Development of a solid-phase padlock probe technology using microfluidics

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Abstract

The padlock probe technology is a DNA analysis tool with biological applications such at DNA analysis *in situ*, detection of single-nucleotide polymorphisms, DNA analysis in parallel and microbial detection. Briefly, the method utilizes short oligonucleotide probes, so-called padlock probes, for a highly specific dual-recognition event of a particular target sequence, followed by ligase-mediated circularization of the padlock probes. In the present work the padlock probe method, using microbial detection as a model system, has been combined with solid-phase and microfluidics to make the padlock probe hybridization and circularization procedure more efficient in terms of increased sensitivity, as well as decreased analysis time. To achieve this, the idea is to capture fragmented microbial DNA on beads trapped inside a microfluidic column, followed by padlock probe hybridization and circularization.

Keywords

Padlock probe, circularization, solid phase, microfluidics, microbial detection

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Sammanfattning

Under de senaste femtio åren har vår förståelse för hur den genetiska koden, det vill säga vårt DNA, fungerar drastiskt ökat. Man har bland annat kunnat konstatera att många sjukdomar härrör från förändringar i just den genetiska koden. Det mesta utav den kunskap vi har idag bygger på data från diverse DNA-analysverktyg, såsom sekvensering och genotypning. Dock behöver nya sådana analysverktyg ständigt utvecklas, liksom de gamla förbättras, i takt med att nya biologiska frågeställningar formuleras.

Ett DNA-analysverktyg är den så kallade Hänglåsprobteknologin, en analysmetod som bland annat kan användas för att studera enskilda nukleotidvariationer i DNA samt att fastställa närvaron av specifika bakterier i ett givet prov. Metoden ger även möjlighet att utföra flera sådana analyser parallellt i samma provrör.

Målet med detta projekt var att försöka effektivisera Hänglåsprobteknologin med avseende på bland annat kortare analystid. För att nå detta mål introducerades i det redan existerande Hänglåsprobprotokollet en fast fas i form av mikrokulor samt mikrofluidik, där mikrofluidik är en term syftande på handhavandet av vätskor i mycket små kanaler belägna i till exempel mikrochip. Som ett modellsystem för Hänglåsprobteknologin har detektion av specifika bakterier använts.

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1 Introduction

Improvement of DNA analysis tools, such as DNA sequencing and genotyping, has greatly enhanced our understanding of, for instance, genome organization and genetically related diseases. However, new analysis technologies, as well as improvement of older ones, have to be sought for as new biological questions arise, or even to be able to answer already existing ones. I would like to start by addressing some biological questions, on the DNA level, that might be relevantly asked by a molecular geneticist, as well as people in other scientific areas.

Having a genome, one might want to know whether that genome possesses a particular gene or DNA sequence, or if any single-nucleotide variations exist compared to other related genomes. It would also be rather valuable to be able to ask the same questions for individual cells instead of performing global genetic analyses, as the case is to date (1). Moreover, probing for species-specific DNA sequences could reveal important information concerning the presence of specific microbes in a given sample. Finally, detection of DNA molecules *in situ* conveys information about physical locations of the same molecules (2). This is only a selection of relevant biological questions to which new technologies can be applicable.

This report will present a DNA analysis tool, based on the padlock probe technology, which can be used to answer questions like the ones asked above. Shortly, the method utilizes short oligonucleotide probes, so-called padlock probes, for a highly specific dual-recognition event of a predefined target DNA sequence, followed by a ligase-mediated circularization reaction of the padlock probes (3). The concept of padlock probes will be further discussed in this report and some important applications of padlock probing, such as DNA analysis *in situ*, detection of single-nucleotide polymorphisms, DNA analysis in parallel and microbial detection will briefly be mentioned.

1.1 The padlock probe technology

The concept of using short oligonucletides in an assay for the identification of a given DNA sequence was first presented in 1988 (4). The method and detection procedure were then further developed and the concept as it is used today was presented in 1994 (5).

1.1.1 Padlock probe design

Padlock probes are linear oligonucleotides, typically 70-100 nucleotides in length (6), of which approximately 20 nucleotides at both 5′ and 3′ ends are designed to hybridize to a target DNA sequence in juxtaposition (7) (**Figure 1**). The sequence between the two hybridization arms is called a linking segment, commonly possessing sequences for detection and identification (1).

Figure 1. Padlock probe design. A padlock probe is a 70-100 nucleotide long linear oligonucleotide. The outermost 20, or thereabout, 5′ and 3′ nucleotides (blue) of the probe are designed to hybridize next to each other to a target DNA sequence. In the area in-between the hybridization arms (black, red and green), referred to as a linking segment, typically sequences for detection and identification are situated.

1.1.2 Padlock probing

When a padlock probe has found its target DNA, the outermost hybridization arms bind to it in a very sequence-specific manner (**Figure 2A**), due to the requirement of two simultaneous binding events (5). The arms bind to the target head-to-tail, meaning that there are no unhybridized nucleotides in the target sequence in-between the first and last nucleotide of the padlock probe (2). However, depending on the padlock probe application, unhybridized nucleotides may occur in the target between the two hybridization arms (8), as described later.

After probe hybridization to the target sequence, a ligase-mediated reaction closes the nick between the hybridization arms (**Figure 2B**), circularizing the padlock probe and thus making it topologically locked to the target (5). This closing reaction is also very sequence specific, due to the fact that only target-complementary hybridization arms allow closing of the padlock probe by the DNA ligase (2, 5). Hence, a circular padlock probe indicates the presence of that particular DNA sequence the padlock probe was designed to recognize. Finally, detection of the circularized padlock probe can be performed.

1.1.3 Detection of circularized padlock probes

For highly sensitive detection of circularized padlock probes, replication of individual probes is used to amplify the final signal strength, and thus making it possible to distinguish background from true signals (2, 6). Although PCR is widely used for amplifying circular padlock probes (9-11), this report will entirely focus on an amplification mechanism called rolling-circle amplification (RCA), due to its ability to specifically amplify circular DNA strands. In RCA, the highly processive bacteriophage Φ29 DNA polymerase from *Bacillus subtilis* (12-14) utilizes the circular padlock probe DNA as template for replication (3, 15), presumed that the polymerase can start the replication from somewhere, like from a provided primer (16) (**Figure 2C**). A one-hour RCA reaction produces approximately 1,000 single-stranded complementary copies of a circularized padlock probe, concatenated within a single DNA molecule (3, 17) (**Figure 2D**).

In the actual detection event, fluorescence-labeled oligonucleotides, complementary to a specific sequence within the linking segment of each padlock probe copy, hybridize to the RCA product (**Figure 2E**). All fluorescently labeled RCA products are then visible in a fluorescence microscope (18) as discrete, bright objects.
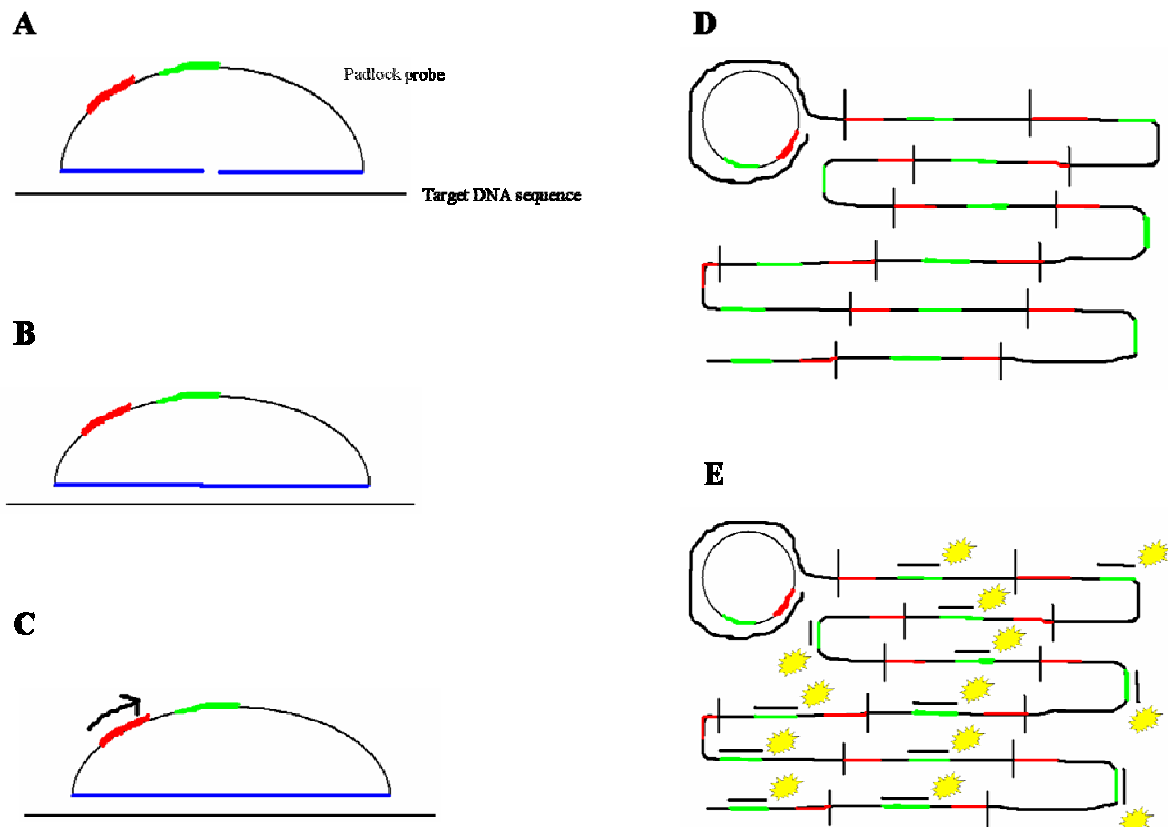
Figure 2. Padlock probing. The picture outlines the general steps in padlock probing, a method developed to reveal, for example, the presence of predefined DNA sequences in a DNA molecule. (**A**) The ~20 nucleotides of the 5′ and 3′ end (blue) of a padlock probe are designed to hybridize to a target DNA sequence in juxtaposition. (**B**) If a correct DNA sequence has been targeted, the padlock probe is circularized upon addition of a DNA ligase in a highly sequence-specific reaction. (**C**) A provided primer (black arrow), binding within the linking segment of the padlock probe, constitute a starting site for a replication reaction. (**D**) The circularized padlock probe can now act as template for a rolling-circle amplification (RCA) mechanism, in which the padlock probe sequence is copied in tandem within a single DNA molecule. RCA is usually performed by the phage Φ29 DNA polymerase from *Bacillus subtilis*. (**E**) To detect the RCA product, fluorescence-labeled oligonucleotides (yellow stars) are allowed to hybridize to a complementary sequence within each padlock probe copy (green). Finally, the RCA product is visualized in a fluorescence microscope as a bright spot.

1.1.4 Amplification of circularized padlock probes by circle-to-circle amplification (C2CA)

Sometimes when probing for a particular DNA sequence using the padlock probe technology the target sequence is present at a very low copy number in the genome, sometimes as a single copy per genome, or the amount of target genome is low. It can in those cases be valuable to be able to amplify the final signal in order to distinguish it from background signals. One way of such signal amplification is a mechanism called circle-to-circle amplification (C2CA), in which circularized padlock probes are specifically amplified into very large numbers (3).

The C2CA procedure is outlined in **Figure 3**, where the starting material is circular DNA strands, like closed padlock probes. First, the C2CA mechanism is initiated by performing ordinary rolling-circle amplification, commonly producing 1,000 complementary copies in one hour. Second, the RCA-synthesized DNA strand is digested by a particular restriction enzyme. This is achieved by adding restriction oligonucleotides complementary to a

sequence situated within each copy of the padlock probe, and produces about 1,000 DNA monomers per RCA product. Third, the DNA monomers are ligated using the restriction oligonucleotide, which was added in excess in the previous step, as template. Finally, RCA of the circularized DNA strands is performed, completing the first round of C2CA (1, 3). If necessary, additional rounds of C2CA can be performed. If a C2CA is initiated having, for example, 1,000 closed padlock probes, one round of C2CA will theoretically generate 1,000,000 closed DNA strands.
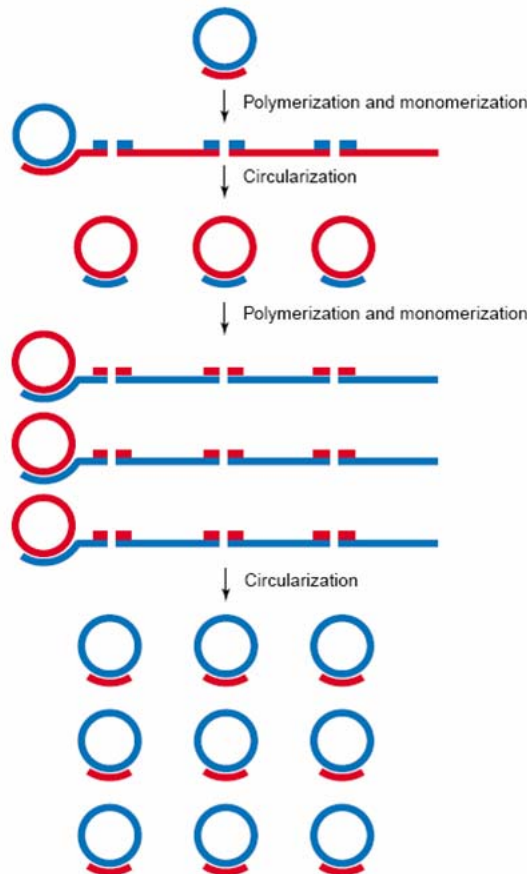
Figure 3. Circle-to-circle amplification (C2CA). In order to specifically amplify true signals generated from the padlock probing procedure, a method called circle-to-circle amplification (C2CA) can be utilized. In this method circularized padlock probes act as starting material, and the first step in the C2CA procedure is to perform an ordinary rolling-circle amplification (RCA) reaction. After RCA, the ~1,000 concatenated complementary copies of one padlock probe are restriction digested with the aid of an added restriction oligonucleotide, complementary to a specific sequence within each copy of the padlock probe. Following the restriction digestion, each DNA monomer is ligated using the restriction oligonucleotide as template. This is possible as the restriction oligonucleotide was added in excess during the restriction-digestion step. The first round of C2CA is now complete with the end product being DNA circles of 1,000 times the number of that of the initial padlock probes, and a second round of C2CA can be initiated if necessary. Picture used with permission from Elsevier Limited.

1.2 Biological applications of padlock probes

1.2.1 Padlock probes for *in situ* DNA analysis

DNA analysis performed *in situ* can be motivated for a number of reasons. For example, one may want to reveal the exact physical locations of different DNA molecules on chromosomes in fixed cells (2), or monitor tumor progression in patients by analyzing tissues (1). Padlock probes, indeed, offer the opportunity to locate specific DNA sequences

in situ, owing to a mechanism called target-primed rolling-circle replication, in which the 3´ target DNA serves as primer for replication (16). Thereby, the padlock probe can be replicated without topological inhibition (2) and the RCA product will be covalently attached to the target DNA sequence on, for instance, a chromosome.

1.2.2 Padlock probes for detection of single-nucleotide polymorphisms

As single-nucleotide polymorphisms (SNPs) constitute a major part of the genetic variations in humans, it remains a great challenge trying to assess the relevance of such mutations in human disease (9). The design of padlock probes makes them well suited for SNP analysis, and a few different padlock probing approaches have been reported to observe SNPs. In one design (3), the hybridization arms of the padlock probe hybridized head-to-tail to the target DNA containing the single mutation, if and only if, the mutated nucleotide was complementary to the corresponding nucleotide at the very last 3´ position of the padlock probe, i.e. the last nucleotide of the 3´ hybridization arm. In another design (10), the two probe arms of the padlock probe were hybridized to the DNA target, so that a one-nucleotide gap at the mutated position of the target was present between the arms. Then, the gap was filled in a sequence-specific polymerization event, using four reaction vessels with one of the four nucleotides (A, G, T, and C) added to each reaction, respectively. The former design requires four padlock probes to be prepared per polymorphism, but the genotyping can be performed in a single reaction, whereas in the latter design, only one padlock probe has to be prepared, but instead four individual reactions have to be performed per polymorphism (9).

1.2.3 Padlock probes for DNA-sequence detection in parallel

To increase throughput, it is worthwhile considering the use of a multiplex analysis technology, i.e. a technology allowing several analyses to be performed in parallel (9). Such a method would certainly help investigating, for instance, the great number of SNPs localized within the human genome. Multiplexed padlock probing has been utilized for several biological applications, like SNP genotyping, microbial detection and expression profiling, all using tag microarrays for parallel analyses (1). The reason why padlock probes show such great potential for multiplexed analyses is mainly due to the minimal risk of cross-reactivity between different padlock probes upon mixing (10). Furthermore, the design to distinguish padlock probes targeting different DNA sequences is based on having different identification sequences situated within the linking segment of each padlock probe type (2).

1.2.4 Some additional padlock probe applications

In addition to the applications of padlock probes stated above, some further ones will briefly be mentioned. First, padlock probes can be used to relatively quantify specific DNA sequences among genomes (1), ultimately leading to conclusions about relative gene-copy numbers for different genes. Second, probing for the presence of specific bacteria in a given sample, an important task in clinical diagnostics, can also be achieved by padlock probing (11, 18). Third, *in situ* RCA might be considered useful in the future for studying lateral gene transfer in bacteria (19). Moreover, the ability of padlock probing to study individual cells opens the opportunity to increase our understanding of, for instance, multicellular organisms and various human diseases (1).

1.3 Microfluidics

In the last twenty years much attention has been paid towards the science and technology of manipulating fluids in channels having very small dimensions, typically 5-500 μm (20, 21). The reasons for this tremendous increase in focus of these micrometer-scale devices, compared to their macroscale counterparts, are decreased cost in manufacture, use and disposal, decreased time of analysis, reduced consumption of sample and reagent volumes, and increased portability, to mention a few (22-24). Moreover, the biological applications of these microfluidic devices are several, ranging from separations coupled to mass spectroscopy, to numerous other bioanalyses (25), as will be further discussed below.

1.3.1 Fabrication of microfluidic devices

In order for the microfluidic technology to be fully integrated into the daily laboratory work worldwide, a first requirement is rapid and inexpensive fabrication of the microfluidic devices. Hitherto, the elastomeric polymer poly(dimethylsiloxane) (PDMS) has been the major standard for fabrication in microfluidics (26), mainly because fabrication in PDMS is easy and rapid, and that devices can be cast with sub-0.1-μm fidelity (27). There are, however, other advantages of using PDMS than the structural properties, like its non-toxicity to cells and permeability to gases, permitting cells to be grown inside microfluidic channels (28), and its optically transparent behaviour (25, 26), allowing for various fluorescent detection techniques.

1.3.2 Biological applications of microfluidics

If one goes through the available microfluidic literature, one can find microfluidic devices for a myriad of different biological applications, the majority of them probably unheard of for most people not working within the microfluidic field. A small selection of such devices will briefly be mentioned below.

Growing cells inside microchannels can be considered rather awkward but can, in fact, be a very good approach if one wants to study how cells react to different environments. For example, in optimizing the conditions for yeast and *Escherichia coli* fermentation and growth, microfluidic bioreactors with nanolitre growth chambers can screen many different conditions in parallel (29).

Micro Total Analysis Systems (μ-TAS), or 'lab-on-a-chip', are microfluidic systems integrating elements for acquisition, pre-treatment, separation, post-treatment and detection of a variety of samples (30). One example of the use of μ-TAS is a microfluidic chip functionally combining single cell injection, cell lysis and capillary electrophoretic (CE) separation of intracellular constituents (31, 32). Other examples of lab-on-a-chip devices include devices for genetic analyses involving on-chip PCR (33, 34), purification of microbial nucleic acids (35), Enzyme-Linked Immuno-Sorbent Assay (ELISA) (36) and detection of food poisoning bacteria (37).

1.3.3 Microfluidics as a tool for shorten diffusion times

Imagine that ligand molecules are immobilized on a surface, and that these ligands are designed to bind a particular type of analyte. In order for the ligands to be able to bind analytes, the analytes have to come into close proximity to the ligands, a procedure commonly relying upon diffusion. However, diffusion can sometimes take quite a time and thus making the assay more time-consuming. For example, if an analyte solution is applied

to a glass slide with immobilized ligands, the system will be mass-transport limited to some extent, meaning that ligand-analyte binding is dependent upon the diffusion of analytes to the surface with immobilized ligands. This mass-transport limitation can, however, be reduced by stirring, but the analytes always have to travel some distance to reach the ligands at the surface.

Microfluidics offers a unique opportunity to decrease the diffusion times for analytes to ligands, drastically decreasing the mass-transport limitation and turning the system into an almost reaction-limited one instead. The way that microfluidics manage this task is by flowing the analyte solution inside a small microchannel, in which ligands somehow are trapped, for instance by immobilization to the channel walls. In this way analytes do not have to travel by themselves to the ligands, relying upon diffusion, but new analytes will continuously be brought to the ligands by the microfluidic flow. This, indeed, will shorten the total analysis time of such an assay.

1.4 Aim of the project

In this project the padlock probe technology has been combined with solid-phase and microfluidics in order to make the padlock probe hybridization and ligation procedure more efficient. As a padlock probing model system, probing of a specific bacterial target sequence in a given sample has been used throughout the project. More specifically, this project aimed at making the assay more sensitive, i.e. all target molecules present should be found, to make the assay more specific due to that separation of ligated and unligated padlock probes decreases the possibility of having false ligations, and to reduce padlock probe hybridization and ligation times. These goals should be achieved by performing the padlock probe hybridization and ligation on a solid phase, like beads packed inside a microfluidic channel, by first capturing microbial DNA at a sequence in close proximity to the padlock probe binding site and then apply and ligate padlock probes (**Figure 4**).
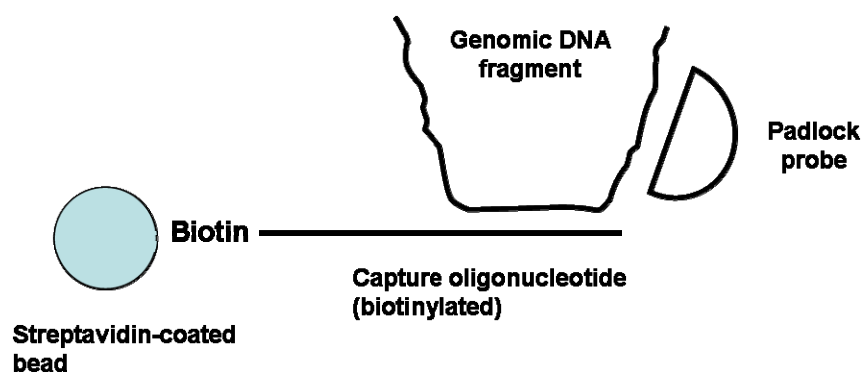
Figure 4. Microbial detection using a solid-phase padlock probe technology. The picture outlines how the padlock probe technology can be combined with solid-phase to detect the presence of specific bacteria. First, the microbial DNA, probably fragmented in some way, is captured on a capture oligonucleotide. Due to the biotinylation of the capture probe, it has initially been coupled to a streptavidin-coated bead, which can be packed inside a microfluidic column. Second, padlock probes will be applied and circularized to the target sequence, one that is situated in close proximity to the capture sequence. By utilizing the padlock probe technology together with solid-phase and microfluidics, one can gain advantages such as higher sensitivity and faster reaction kinetics.

The advantages of such a microfluidic, solid-phase approach would be that it allows for (*i*) washing of unreacted padlock probes, leading to the possibility of applying high concentrations of padlock probes during the hybridization and ligation step, making the assay more sensitive and specific, and also faster, (*ii*) washing of reagents, otherwise

interfering with later assay steps, (*iii*) decreased analysis time, due to that the physical properties of microfluidics leads to shorter diffusion times of padlock probes to target molecules and finally (*iv*) increased surface-to-volume ratio, owing to the beads, leading to even shorter diffusion times and an increased possibility of capturing all target sequences.

2 Materials and Methods

2.1 Generation of a fully functional padlock probe system

In order to generate a functional padlock probe system, two different experiments were performed. One experiment used a synthetic DNA target with a 16S rRNA sequence specific for the bacterium *Salmonella enterica* serovar *typhimurium*, while the other experiment used purified genomic DNA from the same bacterium, where the same target sequence as the synthetic one is present seven times per genome. Furthermore, both experiments used a one-round C2CA in order increase the sensitivity.

Five µl of a dilution series of genomic DNA, generating total DNA amounts of 100 pg, 1 ng, 10 ng and 100 ng, were preheated for 10 min at 95°C. The synthetic target DNA, with a corresponding dilution series of 1 fM, 10 fM, 100 fM and 1 pM in the ligation, were not preheated in any way. In addition to the DNA samples, two negative controls, one for synthetic and one for genomic DNA, were also present in which DNA was replaced by sterile-filtered MilliQ water (dH$_2$O).

2.1.1 First ligation and rolling-circle amplification (RCA)

Padlock probe phosphorylation was performed in a total volume of 50 µl by mixing 1 µl T4 Polynucleotide Kinase (PNK, 10 U/µl, Fermentas), 5 µl PNK buffer A (10×, Fermentas), 5 µl ATP (10 mM), 5 µl padlock probe (P2732, unphosphorylated, 10 µM) and 34 µl dH$_2$O. The reaction was then proceeded for 30 min at 37°C, followed by 10 min at 75°C. To each 5-µl DNA sample a 5-µl ligation mixture [1 µl Bovine Serum Albumin (BSA, 2 µg/µl, sterile filtered), 0.5 µl Ampligase® thermostable DNA ligase (5 U/µl, Epicentre), 1 µl Ampligase buffer (10×, Epicentre), 0.75 µl KCl (1 M, only for genomic DNA reactions), 1 µl padlock probe (P2732, phosphorylated, 10 nM, see Appendix) and 0.75 µl dH$_2$O (1.5 µl was added to the synthetic DNA reactions)] was added, followed by an incubation for 30 min at 30°C.

After the first ligation, a 10-µl RCA solution [1 µl BSA (2 µg/µl, sterile filtered), 0.3 µl Φ29 DNA polymerase (10 U/µl, Fermentas), 2 µl Φ29 buffer (10×, Fermentas), 1 µl dNTP (2.5 mM), 0.5 µl replication oligonucleotide (X01498, 1 µM) and 5.2 µl dH$_2$O] was added to each DNA sample. The samples were then incubated for 1 h at 37°C, followed by 5 min at 65°C.

2.1.2 Second ligation and RCA

A 5-µl restriction-digestion solution [0.5 µl BSA (2 µg/µl, sterile filtered), 1 µl Rsa I (10 U/µl, Fermentas), 0.5 µl Φ29 buffer (10×), 1.5 µl restriction oligonucleotide (L8764, 2 µM) and 1.5 µl dH$_2$O] was added to each DNA sample, containing RCA products, and incubated for 10 min at 37°C, followed by 10 min at 95°C. To each of the restriction-digested DNA samples, a second ligation solution [0.5 µl BSA (2 µg/µl, sterile filtered), 1 µl T4 DNA ligase (1 U/µl, Fermentas), 0.5 µl Φ29 buffer (10×), 1 µl ATP (20 mM) and 2 µl dH$_2$O] was added, followed by incubation for 10 min at 37°C.

A second RCA reaction was performed by mixing 0.5 μl BSA (2 μg/μl, sterile filtered), 0.3 μl Φ29 DNA polymerase (10 U/μl), 0.5 μl Φ29 buffer (10×), 1 μl dNTP (2.5 mM) and 2.7 μl dH$_2$O with every DNA sample, and incubate for 1 h at 37°C, followed by 5 min at 65°C.

2.1.3 Fluorescence detection

Fluorescently-labeled oligonucleotides were hybridized to the RCA products by mixing each sample with a hybridization solution [5 μl detection oligonucleotide (L8883, 100 nM), 4 μl EDTA (0.5 M, pH 8.0), 2 μl TRIS-HCl (1 M, pH 8.0), 25 μl NaCl (2 M), 1 μl Tween-20 (10%, sterile filtered) and 28 μl dH$_2$O] to a final volume of 100 μl, followed by incubation for 2 min at 70°C and 15 min at 55°C.

For actual detection of labeled RCA products, each sample was pumped through a flow channel of a microfluidic chip at a flow rate of 5 μl/min, using a syringe pump. A confocal microscope (LSM 5 META, Zeiss) was then used to fluorescently excite the fluorophores on the RCA products, whereafter a small volume of the flowing fluid was scanned (30,000 line scans of 512 pixels) at 40× magnification by the microscope, and the emission registered. The generated data files were converted to TIF format and the number of RCA products was counted by software written in MATLAB 7.0, in which the user sets a threshold value corresponding to the lowest fluorescent intensity that will be recorded as a RCA product.

2.2 Ligation of padlock probes on magnetic beads off-chip

Ten μl of magnetic, streptavidin-functionalized beads (Dynabeads® M-280 Streptavidin, Dynal) per reaction were washed two times in 100 μl of 1×Binding and Washing (1×B/W) buffer in an Eppendorf tube with the aid of a magnetic rack. A biotinylated oligonucleotide (L9355), serving as the padlock probe target, was coupled to the streptavidin beads in a total volume of 10 μl to a final concentration of 1 nM by mixing equal volumes of the biotinylated probe with 2×B/W buffer [1 mM EDTA, 2 M NaCl, 10 mM TRIS-HCl (pH 7.5)]. The biotin-streptavidin coupling was then performed for 1 h at room temperature, with shaking, after which the beads were washed two times in 100 μl of 1×B/W buffer.

Padlock probe hybridization and ligation were performed in a single reaction for a dilution series (1 nM, 10 nM, 100 nM, 1 μM and negative control (dH$_2$O)) of padlock probes in the ligation by mixing 1 μl BSA (2 μg/μl, sterile filtered), 0.5 μl Ampligase® thermostable DNA ligase (5 U/μl), 1 μl Ampligase buffer (10×), 1 μl padlock probe (L8864, dilution series) and 6.5 μl dH$_2$O with the beads. The ligation reaction was then continued for 5 min in a 55°C water bath, followed by washing two times in 50 μl of 1×B/W buffer. Circularized padlock probes were released from the beads by the addition of 10 μl 0.1 M NaOH, and the resulting 10-μl supernatant was then transferred to a PCR tube. The pH value was thereafter adjusted to approximately 7.0-7.5 by adding 10 μl of TRIS-HCl (5.5 μl 50 mM TRIS-HCl (pH 7.4), 4.5 μl 0.25 M HCl).

A single rolling-circle amplification was achieved by mixing a 10-μl RCA solution [1.5 μl BSA (2 μg/μl, sterile filtered), 0.3 μl Φ29 DNA polymerase (10 U/μl), 3 μl Φ29 buffer (10×), 1.5 μl dNTP (2.5 mM), 0.5 μl replication oligonucleotide (X01498, 1 μM) and 3.2 μl dH$_2$O] with the circularized padlock probes for each reaction. The RCA was conducted for 1 h at 37°C, followed by heat inactivation for 5 min at 65°C. Finally, detection was performed by the addition of a hybridization solution [5 μl detection oligonucleotide (L8748, 100 nM), 4 μl EDTA (0.5 M, pH 8.0), 2 μl TRIS-HCl (1 M, pH 8.0), 25 μl NaCl

(2 M), 1 μl Tween-20 (10%, sterile filtered) and 33 μl dH$_2$O] to a final volume of 100 μl per reaction. The samples were then incubated for 2 min at 70°C, followed by 15 min at 55°C, and RCA products registered with a confocal microscope as described above.

2.3 Ligation on beads with a sandwich oligonucleotide off-chip

Magnetic, streptavidin-coated beads, at a volume of 10 μl per reaction, were washed two times in 100 μl of 1×Binding and Washing (1×B/W). Biotin-streptavidin bead coupling of a biotinylated capture oligonucleotide (L9355) to a final concentration of 100 nM in a total volume of 10 μl, was achieved by mixing equal volumes of capture probe and 2×B/W buffer, followed by incubation for 1 h at room temperature, with shaking. After washing of the beads two times in 100 μl 1×B/W buffer, a sandwich oligonucleotide (L9545) was allowed to hybridize to the capture oligonucleotide by adding 5 μl sandwich oligonucleotide to 5 μl of 2×B/W buffer, so that the final concentration of sandwich probe in the reaction was 1 nM. The hybridization was then continued for 30 min in a 55°C water bath, after which the beads once again were washed two times in 100 μl 1×B/W buffer.

Ligation of a serial dilution (1 nM, 10 nM, 100 nM, 1 μM and negative control (dH$_2$O)) of padlock probes in the ligation was then performed by the addition of a 10-μl ligation mixture [1 μl BSA (2 μg/μl, sterile filtered), 1.5 μl Ampligase® thermostable DNA ligase (5 U/μl), 1 μl Ampligase buffer (10×), 1 μl padlock probe (P2732, dilution series) and 5.5 μl dH$_2$O] to the beads. Each ligation reaction was incubated for 5 min in a 55°C water bath and then the beads were washed two times in 50 μl of 1×B/W buffer.

Release and neutralization of circularized padlock probes, rolling-circle amplification and detection of RCA products were carried out as described in previous section, with the only exception that another detection oligonucleotide (L8825) was used.

2.4 Preparation of a microfluidic channel packed with beads

From a positive-relief, nickel master containing the pattern of the microfluidic microchannel, channels of dimensions 10,000 μm × 1050 μm × 100 μm (L × W × H) were molded by mixing poly(dimethylsiloxane) (PDMS, Elastosil RT 601A, Wacker) and curing agent (Elastosil RT 601B, Wacker) in a ratio of 10:1, pouring the solution over the master. The PDMS was then cured for 30 min at 55°C, after which the elastomer was peeled off the master and holes, serving as inlets and outlets for fluid, pierced. The PDMS and a glass slide were carefully rinsed with 70% ethanol, followed by water. To seal the microfluidic system, the PDMS and the glass slide were first activated by air plasma (PDMS, 25 sek; glass slide, 2 min) in order to oxidize the surfaces and then brought into contact, facing the channel pattern of the PDMS towards the glass slide, creating a waterproof, irreversible bond. The channel was finally incubated for 30 min at 55°C to further strengthen the bond.

The microchannels were consecutively packed with two types of beads. First, 1 mg/ml glass beads (Polysciences, Inc.) with diameters ranging from 10-30 μm were used as packing material at a volume enough just to cover the outlet structure of the microfluidic device. Second, ~50 μl of a five-fold dilution of 6.4-μm diameter, streptavidin-functionalized beads (1.36% solids-latex, Polysciences, Inc.) was used as the main-packing material. Packing was achieved using an ordinary syringe with a compatible needle. Furthermore, the used amount of streptavidin beads can bind approximately 800 fmol of biotinylated oligonucleotides. For every new experiment in a microchannel, a freshly packed chip was used.

2.5 Ligation on beads with a sandwich oligonucleotide on-chip

Two microfluidic channels were packed with both glass and streptavidin beads as described above. Tubings were connected to the channels' fluid inlets and outlets, and the inlet tubings were then coupled to ordinary syringes, placed on a syringe pump.

Biotin-streptavidin coupling between streptavidin beads and biotinylated capture probes was performed at room temperature by flowing (2 μl/min) a 20-μl capture probe solution [10 μl 1×TNT buffer (0.15 M NaCl, 0.01 M TRIS-HCl, 0.05% Tween-20), 2 μl capture oligonucleotide (L9355, 200 nM), and 8 μl dH$_2$O] through the channels, over the beads. The bead surfaces were then blocked for unspecific binding by applying 20 μl of 0.1 μg/μl BSA (sterile filtered) dissolved in 1×TNT buffer, at a flow rate of 2 μl/min. Sandwich-oligonucleotide hybridization to capture probes was carried out in a total volume of 20 μl by flowing (2 μl/min) a mixture of 10 μl 1×TNT buffer, 2 μl sandwich oligonucleotide (L9545, 1 μM) and 8 μl dH$_2$O through both microchannels at room temperature. Hybridization of padlock probes to the sandwich oligonucleotides, possessing the target sequence, was thereafter accomplished by a 2-μl/min flow of a 20 μl hybridization solution [10 μl 1×TNT buffer, 2 μl padlock probe (P2732, 10 μM) and 8 μl dH$_2$O] at room temperature. Padlock probe ligation was performed with added DNA ligase in one of the microchannels and without in the other channel, as a negative control reaction, by flowing a 20-μl ligation mixture [0.4 μl BSA (2 μg/μl, sterile filtered), 1 μl T4 DNA ligase (1 U/μl, only added in one of the microchannels), 2 μl T4 buffer (10×, Fermentas), 2 μl ATP (10 mM), 0.2 μl Tween-20 (10%, sterile filtered) and 14.4 μl dH$_2$O (15.4 μl was added to the microchannel without added DNA ligase)] through the respective microchannel at room temperature and at a flow rate of 2 μl/min. After a 20-μl washing step with 1×TNT buffer, conducted at a flow rate of 2 μl/min at room temperature, the sandwich oligonucleotides, harboring circularized padlock probes, were restriction digested from the capture probes in a total volume of 20 μl by flowing (2 μl/min) through each channel a mixture of 0.4 μl BSA (2 μg/μl, sterile filtered), 1 μl Mnl I (10 U/μl, Fermentas), 2 μl NEB2 buffer (10×, New England Biolabs), 0.2 μl Tween-20 (10%, sterile filtered) and 16.4 μl dH$_2$O. The resulted two restriction solutions, containing circles, were collected in a PCR tube each, followed by thermal inactivation of the restriction enzyme Mnl I for 20 min at 65°C.

Rolling-circle amplification, hybridization of detection oligonucleotides and confocal microscopy were performed as in section *2.2 Ligation of padlock probes on magnetic beads off-chip*, with the one exception that another detection oligonucleotide was used, namely L8825.

2.6 Ligation on beads off-chip, using microbial DNA as target

Sixty μl of magnetic, streptavidin-coated beads were transferred to an Eppendorf tube and then washed twice in 100 μl 1×B/W. Streptavidin-biotin coupling of beads to a biotinylated capture oligonucleotide (L9584), designed to capture genomic DNA from *Escherichia coli*, was then performed by mixing the beads with 5 μl capture probe and 5 μl 2×B/W, the final concentration of the capture probe being 100 nM, and incubate for 1 h at room temperature. The beads were again washed twice in 100 μl 1×B/W, after which 30 μl of fragmented (20 min boiling), 9.4 ng/μl genomic DNA from *E. coli* was mixed with 30 μl of 2×B/W, added to the beads and then the DNA was allowed to be captured for 30 min in a 55°C water bath. After DNA capture, the beads were washed once in 100 μl 1×B/W, followed by the addition of 60 μl of 1×B/W and the subsequent transfer of 10 μl bead

solution to each of five new Eppendorf tubes, which is equal to DNA capture in a total amount of 47 ng of *E. coli* DNA per reaction.

Padlock probe hybridization and ligation were performed by applying a serial dilution of padlock probes (1 nM, 10 nM, 100 nM, 1 µM and negative control (dH$_2$O)). For each of the five reactions, a 10-µl ligation solution [1 µl BSA (2 µg/µl, sterile filtered), 1 µl Ampligase® thermostable DNA ligase (5 U/µl), 1 µl Ampligase buffer (10×), 1 µl padlock probe (P3383, dilution series) and 6 µl dH$_2$O] was mixed with the beads and the circularization reaction allowed to proceed for 30 min in a 55°C water bath. The beads were then washed two times in 50 µl 1×B/W buffer.

The circularized padlock probes were released from the beads and neutralized as described in section *2.2 Ligation of padlock probes on magnetic beads off-chip*, whereas a one-round C2CA and detection were performed as described in section *2.1 Generation of a fully functional padlock probe system*, with the two exceptions that (*i*) the 10-µl RCA solution for the first RCA was composed of 1.5 µl BSA (2 µg/µl, sterile filtered), 0.3 µl Φ29 DNA polymerase (10 U/µl), 3 µl Φ29 buffer (10×), 1.5 µl dNTP (2.5 mM), 0.5 µl replication oligonucleotide (X01498, 1 µM) and 3.2 µl dH$_2$O, (*ii*) the 55-µl hybridization solution added to each sample was composed of 5 µl detection oligonucleotide (L8382, 100 nM), 4 µl EDTA (0.5 M, pH 8.0), 2 µl TRIS-HCl (1 M, pH 8.0), 25 µl NaCl (2 M), 1 µl Tween-20 (10%, sterile filtered) and 18 µl dH$_2$O.

2.7 Showing proof of principle with and without a solid phase, using a serial dilution of padlock probes

A dilution series of padlock probes (1 nM, 10 nM, 100 nM, 1 µM and negative control (dH$_2$O)) was used for ligation on a synthetic DNA target in a homogeneous phase. For each of the five reactions, 5 µl of a biotinylated target-DNA oligonucleotide (L9355) was mixed with a 5-µl ligation solution [1 µl BSA (2 µg/µl, sterile filtered), 1 µl Ampligase® thermostable DNA ligase (5 U/µl), 1 µl Ampligase buffer (10×), 1 µl padlock probe (L8864, dilution series) and 1 µl dH$_2$O], so that the final concentration of the target oligonucleotide was 1 nM in the ligation reaction. The ligation step was then continued for 1 h at 55°C.

Parallel to the homogeneous-phase experiment, a similar solid-phase experiment was performed, in which the same biotinylated target oligonucleotide (L9355) as used above was coupled to magnetic, streptavidin-coated beads to a final concentration of 1 nM in a procedure described in the previous section, *2.7 Solid-phase padlock probe ligation using microbial DNA*. The same dilution series of padlock probes as in the homogeneous-phase experiment was then used for a ligation reaction by the addition of 1 µl BSA (2 µg/µl, sterile filtered), 1 µl Ampligase® thermostable DNA ligase (5 U/µl), 1 µl Ampligase buffer (10×), 1 µl padlock probe (L8864, dilution series) and 1 µl dH$_2$O to the beads. Padlock probe circularization was thereafter allowed for 5 min in a 55°C water bath, after which the closed DNA circles were released from the solid support and neutralized according to section *2.2 Ligation of padlock probes on magnetic beads off-chip*.
A single RCA reaction was performed for the homogeneous-phase experiment by the addition of 1 µl BSA (2 µg/µl, sterile filtered), 0.3 µl Φ29 DNA polymerase (10 U/µl), 2 µl Φ29 buffer (10×), 1 µl dNTP (2.5 mM), 0.5 µl replication oligonucleotide (X01498, 1 µM) and 5.2 µl dH$_2$O to the ligation mixture. For the solid-phase experiment, the corresponding RCA reaction was carried out by the addition of 1.5 µl BSA (2 µg/µl, sterile filtered), 0.3

µl Φ29 DNA polymerase (10 U/µl), 3 µl Φ29 buffer (10×), 1.5 µl dNTP (2.5 mM), 0.5 µl replication oligonucleotide (X01498, 1 µM) and 3.2 µl dH$_2$O to the beads. RCA was conducted for 1 h at 37°C, followed by 5 min at 65°C at for both experiments. Finally, detection of RCA products was performed as describes in section *2.2 Ligation of padlock probes on magnetic beads off-chip* with the one exception that the volume of dH$_2$O was 43 µl per sample for the homogeneous-phase experiment.

2.8 Evaluation of DNA fragmentation by boiling

Five samples of genomic DNA (150 ng/µl), purified from *Salmonella enterica* serovar *typhimurium*, were boiled (i.e., 100°C) for 0, 1, 5, 10 and 20 minutes, respectively. Each DNA sample was spun down in a bench-top centrifuge every 1.5 minute in order to avoid evaporation of DNA. The heat-treated DNA samples were then loaded and run onto a 1% agarose gel to a final concentration of 1.5 µl DNA per reaction, along with a 1-kb DNA ladder (GeneRuler™ 1kb DNA Ladder, Fermentas).

3 Results

The project was in the beginning divided into two distinct parts, padlock probing on solid phase in macroscale and preparation of a microfluidic column for solid-phase padlock probing in microscale. The final goal was to combine these two parts and perform the padlock probing procedure inside the microfluidic column achieving higher sensitivity and faster kinetics.

3.1 Generation of a fully functional padlock probe system

The idea was to first get to know the padlock probing method as it is mostly performed in our group, i.e. in a homogeneous solution with sequential addition of the reagents, and to generate a functional padlock probe system, meaning that it is verified that all reagents work properly together.

Two different sets of reactions were performed, one with a dilution series of synthetic DNA as template and one with purified genomic DNA from *Salmonella enterica* serovar *typhimurium*. Both reaction sets used a 16S rRNA target sequence specific for *Salmonella enterica* serovar *typhimurium* as template in a one-round C2CA padlock reaction. For the synthetic target DNA, it can be concluded that a ten-fold dilution of target DNA corresponds to a rather nice ten-fold change in the number of counted objects (**Figure 5A**), as can be predicted from theory. The same nice ten-fold change in the number of counted objects can be seen using genomic DNA from *Salmonella enterica* serovar *typhimurium* as template for padlock probe hybridization and ligation (**Figure 5B**), except for the largest amount of genomic DNA (i.e., 100 ng). The decrease in the number of counts for the 100-ng data point can be explained by the fact that saturation has been reached, resulting in object overlap and thus fewer objects is registered than the actual number.
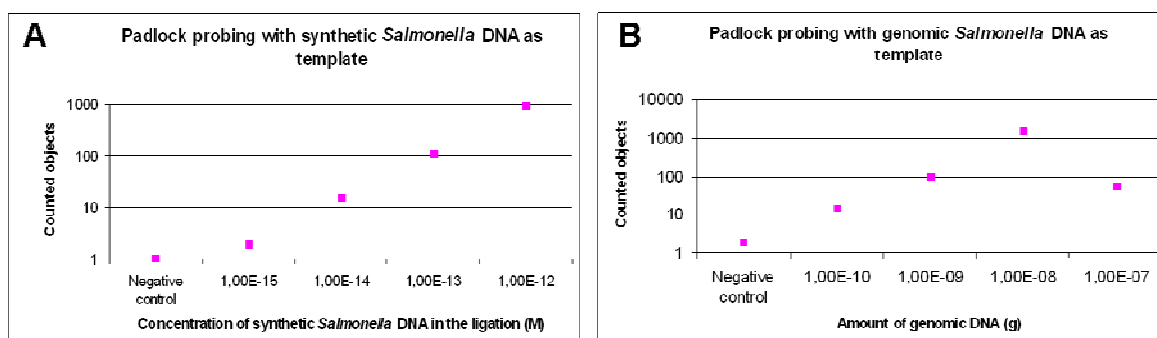
Figure 5. Padlock probing with synthetic and genomic *Salmonella* DNA as template. Padlock probing was performed on two sets of reactions to establish whether a functional padlock probing system had been generated or not. One reaction set (**A**) used a dilution series (1 fM, 10 fM, 100 fM and 1 pM) of synthetic *Salmonella* target DNA in the ligation, possessing a 16S rRNA target sequence specific for *Salmonella enterica* serovar *typhimurium*. The other reaction set (**B**) used a dilution series of purified genomic *Salmonella enterica* serovar *typhimurium* DNA, possessing the same target sequence as the synthetic DNA. Padlock probing was then performed with one round of C2CA to increase the sensitivity by about 1,000 fold. For both reaction sets, data was generated by confocal microscopy. (**A**) For the synthetic target, a ten-fold increase in the target DNA concentration corresponded nicely to a same ten-fold increase in number of counted objects. This, indeed, indicates that all reagents in this experiment actually work properly together. (**B**) Using genomic *Salmonella* DNA instead of the synthetic one, gave the same nice ten-fold change in the number of counted objects for the corresponding ten-fold dilution of genomic DNA. However, for the largest amount of genomic DNA (100 ng), RCA-product overlap occur due to the high concentration of target sequences, leading to fewer registered object than the actual number. Taken together, these two experiments establish that the used padlock probing reagents are functional together.

The data from these two initial experiments revealed that a functional padlock probe system has been generated, meaning that the padlock probe, the available genomic *Salmonella enterica* serovar *typhimurium* DNA and all other padlock probing reagents work properly together. This information is to be considered very valuable in the future if the assay, for whatever reason, do not work at some stage.

3.2 Ligation of padlock probes on magnetic beads off-chip

The introduction of a solid phase in the padlock probing protocol gives us the possibility to wash away unreacted padlock probes after the ligation step, which, in turn, makes it possible to add a huge excess of padlock probes during the ligation. To be able to have a huge excess of padlock probes compared to target sequences increase the possibility of finding all targets present, i.e. the sensitivity increases. The washing of unreacted padlock probes also prevent inhibition of later enzymatic steps, that otherwise will be the case in a solution-phase assay. Moreover, the padlock probe hybridization and ligation time will decrease because the diffusion time for the nearest padlock probe to a specific target sequence will be inversely proportional to the padlock probe concentration, i.e. the total analysis time decreases.

In this initial solid-phase experiment, magnetic, streptavidin-functionalized beads were used. The magnetic properties of the beads allow for washing, and to the streptavidin sites different biotinylated oligonucleotides can be coupled. In this particular assay a biotinylated oligonucleotide, possessing the target sequence for the padlock probes, was coupled to the beads, after which a dilution series (1 nM, 10 nM, 100 nM, 1 μM and negative control (dH$_2$O)) of padlock probes in the ligation was applied and a slightly modified padlock probe protocol followed. Notable is that the ligation step proceeded for only 5 min, compared to the usual 3 h for genomic DNA, in order to speed up the assay.

In the resulting graph (**Figure 6**) a ten-fold increase in padlock probe concentration from 1 nM to 10 nM also corresponds to a ten-fold increase in the number of counts. However, a rather drastic drop in the number of counted objects can bee seen for the two highest concentrations of padlock probe in the ligation, i.e. 100 nM and 1 μM, and this drop could potentially originate from two different scenarios. Either, the concentration of the ligation enzyme is too low for these two target concentrations, meaning that some padlock probes that have found a correct target cannot be ligated due to the absence of ligation enzyme. In this case a larger amount of enzyme would solve the problem. Or, all target molecules have actually been found, meaning that it does not matter how much more padlock probes one adds to the ligation reaction, the system is already saturated. Which scenario that best describes the actual situation in the system has to be further investigated by, for example, add different amounts of ligation enzyme and compare the number of counted objects.
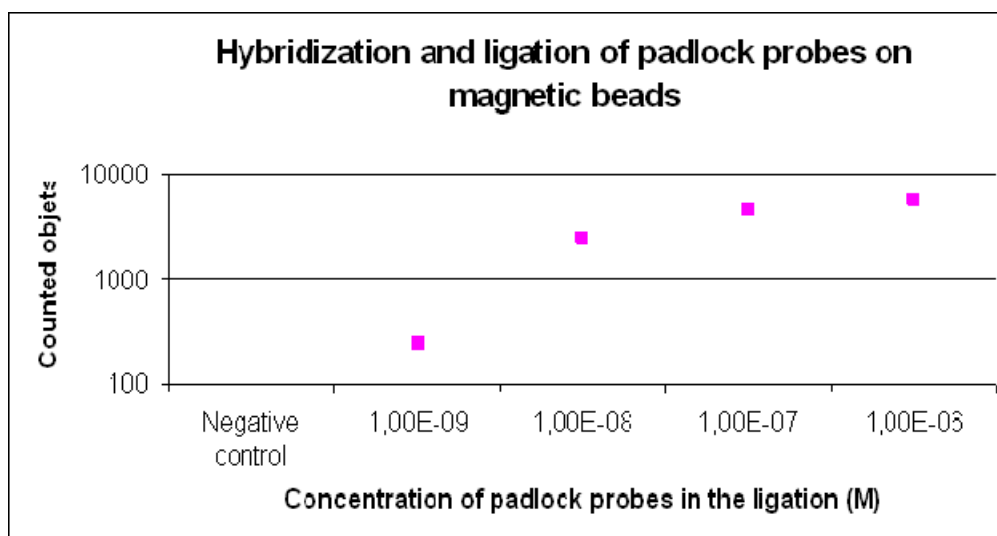

Figure 6. Hybridization and ligation of padlock probes on magnetic beads. A solid phase was introduced in the padlock probe ligation step in order to be able to wash away unreacted padlock probes after the ligation reaction and shorten the ligation time. As the padlock probe target, a biotinylated oligonucleotide was used. This oligonucleotide was coupled to magnetic, streptavidin beads, and due to the magnetism of the beads a simple washing procedure could be performed. After biotin-streptavidin coupling of the oligonucleotide to the beads, a dilution series (1 nM, 10 nM, 100 nM, 1 μM and negative control (dH₂O)) of padlock probes in the ligation was applied and the ligation reaction was carried out for only 5 min. Thereafter, rolling-circle amplification and detection were performed as described above. The last two data points, corresponding to 100 nM and 1 μM padlock probes in the ligation, show a drastic drop in the number of counted objects compared to the 1-nM and 10-mM data points, showing a ten-fold increase in the number of counts for the same increase in padlock probe concentration. One conclusion to draw from these data is that the drop in counts is due to the fact that all target sequences have been found. Another conclusion is that the amount of ligation enzyme is too low for the two highest padlock probe concentration, thereby the drop in the number of counted objects. The negative control cannot be presented in the logarithmic graph, simply because its value is zero.

3.3 Ligation on beads with a sandwich oligonucleotide off-chip

In a real padlock probe assay, probing for a pathogenic microbe, for example, the biotinylated oligonucleotide coupled to the beads will serve as a capture probe for the genomic DNA of the microbe. The capture probe will be designed to bind the microbial DNA at a sequence situated in close proximity to the target sequence of the padlock probe. The capture event of the microbial DNA does not have to be very specific as the padlock probes contribute the desired specificity.

To test this approach, a biotinylated capture oligonucleotide was biotin-streptavidin coupled to beads, just as before. Then, a 'sandwich' oligonucleotide, simulating a microbial DNA fragment, was hybridized to the capture oligonucleotide through their designed base complementarity. Ligation was then performed by applying padlock probes in various concentrations (1 nM, 10 nM, 100 nM, 1 μM and negative control (dH$_2$O)) for the ligation, which again was proceeded for only 5 min. Rolling-circle amplification and detection were then performed as described.

The resulted graph (**Figure 7**) actually shows the same trend as in the experiment without the sandwich oligonucleotide, i.e. the two lowest padlock probe concentrations (1 nM and 10 nM) correspond to an approximately ten-fold change in the number of counts, whereas the two highest concentrations (100 nM and 1 μM) result in a drastic drop in the number of counted objects. There is, however, an overall drop in the number of counted objects by about five fold in this assay, compared to the one without the sandwich probe. It is really hard to tell, at this stage, what has caused this overall drop, but maybe it has something to do with the extra hybridization step introduced in this experiment, namely the hybridization of sandwich probe to capture probe. Finally, it should be noted that the drop in the number of counts is for both this assay and the assay without sandwich probe, appearing between the 10-nM and 100-nM padlock probe concentration. Since this approach seems to work, the next step in the assay development will be to replace the sandwich oligonucleotide with real genomic DNA and see how that works.
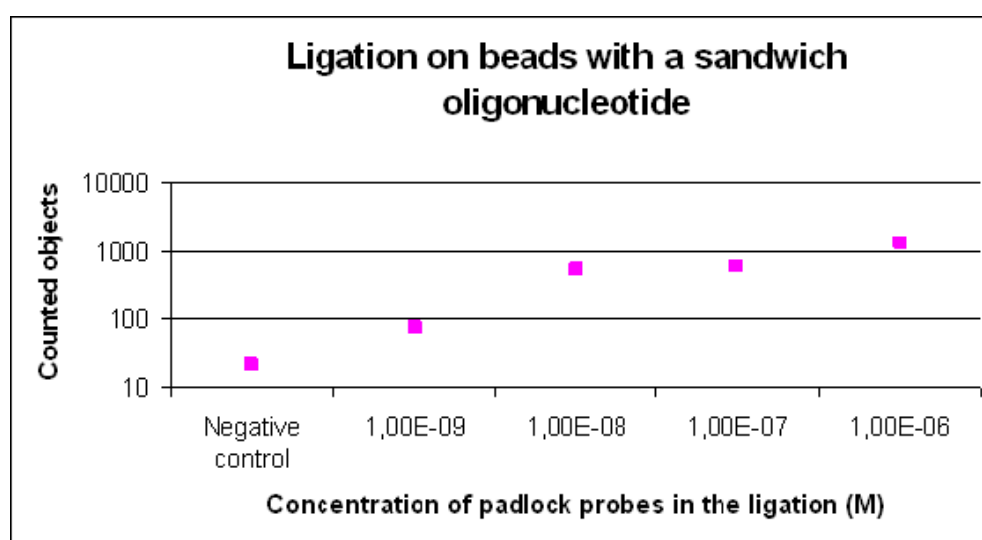

Figure 7. Ligation on beads with a sandwich oligonucleotide. In this assay a sandwich oligonucleotide was introduced that had been specifically designed to bind to the biotinylated probes attached to the beads. The aim of the sandwich probe is to act as a genomic DNA fragment from, for instance, a microbe, and it will be captured to the beads by the biotinylated capture oligonucleotide. The sandwich probe sequence, by which the same probe hybridizes to the capture probe, is situated in close proximity to the padlock probe target sequence, harbored by the sandwich probe. The experiment was conducted by biotin-streptavidin coupling of capture oligonucleotides to beads, sandwich probe hybridization to capture probes, ligation of padlock probes [serial dilution: 1 nM, 10 nM, 100 nM, 1 μM and negative control (dH$_2$O)], followed by release of closed circles, RCA and detection. The figure shows concentration of padlock probes in the ligation plotted against number of counted objects, and the 1-nM and 10-nM padlock probe concentrations correspond to ten-fold difference in number of counted objects. On the other hand, a rather significant drop in the number of counts appears for the two highest padlock probe concentrations, i.e. 100 nM and 1 μM. The overall shape of the curve, with the characteristic drop-off in counts, is similar to that of the experiment where no sandwich probe was used, although an approximately five-fold overall decrease in counted objects appears with sandwich probe, compared to without. This observation may be interpreted as to be a cause of the extra hybridization step introduced between the capture and sandwich probes.

3.4 Preparation of a microfluidic channel packed with beads

The major benefits from introducing microfluidics into the padlock probing ligation step are, besides the ones discussed in the introduction part, that the physical properties of a microfluidic flow allow for shorter diffusion times of mobile padlock probes to stationary target DNA sequences. This should be compared to applying padlock probes to a surface, immobilized with target molecules, and perform simple shaking of the reaction solution. Moreover, performing the ligation step onto the surfaces of beads will drastically increase the surface-to-volume ratio, compared to having just a plane surface, allowing for even shorter diffusion times and the possibility of having huge concentrations of capture probes, increasing the possibility of capturing all target sequences present in a sample. These two advantages, derived from the physical nature of microfluidics, will substantially decrease the overall analysis time.

Microchannels were fabricated in poly(dimethylsiloxane) (PDMS) as described previously, and the channel dimensions were $10,000 \times 1050$ μm $\times 100$ μm (L × W × H). The generated microchannels acted as tiny columns that were packed with two different kinds of beads, serving different purposes. On one hand, pre-packing with glass beads (10-30 μm in diameter) effectively ensured that the smaller streptavidin beads got trapped inside the column and did not flow through it. On the other hand, the obvious purpose of the 6.4-μm streptavidin beads was to couple biotinylated oligonucleotides, one of the key players in the present assay development. In **Figure 8**, an outline of the microchannel packing concept can be viewed.
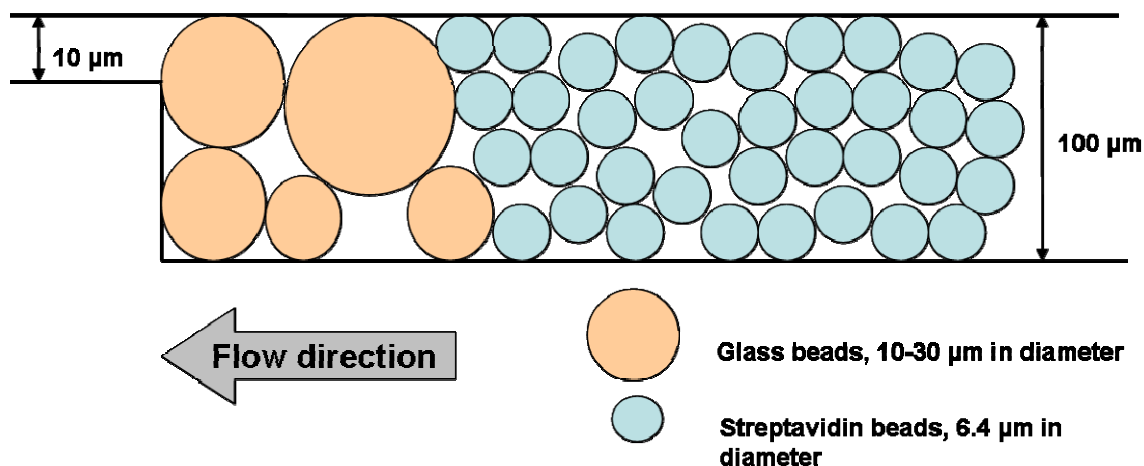
Figure 8. Packing of a microfluidic channel with two different types of beads. The picture shows a microchannel of dimensions $10,000 \times 1050$ μm $\times 100$ μm (L × W × H), fabricated in poly(dimethylsiloxane) (PDMS) viewed along the side. The present design of the microchannel, with a 10-μm high subchannel connecting to the outlet, requires a pre-packing of 'large' beads in order to trap a second type of beads, with smaller dimensions, inside the column. For pre-packing, a mixture of glass beads with diameters ranging from 10-30 μm was used, whose only purpose is to trap the smaller beads. Streptavidin-functionalized beads (1.36% solids-latex, 6.4 μm in diameter) were then used as the main packing material, serving as solid-phase capturers of biotinylated oligonucleotides.

3.5 Ligation on beads with a sandwich oligonucleotide on-chip

It was established above, in macroscale, that ligation of padlock probes on a sandwich-oligonucleotide target attached to a biotinylated probe, immobilized onto magnetic beads, seemed to work. The aim of this experiment was thus to reveal if the same approach can work in a microfluidic channel packed with beads, i.e. on-chip.

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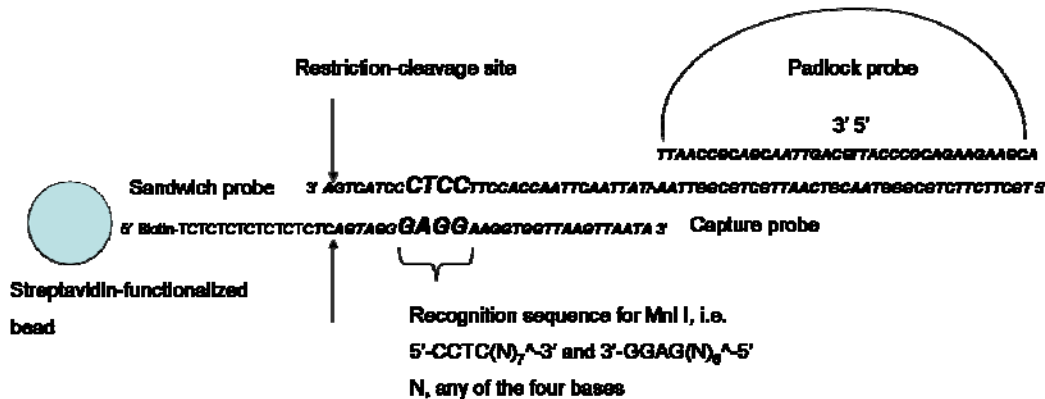The experiment was initiated by pre-packing two microchannels with glass and streptavidin beads, whereupon biotinylated capture probes, sandwich oligonucleotides, harboring the padlock probe target sequence, and finally padlock probes were allowed to flow through the two microchannels in the relative order indicated. For padlock probe circularization, DNA ligase was added to one of the channels but not to the other, the one without added ligase working as a negative control reaction. After padlock probe ligation, a restriction enzyme, Mnl I, was used to release the sandwich oligonucleotides, with attached DNA circles, from the beads (**Figure 9**). Ordinary RCA, hybridization of detection probes and counting of RCA products by confocal microscopy were finally performed for both microchannel experiments.

Figure 9. Release of sandwich oligonucleotides, with attached circularized padlock probes, from beads by restriction digestion. The schematic picture shows a particular state in the microchannel where, in turn, a biotinylated capture probe has coupled to a streptavidin bead, the capture probe has captured a sandwich oligonucleotide, and finally, a padlock probe has been ligated to its target sequence, possessed by the sandwich oligonucleotide. In order to release the circularized padlock probe from the bead, and thus also from the microchannel, a restriction enzyme called Mnl I was used, that recognizes double-stranded DNA with the particular sequences 5'-CCTC and 3'-GGAG, sequences that can be found in the hybridization region between the capture probe and the sandwich oligonucleotide. Mnl I then cleaves the double-stranded DNA a few bases away from the respective strand, or more precise 5'-CCTC(N)$_7$^-3' and 3'-GGAG(N)$_6$^-5', where N represents any of the four bases and ^ the position for cleavage.

The results from this experiment revealed 7,208 counted objects in the reaction with added DNA ligase and 39 counted objects in the reaction without the addition of DNA ligase, i.e. in the negative control experiment. Hence, the conclusion can be drawn that the assay approach to capture a piece of DNA, functioning as a target for padlock probe ligation, and then actually perform padlock probe ligation has worked, although the reaction conditions have not been optimized in any way, whatsoever. The next step will be to replace the sandwich oligonucleotides with genomic DNA fragments and evaluate the outcome of such an experiment. But, of course, replacement of the sandwich probe with genomic DNA will first be evaluated in a macroscale experiment.

3.6 Ligation on beads off-chip, using microbial DNA as target

It has already been shown to work to capture a synthetic DNA target, a so-called sandwich oligonucleotide, and then ligate padlock probes against a target sequence situated a few bases away from the capture sequence on the sandwich oligonucleotide. In a real situation, probing for the presence of a specific bacterial DNA in a given sample, it also has to work to capture genomic DNA instead of a synthetic target. These experiments has investigated the situation of trying to capture genomic DNA from *Escherichia coli*, fragmented 20 min by boiling. The padlock probe, targeting a sequence within strains of *E. coli* as well as

other species, has from previous experiments (Jenny Göransson, unpublished experiment) been shown to work properly.

To explore the situation with real genomic DNA, a capture oligonucleotide was coupled to beads by streptavidin-biotin interactions, followed by DNA capture of genomic DNA from *E. coli*, fragmented for 20 min by boiling. A serial dilution (1 nM, 10 nM, 100 nM, 1 μM and negative control (dH$_2$O)) of padlock probes was used in the ligation, which was performed on the solid phase, followed by rolling-circle amplification and detection in a homogeneous phase. For each sample there were between one and ten RCA products in total (data not shown), suggesting that the principle with a solid support has not worked with genomic DNA.

3.7 Showing proof of principle with and without a solid phase, using a serial dilution of padlock probes

From previous experiences within my research group, solution-phase padlock probing will not work with padlock probe concentrations higher than approximately 10 nM in the ligation reaction. This is mainly due to two different phenomena. First, at high padlock probe concentrations, i.e. over ~10 nM, unreacted padlock probes will interfere with the Φ29 DNA polymerase in the rolling-circle amplification mechanism and this interference is due to the 3´ → 5´ exonuclease activity of the polymerase. Second, unreacted padlock probes will also interfere with the RCA detection, since they will compete with the fluorescently labeled detection oligonucleotides of binding the RCA products. As discussed above, there are many advantages of applying high concentrations of padlock probes in the ligation reaction, and the negative effects of unreacted padlock probes in later assay steps can be avoided by washing away the unreacted padlock probes.

The aim of this experiment was to compare solution-phase and solid-phase padlock probing together with a serial dilution of padlock probes (1 nM, 10 nM, 100 nM, 1 μM and negative control (dH$_2$O)). In the solution-phase assay, all reagents were sequentially added to the tubes with no removal of unreacted padlock probes, whereas in the solid-phase assay unreacted padlock probes were washed away before RCA. In both experiments a biotinylated oligonucleotide served as a DNA target for the serial dilution of padlock probes. In the homogeneous-phase experiment (**Figure 10A**) the number of counted objects shows a parabolic relationship to the serial dilution of padlock probes. From 10 nM to 100 nM of padlock probes, the increase in counts drops off and from 100 nM to 1 μM there is a drastically decrease in counts so that the total number of counts for the 1 μM concentration corresponds to that of the negative control, meaning that the reaction simply dies. For the solid-phase experiment (**Figure 10B**), on the other hand, there is a linear relationship between the number of counted objects and the concentration of padlock probes, in that sense that the counts is increasing with increased padlock probe concentration. However, a ten-fold increase in padlock probe concentration does not correspond to a ten-fold increase in the number of counted objects for every data point.
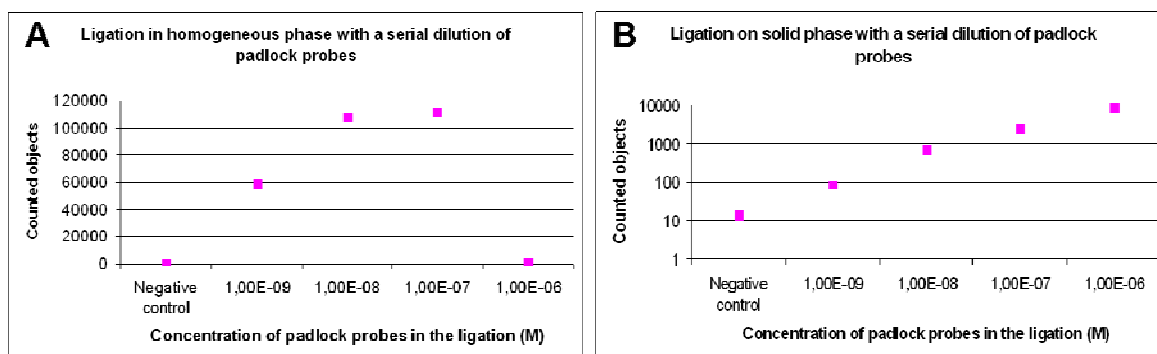
Figure 10. Ligation in homogeneous phase and on solid phase applying a serial dilution of padlock probes. In order to show the difference between washing and not washing of unreacted padlock probes, using high concentrations of padlock probes in the ligation step, one homogeneous-phase and one solid-phase experiment were performed. In the homogeneous-phase experiment (**A**) a biotinylated target oligonucleotide was used together with a serial dilution of padlock probes (1 nM, 10 nM, 100 nM, 1 μM and negative control (dH$_2$O)), with sequential addition of the reagents. For the solid-phase experiment (**B**) the same DNA target and serial dilution of padlock probes were used, but the biotinylated target oligonucleotide was in this experiment coupled to streptavidin-coated beads instead of being free in a solution. (**A**) The relationship between the number of counted objects and the concentration of padlock probes is in the homogeneous-phase experiment parabolic, with the reaction being dead at 1 μM padlock probes. From earlier solution-phase experiments, reactions use to die at a concentration of 100 nM padlock probes. (**B**) In the solid-phase experiment, where unreacted padlock probes have been washed away prior to RCA, an increase in the padlock probe concentration also corresponds to increase in counts, although not a ten-fold increase for every data point as could be excepted. In conclusion, washing of uncircularized padlock probes prior to RCA can allow for having higher padlock probe concentrations in the ligation, compared to a solution-phase assay, without risking the reaction to die.

In this experiment it was shown that washing of unreacted padlock probes enabled addition of high concentrations of padlock probes in the ligation step without risking that the reaction will stop. Furthermore, it might be something strange with my padlock probe concentrations as the solution-phase reaction did not stop at 100 nM padlock probe, a concentration at which reactions usually stop according to previous experiences of my colleagues. However, introducing a washing step that eliminates uncircularized padlock probes has here led to the conclusion that high concentrations of padlock probes during the ligation procedure can be used in order to gain all the advantages of that, for example, higher sensitivity and shorter assay time.

3.8 Evaluation of DNA fragmentation by boiling

A fully functional solid-phase padlock probe assay will include a protocol for extracting total DNA from a given sample, for instance, a soil or water sample. Then, the purified DNA sample will be treated in any way that allow the DNA of interest, if present, to be broken into fragments of appropriate length. Such a DNA fragmentation will allow for a more effective probing, due to the fact that shorter fragments is easier to make single stranded than longer ones, therefore the extent of DNA fragmentation has to be optimized. One simple approach to fragment genomic DNA could be to treat it with heat, and a particular variant of that could be to boil (i.e., 100°C) the DNA for a predetermined period of time.

To investigate to what extent genomic DNA fragment upon heat treatment by boiling, purified genomic DNA from *Salmonella enterica* serovar *typhimurium* was used in five different reactions for boiling in 0, 1, 5, 10 and 20 minutes, respectively. The fragmented DNA was then analyzed on a 1% agarose gel. The gel picture (**Figure 11**) shows six lanes,

representing (*A*) untreated DNA, DNA boiled for (*B*) 1 min, (*C*) 5 min, (*D*) 10 min, (*E*) 20 min and (*F*) a 1-kb DNA ladder. It can be concluded that a 1-min boiling do not fragment the DNA in any way, whereas boiling for 5, 10 and 20 min generate a smear of fragments of around 10,000-500; 4,000-250; and 500-100 base pairs (bp) in length, respectively. This experiment shows that genomic DNA can be fragmented by simple boiling instead of using more time-consuming methods, like restriction digestion or sonication. To what extent the DNA has to be fragmented in order to constitute the best target for effective padlock probing has to be investigated in more detail.
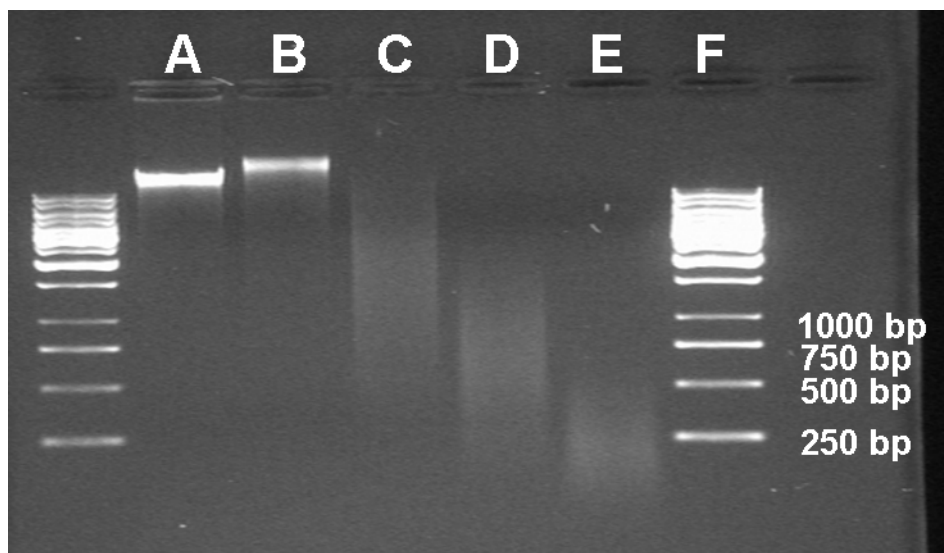

Figure 11. Evaluation of DNA fragmentation by boiling. In order to investigate to what degree genomic DNA fragment by heat treatment, genomic DNA from *Salmonella enterica* serovar *typhimurium* was boiled in water for 0, 1, 5, 10 and 20 minutes in five independent reactions. The heat-treated DNA samples were then analyzed on a 1% agarose gel, with a total amount of 1.5 µg of DNA loaded for each sample. The gel was loaded as follows: (*A*) untreated DNA, (*B*) DNA boiled for 1 min, (*C*) DNA boiled for 5 min, (*D*) DNA boiled for 10 min, (*E*) DNA boiled for 20 min and (*F*) a 1-kb DNA ladder. As can be seen, boiling for 1 min does not seem to fragment the DNA at all, compared to untreated DNA. However, boiling for 5, 10 and 20 min generate DNA fragments ranging from approximately 10,000-500; 4,000-250; and 500-100 bp, respectively. Hence, genomic DNA can rapidly be fragmented to pieces of an appropriate length by boiling it for a predetermined period of time.

3.9 Design of new microfluidic columns

The final goal of this project was to perform the padlock probing procedure inside a microfluidic column, as described earlier, and in order to do so there has to be a rapid and reproducible protocol for the bead-packing procedure. The most critical aspect of the bead packing, except from having a rapid packing protocol, is to be able to pack each microcolumn with approximately the same number of beads every single time. This is important to ensure that the same conditions in terms of, for example, flow resistance exists in every microfluidic channel. Also, it can be important to couple similar amounts of capture probes to the beads in every microchannel. Shortly, it is important to have as similar conditions as possible between different experiments to be able to compare them.

In this project the microcolumns have been packed with beads using ordinary syringes and needles, an approach that has been shown to be very time-consuming and irreproducible. For this reason I started to think about new designs of the microcolumns that would facilitate the packing procedure. The hallmark of a good microfluidic design is that it allows for a passive packing, meaning that after the packing has been started it proceeds by

itself and when the defined volume of the column is fully packed with beads the excess beads will automatically be guided to a waste container. Two such passive-packing microfluidic designs can be seen in **Figure 12**. In the first one (**A, I-III**) beads (blue) are collected in a reservoir on the chip (**I**), guided to the actual microcolumn by an ordinary flow (**II**) and finally excess beads are flown to a waste container (**III**). The last step of eliminating excess beads (**III**) is not a passive step, as someone has to change the flow direction. In another, truly passive, approach (**Figure 12B**) the beads are guided to the microcolumn by an ordinary flow and when the microchannel is fully packed, as is the situation in the picture, the beads are guided through a waste outlet rather than further accumulate on top of the fully packed microcolumn.
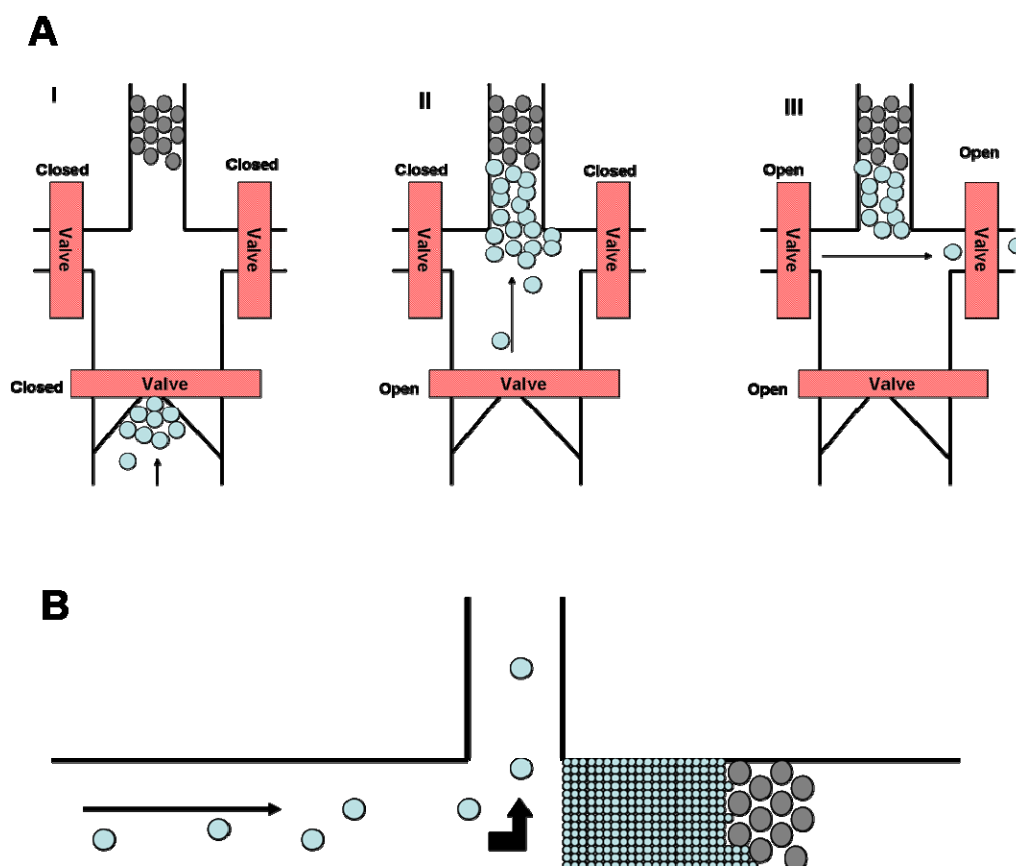

Figure 12. Design of new microfluidic columns. When performing the padlock probing technology on beads packed inside a microfluidic channel, it is very important to be able to compare the results from many microcolumns in order to screen for the most optimal padlock probing conditions. It can be considered crucial to be able to pack approximately the same number of beads in every microcolumn in order to achieve the same flow resistance, for example. Also, a passive-packing mechanism is to be preferred, meaning that when the bead packing has been initiated it will proceed by itself and when the microchannel is fully packed, the excess beads will be guided to a waste container. The picture shows two novel microfluidic channel design. The first design (**A, I-III**) is almost totally passive, with beads (blue) being collected in a reservoir-like structure in the chip (**I**), bead-packing by guidance of an ordinary liquid flow (**II**) and elimination of excess beads by guidance of a liquid flow from another direction (**III**). The part in this design that is not passive is the last step of eliminating excess beads. In the other design (**B**) the microcolumn is packed with beads with the aid of a liquid flow, and when the microchannel is packed excess beads are guided to a waste container by the same liquid flow. Grey circles symbolize a 'pillar forest', or some other structure, capturing the beads inside a microcolumn.

4 Discussion

4.1 The macroscale experiments

In this project the macroscale experiments refer to padlock probe hybridization and ligation on solid phase in Eppendorf tubes, where the solid phase consisted of magnetic, streptavidin-coated beads. The magnetic properties of the beads enabled different washing steps to be introduced that, for example, could eliminate unreacted padlock probes after the ligation step. Also, the streptavidin coating enabled biotinylated oligonucleotides to be coupled to the beads. The idea with the macroscale experiments was to investigate whether the outlined principle shown in **Figure 4** for microbial detection did work at all, namely to capture microbial DNA fragments and then use padlock probes in a targeting process. Although, the final goal is to combine this solid-phase padlock probe technology with microfluidics, it is first necessary to verify in macroscale that the capturing and probing principle really works by itself, without having to account for the extra parameters that microfluidics bring into play.

In the first macroscale experiment a capture oligonucleotide, coupled to beads, possessed a target sequence for a particular padlock probe. The results from this experiment showed that the first half of the principle, tested in this experiment, works. To actually test the capturing procedure, a so-called sandwich oligonucleotide was designed to bind to the capture probe. Furthermore, the sandwich probe also possessed a target sequence for another particular padlock probe. The capture probe was streptavidin-biotin coupled to the beads, the sandwich oligonucleotide hybridized to the capture probe and finally padlock probes bound and circularized at the target sequence. The conclusion of this experiment was that also the capturing part of the 'principle' works, at least with a synthetic sandwich oligonucleotide instead of microbial DNA.

As a final test of the new solid-phase padlock probe technology, one of course wants to replace the synthetic sandwich oligonucleotide with real microbial DNA and see whether that works. This was performed by using genomic DNA from *Escherichia coli*, fragmented 20 min by boiling, but the result showed that it did not work at all. Most probably it is something with the capturing process of microbial DNA that is not working properly, since the used padlock probe has previously been shown to work for targeting *E. coli* in homogeneous-phase assays according to my supervisor Jenny Göransson.

4.2 The microscale experiments

After the novel solid-phase padlock probe technology (**Figure 4**) had been proven to work properly in macroscale, the idea was that the same experiments would be performed in microscale. By microscale, it is here meant padlock probe hybridization and ligation on solid phase in a microfluidic column, where the solid phase consists of streptavidin-coated beads trapped inside the microcolumn (**Figure 8**). As in the macroscale counterpart experiments, the streptavidin coating of the beads is used to couple biotinylated capture oligonucleotides, whereas the design of the microcolumn allow for bead trapping, enabling different washing procedures to be introduced. If the new padlock probe approach has already been shown to work satisfactory in macroscale and is not working in microscale, the conclusion can be drawn that the failure in the microscale experiment is somehow due to the microfluidic parameters, like flow rate and/or duration of the enzymatic steps. However, if the microscale experiments had failed without prior testing of the principle in

macroscale, it had been impossible to know whether it was the principle itself or the microfluidic part that failed.

The first microcolumn experiment (not presented in this report) used a capture oligonucleotide, coupled to beads, possessing a target sequence for a particular padlock probe type. In this experiment rolling-circle amplification (RCA) and detection were both performed on the solid phase, i.e. on-chip. There was significantly stronger signal in a microcolumn where Φ29 DNA polymerase was added compared to a negative-control chip without added Φ29 DNA polymerase, meaning that the first half of the new principle also worked on-chip. However, it was decided for future experiments not to perform RCA and detection on-chip as it was impossible to quantify the signal strength in any proper way, which otherwise can be done by counting RCA products with MATLAB software, a procedure that has been used throughout this report. Moreover, the signal strength for the same type of experiment altered from one time to another, making it impossible to compare results between different experiments.

To test the capturing procedure in the microfluidic assay, a synthetic sandwich oligonucleotide was captured by a capture oligonucleotide, coupled to beads, and padlock probes ligated against a target sequence within the sandwich oligonucleotide. It could be concluded that also the capturing part of the new principle worked satisfactory in the microfluidic assay.

4.3 Concluding remarks

This project has shown that the novel solid-phase padlock probe technology for microbial detection, outlined in **Figure 4**, has worked in both macro- and microscale, using a synthetic sandwich oligonucleotide instead of microbial DNA. Although the padlock probing model system used in this project has been to detect the presence of a specific bacterium in a given sample, all biological applications of the padlock probe technology should be compatible with this solid-phase approach.

Furthermore, this project has also investigated the effects of DNA fragmentation upon heat treatment by boiling, and it has been concluded that boiling show a great potential for rapid and efficient DNA fragmentation. Finally, new microfluidic column designs have been generated on paper, hopefully overcoming the difficulties of rapid and reproducible bead packing.

5 Acknowledgements

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7 Appendix

In **Table 1**, the names of the different primers and probes used throughout this project are listed. Also, sequences and added modifications are indicated.

Table 1. Oligonucleotides used throughout the project. The table shows names, added modifications and sequences of the primers and probes used in this project. Cy3 and Cy5 represent fluorophores with different excitation and emission spectra.

Oligonucleotide	Modification	Sequence (5'-3')
Padlock probes		
L8864	5´-Phosphorylation	CTTCCTCCCTACTGAAGAGTGTACCGACCTCTCGTCGAAGTAGCCGTGACTATCGACTTGCGTCTATTTAGTGGAGCCTATTAACTTAACCAC
P2732	5´-Phosphorylation	TTACCCGCAGAAGAAGCAGAGTGTACCGACCTCAGTAGCCGTGACTATCGACTACTACGGATTCATGCGCTCTTAACCGCAGCAATTGACG
P3383	5´-Phosphorylation	CTTTGGTCTTGCGACGTCAGTGGATAGTGTCTTACACGATTTAGAGTGTACCGACCTCAGTAGCCGTGACTATCGACTAGGTCCCCCT
Capture oligonucleotides		
L9355	5´-Biotinylation	TCTCTCTCTCTCTCTCAGTAGGGAGGAAGGTGGTTAAGTTAATA
L9584	3´-Biotinylation	TACCGTTTCCAGTAGTTATCCCCCTCCATCTTTTT
Sandwich oligonucleotides		
L9545	Not modified	TGCTTCTTCTGCGGGTAACGTCAATTGCTGCGGTTAAATATTAACTTAACCACCTTCCTCCCTACTGA
Replication oligonucleotides		
X01498	Not modified	TACTGAGGTCGGTACACTCT
Restriction oligonucleotides		
L8764	Not modified	AGAGTGTACCGACCTCAGTA
Detection oligonucleotides		
L8382	5´-Cy3	AGTCGATAGTCACGGCTACT
L8748	5´-Cy3	TGCGTCTATTTAGTGGAGCC
L8825	5´-Cy5	ACTACGGATTCATGCGCTCT
L8883	5´-Cy3	AGAGCGCATGAATCCGTAGT