Functionalized \( \text{Co}_2\text{FeAl} \) Nanoparticles for Detection of SARS CoV-2 Based on Reverse Transcriptase Loop-Mediated Isothermal Amplification

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ABSTRACT: Loop-mediated isothermal amplification (LAMP) is a sensitive, efficient, and rapid nucleic acid amplification technique resulting in a large number of amplicons; however, it suffers from a high incidence of false positives due to carry-over and aerosol. Herein, we report a \( 10 \) min nano-capture system that is used in conjunction with a modified reverse transcriptase-LAMP (RT-LAMP) assay for the accurate detection of SARS CoV-2 virus. The nano-capture system employs in-house-designed probe-functionalized magnetic nanoparticles \( \text{Co}_2\text{FeAl} \) (cobalt-based Heusler alloy) for efficient capture of contaminating amplicons from the reaction mixture preceding RT-LAMP. The nano-cleaned RT-LAMP assay along with engineered primers successfully detected the presence of \( 10 \) copies of SARS CoV-2 virus while completely eliminating the incidence of false positives. The presented contaminant-capture method has been compared with other approaches for elimination of contaminants and was found to be more effective. The insight brought in this work is the design of a rapid nano-capture system that hybridizes with contaminating amplicons (carry-over) with high specificity to enable easy removal from the assay for elimination of false positives. The method has been proven to be successful for RT-LAMP assays in the rapid and highly specific detection of SARS CoV-2, which is currently a major challenge for global health. To the best of our knowledge, this is the first work involving a nano-based cleaning strategy for reliable and rapid diagnosis using isothermal amplification approaches.

KEYWORDS: SARS CoV-2, false positives, \( \text{Co}_2\text{FeAl} \) nanoparticles, Heusler alloy, RT-LAMP, contaminant capturing

1. INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS CoV-2) is a recently identified viral infection that turned into a pandemic, causing infections to over \( 100 \) million people and leading to \( 2 \) million fatalities as of February 2021. A large demand for diagnostic assays for mass screening and management of COVID-19 has led to the development of numerous molecular and serological methods. Though laboratory-based reverse transcriptase-polymerase chain reaction tests remain the gold standard for confirmation of SARS CoV-2 infection, many molecular diagnostic methods including reverse transcriptase-loop-mediated isothermal amplification (RT-LAMP) assays have been reported. However, we strongly believe that field deployment of these RT-LAMP assays is challenged by the high incidence of false positives, just like in the case of other LAMP assays explored for viral detections.

LAMP is a rapid and ultrasensitive nucleic acid amplification technique, which allows DNA amplification at a constant temperature. Strong strand displacement activity (\( 5'\rightarrow 3' \)) of \( \text{Bst} \) polymerase enzyme enables isothermal amplification and makes the technique suitable for field applications and point-of-care devices. LAMP offers a \( 100 \)-fold increase in amplification efficiency well within an hour in comparison with polymerase chain reaction (PCR), recombinase polymerase amplification, strand displacement amplification, helicase-dependent amplification, etc. Using a premix of suitable reverse transcriptase enzyme along with LAMP reagents enables amplification of target nucleic acid sequences and subsequent detection of viruses in a single-step reaction.

The underlying high amplification efficiency in LAMP assays leads to rapid accumulation of inverted repeats bearing cauliflower-type structures of amplicons, which can be promptly used as a template in subsequent reactions. However, these amplicons have been reported to contaminate the reagents in the laboratories via aerosols up to weeks, thus jeopardizing the

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outcome of subsequent reactions. Once contamination begins, all the reagents need to be changed or else the assay needs new primers, thus making the LAMP technique very susceptible and fragile. In short, the commercial feasibility of LAMP/RT-LAMP-based assays in the field is limited due to the high incidence of false-positive amplifications, a fact well recognized in the literature. Over the years, researchers have proposed and validated innovative approaches to reduce false-positive amplifications.

Closed-tube monitoring methods have been reported where all reagents required for both amplification and detection (such as intercalating dyes or pH change indicators) are incorporated in the same reaction tube, thereby avoiding the need for opening of the vial post amplification. The method was found to be effective in reducing the contaminants, though it could not eliminate the false positives. However, the method does not allow for distinction of the target amplicons (product) from primer dimers or spurious amplicons. In another well-known method, researchers have employed uracil-DNA-glycosylase (UDG) enzymes for degradation of the contaminants during the primer extension reaction. However, the method does not eliminate the false positives. However, the method does not allow for distinction of the target amplicons (product) from primer dimers or spurious amplicons. In another well-known method, researchers have employed uracil-DNA-glycosylase (UDG) enzymes for degradation of the contaminants during a prereaction protocol (at the site of deliberately incorporated uracil residues). Modifications to this method, such as same-pot amplification and degradation (thus bypassing the need of separate tubes), have also been explored, though the complexity of the system along with residual UTPs (in the reaction mixture) affects the amplification efficiency. Ma et al. in 2017 reported a restriction endonuclease-based method for isolating and removing carry-over contamination in isothermal amplification reactions by an addition of restriction sites to the sequences of forward inner primers (FIP) to enable digestion of contaminants possessing the restriction site in a prereaction step. However, inclusion of restriction site sequences complicates the primer design by restricting the design parameters. The proposed methodology involves digestion of amplicons bearing the restriction site followed by LAMP amplification. However, due to the high inactivation temperature (85 °C) of restriction endonucleases at the end of the preamplification step, Bst enzymes are excluded to avoid denaturation. In other words, the contamination introduced via the LAMP enzyme cannot be eliminated.

Recently, a contamination-free LAMP method was reported that utilizes a CRISPR/Cas9 cleavage technique post incorporation of protospacer-adjacent-motif (PAM) sites in LAMP amplicons by modified primer sequences. The method was based on the engineered spCas9 protein capable of recognizing NG-PAM. Although the report showed specific cleavage of LAMP amplicons (Zika virus detection assay), the use of CRISPR/Cas9 biology and engineered primers makes the system complex, time-consuming, and unsuitable for point-of-care devices. Nonetheless, new innovative approaches with less complexity and steps are warranted to achieve a robust LAMP-based assay suitable for field applications in a reliable manner.

Magnetic nanoparticles have been earlier employed for enriching bioanalytes in many diagnostic assays and biomedical applications. However, the applications for capturing of target-specific nucleic acid sequences in diagnostics have not been explored yet. Heusler alloys are the ternary intermetallic compounds with a stoichiometric composition of A2BC, where A and B represent transition elements such as Fe, Co, Ni, Mn, Cu, V, Ti, etc., while C indicates a main group element from group III, IV, or V including Ga, Al, Ge, In, As, etc. Nanoparticles of Heusler alloys are highly magnetic and are less studied in biological applications. Heusler alloys show 100% spin polarization at room temperature and, above, high Curie temperature, and large magnetic moments, though none of the constituent elements are ferromagnetic. Among these, cobalt-based Heusler compounds show significant magnetic moments and hence find applications in spintronics, ferromagnetic shape memory alloys, and thermoelectric and topological insulators. A change in the dimensions from a macro- to nanoscale leads to changes in the magnetic properties due to the transition from multidomains to single domains, a behavior well utilized in storage devices and other applications. In this work, we report synthesis and surface functionalization of nanoparticles of the Co3FeAl alloy, which showed significant magnetic moment at room temperature.

Here, we report a robust, contaminant-free RT-LAMP assay for detection of SARS CoV-2. An RT-LAMP assay for robust detection of SARS CoV-2 was designed with modified primer sequences that introduce specific linker sequences in the amplicons (potential carry-over and aerosol contaminants) during amplification. These amplicons were later targeted using in-house-developed oligonucleotide antisense probes that were surface-functionalized on highly magnetic nanoparticles of Co3FeAl. The 10 min nano-capture system concentrates and removes the contaminants using a simple magnet. The work showcases the first-of-its-kind, easily deployable, clean RT-LAMP assay to tackle false positives.

2. MATERIALS AND METHODS

2.1. Apparatus and Reagents. A constant-temperature incubator, real-time PCR system (BioRad Inc.), oscillator, electrophoresis agarose gel system (BioRad Inc., CA, USA), centrifuge, gel doc system, NanoDrop 2000, constant-temperature heat blocks, and UV–visible spectrophotometer (Thermo Scientific, USA) were used. The real-time RT-LAMP master mix was supplied by New England Biolabs Inc. (MA, USA); the magnetic stirrer, dialysis bags, tetraethyl orthosilicate (TEOS), and 3-(aminopropyl)-triethoxysilane (APTES) were procured from Sigma-Aldrich Corp. (MO, USA). Ethanol was purchased from Himedia (Himedia, India). All the primer sequences for LAMP assays as well as 5′ amino-modified probes with a C6 modifier were custom-synthesized from Integrated DNA Technologies, Inc. (IA, USA). The Twist RNA control for SARS CoV-2 genome was obtained from Twist Bioscience (CA, USA).

2.2. Primer Design. A conserved domain sequence within the N gene of SARS CoV-2 was identified at position 28,285–28,529 base pairs by multiple sequence alignment (NCBI MSA tool) of the genome. Using a reference sequence for SARS CoV-2 (Gen-Bank accession no: NC_045512) and PrimerExplorer V5 (Eiken Genome Co. Ltd.) software, the initial primers for LAMP assays were obtained. Six sets of primers, viz., forward outer (F3), backward outer (B3), forward inner primer (FIP), backward inner primer (BIP), loop forward (LF), and loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1). Engineered inner primer sets with variable T-loop backward (LB), were designed using a two-step process with default settings (Table 1).

Linker sequences of different numbers of thymidine residues (T-4 to T-16) were placed between F2 and F1c in the case of FIP and B2 and B1c in the case of BIP (Figure 1). The BLAST program was used for verification of primer specificity prior to experimentation.

2.3. Real-Time RT-LAMP Reactions for SARS CoV-2 Detection. All RT-LAMP reactions were set up in a 15 μL reaction volume (7.5 μL of 2× master mix, 3 μL of 5× primer mix, 0.3 μL of fluorescent dye, 0.2 μL of nuclease free water (NFW), and 4 μL of template) in 96-well PCR plates. The real-time amplification was tracked using real-time PCR equipment (CFX96, BioRad Inc., CA, USA). The thermal profile for amplification and melt curve are briefly described in Table S1.

2.4. Effect of T-Linker Lengths. Different primer mixes with different inner primers (FIP and BIP) having T-0 (no linker), T-4, T-6, T-8, T-10, T-12, T-14, or T-16 were mixed with F3, B3, LF, and LB primers (Table 1). Multiple RT-LAMP reactions were set up as...
Table 1. List of Primer Sequences Designed Using PrimerExplorer v5 for RT-LAMP-Based Detection of SARS CoV-2 Infection

| Primer ID | Sequence (5′–3′) |
|-----------|-----------------|
| F3-N      | TGGATCCGGCAGCCACTG-TTTTATTACCAGTTGGTGGAGCTC |
| B3-N      | AGCCTATTTGGTGGAGCCCT |
| FIP-N     | TGGTTTGATCCGGCCACCCG-ATTACGGTGTGGTGGACCCCTC |
| BIP-N     | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| LF-N      | CTGGTTACTGCGTGGAGAC |
| LB-N      | TCTACCTCAGAATGGCAAGGAA |

Engineered primers

| FIP-N-4  | TGGTTTGATCCGGCCACCCG-ATTTATTACCAGTTGGTGGAGCTC |
| BIP-N-4  | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-6  | TGGTTTGATCCGGCCACCCG-ATTATTACCAGTTGGTGGAGCTC |
| BIP-N-6  | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-8  | TGGTTTGATCCGGCCACCCG-ATTATTATTACCAGTTGGTGGAGCTC |
| BIP-N-8  | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-10 | TGGTTTGATCCGGCCACCCG-ATTATTATTATTACCAGTTGGTGGAGCTC |
| BIP-N-10 | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-12 | TGGTTTGATCCGGCCACCCG-ATTATTATTATTATTACCAGTTGGTGGAGCTC |
| BIP-N-12 | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-14 | TGGTTTGATCCGGCCACCCG-ATTATTATTATTATTATTACCAGTTGGTGGAGCTC |
| BIP-N-14 | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-16 | TGGTTTGATCCGGCCACCCG-ATTATTATTATTATTATTATTATTACCAGTTGGTGGAGCTC |
| BIP-N-16 | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |

Engineered primers

| FIP-N-4  | TGGTTTGATCCGGCCACCCG-ATTATTACCAGTTGGTGGAGCTC |
| BIP-N-4  | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-6  | TGGTTTGATCCGGCCACCCG-ATTATTACCAGTTGGTGGAGCTC |
| BIP-N-6  | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-8  | TGGTTTGATCCGGCCACCCG-ATTATTATTACCAGTTGGTGGAGCTC |
| BIP-N-8  | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-10 | TGGTTTGATCCGGCCACCCG-ATTATTATTATTATTACCAGTTGGTGGAGCTC |
| BIP-N-10 | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-12 | TGGTTTGATCCGGCCACCCG-ATTATTATTATTATTATTATTATTATTACCAGTTGGTGGAGCTC |
| BIP-N-12 | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-14 | TGGTTTGATCCGGCCACCCG-ATTATTATTATTATTATTATTATTATTATTATTATTATTACCAGTTGGTGGAGCTC |
| BIP-N-14 | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |
| FIP-N-16 | TGGTTTGATCCGGCCACCCG-ATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTACCAGTTGGTGGAGCTC |
| BIP-N-16 | ATACCTGCTGTGGACCAACCG-ATTGGAAGCCTTGTTCCTC |

As described earlier using these different primer mixes in the same reaction plate. Based on preliminary reactions, all the RT-LAMP assays were run for 45 min at 60 °C. The fluorescence changes were recorded using a BioRad CFX96 machine. For assessing the optimal length of the linker, RT-LAMP profiles of all the reactions were compared.

2.5. Limit of Detection (LOD) Assays for SARS CoV-2 Detection. RT-LAMP reactions as described above were set up for determining the limit of detection for the assay using varied copies of Twist genomic RNAs of SARS CoV-2. As per the manufacturer’s details, the Twist RNA control was supplied at a concentration of 10^6 copies/μL SARS CoV-2 ssRNA genome (fragmented in 5000 bp fragments). Accordingly, a series of dilutions were made for the control RNA from 25,000 to 0.025 copies/μL so that reactions contain 10^3–10^10 copies and template LOD was determined for both normal primer set and primer set with a T-10 linker in FIP and BIP (Table 1).

2.6. Design of Antisense Probes. As described earlier, primers were engineered by incorporating different linker sequences. Such placement of linker sequences not only provides flexibility for inner primers’ binding but also gives an opportunity for antisense probes that specifically target and bind to amplicons from earlier LAMP reactions. The antisense ssDNA probes were designed to target the linker region along with the flanking 4–8 base pairs (Table 2). Three sets of four antisense probes (P-FIP, P-BIP, P-FIPr, and P-BIPr) were designed to target the amplicons inclusive of linkers T-6, T-10, and T-14, respectively. For enabling conjugation of probes to nanoparticles, S’-amino modification was incorporated in the probes.

2.7. Synthesis of Co2FeAl Nanoparticles. Nanoparticles of Co2FeAl were prepared by a coprecipitation method. Precursors used as are as follows: 5 mol of cobalt(II) chloride hexahydrate (CoCl2·6H2O), 2.5 mmol of iron(III) nitrate nonahydrate (Fe(NO3)3·9H2O), and 2.5 mol of aluminum(III) chloride hexahydrate (AlCl3·6H2O). A solution in 10 mL of distilled water was prepared of each precursor separately. The prepared solutions were mixed while stirring, while the pH of the solution was adjusted to ~7.0 by slowly adding NaOH in order to produce the precipitation of the metal ions. The solution was filtered, washed with distilled water, and subsequently dried overnight. The dried solid was ground and annealed up to 800 °C for 5 h with a heating rate of 300 °C/h under a gas mixture of 85% of argon and 15% of H2.

2.8. Surface Modification of Co2FeAl HNPs and Their Characterization. Co2FeAl nanoparticles were surface-decorated with ampiclon-capturing probes through a series of chemical conjugation steps. A detailed protocol for serial surface modification of nanoparticles and final probe conjugation is given in the Supporting Information. Briefly, 10 mg of modified nanoparticles was mixed with 77 nmol of NH2-modified probes (i.e., T6-FIP (19.5 nmol), T6-BIP (19.5 nmol), T6-FIPr (19.5 nmol), and T6-BIPr (19.5 nmol) for T-6 probe conjugation) in an EDC/NHS catalyzed reaction. The probe-functionalized particles shall be termed as Fn-HNP (T-6), Fn-HNP (T-10), and Fn-HNP (T-14), respectively, for the remainder of the manuscript. The modified nanoparticles were then subjected to three cycles of washing and redispersion in ethanol to remove excess reagents and dried overnight under vacuum.

2.9. Surface Modification of Fe2O3 Nanoparticles. For the purpose of comparison, commercially available iron oxide nanoparticles (Nanoshell Inc., USA) were procured and surface-conjugated with T-6, T-10, and T-14 probes. Iron oxide particles were reacted with APTES for amino functionalization as described earlier. Finally, the EDC/NHS catalyzed reaction as described above was used for attaching probes, and Fn-ION (T-6), Fn-ION (T-10), and Fn-ION (T-14) were prepared.

2.10. Characterization of the Size, Surface Charge, and Lattice Structure of HNPs. Co2FeAl NPs were synthesized by coprecipitation methods, and the sizes of nanoparticles were characterized by transmission electron microscopy (TEM). HNP powder was dissolved in water to prepare a 5 mg/mL solution of suspended nanoparticles, which was then drop-casted on a copper grid for TEM imaging. Imaging was performed using a JEM-1400 TEM instrument (JEOL USA Inc.), and multiple fields were captured at a magnification scale of 200 nm. ImageJ was used for estimation of the size of nanoparticles. Experiments were conducted in duplicate. Surface charges of HNPs were estimated by measuring the zeta potential at a concentration of 5 mg/mL using a Zetasizer ZS90 (Malvern Instrument, U.K.) at a fixed angle of 90°. XRD of the prepared samples of Co2FeAl was performed using Cu Kα (λ = 1.5406 Å). The range of 2θ was taken from 10 to 90°.

2.11. Magnetic Moment Characterization of Basic and Surface-Modified HNPs. Magnetic moment measurements for bare and modified HNPs were performed using a physical property measurement system (PPMS EVERCOOL-H) from Quantum Design, USA. M–H measurements for the nanoparticles were recorded at room temperature (18 °C) and at a magnetic field of −1 to 1 Tesla.

2.12. Simulating Carry-Over Contamination and Validating the Capturing of Amplicons. For simulating the carry-over contamination, the products of the RT-LAMP reaction (10^6 copies of Twist RNA were amplified using T-10-modified primers) were diluted to 10 and 100 times. RT-LAMP products were incubated with different concentrations of Fn-HNP (T-10) (5, 25, 100, and 300 ng/μL)
and 1 μg/μL) at 45 °C for 10 min. Diluted LAMP products without HNP treatment served as the control. After incubation and magnetic separation, the supernatant was transferred to fresh tubes. Finally, 2 μL of 100X SYBR dye was added to each tube and fluorescence intensity was recorded using a Victor Nivo plate reader (Perkin Elmer USA Inc.).

2.13. Elimination of the Carry-Over Contamination System Using Functionalized Particles. To evaluate the working of the proposed contamination capture system (functionalized nanoparticles) in reducing false positives in an RT-LAMP reaction, RT-LAMP reactions (positive and negative) with Fn-HNPs and Fn-IONs were conducted. A 13.5 μL reaction mix (7.5 μL of NEB MM, 3 μL of PM, 0.3 μL of dye, 0.2 μL of NFW, and 2.5 μL of NFW/Twist RNA) was treated with 1.5 μL of functionalized nanoparticles (1 μg/μL) so that the final concentration of particles is 100 ng/μL in the reaction. Capturing of contamination was performed by incubating the reactions at 45 °C for 10 min with intermittent mixing followed by magnetic pull down and transferring the reaction mix to fresh PCR tubes. For comparison with a UDG-based system, an RT-LAMP reaction mixture with individual components having dUTP, UDG, and MuLV (reverse transcriptase) was prepared (Table S2). UDG-mediated digestion of contamination was allowed at room temperature for 5 min. Untreated LAMP reactions of 15 μL were set up as described earlier and served as the control. The complete assay was performed using T-6-, T-10-, and T-14-modified primers after cleaning the reactions with Fn-HNP (T-6), Fn-HNP (T-10), and Fn-HNP (T-14), respectively. All the positive RT-LAMP reactions were set up using 1000 copies of the Twist RNA control under conditions as described above.

3. RESULTS AND DISCUSSION

An RT-LAMP assay for the detection of SARS CoV-2 infection was designed (Figure 1) by identifying 359 bases of conserved domain in SARS CoV-2 genome (2828−28,645 bases) through multiple sequence alignment of various isolates of SARS CoV-2 from across the globe (Figure S1). RT-LAMP assay conditions using normal primers were standardized, and typical amplifica-
tion curves for N gene amplification are shown in Figure 2. As seen from Figure 2a−c, the assay was able to amplify and detect 10 copies of N gene of SARS CoV-2 (limit of detection) in 45 min. The heat map shown in Figure 2b indicates the sensitivity and dynamic range of quantification of SARS CoV-2 copy numbers. It is imperative to mention here that false-positive

| probe ID    | sequence (5′−3′)           | target                                      | GC content (%) | Tₘ (°C) | average Tₘ |
|-------------|----------------------------|---------------------------------------------|----------------|---------|------------|
| P-FIP-T6    | CACGTGGTTTTTTATTACG        | T-6 primer-amplified RT-LAMP products       | 33             | 41.2    | 42.35      |
| P-BIP-T6    | CACCGCTTTTTTATTGGGA        |                                             | 39             | 43.5    |            |
| P-FIPr-T6   | CGTAATAAAAAACACTGG         |                                             | 33             | 41.2    |            |
| P-BIPr-T6   | TCCAATAAAAAAGCGGTG         |                                             | 39             | 43.5    |            |
| P-FIP-T10-L18 | ACTGTTTTTTTTTTATTA       | T-10 primer-amplified RT-LAMP products      | 11             | 32.1    | 35.5       |
| P-BIP-T10-L18 | CCGCTTTTTTTTTATTTG       |                                             | 28             | 38.9    |            |
| P-FIPr-T10-L18 | TAATAAAAAAACAGTT         |                                             | 11             | 32.1    |            |
| P-BIPr-T10-L18 | CAATAAAAAAAAGCGG         |                                             | 28             | 38.9    |            |
| P-FIP-T14   | TGGTTTTTTTTTTTTTAT        | T-14 primer-amplified RT-LAMP products      | 6              | 29.8    | 30.95      |
| P-BIP-T14   | GCTTTTTTTTTTTTTAT         |                                             | 11             | 32.1    |            |
| P-FIPr-T14  | ATAAAAAAAAAACA            |                                             | 6              | 29.8    |            |
| P-BIPr-T14  | ATAAAAAAAAAAAGC           |                                             | 11             | 32.1    |            |

Figure 2. Performance of RT-LAMP-based detection of SARS CoV-2 using normal LAMP primers (without a linker). (a) Limit of detection (LOD) assay for RT-LAMP-based N gene amplification. (b) Heat map showing the sensitivity and dynamic range of quantification of SARS CoV-2 copy numbers. (c) Agarose gel electrophoresis of amplified products. (d) False-positive amplification was observed in negative control samples from the fourth to fifth subsequent runs of the assay.
amplification was observed in negative control samples from the fourth to fifth subsequent runs of the assay due to aerosol contamination or cross contamination in reagents, as seen in Figure 2d. The incidence of false amplification worsened with each run of RT-LAMP assay.

Such observations of false amplification in LAMP and RT-LAMP assays have been related to the accumulation of large numbers of amplicons that cause contamination via aerosol and reagents. The negative impact of carry-over amplicon-related contamination in LAMP assays is well recognized, along with reports on tackling the problem. Methods such as a single closed-tube LAMP/RT-LAMP method, use of restriction endonucleases, use of a UDG system, etc. have shown limited success, though the system involved extensive modifications to the LAMP/RT-LAMP system. This has led to unrepeatability of results, thereby challenging the feasibility for field applications. The present work reports a first-of-its-kind amplicon nano-capturing system prior to RT-LAMP based on engineered LAMP probes on highly magnetic Co2FeAl nanoparticles in the detection of SARS CoV-2. For the sake of

Figure 3. Effect of length of T linkers inserted in primer sequences toward the efficiency of N gene amplification. (a) RT-LAMP curves with different T-linker lengths on RT-LAMP-based amplification of N gene. (b) Agarose gel electrophoresis of RT-LAMP products.

Figure 4. Schematics showing sequential surface modification of Co2FeAl nanoparticles. Amine functionalization of the surface was done through TEOS and APTES conjugation. This was followed by attachment of probes through a succinic acid linker.
clarity, the RT-LAMP products from the previous reactions shall be referred as contaminants hereafter.

For enabling specific capturing of contaminants using functionalized nanoparticles, engineered inner primers (FIP and BIP) were designed encompassing T linkers of different lengths, as given in Table 1. Though linkers were encompassed, the primers amplified the target sequences with minimal effect on the efficiency of the assay and are described in detail later. Two hybridization probes were designed with thymidine (T) linkers and two with adenine (A) linkers to selectively bind to contaminants but not to the template nucleic acid sequence. The two probes with A linkers can bind to both contaminants and inner primers, while the other two probes with T linkers specifically bind to contaminants.

The effect of the length of T linkers inserted in the primer sequences toward the efficiency of N gene amplification was studied by RT-LAMP reactions in duplicate. The results of amplification are given in Figure 3. The amplification was observed in all combinations of primers with T linkers except with T-14 and T-16. Longer than linker lengths of 4 T, the amplification was increasingly delayed with the length of linkers.

To counter the issue of cross contamination from aerosol and other modes, four probes for each linker length were designed. Three sets of four antisense probes were designed for primers with T-6, T-10, and T-14 linkers (Table 2). The probes were of

Figure 5. (a) FTIR spectra for basic HNPs and surface-modified particles at different stages of functionalization with probes. Size characterization of Co2FeAl nanoparticles (HNPs) by TEM. (b) Representative image of bare HNPs. (c) Probe-functionalized HNPs. (d) Size characterization and distribution of bare and modified particles using ImageJ software. For quantification of the average particle diameter, 100 particles were selected from more than five fields. (e) Magnetic moment plots (M−H measurement) of nanoparticles as recorded using a PPMS. (f) Zeta potential of HNPs before and after surface functionalization.
18 bases length, targeting the linker sequences including the flanking regions. For instance, in the P-T6-FIP probe, six bases of T linkers were flanked by six bases from either side of the resulting amplicon sequence. This allows the probes to specifically detect and bind to amplicons only and not to the template RNA of the pathogen. The melting temperature of each of the probes, as mentioned in Table 2, is less than 45 °C, thereby allowing the nano-capture system to be run at a significantly different temperature than the RT-LAMP reactions.

For each T-linker length assay, say T-6, the corresponding probes such as P-FIP-T6, P-BIP-T6, P-FIPr-T6, and P-BIPr-T6 were decorated on the surface of HNPs via a series of conjugation reactions, as described in Figure 4 and Figure S2. Similar procedures were repeated for T-10 and T-14 probes. It is imperative here to mention that a longer linker sequence would affect the rate of amplification of the assay; however, choosing a shorter length will affect the specificity of the probe and thus the efficacy of the nano-capture system.

Figure 6. (a) Amplicon capturing and cleansing (under a magnetic field) of RT-LAMP reactions for contamination-free detection of SARS CoV-2. Capturing efficiency of functionalized nanoparticles for cleaning the amplicons. Diluted RT-LAMP products were incubated with different concentrations of functional nanoparticles and cleaned by magnetic pull down. The change in SYBR fluorescence (indicating the presence of dsDNA) was (b) measured using a multiplate reader and (c) visualized under a UV transilluminator.
HNPs were surface-modified with oligos to capture only the amplified LAMP products and not the template RNA sequences. Successful conjugation at each step was confirmed by recording the FTIR spectra for the modified particles (Figure 5a) and identifying the characteristic peaks. TEOS-modified particles showed intensification of the SiO₂ peak at 1039 cm⁻¹ because of formation of a silane layer. APTES modification resulted in distinct peaks at 1455 cm⁻¹ (C=O stretching because of amide bonds), 1633 cm⁻¹ (N–H vibration), and 3404 cm⁻¹ (N–H vibration). Succinic acid group addition to APTES-modified particles was confirmed by deepening of amide stretching (≈1440 cm⁻¹) and significant broadening of the peak at 3404 cm⁻¹ due to the carboxyl group (O–H stretching) presence. Finally, attachment of oligo probes through amide bond formation was indicated by the narrowing and decrease in the peak at 3404 cm⁻¹ (because of removal of –OH groups), narrowing of the peak at 1402 cm⁻¹ (carboxyl to amide change), and presence of a distinct phosphate peak at 1200 cm⁻¹ as seen in Figure 5a.

The physical sizes of HNPs were characterized by transmission electron microscopy, and the results before and after functionalization are given in Figure 5b–d. It was observed that post functionalization, the aggregation of the nanoparticles was significantly reduced. Further, the size distribution also narrowed post functionalization. As seen from Figure 5d, 75–90% of the nanoparticles were between the size range of 20–50 nm. The magnetic moments of HNPs and functionalized HNPs are shown in Figure 5e. For the sake of comparison, commercially available Fe₂O₃ nanoparticles were also characterized. As observed, the magnetic moments of HNPs as well as Fn-HNPs were significantly higher than those of iron nanoparticles. The placement of the ferrous ion at the center of the cubic structure of these metallic alloys results in a double-exchange mechanism that further facilitates ferromagnetic alignment of neighboring ions. In the presence of small magnets of neodymium whose magnetic field is in the range of ≈0.01 T, the surface functionalization does not decrease the magnetic moment significantly; however, functionalization leads to an apparent increase in the surface area by decreasing the aggregation propensity. The surface charge of bare nanoparticles changed from +30 to −25 mV (for TEOS-modified particles) and then to −17.8 mV for the probe-conjugated particles. The sequential change in the surface charge of particles with the stage of surface modification corroborates the FTIR data in confirming successes of the conjugation process, as given in Figure 5f.

The working phenomena of the nano-capture are shown in Figure 6a. For cleaning the RT-LAMP reactions from potential contaminants, the reaction mix was incubated with functionalized particles and the captured amplicons were pulled down in
the tube. The clean mixture was pipetted to a fresh tube. The capturing efficiency of the Fn-HNP (T-10) and the effective concentration were assessed by recording the change in fluorescence (ds DNA) after incubating the diluted RT-LAMP products with different concentrations of Fn-HNPs.

To optimize the concentration of functionalized NPs for effective nano-capture, simulated contaminant solutions of 10 and 1000× diluted RT-LAMP products were prepared. The nano-capture system was used to clean the mixture by using different concentrations of Fn-HNPs. Post cleaning of the mixture, the amount of DNA was estimated using SYBR fluorescence and a plate reader under UV light. The results are shown in Figure 6b,c. It is imperative to mention here that these dilutions would have contained much higher amounts of contaminants than the actual amounts of LAMP contaminants (in aerosol-soiled reagents). Significant capturing of diluted RT-LAMP amplicons (T-10 primer-amplified) was seen at a concentration of 100 ng/μL and above by Fn-HNP (T-10) (Figure 6). Amplicon capturing was also seen after incubation with 1 μg/μL Fn-ION (T-10). It is also noted that the Fn-ION effective concentration required is at least 10 times that of Fn-HNPs.

The performance of functionalized Heusler nanoparticles and other treatments including the UDG system and functionalized ferrous nanoparticles in reducing negative amplification was assessed by setting up RT-LAMP reactions for amplification of N gene (SARS CoV-2) with T-6-, T-10-, and T-14-modified primers. Significant amplification in both positive and negative controls (due to contamination) was observed in all untreated RT-LAMP reactions (T-6-, T-10-, or T-14-based) (Figure 7). For T-6 primer-based amplification, the UDG cleavage system prevented amplification in the negative control, Fn-ION (T-6) treatment showed no effect, and Fn-HNP (T-6)-based capturing resulted in limited reduction in amplification of negatives samples (Figure 7a,d). All three treatments resulted in prevention of negative amplification in T-10 primer-based amplification; however, Fn-HNP (T-10) was the most effective one, as evidenced by the amplification curves (Figure 7b,d). T-14 primer-based reactions showed no negative amplification in the case of Fn-HNP (T-14) capturing, while limited reduction was observed after UDG cleavage and Fn-ION (T-14) treatment (Figure 7c,d). In summary, Fn-HNP (T-10)- and Fn-HNP (T-14)-based capturing was found to be effective in preventing negative amplification, while Fn-HNP (T-6)-based capturing showed limited working (Figure 7).

In contrast to available approaches, the proposed nano-capture system is based on metallic alloy nanoparticles and oligonucleotide probes, which are stable in broad ranges of temperatures. This enables the easy extendibility of the approach and its suitability for point-of-care and field applications. Furthermore, as the approach is based on oligonucleotide probes, it is expected to be much cheaper than molecular enzyme-based methods. On average, 77 nmol of oligonucleotide is required for surface modification of 10 mg of nanoparticles that is sufficient for nearly 6600 reactions (considering a 15 μL volume per reaction). A rough estimation of the cost of functionalized iron oxide nanoparticles is as follows: the cost of 77 nmol of amino-modified oligo is $42 (cost of 100 nmol of amino-modified oligo, $54) and the cost of 50 mg of iron oxide nanoparticles is $66 (approximately). Thus, there is a negligible cost for 10 mg. Therefore, the approximate cost of reagents for 6600 reactions is $42, i.e., less than 1 cent per reaction. As Heusler alloy nanoparticles are lab-synthesized, the cost of iron oxide nanoparticles was considered.

The new insight brought in this work is the design of a rapid nano-capture system that hybridizes with contaminating amplicons (carry-over) with high specificity to enable easy removal from the assay for elimination of false positives. The method has been proven to be successful for RT-LAMP assay in the rapid and highly specific detection of SARS CoV-2, which is currently a major challenge for global health.

4. CONCLUSIONS

RT-LAMP assays are known to yield false-positive results due to carry-over contamination. In this work, a nano-capture system is developed to yield a contaminant-free RT-LAMP assay. The nano-capture system shall be carried out at 45 °C for 10 min prior to the amplification assay. The working of the proposed approach is validated by designing the RT assay using specially engineered primers for amplification of the target N gene sequence of SARS CoV-2 virus. The developed RT-LAMP assay was found to be sensitive enough to detect 10 copies of viral genomes without the incidence of false positives within 45 min at 60 °C.

The indigenously developed nano-capture system uses in-house-designed probe-functionalized Heusler alloy (Co3FeAl) nanoparticles. These highly magnetic particles of Heusler alloys were chemically manufactured using precursors, characterized, and then surface-decorated with four antisense probes through a series of conjugation reactions. The probe-functionalized nanoparticles specifically bind to the carry-over contaminants from aerosol and RT-LAMP reagents. The working of the nano-capture system was validated by high concentrations of diluted RT-LAMP products (10 and 1000 times), acting as contaminants, capturing, and magnetically separating the contaminants via functionalized nanoparticles. The proposed system is quite robust and effective in preventing false-positive results. With suitable design of probes and primers, the technique can be effectively extended to LAMP/RT-LAMP assays of other pathogens. Since the capturing method is not dependent on temperature-sensitive molecular reagents, the proposed nano-capture system can be incorporated in point-of-care devices and field testing.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsanm.1c00782.

Multiple sequence alignment toward identifying the target region, protocol for surface modification, limit of detection with T-10-modified primers, XRD spectra for Co2FeAl nanoparticles, amplification conditions for the RT-LAMP reaction, and comparative tables for the limit of detection of different approaches (PDF)
Magnetic property of Fn-HNPs (MP4)
Magnetic property of HNPs (MP4)
Magnetic property of Fe3O4 NPs (MP4)

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