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Hydrogel micropost-based qPCR for multiplex detection of miRNAs associated with Alzheimer's disease

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ABSTRACT

Quantitative polymerase chain reaction (qPCR) renders profiling of genes of interest less time-consuming and cost-effective. Recently, multiplex profiling of miRNAs has enabled identifying or investigating predominant miRNAs for various diseases such as cancers and neurodegenerative diseases. Conventional multiplex qPCR technologies mostly use colorimetric measurements in solution phase, yet not only suffer from limited multiplexing capacity but also require target-screening processes due to non-specific binding between targets and primers. Here, we present hydrogel micropost-based qPCR for multiplex detection of miRNAs associated with Alzheimer's disease (AD). Our methodology promises two key advantages compared with the conventional solution-based PCR: 1) nearly no non-specific crosstalks between targets and primers, and 2) practically valuable multiplexing by spatial encoding within a single microchamber. Specifically, we immobilized hydrogel microposts (~ 400 μm in diameter) within commercially available polycarbonate PCR chips by multi-step ultraviolet (UV, 365 nm) exposure. We optimized this photoimmobilization for thermal cycles of PCR as well. Acrylated forward primers incorporated in polyethylene glycol diacrylate (PEGDA) posts played a crucial role to confine fluorescent signal of cDNA amplification within the PEGDA hydrogel. To demonstrate the potential of our platform, we successfully verified multiplex detection of five miRNAs, which were reported to be highly correlated with AD, from a complex buffer of human plasma.

1. Introduction

Alzheimer's disease (AD) is a type of dementia and progressive brain disorder that provokes serious problems in one's memory, thinking, and behavior. Although AD is ranked in the sixth leading cause of death in the United States, it has no reliable remedy, yet. Thus, early diagnosis of AD is significantly important to relieve soon-emerging neurodegenerative progression in a patient and allow the patient to prepare for the onset of the disease. Positron emission tomography (PET) (Foster et al., 1983) and profiling of AD-associated proteins such as beta-amyloid peptide (Aβ) in cerebrospinal fluid (CSF) (Blennow et al., 2001) have been demonstrated as clinical diagnostic methods for the early diagnosis of AD. However, high costs for PET and invasiveness to acquire CSF limit their utility in clinical examinations.

As an alternative, much attention has been paid to microRNAs (miRNAs) that modulate translation of messenger RNAs (mRNAs) to proteins by cleaving or destabilizing the mRNAs (He and Hannon, 2004; Krol et al., 2010). Meanwhile, recent research has reported that circulating miRNAs, enriched in extracellular vesicles (e.g., exosomes), can serve as promising biomarkers of AD because of beneficial sample acquirement from subjects with low costs in a minimally invasive manner (Cheng et al., 2014; Geekiyanage et al., 2012; Maes et al., 2009;

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Schonrock et al., 2010). In other words, profiling of miRNAs corresponding to certain diseases allows for designating gene expressions which provoke or accelerate progressions of the diseases (Calin and Croce, 2006). However, the identification of disease-specific miRNAs, especially circulating ones in various body fluids such as blood, has been at its early stage and still under investigation. Therefore, it is thoroughly necessary not only to create panels of miRNAs uniquely associated with certain diseases but also to analyze them in a multiplex manner with high specificity and selectivity for rapid, but credible (Galimberti et al., 2014; Kumar et al., 2013; Leidinger et al., 2013; Tan et al., 2014).

Existing technologies to profile miRNAs include microarray (Yin et al., 2008), quantitative polymerase chain reaction (qPCR) (Benes and Castoldi, 2010; Zhang et al., 2009), and next-generation sequencing (NGS) (Creighton et al., 2010), among which qPCR has been used most widely. We note that conventional end-point PCR requires an inevitable post-PCR process; that is, gel electrophoresis for both visualization and identification of PCR products (i.e., amplicons) with particular molecular weight. This gel electrophoresis provides a relatively poor sensitivity: distinguishable bands from ~ 10-fold changes in gene expression level (Cseke et al., 2011). Because changes in the level of miRNAs between AD patients and healthy controls have been reported to range within a 10-fold (Cheng et al., 2014; Galimberti et al., 2014; Kumar et al., 2013), the end-point PCR is not applicable to profile miRNAs. Therefore, qPCR has served as an increasingly appealing technique to observe results in real-time owing to its reproducibility, reliability, and sensitivity (Git et al., 2010; Hardikar et al., 2014; Jensen et al., 2011). Strategies for qPCR have been recently evolving towards efficient multiplexing capability that allows for obtaining meaningful information in a less time-consuming and thus, cost-effective manner. To accomplish multiplex profiling of miRNAs via qPCR, either spatially isolated systems (Phaneuf et al., 2015; Prakash et al., 2014) or fluorophore-tagged probe-based chemistry (Barros et al., 2013; Brault et al., 2015; Fuentes et al., 2014; Su et al., 2013; Zhang et al., 2014; Zink et al., 2013) has been utilized. However, the spatially isolated systems require independent multiple PCR chambers integrated into a single substrate, which is intrinsically not multiplex but singleplex in parallel. Meanwhile, fluorophore-tagged probe-based technologies such as TaqMan and AllGlo have been used more dominantly and are suitable for multiplex analyses. Both techniques utilize spectrally-resolvable fluorophores to differentiate between signals of target genes. Unfortunately, both techniques reveal intrinsic limitations in the number of multiplexing (typically 3–4) due to inevitably overlapping wavelengths of both excitation and emission. Moreover, a significant issue of conventional qPCR for multiplexing lies on 'off-target' amplifications, which is attributable to the formation of primer-dimers and mismatched bindings between primers and targets. Hence, the conventional approaches should accompany adequately exquisite primer designs and operational conditions for chain reactions, to evade non-specific bindings (Edwards and Gibbs, 1994). We note that the issue above and requirements fall under limitations of the end-point PCR for multiplexing as well.

Hydrogels have drawn attention as promising scaffold biomaterials for detection of various biomolecules including RNAs, owing to their biocompatibility as well as tunability capable of adding various physical and (bio)chemical functionalities (Le Goff et al., 2015). With these

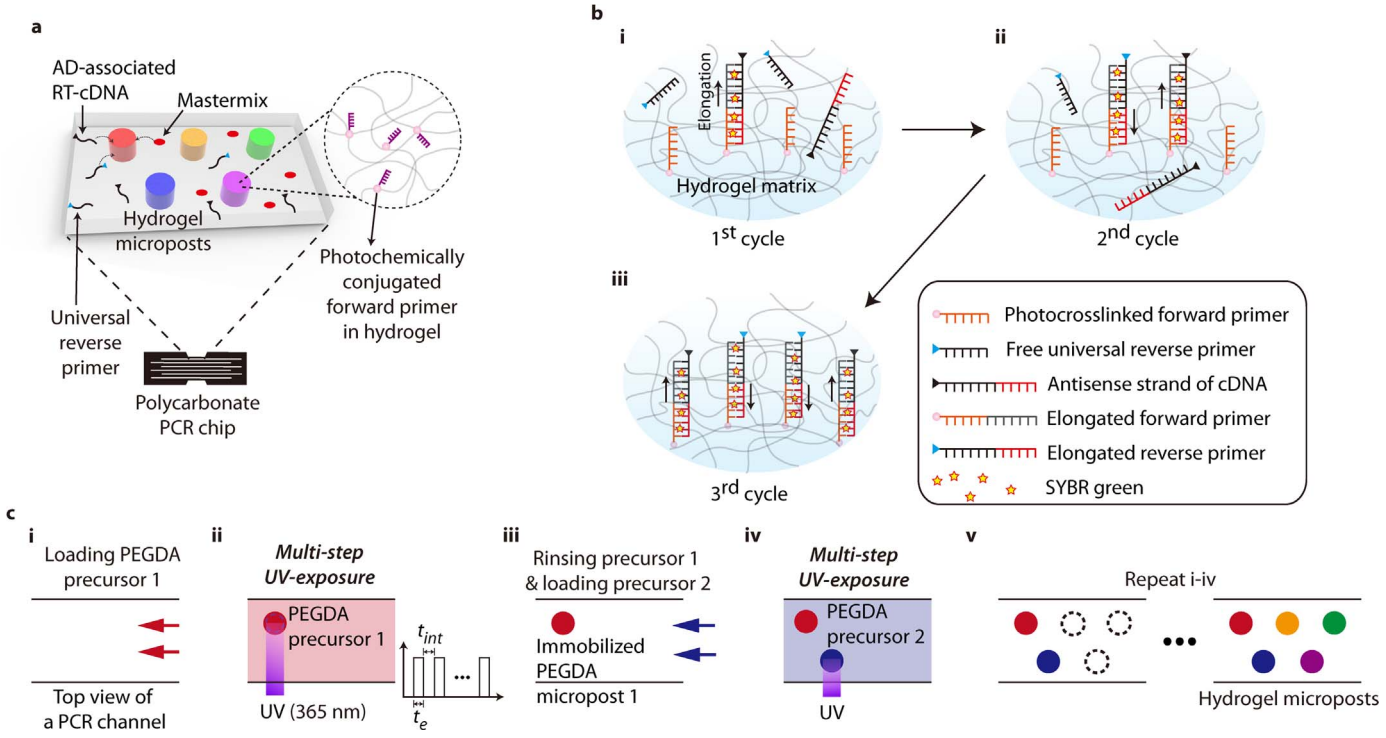

Fig. 1. Overview of hydrogel micropost-based multiplex qPCR. (a) Schematic illustration of hydrogel microposts immobilized in a polycarbonate (PC) PCR chip in the presence of universal reverse primers and reverse transcribed (RT)-cDNAs associated with Alzheimer's disease (AD). Color-coded cylinders represent five different hydrogel microposts in which target-specific forward primers are crosslinked photochemically within the hydrogel matrices. (b) Schematic illustration depicting the principle of PCR confined within a hydrogel matrix. (i) During the 1st cycle of qPCR, antisense strands of cDNAs hybridize with photoconjugated forward primers from which elongation occurs by DNA polymerases to synthesize sense strands of cDNAs leading to double-stranded amplicons. (ii) During the 2nd cycle, universal reverse primers hybridize onto a 3′-end of the elongated forward primers (a sense template), followed by the elongation. (iii) From the 3rd cycle, both the antisense strands of cDNAs and elongated forward primers, which serve as sense strands, continue PCR. (c) Fabrication process to immobilize polyethylene glycol (PEGDA) microposts in the PCR chip made with PC. (i) PEGDA precursor 1 (red) containing acrylated forward primer 1 is loaded. (ii) Ultraviolet (UV; 365 nm) is periodically irradiated through a photomask via *multi-step UV-exposure* with unit exposure time (t_e) and interval time (t_{int}) to fabricate an immobilized micropost (dark red). (iii) Uncrosslinked precursor 1 is rinsed, and then precursor 2 (blue) containing acrylated forward primer 2 is loaded. (iv) Micropost 2 (dark blue) is immobilized via the multi-step UV-exposure. (v) Steps i-iv are repeated to fabricate five different microposts as color-coded schematically. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)