Charge-shifting polyplex as a viral RNA extraction carrier for streamlined detection of infectious viruses†

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The recent outbreak of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has highlighted the need for rapid, user-friendly nucleic acid testing that involves simple but efficient RNA extraction. Here, we present a charge-shifting polyplex as an RNA extraction carrier for advanced diagnosis of infectious viral diseases. The polyplex comprises poly(2-(dimethylamino) ethyl acrylate) (pDMAEA) electrostatically conjugated with RNA. The pDMAEA film can rapidly dissolve in the viral RNA solution, promoting immediate binding with RNA to form the polyplex, which enables the efficient capture of a substantial quantity of RNA. Subsequently, the captured RNA can be readily released by the quick hydrolysis of pDMAEA at the onset of quantitative reverse transcription-polymerase chain reaction (qRT-PCR), streamlining the entire process from RNA extraction to analysis. The developed method requires only 5 min of centrifugation and enables the detection of RNA in a one-pot setup. Moreover, the proposed method is fully compatible with high-speed qRT-PCR kits and can identify clinical samples within 1 h including the entire extraction to detection procedure. Indeed, the method successfully detected influenza viruses, SARS-CoV-2, and their delta and omicron variants in 260 clinical samples with a sensitivity of 99.4% and specificity of 98.9%. This rapid, user-friendly polyplex-based approach represents a significant breakthrough in molecular diagnostics.

New concepts

Polyplexes are electrostatic complexes formed by the combination of cationic polymers and nucleic acids (NA), which have been extensively utilized in gene delivery applications. Despite their distinct advantage of creating strong electrostatic interactions that result in robust binding of NA, their binding affinity was often too strong, leading to difficulties in NA release and compatibility with polymerase chain reaction (PCR), which hindered their application in molecular diagnostics. In this article, we propose a streamlined viral RNA detection method utilizing a charge-shifting polyplex as an RNA extraction carrier. The charge-shifting polyplex is comprised of a cationic poly(2-(dimethylamino)ethyl acrylate) (pDMAEA) and RNA complex. By leveraging the unique properties of self-catalyzed hydrolysis of pDMAEA, we can modulate electrostatic interactions with target viral RNA, enabling efficient RNA capture and release. This approach allows for a high RNA capture rate of over 90% within 5 min. Subsequently, 85% of the captured RNA can be readily released by the quick hydrolysis of pDMAEA at the onset of quantitative reverse transcription-polymerase chain reaction, streamlining the entire process from RNA extraction to analysis in a one-pot manner with minimal steps, reagents, and consumables. This rapid, user-friendly polyplex-based approach represents a significant breakthrough in molecular diagnostics.

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† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d3mh00861d
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1. Introduction

Modulating nucleic acid (NA) capture and release plays a key role in maximizing NA extraction yields in molecular diagnostics to facilitate the subsequent amplification process. However, traditional NA testing methods (e.g., quantitative reverse transcription-polymerase chain reaction, qRT-PCR) heavily rely on complicated extraction protocols associated with various issues, including yield variability, time-consuming extraction procedures requiring well-trained technical expertise, sample contamination, and high costs.1-5 Although extraction-free nucleic acid testing methods have attracted much attention recently in response to these challenges,6-8 amplifying target genes from heterogeneous patient samples without an extraction process often sacrificed the amplification efficiency or accuracy substantially due to the potential inhibitory effects of nucleases, proteins, salts, or other molecules.7,8 Therefore, it is still imperative to secure a robust extraction method that can effectively purify and enrich the target NA without perturbing the amplification process for rapid, accurate, and reliable NA-based diagnostics. To enhance the NA extraction efficiency from samples, several strategies have been devised by harnessing chemical forces such as electrostatic interaction,9-10 covalent bonding,11 or ion exchange forces.12,13 However, the strong NA affinity created by these strategies is in trade-off relation with the efficient release of NA. In other words, for optimized NA extraction, the NAs must be captured tightly from the sample, and at the same time, they must also be fully released to facilitate the following NA amplification. Indeed, it is not a simple task to achieve both goals simultaneously. Therefore, there is an unmet need for a groundbreaking approach that can control the capture and release of NA to maximize the NA extraction yield.

Polyplex, a complex system consisting of cationic polymers and negatively charged NAs, is typically formed by electrostatic interaction.14-16 Various types of polyplexes have been developed by combining NAs with a variety of cationic polymers such as polylysine,17 poly(ethyleneimine),18 and stimuli-responsive polymers,19 and have been widely utilized for gene delivery.20,21 The binding strength of the polymer/nucleic acid complex can be optimized by adjusting the number of positive charges or the molecular weight of the polymer.22,23 Recently, charge-shifting polyplexes have been suggested, where their surface charge can be altered from positive to neutral or negative by hydrolysis in aqueous environments to control the nucleic acid loading and release kinetics.19-22 Given the ability of charge-shifting polyplexes to modulate the capture and release of NA, we hypothesized that the polyplex could serve as a versatile, controllable NA extraction matrix in qRT-PCR processes.

Here, a charge-shifting polyplex-assisted direct qRT-PCR (PAD-qRT-PCR) method was newly developed for one-pot detection of infectious viruses. The method employed a cationic poly(2-(dimethylamino)ethyl acrylate) (pDMAEA) film as an RNA extraction carrier, which was deposited on a PCR tube directly in the vapor phase via initiated chemical vapor deposition (iCVD). Upon introduction of the RNA-containing sample into the PCR tube, the pDMAEA immediately dissolved to form electrostatic pDMAEA–RNA conjugates (polyplexes), which turned out to capture a large amount of RNA immediately – within 5 min – with a capture efficiency greater than 90% from the input RNA. One of the biggest advantages of the charge-shifting polyplexes is that the captured RNA can be released readily by quick hydrolysis of pDMAEA at the initial stages of the reverse transcription reaction, enabling the rapid, but reliable and efficient RNA extraction in one-pot manner. We also verified that the polyplexes did not hamper the qRT-PCR process. The PAD-qRT-PCR method was capable of detecting influenza viruses as well as identifying wild-type (WT), delta, and omicron variants of SARS-CoV-2 with high accuracy in 260 clinical samples. To the best of our knowledge, no attempt has been made to employ polyplexes in molecular diagnostics thus far. It is anticipated that the PAD-qRT-PCR method will simplify and improve the current molecular diagnostic procedures.

2. Results

2.1. Workflow of PAD-qRT-PCR method

One of the primary targets of the PAD-qRT-PCR method is to reduce the resources and time while enhancing RNA detection sensitivity by processing RNA purification and qRT-PCR procedures in a single tube (Fig. 1(a)). Unlike the traditional spin column-based extraction method widely used in qRT-PCR, which involves multiple steps of binding, washing, elution, and centrifugation, PAD-qRT-PCR requires only one 5 min centrifugation step for whole extraction procedure. The workflow...
of PAD-qRT-PCR is depicted in Fig. 1(b). In brief, an RNA-containing sample is loaded into the pDMAEA-coated PCR tubes, where the pDMAEA quickly dissolves to form polyplexes with RNA through electrostatic interaction. After 5 min of centrifugation, the polyplexes are precipitated, allowing for the easy removal of the supernatant including any impurities. Then a qRT-PCR mixture was added to the PCR tube to proceed the following RNA reverse transcription steps. At the early stage of reverse transcription reaction at 50 °C, the pDMAEA polyplex undergoes a charge shift from positive to negative, which substantially alleviates the electrostatic attraction, and thereby prompts the release of RNA from the polyplex. The RNA is then subjected to reverse transcription, amplification, and analysis. The RNA extraction process can be completed in 5 min with minimal extraction steps. Most of all, the whole PAD-qRT-PCR can be performed in a single tube, an 8-stripe tube, or a 96-well plate. The streamlined workflow of PAD-qRT-PCR reduces RNA loss, extraction time, and plastic wastes remarkably.

2.2. Characterization of charge-shifting polyplex

The central hypothesis of the PAD-qRT-PCR approach is that the cationic pDMAEA film with high water solubility can efficiently capture RNA by forming polyplexes via electrostatic attraction with RNA. Then, rapid hydrolysis of pDMAEA (Fig. 2(a)) leads to fast charge-shift, which in turn releases RNA from the polyplexes promptly via electrostatic repulsion, thereby facilitating qRT-PCR. To assess this hypothesis, pDMAEA was synthesized in solvent-free way in the vapor phase by iCVD process, which forms the highly soluble pDMAEA film on PCR tubes without damaging them. The Fourier transform infrared (FTIR) spectroscopy results (Fig. 2(b)) showed that the C==C vinyl stretching peak from DMAEA monomer (1635 cm\(^{-1}\)) disappeared after polymerization, indicating the complete consumption of the vinyl group through the free radical addition reaction.\(^{24}\) Meanwhile, the peaks representing the tertiary amine-methyl moieties (2800–3000 cm\(^{-1}\)) remained intact, proving the retention of the tertiary amine functional group in pDMAEA during the iCVD process.

The formation of polyplexes between the cationic pDMAEA and RNA was investigated by zeta-potential analysis. Fig. 2(c) displays the zeta-potential analysis results for polyplexes with each nitrogen (N)/phosphorous (P) ratio from 0.5 to 200. The N/P ratio is contributed by the amine group of pDMAEA and the phosphate group of RNA, respectively. Purified RNA showed a zeta-potential value of \(-12\) mV, while polyplex with an N/P ratio of 0.5 showed \(-10\) mV. Notably, polyplex with an N/P ratio of 1 showed a positive zeta-potential value of 1.5 mV. As the N/P ratio increased from 10 to 200, the zeta potential of polyplex gradually increased from 6.0 to 11.4 mV due to the excess amount of cationic pDMAEA. The pure pDMAEA displayed a zeta-potential value of 19.4 mV. The formation of polyplex was further confirmed by observing RNA condensation. A dramatic reduction in RNA volume can occur when RNA interacts with a positively charged polymer electrostatically, resulting in the reduction of interchain electrostatic repulsive forces.\(^{25}\) RNA condensation was observed by monitoring the hydrodynamic size of the polyplex at each N/P ratio (Fig. 2(d)). The size of polyplexes increased with the N/P ratio; the hydrodynamic sizes of polyplex with N/P ratios of 1 and 2 were 20-fold smaller than that of purified RNA, which is fully consistent with the transmission electron microscopy (TEM) observation of the polyplex (Fig. 2(e) and Fig. S1a, b, ESI†). These findings clearly verify the successful formation of polyplexes between pDMAEA and RNA. Considering the N/P ratio threshold, we proceeded to coat a far excessive amount of pDMAEA (average coating mass to be 1.2 ± 0.085 µg) on a PCR tube to ensure that the threshold N/P ratio was exceeded (Fig. S2, ESI†). Concurrently, we evaluated the reproducibility of the pDMAEA-coated tubes manufactured via the iCVD process (Fig. S2, ESI†).

Prior to further quantitative analysis, we compared the PCR amplification curves for template RNA (0.1, 1, and 10 ng µL\(^{-1}\)) in both pristine tubes and pDMAEA-coated tubes (Fig. S3a–c, ESI†) to ensure that the use of charge-shifting polyplex as an RNA extraction carrier does not interfere with the qRT-PCR reaction. The results confirmed that the pDMAEA-coated tubes showed equivalent PCR amplification to the standard PCR tubes.

The RNA capture efficiency of the pDMAEA-coated PCR tube was evaluated using influenza A virus (H1N1) RNA. Primers for...
H1N1 are listed in Table S1 (ESI†). The sample solution was loaded into the pDMAEA-coated tube, and centrifuged, and the supernatant was collected and analyzed by qRT-PCR. The plot of RNA capture efficiency (the percentage of the captured RNA from the input RNA) as a function of centrifugation time is shown in Fig. 2(f). The plot suggests that 99% of RNA could be captured by the pDMAEA-coated tube within 15 min. The results also show that even within 5 min of centrifugation, 93% of RNA could be captured. The efficiency of this process is further highlighted by the fact that centrifugation at speed above 3000 rpm is sufficient for efficient recovery of the polyplexes (Fig. S4, ESI†), making it a cost-effective and efficient method without the need for large-scale equipment. The markedly rapid RNA capture property is attributed to the immediate dissolution of pDMAEA from the tube surface into the RNA-containing sample solution. The RNA capture efficiencies exceeding 90% were maintained for various initial RNA amounts (3, 30, 60, and 150 ng) under 5 min centrifugation (Fig. 2(g)). Additionally, the linearly increased amount of captured RNA with respect to the input RNA supports the effective RNA capture ability of the pDMAEA-coated tube (Fig. 2(h)).

2.3. RNA release from the charge-shifting polyplexes

pDMAEA can undergo a self-catalyzed hydrolysis reaction of the dimethylamino substituent to acrylic acid (AA) (Fig. 2(a)). This reaction can occur by both base- and acid-catalyzed mechanisms involving intramolecular interactions of the dimethylamino substituent with the ester carbonyl group (Fig. S5, ESI†). The rapid hydrolysis of pDMAEA is of crucial importance for the rapid and efficient release of RNA during the reverse transcription reaction (Fig. 1(b)). Therefore, the hydrolysis behavior of pDMAEA was monitored using proton nuclear magnetic resonance (NMR) spectroscopy at 5 min intervals under reverse transcription conditions (50 °C and pH 8.0) (Fig. 3(a) and Fig. S6a–c, ESI†). The results showed that about 80% of pDMAEA was hydrolyzed at 50 °C within 25 min, while only less than 13% was hydrolyzed at 25 °C under the same pH condition. The half-life of the hydrolysis of pDMAEA was 5.2 min at 50 °C and 158 min at 25 °C, estimated from pseudo-first-order kinetic plots (Fig. S7a and b, ESI†). The initial hydrolysis rate constant of pDMAEA was calculated to be $10^{-4.1}$ s$^{-1}$ at 25 °C and was greatly accelerated to $10^{-2.5}$ s$^{-1}$ at 50 °C, demonstrating the rapid hydrolysis of pDMAEA at the early stage of the reverse transcription reaction (Fig. 3(b)).

The self-catalyzed hydrolysis of the dimethylamino substituent to AA was further confirmed by pH back-titration analysis (Fig. S8, ESI†). After hydrolysis at 50 °C for 30 min, the hydrolyzed pDMAEA displayed high buffering capacity at around pH 4 due to the production of AA moieties with a $pK_a$ of approximately 4. At the same time, the buffering capacity at pH 8–9 was also retained because the hydrolyzed dimethylamino ethanol substituents remained in the solution. The charge-shifting effect upon hydrolysis of the pDMAEA was investigated using zeta-potential analysis (Fig. 3(c)), which showed a dramatic change from 20 mV to ~22 mV after the hydrolysis, mainly due to the extensive reduction of the net positive charge resulting from the production of AAs by the hydrolysis reaction.

To evaluate the quantity of RNA released from the polyplexes, a series of PAD-qRT-PCR procedures including (1) sample loading followed by 5 min centrifugation, (2) supernatant removal, and (3) addition of PCR mixture to proceed qRT-PCR was performed in the pDMAEA-coated tube. Fig. 3(d) shows that the recovery yield of the pDMAEA-coated tube (the percentage of released RNA from the captured RNA by polyplex) was as high as 85%. The overall extraction yield of the tube (the percentage of released RNA from polyplexes to input RNA) also surpassed 80% (Fig. 3(e)), comparable to the extraction yield (90%) from commercial spin-column extraction kits (Fig. S9, ESI†). The polyplex-based extraction method exhibited an extraordinary RNA extraction yield with minimal steps and time, and without any additional chemical or temperature control to release the captured RNA, rendering the method a highly attractive option for real-world applications in the simple diagnosis of infectious diseases.

2.4. Detection of influenza viruses using the PAD-qRT-PCR method

The performance of the PAD-qRT-PCR method in detecting H1N1 was evaluated. Fig. 4(a) is the amplification curves of PAD-qRT-PCR for various H1N1 concentrations. As the concentration of H1N1 lysate decreased, the amplification curve shifted to the right at regular intervals. The corresponding cycle threshold ($C_t$) values were plotted along with the input H1N1 concentration (Fig. 4(b)), revealing a dynamic detection range of 10 to 10⁴ plaque-forming units (PFU) mL⁻¹. The linear fit exhibited a high correlation coefficient ($R^2$) value of 0.9983, demonstrating the quantitative detection of H1N1. The detection sensitivity of the PAD-qRT-PCR for H1N1 was calculated to...
be 3.3 PFU mL\(^{-1}\), according to 3.3σ/slope, where σ is the standard deviation at the lowest concentration of the linear range. This sensitivity value is lower than the minimum infective dose (MID) of H1N1 (\(\approx 10^{0.5-1.5}\) PFU),\(^{30}\) supporting the applicability of the PAD-qRT-PCR method for detecting H1N1 in a clinically relevant range.

The simple, one-pot PAD-qRT-PCR method was utilized to detect influenza viruses in clinical samples. Nasopharyngeal swabs (NPS) and sputum samples were collected from patients with flu symptoms. A total of 20 clinical samples were diagnosed as flu (11 for H1N1 and 9 for influenza B virus (IBV)), and 15 samples were diagnosed as negative by the hospital (Table S2, ESI\(^{†}\)). The PAD-qRT-PCR method was used to diagnose the same samples (stored at \(-70\) °C) and provided 100% positive predictive agreement (PPA) for H1N1 (11 out of 11), 100% PPA for IBV (9 out of 9), and 100% negative predictive agreement (NPA) for both H1N1 and IBV (Fig. 4(c)). The PAD-qRT-PCR method was able to classify the flu-positive and negative samples based on the \(C_t\) values obtained (Fig. 4(d)). A receiver operating characteristic (ROC) analysis also provided an area under the curve (AUC) value of 1.0, indicating full accuracy of the PAD-qRT-PCR results compared to the hospital results (Fig. S10, ESI\(^{†}\)). The scatter plot of the \(C_t\) value comparison showed a high degree of correlation, with a Spearman’s rank correlation coefficient (\(\rho\)) of 0.889 (Fig. 4(e)). The hospital \(C_t\) values were obtained from freshly collected clinical samples, whereas the PAD-qRT-PCR \(C_t\) values were obtained from the same samples after 3 to 4 years. Despite the inevitable increase in \(C_t\) values due to the long sample storage, the PAD-qRT-PCR assay was capable of accurate diagnosis of flu from NPS and sputum samples, which was attributed to the high RNA extraction and recovery rates of the PDMAEA-coated tube.

### 2.5. Detection of SARS-CoV-2 and variants using PAD-qRT-PCR method

The PAD-qRT-PCR method was evaluated for the detection of SARS-CoV-2, the causative agent of coronavirus disease 2019 (COVID-19) pandemic. Fig. 5(a) depicts the structure of SARS-CoV-2. First, a test was performed using the primers specific for the spike (S) gene of the virus (Table S1, ESI\(^{†}\)). Fig. 5(b) shows a significant fluorescence signal in the presence of SARS-CoV-2 (10\(^5\) PFU mL\(^{-1}\)) and no signal in the absence of the virus. To verify the specificity of the PAD-qRT-PCR method for SARS-CoV-2, several respiratory viruses (each at 10\(^4\) PFU mL\(^{-1}\)), including influenza A subtypes H1N1, H3N2, H5N2, H1N2, and H3H8, and respiratory syncytial virus A (RSV A) were examined (Fig. 5(c) and Fig. S11, ESI\(^{†}\)). The results indicated that the PAD-qRT-PCR method exclusively detected SARS-CoV-2, with a \(C_t\) value of 25, while the other viruses were not detected.

Next, the target genes for the PAD-qRT-PCR method were expanded to include the open reading frame 1 (ORF1) and nucleocapsid (N) genes of SARS-CoV-2 (Table S1, ESI\(^{†}\)). The Centers for Disease Control and Prevention (CDC) recommends the detection of at least two viral genes to avoid false results in the diagnosis of COVID-19.\(^{31}\) Fig. 5(d)–(f) depicts the relationship between the \(C_t\) values and SARS-CoV-2 concentration for each of the three genes (ORF1, N, and S). The real-time curves for these genes are presented in Fig. S12a–c (ESI\(^{†}\)). As the concentration of SARS-CoV-2 increased, the \(C_t\) values decreased. A strong linear relationship (\(R^2 = 0.9801, R^2 = 0.9979, and R^2 = 0.9932\) for ORF1, N, and S genes, respectively) was also observed in the concentration range of 10\(^3\)–10\(^4\) PFU mL\(^{-1}\). The limit of detection (LOD) was attributed to the high RNA extraction and recovery rates of the PDMAEA-coated tube.
The PAD-qRT-PCR method was utilized to diagnose COVID-19 from a total of 135 clinical samples (Table S4, ESI†). The results of the PAD-qRT-PCR method on the clinical samples are plotted as green dots in Fig. 6(a), with a PPA of 74 out of 75 and an NPA of 59 out of 60 compared to the hospital results. Two samples showed false negative and false positive results (indicated by a red star in Fig. 6(a)). The diagnostic results of PAD-qRT-PCR were also compared with those obtained by conventional qRT-PCR with a spin column-based extraction method (black dots in Fig. 6(a)). A strong correlation was observed between the average \( C_t \) values obtained by the two methods, as shown in the scatter plot in Fig. 6(b) \( (\rho = 0.972) \). Interestingly, the PAD-qRT-PCR method showed an average \( C_t \) value 1.35 lower than the conventional qRT-PCR, implying that it has 2.5 times higher sensitivity in detecting RNA. The high correlation between the \( C_t \) values obtained by PAD-qRT-PCR and those provided by the hospital \( (\rho = 0.936) \) was demonstrated in Fig. 6(c). The results of the ROC curve analysis showed an AUC value of 0.989, indicating excellent accuracy of the PAD-qRT-PCR method in relation to the clinical status of the patients (Fig. 6(d)).

In conclusion, the one-pot PAD-qRT-PCR method demonstrated a remarkable clinical diagnostic accuracy of 99.3% PPA and 98.8% NPA for SARS-CoV-2 (Table 1). In addition, the method can also discriminate SARS-CoV-2 variants with convenience. These results demonstrate that the one-pot PAD-qRT-PCR method can be a robust and effective strategy for identifying infectious viruses in clinical samples.

3. Discussion

Current standard molecular diagnostic tests commonly involve a complicated nucleic acid extraction protocol and require technical expertise, which substantially limited the rapid deployment of diagnostic tests during outbreaks of new infectious diseases. The PAD-qRT-PCR method was developed to
The conventional qRT-PCR using extraction kits. The developed method requires only 5 min of centrifugation, enabling the detection of RNA in a one-pot setup. Furthermore, the PAD-qRT-PCR method not only simplifies the methodology but also enhances the sensitivity compared to the conventional method. This is due to the superior extraction efficiency of the charge-shifting polyplexes, as evidenced by an average 1.35-fold reduction in Ct values compared to the conventional method using the same samples. Moreover, the extraction method is fully compatible with various kinds of NA amplification methods and instrumentations. All the extraction and NA amplification procedures occur in a single tube, which could maximize RNA utilization. These highly advantageous characteristics demonstrate that the PAD-qRT-PCR method is a promising approach for RNA detection with improved sensitivity and streamlined methodology.

The use of polyplexes as RNA extraction carriers offers a promising solution to the complexity and low extraction efficiency issues that often impede molecular diagnostics. Although polyplexes have been utilized extensively for drug delivery applications, their potential in molecular diagnosis has yet to be explored. In this study, we demonstrated that the pDMAEA thin film can effectively capture and release RNA without compromising qRT-PCR performance, leading to an exceptional RNA extraction efficiency of over 80%. Considering that the previous insoluble cationic polymer films show less than 20% extraction efficiency under the same conditions, the pDMAEA films have greatly improved in efficiency and convenience. The PAD-qRT-PCR method also allows for a wide range of sample volumes from 3 μL to 30 μL allowing for RNA enrichment (Fig. S13, ESI†). This increased sample volume is beneficial compared to typical PCR approaches, which limit the sample input to less than 10 μL. Most other extraction-free molecular diagnostic assays cannot afford to increase sample volume beyond 10 μL due to inhibitory effects, either. Thus, increasing the input volume by PAD-qRT-PCR is highly advantageous for detecting low-copy number samples. Furthermore, we verified that the proposed PAD-qRT-PCR method is fully compatible with high-speed qRT-PCR kits and can identify clinical samples within 1 h including the whole extraction to detection procedures (Fig. S14, ESI†). With its numerous advantages, PAD-qRT-PCR has the potential to be applied to various genetic analysis fields such as food safety testing, forensic analysis, and genome sequencing. Minimizing the use of industrial kits is crucial to avoid supply shortages and reduce medical waste during pandemics. The whole PAD-qRT-PCR operates in a one-pot system, which can also alleviate the supply shortages substantially by reducing plastic waste per sample compared to commercial kits (Fig. 1(a)). The cost of molecular diagnostics is often a limiting factor for low-income and middle-income countries. The estimated material cost of the PAD-qRT-PCR is $107 per 100 reactions, whereas the market cost of commercial extraction kits is estimated to be between $400 and $600 per 100 reactions (Tables S7 and S8, ESI†). The cost-effectiveness of the PAD-qRT-PCR can be attributed to the minimal use of organic solvents and plasticware wastes. In addition, PAD-qRT-PCR requires a shorter process time, less cost, while similar recovery yield compared to the commercial kit. (Table S9, ESI†) It follows from the cost analysis results that the newly developed polyplex-based method makes molecular diagnostics more affordable and accessible, even in developing countries.

Human-associated respiratory viruses pose a significant threat to public health and the economy worldwide. The COVID-19 pandemic continues to spread due to its high transmission rate and the emergence of various variants such as delta and omicron. As of the beginning of January 2023, the CDC reported more than 650 million officially confirmed cases and greater than 6.6 million casualties. In addition to COVID-19, seasonal influenza is another major global health concern, with approximately 20% of children and 5% of adults worldwide developing symptomatic illness each year. Given the ongoing need for COVID-19 and influenza testing and the potential for another pandemic wave, there is a strong demand for user-friendly and affordable molecular diagnostic tools.

The PAD-qRT-PCR method still has technical challenges that should be addressed before practical implementation. There is still room for further improvement of the current PAD-qRT-PCR method for point-of-care testing. While we streamlined the extraction process by using polyplex as RNA extraction carriers, the qRT-PCR amplification reaction requires a bulky thermal cycler. To address this, we plan to explore alternative amplification assays, such as isothermal NA amplification with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) systems, to eliminate the need for a thermal cycler. We are committed to continuously improving the method and making it a widely used diagnostic tool.

4. Experimental section

4.1. Materials

All oligonucleotides were synthesized by Bioneer Co. (Daejeon, Korea), and their sequences are listed in Table S1 (ESI†).
Tris(2-carboxyethyl) phosphine (TCEP) and ethylenediamine-tetraacetic acid (EDTA) solutions were purchased from LPS Solution (Daejeon, Korea) and Dynebio (Seongnam, Korea), respectively. The RNA Extraction kit used in this experiment was G-spin genomic RNA extraction kit (iNTRON Biotechnology, Seoul, Korea). qRT-PCR kits used in this experiment were Qiagen one-step qRT-PCR kit (Qiagen, Hilden, Germany) and TaqPath™ 1-Step Multiplex Master Mix (Thermo Fisher, Waltham, MA, USA). SARS-CoV-2 strains (BetaCoV/Korea/KCDC03/2020, hCoV-19/Korea/KDCA119861/2021; G: B.1.617.2, hCoV-19/Korea/KDC4A47321/2021; GRA: B.1.1529) were provided by the National Culture Collection for Pathogens (NCCP, Cheong-Ju, Korea). The influenza H1N1 strain (A/California/04/2009) was provided by the Korea Research Institute of Bioscience and Biotechnology (KRIIB, Daejeon, Korea). The viral genomic RNA of SARS-CoV-2, RSV A, and the influenza A virus subtypes H1N1, H3N2, H5N2, H1N2, and H3N8 were provided by NCCP. DNase/RNase-free distilled water was purchased from Bioneer Co. and used in all the experiments. All other compounds were of analytical grade and were used directly.

4.2. Preparation of pDMAEA-coated PCR tubes

DMAEA (98%, Merck, Darmstadt, Germany), tert-butyl peroxide (TBPO, 98%, Merck, Darmstadt, Germany), and PCR tubes (Bio-Rad, Hercules, CA) were prepared. Next, the DMAEA monomer and TBPO were vaporized by heating to 35 and 25 °C, respectively, and introduced into the iCVD chamber (160 mTorr). The substrate and filament temperatures were set to 30 and 140 °C, respectively. When the filament temperature was increased to 140 °C, vapor-phase free radical polymerization started, resulting in the formation of a pDMAEA thin film on the surface of PCR tubes.

4.3. Characterization of charge-shifting polyplexes

FTIR spectra were measured in the wavenumber range of 400–4000 cm⁻¹ with a resolution of 1.4 cm⁻¹ (Alpha, Bruker Optics, Billerica, MA). Zeta-potential values and hydrodynamic sizes of the samples were measured using a Nano Zetasizer 3600 (Malvern Instruments Ltd, Malvern, UK) equipped with a He–Ne laser source (633 nm). Field emission-TEM operating at 80 kV (FE-TEM, Tecnai G2 F30 S-TWIN, FEI Company, Netherlands) was used to observe the morphology of polyplexes with a 2 N/P ratio. FE-TEM samples were prepared by dropping the polyplex onto carbon-coated copper grids. NMR samples were prepared by dissolving the pDMAEA in phosphate D₂O buffer solutions (pH 8.0). 1H NMR spectra were recorded at 5 min intervals at 25 and 50 °C using a Bruker Avance Neo 600 MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany). The degree of hydrolysis was calculated by comparing the integrated signals for the methylene of the ester in pDMAEA (ca. 4.41 ppm) and the alcohol of dimethylaminoethanol (DMAE), a small-molecule by-product, (ca. 4.00 ppm). Initial hydrolysis rates (k_initial) were obtained from the slopes of the linear lines in the pseudo-first-order kinetic plots. pH backtitration was performed as follows. First, the pDMAEA solution of 2 mg mL⁻¹ (1 μmol in 5 mL H₂O) was adjusted to pH 2.0 with 0.1 M HCl using an Orion Versa Star Pro pH meter (Thermo Fisher, Waltham, MA). The pH was then monitored by adding 0.1 M NaOH at the flow rate of 100 μL min⁻¹.

4.4. Quantification of RNA

To measure the amount of captured RNA, 3 μL of H1N1 RNA (3, 30, 60, and 150 ng) was added to the pDMAEA-coated tubes. The supernatant was then collected after centrifugation (1, 5, 10, and 15 min). The concentration of RNA in the supernatant was measured by qRT-PCR. Twenty-five μL of PCR mixture contained 1 μL of supernatant, a primer set (0.6 μM each), 1× QIAGEN OneStep RT-PCR buffer (tris–Cl, KCl, (NH₄)₂SO₄, 12.5 mM MgCl₂, DTT, pH 8.7), 1 μL of QIAGEN OneStep RT-PCR Enzyme Mix, and dNTP Mix (400 μM). PCR amplification was performed using CFX Connect™ Real-Time System (Bio-Rad) under the following conditions: reverse transcription at 50 °C for 30 min, initial denaturation at 95 °C for 5 min, 40 thermal cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The amount of captured RNA was calculated by subtracting the amount of RNA in the supernatant from the input RNA.

To measure the amount of RNA released from the polyplexes, 3 μL of H1N1 RNA (3, 30, 60, and 150 ng) was added to the pDMAEA-coated tubes, centrifuged for 5 min, and the supernatant was removed. qRT-PCR was then performed in the pDMAEA-coated tubes after adding the PCR mixture without supernatant. PCR amplification conditions were as described above.

4.5. Detection of influenza viruses using the PAD-qRT-PCR method

Ten μL of influenza virus samples were mixed with 90 μL of proteinase K (0.8 U per reaction) and RNase inhibitor (10 U per reaction), heated at 50 °C for 5 min and 64 °C for 5 min, pulse-vortexed for 15 s, and incubated at 25 °C for 10 min. Three μL of viral lysate was added to the pDMAEA-coated tubes, centrifuged for 5 min, and the supernatant was removed. qRT-PCR was then performed in the pDMAEA-coated tubes after the addition of PCR mixture without supernatant. PCR amplification conditions were as described above.

A total of 35 clinical samples were collected from Yonsei University Health Service Center, Severance Hospital (IRB approval number: 4-2020-0465). All samples were stored at −70 °C prior to use. Three μL of clinical samples were added to the pDMAEA-coated tubes, centrifuged for 5 min, and the supernatant was removed. qRT-PCR was then performed in the pDMAEA-coated tubes after the addition of the PCR mixture. PCR amplification conditions were as described above.
4.6. Detection of SARS-CoV-2 and variants using PAD-qRT-PCR method

Ninety μL of SARS-CoV-2 samples were mixed with 10 μL of TCEP/EDTA (final concentrations of 100 and 1 mM, respectively) and heated at 50 °C for 5 min and 64 °C for 5 min. Three μL of viral lysate was added to the pDMAEA-coated tubes, centrifuged for 5 min, and the supernatant was removed. qRT-PCR was then performed in the pDMAEA-coated tubes after the addition of PCR mixture including a primer set (0.6 μM each), 1 × QIAGEN OneStep RT-PCR Buffer (tris–Cl, KCl, (NH₄)₂SO₄, 12.5 mM MgCl₂, DTT, pH 8.7), 1 μL of QIAGEN OneStep RT-PCR Enzyme Mix, and dNTP Mix (400 μM). PCR amplification was performed using CFX Connect™ Real-Time System (Bio-Rad) under the following conditions: reverse transcription at 50 °C for 30 min, initial denaturation at 95 °C for 5 min, 40 thermal cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min.

A total of 225 clinical samples were collected from Gyeongsang National University College of Medicine (IRB approval number: 2022-10-012). All samples were stored at −70 °C prior to use. The clinical samples, containing virus transfer media, were then mixed with 10% v/v RLT lysis buffer (Qiagen, 79216). After lysing, three μL of the samples were added to the pDMAEA-coated tubes, centrifuged for 5 min, and the supernatant was removed. qRT-PCR was then performed in the pDMAEA-coated tubes after the addition of the PCR mixture. PCR amplification conditions were as described above. For fast qRT-PCR, 20 μL of PCR mixture including 2 μL of ORF1 gene primer set (0.6 μM each), 1 × QIAGEN OneStep RT-PCR buffer (tris–Cl, KCl, (NH₄)₂SO₄, 12.5 mM MgCl₂, DTT, pH 8.7), and 2 μL of TaqPath™ 1-Step Multiplex Master Mix (4 ×) was used. PCR amplification was performed using CFX96 real-time PCR machine (Bio-Rad) under the following conditions: UNG incubation at 25 °C for 2 min, reverse transcription at 53 °C for 10 min, polymerase activation at 95 °C for 2 min and 40 thermal cycles of denaturation at 95 °C for 3 s and annealing at 60 °C for 30 s.

5. Conclusions

In this study, we leveraged the charge-shifting polyplex for a simple molecular diagnosis of human-associated respiratory viruses. The pDMAEA film prepared by iCVD technology was used as an RNA extraction carrier, allowing for rapid but highly efficient, reliable RNA capture and release without interfering with qRT-PCR. The PAD-qRT-PCR method resulted in a user-friendly, one-pot diagnostic procedure, requiring only a 5 min centrifugation step prior to qRT-PCR. This method demonstrated high sensitivity and specificity in identifying 260 clinical samples, including the influenza virus, SARS-CoV-2, and its variants. In addition, the method was economical due to the minimal use of plasticware. We anticipate that the present method has potential for improvement by combining simple amplification assays, which holds promise for its future application in various nucleic acid analyses beyond infectious virus detection.

Author contributions

Younseong Song and Jayeon Song: conceptualization, data curation, formal analysis, validation, investigation, visualization, writing – original draft. Seongeun Kim, Hyowon Jang, Hogi Kim, Bookseok Jeong, and Nahyun Park: data curation, validation, visualization. Sungjoo Kim, Dongeun Yong, and Eun-Kyung Lim: methodology, resources. Kyung G. Lee, Taejoon Kang, and Sung Gap Im: project administration, supervision, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This research was supported by National R&D Program through the National Research Foundation of Korea (NRF) funded by Ministry of Science and ICT (MIST) (grant no. NRF-2021 M3H4A4079293, 2021R1A2B5B03001416, 2021M3E5E3080379, 2021M3H4A1A02051048, and 2023R1A2C2005185). This work was also supported by Nanomedical Devices Development Project of NNFC in 2023, Technology Development Program for Biological Hazards Management in Indoor Air through KEITI funded by Korea government [ME] (202100373003), KEITI grant funded by Korea government (MOTIE) (RS-2022-00154853), and KRIBB Research Initiative Program (KGM5472322). This work was supported by the Technology Innovation Program funded by the MOTIE (22025649).

Notes and references

8 J. Arieti-Sanz, A. D. Bradly, Y. B. Zhang, C. K. Boehm, C. A. Freije, M. E. Grunberg, T.-S. F. Kosoko-Thoroddsen,
31 C. F. D. Control, Prevention, 2020.