Colloids and Surfaces B: Biointerfaces 158 (2017) 1–8

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Magnetic particles for *in vitro* molecular diagnosis: From sample preparation to integration into microsystems

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ARTICLE INFO

Article history:
Received 21 March 2017
Received in revised form 5 June 2017
Accepted 19 June 2017
Available online 22 June 2017

Keywords:
Magnetic particles
Nucleic acids
Polymerase chain reaction
Microfluidic biosensor
Lab-on-a-chip microsystem

ABSTRACT

Colloidal magnetic particles (MPs) have been developed in association with molecular diagnosis for several decades. MPs have the great advantage of easy manipulation using a magnet. In nucleic acid detection, these particles can act as a capture support for rapid and simple biomolecule separation. The surfaces of MPs can be modified by coating with various polymer materials to provide functionalization for different applications. The use of MPs enhances the sensitivity and specificity of detection due to the specific activity on the surface of the particles. Practical applications of MPs demonstrate greater efficiency than conventional methods. Beyond traditional detection, MPs have been successfully adopted as a smart carrier in microfluidic and lab-on-a-chip biosensors. The versatility of MPs has enabled their integration into small single detection units. MPs-based biosensors can facilitate rapid and highly sensitive detection of very small amounts of a sample. In this review, the application of MPs to the detection of nucleic acids, from sample preparation to analytical readout systems, is described. State-of-the-art integrated microsystems containing microfluidic and lab-on-a-chip biosensors for the nucleic acid detection are also addressed.

Contents

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http://dx.doi.org/10.1016/j.colsurfb.2017.06.024
0927-7765/

1. Introduction

In recent decades, miscellaneous colloidal particles have been used extensively in various applications of biomedicine, e.g., biological imaging, targeted drug delivery, medical therapy, and biomedical diagnosis [1–3]. Among these colloidal particles, colloidal magnetic particles (MPs) have been the most extensively used in biomedical applications since they can be manipulated using a magnet. MPs can play important roles in the quantification of biomolecules due to their various advantages, e.g., biocompatibility, high dispersion, and high surface to volume ratio [4–6]. Several detection methods can be applied by using MPs with various types of terminal functionalization [7,8]. Modification with polymer coating materials and additional biological components has been described for specific diverse applications.

Since the discovery of circulating nucleic acids, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in plasma and serum, they have been the most important targets for molecular diagnosis of various diseases [9]. The presence of these nucleic acids due to pathogens or genetic mutations can differentiate patients from the healthy population. However, the detection of nucleic acids must have high sensitivity to recognize very low amounts of the target in the sample. In some diseases, highly sensitive and specific quantification of target molecules is a key factor in accurate and precise diagnosis [10,11]. The development of polymerase chain reaction (PCR), a molecular technique that can amplify a few copies of a DNA sequence to millions of copies, has expanded the horizon of molecular diagnosis. PCR has become the basis for latter developments, e.g., real-time PCR (RT-PCR), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), and circle-to-circle amplification (C2CA), which improved many aspects of PCR [12–14]. Although PCR has been enhanced its usability through minor procedural changes throughout the past, the post-amplification readout system of PCR products using gel electrophoresis is complex, time consuming and may exhibit non-specific and contaminated products [15]. While the sensitivity of RT-PCR represents the most advanced PCR-based application to date, the real-time analysis using fluorescent DNA intercalating dyes requires optimization of the conditions and consideration of non-specific binding between the dyes and amplified double-stranded DNA. Herein, the development of colloidal MPs has advanced several applications in the analysis of biomolecules for better specificity, e.g., bioseparation and specific-tagged particles [6,16]. In addition to specificity, highly sensitive detection is also achieved in applications based on individual particles, e.g., fluorescence microarrays [17], functionalized biosensors [18], and microfluidic systems [19].

In this review, we focused on the application of colloidal MPs in molecular detection, especially in PCR-based methods and their related procedures. The review sheds light on the state-of-the-art techniques for two main applications. The first is applications of MPs in sample preparation and extraction of target molecules using particle-based separation methods. The second is the molecular detection and readout system with functionalized MPs, including magneto immuno-PCR, microfluidic biosensors, and lab-on-a-chip microsystems. Synthesis and functionalization of colloidal particles in molecular detection were also discussed.

2. Colloidal MPs in sample preparation

Genetic materials that contain hereditary nucleic acid sequences, including DNA and RNA, have become the major target of PCR-based detection for various diseases. In PCR-based detection, extraction and purification of target DNA/RNA are required to eliminate unwanted PCR inhibitors and residual contamination in the sample. Conventional methods to extract and purify nucleic acids are dependent on treatment with a chemical solution, e.g., phenol-chloroform and spin column chromatography with centrifugation [20]. However, these conventional methods require a large volume of sample and lose some of the extracted DNA in the washing step. Colloidal particles have been applied to the sample to enhance the efficiency of extraction and purification methods. Spherical particles are suitable for molecular biology applications because they provide several advanced properties [6,7]. Colloidal particles have been implemented for extraction, purification, and pre-concentration *via* rapid and simple processes [21–23]. Depending on the purpose of the application, several types of material have been applied in the synthesis of colloidal particles.

MPs are also of great interest in sample preparation as a separating agent. Iron oxides, including magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3), have attracted substantial attention due to their biocompatibility, low toxicity, and simple preparation. Fe_3O_4 MPs can adsorb DNA sequences without modification [24,25]; however, negatively charged MPs may reduce the binding capacity to DNA and slightly increase inhomogeneous aggregation [26]. To enhance the efficiency and diversity of MPs, coating substances onto the particle surface has revealed a new dimension of the particles for a variety of applications.

2.1. Coating with aminated polymer

Coating MPs with a polymer can increase their stability, protect their surfaces from oxidation and reduce aggregation in the absence of a magnetic force [27]. The coating of MPs with aminosilane compounds, e.g., [3-(2-aminoethylamino)propyl]trimethoxysilane (AEEA), resulted in higher DNA binding and releasing capacities [28]. Silanization of AEEA was subsequently modified with dendrimer, i.e., polyamidoamine, which increased the cationic characteristics, reduced agglomeration and provided a high surface area for DNA binding [29]. Moreover, the efficiency of DNA binding and releasing was directly related to the generation of the dendrimer coating (Fig. 1) [30].

2.2. Coating with carboxylated polymer

MPs modified with a carboxylic functionalized surface were reported to adsorb DNA under a high concentration of sodium chloride [23,31–33]. DNA interacts with the carboxyl group on the surface of MPs through hydrogen bonding [31,34]. The concentration of sodium chloride also promoted salting out of the DNA containing hydrophobic nitrogen bases from the aqueous phase to the particles. Functionalization of the carboxyl groups was also performed using several compounds, e.g., methacrylic acid, phosphonic acid and dimercaptosuccinic acid [35–37]. Another interesting modification was the covalent grafting of poly(maleic anhydride-*alt*-methyl vinyl ether), which provided rapid functionalization of the carboxyl groups onto the cationic MPs [38,39].

2.3. Covalent coupling

The separation of nucleic acids using MPs was enhanced by immobilization of specific oligonucleotide sequences on the activated surfaces of the particles, primarily achieved by surface modification with specific biomolecules that can be covalently bound *via* activation of a crosslinking agent, e.g., glutaraldehyde and carbodiimide [40–42]. Amino-functionalized Fe_3O_4 MPs were immobilized on oligonucleotide sequences with 5′-amino modification in the presence of glutaraldehyde solution in sodium chloride-sodium citrate buffer (SSC) (Fig. 2). The immobilized MPs were able to capture DNA from various samples by hybridiza-

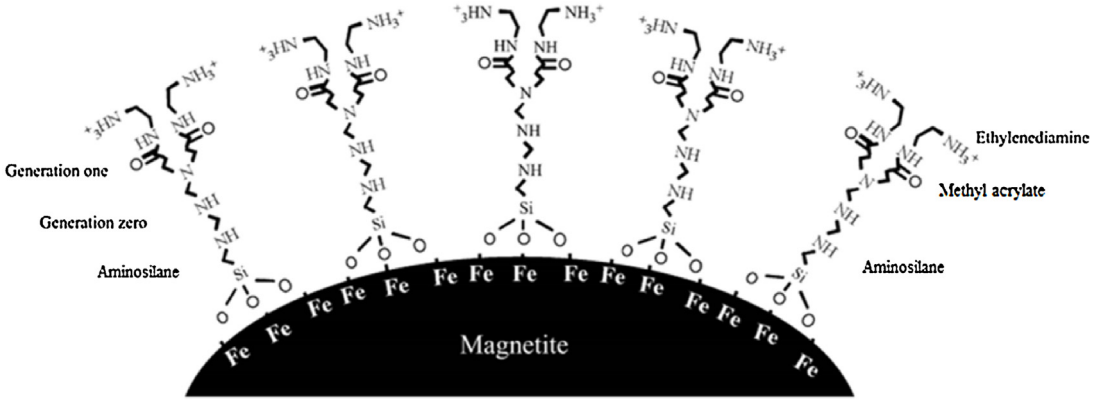

Fig. 1. Generation of dendrimer coating on the surface of MPs with silanization for the DNA binding support. Reprinted from Ref. [30], Copyright © 2002 with permission from Elsevier.

tion with the complementary target sequence. The efficiency of hybrid capture was directly correlated to the amino group density on the surface of the MPs [43,44]. The captured DNA could be eluted from the probe-immobilized MPs by dehybridization at 85 °C [40]. Direct amplification of the hybridized target DNA in the presence of probe-immobilized MPs at various quantities was subsequently characterized without the elution step. Although the probe-immobilized MPs potentially reduced the efficiency of PCR amplification in a dose-dependent manner, treatment with a blocking solution containing bovine serum albumin and glycine was responsible for full recovery of the amplification capacity [43,44].

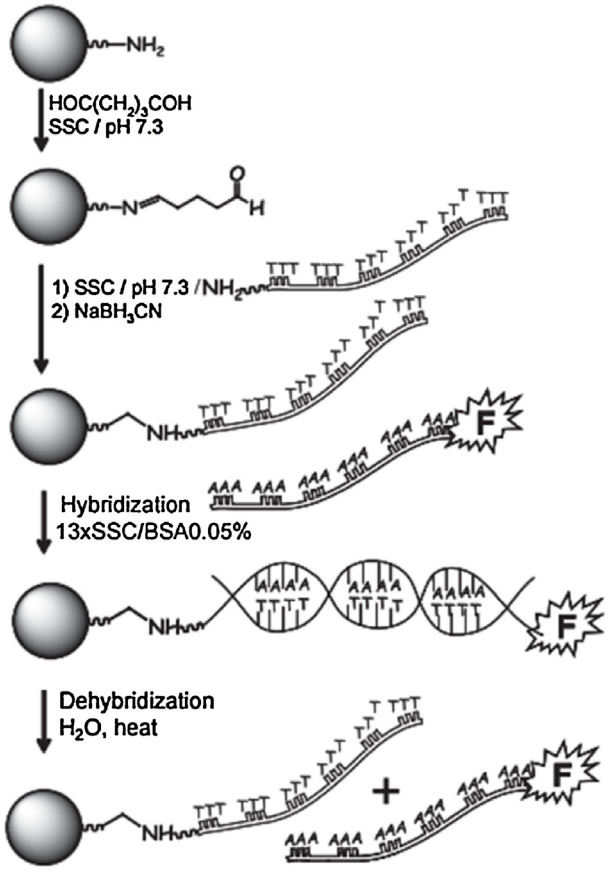

Fig. 2. Immobilization of an oligonucleotide sequence onto amino-modified MPs by activation with glutaraldehyde. Reprinted from Ref. [40], Copyright © 2005 with permission from Elsevier.

These developments have indicated adaptability to not only versatile sample preparation but also novel detection methods.

3. Functionalized colloidal MPs in molecular detection and readout systems

The application of readout systems using antigen- and antibody-conjugated colloidal MPs for specific tagging and sensitive analysis has been described elsewhere. Herein, the functionalized MPs have been implemented from various aspects, e.g., separating and signaling supports of analytical processes.

3.1. Separating support in DNA amplification

Immuno-PCR (IPCR), which incorporated immunological materials, was first described in the 1990s and was subsequently developed for highly sensitive nucleic acid detection [45,46]. IPCR adopted principles similar to enzyme-linked immunosorbent assay (ELISA), that is, colorimetric quantification depending on immuno-conjugation and an enzyme-substrate system. IPCR-based methods were subsequently enhanced by the use of functionalized MPs to support effective magnetic enrichment and increase the detection performance. A number of literatures reports demonstrated specific capture and powerful enrichment of biological substances *via* durable interaction of an antigen and antibody-immobilized MPs [47–51]. For the detection of an antigen by magneto immuno-PCR (M-IPCR), specific antibody-MPs and antibody-DNA conjugates were used for magnetic separation and signal-generating detection by RT-PCR, respectively, providing higher sensitivity than conventional and magneto-ELISA [52,53]. The application of antigen-antibody interactions eventually contributed to the use of biotin-streptavidin for signaling conjugation in DNA amplification. Magnetic nanoparticles (MNPs)-PCR enzyme-linked gene assay (MELGA), which is composed of PCR amplification using an MPs-immobilized forward primer and a biotinylated reverse primer and is combined with a colorimetric readout system using streptavidin-conjugated horseradish peroxidase (SA-HRP) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), was developed to overcome some negative characteristics, e.g., laborious workload, time consuming, and use of a costly apparatus and hazardous reagents [54]. The amplification resulted in double-labeled PCR amplicons that can be rapidly separated by magnet and analyzed based on colorimetric enzyme-substrate interactions (Fig. 3). MELGA can be used in biomedical diagnostics to detect several target genes [54–57]. For example, detection of the *BCR/ABL* fusion gene in chronic myelogenous leukemia patients using MELGA was reported with high

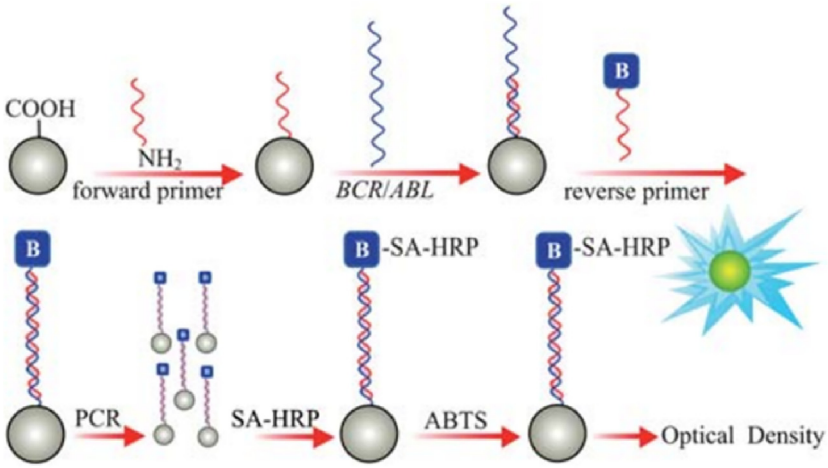

Fig. 3. Schematic of MELGA. Reproduced from Ref. [54], Copyright © 2010 with permission from the Royal Society of Chemistry.

specificity and enhanced sensitivity to the femtogram level, which was a thousand-fold higher than the sensitivity of conventional methods [54,55]. MPs have also been applied in several isothermal amplifications, e.g., LAMP, RCA, and C2CA [58–61], enabling more rapid separation and higher DNA detection sensitivity.

In addition to the conjugation of biotin-streptavidin, sensing support for DNA detection was characterized using non-MPs. As a double-labeling of the target sequence, DNA probe-modified MPs and non-magnetic materials were applied as capturing and sensing probes, respectively, while amino-functionalized MPs were used for target separation, and multi-walled carbon nanotubes (MWNTs) with carboxyl groups were used as recognition elements for DNA hybridization detection [62]. To provide signaling support, carboxyl MWNTs were covalently attached to amino-modified DNA probes using *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride and *N*-hydroxysuccinimide as crosslinking agents [63]. The sandwich structure of the target DNA with the MPs and the MWNTs was determined by the light scattering (LS) signal using a UV–vis spectrofluorometer (Fig. 4). Another interesting DNA quantification method using gold nanoparticles (AuNPs) as a labeling probe was recently reported [64,65]. Isothermal amplification of *Leishmania* DNA was performed using primers labeled with MPs and AuNPs as capturing and sensing supports, respectively. The double-labeled isothermal amplified DNA was enriched using a magnet on a screen-printed carbon electrode, which was designed to detect the electrocatalytic activity of AuNPs after amplification. These results demonstrated that MPs can be applied in combination with non-MPs to detect DNA sequences with high specificity and reproducibility.

3.2. Signaling support in the analytical process

MPs can be used as a sensing support to characterize DNA using magnetic tweezers, i.e., a measurement of force and torque generated from an individual molecule of DNA [66]. Probe-immobilized MPs can recognize a target molecule and produce torque from the rotation of the MPs after applying a magnetic field. This procedure has been widely used in the analysis of DNA, proteins, and enzymatic mechanisms [67–69]. Moreover, in addition to using MPs as the capture phase, a detectable colorimetric signal for downstream applications can be generated from the intrinsic catalytic activity of MPs [70–72]. Similar to the activity of an enzyme, several studies have reported that functionalized MPs were able to catalyze the oxidation of a peroxidase substrate in the presence of hydrogen peroxide, producing a detectable colored product. Amplification of *Chlamydia trachomatis* nucleic acid combined with colorimetric signaling was performed using hydroxyl-functionalized MPs [73]. The separation and amplification of *Vibrio cholerae* DNA, followed by the assignment of intrinsic catalytic activity on the surface of carboxylated magnetic polymeric particles (MPNPs), showed high efficiency for bacterial detection, with a sensitivity of 10^3 CFU/mL (Fig. 5) [74]. Furthermore, the detection of PCR products

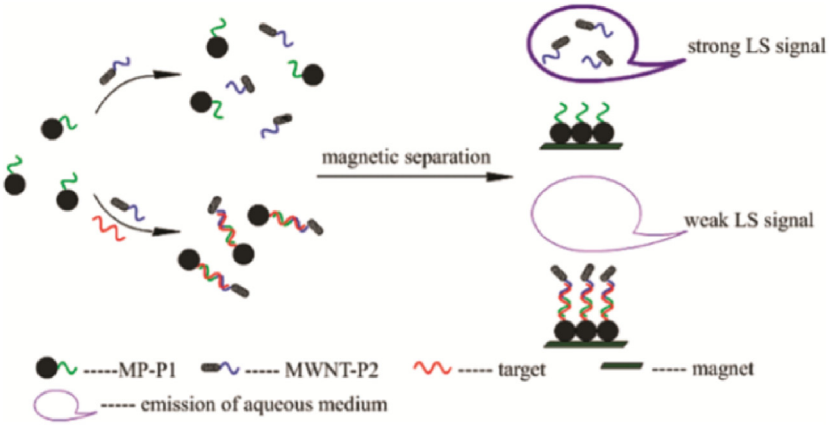

Fig. 4. Detection of a LS signal from the sandwich structure of target DNA with MPs-conjugated probe 1 (MP-P1) and multi-walled carbon nanotubes-conjugated probe 2 (MWNT-P2). Reprinted with permission from Ref. [62]. Copyright © 2008 American Chemical Society.

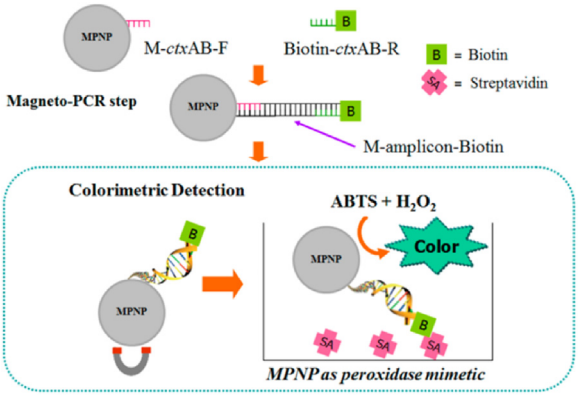

Fig. 5. Detection of a colorimetric signal from the intrinsic catalytic activity of MPNPs. Reprinted with permission from Ref. [74]. Copyright © 2013 American Chemical Society.

from the amplification of *Salmonella typhimurium* using MPs conjugated with DNA aptamer and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate provided a robust and rapid system that required only 10 min to complete the assay [75]. Interestingly, the oxidase activity of CeO_2 nanoparticles enabled simple and ultrafast colorimetric detection by the naked eye within 1 min [76]. As a result of the multifunctional properties and beneficial characteristics, MPs have been evolved into a promising material in the current era of molecular applications.

4. Functionalized colloidal MPs in microfluidic and lab-on-a-chip biosensors

Biosensors are an extensive group of transformative apparatus used to detect miscellaneous chemical substances and biological molecules [77,78]. There are 3 major stages in the development of a biosensor. First, a specific biological receptor (or bioreceptor), e.g., an enzyme, antibody or nucleic acid, is adopted. The bioreceptor is required to characterize a specific target molecule among a mixture of contaminants in the sample. Second, a biotransducer detects and subsequently converts a biochemical change, e.g., mass, pH, photon, temperature, or electrical charge, into a measureable signal that is proportional to the concentration of the analyte. The biotransducer type is defined according to the application, e.g., gravimetric, potentiometric, thermometric, and amperometric. Finally, the associated signal processor consolidates and evaluates the signal from the transduction process to provide accurate and simple information.

Based on multidisciplinary technology, biosensors with various practical functions have been assembled to consolidate the entire process into a single unit. Colloidal MPs have been applied in biosensors for several purposes, e.g., separation, manipulation, and detection of target molecules [79,80]. Furthermore, the most recent generation of biosensors has been designed for micro-scale detection, including microfluidic and lab-on-a-chip microsystems, which require low fluid sample volumes and provide high-throughput analysis. In microfluidic platforms, functionalized MPs can act as a solid support for DNA hybridization, as well as the intermediate in several steps, e.g., DNA intercalation and purification, using an external magnet and a group of reagents. The combination of microfluidic on-chip microsystems and magnetic manipulation represents an automated continuous platform that facilitates simple and rapid DNA analysis [81].

The most important function of MPs in lab-on-a-chip biosensors for molecular diagnosis is magnetic manipulation of a target molecule in a microfluidic droplet. To achieve efficient manipulation, the interaction between the MPs and microfluidic droplet was investigated for several applications, e.g., particle extraction, droplet deformation, and transport of droplets containing particles [82,83]. A microfluidic droplet platform for cancer biomarker detection was described using silica superparamagnetic particles (SSPs) as the solid support for sample preparation and target analysis [84]. SSPs were also used in an integrated DNA extraction and purification system, for droplet handling, and for the identification of biomarkers using RT-PCR and helicase-dependent amplification (Fig. 6). Highly efficient DNA extraction and purification were subsequently reported using immiscible filtration assisted by surface tension (IFAST) [85]. IFAST used the MPs as a separation support, a magnet for sample manipulation, a well-defined chamber containing an immiscible oil solution and an elution buffer to wash and remove the target DNA (Fig. 7). IFAST provided an extremely fast procedure, completing the entire DNA extraction process in 7 min, and required a very small amount of sample (10 microliters) [85]. These miniaturized platforms combined various sample preparation steps into a simple, convenient, and real-time detection system.

Biosensors using microfluidic platforms and lab-on-a-chip microsystems have recently played a significant role in the development of point-of-care (POC) diagnosis due to their simplicity, rapidity, and convenience. Especially when taking advantage

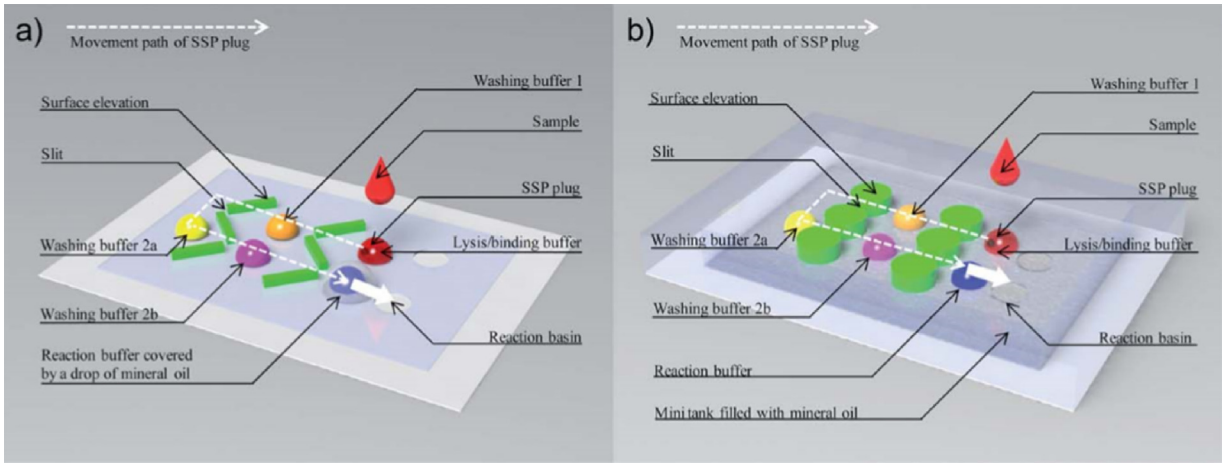

Fig. 6. Integrated microfluidic droplet platform for sample preparation, droplet manipulation, and biomarker detection using SSP. (a) Microfluidic device with V-shape slits and open-air chamber system. (b) Microfluidic device with micropillars and a mini tank with mineral oil. Reproduced from Ref. [84], Copyright © 2010 with permission from the Royal Society of Chemistry.

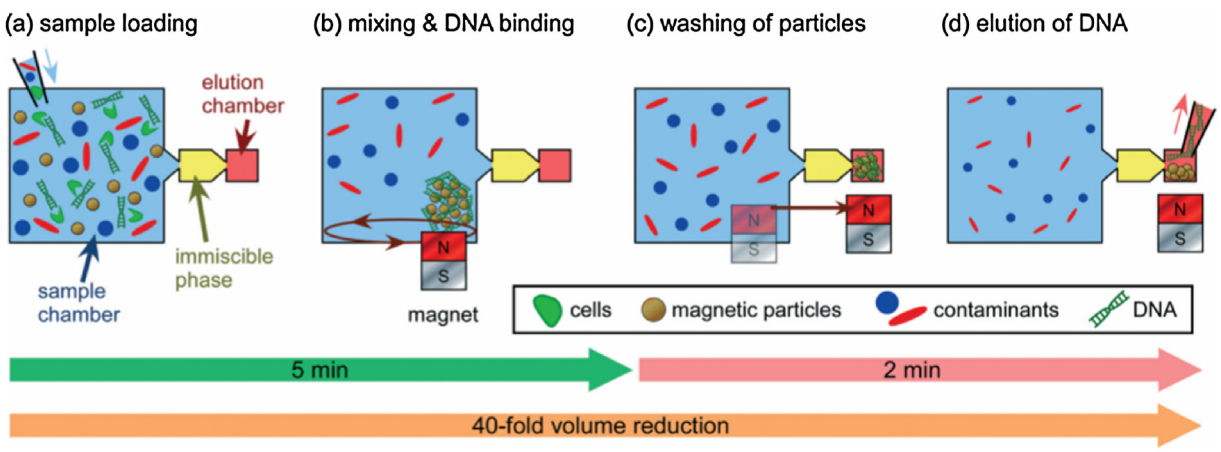

Fig. 7. DNA extraction and purification using immiscible filtration assisted by surface tension (IFAST). Reproduced from Ref. [85], Copyright © 2016 with permission from the Royal Society of Chemistry.

of PCR-based detection, POC testing can solve the limitations of turnaround time and complexity. The most recent advance in microfluidic-integrated lab-on-a-chip biosensors introduced a single microdevice for POC amplification of a DNA sequence. Infrared-mediated PCR (IR-PCR) integrated hybridization-induced aggregation (HIA) was performed on-chip for the rapid amplification of *Salmonella enterica* DNA and simple optical analysis. IR-PCR amplification can be completed within 35 min, HIA detection can demonstrate MP aggregation in 30 s of enhancement by a rotating magnetic field, and semi-quantitative analysis of the particle aggregation can be performed using Mathematica™ software [86]. Another interesting POC application using magnetic-silica particles for label-free DNA quantification was reported [87]. DNA quantification was performed using a "pipette, aggregate, and blot" assay *via* simple mixing of MPs with a DNA sample in a pipette tip. The aggregation of MPs in the presence of DNA was induced using a magnetic field. After exposure to the magnetic field, the aggregate was transferred onto filter paper, and an image was taken using a photo scanner or mobile phone and subsequently analyzed with respect to the saturation histogram using Mathematica™ software in hue-saturation-brightness mode. These POC applications achieved sensitive and straightforward detection, represented portable and label-free DNA quantification and accommodated the next generation of simple, rapid, and sophisticated molecular diagnosis.

5. Summary

In the past, colloidal MPs have been thoroughly investigated in material science and biomedical diagnosis. MPs have been widely used in various applications as a separating solid support for several biomolecules, including DNA. In addition, surface functionalization of MPs provides diverse advantages and versatile properties, and the functionalized MPs can be used in any step of molecular diagnosis.

The ability to customize MPs for a wide range of applications is one of the most important features of colloidal MPs. Immobilization of biomolecules provides greater detection sensitivity and specificity. In addition, the combination of MPs with various types of non-magnetic materials, e.g., silica particles, gold nanoparticles (AuNPs), and multi-walled carbon nanotubes (MWNTs), enhances the range of molecular application. Moreover, the age of nanotechnology has introduced MPs into microfluidic platforms with lab-on-a-chip analytical microsystems. The most advanced applications have been proposed to solve the limitations of conventional methods. However, the latest technology still has some issues that need to be further characterized and improved. Moreover, each method requires optimization and continuous improvement.

In conclusion, the development of MPs for PCR-based detection is an intense research focus. The application of colloidal MPs has been continuously reported since their development. Applications from sample preparation to analytical readout systems have proved that MPs are indispensable for molecular diagnosis. Furthermore, MPs have been implemented in current microfluidic and lab-on-a-chip biosensors. With respect to future trends, MPs will definitely play important roles in the development of the modern generation of diagnostic methods. Modification of MPs with novel materials, e.g., stimuli-responsive polymer and ultrahigh sensitivity semiconductor quantum dots, is expected to expand the boundary of research in materials science and related studies.

Acknowledgements

The authors are thankful for the scholarship from the Thailand Research Fund, Mahidol University, and the Embassy of France in Bangkok, Thailand through the Royal Golden Jubilee Ph.D. Program (Grant No. 5TMU57T1.PHD/0007/2557) to the Ph.D. student T.T.

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