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Comparison of Three Magnetic Bead Surface Functionalities for RNA Extraction and Detection

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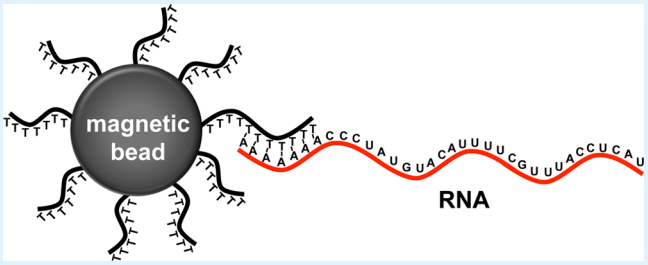
Supporting Information

ABSTRACT: Magnetic beads are convenient for extracting nucleic acid biomarkers from biological samples prior to molecular detection. These beads are available with a variety of surface functionalities designed to capture particular subsets of RNA. We hypothesized that bead surface functionality affects binding kinetics, processing simplicity, and compatibility with molecular detection strategies. In this report, three magnetic bead surface chemistries designed to bind nucleic acids, silica, oligo (dT), and a specific oligonucleotide sequence were evaluated. Commercially available silica-coated and oligo (dT) beads, as well as beads functionalized with oligonucleotides complementary to respiratory syncytial virus (RSV) nucleocapsid gene, respectively recovered ∼75, ∼71, and ∼7% target RSV mRNA after a 1 min of incubation time in a surrogate patient sample spiked with the target. RSV-specific beads required much longer incubation times to recover amounts of the target comparable to the other beads (∼77% at 180 min). As expected, silica-coated beads extracted total RNA, oligo (dT) beads selectively extracted total mRNA, and RSV-specific beads selectively extracted RSV N gene mRNA. The choice of bead functionality is generally dependent on the target detection strategy. The silica-coated beads are most suitable for applications that require nucleic acids other than mRNA, especially with detection strategies that are tolerant of a high concentration of nontarget background nucleic acids, such as RT-PCR. On the other hand, oligo (dT) beads are best-suited for mRNA targets, as they bind biomarkers rapidly, have relatively high recovery, and enable detection strategies to be performed directly on the bead surface. Sequence-specific beads may be best for applications that are not tolerant of a high concentration of nontarget nucleic acids that require short RNA sequences without poly(A) tails, such as microRNAs, or that perform RNA detection directly on the bead surface.



KEYWORDS: *nucleic acid extraction, RNA extraction, silica-coated magnetic particles, oligo (dT) magnetic particles, sequence-specific magnetic particles*

INTRODUCTION

Molecular detection of nucleic acid biomarkers has increasingly become the diagnostic modality of choice due to its inherent specificity, sensitivity, and rapid time-to-diagnosis. In patient samples, however, high concentrations of contaminants inhibit molecular detection. Consequently, most detection schemes require nucleic acids to be extracted into a contaminant-free buffer or water prior to performing the assay.[1] Nucleic acid capture using surface functionalized magnetic beads is one widely utilized technique for purifying nucleic acid biomarkers from patient samples. Once nucleic acids are captured onto the magnetic bead surface, they can be isolated, washed, and eluted quickly and easily by passing the beads through processing solutions. For this reason, magnetic bead-based purification has been adapted to a variety of formats, including manual extraction performed on a magnetic rack,[2] microfluidic chips[3] and other self-contained formats,[4−6] as well as automated robotic processing.[7]

In nucleic-acid-based diagnostic assays that use magnetic beads, the appropriate surface chemistry must be carefully considered with respect to the target biomarker, the sample type, and the downstream detection method. Three general magnetic bead surface functionalities are used to extract nucleic acid biomarkers: silica, oligo (dT), and specific probe sequences. Silica-coated magnetic beads nonselectively bind nucleic acids in the presence of chaotropic salts via electrostatic interactions and are commonly used to extract DNA or RNA prior to PCR or RT-PCR, respectively.[2,4,5,8] Oligo (dT) functionalized beads use complementary base pairing to capture mRNA, which makes up only ∼3−5% of the total RNA in biological samples, and are also commonly used prior to RT-PCR.[9,10] Sequence-specific beads are much less common but have been used for specialized applications that require highly purified RNA sequences.[11]

In many RNA extraction applications, there is not always a clear justification for the use of a particular surface functionality,

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despite clear distinctions in RNA binding specificity and yield, as well as assay duration. Each of the three bead types can be used to prepare RNA for RT-PCR detection, as PCR reliably amplifies low concentrations of target RNA in the presence high concentrations of nontarget nucleic acids. Some detection schemes, however, require the capture of unique RNA sequences or subsets of RNA and are not tolerant of the presence of nontarget nucleic acids. Some of these assays also require hybridization of detection probes to the captured RNA on the bead surface. For example, following extraction with oligo (dT) magnetic beads, captured mRNA has been detected on a bead surface using the isothermal amplification strategy quadruplex priming amplification.[12] Similarly, the biobarcode assay relies on the detection of specific nucleic acid sequences by hybridizing complementary DNA-functionalized gold nanoparticles to captured nucleic acids.[13,14] These approaches are not possible with silica-coated magnetic beads since the nucleic acids are precipitated onto the bead surface, preventing detection probes from hybridizing to them until eluted from the bead surface. Therefore, it is critical to consider the appropriate magnetic bead surface functionality in the context of the detection scheme.

In this report, we directly compare the extraction performance, target selectivity, and binding kinetics of the three most common magnetic bead surface functionalities for extracting nucleic acids (i.e., silica, oligo (dT), and sequence-specific). The results of these studies highlight the advantages and disadvantages of each surface functionality and provide a clearer understanding of appropriate applications for each type.

MATERIALS AND METHODS

Description of the Silica-Coated, Oligo (dT), and Sequence-Specific Beads. The silica-coated beads used in these studies were Dynabeads MyOne SILANE (Life Technologies, cat no. 37005D), and the oligo (dT) beads were Dynabeads Oligo $(dT)_{25}$ (Life Technologies). The silica-coated beads are described by the manufacturer to be 1 μm in diameter with a surface area of ~20 m^2/g. The oligo (dT) beads are described by the manufacturer to be 2.8 μm in diameter with a surface area of 3−7 m^2/g. The specific-sequence, or RSV-specific, beads were prepared by functionalizing Dynabeads M-270 Amine magnetic beads (Life Technologies, cat. no. 14307D) with a 22 nucleotide ssDNA sequence complementary to a region of the N gene mRNA of RSV. The thiolated DNA sequence consisted of a 9 nucleotide poly(dA) linker sequence followed by 22 nucleotides complementary to the RSV N gene as follows: 5′-/5ThioMC6-D/AAA AAA AAA ATC ATG TAA AAG CAA ATG GAG TA-3′. The oligos were attached to the amine-coated beads by SMCC coupling according to the protocol provided in Hill et al.[13] The number of binding sites available to bind complementary oligonucleotides were quantified and compared to oligo (dT) beads in the Supporting Information (SI; see Figure S1). The amine-coated beads are described by the manufacturer to be 2.8 μm in diameter with a surface area of 2−5 m^2/g.

Preparation of RSV N Gene-Spiked HEp-2 Cell Lysates. HEp-2 cell lysates were prepared from a confluent monolayer of HEp-2 cells grown in a T-150 flask. The cells were harvested by scraping them from the flask and centrifuging at 500*g* for 5 min. The cell pellet was resuspended in 8 mL of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0] 0.5% *N*-lauroylsarcosine [Sarkosyl], 0.1 M 2-mercaptoethanol) and passed through a pipet tip 10 times. The cell lysates were stored at a concentration of approximately 3×10^6 lysed cells/mL in 1 mL aliquots at −80 °C until ready for use. Full-length RSV N gene mRNA (~1600 nucleotides, including poly(A) tail) was synthesized by Bio-Synthesis, Inc. Spiked HEp-2 cell lysates were prepared by adding 5 μL of RSV N gene mRNA at a concentration of 1×10^6 copies/μL (~840 fg/μL) to 20 μL of HEp-2 cell lysate. The resulting 25 μL samples were used immediately following preparation.

RSV N Gene Detection by RT-PCR. RSV N gene mRNA was amplified using forward primer 5′-GGAACAAGTTGTTGAGGTTTAT-GAATATGC-3′ and reverse primer 5′-CTTCTGCTGTCAAGTCTAG-TACACTGTAGT-3′.[15] PCR reactions were performed in 20 μL volumes using 5 μL of RNA template or eluate and the Power SYBR Green RNA-to-C_T 1-Step Kit (Life Technologies, cat. no. 4389986) according to manufacturer's instructions. Thermal cycling consisted of 48 °C for 30 min to synthesize cDNA, 95 °C for 10 min to inactivate the reverse transcriptase and activate QTaq DNA polymerase, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s using a Rotor-Gene Q thermal cycler (Qiagen, cat. no. 9001580). Product specificity was confirmed using melting curve analysis. Data were collected, and C_t values were quantified by Rotor-Gene Q Software using a standard curve of serial diluted RSV N gene mRNA at 10^8, 10^7, 10^6, 10^5, and 10^4 copies/μL.

RNA Binding Capacity Using Silica-Coated, Oligo (dT), and RSV-Specific Beads. The binding capacities of the silica-coated, oligo (dT), and RSV-specific magnetic beads were determined by quantifying the amount of RNA extracted from 25 μL of TE buffer containing increasing quantities (200, 400, 600, 800, 1000, 1200, 1400, or 1600 ng) of RSV N gene mRNA. For extraction with silica-coated beads, samples were combined with 300 μL of RNA-silica adsorption buffer (5.7 M guanidine thiocyanate [GuSCN], 25 mM sodium citrate, 1% 2-mercaptoethanol), 1×10^8 silica-coated magnetic beads, and 300 μL of ethanol and placed on a laboratory rotisserie for 30 min. Following nucleic acid capture, magnetic beads were washed with 300 μL of precipitation buffer (80% ethanol, 5 mM potassium phosphate, pH 8.0) and 300 μL of 70% ethanol. Captured nucleic acids were eluted by vortexing beads in 50 μL of nuclease-free water for 15 s. For extraction with oligo (dT) and RSV-specific magnetic beads, samples were combined with 300 μL of lysis/hybridization buffer (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT) and 1×10^8 magnetic beads in a 1.5 mL centrifuge tube, vortexed thoroughly, and placed on a laboratory rotisserie for 30 min. Captured nucleic acids were eluted by heating the beads in 50 μL of nuclease-free water at 65 °C for 2 min. The extracted RNA was quantified by measuring absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer or a BioTek Synergy H4 microplate reader.

Because of the disparity in the size and surface properties, as well as the binding capacity, of the silica-coated and oligo-coated beads, it is difficult to identify the concentrations to use for each that are suitable for direct comparisons. The amount of beads used for the following studies are 5×10^8 of the silica-coated beads and 6.7×10^8 of the oligo (dT) and RSV-specific beads. The binding capacities of the beads at these concentrations are in >2-fold excess of the highest amount of RNA present in the following experiments and that is present in common sample matrices.

RNA Binding Kinetics Using Silica-Coated, Oligo (dT), and RSV-Specific Beads. The RNA binding kinetics for silica-coated, oligo (dT), and RSV-specific magnetic beads were determined by incubating each bead type with 25 μL of the RSV spiked HEp-2 cell lysates and the appropriate buffer for 1, 3, 5, 30, 60, 120, or 180 min. For silica-coated beads, the beads and lysate were added to 300 μL of RNA-silica binding buffer and vortexed thoroughly, and then 300 μL of ethanol was added to the mixture before placing on a laboratory rotisserie. For the oligo (dT) and RSV-specific beads, the beads and lysate were added to 300 μL of lysis/hybridization buffer and placed on the rotisserie. Following incubation, the solution was removed by holding the beads to one side of the tube with a magnet and pipetting out the supernatant. The silica-coated beads were subsequently washed with 300 μL of precipitation buffer followed by 300 μL of 70% ethanol. The RNA was eluted from the silica-coated beads by adding 50 μL of nuclease-free water, vortexing the solution for 15 s, and collecting the eluate. For the oligo (dT) and RSV-specific beads, no wash steps were used. The RNA was eluted by adding 50 μL of nuclease-free water to the beads, placing the tube on a 65 °C heat block for 2 min, and collecting the eluate. The RSV N gene mRNA was quantified from the eluate using RT-PCR as described above.

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Selectivity for Target RNA Using Silica-Coated, Oligo (dT), and RSV-Specific Beads. The quantity of extracted RSV mRNA was compared to a background of RNA and nontarget mRNA for extraction with the silica-coated, oligo (dT), and RSV-specific beads. A mixed RNA sample was prepared by combining 1000 ng of background RNA (yeast tRNA, Life Technologies, cat. no. AM7119), 30 ng of nontarget mRNA (influenza A matrix gene mRNA synthetically prepared by Bio-Synthesis), and 0.003 ng of full-length target mRNA (RSV N gene) into 25 μL of TE buffer. These amounts of background RNA, nontarget mRNA, and viral RNA are approximately equivalent to the amounts found in RSV-infected cells collected from a patient nasal wash sample.[10] After a 5 min initial incubation time with the beads, the mixed RNA sample was extracted using the same methods described in the kinetics studies above, with the exception that 25 μL of the mixed RNA solution was used instead of 25 μL of the RSV mRNA-spiked HEp-2 lysates. Extracted total RNA was measured using a NanoDrop ND-1000 spectrophotometer at 260 nm. Nontarget mRNA and RSV N gene mRNA were quantified using RT-PCR. The nontarget control mRNA was quantified by adding 5 μL of a 100-fold dilution of the eluate to 20 μL Power SYBR Green RNA-to-C_T 1-step master mix containing the forward primer 5′-GACCRATCCTGT-CACCTCTGAC-3′ and the reverse primer 5′-AGGGCATTYTGGA-CAAAKCGTCTA-3′.[16] Cycling conditions were as follows: 30 min at 48 °C to synthesize cDNA, 95 °C for 10 min to inactivate the reverse transcriptase and activate QTaq DNA polymerase, and 40 cycles of 95 °C for 15 s and 55 °C for 60 s using a Rotor-Gene Q thermal cycler (Qiagen, 9001580). Product specificity was confirmed using melt curve analysis. Data were collected and C_t values were quantified by Rotor-Gene Q Software using a standard curve of serial diluted influenza A matrix gene mRNA at 10^8, 10^7, 10^6, 10^5, and 10^4 copies/μL. The results were then multiplied by 100 to account for the 100-fold dilution factor. Extracted RSV N gene mRNA was quantified using RT-PCR as described above.

Self-Contained RNA Extraction Using Silica-Coated, Oligo (dT), and RSV-Specific Beads. The self-contained extraction format used in this study is a generalizable method for comparing the fractional recovery of RNA target for each bead in a practical RNA extraction application. Our laboratory has extensive experience using this method for developing sample processing assays for limited-resource settings.[4−6] Details about this device for extracting RNA, including RNA recovery, specificity, and variability assessments as well as comparisons with commercial methods, have been previously published.[4,17]

The self-contained extraction tubing was prepared by preloading the processing solutions for each bead type into 2.4 mm i.d. fluorinated ethylene propylene (FEP) tubing (McMaster Carr, cat. no. 9369T24) in a manner similar to our previously described RNA extraction devices[4,17] and as shown in Figure 1. For silica-coated beads, 50 μL of nuclease-free water, 300 μL of 70% ethanol, and 300 μL of RNA precipitation buffer, separated by ∼6 mm air gaps, were loaded into one end of the tubing. For oligo (dT) and RSV-specific beads, 50 μL of nuclease-free water was loaded. Next, in a 1.5 mL centrifuge tube, silica-coated, oligo (dT), or RSV-specific beads were added to 25 μL of RSV RNA spiked HEp-2 cell lysate and the appropriate RNA-binding buffer (300 μL of RNA-silica binding buffer plus 300 μL ethanol for silica-coated beads and lysis/hybridization buffer for oligo (dT) and RSV-specific beads). The tube was then placed on a laboratory rotisserie for 5 min. Following the incubation step, the contents of the tube were pipetted into the end of the extraction tubing, and both ends of the tubing were capped with Hemato-Seal capillary tube sealant (Fisher Sci, cat. no. 02−678) as shown in Figure 1. A 2.5 cm cubic neodymium magnet was used to gather the magnetic beads from the initial binding solution and transfer them through the first air gap and into the next solution. The magnet was moved rapidly back and forth to disperse the magnetic beads throughout each solution for ∼10 s before transferring the beads to the next solution. For the silica-coated beads, this included the two processing steps before the elution step (Figure 1A), and for oligo (dT) and RSV-specific beads, this included only the elution step (Figure 1B). For the silica-coated beads, the beads were then transferred out of elution solution back to the preceding solution, and the elution solution was transferred to a 0.5 mL centrifuge tube. For the oligo (dT) and RSV-specific beads, the elution solution containing the beads was transferred to a 0.5 mL centrifuge tube and placed on a 65 °C heat block for 2 min, and then the eluate was removed from the beads. Extracted RSV mRNA was quantified by RT-PCR as described above.

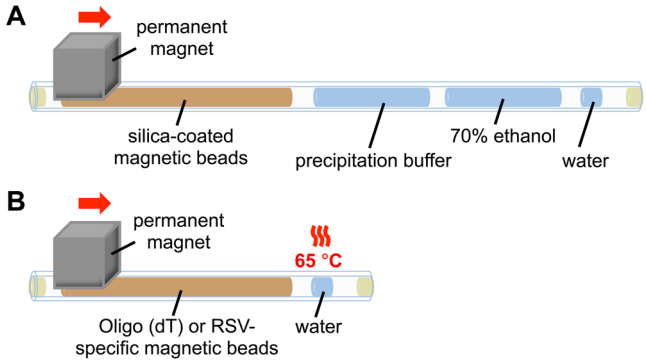

Figure 1. Comparison of the processing steps used for each bead type. (A) Total nucleic acids are captured from a sample using silica-coated magnetic beads and are extracted by pulling the beads through a series of processing solutions using a permanent magnet. Nucleic acids elute from the beads in the final water solution. (B) Sequence-specific nucleic acids are captured on oligo (dT) or RSV-specific beads and extracted by simply pulling the beads from the sample into water. RNA is eluted from the beads by heating the sample at 65 °C for 2 min.

Statistical Analysis. All statistical analyses were performed in SigmaPlot 11.0. Analysis of variance (ANOVA) was used to determine statistical significance for data containing three or more sample populations. In cases where ANOVA determined unequal means among the populations tested, a posthoc Tukey-Kramer analysis was performed. A t test was used for data containing two sample populations. A p value <0.05 was considered statistically significant.

RESULTS

RNA Binding Capacity Using Silica-Coated, Oligo (dT), and RSV-Specific Beads. The RNA-binding capacity was greater for silica-coated beads than for the oligo (dT) and RSV-specific beads. At the concentrations of RNA tested, the maximum quantity of RSV N gene mRNA extracted from TE buffer using 1×10^8 silica-coated magnetic beads was ∼800 ng (Figure 2, black circles). This corresponds to an mRNA binding capacity of ∼8 fg/bead or ∼10 000 RSV N gene mRNA copies/bead for the silica-coated beads. The maximum quantity of RSV N gene mRNA extracted using 1×10^8 oligo (dT) (Figure 2, white circles) or 1×10^8 RSV-specific magnetic beads (Figure 2, gray circles) was the same, at ∼300 ng. This corresponds to an mRNA binding capacity of ∼3 fg/bead or ∼3800 RSV N gene mRNA copies/bead for both the oligo (dT) and RSV-specific beads. Based on this data, it was determined that the concentrations of beads used for the following studies (i.e., 5×10^8 of the silica-coated beads and 6.7×10^8 of the oligo (dT) and RSV-specific beads) are in >2-fold excess of the highest amount of RNA present in the following experiments.

RNA Binding Kinetics Using Silica-Coated, Oligo (dT), and RSV-Specific Beads. For both the silica-coated and the oligo (dT) beads, RNA binding was maximized in less than 60 s, and the amount of RNA recovered remained relatively constant as binding time was increased. As shown in Figure 3, 75 ± 9% of spiked RSV N gene mRNA was recovered using the silica-coated beads (black circles) and 71 ± 26% recovered using the oligo (dT) beads (white circles) following a 1 min binding step. As the nucleic acid binding time was increased

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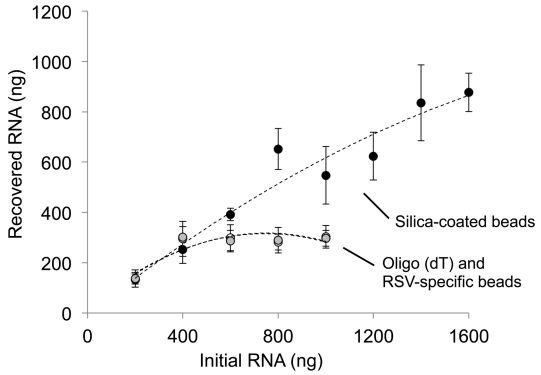

Figure 2. As the amount of spiked RNA is increased, the amount of RNA recovered using silica-coated (black circles), oligo (dT) (white circles), or RSV-specific beads (gray circles) increased until the RNA binding capacity of the oligo (dT) and RSV-specific beads was reached. Within the range of RNA tested, the binding capacity of the silica-coated beads was not reached (mean ± s.d., n = 3). Data points for the oligo (dT) and RSV-specific beads are offset ±10 ng from the silica-coated beads to aid in visibility.

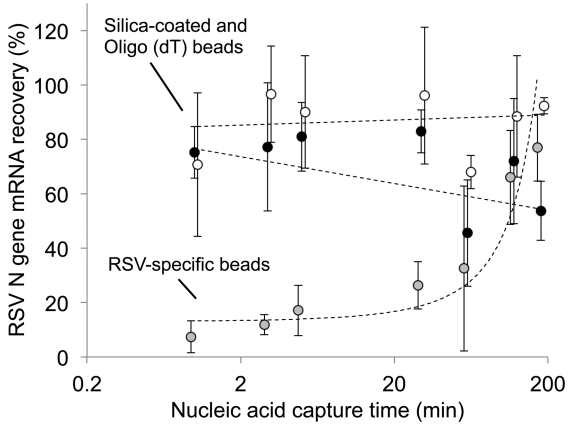

Figure 3. RSV N gene mRNA recovery remains relatively constant as nucleic acid capture time is increased from 1 min to 3 h using silica-coated (black circles) and oligo (dT) (white circles) magnetic beads. RSV N gene mRNA recovery using RSV-specific beads (gray circles) increases exponentially as RNA capture time is increased to 3 h (mean ± s.d., n = 3). Note the logarithmic scale used on the x axis. Data points for the oligo (dT) and RSV-specific beads are offset ±5% from the silica-coated beads to aid in visibility.

from 1 to 180 min, there was no significant increase in RNA recovery. Significantly slower binding kinetics were observed for the RSV-specific beads, which had maximal RNA binding only after ~180 min. The recovery of RSV N gene using the RSV-specific beads increased approximately exponentially as binding time was increased from 1 to 180 min. After 1 min, the recovery of RSV N gene was 7.4 ± 6% and increased to 77 ± 12% after a 180 min binding step.

Selectivity for Target RNA Using Silica-Coated, Oligo (dT), and RSV-Specific Beads. Silica-coated magnetic beads extracted a significant fraction of each type of RNA from the mixed RNA sample after a 5 min bead incubation step: 32 ± 4% of the 1030 ng total RNA, 41 ± 17% of the 30 ng nontarget mRNA, and 70 ± 6% of the 0.003 ng RSV N gene mRNA (Figure 4). From the same initial sample, oligo (dT) beads extracted 4 ± 2% of the total RNA, 79 ± 46% of the nontarget mRNA, and 103 ± 11% of the RSV N gene mRNA. RSV-specific beads extracted 7.2 ± 2% of the total RNA, 1.8 ± 0.2%

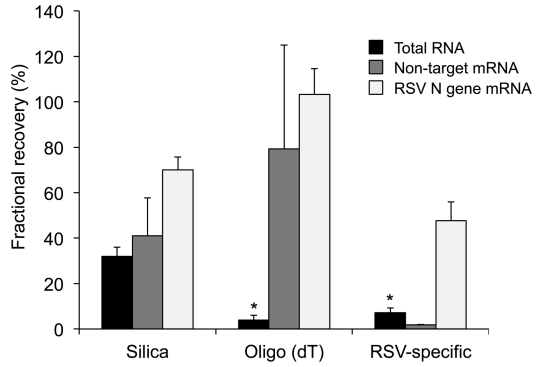

Figure 4. Comparison of RNA target selectivity at 5 min for each bead type. Silica-coated beads extracted a significant fraction of total RNA (black bars), nontarget mRNA (gray bars), and RSV N gene (target) mRNA (white bars). Oligo (dT) beads extracted very little total RNA but a significant fraction of nontarget mRNA and RSV N gene mRNA. RSV-specific beads extracted very little total RNA and nontarget mRNA but a significant fraction of RSV N gene mRNA. Total RNA was quantified using absorbance spectroscopy and nontarget and RSV N gene mRNA was quantified using RT-PCR (mean ± s.d., n = 3). *Not significantly different from background.

of the nontarget mRNA, and 48 ± 8% of the RSV N gene mRNA. This latter recovery value is greater than that reported at the 5 min time point in Figure 3 (17 ± 9%); however, it is important to note that these selectivity experiments of Figure 4 were performed using a buffer matrix and not HEp-2 cell lysate matrix used in the kinetics experiments. The difference in RNA recovery may be due to the presence of cellular material, such as lipids or proteins, which may prevent RNA from binding the beads.

These data correspond to an enrichment of extracted RSV N gene mRNA, relative to the initial and extracted amount of total mRNA from the mixed RNA sample, of ~1.7-fold for silica-coated beads, ~1.3-fold for oligo (dT) beads, and ~26-fold for RSV-specific beads (refer to the SI for calculation method). The enrichment of extracted RSV N gene mRNA, relative to the initial and extracted amount of total RNA from the mixed RNA sample, was not calculated because the total RNA extracted using oligo (dT) and RSV-specific beads was not significantly different from background.

Self-Contained RSV RNA Extraction Using Silica-Coated, Oligo (dT), and RSV-Specific Beads. Using the self-contained extraction methods illustrated in Figure 1 with a 5 min binding step, the silica-coated and oligo (dT) beads recovered 84 ± 48% and 78 ± 38% of spiked RSV N gene mRNA from HEp-2 cell lysates, respectively. Under the same conditions, the RSV-specific beads recovered 8.9 ± 5.4% of spiked RSV N gene mRNA using this method (Figure 5). Notably, these data are consistent with the 5 min time points of the kinetics data presented in Figure 3, which were prepared using the same starting sample and processing solutions but using microcentrifuge tubes versus the self-contained extraction device.

DISCUSSION

In this report, we present a performance analysis of three common magnetic bead surface functionalities used for RNA extraction: silica-coated, oligo (dT), and sequence-specific. Our quantitative assessment of RNA binding kinetics, target selectivity, and extraction yield identifies distinct advantages and disadvantages of each bead type. The results of these

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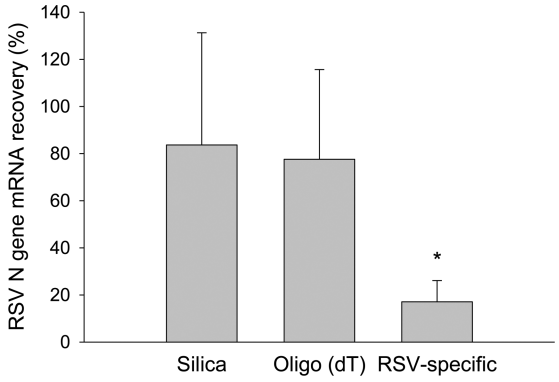

Figure 5. RSV N gene mRNA recovery from HEp-2 cell lysates is shown for the silica-coated, oligo (dT), and RSV-specific magnetic beads used in the self-contained extraction (mean ± s.d., n = 6). * denotes statistically less recovery than silica method.

studies provide a set of guidelines to assist in determining the most appropriate bead surface functionality for a given application.

For many applications, the oligo (dT) beads provide an optimal balance of performance characteristics, including a relatively high target selectivity, short assay duration, and high extraction yield. In these studies, samples processed with the oligo (dT) magnetic beads were highly enriched with mRNA. In an extracted sample containing no background nucleic acids or other potential contaminants, oligo (dT) beads recover over 70% of complementary poly(A) sequence, while recovering less than 3% of a random nontarget RNA sequence not complementary to the poly(T) sequence (Figure S1 in the SI). Notably, this hybridization-based approach extracts relatively short nucleic acid sequences (i.e., 22 nucleotides), which is not possible using silica-coated beads. In more complex samples containing both mRNA and large quantities of background tRNA, the oligo (dT) beads recovered a significant fraction of the mRNA, including approximately all of the RSV N gene target mRNA and less than 4% of the nontarget background tRNA (Figure 4). Although this reduction in nontarget RNA may not be necessary in background-tolerant methods such as RT-PCR, there may be some distinct advantages to reducing the amount of nontarget nucleic acids present in a sample. Furthermore, using the oligo (dT) beads, the captured mRNA sequences are available for amplification or detection strategies to be performed directly on the mRNA sequences while they are still attached to the beads. This advantage enables orthogonal nucleic acid detection strategies, such as quadruplex priming amplification or biobarcode assays, to be performed on the extracted biomarkers directly on the magnetic bead surface.[12,14]

Another major advantage of oligo (dT) beads is that the mRNA extraction protocol is relatively rapid and simple to perform. These are important characteristics for biomarker extraction assays used in diagnostic settings, as time-to-result and assay reproducibility may be critical to the clinical outcome. Our results indicate that the ∼1600 nucleotide mRNA biomarker hybridizes to the oligo (dT) magnetic beads within 1 min (Figure 3, white circles), which is in accordance with the findings from other studies that indicate that repetitive poly A and T hybridization is a fast, first-order reaction.[18] Because of the minimal number of processing steps, the total extraction time is less than 10 min. The streamlined processing also decreases the likelihood of operator error and risk of contamination. The assay was easily adaptable to the self-contained format, recovering ∼70% of RSV N gene mRNA biomarker from a complex biological sample (i.e., HEp-2 cell lysates; Figure 5). These data collected in the self-contained format are consistent with the 5 min data points collected in the kinetics experiments using the same starting samples and processing solutions (refer to Figure 3).

Interestingly, the oligo (dT) bead manufacturer recommends a simple protocol including two magnetic bead wash steps prior to nucleic acid elution. Yet, in these studies we found that the process could be further simplified by eliminating the wash steps and eluting extracted mRNA immediately following the mRNA binding step (Figure 1B). The single step oligo (dT) bead protocol was validated using our previously described self-contained extraction device (SI, Figure S2), which relies on surface tension valves to separate solutions within small diameter plastic tubing.[4,17] One potential disadvantage of this single step protocol used with the oligo (dT) beads is the carry over of residual liquid associated with the beads from the sample to the eluate, which would otherwise be diluted into the additional wash solutions used with other protocols. This residual fluid carried between solutions is ∼1 μL and may account for some of the background RNA detected in the eluate, as well as the presence of inhibitors of PCR.[4] However, in these studies, this carry over fluid had minimal effect on the efficiency of PCR. These results, although performed under different conditions, align with a report published by Sur et al., which demonstrated that PCR inhibitors are effectively removed without bead wash steps by moving magnetic beads through an immiscible fluid separating the sample and the elution solution.[19]

The silica-coated beads also appear to be suitable for a variety of applications. Nucleic acid adsorption to silica is the basis of the most common commercially available nucleic acid extraction kits. RNA extraction with silica-coated magnetic beads is simple and rapid, similar to extraction with oligo (dT) beads. Of the three bead types investigated, silica-coated beads were demonstrated to be the least selective, extracting a significant fraction of total RNA, nontarget-mRNA, and RSV N gene mRNA (Figure 4). Interestingly, the silica beads selectively extracted a greater fraction of RSV N gene mRNA compared to total RNA and nontarget mRNA. One explanation for this result could be that the RSV N gene mRNA exhibits characteristics, such as certain tertiary structures, that cause it to preferentially bind to the electrostatic surface of silica-coated beads. Because silica-based extractions generally extract all types of nucleic acid sequences, they are well-suited for detection strategies that require total nucleic acids or that are tolerant of high backgrounds of nontarget nucleic acids, such as PCR and RT-PCR. Furthermore, using these detection strategies, eluate from a single extraction could potentially be used to detect DNA, RNA, or biomarkers of multiple diseases simultaneously.

The silica-coated magnetic beads rely on electrostatic interactions to capture nucleic acids. This process occurs rapidly and results in a total extraction time of less than 10 min, an advantage in settings where short time-to-diagnosis is important (Figure 3, black circles). The binding capacity is also much greater than the oligo (dT) and RSV-specific beads. For the RNA concentrations tested, the silica-coated beads extracted as much as 8 fg/bead without becoming fully saturated, whereas the other two beads saturated at 3 fg/bead. This high binding capacity is likely an effect of electrostatic layering of RNA and chaotropic salts on the surface of the silica beads, which is in contrast to the finite number of binding sites on the oligo (dT)

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and RSV-specific beads. One disadvantage of the silica-coated beads is that, unlike hybridization-based capture methods such as oligo (dT) and RSV-specific magnetic beads, the minimum nucleic acid length that can be extracted with silica-based extractions is ∼60 nucleotides.[20] In fact, nucleic acid recovery is reduced by more than 4-fold for 60 nucleotide sequences compared to sequences longer than 100 nucleotides.[5] Another potential disadvantage of silica-coated beads is that the electrostatic interactions with nucleic acids require the presence of high concentrations of a strong chaotropic agent (i.e., 5.7 M GuSCN, in these studies). In laboratory settings, GuSCN has been relied on for decades as a rapid, dependable reagent for inhibiting nucleases and extracting nucleic acids. However, GuSCN is a potent inhibitor of RT-PCR and must be removed during the extraction. Additionally, GuSCN is toxic and can be dangerous in settings where the operator does not have the training or personal protective equipment to properly handle and dispose of this chemical.

Extractions performed with silica-coated magnetic beads are relatively simple to complete. The silica-coated beads require thorough washing with ethanol to precipitate nucleic acids onto the bead surface and remove traces of GuSCN that could inhibit downstream processes such as RT-PCR. Even with these additional wash steps compared to the oligo (dT) beads, silica-based extractions using magnetic beads have been adapted to a variety of formats due to their relative simplicity compared to alternative methods such as phase separation and centrifugation columns. The silica-based extraction method was successfully adapted to our previously described self-contained tubing format to extract both RNA and DNA from patient samples.[4,5] The silica-coated magnetic beads performed well in the self-contained format shown in Figure 1A, recovering ∼70% of spiked RSV mRNA biomarker (Figure 5), which is consistent with the 5 min data points collected in the kinetics experiments using the same starting samples and processing solutions (refer to Figure 3).

Our results indicate that sequence-specific magnetic beads may be useful in specialized applications that require highly purified sequences of RNA and that do not require rapid RNA binding kinetics. In these studies, the RSV-specific beads recovered minimal amounts of nontarget, noncomplementary sequences (∼7% of the 1030 ng of total RNA and ∼2% of the 30 ng of nontarget mRNA), while extracting a significant fraction of the 3 pg of target RNA (∼48% RSV N gene mRNA; Figure 4). Based on these values, the RSV-specific beads enriched the RSV N gene mRNA relative to the total mRNA present by ∼26-fold (Figure 4). Because of this capture selectivity, one major advantage of sequence-specific beads is that detection strategies are enabled that are not tolerant of the presence of nontarget background nucleic acids. Therefore, sequence-specific magnetic beads are generally not advantageous for applications that use RT-PCR as the final detection strategy, as RT-PCR is highly tolerant of the presence of nontarget nucleic acids.

The RSV-specific beads used in these studies have many similarities to the oligo (dT) beads, as both bead types rely on nucleic acid hybridization to capture target sequences. The RSV-specific beads performed reasonably well when tested in the self-contained tubing format illustrated in Figure 1B. The RSV-specific extraction was simplified to require no wash steps following nucleic acid capture, reducing the process to a single step (SI, Figure S2). However, the recovery was ∼9%, which is ∼15 fold lower than the oligo (dT) beads using a 5 min RNA binding step (Figure 5). Notably, these data collected in the self-contained format are consistent with the 5 min data points collected in the kinetics experiments using the same starting samples and processing solutions (refer to Figure 3). Also similar to oligo (dT) beads, the RSV-specific beads were demonstrated to capture relatively short nucleic acid sequences (i.e., 22 nucleotides; SI, Figure S1), which would enable the capture of short RNA sequences without poly(A) tails, such as microRNAs. Sequence-specific beads also have the advantage of enabling assays that rely on RNA detection on the surface of the magnetic bead. For example, sequence-specific magnetic beads are used in the biobarcode assay, in which DNA-functionalized gold nanoparticles hybridize to, and detect the presence of, nucleic acids captured on the surface on the magnetic beads.[14]

The trade-off between total extraction time and biomarker yield is an important consideration for the sequence-specific beads. It is well-documented that the binding kinetics for specific, complementary nucleic acid sequences are relatively slow.[21,22] In these studies, RSV-specific capture beads required a binding time of nearly 3 h to maximize RNA target recovery, whereas the oligo (dT) beads and silica-coated beads maximized recovery within 1 min (Figure 3). The disparities in the RNA binding kinetics are not completely explained by the affinities of the targets for the bead surface. The RSV-specific and oligo (dT) beads have similar dissociation constants with their respective targets, in the range of $\sim 10^{-10}$ M;[22] yet, the dissociation constant for DNA to silica is much higher at $\sim 10^{-5}$ M,[23] indicating a much weaker interaction. Another explanation for the disparities in binding kinetics is the differences in the availability of target binding sites. An increase in the number and availability of binding sites is essentially equivalent to an increase in the concentration of target. In these studies, the RSV-specific beads are complementary to a single 22 nucleotide stretch along the length of the ∼1600 nucleotide RSV N gene mRNA target. The oligo (dT) and silica-coated beads, on the other hand, have a much greater availability of target binding positions. Oligo (dT) sequences are available to bind virtually any position along the ∼250 nucleotide poly(A) tail of the target sequence, and the electrostatic silica surface nonselectively interacts with any part of target sequence. Furthermore, nucleotide dimers and hairpins are common in long RNA molecules. If these secondary or other tertiary structures formed in the target region, the kinetics of specific-sequence capture would be significantly impacted.[24] This is less of a consideration for oligo (dT) and silica-coated beads, as poly(A) sequences lack complementarity necessary for dimers and hairpins and silica-coated beads are not hybridization dependent. Based on this explanation, increasing the number of positions on the target that the sequence-specific beads could bind has the potential to improve the binding kinetics. This could be done, for example, by functionalizing the beads with a combination of oligonucleotides complementary to multiple regions of the target sequence.

CONCLUSIONS

Silica-coated, oligo (dT), and specific-capture magnetic beads for RNA extraction have distinct trade-offs with regard to assay effectiveness, simplicity, speed, and compatibility with the desired downstream detection strategy. Depending on the application, each of the three surface functionalities has advantages and disadvantages. Oligo (dT) beads offer the benefits of simplicity, speed, and the flexibility to perform

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mRNA detection directly on the bead surface. Therefore, oligo (dT) beads may be most appropriate for applications that require high yields of purified mRNA, such as the detection of pathogens that are actively replicating and producing mRNA. Silica-coated beads appear to be most appropriate in applications that require rapid isolation of total RNA and that are tolerant of high concentrations of chaotropic salts. RSV-specific beads extract samples highly enriched with RSV N gene mRNA and have the potential to extract short RNA sequences without poly(A) tails, such as microRNAs, or enable on-bead RNA detection. However, the specific-sequence beads appear to suffer from a trade-off between binding kinetics and RNA recovery efficiency.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary details about the characterization of the RSV-specific beads, optimization of the RNA extraction protocols, and calculation of mRNA enrichment values, including Figures S1 and S2 and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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