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Pre-storage of gelified reagents in a lab-on-a-foil system for rapid nucleic acid analysis

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Reagent pre-storage in a microfluidic chip can enhance operator convenience, simplify the system design, reduce the cost of storage and shipment, and avoid the risk of cross-contamination. Although dry reagents have long been used in lateral flow immunoassays, they have rarely been used for nucleic acid-based point-of-care (POC) assays due to the lack of reliable techniques to dehydrate and store fragile molecules involved in the reaction. In this study, we describe a simple and efficient method for prolonged on-chip storage of PCR reagents. The method is based on gelification of all reagents required for PCR as a ready-to-use product. The approach was successfully implemented in a lab-on-a-foil system, and the gelification process was automated for mass production. Integration of reagents on-chip by gelification greatly facilitated the development of easy-to-use lab-on-a-chip (LOC) devices for fast and cost-effective POC analysis.

Introduction

Point-of-care (POC) testing allows inexpensive and rapid detection of infectious diseases, contaminants, and biowarfare agents.[1] Although many POC devices utilize immunoassays involving antigen–antibody binding, nucleic acid-based diagnostics, which measure DNA or RNA, are becoming more popular because of their high specificity and sensitivity.[2,3] The method usually involves several procedures, including nucleic acid extraction and purification, polymerase chain reaction (PCR) amplification, and size- or sequence-dependent detection.[4] The design of nucleic acid-based assays is therefore more challenging than standard lateral flow immunoassay (LFA) strips.

Microfluidic platforms represent a promising technology for nucleic acid-based POC devices.[5,6] Disposable chips or cartridges that include features of sample preparation, reagent mixing, thermal cycling, and real-time detection have been reported.[7,8] However, such integrated devices often suffer from quite complicated flow control, since numerous pumps/valves need to be designed to transfer liquid and reagents for cell lysis, DNA purification, and PCR amplification.[9,10] As a result, these devices are more expensive, less reliable, and not so easy-to-use, which basically renders them inappropriate for POC testing.

To make the leap from lab-on-a-chip to practical POC devices, it is desirable to preload and store all the required reagents on chip.[11] Such self-contained devices would eliminate the time needed to load reagents, reduce the risk of cross-contamination, reduce the cost of storage and shipment, and enhance portability. Although on-chip storage of dry reagents is well developed for LFA strips, it has rarely been used for nucleic acid-based POC assays.[12] This is largely due to the lack of reliable techniques to dehydrate and store fragile molecules (*e.g.* polymerase enzymes required for PCR) without causing loss of activity.[13] A few attempts have been made to keep reagents on-chip for an extended time period in either a wet[14] or dry state.[12,15–18]

Fluids can be stored directly within the device in blister packs, which is a concept borrowed from the pharmaceutical industry. Findlay *et al.*[14] demonstrated encapsulation of reagents within containers of an inert material. Using this storage concept brings its own typical challenges. Many enzymes have very limited shelf-life at room temperature, thus chips need to be refrigerated. Besides, care must be taken for the sealing and releasing methods. Moreover, it is not practical on polymers that have significant permeability to water vapor.

Compared to the liquid form, dry reagents exhibit improved stability and are thus preferred for POC applications. PCR reagents can be dried at room temperature. Furuberg *et al.*[16]

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spotted NASBA enzymes in the microchip. The enzyme was then dried at room temperature for up to 2 days and sealed with adhesive tape. Kim *et al.*[15] pre-stored PCR reagents by drying the mixture at room temperature in a moderate vacuum. The PCR amplification was demonstrated on a polycarbonate microchip, however, the lengthy process at room temperature was not ideal for enzyme activity and caused 30% loss in the PCR product.[15]

To better preserve the enzyme's integrity, more sophisticated drying techniques were explored. Ahlford *et al.*[17] utilized freeze-drying for the storage of multiplex PCR reagents. The mixture was frozen at −80 °C for one hour and freeze-dried overnight. The process was gentle to the enzymes as no significant loss of enzyme activity was observed. However, the freeze-dried reagents are prone to humidity interference, and they have to be either used immediately or stored in vacuum-packed bags at −21 °C until use. This is not applicable in environments with high local temperatures and a lack of refrigeration.

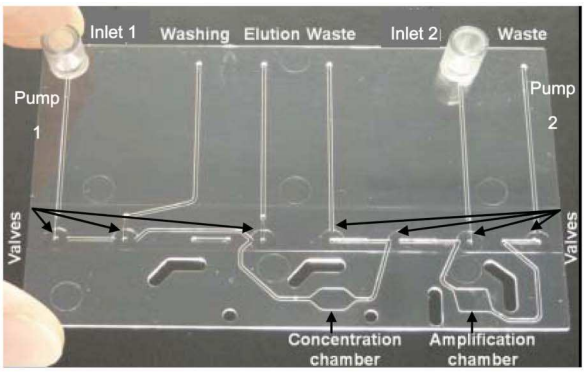
For on-field POC applications, the ability to preserve reagent function in harsh conditions is particularly important for dry-form reagent storage. In the LABONFOIL EU project,[19,20] a totally integrated microfluidic platform for PCR-based POC diagnosis is being developed. New possibilities to simplify the on-chip storage process are being envisioned by using an advanced technique called "gelification". The strategy was developed and patented by one of the EU project partners – Biotools, Spain.[21] The reaction mixtures, containing all necessary reagents including enzymes, primers, *etc.*, are stabilised for long time storage by the addition of gelifying and stabilising agents and desiccation at room temperature. Gelification minimizes liquid handling steps, and the reaction can start immediately by rehydrating with sample solution containing DNA template. Moreover, it allows for convenient room temperature shipment and extended 4 °C storage. This technology is especially advantageous for POC diagnostic systems and other portable devices.

In this paper, the application of gelified reagents for nucleic-acid based POC assays was investigated. The PCR master mixture was gelified on the microchip and multiplex PCR was performed for rapid detection and identification of a food-borne pathogen – *Campylobacter* spp. at sub-species level. The gelification procedures were optimized for on-chip reagent storage. The approach was successfully implemented in the lab-on-a-foil system. The enzyme activity and long-term stability were studied. No loss of enzyme efficiency was observed after gelification. The half life of gelified reagents at room temperature was found to be at least 3 months. Large-scale production was also made possible by automating gelification and sealing steps.

Experimental

Lab-on-a-foil system

The developed lab-on-a-foil system (Fig. 1) integrates various functional components, such as DNA isolation and purification, PCR amplification and real-time fluorescence detection of PCR product, on a single platform. It consists of a disposable polymeric labcard (Fig. 1a) and a reusable control unit for the process operations (Fig. 1b). The labcard contains two microchambers, one for DNA purification and the other for PCR reaction. The control unit, developed by another partner, Ikerlan, Spain, provides micropumps/valves for liquid pumping and flow control, magnets to capture magnetic beads, heaters and temperature sensors for thermal cycling, and a detector to read the fluorescent signal.



(a)

(b)

Fig. 1 (a) Image of the polymeric labcard, which has a credit-card size and consists of a sample preparation chamber and a PCR amplification chamber. (b) Prototype of the LabCardReader.

The cyclic olefin copolymer (COC) labcards were provided by Ikerlan. They were fabricated by injection molding. All the required PCR reagents were gelified on a thin pressure sensitive adhesive foil (Progene, Ultident Scientific, Canada). The labcard was then sealed with the adhesive foil. Alignment was made to ensure that gelified product was situated in the PCR chamber.

The system was designed in a very flexible way. The PCR reaction chamber can be either used as a stand-alone unit or integrated with upstream sample preparation. As the focus of this work is to study the feasibility of gelification for PCR reagent storage, pure DNA sample was loaded to inlet 2 and pumped to the PCR chamber directly.

DNA samples

The bacteria strain used in this study was *Campylobacter jejuni* reference strain NTCC-11284 from a bacterial collection at the National Veterinary Institute, Technical University of Denmark (DTU-Vet) in Aarhus Denmark. The campylobacter chromosomal DNA was isolated using QIAamp DNA mini kit (Qiagen, Germany). The DNA concentration was determined by Nano drop (Thermo Scientific, USA).

Primers

Two sets of primers were designed for identification of *Campylobacter* spp. and their subtype.[22] The universal *Campylobacter* primers (UC) targeted a 300 bp 16sRNA gene fragment of *Campylobacter* spp., while the hippuricase (HIP) primers aimed at a 150 bp *hipO* gene sequence specific for *Campylobacter jejuni* species. All the primers were synthesized at DNA Technology A/S, Denmark (Aarhus Denmark).

PCR mixture

The master mixture for multiplex PCR consisted of 2× Promega mixture (PCR Master Mix, Promega, USA, with 2 mM $MgCl_2$; 0.4 mg μl^{-1} BSA; 0.04 U μl^{-1} FastTaq DNA Polymerase), and two pairs of primers, UC with final concentration of 160 nM, and HIP with final concentration of 240 nM.

Gelification

Gelification was utilized for the stabilization and storage of the PCR reagents. The PCR mixture was mixed with the stabilising and gelifying agents developed by Biotools A/S, Spain (7 : 1 v/v according to the suppliers instruction). The gelification process was carried out at 30 °C under a pressure of 30 mbar for 30 min in a vacuum drying oven (VO400, Memmert, Germany). The use of expensive equipment as in the lyophilisation case was avoided. A small percentage of water molecules remained in the mixture after the dehydration process in order to preserve the enzymatic activity. After that, the chips were quickly bonded to prevent the gelified PCR mixture from contacting with air. The chips were stored at both 4 °C and room temperature (25 °C) for up to 3 months.

Different volumes of the mixture ranging from 5 to 10 μl were deposited either on the PCR chamber (10 μl) of the labcard or on the adhesive foil. Optimized conditions for making gelified PCR mixture on-chip were explored in order to create a droplet in the middle of the chamber, and at the same time avoid blocking the inlet and outlet channels.

On-chip PCR with liquid or gelified reagents

On-chip real-time PCR experiments were performed on the lab-on-a-foil system with both liquid and gelified reagents. For PCR using liquid reagents, a mixture of 7.5 μl PCR master mixture, 0.5 μl of 10 μM To-pro-3 and 2 μl 0.2 ng μl^{-1} or 2 ng μl^{-1} DNA (total volume 10 μl) was prepared and pumped directly into the empty PCR chamber using pump 2. For PCR using pre-stored gelified reagents, a mixture consisting of 7.5 μl PCR graded water, 0.5 μl of 10 μM To-pro-3 and 2 μl 0.2 ng μl^{-1} or 2 ng μl^{-1} DNA (total volume 10 μl) was injected into the PCR chamber. The gelified reagents were rehydrated instantly and ready for PCR.

Before thermal cycling, the PCR chamber was sealed by closing the valves at the in- and outlets to the chamber. On-chip real-time PCR was performed with a thermal-cycling program consisting of an initiating step at 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 15 s, annealing at 54 °C for 15 s, extension at 72 °C for 8 s, and finally an additional extension step at 72 °C for 1 min. The signal was recorded by an ESE log detector integrated in the system. Three repetitive experiments were carried out for each condition.

Long-term stability study

The enzyme activity of the PCR master mixture gelified on labcard and stored for up to three months was studied using quantitative real-time PCR. After storage for the desired period, the gelified PCR master mixture was rehydrated by a mixture consisting of 7.5 μl PCR graded water, 0.5 μl of 10 μM To-pro-3 and 2 μl 2 ng μl^{-1} DNA (total volume 10 μl). The mixture was then pumped out of the chip and real-time PCR was performed on a Stratagene MX3005PH (Agilent Technologies, USA) using the same thermocycling protocol described above. Immediately after amplification, the melting curve was measured to verify the two PCR amplicons. The PCR efficiency of the gelified master mixture was determined at day 0 (the day the gelification took place), day 7 (after one week), day 21 (after three weeks), after a month, and three months of storage.

Results and discussion

On-chip gelification

The gelification technology was, for the first time, applied for on-chip reagent storage to facilitate the use of the lab-on-a-foil system and simplify the system design. There were two ways to make the gelification mixture in the microchip, directly on the labcard or on the adhesive foil which was used for sealing. We decided to choose the latter for two reasons. Firstly, if placed directly in the microchamber, the mixture will migrate fast to the border of the chamber, which may easily block the inlet and outlet channels of the microchamber. Secondly, since the adhesive foil has a very hydrophobic surface with contact angle of 81°, perfect round droplets can be formed on the foil. This is important for quality control in mass production.

The optimum conditions for gelification on foil were explored. Five different volumes of 6, 7, 8, 9 and 10 μl of the PCR gelification mixture were tested. It was found that 8, 9 and 10 μl gave too big droplets, which in many cases blocked the input/output channels and resulted in poor loading capabilities. When using only 6–7 μl droplets on the foil, it was easy to centre the droplet in the chamber, which ensures a good flow through. Therefore, the optimized volume of gelification mixture should be 60%–70% of the total volume of the chamber. Fig. 2 shows a photograph of a gelified PCR reagent in the PCR chamber of the labcard.

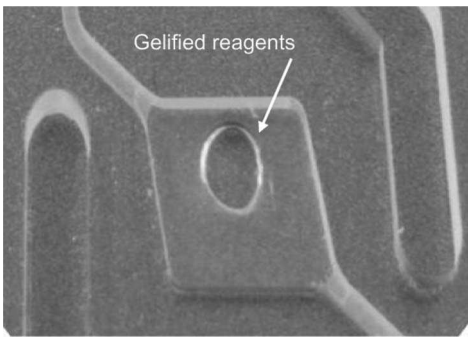


Fig. 2 Droplet of 6 μl gelified master mixture in the PCR amplification chamber of the labcard.

Comparison of on-chip PCR with liquid and gelified reagents

To test the effect of gelification on enzyme activity, on-chip multiplexed real-time PCR was performed with freshly prepared gelified reagents as well as conventional liquid reagents. The gelified reagents were rehydrated by loading rehydrating mixture (containing DNA template) into the PCR chamber. The reagents were easily dissolved in the solution, and no extra mixing was needed. PCR was then carried out by thermal cycling the reaction chamber. The real-time PCR products were also analyzed on a 2% agarose gel. The PCR efficiency using gelified reagents was compared to that with only liquid reagents. As shown in Fig. 3, for both 0.2 ng μl^{-1} and 2 ng μl^{-1} DNA templates, no differences were observed in the amount of PCR products using the liquid and the gelified reagents, suggesting that the integrity of enzyme was well maintained during the gelification process. The results demonstrated the feasibility of the gelification for reagent storage in lab-on-a-chip systems.

The method shows a clear advantage over other room-temperature drying technique where at least 30% loss of PCR product was reported.[15] This is greatly attributed to the addition of stabilizing agents which stabilize enzymatic reactions at room temperature. The gelification process does not alter protein structures, and interactions between reagents are avoided until the reaction is activated by the user either by re-suspending in a liquid medium or by heat activation. Thus, the technology significantly reduces the risk of reagent degradation and cross contamination. It provides a simple, fast and robust way to make on-chip PCR.

Long-term stability

The shelf life of the gelified PCR reagents was also studied by carrying out real-time PCR with gelified reagents stored at 4 °C and at room temperature for up to 3 months. Two PCR products were amplified by HIP and UC primers. To test the effect of gelification on enzyme activity, fluorescence intensities of the two PCR amplicons were measured from the melting curves as shown in Fig. 4. The percentage of reduction of the enzymatic activity was calculated and the data is presented in Table 1.

After one week of storage at room temperature, 12–16% reduction of the enzymatic activity was observed for both primer pairs. While when preserved at 4 °C, only 3–4% loss was found. Interestingly, after 4 weeks of storage, enzymatic activity only reduced 12% for 4 °C storage and 30% for room temperature storage. After 3 months, the activity of the gelified PCR reagents reduced 37% when stored at 4 °C, while 50% loss was observed for those that were kept at room temperature.

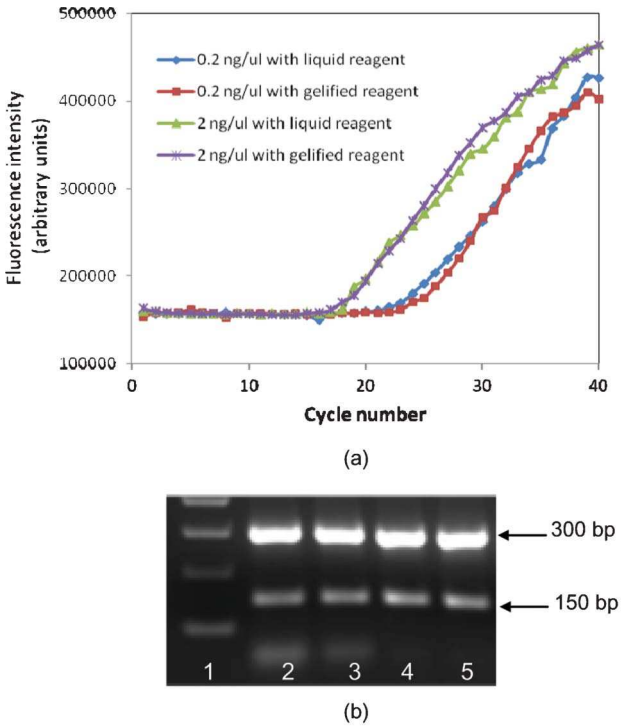


Fig. 3 (a) On-chip real-time PCR results for 0.2 ng μl^{-1} and 2 ng μl^{-1} DNA templates using both freshly prepared gelified reagents and conventional liquid reagents. (b) Agarose gel (2%) image of amplified products from the on-chip PCR. Lane 1: 100 bp DNA marker; lane 2: 0.2 ng μl^{-1} DNA with liquid reagents; lane 3: 0.2 ng μl^{-1} DNA with gelified reagents; lane 4: 2 ng μl^{-1} DNA with liquid reagents; lane 5: 2 ng μl^{-1} DNA with gelified reagents.

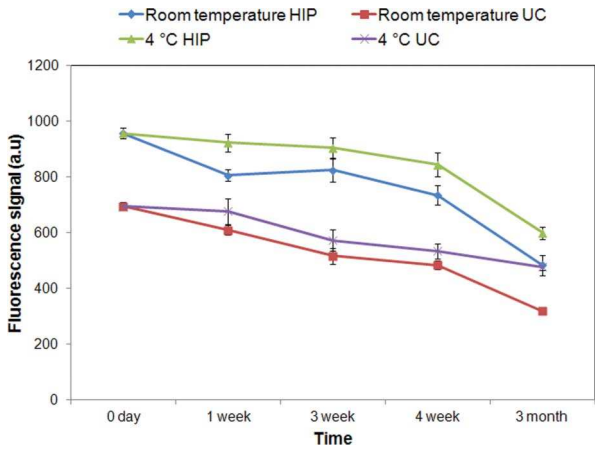


Fig. 4 Effects of time (0–3 months) and storage temperature (4 °C and 25 °C) on the activity of the gelified PCR mixture. Intensity of fluorescence signal of each PCR product was measured.

Table 1 Reduction of the Taq polymerase enzymatic activity of the gelified PCR master mixture over time of storage at room temperature and at 4 °C

	Room temperature		4 °C	
Time	UC	HIP	UC	HIP
1 week	12%	16%	3%	4%
3 weeks	26%	14%	18%	6%
4 weeks	30%	23%	23%	12%
3 months	55%	50%	31%	37%

Generally, the enzyme activity was better kept at 4 °C. The half-life of the gelified PCR reagents at room temperature was 3 months, which was comparable to freeze-dried reagents,[17] but the latter need to be stored at −21 °C. The long-term stability provided by gelification is extremely important for a robust and stable POC diagnostic system.

Large-scale fabrication

In order to keep up with demand for commercialization of POC analysis systems, we need a fast, reliable and cheap manufacturing process. The labcard is made of COC by injection molding. Both the material and the fabrication method are compatible with mass production. To pave the way for constructing reproducible and low-cost devices, it is essential that gelification and bonding processes can also be done on a large scale.

High-volume production of on labcard gelification has been achieved by EVG (Austria), who is also one of the EU project partners. EVG 510 bonding system (Fig. 5a,b) was modified and used as a tool for large scale gelification on labcard (Fig. 5c). Six μl of gelification mixture is deposited on the adhesive tape at 12 programmed positions. Twelve labcards are then aligned by the machine and in-line sealing is realised by bonding to the adhesive foil in one batch. Finally, individual labcard cartridges are trimmed, labelled and packed. Automation of the gelification and bonding processes greatly improves the yield of the fabrication and facilitates the mass production of the labcard.

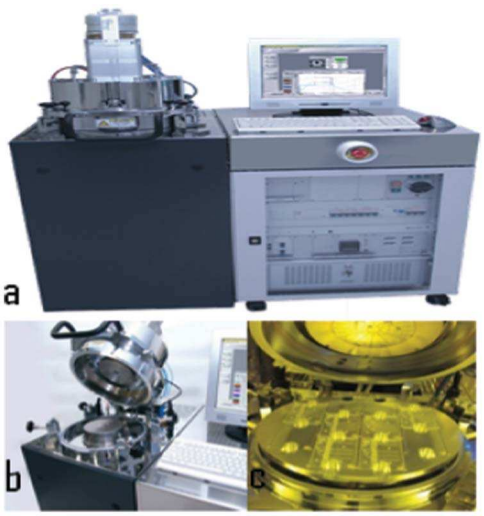


Fig. 5 In a and b: EVG 510 bonding system. c: The bonding equipment was modified for on large scale on labcard gelification.

Conclusion

On-chip storage is a critical factor for the successful development of a microfluidic device for POC diagnosis. In this paper, we addressed the problem by applying an advanced gelification technique to dehydrate PCR reagents for prolonged storage. The experiments indicate that gelification has no adverse effect on the Taq polymerase enzyme activity. The reagents were stable at room temperature for 3 months, allowing for convenient shipment and storage. Moreover, the on-chip gelification process can be automated, which is extremely important for commercialization of the integrated lab-on-a-chip system. The results obtained in this study clearly demonstrate the feasibility of on-chip storage of gelified reagents, and the technology is generally applicable for other lab-on-a-chip devices.

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