 100mM 2-(N-morpholino) ethane sulfonic acid buffer (MES; pH 4.7) containing 1 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). One micrometer polybead carboxylate microspheres (Polysciences, Inc., Warrington, PA) were added at a final concentration of 2.28×10^4 particles mL^{-1}. For VACNFs, 100 ng of pd2EYFP-N1 were placed in 200 uL of 100 mM 2-(N-morpholino) ethane sulfonic acid buffer (MES) (pH 4.7) containing 1 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) along with one carbon nanofiber chip (2 mm × 2 mm dimensions). The carboxylate microspheres and nanofiber chips were incubated for 18 h at 25°C on an orbital shaker overnight to condense primary amines of the

DNA to the carboxylic acid sites of the solid substrates (Dwyer et al., 2002; McKnight et al., 2003). The carboxylate microspheres and nanofiber chips were then incubated in 1 mL of HPLC grade sterile H_2O for 15 min followed by vortexing. This washing step was repeated in 1 mL of 2 M NaCl in PBS and once again in 1 mL of HPLC grade sterile H_2O. This stepwise wash procedure was elected due to heterogeneity of the silicon and VACNF surfaces. This heterogeneity presents difficulty with effectively removing non-specifically bound DNA during washing. Oxidized silicon behaves as conventional glass or glass-milk, requiring low salt solutions for DNA elution. The heterogeneous charge and high surface area of carbon nanofibers appears to present both ionic as well as physical adsorption. Thus, conventional approaches at optimizing salt concentrations are confounded. Our methods were tested using three different salt concentrations, and a sequential sterile H_2O/2 M NaCl/sterile H_2O was selected for this study. For carboxylate microspheres, the samples were centrifuged after each wash in 1.5 mL microcentrifuge tubes (Eppendorf North America, Westbury, NY) for 2 min. at 16,000*g* in order to remove the microspheres from suspension. Carboxylate microspheres and carbon nanofiber chips were also prepared in the absence of EDC as a negative control. Sham silicon chips, without nanofibers, were also prepared with and without EDC as additional negative controls. These silicon chips without fibers result on each wafer in areas without Ni catalyst sites, as defined by the pregrowth Ni photolithographic patterning step. Without Ni catalyst, these regions are unpopulated with nanofibers, but remain otherwise exposed to all other processing steps, including high temperature plasma, photoresist protection, and acetone stripping.

Primer and Probe Design for PCR and Quantitative PCR

Three sets of primers (Sigma Genosys, St. Louis, MO) were constructed specifically for *qualitative* determination of amplifiable regions of the pd2EYFP-N1 vector when tethered to the carboxylate microspheres or carbon nanofiber chips (Table I). This 4.9 kb plasmid contains a CMV promoter upstream of the enhanced yellow fluorescent protein gene (eYFP). The forward primer (YFP F1) was identical for all three primer sets. The three reverse primers (YFP R1, YFP R2, YFP R3) were made to amplify (1) the CMV promoter region (amplicon = 648 bp), (2) the CMV promoter and eYFP gene (amplicon = 1,603 bp), and (3) a large fragment containing over 80% of the plasmid length (amplicon = 4,053 bp). Additionally, two primer and probe sets (Biosearch Technologies, Novato, CA) were designed for *quantitative* determination of the pd2EYFP-N1 vector when tethered to the carbon nanofiber chips. The first primer and probe set (YFP FQ, YFP RQ, YFP Probe1) amplifies a 72 bp region internal to the CMV promoter upstream of the EYFP gene. The second primer and probe set (YFP FV, YFP RV, YFP Probe2) amplifies a 97 bp region of the SV40 promoter, 200 bp upstream of the kanamycin resistance gene, and 938 bp downstream of the CMV promoter and EYFP gene.

PCR Amplification of pd2EYFP-N1 Covalently-Bound to Carboxylate Microspheres and VACNFs

The PCR setup for the three amplicons was optimized by using temperature gradient protocols (45–60°C), different concentrations of primers and template DNA, as well as the Invitrogen PCR Optimization Kit. The final mix of each PCR tube contained one Ready-To-Go PCR Bead (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), 400 nM forward primer, 400 nM reverse primer, and HPLC grade sterile H_2O. For PCR amplification, the PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA) was used. The optimal annealing temperature for all primer sets was 56°C. Negative controls in the presence and absence of EDC were performed in duplicate via PCR amplification of the 648 bp CMV promoter region using chips selected from regions of the silicon growth wafer that were unpopulated with carbon nanofibers. Negative control chips generated no

Table I. Primers and probes used in this study.

Name	Sequence	Area of amplification	Amplicon size
YFP F1	5′-CCT GAT TCT GTG GAT AAC CGT AT-3′	CMV promoter	648 bp
YFP R1	5′-ATC TGA GTC CGG TAG CGC TA-3′		
YFP F1	5′-CCT GAT TCT GTG GAT AAC CGT AT-3′	CMV promoter and eYFP gene	1,603 bp
YFP R2	5′-AAA TGT GGT ATG GCT GAT TAT GAT C-3′		
YFP F1	5′-CCT GAT TCT GTG GAT AAC CGT AT-3′	80% of plasmid length	4,053 bp
YFP R3	5′-TAT ATA TGA GTA ACC TGA GGC TAT G-3′		
YFP FQ	5′-CAC CAA AAT CAA CG-3′	Internal to CMV promoter	72 bp
YFP RQ	5′-ACG CCT ACC GCC CAT TT-3′		
YFP Probe1	5′-6-FAM d(AAT GTC GTA ACA ACT CCG CCC CA)BHQ-1-3′		
YFP FV	5′-CAA TTA GTC AGC AAC CAG G TG TG-3′	Internal to SV40 promoter	97 bp
YFP RV	5′-CGG GAC TAT GGT TGC TGA CTA A-3′		
YFP Probe2	5′-6-FAM d(CAG GCT CCC CAG CAG GCA GAA GTA T)BHQ-1-3′		

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detectable bands, thereby indicating that DNA binding is occurring to nanofiber sites and not the underlying Si substrate (data not shown). For positive controls of pd2EYFP-N1, 10 ng of the vector was added to each reaction tube. For amplification of pd2EYFP-N1 on carboxylate microspheres, 5 μL of the 2.28×10^4 particles mL^{-1} microsphere suspension were added to the reaction tube. For amplification of pd2EYFP-N1 on a carbon nanofiber chip, the chip was placed directly into the reaction tube containing the Ready-To-Go PCR Bead, primers and HPLC grade sterile H_2O. The final volume for all reaction tubes was 25 uL. For gel electrophoresis, a 1% agarose gel of molecular biology grade agarose (Fisher Scientific Company LLC, Pittsburgh, PA) was run at 70 V for 45 min. For the DNA ladder mix, 1 KB+ Ladder (Invitrogen, Carlsbad, CA) was mixed with HPLC grade sterile H_2O and 5X bromophenol blue.

IVT of pd2EYPF-N1 Covalently Tethered to VACNFs

IVT was performed using the HeLa Scribe Kit (Promega), following the protocol from the manufacturer. Briefly, each nanofiber chip was added to 10 mM rNTP mix, 50 mM $MgCl_2$, 20 mM HEPES (pH 7.9 at 25°C), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, and HeLa nuclear extract and incubated for 1 h at 30°C. After the reaction was terminated by the addition of 0.3 M Tris-HCl (pH 7.4 at 25°C), 0.3M sodium acetate, 0.5% SDS, 2 mM EDTA, and 3 μg/mL tRNA, the generated RNA was isolated using phenol chloroform extraction as described in the HeLa Scribe Kit. Samples were stored at −80°C. Quantification of RNA in each sample was determined using the Ribogreen RNA Quantitation Kit (Invitrogen). Samples were measured in triplicate on the VersaFluor Fluorometer (BioRad Laboratories, Hercules, CA) using VersaFluor Cuvettes (BioRad).

Quantitative PCR of pd2EYFP-N1 on VACNFs

DNA was immobilized on the VACNFs as described above. Once pd2EYFP-N1 had been tethered to the carbon nanofiber arrays, the YFP F1 and YFP R2 primer set was used to amplify the CMV promoter and EYFP gene for three cycles of PCR. Volumes containing carbon nanofiber arrays cannot be measured directly in quantitative PCR reactions due to the opacity of the nanofiber substrate. This initial step allowed amplification of pd2EYFP-N1 that was present on the carbon nanofibers to be amplified into solution, such that the chip could be removed for subsequent q-PCR. Primers designed to amplify the CMV promoter and EYFP gene region were used for these initial PCR cycles because the promoter and gene region are what need to remain accessible to the cellular machinery following impalefection. After the initial three rounds of PCR, the nanofiber chips were removed from the tube and a 5 μL aliquot of the PCR solution was then added to the quantitative PCR mix in place of the template DNA. The quantitative PCR mix consisted of 400 nM of each primer (YFP FQ, YFP RQ), 600 nM of probe (YFP Probe1), and 1X Taq PCR Master Mix (Qiagen, Valencia, CA). The pd2eYFP-N1 plasmid was run as a standard in known amounts using 10-fold dilutions to generate a standard curve. Positive controls of known amounts of template were run against the standard curve to verify the accuracy of this technique. Quantitative real-time PCR assays were performed on the DNA Engine Opticon Real-Time PCR Detection System (BioRad).

Results

PCR Amplification on VACNFs

PCR was used to determine the presence or absence as well as the accessibility of DNA on the nanofiber arrays after extensive washing steps. Figure 1a shows that the CMV promoter region of pd2EYFP-N1 can be amplified when the DNA vector is putatively covalently tethered to nanofibers (lane 6). Light bands can also be seen in the lane 5, where no EDC was present for covalent attachment, indicating retention of non-specifically adsorbed DNA on the nanofibers even after washing. In Figure 1b, it is shown that the larger region of the CMV promoter and EYFP gene sequence of pd2EYFP-N1 is also accessible for amplification after EDC condensation and extensive washing of the nanofiber chip (lane 6). It is worth noting that the nanofiber chips incubated with DNA in the absence of EDC showed undetectable levels of amplifiable CMV promoter and EYFP gene (lane 5). Figure 1c shows the results of using primers (YFP F1, YFP R3) for amplification of approximately 80% of the pd2EYFP-N1 vector sequence. No detectable levels of this length of the vector could be seen from the nanofiber arrays with DNA incubated in the presence or absence of EDC (lanes 5 and 6). Similar results were observed with the carboxylated microspheres using all three primer sets in Figure 1a, b, and c (lanes 3 and 4).

IVT on VACNFs

Although the DNA immobilized on carbon nanofibers is available for amplification, this does not mean that the DNA sequence of interest is also available for transcription in the cell. To evaluate the transcriptional efficiency of pd2EYFP-N1 covalently bound to the nanofiber arrays, IVT was performed. Table II shows a comparison of IVT of pd2EYFP-N1 in solution and on nanofibers in the presence or absence of EDC. There was no significant difference between the transcription of RNA from nanofiber arrays bound with pd2EYFP-N1 in the presence of EDC (135 ± 44 μg/μL) and the transcription of RNA from pd2EYFP-N1 in solution (177 ± 40 μg/μL). Conversely, the transcriptional levels of RNA from pd2EYFP-N1 on nanofibers bound in the absence of EDC were significantly lower (70 ± 4 μg/μL). These data are consistent with the results from the PCR assays. Likewise, these results confirm

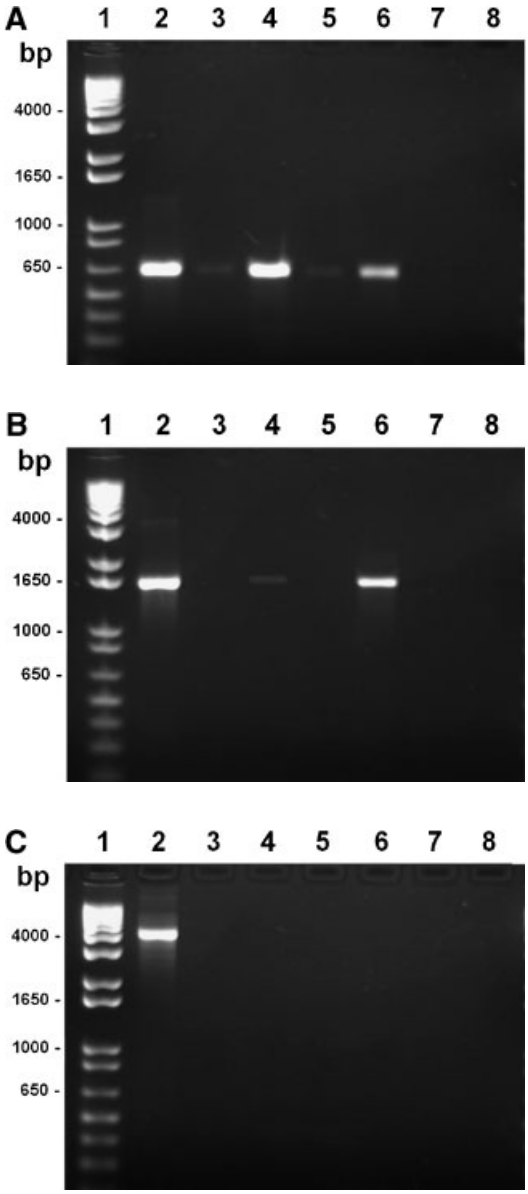

Figure 1. Amplified regions of pd2EYFP-N1 on both carbon nanofiber arrays and carboxylated latex beads using different primer sets. Amplification of (**A**) the CMV promoter region (648 bp), (**B**) the CMV promoter and EYFP gene region (1,603 bp), and (**C**) 80% of the vector length (4,051 bp) of pd2EYFP-N1. Lanes are identical for each PCR gel. **Lanes**: (**1**) 1 KB+ Ladder (Invitrogen), (**2**) 10 ng of non-tethered DNA, (**3**) carboxylated microspheres without EDC, (**4**) carboxylated microspheres with EDC, (**5**) carbon nanofiber chip without EDC, (**6**) carbon nanofiber chip with EDC, (**7**) non-tethered DNA without primers, (**8**) primers without DNA.

Table II. Quantification of RNA after in vitro transcription of pd2EYFP-N1 on VACNF arrays.

Sample	RNA (μg/μL)	Std. Dev.
Positive control (no nanofibers)	176.7	± 40.3
Nanofibers with EDC	134.8	± 44.2
Nanofibers without EDC	69.9	± 4.3

previously reported data that tethered DNA is available to the transcriptional machinery of mammalian cells and can be expressed by these cells when introduced by means of impalefection (McKnight et al., 2003, 2004).

Quantitative PCR Amplification on VACNFs

Figure 1 indicates that pd2EYFP-N1 is covalently tethered to the carbon nanofiber arrays when incubated in the presence of EDC. However, similar to a previous examination of the accessibility of immobilized DNA using polymerase chain reaction, the amount of immobilized DNA cannot be accurately estimated via conventional PCR (Bulyk et al., 1999). These data only provide a qualitative indication of DNA present on the nanofibers. In order to assess how much DNA (e.g., copies of accessible CMV promoter and EYFP genes) remains on the nanofibers, we used quantitative PCR methods coupled to an initial three cycles of PCR as illustrated in Figure 2 and described in the methods. These results are presented in Figure 3a. Amplification yields of the CMV promoter and EYFP gene region of tethered DNA were quantified and compared to controls which were incubated in the absence of EDC. During the first round of quantification, approximately 1.6×10^9 gene copies were amplified from each nanofiber chip in the presence of EDC, while 7.9×10^8 gene copies were amplified from nanofiber chips in the absence of EDC. In subsequent reactions of the same nanofiber chips, DNA yields decreased dramatically (1.2×10^6 gene copies) on chips without EDC, while chips incubated in the presence of EDC retained DNA (5.6×10^8 gene copies) up to 10 days and 3 qPCR reaction cycles after initial covalent attachment. The subsequent decrease in gene copies from these samples suggests that non-tethered DNA can remain non-specifically adsorbed to the nanofibers during washing steps, but can be removed during thermal cycling.

The results from Figure 3a also suggest that some portion of the pd2EYFP-N1 measured was the result of unbound plasmid, not amplification product. Therefore, a set of primers and probe were designed which bound external to the CMV promoter and EYFP gene region that was being amplified during the three initial PCR cycles illustrated in Figure 2. This method was used to determine if the DNA measurements from Figure 3a were in fact products of the preliminary PCR amplification, or of pd2EYFP-N1 plasmid released from the nanofibers during the reaction. After these initial PCR cycles, this primer and probe set (YFP FV, YFP RV, YFP Probe2) were used for Taqman assays of real-time quantitative PCR on the same samples used in Figure 3a. The results are shown in Figure 3b. During the first reaction, there were high levels of DNA copies for both samples (EDC and no EDC). These copies of DNA cannot be attributed to the first three PCR cycles, because the primer and probe set used bind to a region of the vector sequence found outside the CMV promoter and EYFP gene region that were initially amplified. These data would imply that those copies of DNA being quantified are equivalent to the number of

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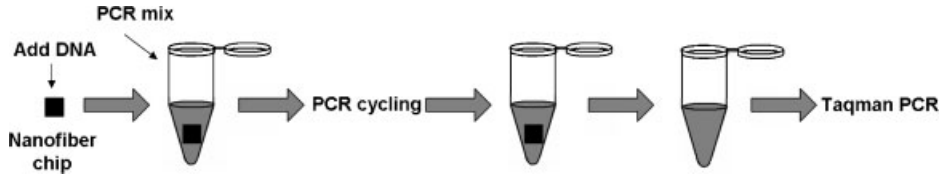

Figure 2. Illustration of the methods used for quantification of accessible DNA bound to the nanofiber arrays. Detailed steps are explained above in the methods.

pd2EYFP-N1 vectors being entirely released from the nanofiber arrays during the first three cycles of PCR. These copies of the vector were not removed during the extensive washing steps. The subsequent data from reaction 3 are consistent with the data in Figure 3a, showing that no significant amounts of DNA vector were amplified outside of the CMV promoter and EYFP gene region for either sample (EDC or no EDC), suggesting that the majority of DNA being amplified in reaction 3 remained covalently tethered to the nanofiber arrays.

Discussion

Nanofibers have been previously used for DNA delivery into Chinese Hamster Ovary cells (McKnight et al., 2003, 2004). Penetration and residence of DNA-modified nanofibers within the nucleus offers numerous possibilities for both gene delivery applications and the fundamental study of gene expression and transcriptional phenomenon. For example, delivery of nanofiber-tethered DNA into a cell can offer a higher level of control over the fate of introduced genes, including the potential to remove these genes from a system after a period of transient expression simply by removing the cells from the nanofiber array. Nuclear delivery of tethered DNA on a parallel basis may also provide more efficient methods for studying the impact of template length and topology on transcriptional activity, which has traditionally been investigated through application of the serial method of microinjection (Harland et al., 1983; Krebs and Dunaway, 1996; Weintraub et al., 1986). Similarly, nuclear-penetrant nanofiber arrays might be used for in-cell transcriptional assays based on immobilized-template methods that have been developed for the study of IVT

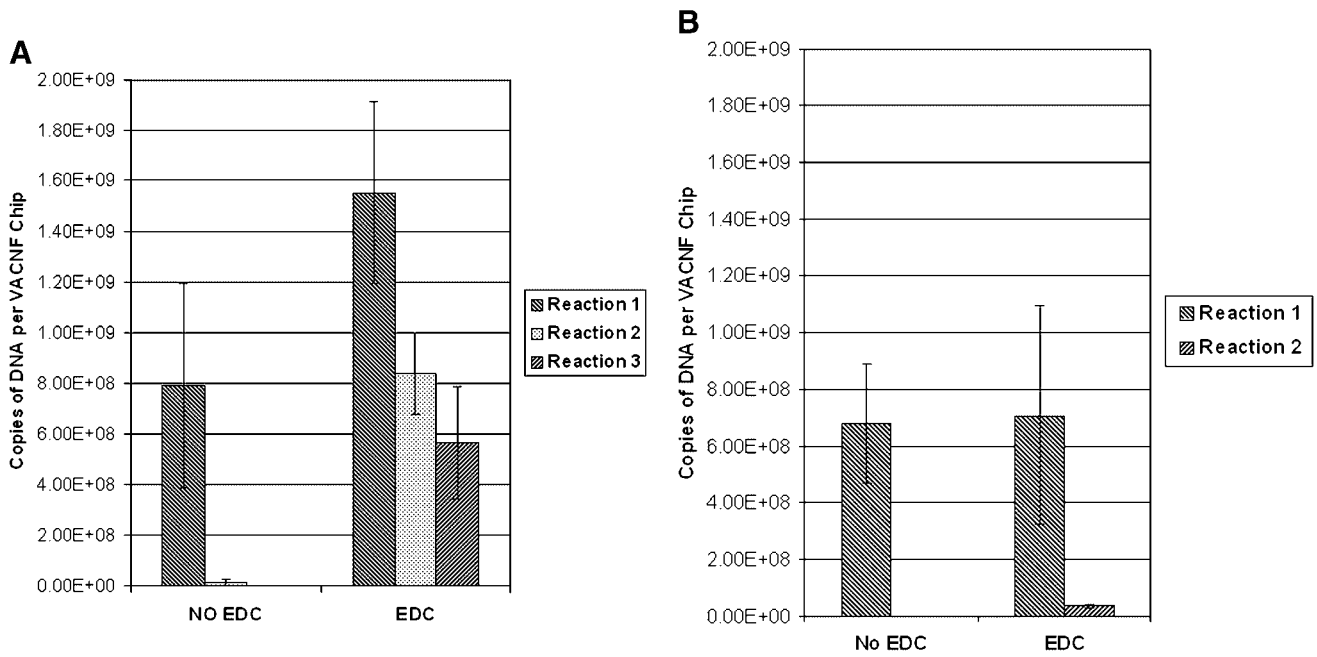

Figure 3. Quantitative PCR of pd2EYFP-N1 on carbon nanofibers. These assays were used to determine how much DNA remains on the carbon nanofibers over time in the presence or absence of EDC. The subsequent decrease in gene copies from carbon nanofibers incubated in the absence of EDC suggests that this DNA remained non-specifically adsorbed to the nanofibers after the wash steps and was removed during thermal cycling. **A**: Shows the number of EYFP gene copies amplified from the nanofiber using the internal primer and probe set (YFP FQ, YFP RQ and YFP Probe1) and (**B**) shows the number of DNA copies released from the nanofiber arrays into solution and amplified using the external primer and probe set (YFP FV, YFP RV, and YFP Probe 2).

using nuclear extracts (Adamson et al., 2003), thereby providing new levels of insight into the fundamental processes of transcription and transcriptional regulation.

In prior work, DNA was typically bound to the nanofiber arrays using EDC condensation. While this method for DNA attachment is rapid and simple, it is also relatively random in where it will attach the amine groups of the individual DNA strand to the carboxyl groups available on the carbon nanofiber. This study shows that simple molecular techniques such as PCR, IVT, and quantitative PCR can be used to efficiently evaluate EDC condensation and other DNA immobilization methods on nanoscale substrates. The PCR results provided a qualitative evaluation of how accessible the DNA remains after condensation. The IVT results were consistent with PCR, showing that after extensive washing, high levels of DNA remained accessible and could be utilized by both DNA and RNA polymerase. The results of the quantitative PCR showed that in the presence of EDC, large quantities of accessible DNA remained tethered to the VACNF arrays for at least 10 days and through three subsequent qPCR thermal cycling sessions. While 5.6×10^8 gene copies per chip is only a fraction of the DNA that was originally attached (approximately 2.98 ng of the initial 100 ng), it is still a substantial amount. For example, the VACNF array samples used in this study contained approximately 1 million fibers. Therefore every fiber likely contained more than 500 accessible gene copies.

The PCR results in Figure 1 show that amplification decreases as the amplicon becomes larger. While tethered pd2EYPF-N1 vector on these nanofiber chips is evidenced by the yields of shorter amplicons, long stretches of DNA cannot be amplified, indicating that they are not accessible to the polymerase enzyme during PCR. It is possible that this is due to steric hindrance or stalling of bound RNA and DNA polymerases. Stalling of RNA polymerase II can occur in vivo during transcription due to DNA lesions (Yu et al., 2003) or nucleotide-specific binding of proteins (Sunstrom et al., 1992). Likewise, DNA polymerase has been shown to stall during DNA amplification due to stable secondary structures from base repeats (Krasilnikova et al., 1998) or bulky DNA lesions (Yan et al., 2004). Although no studies have examined the integrity of DNA for polymerization or transcription following covalent immobilization on a substrate, it can be assumed that stalling of the polymerase enzyme does occur under these circumstances. This explanation would be consistent with the results of Figure 1, where amplification of the DNA template decreased as the amplicon became larger. As the amplicon increases in size and more nucleotide bases are required during elongation, there is a higher probability that these base amines will be randomly immobilized to the available carboxyl groups on the VACNFs due to stochastic fluctuations in the helical structure of the DNA. Figure 4 illustrates various binding modalities of DNA with VACNFs. In Figure 4a, the template DNA is initially tethered to the nanofiber, but will be removed during the washing steps or subsequent experimentation. Figure 4b illustrates DNA template that is tethered to the nanofiber, but the gene region is not fully accessible for primer annealing or polymerase binding. In this case, fragments of the gene may have base amines bound to carboxyl sites of the carbon nanofiber, restricting access to the DNA sequence. Large stretches of DNA (i.e., the large 80% plasmid amplicon of pd2EYFP-N1) may not be amplified or transcribed due to the limited binding to the nanofiber that is required for these sequences to still be accessible. Figure 4c shows the desired scenario, when DNA is covalently bound to the nanofiber in multiple locations, giving it resilience during the experimentation, while retaining an available gene sequence that can be accessed by the cellular machinery.

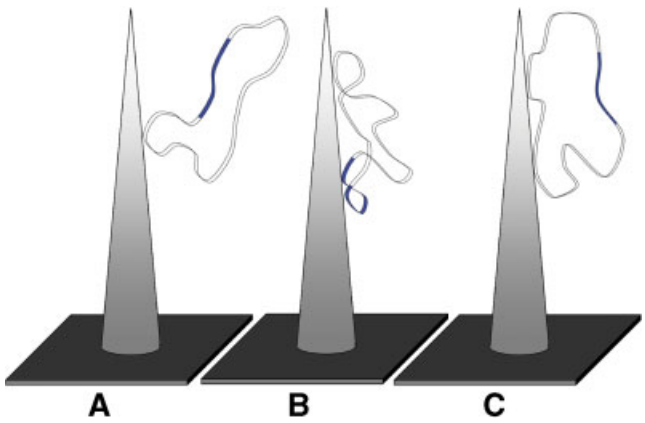

Figure 4. Illustration of potential immobilization of DNA on a vertically aligned carbon nanofiber. **A**: The template DNA is weakly tethered to the nanofiber at only one or two binding sites, and will be removed during the washing steps or subsequent experimentation. **B**: The DNA is tethered to the nanofiber, but the gene region is not fully accessible for primer annealing or polymerase binding. **C**: This is the desired scenario, where DNA template is covalently bound to the nanofiber in multiple locations, giving it resilience during the experimentation, while retaining an accessible gene sequence. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

The results of the real-time quantitative PCR show that over a period of 10 days and 3 subsequent thermal cycling sessions, there were three orders of magnitude more DNA copies remaining on the nanofiber chips in the presence of EDC compared to the nanofiber chips incubated without EDC. This is consistent with the qualitative data from the PCR gels, verifying that more accessible DNA is retained on the nanofibers when incubated in the presence of EDC. However, the qPCR results from Day 1 show that a large amount of DNA is initially adsorbed to the nanofiber chips without EDC, even after extensive washing. These results were not observed in Figure 1, although they have been confirmed from multiple experimental reactions (data not shown). These results could be a result of differences in salt concentrations and oligonucleotide content between the PCR and qPCR mixes, both of which have been shown to affect the binding efficiency of DNA (Castelino et al., 2005; Huang et al., 1996).

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Other DNA binding strategies are currently being evaluated, including the use of short linear DNA constructs featuring biotin, thiol, and amine terminations. Enhancing the binding strategies could result in fewer DNA molecules being needed and less DNA being released from the nanofiber arrays, as well as yielding a higher percentage of successful delivery and expression inside the cell. It is anticipated that the combination of PCR, IVT, and qPCR assays presented in this manuscript along with the evaluation of engineered linear constructs with well defined site-specific binding properties, will provide insight into steric hindrances and other interfering properties of the solid scaffolding. In addition to their evaluation with nanofiber based DNA delivery systems, it is anticipated that these same analysis methods will prove useful for other nanostructured systems used for DNA delivery.

Conclusions

These results conclusively show that DNA covalently bound to carbon nanofibers can be amplified in PCR reactions. In these experiments, the CMV promoter and EYFP gene region of tethered pd2eYFP-N1 were readily amplified, while the longer sequence consisting of 80% of the vector length could not be amplified from the carbon nanofiber arrays. The IVT assay showed that DNA bound to carbon nanofibers can also be accessed by transcriptional machinery. Although the IVT assay does not directly measure the success of nanofiber-tethered DNA transcription within the cell, it does suggest that coding regions of the DNA remain accessible to RNA polymerase and other transcriptional factors. Likewise, these results show that DNA bound to carbon nanofibers can be quantified using quantitative real-time PCR assays. The promoter and gene region of tethered pd2eYFP-N1 were easily amplified and the number of DNA copies bound to carbon nanofibers were compared in the presence or absence of EDC. These results also demonstrate that DNA can adsorb non-specifically to carbon nanofibers in the absence of EDC and can remain adsorbed even after extensive washing. However, this non-specifically adsorbed DNA is rapidly removed over time, possibly due to thermal cycling or storage conditions. These techniques provide a means of efficiently evaluating the site specific activity of tethered DNA constructs and thus provide a means for optimizing DNA attachment methods such as EDC condensation or biotin labeling, which are both currently being used in biological applications. Optimization of current protocols and investigations of other binding strategies are presently being assessed to create more efficient DNA delivery systems.

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