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Streptavidin-modified monodispersed magnetic poly(2-hydroxyethyl methacrylate) microspheres as solid support in DNA-based molecular protocols

Tagrid Salih [a], Annika Ahlford [a], Mats Nilsson [a], Zdeněk Plichta [b], Daniel Horák [b,*]

[a] Science for Life Laboratory, Department of Biochemistry and Biophysics, Stockholm University, Tomtebodavägen 23B, 171 65 Solna, Sweden
[b] Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovského Sq. 2, 162 06 Prague 6, Czech Republic

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ABSTRACT

Molecular diagnostics may provide tailored and cost efficient treatment for infectious disease and cancer. Rolling circle amplification (RCA) of padlock probes guarantees high specificity to identify nucleic acid targets down to single nucleotide resolution in a multiplex fashion. This makes the assay suitable for molecular analysis of various diseases, and interesting to integrate into automated devices for point-of-care analysis. A critical prerequisite for many molecular assays is (i) target-specific isolation from complex clinical samples and (ii) removal of reagents, inhibitors and contaminants between reaction steps. Efficient solid supports are therefore essential to enable multi-step, multi-analyte protocols. Superparamagnetic micro- and nanoparticles, with large surface area and rapid liquid-phase kinetics, are attractive for multi-step protocols. Recently, streptavidin-modified magnetic monodispersed poly(2-hydroxyethyl methacrylate) (STV-mag.PHEMA) microspheres were developed by multiple swelling polymerization. They are easily separated by a magnet and exhibit low non-specific protein sorption. In this study, the performance and the binding efficiency of STV-mag.PHEMA was addressed by circle-to-circle amplification (C2CA). A lower number of RCA products were detected as compared to the gold standard Dynabeads. Nevertheless, this study was the first to successfully adapt STV-mag.PHEMA microspheres as solid support in a DNA-based protocol, which is an important finding. The STV-mag.PHEMA microspheres were larger with about 16 times less surface area as compared to the Dynabeads, which might partly explain the lower rolling circle product (RCP) count obtained. Further research is currently ongoing comparing particles of similar sizes and optimizing reaction conditions to establish their full utility in the field. Ultimately, low cost and versatile particles are a great resource to facilitate future clinical molecular diagnostics.

1. Introduction

There is increasing demand for the development of cost effective and user friendly lab-on-chip medical devices for *in vitro* molecular diagnostics of infectious and neurodegenerative diseases as well as cancer [1,2]. Analysis of clinically relevant nucleic acid targets is thus becoming an integral part of such novel point-of-care testing [3]. Innovative, ligation-based assays, using highly specific padlock probes, are attractive for diagnostics of pathogens or detection of mutations [4,5]. Probed targets can be amplified by rolling-circle amplification (RCA) [6] forming large DNA bundles which can be fluorescently labeled and digitally counted [7]. Detection sensitivity is greatly improved by applying two rounds of RCA, so called circle-to-circle amplification (C2CA) [8].

Existing single-step molecular assays are easy to perform but do not allow for any reagent exchange or washing steps to remove contaminants. This restricts the analysis to less complex samples using simple protocols and consequently limits the number of analytes to be interrogated simultaneously which limits the value of homogeneous tests for advanced next generation molecular analysis. Such a simple assay was previously published for fast and sensitive detection of single rolling circle products [9]. Diagnostic devices for sophisticated and multiplex DNA-based molecular protocols, such as C2CA, should optimally be compact and fully integrated for maximal ease of use. More advanced systems often make use of multi-functional magnetic nano- or microparticles as a solid reaction support. The application of particles in multi-step protocols has several benefits as compared to standard surface-based formats. These include large surface area, improved liquid-phase reaction kinetics, small reaction volumes, localized bead collection, and a range of established surface functionalization protocols for molecule immobilization. Moreover, it is feasible to adapt their usage in automated protocols. Recently, we published the first integrated version of the C2CA protocol based on digital microfluidics and employing commercially available beads [10].

Most magnetic polymer particles on the market today are based on hydrophobic polystyrene (PS). Polystyrene generally lacks specific

* Corresponding author.

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interactions between the support and the analyte, making it difficult to introduce desired reactive groups on the PS surface. In this study we present the development of novel magnetic poly(2-hydroxyethyl methacrylate) (PHEMA)-based microspheres containing reactive carboxyl groups that enable easy washing and separation of reaction mixtures required in multi-step molecular protocols for diagnostic analysis of clinically relevant nucleic acids. PHEMA microspheres are advantageous over PS beads due to their inert nature and relatively low nonspecific protein adsorption, related to the lack of hydrophobic interactions. The main objective of this study was to investigate the performance and the binding efficiency of the novel streptavidin magnetic PHEMA microspheres (STV-mag.PHEMA) using the C2CA protocol to detect DNA sequences from *Escherichia coli* (*E. coli*).

2. Experimental

2.1. Materials

2-Hydroxyethyl methacrylate (HEMA; Röhm, Darmstadt, Germany) and ethylene dimethacrylate (EDMA; Ugilor, France) were vacuum-distilled before use. 2-(Methacryloyl)oxyethyl acetate (HEMA-Ac) was prepared from HEMA and acetic anhydride. Cyclohexyl acetate (CyAc), 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), streptavidin and the phosphate buffer solution (PBS) were all from Aldrich (St. Louis, USA). 2-[(Methoxycarbonyl)methoxy]ethyl methacrylate (MCMEMA) was prepared according to an earlier described procedure from ethylene glycol, chloroacetic acid, methanol and methacrylic anhydride [11]. [(2-Hydroxy-propyl)methyl]cellulose (Methocel 90 HG), sodium chloroacetate, benzoyl peroxide (BPO), dibutyl phthalate (DBP) and dioctyl adipate (DOA) were from Fluka (Buchs, Switzerland). Polystyrene (PS) latex was prepared according to an earlier report [12]. All other solvents and reagents were from Aldrich or Lachner (Neratovice, Czech Republic).

2.2. Preparation of magnetic PHEMA-COOH microspheres

PS latex (0.3 g) was activated with a mixture of DBP (3.8 g) and DOA (0.2 g) in 0.25 wt.% aqueous sodium dodecyl sulfate (SDS; 13 ml). The activation was performed in six steps and the final volume of the activated PS seeds was adjusted to 17.5 ml. A mixture of BPO (30 mg), HEMA-Ac (1.5 g), MCMEMA (0.3 g) and EDMA (1.2 g) in 0.1% aqueous SDS (7.5 ml) was sonicated (10 W; 4710 Series Ultrasonic Homogenizer; Cole-Parmer Instruments, Chicago, USA) for 3 min and added to DBP- and DOA-activated PS latex (3 ml). The dispersion was allowed to swell for 16 h and CyAc porogen (4.2 g), sonicated in 0.1% SDS solution (10 ml), was added. The mixture was stirred (500 rpm) in a 45-ml glass vessel for 1 h under a CO_2 atmosphere, after which 2 wt.% aqueous [(2-hydroxypropyl)methyl]cellulose (4 ml) was added and the polymerization proceeded at 70 °C for 16 h under a CO_2 atmosphere. Resulting P(HEMA-Ac-MCMEMA) microspheres were filtered off, washed with 0.05% Tween 20 solution to remove stabilizers and then washed with ethanol. The microspheres were then hydrolyzed by 0.4 M NaOH solution (50 ml) at 70 °C for 16 h, washed ten times with water (150 ml each) and the resulting PHEMA-COOH particles were kept in 20% aqueous EtOH at 4 °C.

To render the PHEMA-COOH microspheres magnetic, iron oxide was precipitated inside their pores. The microspheres (2 g) were washed with 0.04 M HCl (15 ml), separated by centrifugation and 1.5 M aqueous $FeCl_2$ (5 ml) was added and the suspension was allowed to stand for 5 min. The microspheres were separated by centrifugation and the imbibition of the $FeCl_2$ solution was repeated. The microspheres were then filtered off and 1.35 M ammonium hydroxide (5 ml) was added, the mixture vortexed, water (5 ml) added and the mixture stirred for 1 h using a rotator under an Ar atmosphere. The particles were separated, water (12 ml) added, the mixture stirred for 5 min and the particles washed with water until pH 8 was reached. Magnetite was formed in air oxygen for 16 h while stirring. Resulting magnetic PHEMA-COOH microspheres were thoroughly washed with water until formation of iron oxide colloid which was removed by centrifugation. Saturation of the microspheres with $FeCl_2$ and precipitation of iron oxide was performed once more. Finally, the microspheres were classified via a 25 μm sieve to remove aggregates and other impurities. Non-magnetic particles were removed by magnetic separation.

2.3. Attachment of streptavidin on the magnetic PHEMA-COOH microspheres

Magnetic PHEMA-COOH microspheres (0.3 g) were washed with water and 0.1 M MES buffer (pH 5; 3 × 4 ml each) for 20 min and separated. The microspheres were activated with EDC (5 mg) and NHS (17 mg) in MES buffer (pH 5; 3 ml) at RT for 15 min while stirring (100 rpm). The activated microspheres were washed with 0.1 M MES (5 ml) and three times water (5 ml each) for 15 min and finally magnetically separated. A solution of streptavidin (STV; 2.5 mg) in 0.07 M phosphate buffer (pH 7.4; 3 ml) was added to the activated microspheres and the reaction proceeded at RT for 30 min while stirring (100 rpm). The resulting STV-mag.PHEMA microspheres were magnetically separated and five times washed with PBS (pH 7.4).

2.4. Characterization of the microspheres

The microspheres were observed by a Vega Plus TS 5135 scanning electron microscope (SEM; Tescan; Brno, Czech Republic). Thin sections of the particles embedded in LR white resin were cut using an LKB III ultramicrotome and observed on a Spirit G [2] Tecnai transmission electron microscope (TEM; FEI; Brno, Czech Republic). The microspheres were analyzed with a Paragon 1000 PC FT-IR spectrometer (Perkin Elmer) equipped with a Specac MKII Golden Gate Single Reflection ATR System containing a diamond crystal and a ray angle of incidence of 45°. The Fe content was determined by atomic absorption spectrometry (AAS Perkin-Elmer 3110) of an extract from a sample obtained by treatment with 70% perchloric and 65% nitric acid at 100 °C for 30 min. The reaction solutions were measured with a Specord Plus UV–VIS spectrophotometer (Analytik Jena, Jena, Germany).

2.5. Evaluation of the STV-mag.PHEMA microsphere performance by C2CA

The padlock probe was designed to detect the *beta-D-glucuronidase* gene in *E. coli.* Sequences for all oligonucleotides used in this study are given in Table 1 and ordered from Integrated DNA Technologies (Munich, Germany). All reagents and enzymes were purchased from Fermentas (Hanover, USA) unless stated otherwise. In this study the performance of STV-mag.PHEMA microspheres was evaluated by

Table 1
Oligonucleotides used in this study.

Type of oligonucleotide	Sequence	5′-modification
E. coli target	TAAAGCCGACAGCTGCAGTTTCATCAATCACC ACGATGCCATGCTCATCTGCCAGTCGAGCATC TCTTCAGCGTAAGGGTAATGC	None
Padlock probe	AAGAGATGCTCGACTGGTGTGTATGCAGCTCC TCAGTAATAGTGTCTTACGCTGCTCGTGGTTA AGCTCTGCATTACCCTTACGCTG	Phosphate group
Capture probe	CTCTCTCTCTCTCTCTCTGGCATCGTGGTGAT TGAGAAACTG CAGCTGTCGGCTTTA	Biotin
Restriction oligonucleotide	GTGTATGCAGCTCCTCAGTA	None
Detection oligonucleotide	TTTTTGTAAGACACTATTACTGAG	Cy3 label