Anal Bioanal Chem (2006) 386:1975–1984
DOI 10.1007/s00216-006-0872-9

ORIGINAL PAPER

Detection and identification of IHN and ISA viruses by isothermal DNA amplification in microcapillary tubes

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Received: 20 July 2006 / Revised: 15 September 2006 / Accepted: 19 September 2006 / Published online: 28 October 2006

Abstract Unique base sequences derived from RNA of both infectious hematopoietic necrosis virus (IHNV) and infectious salmon anemia virus (ISAV) were detected and identified using a combination of surface-associated molecular padlock DNA probes (MPPs) and rolling circle amplification (RCA) in microcapillary tubes. DNA oligonucleotides with base sequences identical to RNA obtained from IHNV or ISAV were recognized by MPPs. Circularized MPPs were then captured on the inner surfaces of glass microcapillary tubes by immobilized DNA oligonucleotide primers. Extension of the immobilized primers by isothermal RCA produced DNA concatamers, which were labeled with fluorescent SYBR Green II nucleic acid stain, and measured by microfluorimetry. Molecular padlock probes, combined with this method of surface-associated isothermal RCA, exhibited high selectivity without the need for thermal cycling. This method is applicable to the design of low-power field sensors capable of multiplex detection of viral, bacterial, and protozoan pathogens within localized regions of microcapillary tubes.

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Keywords Bioanalytical methods · Biosensors · Fluorescence/luminescence · Virus · Molecular padlock

Introduction

Infectious viral disease is responsible for significant economic losses in aquaculture ventures that rely on the cultivation of finfish. Two viruses of major concern are infectious hematopoietic necrosis virus (IHNV) and infectious salmon anemia virus (ISAV). Early virus detection and confirmation, through rapid, sensitive, and selective molecular on-site methods, is crucial to the reduction of economic loss. Sensing methods based on the recognition of nucleic acid sequences isolated from pathogenic microorganisms are highly selective, and detectable by a variety of means, including optical, electrochemical, and acoustic wave sensors. A sensitive, low-power sensor solution with capability to test for a wide range of pathogens is needed, especially in field applications. This need is currently addressed in laboratory settings by techniques incorporating polymerase chain reaction (PCR) [1–5]. PCR-based diagnostics have demonstrated a high degree of sensitivity [6], and these techniques are used in several recently described portable biosensing devices [7–10]. The requirement for thermal cycling in PCR increases the power consumption and places limitations on the materials used in these platforms, factors that are of critical importance in field sensors.

Isothermal methods such as loop-mediated isothermal amplification (LAMP) [11–14] and rolling circle amplification (RCA) [15–17] offer several advantages over PCR in field applications, including relative insensitivity to contamination [18], and the capability for use in polymer

microchips that cannot withstand the high temperatures required for PCR [12]. LAMP, a technique that generates a looped and branched product, requires an additional reverse-transcriptase step (as does PCR) for the detection of RNA [11]. In contrast, RCA works in conjunction with molecular padlock probes (MPPs) that are capable of detecting either DNA or RNA directly.

The use of MPP technology and RCA has recently been shown to be a viable approach for the sensitive detection of viral RNA in tissues taken from infected fish [19]. This approach is potentially inexpensive and easily adapted to field use by individuals without specialized training in molecular techniques. Surface-associated RCA provides a high degree of selectivity, while at the same time reducing the frequency of false positive results. Since MPP can detect either RNA or DNA directly and interchangeably, the technique can be used to report the presence, and potentially the state of growth, of a target organism.

A MPP is a synthetic single-stranded linear DNA oligonucleotide that is complementary to a DNA or RNA target sequence. A specified number of bases at both the 5′ and 3′ ends of the MPP, which are connected by a specified linker sequence, are complementary to the 5′ and 3′ segments of a target sequence. Upon annealing and ligation, a circular oligonucleotide construct with a single-stranded region of linker sequence and a double-stranded segment located at the MPP–target annealing region is generated [20]. With the addition of an appropriate polymerase and primer oligonucleotide, the circularized MPP (cMPP) can undergo RCA, forming a concatamer of the complement of the cMPP and thus confirming the detection of a specific nucleic acid sequence.

The MPP/RCA technique may be executed in a surface-immobilized format by attachment of the RCA primer to a functionalized self-assembled monolayer (SAM) enabling multiplex detection of nucleic acid sequences or proteins [17, 21, 22]. MPP/RCA has been used to amplify surface-bound cMPP on functionalized gold surfaces [23] and glass surfaces [24], and achieved discrimination between cMPP formed against either ISAV or IHNV base sequences.

This report describes an analysis approach, with multiplex capabilities, that is realized by the immobilization and subsequent extension of RCA primers on the inner surfaces of glass microcapillary tubes, allowing fluorescence detection. Circularized MPP formed to different targets are detected by surface-associated RCA achieved using selective primers that hybridize solely to complementary cMPP. This study serves as an essential first step toward the integration of MPP/RCA technology into a miniaturized, multiplex molecular sensing system with broad applicability to the accurate detection of multiple microbial pathogens.

Methodology

Oligonucleotide sequences

Table 1 shows the sequences of all of the oligonucleotides used in this study. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and subsequently purified by polyacrylamide gel electrophoresis. Synthetic target sequences were 3′-phosphorylated to prevent self-priming during RCA, and MPP sequences were 5′-phosphorylated to permit ligation. Corresponding to nucleotides 473–517 within the N gene, the IHNV target (*IHN-Target*) sequence was chosen from the published genomic sequence of the IHNV genome (GenBank L40883) [25]. A 90-base IHNV MPP (*IHN-MPP*) was designed based on *IHN-Target*. The 47-base ISAV target (*ISA-Target*) sequence represents nucleotides 638–684 of the protein PB1 gene of the North American isolate of ISAV (GenBank AF095254) [26]. A 95-base ISAV MPP (*ISA-MPP*) was designed to allow proper alignment with the target for cMPP construction. For RCA of cMPP formed to *ISA-Target* or *IHN-Target*, two unique 18-base rolling circle primers were designed to be complementary to bases within the single-stranded portion of formed cMPP.

Molecular padlock ligation reactions

MPP ligation reactions using one or more MPP with one or more target oligonucleotide(s) were carried out in 50 μL of exonuclease I buffer (67 mM glycine-KOH, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, pH 9.5) supplemented with 1 mM ATP [19]. For target recognition, 100 nM total probe and 400 nM total target were incubated with 40 units of T4 DNA ligase at 23 °C for 30 min. The reaction was terminated by heating at 65°C for 15 min. Twenty units of exonuclease I (*E. coli*) were subsequently added to degrade any non-ligated reactants. After incubation at 37 °C for 30 min, the exonuclease reaction was halted by heating at 80 °C for 15 min. cMPP-containing reaction mixtures were stored at −20 °C until used in the amplification process. All enzymes and buffers for cMPP formation were purchased from New England Biolabs (Beverly, MA). The construction of cMPP to *IHN-Target* or *ISA-Target* was confirmed by gel electrophoresis on 4% Nu-Sieve agarose (Cambrex Bioscience, Rockland, ME). Gels were run at 1.5 V/cm for 30 min in TAE buffer (40 mM TRIS-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.3) with 0.25 μg/mL ethidium bromide.

To test the ability of two or more unique MPPs to work in a single assay, the cMPP formation protocol was carried out using a mixture of *IHN-MPP* and *ISA-MPP*, and the following target sequences: *IHN-Target*, *ISA-Target*, or a mixture of *IHN-Target* and *ISA-Target*. For comparison, cMPP preparations were made individually by ligation of

Table 1 Oligonucleotide probes, targets, and primers

Oligonucleotide	Sequence
IHNV Target (*IHN-Target*)	5′-GGGCACCACCAGACGCTACCTTCGCAGATCCCAACAACAAGCTTG-3′
IHNV MPP (*IHN-MPP*)	5′-GTAGCGTCTGGTGGTGCCCATTCAA**TGTGCCTTGTCCGTACT**TCTAGTACTCTAGTACGCTTAGCAAGCTTGTTGTTGGGATCTGCGAAG-3′
IHNV RCA Primer (*IHN-Primer*)	Biotin-C6-5′-AGTACGGACAAGGCACAT-3′
ISAV Target (*ISA-Target*)	5′-AGGACCAAACTCGAGCCGAGAGCAGTGTTCACAGCAGGAGTTCCATG-3′
ISAV MPP (*ISA-MPP*)	5′-CTCTCGGCTCGAGTTTGGTCCTAATCTCAG**TGCCATGGTCACTCTTCGT**TCTAGTACTCTAGTACGCTTAG CATGGAACTCCTGCTGTGAACACTG-5′
ISAV RCA Primer (*ISA-Primer*)	Biotin-C6-5′-AAAAAAACGAAGAGTGACCATGCA-3′

The region of the probe complementary to the target is underlined. The region of the probe complementary to the primer appears in boldface type only

either MPP in the presence of its complementary target. A negative control was included in which both MPPs were present, but neither *IHN-Target* nor *ISA-Target* was present. The ligation step was always followed by incubation with exonuclease I (*E. coli*) to degrade mismatched MPPs and target sequences.

Amine modification of glass microcapillary tubes

The surfaces of microcapillary tubes were derivatized to yield free amine moieties according to the two-step gas-phase method described by Kanan et al. [27] using pre-adsorbed ethylenediamine (EDA) to catalyze the reaction of (3-aminopropyl)dimethylethoxysilane (APDMES) with the silica surface. EDA was obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification. APDMES was purchased from United Chemical Technologies (Bristol, PA). EDA and APDMES were transferred to evacuated glass bulbs and delivered as gaseous compounds to an evacuated reaction cell using standard vacuum line techniques. Twenty-μL capacity, 32-mm-long glass microcapillary tubes (Microcaps; Drummond Scientific Company, Broomall, PA) were cleaned by UV-ozone treatment for 10 min in a Model 342 UVO cleaner (Jelight Company, Irvine, CA) immediately prior to derivatization. Microcapillary tubes were placed in an open 50-mL glass beaker which was placed in a custom-built vacuum cell with valve ports to permit addition of gaseous reagents. After the chamber pressure reached 60 mTorr, the pump valve was closed and the sample exposed to EDA for 30 min, followed by APDMES for 60 min. The vacuum was released and amine-derivatized microcapillary tubes were removed and stored in a vacuum desiccator until use. Amine-derivatized microcapillaries could be stored in this manner for at least 1 week before use.

Addition of biotin and biorecognition components to microcapillary tubes

Biotin was covalently coupled to the inner surfaces of microcapillary tubes by filling amine-derivatized tubes with a 10 μM solution of succinimidyl-6′-(biotinamido)-6-hexanamido hexanoate (NHS-LC-LC-biotin; Pierce Biotechnology, Inc, Rockford, IL) in phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 11.9 mM Na_2PO_4), pH 7.4, followed by 2-h incubation at room temperature. The tubes were subsequently rinsed with 20 volumes of blocking buffer comprising PBS with 1% bovine serum albumin (Sigma) and 0.05% polyoxyethylenesorbitan monolaurate (Tween-20; Sigma), using a Nunc-Immuno Wash 8 plate washer (Nunc, Denmark) with attached extended-length, low-volume plastic pipet tips to permit multiple tubes to be rinsed simultaneously. NeutrAvidin (Molecular Probes/Invi-

trogen, Eugene, OR), 30 μg/mL in blocking buffer, was added and allowed to bind for 1 h and the microcapillary tubes were again rinsed with 20 volumes of blocking buffer. The tubes were filled with 1.5 μM biotinylated single-stranded oligonucleotide primers in blocking buffer, incubated for 1 h at room temperature, and then rinsed with 20 volumes of blocking buffer.

Spatial localization of biorecognition components

The feasibility of using microcapillary tubes with internally localized molecules for multiplex detection of nucleic acid targets was examined first by sequential derivatization of microcapillary tube end regions, each comprising one third of the tube length, with 6-(fluorescein-5-(and-6)-carboxamido) hexanoic acid, succinimidyl ester (fluorescein-SE; Molecular Probes/Invitrogen, Eugene, OR). Following amine derivatization, a 32-mm-long, 20-μL glass microcapillary tube was approximately one third-filled by taking up approximately 7 μL of 10 μM amine-reactive fluorescein-SE in PBS from one end. The fluorescein-SE was allowed to react with the amine surface for 30 min, and was then removed. The tube was washed by flowing 20 volumes PBS through from the unlabeled end. A solution of 10 μM fluorescein-SE in PBS was then reacted with the unlabeled end of the microcapillary tube for 30 min, removed, and the tube rinsed by flowing through 20 volumes PBS from the previously labeled end. The efficacy of gas-phase amine derivatization of glass microcapillary tubes, and the spatially constrained labeling of this surface, was evaluated by measurement of the extent of covalent attachment of fluorescein to the inner surface of the tubes relative to an unexposed area of the same tube.

The procedure developed using fluorescein-SE, described above, was then used to derivatize segments of microcapillary tubes with biotinylated oligonucleotide primers for cMPP formed to either *ISA-Target* or *IHN-Target*, to determine whether multiplex surface-associated RCA could be carried out within single tubes. Amine-derivatized microcapillary tubes were exposed to NHS-LC-LC-biotin (10 μM in PBS for 2 h), rinsed with 20 volumes blocking buffer, and then exposed to NeutrAvidin (30 μg/mL in blocking buffer for 1 h). The same tubes were then partially labeled by taking up approximately 7 μL of 1.5 μM biotinylated *IHN-Primer* in blocking buffer for 1 h, followed by rinsing with 20 volumes blocking buffer from the opposite end. The opposite ends of the microcapillary tubes were labeled in the same way with biotinylated *ISA-Primer*, leaving an unlabeled region in the middle third of the tubes.

cMPP binding and rolling circle amplification

Binding of cMPP was carried out by diluting 1 μL of cMPP ligation product (≤100 nM cMPP) into 19 μL of blocking buffer and allowing the entire amount to be drawn into the microcapillary tube. cMPP solutions in blocking buffer were allowed to anneal to surface-bound primers for up to 1 h at 25 °C. The microcapillary tubes were subsequently rinsed with 20 volumes of blocking buffer. The RCA reactions were carried out in Φ-29 reaction buffer (50 mM TRIS-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 4 mM dithiothreitol, 0.1% Triton X-100, pH 7.5), supplemented with 200 μM (each dNTP) dNTPs, and 100 U/mL of Φ-29 DNA polymerase (NEB). SYBR Green II nucleic acid stain (Molecular Probes/Invitrogen) was included in the reaction mixture, at a final dilution of 1:5,000 of the stock solution provided by the manufacturer, to permit fluorescence monitoring of the development of RCA product. Figure 1 summarizes the formation of cMPP, immobilization of specific cMPP on the functionalized surface, and surface-associated RCA (see Fig. 1 caption).

A multiplex detection platform was created by derivatization of an array of microcapillary tubes with RCA primers for cMPP formed to *ISA-Target* (top third of tubes) and RCA primers for cMPP formed to *IHN-Target* (bottom third of tubes). The cMPP stocks (Molecular padlock ligation reactions) were incubated in the microcapillaries to permit binding to the surfaces. A solution of RCA components, including SYBR Green II nucleic acid stain, was then added and the microcapillary tubes were held at 30 °C for 1 h. After rinsing the capillary tubes with PBS, the fluorescence of RCA product in the top and bottom segments of the microcapillary tubes was measured with a microscope-coupled photomultiplier tube (PMT) detector (Microfluorometric measurements).

An important rate-liming step in cMPP sensing is the diffusion and binding of cMPP to the inner surfaces of microcapillaries previously modified with biotin, NeutrAvidin, and biotinylated RCA primer oligonucleotides. The minimum time required for optimum cMPP capture under saturating conditions was tested by exposing identical primer-linked microcapillary tubes to specific cMPP for 20, 40, and 60 min prior to washing and microfluorimetric RCA. No significant difference between association times was indicated by these tests, and so a 20-min association period was adopted for cMPP annealing to microcapillary-bound capture primers for all subsequent tests.

Microfluorometric measurements

Fluorescence analysis of nucleic acid product bound to the inner surfaces of microcapillary tubes was performed using an Olympus BX-51 upright fluorescence microscope (Optical Analysis Corporation, Nashua, NH) equipped with a 100-W Hg arc lamp, epifluorescence illuminator, ×10 objective lens, Spot-2 cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI), dual PMT detectors (Photon Technologies Intl.,

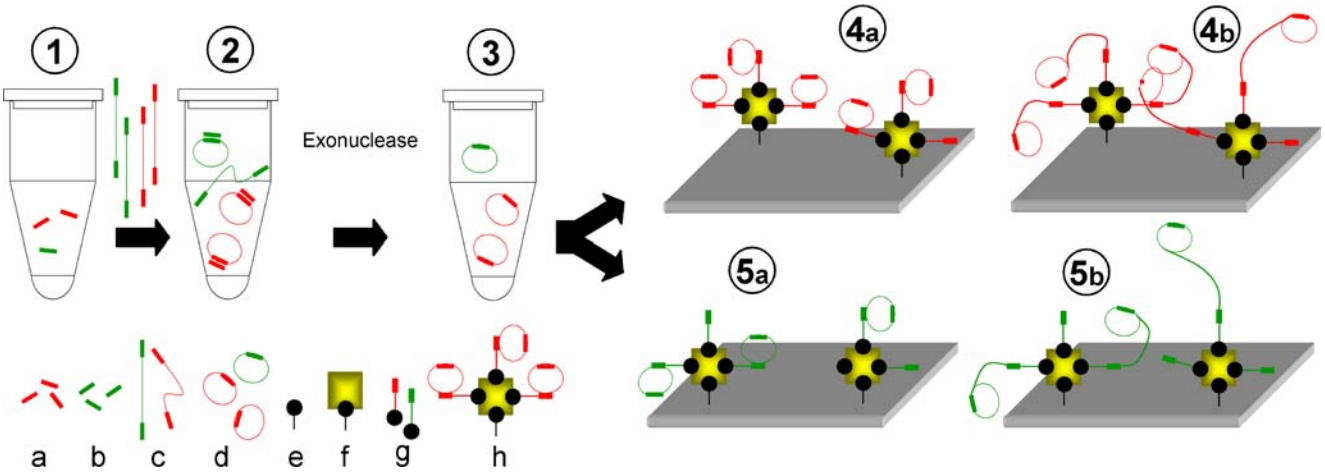

Fig. 1 Microcapillary-based target detection. General steps in cMPP formation and microcapillary surface-associated RCA. *1* Tube containing *IHN-Target* (*red*) and *ISA-Target* (*green*); *2* annealing of MPP with specific target only, followed by ligation to form cMPP; *3* exonuclease digestion to remove excess single-stranded MPP and target, leaving only cMPP product; *4a* capture of cMPP formed upon recognition of *IHN-Target* by biotinylated *IHN-Primer* bound to surface-associated NeutrAvidin; *4b* RCA of captured cMPP; *5a* capture of cMPP formed upon recognition of *ISA-Target* by biotinylated *ISA-Primer* bound to surface-associated NeutrAvidin; *5b* RCA of captured cMPP. Key to symbols: *a IHN-Target* oligonucleotides (*red*), *b ISA-Target* oligonucleotides (*green*), *c* specific linear *IHN-MPP* (*red*) and ISA-MPP (*green*) oligonucleotides, *d* annealed and ligated cMPP, *e* NHS-LC-LC-biotin immobilized via $-NH_2$ moieties in glass microcapillary tubes, *f* NeutrAvidin bound monovalently to surface biotin, *g* biotinylated primer oligonucleotides, *h* complete surface structure with immobilized cMPP prior to RCA

Princeton, NJ), MS-2000 computer-controlled servo-driven 3-axis stage (Applied Scientific Instruments, Eugene, OR), computer-controlled combination electronic shutter and filter wheel combination (Lambda-10/2, Sutter Instrument Company, Novato, CA), and MetaMorph v6.2r4 digital imaging acquisition and analysis software (Universal Imaging Corp., West Chester, PA). Glass microcapillaries were placed in a home-built black-anodized, grooved aluminum holder for fluorimetric measurement (Fig. 2).

The temperature of reactions carried out on the microscope stage was held at 30±1 °C with a circulating water bath (Model 1197, VWR Scientific, Boston, MA) coupled to a 2.5″×3.5″×0.5″ copper liquid heat exchanger (Model LI-301, Melcor, Trenton, NJ). The capillary tube holder was mounted on the upper surface of the heat exchanger. Changes in the intensity of SYBR Green II nucleic acid stain fluorescence in the microcapillary tubes were quantified by epifluorescence imaging using the cooled CCD camera. The capillary holder was positioned on the stage-mounted heating block to permit fluorescence imaging of each tube sequentially while driving the stage under program control. This configuration permitted the sequential acquisition of data from up to 40 individual microcapillary tubes in a single experiment. A macro (journal) was created in Metamorph to position the stage so that each capillary tube could be imaged in sequence, with a programmable delay between samples and between sets of readings. Microcapillary tubes were illuminated using a 485±12 nm excitation filter, 505 nm dichroic mirror, and 535±12 nm barrier filter (Chroma Technologies, Brattleboro, VT). Each image acquisition involved a short 80-ms camera exposure to minimize photobleaching and ×4 pixel binning of the image of the microcapillary tube. Individual images were acquired over the duration of the experiment and subsequently analyzed as a series by manually selecting an area of the image of each microcapillary tube and

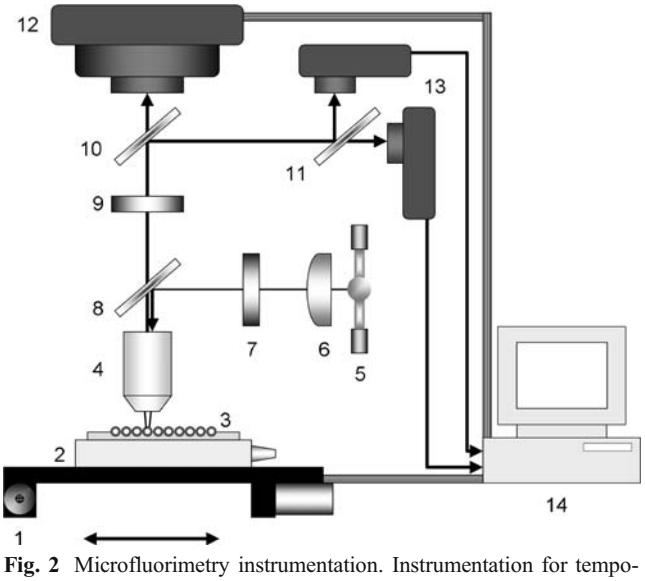

Fig. 2 Microfluorimetry instrumentation. Instrumentation for temporal and spatial measurement of fluorescence in microcapillary tubes:*1* computer-controlled X-Y stage, *2* copper heating block, *3* microcapillary holder with tubes, *4* ×10 objective lens, *5* 100-W Hg arc lamp, *6* condenser lens, *7* excitation filter, *8* dichroic beamsplitter, *9* emission filter, *10* and *11* 50/50 beamsplitters, *12* cooled CCD camera, *13* photomultiplier detectors, *14* personal computer for device control and data acquisition

automatically recording to a file the pixel intensity of this region at each time during acquisition.

PMT fluorescence measurements were obtained by sampling PMT voltage output at 10-ms intervals while driving the stage at a constant speed of 0.47 mm/s. PMT readings were acquired and stored using Felix software (Photon Technology International, Monmouth Junction, NJ). An adjustable rectangular aperture was set to acquire an optical window of approximately 0.1 mm in width.

Results

Detection of surface fluorescence in microcapillary tubes

The fluorescence of the interior fluorescein-labeled surface of the capillary was measured along the length of the capillary, and in both derivatized segments of the tube averaged 1.61±0.24 relative fluorescence units (RFU), while the background fluorescence in the underivatized region averaged 0.24±0.07 RFU (Fig. 3a).

Surface-associated RCA product, measured by microfluorimetric scanning of the cross-sections of tubes after replacing RCA reaction solution with PBS, is shown in Fig. 3b. A representative set of scans is shown that indicates the amount of surface-associated dye remaining in microcapillaries containing *ISA-Primer*, incubated with either the complementary circularized *ISA-MPP* (Fig. 3b, open circles), or non-complementary circularized *IHN-MPP* (Fig. 3b, closed circles), amplified by RCA in the presence of SYBR Green II, and rinsed with PBS. For comparison, a cross-sectional scan of a microcapillary tube containing a 100 nM solution of 5-carboxyfluorescein in PBS was performed to measure the distribution of fluorescence signal under conditions where the majority of fluorophore is in the bulk solution, rather than immobilized on the glass surface (Fig. 3b, inverted triangles/dotted line).

The walls of microcapillary tubes in cross-sectional scanning after removal of SYBR Green II in the RCA reaction solution displayed fluorescence intensity peaks due to the association of fluorescence with the inner surfaces of the capillaries, and the greater contribution of signal from above and below the plane of focus at the outer dimensions of the tubes (Fig. 3b, open circles). Peaks due to surface-bound fluorescent product that were apparent in tubes with immobilized, amplified cMPP product were not observed in tubes in which fluorophore was present solely in solution within the microcapillary tubes. Thus, the former represent surface-bound RCA product.

RCA of MPP immobilized in microcapillary tubes

Surface-associated RCA was carried out as described in cMPP binding and rolling circle amplification and fluorescence of microcapillary tubes measured using the cooled CCD camera (Fig. 4). In order to correct for the intrinsic background fluorescence of the samples, the fluorescence after 2 min was subtracted from all subsequent fluorescence readings.

The initial rate of RCA in microcapillary tubes containing immobilized cMPP was quantified by evaluating the slope of a tangent line to fluorescence data generated within the first 10 min of RCA. The initial rates of generation of fluorescent products were 6.25×10^{-2} RFU/min in the test solution (Fig. 4, closed circles), 3.19×10^{-3} RFU/min in the

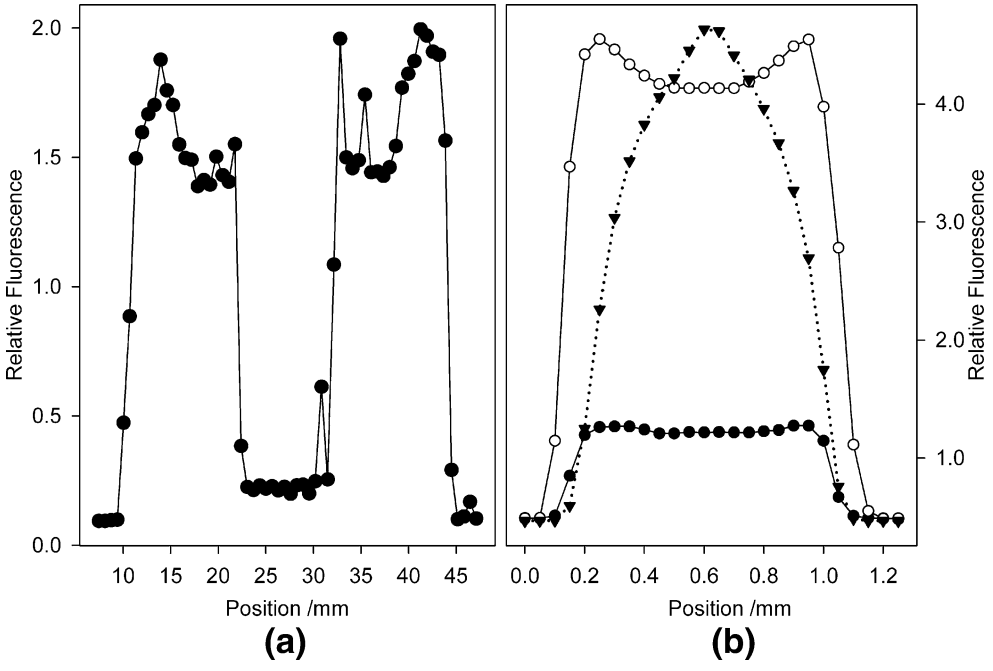

Fig. 3 Spatial resolution in microcapillary surface fluorescence measurements. **a** Fluorescence scan of a 32-mm, 20-μL volume glass microcapillary tube in which the ends only were derivatized with fluorescein-SE, leaving an unlabeled center region. **b** Surface labeling of PBS-rinsed tubes after 30 min RCA of complementary cMPP (*open circles*), or non-complementary cMPP (*closed circles*). The *dotted line* shows the profile of a tube containing fluorescein in the bulk liquid, with no observable peaks in fluorescence at the sides of the tube (bulk measurements were scaled to show only the fluorescence profile and not the relative intensity of bulk and surface fluorescence). All measurements were acquired with a PMT detector

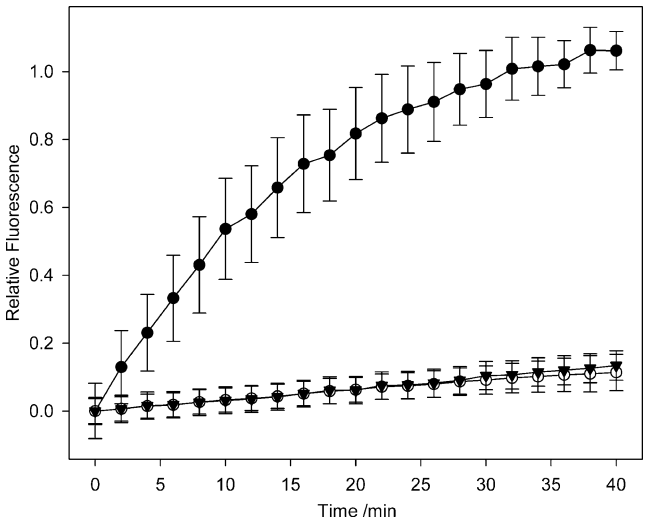

Fig. 4 RCA of MPP immobilized in glass microcapillary tubes. Two sets of 20 μL capillary tubes derivatized with NHS-LC-LC-biotin and treated with NeutrAvidin. Microcapillary tubes were labeled with *IHN-Primer* (open and closed circles), or left untreated (triangles). cMPP were then added (closed circles, triangles) or the microcapillaries were left untreated (open circles). Fluorescence was measured over time using the cooled CCD camera. Fluorescence values after 2 min were subtracted from all fluorescence readings. Error bars represent the standard deviations of readings from triplicate microcapillary tubes

no primer control (Fig. 4, triangles) and 3.00×10^{-3} RFU/min in the no padlock control (Fig. 4, open circles). The ratio of the average rate of specific product generation to average nonspecific amplification was ~20.2.

Discrimination of specific cMPPs using selective microcapillary surfaces

Surface-associated RCA was initiated in microcapillary tubes that were labeled at one end with *IHN-Primer* and at the other with *ISA-Primer*, as described in Spatial localization of biorecognition components. After RCA at 30 °C for ~30 min, the reaction buffer was replaced with PBS, and fluorescence peaks were measured at the inner microcapillary walls (Fig. 5). These peak values were representative of the relative level of the surface-associated RCA. Fluorescence measurements from the walls of cross-sectional scans of duplicate adjacent microcapillary tubes (4 peaks per control set) were averaged to demonstrate the capacity for simultaneous detection of two target nucleic acid sequences, proving the concept for future multiplex target detection.

The average background fluorescence from negative controls (neither *ISA-Target* nor *IHN-Target* present) was 1.47±0.166 RFU on surfaces derivatized with *ISA-Primer*, and 1.401±0.135 RFU on surfaces derivatized with *IHN-Primer*. Figure 5 represents the experimental data with these background signals subtracted. Amplified cMPP made from a mixture of ISA-MPP and IHN-MPP in the presence of *IHN-Target* (Fig. 5, IHN-Target) had an average fluorescence of 1.96±0.154 RFU higher than background when incubated on surfaces derivatized with *IHN-Primer*, but displayed negligible fluorescence on surfaces derivatized with *ISA-Primer*. The results were comparable to cMPP formed by only *IHN-MPP* in the presence of *IHN-Target* (Fig. 5, IHN-Target*). Amplified cMPP formed to *ISA-Target* in the presence of both *ISA-MPP* and *IHN-MPP* were 3.07±0.171 RFU higher than background on the microcapillary segments derivatized with *ISA-Primer*, but displayed negligible fluorescence on those derivatized with *IHN-Primer* (Fig. 5, ISA-Target). As expected, cMPP formed by the combination of *IHN-MPP* and *ISA-MPP* in the presence of *IHN-Target* and *ISA-Target* underwent surface-associated RCA on both

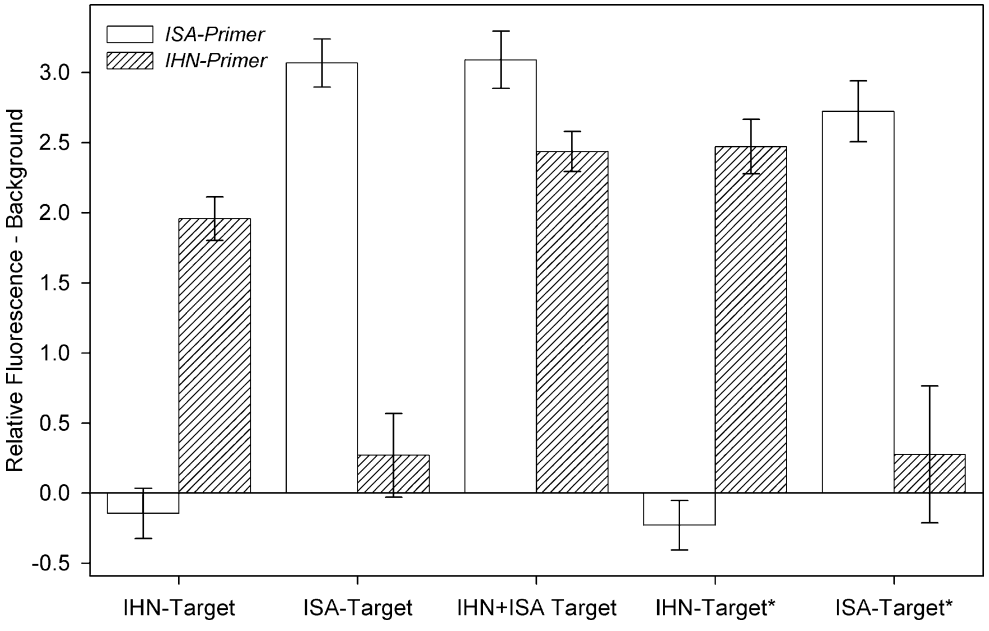

Fig. 5 Generation of cMPP directed against both *ISA-Target* and *IHN-Target* in a single reaction. Selective capture was performed in microcapillaries with single bound capture primers for cMPP formed in response to *ISA-Target,IHN-Target*, or a mixture of both target oligonucleotides. Target sequence represents the target present in the cMPP-generating reaction and *ISA-Primer* or *IHN-Primer* represents the primer immobilized in the microcapillary tube. *Error bars* represent the standard deviations of two measurements performed on duplicate microcapillary tubes. *Formation of cMPP with only the complementary MPP and target pair

surfaces (Fig. 5, IHN+ISA Target). Slightly greater fluorescence was observed for amplified cMPP formed to *ISA-Target* (3.09±0.205 RFU) compared with amplified cMPP formed to *IHN-Target* (2.44±0.143 RFU). These results indicate that the cMPP formation process is specific and can be adapted to use with more than one probe for the detection of multiple nucleic acid sequences, and that RCA is initiated selectively by binding of cMPP by primers bound to the inner surfaces of the microcapillary tubes.

Discussion

The feasibility of using microcapillary tube-immobilized cMPP in conjunction with RCA and fluorescence detection has been demonstrated. Prior work by Millard et al. [19] showed that IHN and ISA virus RNA-specific cMPP could be generated in response to RNA extracted from isolated virus, RNA from virus-infected fish tissue, or DNA oligonucleotides with base sequences identical to those selected from viral RNA. It is shown here that gas-phase derivatization of microcapillary tubes yielded an effective density of amine moieties, which could be reacted with fluorescein-SE or NHS-LC-LC-biotin under mild conditions.

Reaction of surface amine moieties with NHS-LC-LC-biotin resulted in a sufficient number of NeutrAvidin binding sites to permit surface association of biotinylated primer oligonucleotides for the capture and amplification of circular, ligated MPP. Following recognition of target oligonucleotides by MPP, exonuclease treatment of the ligation mixture was always carried out to eliminate linear DNA, including excess MPP, thus permitting only cMPP to be captured by immobilized primer oligonucleotides and subsequently amplified by RCA. A 9.3-fold enhancement in total fluorescence after 40 min, and a 20-fold increase in reaction rate above that in identical microcapillary tubes without surface-associated capture/primer oligonucleotides within 10 min showed that the method can be used effectively as an end-point assay in less than 1 h, or in a kinetic mode to yield results within a significantly shorter period of time. Conventional methods of detection of ISAV and IHNV include virus isolation (VI), indirect immunofluorescent antibody testing (IFAT) and PCR in combination with a reverse transcriptase (RT-PCR). Diagnostic procedures involving VI, while highly accurate, are typically very time consuming and expensive to conduct. IFAT is a low-cost method, but lacks the selectivity inherent in RNA sequence detection methods. A complete assay for ISAV detection using RT-PCR that required only 80 min was reported by Munir et al. [28]. The time requirement for the MPP/RCA technique described here, including cMPP formation, capture of cMPP on the prepared surface, and kinetic fluorescence measurement, is approximately 2 h. The lack of a detectable difference in the amount of RCA product formed when cMPP capture incubation time was varied between 20 min and 60 min suggests that this annealing step can be carried out in a more abbreviated format, further contributing to the rapidity of the measurement.

The incorporation of fluorophore-labeled deoxyribonucleotides into the growing RCA concatamers could serve as an alternative to the incorporation of SYBR Green II nucleic acid stain, thereby eliminating one reagent from the assay. However, the fluorescence enhancement observed when the SYBR stain goes from the unbound to the DNA-bound state facilitates the kinetic assay by permitting continuous fluorescence measurements to be made. This mode of kinetic data acquisition would not be possible by measuring fluorescent dNTP incorporation unless the bulk fluorescence of the reaction mixture could be eliminated from the measurement.

The ability to discriminate between closely related strains of pathogens is an important aspect of a molecular detection system, permitting more general use and greatly reducing the cost of each analysis. The feasibility of an approach in which a number of MPPs are used simultaneously for recognition of multiple target sequences, in conjunction with a detection system employing specific immobilized capture oligonucleotides, is supported by experiments in which only cMPP formed in response to *IHN-MPP* or *ISA-MPP* were bound by microcapillaries in defined regions decorated with their respective primers. The ability of MPP to discriminate closely related viral strains is supported by the finding that even single base substitutions can be discriminated [17], results that have been confirmed in the experimental system used here for the detection of ISAV and IHNV [19]. It was noted that the RCA of *ISA-Target* cMPP was more efficient than RCA of cMPP formed to *IHN-Target* (ca. 26% increase, Discrimination of specific cMPPs using selective microcapillary surfaces). This difference could have been due to a change in hybridization efficiency resulting from the differences in base composition of the different capture oligonucleotides on the surface [29]; however, RCA was clearly observed in either case. By designing a range of MPP molecules, each with a different primer/capture oligonucleotide sequence, it will be possible to perform a number of diagnostic tests simultaneously.

When surface labeling with fluorescein was controlled by partial filling of amine-derivatized microcapillaries with fluorescein-SE, the position within the microcapillary tube of fluorescein derivatization was spatially resolved with a complete transition between the unlabeled and labeled region occurring in less than 0.85 mm

(0.15 mm from minimum to half-maximum value), measured using a low power (×10) microscope objective lens. Spatial localization of the surface-associated fluorescent signal was indicative of labeling patterns that were subsequently generated with RCA primers specific for virus-directed cMPP. Uniform surface labeling with biotin and NeutrAvidin, followed by spatially separated labeling with biotinylated capture primers for cMPP formed to *IHN-Target* or *ISA-Target*, was an effective means to discriminate the presence of these two cMPP products within a single microcapillary. The success of this labeling procedure illustrates the feasibility of using this approach for multiplex virus detection within a single microcapillary tube.

Earlier work by Millard et al. [19] involving detection of cMPP in solution demonstrated sensitivity to as few as 10^4 target molecules. RCA within microcapillary tubes has been demonstrated using a cMPP concentration, based on complete circularization of MPP, of 5 nM (6×10^{10} target molecules in a 20-μL microcapillary). Assuming even distribution of the cMPP along the length of the microcapillary (32 mm), this corresponds to about 2×10^9 cMPP/mm. The measurement system described, with a standard fluorescence microscope and measurement window size of approximately 0.1 mm^2, was therefore capable of measuring about 5×10^6 cMPP undergoing RCA under standard conditions. With further optimization of capillary size and geometry, surface structure and biochemistry, and fluorescence collection optics, it is expected that microcapillary-based assays will exhibit similar sensitivity for ISA and IHN virus detection to that observed in solution-based assays. While RT-PCR has already been demonstrated to achieve high sensitivity for ISAV detection, the technique has been widely reported to yield variable results in sensing applications, with a relatively high frequency of false-positive results [30, 31].

Based on earlier studies [23], it is expected that the sensitivity of MPP/RCA on surfaces will be largely dependent on the rate of diffusion of cMPP to the surface-immobilized oligonucleotide primers and the time permitted for this to occur. Hence, the size and dimensions of derivatized glass microcapillary tubes are important factors limiting the sensitivity of the method. The microcapillaries used here, capable of holding up to 20-μL reagent volumes, had an inner diameter of 0.89 mm, so the maximum diffusion distance to a wall for a molecule under static conditions is equal to the radius of the tube, 0.445 mm. Diffusion time is commonly expressed by the equation: $\langle x^2\rangle = 6\cdot D\cdot t$ (for a random walk in 3 dimensions), where x is the mean displacement of a particle in solution, D is the diffusion coefficient, and t is the diffusion time. The diffusion coefficient (D_C) for a cMPP can be estimated based on the value for linear oligonucleotides (D_L) of similar length, 4.04±17 ($\times10^{-7}$ cm^2/s) [32], and a linear dependence on topology, $C=D_C/D_L=1.32\pm0.014$ [33]. Using a calculated D_C for cMPP of 5.33±0.043 ($\times10^{-7}$ cm^2/s) the maximum diffusion time is approximately 10.3 min, which is less than the shortest incubation time of 20 min used throughout this work. It may be concluded that complete diffusion has taken place after 20 min under these conditions.

The preceding calculations assume a sub-saturating amount of cMPP on the glass surface. Previous research has shown glass surfaces to be capable of accommodating 10^{12}–10^{13} oligonucleotide probes per cm^2 [34, 35]. The cMPP concentration used, based on complete circularization of MPP, was 5 nM, or 6×10^{10} cMPP in the 20-μL sample volume, so sub-saturation of the surface is most likely a valid assumption. It is expected that the required incubation times could be reduced substantially by using higher concentrations of molecular components, in excess of the surface binding sites, or by using microcapillary tubes with smaller inner diameters. Since diffusion time is proportional to the square of the distance traveled, a reduction of the inner diameter by a factor of two should reduce the time requirement to about 2.6 min. The surface area to volume ratio is increased as capillary diameter is decreased, however, leading to further sub-saturation of surface sites. Higher concentrations of cMPP (above the amount required to saturate surface sites) would also reduce the time requirement, since it is expected that molecules near the inner surface would bind first, filling available sites and thereby reducing the *effective* maximum diffusion distance. An approximation of concentration-dependent diffusion distance was found by subtracting the radius of a cylinder containing the volume of molecules in excess of surface sites from the effective radius of the microcapillary. A two-fold increase in cMPP concentration using this model leads to a 70.7% decrease in the diffusion length, dropping the diffusion time to about 53.2 s. It is clearly advantageous to work with concentrations greater than those required for surface saturation to reduce the diffusion time, but this would likely necessitate a separate amplification step. Furthermore, densely packed surfaces may be prone to inefficient hybridization [36], or may demonstrate reduced RCA due to steric effects, as has been shown with solid-phase PCR [5]. The tradeoff between miniaturization and concentration effects must be considered to determine optimal physical dimensions of microcapillaries for rapid binding and measurement. The 20-μL reagent volume may be easily reduced by at least 10-fold by using smaller microcapillaries. The cost of the present assay based on the costs of components is already minimal, estimated at about 50¢ per assay, regardless of the number of individual sequences detected simultaneously.

Conclusion

A novel approach to the detection of specific nucleic acid sequences in pathogens has been demonstrated. The use of MPP, in conjunction with immobilized capture oligonucleotides in a microcapillary-based fluorescence assay, will be useful for a range of microbial analyses. The surface-associated RCA technique described here provides the basis for future development of biosensors employing rapid molecular recognition, amplification, and detection of multiple targets with surface-bound oligonucleotides that recognize a variety of unique target sequences simultaneously. The method described here provides the basis for future development of field-adaptable devices for multiplex detection of a variety of viral, bacterial, and protozoan pathogens.

Acknowledgements We gratefully acknowledge Drs. Carl Tripp and Ben McCool for their help with procedures for amine derivatization of glass microcapillaries, and Dr. Carol Kim for helpful discussion and reading of the manuscript. This work was supported in part by Maine Sea Grant NA16RG1034 and National Science Foundation grants ECS-0329913 and EEC-0452021.

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