ENHANCING/MULTIPLEXING PROTEASE ASSAY WITH DROPLET BASED MICROFLUIDICS USING BIOMOLECULE CONCENTRATOR

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ABSTRACT

We introduce an integrated microfluidic device consisting of a biomolecule concentrator, picoinjector and a micro droplet generator. We used this platform to detect ultra-low levels of matrix metalloproteinases (MMPs) from diluted cellular supernatant and showed that it significantly (~10-fold) reduced the time required to complete the assay and the sample volume used. Biomolecule concentrator enhances the limited sensitivity of low-abundance enzyme assays by concentrating sample solutions, while droplet generator confines concentrated reactants into a pico-liter volume for sustained high sensitivity enzyme reaction as well as enabling multiplexed assays with a limited sample volume. We demonstrated to screen five different protease sensors simultaneously to indicate specific protease MMP-9 with high sensitivity.

KEYWORDS: Protease, Emulsions, Biomolecule concentrator, Multiplexed activity bioassay

INTRODUCTION

Cell-secreted proteases, from families of enzymes like matrix metalloproteinases (MMPs), participate in diverse biological and pathological processes[1], such as cancer metastasis. However, activity of these secreted enzymes has been difficult to measure, due to their low abundance and hence relatively long reaction times required to turn over enough substrates for detection. Existing enzyme activity assays either lack the sensitivity required to directly detect the protease activity in limited sample quantities, or suffer from low throughput and/or inability to measure under physiological conditions[2]. Here, we introduce an integrated microfluidic platform to detect very low levels of MMPs' activity directly from cellular supernatant[3]. The combination of biomolecule concentrators, picoinjector and droplet-based assays are highly synergistic, since droplet confinement provides an effective way to offer highly multiplexed, equilibrium activity assay, while concentration device provides a solution to low droplet sampling rate for detecting low-abundance biomolecules. Detection of many MMP targets from physiological samples with low abundant protease concentrations are possible, by incorporating controllable droplet picoinjector systems[4]. While the specificity of MMP sensing fluorogenic substrate is limited, multiplexing several (~5) sensors for selected MMP targets can provide a comprehensive picture of biological protease activity 'state' of a given cell culture (or clinical samples), therefore potentially providing a more accurate picture of cancer cells' metastatic potential. We believe this platform can also benefit other droplet-based assays, such as droplet-PCR[5] or droplet-based single cell assays[6].

EXPERIMENTAL RESULTS AND DISCUSSION

The device shown in the schematic diagram (Figure 1A) was fabricated as a Polydimethylsiloxane (PDMS) chip bonded to a PDMS-coated glass slide. We introduced three aqueous streams into the device using syringe pumps (PHD 2000, Harvard Apparatus, Holliston, MA). The sample flow rate (0.03 L/min) and the applied voltage (~50V) were adjusted so that the electrokinetic force balanced the pressure-driven flow to trap the biomolecules at the boundary and continuously accumulate them into a plug. This plug was monitored by the fluorescence (λ_{ex}=561nm; λ_{em}=572nm) of the added tracer, as shown in Figure 1B. After sufficient accumulation, the plug was released by turning off the voltage, transported by pressure-driven flow, and mixed with the substrate coming in from the side channels in the mixing zone (Figure 1C). A flow-focusing geometry was used as the droplet generator with fluorocarbon oil as the carrier fluid (Figure 1D). The hydrophobic PDMS surfaces in the device helped the incoming aqueous solution (enzyme-substrate mixture) become encapsulated in the oil, forming monodisperse water-in-oil emulsions. Because the volume of the reaction mixture plug was much larger than the individual droplet size, the plug was divided into many droplets with different (concentrated) enzyme concentrations, but with a constan, predetermined substrate concentration. All droplets were monitored using the tracer fluorescence (in order to decide concentration of enzyme) and reaction product fluorescence (Figure 1E). This scheme enabled the simultaneous observation of the activities at different enzyme concentrations, therefore providing important parameters on reaction kinetics in a single experiment with limited sample volumes (Figure 1F and G).

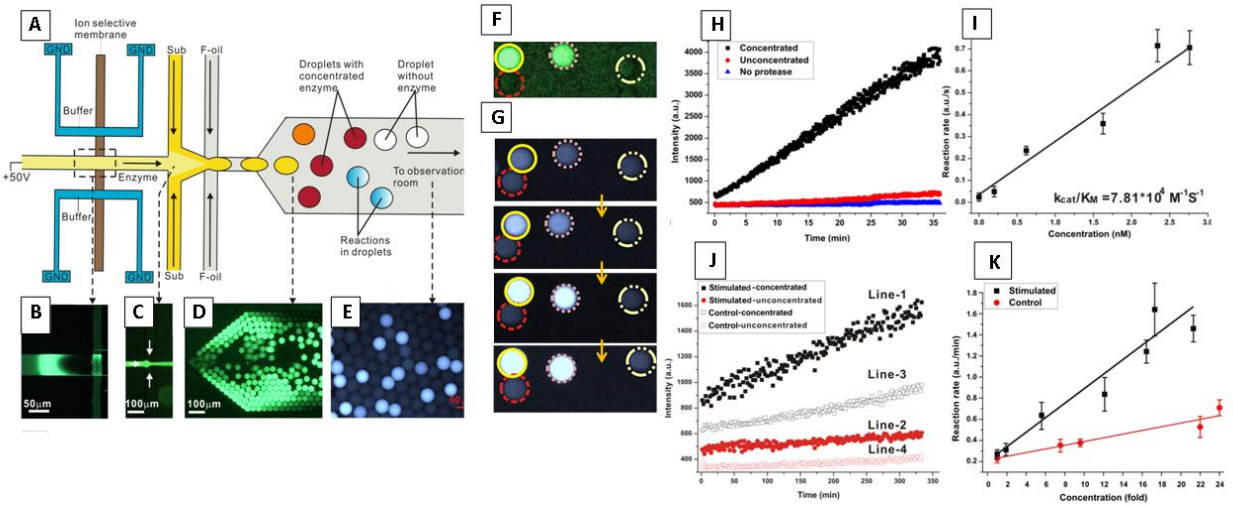

Figure 1: (A) Schematic representation of the integrated nanofluidic biomolecule concentrator and microfluidic droplet generator chip. (B) The enzyme molecules were accumulated by a concentrator into a plug that (C) was mixed with the substrate and (D) then encapsulated into monodisperse microdroplets for time-dependent observation. (E) The reaction to turn over the fluorogenic substrate was monitored as a function of time in the droplets. (F) The tracer dye to indicate the enzyme concentrations in each droplet. (G) The reaction in each droplet. (H) The product fluorescence intensity increase in each individual droplet with reaction time (after mixing the MMP and the sensor) is shown. (I) The reaction rate increased as the MMP concentration in the droplets increased. (J) Reactions of cellular supernatant in different conditions is shown by the increase in the product fluorescence intensity in an individual droplet with reaction time. (K) The reaction rate increased with increasing cellular supernatant concentrations in the droplets. Each data point represents the average of five droplets, and the error bar represents the standard deviation.

To characterize the microfluidic platform, we employed this platform to study the activity of a recombinant matrix metalloproteinase (MMP-9, 0.2nM) which fluoresces upon cleavage as an indicator of proteolytic activity. For droplets containing the protease, the fluorescence intensities of the turned-over substrates increased linearly with assay time, with and without the concentration step (Figure 1H). After the preconcentration step, the concentration of MMP-9 in the droplets increased up to 16-fold (inferred by the tracer fluorescence) which correlated with the identical increase in activity measured using product fluorescence. Additionally, different enzyme concentrations (from 0.2nM to 3.2nM) were screened in a single experiment to obtain information on reaction kinetics, as shown in Figure 1I. As expected, the reaction rates showed an almost linear increase with MMP-9 concentration. After calibration of the fluorescence intensity of the product, the value of the kinetic constant ($k_{cat}/K_m = 7.81*10^4 M^{-1}S^{-1}$) was obtained by assuming that Michaelis-Menten kinetics were obeyed. We then measured secreted MMP-9 activity directly from cell culture supernatant from *in vitro* tissue culture samples (0.5X cellular supernatant dilutions in MMP buffer). For both stimulated and untreated cell culture supernatants, the proteolysis reaction caused the fluorescence intensity to increase linearly over time (Figure 1J). After concentrating the sample, the difference in activity between the stimulated and untreated samples was amplified ~10-fold compared to difference between the unconcentrated samples. The detection sensitivity for the stimulated and untreated conditions was thus improved, allowing us to clearly differentiate these conditions by the slopes of the fluorescence intensity increase over 5 min. Figure 1K shows the reaction rates over a range of concentration enhancements, determined by a single experimental run, to obtain the parameters related to the reaction kinetics (stimulated sample: $(k_{cat}/K_M)[E] = 1.61*10^{-4}S^{-1}$; untreated sample: $(k_{cat}/K_M)[E] = 4.68*10^{-5}S^{-1}$).

We combine the picoinjector with the biomolecule concentrator in a microfluidic device for multiplexed assay (Figure 2A). The droplet screening workflow involves three steps. First, the sensor library was formed using the droplet generator chips. Different protease sensors were encapsulated into different droplets with unique fluorescent dye intensity as the optical label. Second, the droplets were driven into the device, where the concentrated sample solution is (Figure 2C) injected into the droplets to mix with the sensors (Figure 2D) in a fast (~2.5k droplets / sec) and controllable manner. Finally, the resulting droplets were sent to a 'storage chamber' for a long term observation (Figure 2E). Each droplet's fluorescence signals (multiple colors) were monitored for activity measurement, as well as the identification of the type of the sensors and the concentration of the sample in individual droplets. We screened five different kinds of protease sensors (Substrate-5, 8, 11, 13, 22) [2] to react with concentrated protease MMP solutions in Figure 2F. By combining the Proteolytic Activity Matrix Analysis (PrAMA), the specific MMP-9 activity can be measured with high specificity, in spite of the cross-reactivity of different activity sensor molecules used. Our experiment showed that the reaction activities of substrate 8 and 11 with MMP-9 were more active than others (substrate 5, 13, 22) to give the sharp reaction rate (~2.5a.u./min), which is consistent with the previous MMPs studies [2].

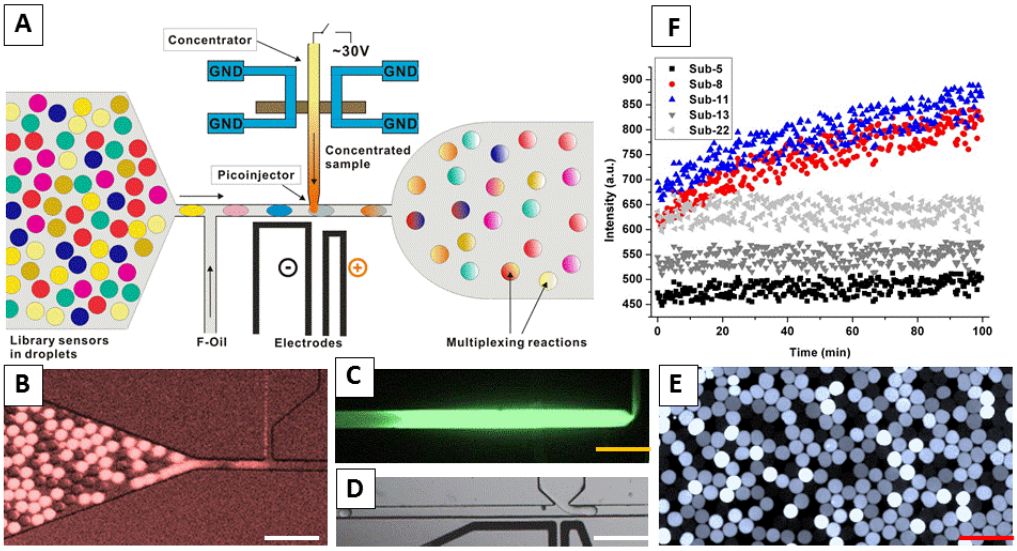

Figure 2 (A) Schematic representation of the picoinjector integrated device for multiplexing assay. (B) The library droplets with different protease sensors were reinjected into the device. (C) The concentrated plug was released to inject into the droplets with different sensors. (D) The merging of sample solutions and droplet with sensors. (E) The resulting droplets with different protease sensors and a range of sample concentrations are observed for a long time in the storage room. (F) We screened five different protease sensors simultaneously (without concentration step) to indicate the specific protease MMP-9 in the sample solutions with high accurate. The scaling bars above are 150μm.

CONCLUSION

In summary, we developed an integrated microfluidic platform that combines a biomolecule concentrator, picoinjector and a droplet generator to detect enzyme activity with high sensitivity. This system can be used generally, to analyze different enzyme reactions, such as those catalyzed by reporter enzymes, kinases and proteases. Using the system, we specifically characterized the activity of protease MMPs which is the key biomarkers for cancer metastasis. The concentrator amplified the difference between the stimulated and untreated conditions, and allowed a significant reduction in the reaction time (~10-fold). The protease-substrate reaction kinetics could be determined by a parallel analysis of droplets with different amplified enzyme concentrations in a single experiment to significantly reduce the sample volume used (~100-fold). Moreover we demonstrated the screening of five different kinds of protease sensors to indicate a specific protease type (MMP-9) from the sample solutions. This device, with its ability to assay biochemical reactions catalyzed by low-abundance enzymes and other relevant biomarkers in physiologically complex samples, is a generic and useful platform for systems biology research and medical diagnostics.

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