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Analytical Methods

Microfluidic Isolation of Nucleic Acids

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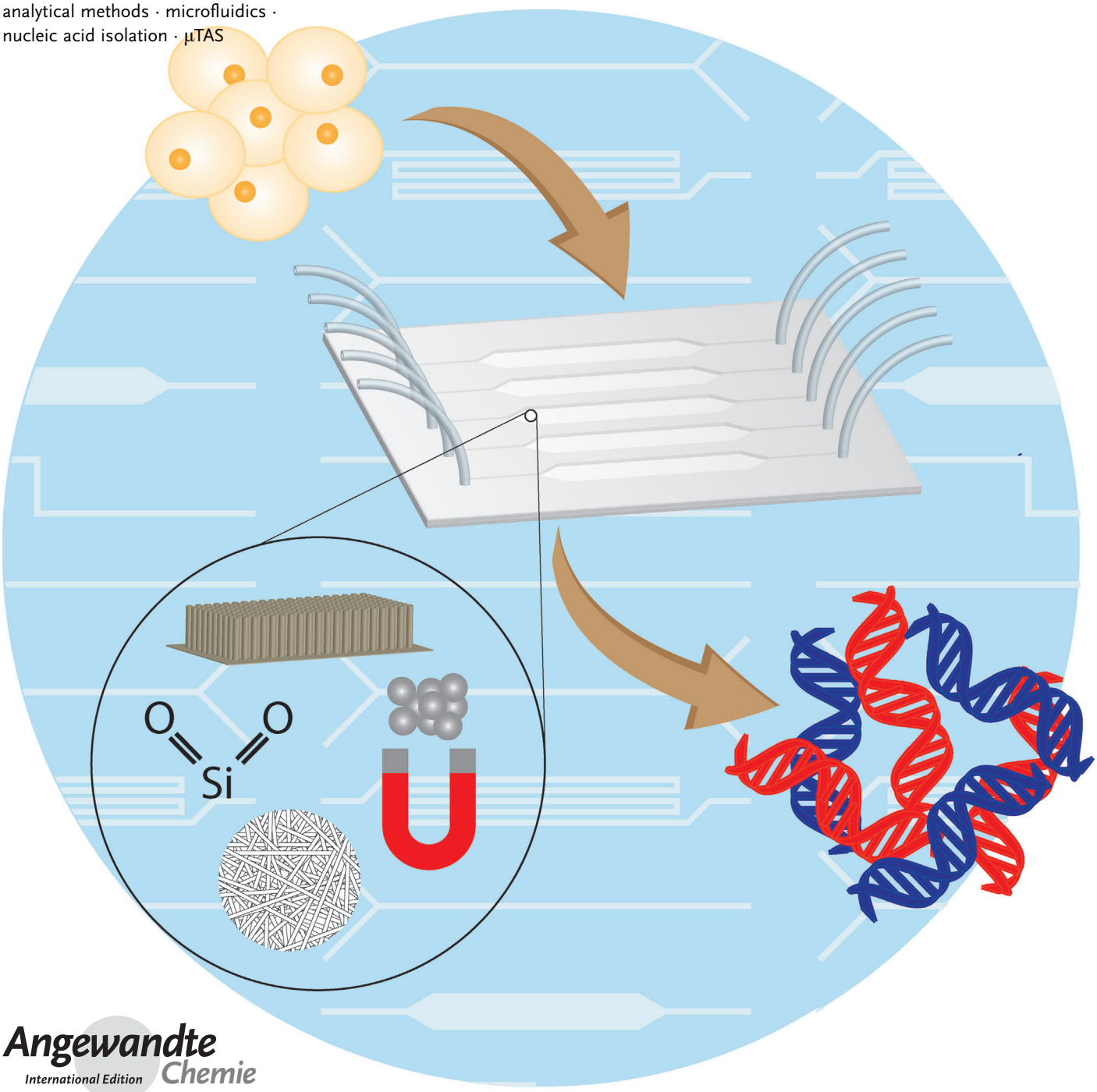

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The detection of nucleic acids (NAs) within micro total analysis systems (μTASs) for point-of-care use is a rapidly developing research area. The efficient isolation of NAs from a raw sample is crucial for these systems to be maximally effective. The use of microfluidics assists in reducing sample sizes and reagent consumption, increases speed, avoids contamination, and enables automation. Through miniaturization into microchips, new techniques have been realized that would be unfavorable and inconvenient to use on a macroscopic scale, but provide an excellent platform for the purification of NAs on a microscopic scale. This Review considers the complexities of NA isolation with miniaturized and microfluidic devices, as well as the considerations when choosing a technique for microfluidic NA isolation, along with their advantages, disadvantages, and potential applications. The techniques presented include using silica-based surfaces, functionalized paramagnetic beads, oligonucleotide-modified polymer surfaces, pH-dependent charged surfaces, Al_2O_3 membranes, and liquid-phase isolation. This Review provides a basis to develop the chemistry to improve NA isolation and move it toward achieving 100 % efficiencies.

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1. Introduction

The development of detection methods for nucleic acids (NAs) has provided a monumental technological advancement in numerous fields of study, with applications including pathogen detection in food, environmental, and clinical samples, as well as genetic analysis.[1–9] A crucial part of NA detection assays is the isolation of the NAs. Without adequate purification of the NAs from raw samples, the device sensitivity is greatly decreased, and downstream processes, such as amplification and detection, can be inhibited.

The detection of NAs by using bench-top, macroscale techniques has been well-established; however, these techniques often require large specialized equipment, trained personnel, large sample sizes, and are generally time-consuming and expensive, thus limiting them to usage in centralized laboratory facilities. Consequently, there has been a strong interest in developing portable point-of-care devices that can be used in resource-limited settings. As a result, the concept of a micro total analysis system (μTAS, or lab on a chip) was presented, which provides a significant improvement in performance.[10,11] The major advantages of miniaturizing NA detection assays are that they require much smaller sample volumes, consume smaller quantities of reagents, and are faster and more sensitive. Additionally, because these assays are carried out completely within a single device, the risk of contamination is almost eliminated and the system can be automated. To take full advantage of miniaturized lab-on-a-chip systems for NA detection, the isolation of the NAs must be done effectively inside a device. Furthermore, the unique advantages of miniaturized devices can and should be exploited, rather than simply shrinking macroscale processes down to the microscale, so that lab-on-a-chip systems can reach their full potential. Here, automation, lack of contamination, short diffusion/reaction distances, fast heat transfer, and simple liquid handling capabilities are of ultimate importance.

The isolation of NAs is a crucial step in a μTAS for the detection of cells and specific NA sequences in disease control, pharmacological studies, environmental protection, and fundamental studies in which findings are tied to the identification of specific NA sequences. Critical sample aspects in the typically complex clinical[12] and environmental[13] samples in which cells are contained have to be considered prior to choosing the most appropriate method for isolating the NAs. Cell lysis is the first step in NA isolation, and a variety of techniques both on- and off-chip have been reviewed extensively.[12,14] The lysate contains several constituents, including proteins, cellular membrane debris, polysaccharides, metabolites, ions, and other NAs aside from the target NA,[15–17] which must be removed in the NA isolation process prior to subsequent analysis stages. Downstream processes frequently include an amplification step, and while the polymerase chain reaction (PCR) is the most commonly used on-chip amplification method,[18,19] isothermal amplification techniques are becoming more abundant, since it is difficult to integrate thermocycling into a microchip.[20]

Other critical considerations include the type of NA to be isolated, the purpose for its isolation, and the complexity of the required operations, as these need to be realized within a limited space on a μTAS chip. There are different types of NAs present in cells, and each have their own unique characteristics, such as function, mass, abundance, and location within the cells, which present unique challenges in

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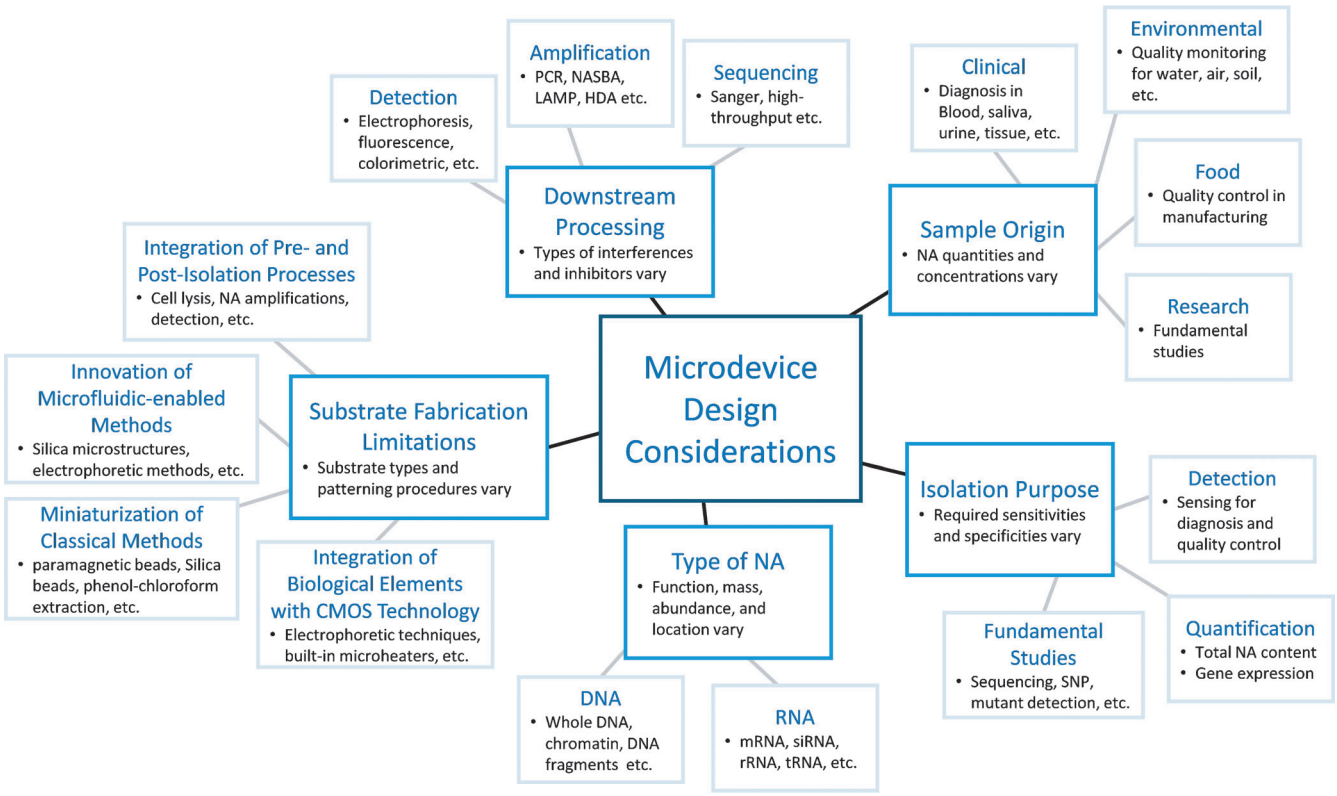

Figure 1. Criteria for choosing the appropriate technique to isolate NAs. The aspects that must be considered when deciding on a technique include the sample type, the specificity of the isolation, the isolation purpose, the upstream and downstream processes, and the device characteristics and restrictions. The downstream processing is closely related to the purpose of the isolation, as the post-isolation steps are necessary to attain the ultimate results of the assay.

their detection.[20] Figure 1 summarizes relevant design aspects that guide the researcher and user in determining the critical device and assay characteristics. This Review delivers a comprehensive overview of NA isolation techniques carried out within microdevices. The different methods are compared in terms of advantages and disadvantages as well as their performance in specific applications. The integration of microfluidic isolation of NAs into complete μTASs enables the development of rapid, sensitive, and portable NA detection assays that can be used in resource-limited settings. A brief summary of the reviewed techniques, critical analysis of their advantages and disadvantages, as well as many demonstrated applications is given in Table 1.

2. Techniques for the Microfluidic Isolation of Nucleic Acids

2.1. Silica-Based Techniques

Silica-based techniques were developed and investigated early on in microfluidic devices, mimicking the successful silica-based isolation techniques of the Boom technology, currently commercialized by bioMérieux.[21–23] Here, the nonspecific binding of NA molecules to silica is exploited for their separation from other sample components. Chaotropic salts, such as guanidinium and sodium iodide, shield the

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Table 1: Summary of techniques for the isolation of nucleic acids.

Isolation technique	Advantages	Disadvantages	Application	Ref.
packed silica beads	• well-established • simple concept	• bead compaction leading to increased back pressure and decreased flow • not reliably reusable • uses solutions that inhibit amplification • device restrictions	• RNA from neat semen, mock semen stain, and alveolar rhabdomyosarcoma • *B. anthracis* DNA in whole blood and *B. pertussis* DNA in nasal aspirate • short tandem repeat (STR) DNA fragments in whole blood and semen	[37] [38] [39]
sol–gels incorporating silica beads	• large SA/V ratio • generally reusable	• requires post-fabrication processing • high fabrication temperature • expensive substrate • uneven bead distribution • uses solutions that inhibit amplification	• *S. typhimurium* and *B. anthracis* DNA in whole blood • genomic DNA in whole blood and *B. anthracis* DNA in a nasal swab	[24] [41]
porous polymer monoliths incorporating silica beads	• large SA/V ratio • disposable (one-time use) • inexpensive	• requires post-fabrication processing • uneven bead distribution • uses solutions that inhibit amplification	• influenza A viral RNA in cultured mammalian (MDCK) cells • influenza A and B viral RNA in a nasopharyngeal swab and aspirate • *E. coli* DNA in urine • *E. coli*, *B. subtilis*, and *E. faecalis* DNA in whole blood • *C. difficile* DNA in stool	[44] [48] [47] [45] [46]
fabricated silica microstructures	• no post-fabrication processing • reusable • higher flow rates feasible	• expensive substrate • long, complex, expensive fabrication process • lower SA/V ratio • uses solutions that inhibit amplification	• genomic DNA from leukocytes in whole blood • DNA from salmon sperm cells • genomic DNA in whole blood and A549 cell suspensions • bacterial DNA from *L. monocytogenes*	[50] [51] [52] [53]
silicate-based porous monolith	• large SA/V ratio	• requires post-fabrication processing • uses solutions that inhibit amplification	• genomic DNA in whole blood • genomic DNA from buccal cells • RNA from rat hepatocytes • DNA from *Mus musculus* cells	[27, 54] [55] [56] [57]
silica membrane	• easy integration into microfluidic device	• expensive • uses solutions that inhibit amplification	• *B. cereus* DNA and HIV I viral RNA in saliva • G6PDH and BCR-ABL cancer transcripts in K562 cells	[59] [60]
silica-coated paramagnetic beads	• high flow rates possible • large SA/V ratio	• requires magnetic field • device material and dimensions restrictions • uses solutions that inhibit amplification • beads are expensive	• genomic DNA in whole blood • α-thalassemie 1 DNA genetic deletions in saliva • RNA from RSV-infected HEp-2 cells • RNA from HIV-1 virus in plasma	[65] [66] [67] [69]
paramagnetic beads with switchable charges	• high flow rates possible • large SA/V ratio • uses pH instead of PCR-inhibiting solutions	• requires magnetic field • device material and dimensions restrictions • beads are expensive	• RNA from T98 cells • single nucleotide polymorphism genotyping of genomic DNA from leukocytes • DNA from *M. tuberculosis* in saliva	[70] [72] [73]
paramagnetic beads coated with oligo-dT	• only captures mRNA • detects viable organisms • high flow rates possible • large SA/V ratio	• mRNA poly-A tail is necessary • probe density restrictions • requires magnetic field • device material and dimensions restrictions • beads are expensive	• bicoid gene mRNA from *Drosophila melanogaster* • mRNA from a single NIH/3T3 cell • multiplex mRNA isolation from MCF-7-cells	[75] [76] [77]

Table 1: (Continued)

Isolation technique	Advantages	Disadvantages	Application	Ref.
paramagnetic beads coated with specific sequences	• direct capture of target • lower LODs	• probe density restrictions • requires magnetic field • device material and dimensions restrictions • beads are expensive	• circular RNA from *E. coli* • RNA from nervous necrosis virus in water samples • DNA from methicillin-resistent *S. aureus* in sputum, serum, and milk • RNA from influenza A and B viruses	[78] [80] [82] [81]
oligonucleotides on polymer surfaces	• no device restrictions • Large SA:V	• requires post-fabrication processing • uses reactive substances that can inhibit downstream processes	• mRNA from *C. parvum* • mRNA from rat liver cells • *gag* region RNA from HIV viruses in serum	[74] [84] [85]
chitosan-coated beads	• uses pH instead of PCR-inhibiting solutions • fast elution in small volume	• lower NA capacity • bead compaction leading to increased back pressure and decreased flow • device restrictions	• RNA from alveolar rhabdomyosarcoma cancer cells and buccal cells • genomic DNA from whole blood • RNA from influenza A virus in a mock nasal swab	[88] [89] [87]
aluminum oxide membranes	• does not inhibit PCR	• not reusable	• DNA from *B. cereus* cells and synthetic HIV RNA *gag* fragments • DNA from methicillin-susceptible and methicillin-resistent *S. aureus* in saliva	[90] [91]
photoactivated polycarbonate surfaces	• inexpensive • patterned easily • does not bind SPS • disposable	• stability of activated surface over time and range of conditions	• genomic DNA from whole blood and RNA from *E. coli* • multiplex DNA isolation from *B. subtilis*, *S. aureus*, and *E. coli*	[93, 95] [96]
amine-coated surfaces	•uses pH instead of PCR-inhibiting solutions • many ways to generate amine surfaces	• attracts other negative species • high pH value of elution buffer could inhibit downstream processes	• Genomic DNA from whole blood	[97]
electrophoretic techniques	• no need for external pumps (smaller experimental setup) • does not use PCR-inhibiting solutions • insensitive to device material and dimensions	• difficult to integrate downstream processes • electrophoretic mobility of NAs must be known	• genomic DNA from whole blood by ITP • DNA from *P. falciparvum* in whole blood by ITP • chromatin from *E. coli* by DEP trapping • RNA from *E. coli* and *S. thermophilus* by gel-EP	[98] [99] [101] [102]
isolation with an organic liquid	• well-established on macro scale • higher purification efficiency • accommodates very small samples	• uses extremely hazardous organic solvents	• multiplex DNA and RNA isolation from *P. aeruginosa* and *S. aureus*	[104]

negative charge of the silica surface, which decreases repulsion of the NAs, and dehydrates the silica and NAs. This creates a hydrophobic environment, so that favorable hydrogen bonding occurs between the NAs and silanol groups.[24] The bound NAs are washed with an organic solvent, such as ethanol or 2-propanol, thereby resulting in highly purified NAs upon elution into a solution with a low ionic strength.

This chemical principle has been exploited in a variety of microfluidic designs that combine the strengths of microfabrication and silica chemistry. Microscopy images of some of these platforms are shown in Figure 2. Microfluidic devices initially copied the macroscopic principle of packed silica beads or the inclusion of silica beads in polymer matrices. Shortly thereafter, silica microstructures were proposed as the next step in streamlining device fabrication and customization.

2.1.1. *Packed Silica Beads*

Silica beads have been used in the purification of NAs for over two decades, starting with bench-top assays using spin columns packed with silica beads.[21,30–32] This concept was

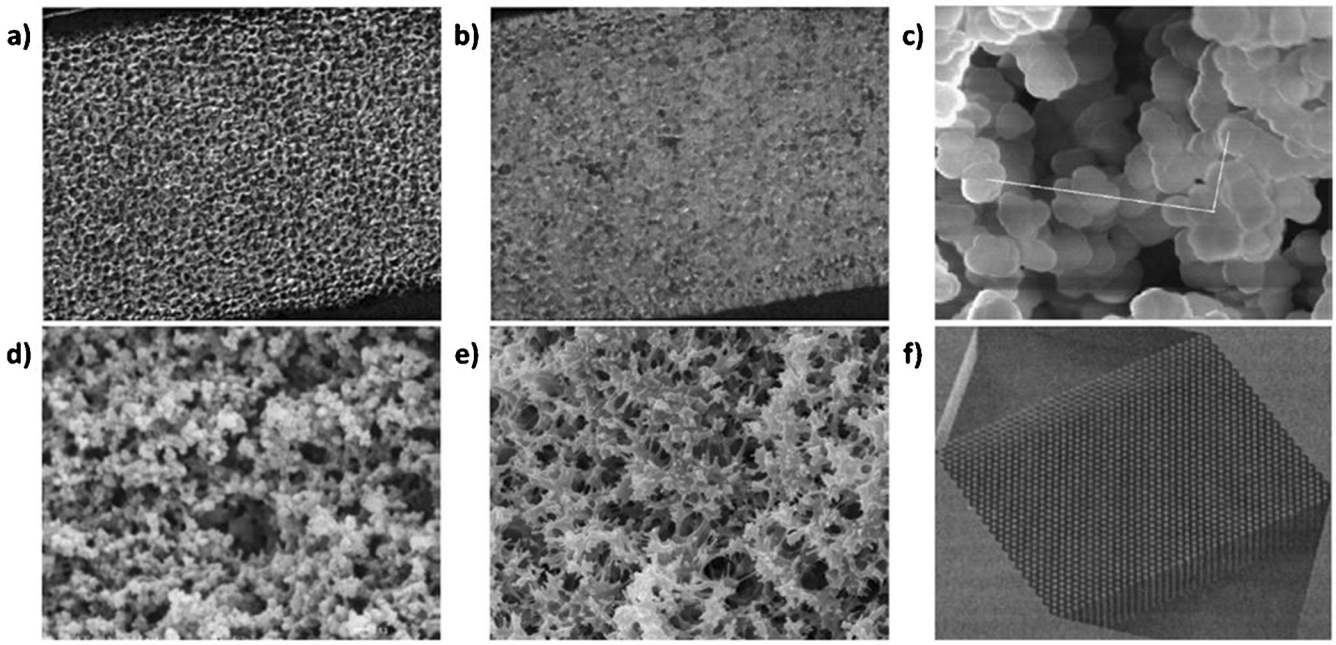

Figure 2. Microscopy images of the different silica-based surfaces used for NA isolation. a) Silica beads within a microchannel (reprinted from Ref. [25] with permission, Copyright 2002 Wiley-VCH). b) Silica beads incorporated into a sol–gel matrix within a microchannel (reprinted from Ref. [25] with permission, Copyright 2002 Wiley-VCH), c) Silica beads incorporated into a porous polymer monolith (reprinted from Ref. [26] with permission, Copyright 2010 American Chemical Society). d) Silica-based sol–gel (reprinted from Ref. [27] with permission, Copyright 2006 American Chemical Society). e) Silica-based porous polymer monolith (reprinted from Ref. [28] with permission, Copyright 2009 Elsevier). f) Silica micropillar array (reprinted from Ref. [29] with permission, Copyright 1999 ASME).

miniaturized by incorporating silica beads into microcapillaries for the more rapid isolation of NAs without the need for lengthy centrifugation.[33,34] The majority of microfluidic devices constructed for the use of packed silica beads incorporate a weir structure, which acts as a frit to confine the beads to a specific location within the channel, while still allowing fluid flow.[35] Both DNA and RNA have been successfully isolated by using this technique.[36,37] Combining NA isolation directly with an amplification reaction is a major step towards a complete μTAS, and has been demonstrated for the isolation of DNA using packed silica beads and PCR amplification on-chip.[38,39] Here, it is important to remove the chaotropic salts and organic solvents thoroughly, as these are known PCR inhibitors.[39] Therefore, these devices contained separate channels/reservoirs for the isolation and PCR amplification to further prevent the inhibitors from interfering with the PCR.[38,39] However, the results were often not reproducible and the stability of the bead bed was poor, which resulted in destruction of the microchip after prolonged use.[25] Additionally, issues such as bead compaction, increasing back pressure, and decreasing flow as a result of the dynamic nature of the bead packing are common drawbacks. A solution for several of these challenges has been found by entrapping the silica beads in polymer matrices within the microfluidic devices.[25]

2.1.2. *Matrices Containing Silica Beads*

An improvement upon packed silica beads has been their confinement within porous materials, such as sol–gel matrices and porous polymer monoliths. In the case of sol—gel matrices, silica particles are incorporated into the gelation or polycondensation process,[40] thereby resulting in a monolith for NA isolation. Investigations revealed that the most relevant factors affecting the efficiency were the size of the silica beads, the matrix material, the type of condensation reaction, and the method used to construct the monolith.[25] Additionally, examinations showed that a decrease in the pH values resulted in decreased repulsion between the silica surface and DNA molecules, which in turn increased the rate of DNA adsorption, thus enabling higher flow rates to be used. As a result, rapid extractions of bacterial and viral DNA as well as DNA from whole blood were accomplished.[24] The successful isolation of DNA and PCR amplification within the same microdevice was subsequently demonstrated.[41] However, a significant limitation of using sol–gel matrices is the high temperature required, which in turn necessitates the use of glass as the microfluidic platform, and glass is a relatively expensive material to pattern.

The Klapperich research group has demonstrated a novel strategy to create porous polymer monoliths containing silica beads within inexpensive plastic-based microfluidic platforms that can be patterned easily by hot embossing. The significant decrease in manufacturing cost also allows these devices to be disposable, thus eliminating the risk of contamination between uses. These monoliths contain micro- and nanoscale pores, which allow fluid flow with low back pressures as well as a large surface area for NA adsorption.[42] Polymers, such as cyclic olefin copolymers, can be chosen that facilitate subsequent in-device detection as a result of their excellent

optical qualities.[43,44] These devices were successfully applied to a variety of sample conditions and targets.[44–47] Some of these assays have even been incorporated into μTASs with integrated on-chip NA amplification.[46,48] While these methods of immobilizing silica beads within porous materials solve many of the problems associated with packed silica beads, they ultimately require additional fabrication steps after the microfluidic device has been constructed, and nondisposable devices can be difficult to reuse reliably.

2.1.3. *Silica Microstructures*

The development of silica microstructures enabled the creation of a NA binding surface during the fabrication of the microdevice itself. Since significantly less back pressure is observed compared to packed beads and matrices, higher flow rates, and in turn higher throughput, can be realized, which allows for the processing of larger volumes. These devices are generally fabricated from silicon or glass substrates using reactive ion etching to pattern the micropillars.[29,49–51] Here, maximizing the available surface area and mixing within the device is key to optimizing the extraction efficiency.

The surface area and mixing within the isolation device is determined by the size, density, and shape of the microstructures as well as the overall size and shape of the isolation chamber. Generally, the surface area/volume ratio (SA/V) is chosen as a compromise between maximization of the surface area, ease of fabrication, and flow-rate limitations because of back pressure. These aspects ultimately affect the performance, with the extraction efficiency scaling positively with the SA/V ratio[49] and negatively with the flow rate.[29,51] The shape of the microstructures and microchannels is important for determining an ideal flow pattern. Wu et al. found when comparing the shapes of microstructures that a bed of pyramidal pillars had a higher extraction efficiency than frustoconical pillars, likely because the pyramidal pillars provide an increased flow disturbance (i.e. increased mixing), and therefore increased the number of DNA molecules that contacted the binding surface.[52] A wider, shorter microfluidic channel will reduce the hydrodynamic resistance and allow higher flow rates to be used, which is better for larger samples with high target concentrations where a compromise in terms of the extraction efficiency can be tolerated. In contrast, a thinner, longer channel increases the resistance, which is more ideal for smaller samples with a low concentration of the target NAs, with lower flow rates used to maximize the NA binding.[51] Figure 3 shows a summary of the design considerations for fabricating silica microstructures within microfluidic channels for the isolation of NAs.

In summary, silica microstructures have been used as effective platforms for the isolation of NAs. This technology has also been integrated into μTASs that include NA amplification and detection steps.[52,53] However, this isolation technique involves devices constructed from relatively expensive materials by using complex manufacturing processes that are time-consuming and require access to a clean room, which is very costly.

2.1.4. *Other Silica-Based Surfaces*

Other silica-based surfaces for NA isolation have been used within microfluidic devices, including silicate-based porous polymer monoliths and silica membranes, to avoid such issues as bead compaction, uneven distribution of beads within matrices, and the high cost of microfabrication. Several research groups have used porous polymer monoliths made from tetramethyl orthosilicate (TMOS)[27,54] and potassium silicate solutions[55–57] to isolate NAs from a variety of samples. Specifically, with small volumes of dilute sample, it is traditionally[58] suggested to use carrier RNA to reach sufficient extraction efficiencies.[28] However, Kashkary et al. were able to extract DNA with high efficiency from samples by using a silica monolith without the use of a carrier.[57] This shows that porous polymer monoliths without silica beads still

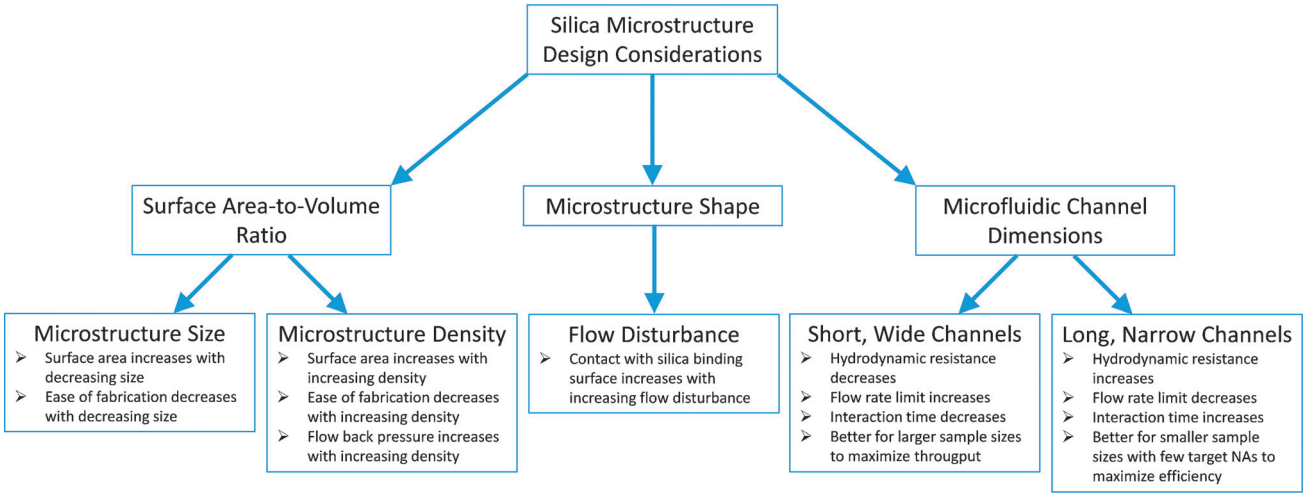

Figure 3. Criteria for the fabrication of silica microstructures for NA isolation. Critical characteristics of the microstructures include their size, density, and shape. The selection of the appropriate dimensions for the microfluidic channels is also very important and should be made on the basis of the sample size and concentration.

possess a very large SA/V ratio, and have been proven to enable the sufficient isolation of NAs.

Another format for silica-based NA isolation is the use of silica membranes consisting of a network of glass fibers. Typically, the membranes are integrated into the microdevices simply by physically securing them within a chamber. This approach was even used within complete μTASs by incorporating lysis, amplification, and detection along with the NA purification step before amplification by PCR.[59] These μTASs have been used to isolate DNA and RNA from several different viruses,[59] bacteria,[59] and other cells.[60] In all cases, the NA isolation step within these devices was essential for achieving such low limits of detection (LODs).

2.2. Techniques Using Paramagnetic Beads

Separation assays using paramagnetic beads have been used extensively since the 1970s, and have been thoroughly reviewed in the literature.[61–64] More recently, paramagnetic beads have been integrated into biological assays within microfluidic devices.[65,66] Paramagnetic beads have several advantages over packed bead beds in NA isolation assays. Crude samples with many solid contaminants of various sizes are more easily dealt with, as there is no issue of clogging because of the presence of densely packed beads. A wide range of sample volumes can also be accommodated, since the beads still possess a large SA/V ratio and the flow rate is not restricted by small pore sizes between the packed beads and the weir structure. These advantages result from the ability to suspend the beads freely in the sample solution, thereby maximizing the interaction between the beads and sample, and collecting the beads simply by using a magnetic field rather than by centrifugation or filtration. Additional benefits of miniaturizing this process in microfluidic devices are that far fewer beads can be used regardless of the sample volume, thereby reducing the cost, and a lower magnetic field strength can be used, since it is a microscale platform. However, the need for a magnetic field puts limitations on the design of the microfluidic chip. The type of material and its thickness will affect the strength of the magnetic field,[65] and the microchannel should be designed to minimize bead loss. Both specific and nonspecific isolation of NAs have been demonstrated through the use of paramagnetic beads with different surface modifications.

2.2.1. Silica-Coated Paramagnetic Beads

The general technique of using silica beads as described above can be enhanced by using silica-coated paramagnetic beads. Instead of being packed and confined in the chamber using a weir, paramagnetic silica beads can be free in solution and collected using a magnetic field, which increases the available silica surface area for binding NAs. A variety of techniques have been employed for the washing and elution phases in the isolation of the NAs. There are conventional techniques that use a magnetic field to hold the beads stationary while washing buffers flow through the microchannels, and there are more unconventional techniques that use a magnetic field to move the paramagnetic beads through stationary washing solutions.

Conventional setups using silica-coated paramagnetic beads within channels have been successfully employed with different substrate materials, and eventually scaled up to eight parallel devices.[65] However, scaling up posed an issue of nonhomogeneity in the magnetic field, so each channel had a different extraction efficiency. A complete μTAS for the detection of genetic deletions in cells from saliva samples has also been demonstrated, thus showing the compatibility of this technique with other processing steps.[66]

Microfluidic devices using stationary solutions and flowing paramagnetic beads have been developed with various geometries and complexities. Washing solutions have been separated by air bubbles,[67] as well as using hydrophobic immiscible liquids[68] that also serve as washing phases between the binding and elution steps to minimize the carry-over.[69] An example of a technique using air to separate solutions can be seen in Figure 4. Ultimately, the main disadvantage of using silica-coated paramagnetic beads is that they still require the use of chaotropic salts and organic solvents, which can interfere with downstream processes such as NA amplification.

2.2.2. Paramagnetic Beads with Switchable Charges

Similar to silica surfaces, charged surfaces can also be used to isolate NAs from complex samples because of the charged nature of NAs. Some materials can possess different surface charges depending on the pH value of the solution in contact with them. This phenomenon has been utilized for the isolation of NAs by using materials that switch between a positive charge for binding the negative NAs and a negative charge for elution. This type of material has been coated onto paramagnetic beads to take advantage of their additional benefits. With this technique, a low pH value is used to create a positive surface charge for binding, and a high pH value is used to create a negative surface charge for elution.[70] Many research groups, especially those focused on the microfluidics aspect, use commercial beads coated with a proprietary charge-switchable material; however, a well-known example also used on magnetic beads is polyethyleneimine. This is a multivalent cationic polymer capable of possessing a positive or negative charge depending on the pH value of the solution.[71] This class of magnetic beads has not only been used within microdevices, but has also been incorporated into complete μTASs.[72] Furthermore, a multiplex μTAS capable of handling 10 samples has been developed, and achieved an excellent LOD of just 10 bacteria,[73] which demonstrates the great potential of using paramagnetic beads with switchable charges to develop highly sensitive μTASs. However, a general disadvantage of these methods is that this process still necessitates the use of DNases and RNases to degrade the unwanted types of NAs.

2.2.3. Paramagnetic Beads Coated with Oligo-dT

The nonspecific isolation of all NAs sometimes requires additional purification steps to narrow down the sample to

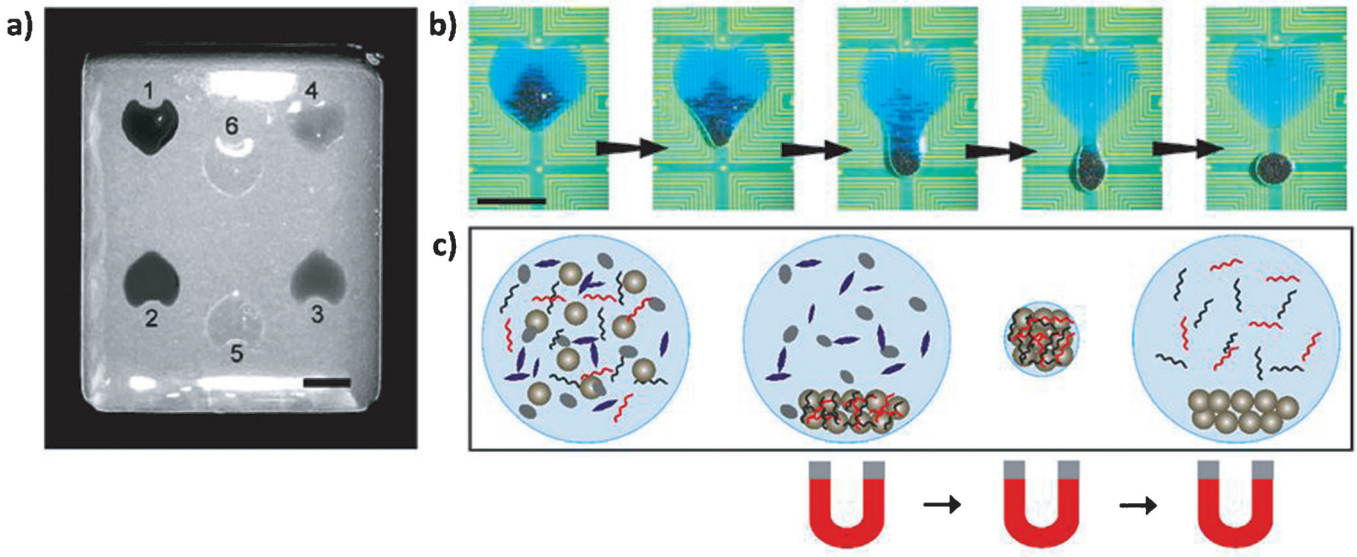

Figure 4. Isolating NAs using stationary solutions separated by air, and by using a magnet to move silica-coated paramagnetic beads through the solutions. a) An image showing the effectiveness of washing using this technique, where the binding buffer was mixed with a dye. As the paramagnetic beads with bound NAs travel from solution to solution, the binding buffer and non-NA constituents are left behind in the droplets of the wash buffer. b) Time lapse images of the paramagnetic beads being drawn out of the buffer droplet. The droplet carry-over is minimal. c) Schematic representation of the NA isolation process. The NAs are bound to the beads, the beads are washed, and the NAs are eluted from the beads. (a) and (b) reprinted from Ref. [68] with permission, Copyright 2006 Wiley-VCH).

a specific type of NA. The large number of NAs in some samples can even saturate the silica surface, thereby lowering the extraction efficiency of the target NAs. However, in eukaryotic organisms, most messenger RNA (mRNA) possess a polyadenosine (poly-A) tail, and an elegant way to increase the specificity of the NA isolation is to use the thymidine oligonucleotide, oligo-dT. The oligo-dT will hybridize with the poly-A tail of all the mRNA molecules, while other types of NAs do not bind. An additional bonus of mRNA detection is the fact that only viable cells will have these present, in contrast to the detection of a general DNA sequence.[74] Specific mRNA extraction is also advantageous, since the quantity of mRNA is very small compared to the total RNA, yet multiple copies exist for each sequence. Consequently, oligo-dT has been immobilized onto paramagnetic beads and used within microfluidic devices to isolate minute quantities of mRNA.[75] A μTAS for preparing sequencing libraries even demonstrated the extraction of picogram and sub-picogram quantities from single cells.[76] Furthermore, isolation using oligo-dT-coated paramagnetic beads has also been scaled-up for high throughput, as well as simplified for rapidity, where standardized microtiter plates were used for extracting 384 or 1536 samples in parallel.[77] In these devices, the beads are magnetically pulled through the binding and elution solutions separated by an immiscible hydrophobic phase in just 10 s. This principle of specific RNA isolation can be further specialized by moving toward the isolation of specific sequences.

2.2.4. *Paramagnetic Beads Coated with Specific Sequences*

Modifying paramagnetic beads with specific oligonucleotide sequences is very advantageous, as it facilitates the possibility of the direct detection of target NA sequences without further purification, since the NA sequences of interest simply hybridize complimentarily to the paramagnetic beads, while the rest of the NAs and contaminants are washed away. An important parameter that needs to be considered when developing these systems is the density of probes on the magnetic bead surface. If the probe density is too high, then isolation can be hindered by steric effects.[78,79] Conversely, if the probes are too sparse, nonspecific binding to the bead surface can occur and contaminate the sample.[78] Several μTASs have been developed that are both rapid and sensitive by using paramagnetic beads modified with specific sequences, which have allowed detection of viral RNA[80,81] and bacterial RNA[78] and DNA[82] from a wide range of sample types. The specific nature of the NA isolation in these systems enabled detection with very low LODs. However, the isolation of specific sequences may be hindered by the presence of large concentrations of bulk DNA and RNA present in a sample, and this requires specific attention, potentially with the development of a two-step procedure.

A general disadvantage of using magnetic beads is the sometimes prohibitive costs involved with high-quality paramagnetic beads, which limits their widespread use in developing areas.

2.3. *Specific Surface Modifications*

The isolation of NAs can also be achieved by modifying a surface directly with oligonucleotides as well as by using specific surfaces that will nonspecifically bind NAs under certain buffer conditions. These methods are discussed below.

2.3.1. *Oligonucleotides on Polymer Surfaces*

Modifying polymer surfaces covalently with oligonucleotides is very advantageous for NA isolation, since restrictions on the dimensions of the device for confining beads (packing or magnetic) as well as the cost of the beads are eliminated. Additionally, the synthesis of these surfaces can be easily added to the manufacturing process; however, limiting the synthesis to isolated surface locations is only achievable by using more complicated procedures, such as masks or alternate assembly designs.[83] Furthermore, microstructures within channels, similar to those described above in the silica-based techniques, could be modified with oligonucleotides, and cohesively create a platform for the highly sensitive isolation of NAs.

Both oligo-dT and specific oligonucleotide probes have been immobilized onto porous polymer surfaces, and used to isolate mammalian mRNA[84] and specific NA sequences from viruses.[85] Creating these modified polymers within microfluidic channels requires additional steps that include reactive ingredients that could inhibit isolation or other processes if they are not adequately washed out; however, these polymers are needed to provide the additional surface area to immobilize a sufficient number of capture probes for effective isolation of the NAs. In contrast, our research group developed a method for modifying microchannels with high immobilization efficiency, without the need for the synthesis of a porous matrix. Oligo-dT probes were immobilized onto the periphery of 5th generation polyamidoamine dendrimers to achieve a high immobilization efficiency, which resulted in a high capture efficiency.[74] Figure 5 illustrates this isolation concept and shows how the microdevices are fabricated. Ultimately, the use of probe-modified surfaces as the isolation media would be very advantageous for μTAS applications in resource-limited locations, as it simplifies the operation and reduces the cost of the device.

2.3.2. *Chitosan-Coated Beads*

The Landers research group has developed an NA isolation technique based on chitosan-coated beads, and used these beads within microdevices to extract RNA and DNA from viruses[87] and mammalian cells.[88,89] This is a nonspecific isolation technique, where chitosan, which has a pH-dependent charge, can bind NAs at pH 5 and elute them at pH 9. This is advantageous compared to silica beads in that all aqueous buffers can be used in this procedure, as opposed to the need for chaotropic buffers and organic solvents that can inhibit downstream processes. Although chitosan surfaces have a lower overall capacity than silica surfaces for NAs (but still sufficiently high for the desired applications), the elution of NAs is much faster with chitosan surfaces within a small elution volume.[88] Additionally, the general advantages and disadvantages of bead-based microfluidic devices naturally also apply here (see Table 1).

2.3.3. *Aluminum Oxide Membranes*

Aluminum oxide membranes (AOMs) can be used to isolate NAs in a similar way as silica membranes. A high salt concentration causes the NAs to bind tightly to the membrane surface. A positive characteristic of these membranes that has been exploited is that they will not inhibit PCR if the membrane volume is maintained below a certain threshold relative to the PCR reaction volume.[90] In fact, the NAs will stay bound to the membrane in the presence of the PCR mix, so it can be used as a template in successive PCR reactions. However, if elution is needed, the NAs can be eluted by adding bovine serum albumin and extra Taq polymerase to the PCR reaction mixture.[91] AOMs have been incorporated into microdevices capable of isolating and amplifying both RNA and DNA,[90] and this technology has even been multiplexed to detect up to seven different targets simultaneously.[91] The compatibility with downstream processing makes AOMs very attractive NA isolation platforms. However, as a result of their tight binding of NAs, it is difficult to replenish the surface of the membrane, and replacement of the membrane with every sample presents its own challenges.

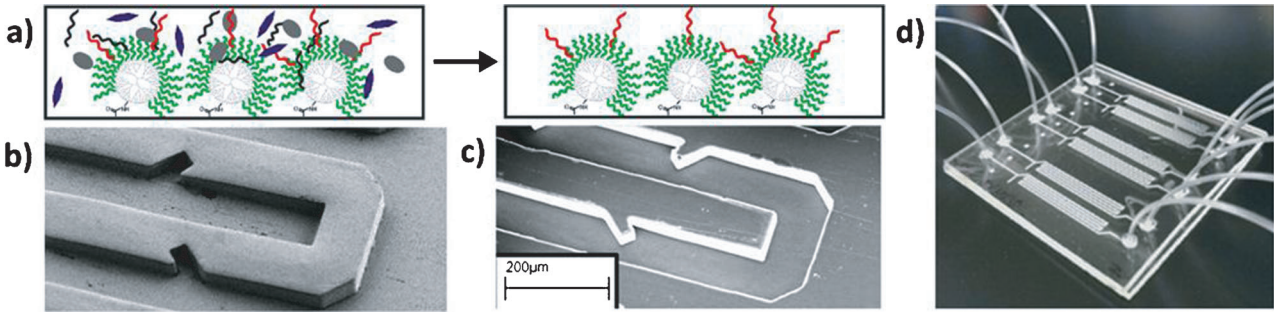

Figure 5. Isolation of mRNA using oligo-dT conjugated to the periphery of dendrimers immobilized within PMMA microfluidic channels. These microchannels are readily and inexpensively fabricated through hot-embossing and UV/ozone-assisted thermal bonding.[86] a) Schematic representation of the isolation process, where mRNA is captured by oligo-dT probes that are more densely immobilized within the microchannel through the use of dendrimers. Here, no elution of the mRNA is needed before isothermal amplification. b) Image of the copper master used to emboss the PMMA microchannels (c; reprinted from Ref. [68] with permission, Copyright 2008 Springer). d) Image of the fully fabricated microfluidic device (reprinted from Ref. [74] with permission, Copyright 2013 American Chemical Society).

2.3.4. Photoactivated Polycarbonate Surfaces

Photoactivated polycarbonate (PC) surfaces have been shown to nonspecifically bind NAs in the presence of high concentrations of polyethylene glycol (PEG) and NaCl, as demonstrated by the binding of DNA to a carboxylated surface.[92] Photoactivation of the PC creates a carboxylated surface, and the NA binding occurs in an analogous way to using silica surfaces. Unlike silica, PC can be patterned using hot-embossing, which is much easier and less expensive. An additional benefit is that sodium polyanethole sulfonate (SPS), a commonly used anticoagulant for whole blood samples and known PCR inhibitor, binds to silica in the presence of chaotropic salts, but not to PC.[93] This technology was implemented into microfluidic devices, and first characterized in a sheet format.[94] On the basis of these promising results, devices with in-channel micropost arrays were fabricated and characterized,[95] and then scaled up to extract gDNA and total RNA in a 96× microplate format.[93,96] These extractions were performed very quickly, with 96 purifications taking less than 30 min, and the inexpensive nature of these devices enables them to be disposable. Figure 6 depicts this isolation technique and highlights the fabrication of the microdevice. However, a limitation of using these surfaces could be the stability of the photoactivated surfaces over time and under various temperature conditions, which is important for applications in resource-limited settings.

2.3.5. Amine-Coated Surfaces

Amine-coated surfaces have also been investigated for the isolation of NAs by using microfluidic techniques. Amine groups are positively charged in buffers with a low pH value, which allows NAs to bind nonspecifically through electrostatic interactions, while buffers with a high pH value will enable elution of the bound NAs.[97] These surfaces do not require buffers that contain species which can inhibit downstream processes, and amine surfaces can be generated in many ways on a variety of surfaces, thus making this technique simple and robust. These surfaces were shown to successfully extract NAs;[97] however, the solely electrostatic nature of the immobilization presents a surface that will attract any species with a negative charge. Moreover, the high pH value of the elution buffer can also present problems for subsequent processes or necessitate adjustment of the pH value.

2.4. Liquid-Phase Isolation Techniques

Other isolation techniques have been developed within microfluidic devices that do not use surfaces or probes as a means for binding NAs. Instead, these techniques utilize mobility in an electric field and solution chemistry to separate NAs from the sample contaminants.

2.4.1. Electrophoretic Techniques

Different techniques based on electrophoresis have been developed and integrated into microfluidic devices. For example, isotachophoresis (ITP) uses a two-buffer system containing a leading electrolyte and a trailing electrolyte that are designed to have electrophoretic mobilities higher and lower, respectively, than the NAs. The trailing electrolyte should have a mobility lower than the NAs, but higher than the negatively charged impurities (positively charged impurities do not migrate in the same direction as the negatively charged NAs). When an electric field is applied across the two buffers, the NAs migrate to the interface between the two buffers, where an electric field gradient exists. This technology has been demonstrated in a microchip, and DNA from whole blood samples was successfully isolated.[98,99]

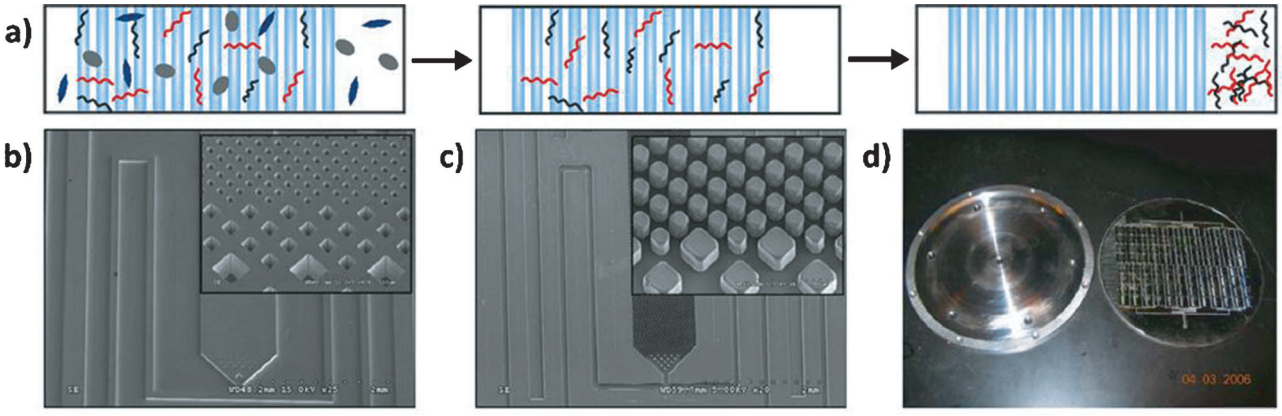

Figure 6. Isolation of NAs using photoactivated PC micropillar arrays. These microdevices are fabricated by hot-embossing and UV-assisted thermal bonding. a) Schematic representation of the NA isolation method, where the photoactivated PC has a carboxylated surface that binds NAs in the presence of high concentrations of PEG and NaCl. The unwanted cell constituents are washed away, and the NAs are eluted from the surface for further processing. b) Image of the nickel mold used to hot-emboss the PC. c) Image of the nicely formed PC micropillars. d) Image of the 96× device that uses these micropillar arrays to isolate NAs from 96 samples in a microtiter plate format. (Reprinted from Ref. [96] with permission, Copyright 2007 Springer).

Another example is dielectrophoretic trapping. In this technique, areas with large electric field gradients are used to trap DNA, through its polar nature. This is the same method used by Nanogen Inc. to trap cells within their microdevices.[100] The use of an alternating current field led to chromatin being trapped, and a small direct current field was used to remove contaminants.[101] Here, the microdevices were designed with a specific geometry that controlled the location of the large electric field gradient, and thus the trapping areas.

Gel electrophoresis has also been demonstrated within a microfluidic device for the extraction of RNA from complex samples. Here, a constant direct current is applied to the microchannel containing the sample, and negatively charged species migrate across the channel, with smaller, higher charged species moving more quickly than the larger, lower charged species. Low molecular weight RNA has been isolated by using this technique, with the gel acting as a cut-off filter for higher molecular weight species.[102] This is another example of an established laboratory-scale process being scaled down into a microfluidic device.

These electrophoretic-based techniques for NA isolation have several advantages over typical solid-phase extraction. Namely, 1) a voltage source is used instead of external pumps, which decreases the overall device size; 2) PCR-inhibiting solutions are avoided, and essentially any substrate material can be used; and 3) in the case of ITP, the geometry of the microchannels does not affect the process. These advantages make electrophoretic techniques attractive for the isolation of NAs, but there are also some concerns. Most importantly, none of these technologies have been integrated into a microchip that also performs on-chip amplification or detection; they all require removal of the sample by pipette. Furthermore, the electrophoretic mobility of the NAs to be isolated must be known, which varies with the charge and hydrodynamic size of the NAs.[103]

2.4.2. *Isolation Using Organic Solvents*

The well-established laboratory-scale technique of liquid-phase phenol/chloroform extraction has also been miniaturized and performed within a microfluidic device. In this process, proteins and cellular debris partition from the aqueous sample phase into the organic phase, as this is an energetically favorable transition. This technique has been integrated with on-chip PCR, and allows not only successful NA isolation but also the removal of inhibitors.[104] Here, sensitivity was likely improved by performing the isolation and amplification within the same chamber, since NA losses during sample transfer are avoided, which was also demonstrated previously in our research.[74] The main advantage of this method over solid-phase extraction in microdevices is that the purification efficiency is higher. Conversely, the main disadvantage of this technique is the use of hazardous organic solvents, which require safe handling and disposal. This disadvantage would clearly limit the use of these devices in most in-field, on-site, and resource-limited applications.

3. Conclusions

The techniques discussed for the isolation of NAs (summarized in Table 1) demonstrate the potential to develop sensitive μTAS devices capable of being used in point-of-care settings. By miniaturizing these processes and integrating them into microfluidic devices, many advantages are realized compared to their macroscale bench-top counterparts, including decreased cost, increased speed, automation, and reduced contamination. Since many different techniques have been demonstrated, the isolation method can be selected on the basis of which technique is most suitable for the assay parameters, such as sample type and size, device material, and fabrication technologies available, as well as the pre- and post-isolation processes. Furthermore, miniaturization enables these technologies to be multiplexed to handle multiple samples simultaneously, and provides a platform for high-throughput sample processing.

In some cases, highly purified NAs are not necessary and isolation may not even be needed. Amplification without prior NA isolation has recently been seen in some single-cell studies. Here, the focus is to isolate cells such that a single cell is in a pure solution, so skipping NA isolation is possible. This has been demonstrated in a chip that integrates cell capture and purification, reverse transcription of RNA, and quantitative PCR in a "one-pot" process.[105] The microfluidic isolation and processing of single cells without NA purification has also been commercialized by Fluidigm for the purpose of high-throughput sequencing.[106] However, NA isolation is still necessary even in single-cell analyses.[20,107,108]

Although the development of techniques for the isolation of NAs within microdevices has advanced significantly, there is still great room for improvement in both the techniques themselves and the integration and compatibility with other processes. For example, the ability to achieve close to 100% extraction efficiency with complex samples has yet to be realized. Wen et al. demonstrated the use of a protein removal step before NA isolation, which substantially increased the NA extraction efficiency.[54] These types of pre-isolation preparation steps can be implemented to improve the effectiveness of the device and move toward 100% efficiency. Ultimately, the challenge for chemists will be to develop reactions that work by the simplest means and avoid harsh conditions to maximize the compatibility between all the sample processing and analysis steps. Furthermore, since NA isolation is quite difficult even on a microchip, an alternative would be to develop assays for NA analysis that do not require isolation—this presents additional challenges. Very few of the techniques reviewed here were integrated into complete μTAS devices able to handle raw samples and take them through to detection. This will be a necessary step toward developing truly point-of-care devices that can be used in a variety of places, including resource-limited locations.

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