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On-nylon membrane detection of nucleic acid molecules by rolling circle amplification

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ABSTRACT

Positively-charged nylon membrane (NM) is a general solid-phase support for nucleic acid detection due to its convenient immobilization of nucleic acid materials by direct electrostatic adherence and simple UV crosslinking. Rolling circle amplification (RCA) is a widely used isothermal DNA amplification technique for nucleic acid detection. Near-infrared fluorescence (NIRF) is a new fluorescence technique with high sensitivity due to low background. This study developed a simple method for detecting nucleic acid molecules by combining the advantages of NM, RCA and NIRF, named NIRF-based solid phase RCA on nylon membrane (NM-NIRF-sRCA). The detection system of this method only need two kinds of nucleic acid molecules: target-specific probes with a RCA primer (P) at their 3′ end and a rolling circle (RC). The detection procedure consists of four steps: (1) immobilizing detected nucleic acids on NM by UV crosslinking; (2) hybridizing NM with specific probes and RC; (3) amplifying by a RCA reaction containing biotin-dUTP; (4) incubating NM with NIRF-labeled streptavidin and imaging with a NIRF imager. The method was fully testified by detecting oligonucleotides, L1 fragments of various HPV subtypes cloned in plasmid, and *E.coli* genomic DNA. This study thus provides a new facile method for detecting nucleic acid molecules.

Rolling circle amplification (RCA) is an isothermal nucleic acid amplification technique referencing circular DNA replication of pathogens with high (or ultrahigh) specificity, speed and efficiency [1,2]. A typical RCA reaction system is constituted of four basic components: DNA polymerase, a DNA or RNA primer, a circular DNA template, and dNTP mixture. Thereamong, the most commonly used DNA polymerase is bacteriophage φ29 DNA polymerase, which can efficiently catalyze reaction for several hours with a high synthesis rate (about 50 bp/s), the longest sequence up to 70 kb [3]. RCA reaction for detection of target molecules is based on two basic principles: one is that target molecules as primers initiate cyclic DNA as templates amplification [4]. The other is that padlock probes are cyclized as templates with DNA ligase catalysis for rolling circle amplification [5–7]. RCA includes linear RCA and exponential RCA according to the amplification efficiency, and contains single primer RCA, double primer RCA, and multiple primer RCA depending on the number of primers [8]. Thereamong, linear RCA means single primer RCA, the system of which contains only one primer that hybridizes complementarily with circular DNA template [9]. The exponential RCA includes multiprimed RCA, hyper branched RCA, and cascade RCA [10,11]. Nowadays, RCA has been widely applied in gene cloning, gene diagnosis, typing and nucleic acid sequencing due to low constant reaction temperature (30 °C) and high sensitivity.

Researchers have established a variety of test platforms based on RCA, mainly including liquid-phase RCA (lRCA) and solid-phase RCA (sRCA) detection systems. lRCA has been widely applied to detect single nucleotide polymorphism (SNP), epigenetic inheritance (such as DNA methylation) and protein, and amplify whole genome DNA for sequencing. In 2001, Faruqi et al. detected 10 SNP genotyping of human genome DNA depending on high-throughput RCA with sensitivity up to 1 ng [12]. In recent years, some researchers establish a new method for protein detection depending on high sensitivity of RCA. For example, Ou et al. invented an immunoRCA method by combining RCA technology with liposomes, the sensitivity of which is up to 0.08 fg/mL [13]. The sRCA reaction is realized on solid supports, including glass substrate, microsphere, nano-sphere, micro plate, micro flow control device, and so on. sRCA captures target nucleic acid molecules through DNA oligonucleotide immobilized on a solid support, which then are used as templates to initiate the RCA reaction. The products are

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always immobilized on the solid support throughout the amplification process. Therefore, sRCA achieves rapid separation of the target products among those complex samples, which also has significant advantages, including high-throughput analysis and point-of-care diagnostics potential. Larsson detected p53 gene mutation in as few as 2 amol DNA by using through RCA reaction on magnetic beads [14]. Some researches focus on RCA in combination with nanogold to assemble directly small molecules such as drugs. Furthermore, nanoparticles (NP) were assembled into Au-NP island structure in a salt solution to made nanoparticles dispersion at a distance from each other maintaining the unique color. Ali established an ATP colorimetric bioassay based on this feature [15–17]. Our laboratory established a new method of near infrared fluorescence (NIRF) sRCA on aldehyde-modified glass substrate to detect the activity of transcription factors [18]. This method detects NF-κB as low as 10 fmol, much more sensitive than electrophoretic mobility shift assays (EMSA) reported by NIRF and ethidium bromide (EB). Although these sRCA detection platforms showed excellent detection performance, they have to rely on costly and tedious chemical modifications of solid support and nucleic acid probes, and chemical coupling processes.

Nylon membrane is a traditional solid support, which can be used to effectively fix the negatively-charged DNA molecules due to its positive charge. Furthermore, DNA molecules can be easily covalently coupled on membrane surface by several minutes ultraviolet (UV) crosslinking, in which no any chemicals is required for the modification and coupling of DNA molecules. Therefore, nylon membrane provides a very facile and cost-effective solid-phase support for immobilizing and detecting DNA molecules. For these reasons, nylon membrane and coupling DNA molecules on nylon membrane had already been widely used for detecting DNA molecules in Southern blot and DNA microarray. We have recently developed several RCA-based methods for detecting nucleic acid molecules [18,19]. However, in these methods, DNA probes have to be modified with amino groups and coupled on solid-phase supports of glass (chip) or polypropylene (microplate) modified with aldehyde or N-oxysuccinimide (NOS) groups. Therefore, these methods may be prohibitive due to their high cost. Also recently, we developed a method for detecting DNA methylation by combining DNA hybridization on nylon membrane and near infrared fluorescence (NIRF) reporting. In this study, we found that the normally used positively-charged nylon membrane showed almost no autofluorescence in NIRF detection [20]. Based on these studies, we conceived that if RCA detection of nucleic acid molecules could be realized on nylon membrane surface. To our knowledge, no investigation reported the RCA detection on nylon membrane surface. Although a recent paper-based RCA detection was realized on nitrocellulose membrane surface [21], it relied on printing biotinylated DNA-streptavidin conjugate on solid surface. However, we expect to realize a RCA detection of target nucleic acid molecules by directly immobilizing the detected nucleic acid samples without any pre-modification on nylon membrane by easy UV crosslinking.

Therefore, we investigated the feasibility of RCA detection of nucleic acid molecules on nylon membrane in this study. Our purpose was to develop a very simple and cost-effective method for detecting nucleic acid molecules by realizing sRCA on nylon membrane. We thus performed proof-of-concept on-nylon membrane RCA detections of various nucleic acid molecules, including chemically synthesized oligonucleotide, plasmid DNA containing human papillomavirus (HPV) of six subtypes, and genomic DNA (gDNAs) of *Escherichia coli* (*E.coli*) bacteria, for fully validating the new method. The results indicated that this method (named NM-NIRF-sRCA) could facilely and cost-effectively detect these various DNA molecules with high sensitivity, specificity, and throughput. This study thus provides a newly applicable tool for the facile and cost-effective detection of various target nucleic acid molecules.

Materials and methods

Immobilization of detected DNA on membrane

The oligonucleotides (Table 1) were dissolved in TE buffer

Table 1
Oligonucleotides used in this study.

Oligo	Sequence and modification
NC	5'- TATTCGGATCGCTCATCAGTTCTGC-3'
PC	5'- TTTTTTTTTTTTCATCATCACGCAGAGCATCATTT-Biotin -3'
Linear RC	5'-P-CGTTATTTGTATTCTACTGTATTCTGTATGTCTCCGTCTGCCTGTCACCTG TGTATCTTTGATTCGTCAGCCCTGTATCC-3'
RC link	5'- CAAATAACGGGATACAGGGC-ddC-3′
Target Oligo	5'- GTTGCTCTACCAATTGAGCTACACCGCTGTACCAAGAGTTTGCTCCTGGCTGCTTTGAT-3'
TO-PP	5'- GGTGTAGCTCAATTGGTAGAGCAACAGGTGACAGGCAGACGGAGACATAC-3'
HPV16-PP	5'- ATATCTACTTCAGAAACTACGTGACAGGCAGACGGAGACATAC-3'
HPV18-PP	5'- CAGGTACAGGAGACTGTGTAGAGGTGACAGGCAGACGGAGACATAC-3'
HPV6-PP	5'- TATGTTAACACCCCAAGCGGGTGACAGGCAGACGGAGACATAC-3'
HPV11-PP	5'- GGGGGTAATAACAGATCATCTGGTGACAGGCAGACGGAGACATAC-3'
HPV33-PP	5'- CCTCCATCTGCTAGTTTACAGGTGACAGGCAGACGGAGACATAC-3'
HPV35-PP	5'- GTAATGCTAACCAGGTAAAAGCGTGACAGGCAGACGGAGACATAC-3'
DH5α-PP1	5'- ACTCCTACGGGAGGCAGCAGTGGGGAATAGGTGACAGGCAGACGGAGACATAC-3'
DH5α-PP2	5'- CATCCACGGAAGTTTTCAGAGATGAGAATGAGGTGACAGGCAGACGGAGACATAC-3'
DH5α-PP3	5'- AAAGTTAATACCTTTGCTCATTGAGGTGACAGGCAGACGGAGACATAC-3'
DH5α-PP4	5'- GATGAGAATGTGCCTTCGGGAACCGAGGTGACAGGCAGACGGAGACATAC-3'