Biosensing enhancement of dengue virus using microballoon mixers on centrifugal microfluidic platforms

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1. Introduction

Neglected tropical diseases (NTDs) are a category of infectious diseases that are inextricably linked to poverty that thrive in tropical and subtropical regions (Dias et al., 2013; Linares et al., 2013). Among the NTDs, dengue virus (DV) is of particular importance and is considered one of the major public health problems in 112 countries (Gurugama et al., 2010). According to WHO, in the first quarter of this year the DV outbreak in Malaysia affected ~27,500 individuals and resulted in 64 deaths (WHO, 2014). In order to reduce the economic burden caused by this disease, it is necessary to employ a highly sensitive, rapid and low cost device as an early detection method. Introduction of such a detection system to diagnose the early stage infection where the concentration of the virus (NS1) is in the range of nanograms per milliliter range is expected to reduce the mortality rates from 20% to below 1% (Allwinn, 2011; Lapphra et al., 2008; Linares et al., 2013).

Lab-on-a-chip (LOC) devices are now revolutionizing molecular diagnostics towards a simpler and more cost effective point of care (POC) diagnostic devices and this will result in tremendous advances in the detection of infectious diseases such as dengue (Chin et al., 2007; Daar et al., 2002). In the LOC category, the centrifugal microfluidics platform integrates and automates a sequence of laboratory unit operations (i.e., liquid metering, reagent separation and mixing) required for a chemical assay on a single platform (Kazemzadeh et al., 2013; Madou et al., 2006). In centrifugal microfluidics, liquid is propelled through sophisticated microfluidic networks via the centrifugal force generated by a spinning motor that assists in the development of series of clinical assays (Gorkin et al., 2010; Kazemzadeh et al., 2014). The detection process on these platforms is carried out by employing a...
stationary optical system or on-disc electrochemical immunosensors to identify biological targets. In centrifugal microfluidics, like in other LOC devices, the efficiency and the rate of chemical reactions strongly depends on the efficiency of the mixing methods used in the fluidic networks (Lynn et al., 2014; Ren et al., 2012). This is of particular importance for the diagnosis of NTD in which the concentration of virus circulated inside a patient’s body in the early stages is in nanoscale range.

In recent years, several active (i.e., requires an external actuator) and passive (i.e., without the need for an external actuator) mixing techniques have been introduced to cope with mixing difficulties prevalent in the predominantly laminar flow regime on centrifugal microfluidic platforms. Examples of developed active mixing techniques on centrifugal microfluidic platforms are magnet stirring and batch-mode mixing techniques. Advection mechanism in both active mixers is induced by the relative motion of magnets that were embedded inside these mixing chambers, in respect to the external magnetic forces (Ducrée et al., 2007; Grumann et al., 2005; Haeberle et al., 2005). Passive mixing principles that have been explored on the centrifugal microfluidics include multilamination technique, reciprocating flow, Coriolis pseudo-force and Stopped-flow (passive batch-mode) mixing (Ducrée et al., 2006; Ducrée et al., 2006, 2007; Grumann et al., 2005; Haeberle et al., 2005; Kinahan et al., 2014; Noroozi et al., 2009; Ren and Leung, 2013a; Steigert et al., 2005). Stopped-flow mode mixing is based on inertia induced by the reversal of the spinning direction, hence it is commonly used in complex bioassays where the efficient use of the limited space on the disk is crucial (Grumann et al., 2005). In a reciprocating flow mixer, compression and expansion of air trapped inside an air chamber generates two dimensional (2D) reciprocating currents to facilitate mixing progress. A reciprocating flow mixer occupies quite a lot of

**Fig. 1.** Breakdown of the centrifugal microfluidic platforms. Design A and design B consisting of PMMA, PSA and latex layers. Design A was employed to evaluate microballoon efficiency in chambers having large-surface-area-to-volume-ratio. Design B is a CD-like well plate equipped with microballoon mixers which was used to assess the effect of microballoon mixing on detection sensitivity of colorimetric ELISA.
space on the disc as an air chamber that it used to generate pneumatic energy must be accommodated. Nevertheless, by means of the same air chamber the mixer could pump the mixed liquids against the centrifugal force to other reservoirs and therefore enable more complex bioassays on the disk (Noroozi et al., 2009, 2011). The mixing techniques on centrifugal microfluidics discussed here are useful approaches that have been employed, for example, in quantifying nitrate and nitrite levels in blood, repara-
tory virus detection, detection of the antibiotic resistance gene mecA of Staphylococcus aureus and several enzyme linked immu-
inosorbent assays (ELISA) (Focke et al., 2010; Kim et al., 2013; Kinahan et al., 2014; Lee et al., 2011; Lutz et al., 2010; Noroozi et al., 2009, 2011; Nwankire et al., 2013, 2014; Siegrist et al., 2010, 2006).

For this research paper, we used microballoons, introduced recently by this team (Aeinehvand et al., 2014), to enhance the efficient mixing on a centrifugal microfluidics. This microballoon technique that, we will validate constitutes a micromixer that combines various mixing principles such as inertial effects and reciprocating flows with a minimal use of the disk space and without the need for an external power source or high rotational frequency. The microballoon mixer generates a three dimensional (3D) reciprocating flow in response to the changes in rotational frequency. Increasing the spinning speed forces liquids to expand the microballoon and decreasing it will push the liquid back to the mixing chamber. Due to the mechanical properties of the micro-
balloon, this newly introduce mixer is also able to pump liquids with efficacy similar to other reciprocating flow mixers, and it is able to pump larger volume of liquids at dramatically lower rotational frequency. In this study, the microballoon mixing mechanism and its efficiency are experimentally analyzed and compared with the Stopped-flow mixing. Finally the effect of microballoon mixing on enhancing the sensitivity of DV detection was experimentally evaluated and assessed. In general, the micro-
balloon mixers can be used in a wide range of applications and they may be a promising approach to help facilitate the early detection of dengue.

2. Methodology

2.1. Designs and fabrications

Two microfluidic designs, A and B were created to evaluate the efficiency of the microballoon mixing in centrifugal microfluidic platforms (see Fig. 1). Design A was introduced for three purposes: (i) to evaluate the microballoon mixing efficiency in a chamber that provides high-surface-area-to-volume-ratio in which diffu-
sional mixing is time consuming, (ii) to compare the efficiency of the newly introduced technique with the Stopped-flow mixing method and (iii) to theoretically analyze the rotational frequencies corresponding to the change of liquid levels inside a mixing chamber. Two compact disk-like (CD-like) well plates (design B) were created to investigate the effects of the microballoon and the Stopped-flow mixing techniques on the colorimetric ELISA results.

Centrifugal microfluidic platforms were fabricated using layerby-layer assembly of poly(methyl methacrylate) disks (PMMA, 2 mm thick, Asia Poly Industrial Sdn. Bhd, Selangor, Malaysia), pressure sensitive adhesive layers (PSA, 100 μm thick, FLEXmount DFM-200-Clear V-95 150 poly V-95 400 poly, FLEXcon, Spencer, MA, USA) and highly elastic latex films (~140 μm thick, N.S. Uni-Gloves Sdn Bhd, Seremban, Malaysia). The mean thickness of the PSA layer that reduces by the pressure applied has been experiment-
ally determined by the calibration of the screw pressure used for assembling platform layers. The mean thickness of PSA layer is ~60 μm that results in producing of a mixing chamber with large-
surface-to-volume-ratio in Design A. Microfeatures on the PMMA discs were either engraved or cut-out using a computer numerical controlled machine (CNC, model Vision 2525, by Vision Engraving & Routing Systems, USA) and PSA microfeatures were cut-out using a cutter plotter machine (model PUMA II, by GCC, Taiwan). The spin test system employed to monitor the liquid movements on the centrifugal microfluidic platform, comprised of a high speed camera and a programmable spin controller system (for more details of the experimental setup please refer to (Aeinehvand et al., 2014)). Design A consists of two loading reservoirs, a mixing chamber partially covered by a latex layer and two microchannels that connect the loading reservoirs to the mixing chamber (see Fig. 1A). Fig. 1A illustrates the six layers of centrifugal microfluidics design A. It is composed of two PMMA disks, three PSA layers and a latex film. The top PMMA disk (first layer) contains venting and inlets/outlets. Unlike conventional microfluidic platforms, instead of using engraved chambers on plastic disk, we cut chambers and microchannels on the upper PSA layer (second layer). Hence sandwiching the upper PSA layer between the top PMMA disk and the middle PSA layer (third layer) produced chambers that have a high surface-area-to-volume-ratio. In order to equalize liquid capacity of all mixing sets, under all chambers masks of 4 mm microballoon were cut-out in the middle PSA layer. How-
ever in the bottom PSA layer (fifth layer), the balloon masks were cut-out only below chambers (under latex layer) in which ballooning action was desired. Arrays of microballoons were fabricated on the platform by sandwiching a latex film (forth layer) between the middle and bottom PSA layers. In order to protect the centrifugal microfluidics, a PMMA disk (sixth layer) is added to the bottom PSA layer (see Fig. 1A).

Design B has an additional PMMA disk when compared to design A to help increase the thickness of the reservoirs (see Fig. 1B). Two CD-like well plates were fabricated to compare the effects of microballoon and Stopped-flow mixing in the ELISA experiments. Both microfluidic platforms had seven layer plat-
forms (see Fig. 1B), but in order to prevent ballooning action in the Stopped-flow mixer platform, the bottom PSA layer (sixth layer) was featureless. During the platform assembly procedure, we further attached either a 3 × 4 mm2 cellulose membranes (CM) or a 3 × 4 mm2 polystyrene (PS) into the specific mixing chambers (cells). Normal mixing chambers with no PS and CM remained as control.

2.2. Microballoon mixings

Mixing experiments using design A was initiated with loading 11 μl deionized water (DI) into the loading chambers and spinning the platform to transfer liquids into the mixing chamber. Then 1 μl black color dye was injected into the source chamber. In order to transfer the color dye into the mixing chamber and conduct the first mixing cycle, the rotational frequency was increased to 13.5 Hz with an acceleration of 25 Hz s⁻¹. The optimum (i.e., maximum) rotational frequency of 13.5 Hz which is the speed required to reduce the liquid level inside the mixing chamber to the top of the microballoon has been experimentally defined (see Fig. 2a). When the spinning speed is increased, the liquid level in the mixing chamber is reduced as it enters into the microballoon (see Fig. 2a). The platform was then stopped until the entire liquid volume was gradually emptied out from the microballoon (see Fig. 2b). The mixing efficiency of the mixing progress has been evaluated by analyzing the image captured at each mixing cycle (Supplementary section A). All images were converted and saved into 8 bit gray scale bitmaps. The part of the recorded images that represent the mixing area was subsequently transferred into an intensity histogram in order to measure the uniformity of the gray-scale distribution in the mixing region. The efficiency of the
microballoon and the Stopped-flow in mixing liquids under the same operational conditions was compared and the results are presented in Section 4.1.

### 2.3. ELISA in CD-like well plate

Conventional colorimetric ELISA assay for the detection of DV was implemented on a CD-like well plate i.e. design B (see Fig. 1B). In order to assess the effect of mixing techniques on ELISA detection sensitivity, several mixing cycles were applied during the assay procedure periodically. The CD-like platforms were mounted on a portable made spin system and mixing cycles were performed for 5 times in each 3 min cycle during the incubation periods. In each mixing cycle, the rotational frequency was increased up to 27.5 Hz with an acceleration of 17 Hz s \(^{-1}\) and the CD-like platforms were stopped after 5 s. Two different mixing systems in the CD-like platforms were applied during the ELISA assay: (1) liquid mixing through ballooning action and (2) liquid mixing via Stopped-flow technique. In last step of the ELISA assay (Supplementary section B.2.4), once the color of the liquids changed from transparent to blue, these colored liquids from CD-like platforms were transferred into a conventional 96 well plates. The detection signal was then obtained using a micro-plate reader (at an absorbance of 570 nm). As a standard reference, conventional ELISA test was performed under the same protocol (Supplementary section B.2).

### 3. Theoretical analysis

This section theoretically describes the maximum required rotational frequency to be performed in a mixing cycle. In general, the rotational frequency corresponding to the level of liquid in the mixing chamber can be described in terms of equilibrium present between the kinetic energy stored in the expanded microballoon and the centrifugal pressure. The maximum rotational frequency is defined when the rotational frequency increases as much as the liquid level in the mixing chamber when it reaches the top of the microballoon (see Fig. 2).

In a mixing cycle, once the speed is increased, the microballoon expansion volume is equal to the change of liquid volume in the mixing chamber. If we denote the cross section area of the mixing chamber as \(S\) and microballoon expansion volume as \(V\), the microballoon expansion reacting to the changing liquid level in the mixing chamber is

\[
V = S(R_c - R_0) \quad \text{or} \quad V = \frac{2\pi r_{mb}^2 z}{3}
\]

(1)

where \(r_{mb}\) is the initial radius of the microballoon, \(z\) is the height of the bulged microballoon, \(R_c\) and \(R_0\) are corresponding liquids level in the mixing chamber when the platform is stopped and spun at the maximum required frequency of rotation, respectively. Stored kinetic energy in the expanded microballoon that is denoted with liquid volume of \(S(R_c - R_0)\) is expressed in terms of bulged height of \(z\) which is

\[
z = \frac{3}{2\pi r_{mb}^2} V = \frac{3(R_c - R_0)}{2\pi r_{mb}^2} z
\]

(2)

and the energy stored in the microballoon expanded can be described as microballoon bulge pressure (Hohlfelder 1999; Madou 2002):

\[
P_{mb} = C_1 \frac{Mz^2}{r_{mb}^4} + C_2 \frac{\sigma t^2}{r_{mb}^2}
\]

(3)

where \(C_1, C_2\) are constants and \(M\) is the biaxial modulus of elasticity defined by \(E(1 - \nu)\) (\(E\) and \(\nu\) are Young’s modulus of elasticity and Poisson’s ratio, respectively), \(t\) is the thickness of the latex of the microballoon and \(\sigma_0\) is its initial stress. The kinetic energy stored in a bulged microballoon is generated from the centrifugal pressure that is based on the liquid density, rotational frequency, liquid length and distance from the center of the platform. Centrifugal pressure that acts on the liquids as well as the microballoon is expressed as follows:

\[
P_c = \rho \omega^2 \Delta r t
\]

(4)

where \(\rho\) is the liquid density, \(\omega\) is the rotational frequency of the spinning platform, \(\Delta r\) is the average distance of the liquid in the mixing chamber from center of the platform and \(t\) is the radial length of the liquid. The density of the liquid is assumed to be constant and the changes of rotational frequency and the parameters related to the liquid position (\(\Delta r\) and \(t\)) determine the amount of centrifugal pressure. The store kinetic energy of the microballoon in all states is proportional to the centrifugal pressure \(P_c = P_{mb}\). Therefore, rotational frequencies corresponding to the changes of the liquid level in the mixing chamber can be yielded from Eqs. (3) and (4) as follows:

\[
\omega = \left\{ \frac{C_1 \frac{Mz^2}{r_{mb}^4} + 2C_2 \sigma_0}{\rho \Delta r t\Delta r t^2} \right\} \left( \frac{IZ}{2(\Delta r t r_{mb}^3)} \right)
\]

(5)

and maximum rotational frequency at which liquid level is reduced to \(R_c\) or when \(t = 2r_{mb}\) yield as

\[
\omega_{max} = \left\{ \frac{C_1 \frac{Mz^2}{r_{mb}^4} + 2C_2 \sigma_0}{\rho \Delta r t^2} \right\} \left( \frac{IZ}{2(\Delta r t r_{mb}^3)} \right)
\]

(6)

Employing Eq. (5), the rotational frequencies corresponding to the change of radial length of liquid (\(t\)) from \(R - R_0\) to \(R - R_c\) was measured. In order to test the accuracy of Eqs. (5) and (6) in predicting liquid levels corresponding to rotational speeds, theoretically calculated values were compared with experimental data.
Table 1  
Value of parameters used in theoretical analysis.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r_{mb}$</td>
<td>4 mm</td>
<td>$E$</td>
<td>3.2 MPa</td>
</tr>
<tr>
<td>$R$</td>
<td>56 mm</td>
<td>$\nu$</td>
<td>0.48</td>
</tr>
<tr>
<td>$R_0$</td>
<td>34 mm</td>
<td>$\sigma_0$</td>
<td>0.55 MPa</td>
</tr>
<tr>
<td>$\rho$</td>
<td>1000 kg/m$^3$</td>
<td>$\ell$</td>
<td>136 $\mu$m</td>
</tr>
<tr>
<td>$C_1$</td>
<td>8/3</td>
<td>$C_2$</td>
<td>2</td>
</tr>
</tbody>
</table>

and the results are presented Section 4.3. Value of the parameters used for the evaluation of Eqs. (5) and (6) are listed in Table 1.

4. Results and discussions

4.1. Analytical evaluation

Microballoon mixers in a similar fashion to Stopped-flow mixing enabled efficient usage of the limited space of CD-like platforms. First the liquid is transferred from a high aspect ratio chamber to fill the microballoon with a large angular acceleration which creates a large magnitude of Euler force. The Euler force acting on the liquids generates vortices that push the liquid samples towards the opposite direction of the disk rotation enhancing the mixing efficiency (Grumann et al., 2005; Ren and Leung, 2013b). Thereafter, the angular velocity is reduce to zero with the same acceleration which reversed the flow. However due to the high hydraulic resistance of the chamber and high elasticity of the microballoon the system acted as a large tank that was emptied via leakage. The ratio of cross-sectional area of the mixing chamber to the microballoon area is $\sim 0.9$% in design A. As per graph shown in Fig. 3B, mixing cycles are composed of system paused periods of $\sim 0.5$ s. In addition considering the liquid exchange between the microballoon and the ultra-thin chamber, it is gleaned that the mixing progress can be also enhanced due to the effects of liquid flowing in periodically converging–diverging passages (A1 Yong and A1 Teo, 2014; Jafari et al., 2014).

Fig. 3 shows the mixing progress in different mixing cycles. The standard deviation obtained from the histogram was normalized according to the first microballoon mixing cycle. Shown in Fig. 3A, distribution of pixels in the 2D-histogram which corresponded to the second mixing cycle does not flow a normal distribution, however the decreased standard deviation indicates changes in the state of mixing. Through further mixing cycles, distribution of pixel intensities became more uniform and shifts toward the grayscale color (see Fig. 3A). This may be gleaned from the histograms in Fig. 3A, showing a Gaussian distributions and the narrowing of peak width in the histograms corresponding to the eighth and fourteenth mixing cycles. Therefore as shown in Fig. 3B, standard deviation ($\sigma$) decreases in tandem with increasing number of efficient mixing cycles. For an experimental evaluation, we have compared the mixing time at which standard deviations for both applied techniques stabilized below 0.3. As a reference, constant spinning mode i.e. diffusional mixing, took 170 min to mix the 12 $\mu$l liquid samples. Excluding the pause times required for microballoon constriction, the Stopped-flow mixing reduce the mixing time to 37.45 s via 35 mixing cycles. The best performance was achieved by the microballoon mixing technique, reaching a mixing time of 22.5 s via 14 mixing cycles (see Fig. 3).

In the case of Stopped-flow mixer, the low standard deviation during the early stages of mixing progress is due to the uniformity of white color (see Fig. 3B). As more mixing cycles is applied, the standard deviation fluctuated until cycle twelve in which the liquid color uniformly turned to gray and standard deviation continuously decreased. It has been reported that the optimum mixing in Stopped-flow has been achieved via acceleration of 32 Hz s$^{-1}$ (Grumann et al., 2005). The increase of acceleration/deceleration rate may intensify vortices and further improve the mixing efficiency; however the maximum angular acceleration of the spinning system used in this study is 25 Hz s$^{-1}$. Further study is required to assess the performance of the microballoon mixing at higher accelerations in comparison with Stopped-flow mixing techniques especially when the direction of disc rotation is reversed. However, at low accelerations, where Stopped-flow mixing can become less effective, microballoon mixers can operate similar to pneumatic based reciprocating technique.

4.2. Detection sensitivity enhancement

The enhancement of the DV detection signal was investigated on different substrates in CD-like well plates by using the
Microballoon mixing technique and the result are presented in **Fig. 4**. In order to demonstrate the cross-reactions due to the host cell proteins, the cell supernatant of non-infected cells was used as a negative control in the ELISA assay. The comparison of the positive and negative control values demonstrates the low cross-reactivity in the assay. A generally accepted approach in biomolecule immobilization relies on heterogeneous interaction between biomolecules and plastic surface. As it can be seen from **Fig. 4**, the microballoon mixing procedure resulted in significantly higher detection signals when compared to the Stopped-flow mode mixing. The dramatic difference in the results corresponds to the type of mixing technique, as in the batch mode mixing technique the advective current has exposed the reaction mixture while in the balooning action, 3D reciprocating flow provides much higher interaction of the antigen and the surface and the subsequent protein attachment. This is in a direct agreement with analytical results of the mixing efficiency (Section 4.1).

It is well known that proteins (or other biomolecules) have high sensitivity toward interactions with solid surfaces (Hosseini et al., 2014). In this research we have investigated only pure and non-functionalized polymeric platforms in order to assess the performance of the centrifugal microfluidic platforms. Please note that those platforms that we have used in this research are used commercially to produce a variety of biosensor devices (CM, PMMA and PS). Possibly the most common application of those plastic materials is the production of the ELISA 96 well plates on industrial scales.

Although the cellulose membrane cell has dramatically higher hydrophilicity in comparison to the other cells (PPMA, PS and PC), results indicates that the highest detection rate was obtained from the PS cells. PMMA cells have also shown the enhanced detection signal by mixing technique, however relatively lower detection signal was generated when compared to the PS. It is important to note that the protein attachment is not only dependent on hydrophilicity of the surface. Surface morphology and chemistry also play a crucial role at the biomolecule/plastic interface. From the results, it is obvious that cellulose membranes have adverse effect on antigen activity in comparison to flat surfaces (see **Fig. 4**). The possible reason for such affinity is the higher specific surface of membranes with inter-connected fibrous morphology. For that reason antigens molecules can be trapped inside such a structure and lose the activity due to the steric interaction. Despite the fact that the CD detection cells have been designed with a relatively similar volume as the wells of ELISA 96 well plate, it can be clearly seen form **Fig. 4**, which the conventional method (ELISA) has resulted in a significantly lower intensity of detection signal. For that reason our results prove that our established methods, based on microballoon mixing can significantly enhance the sensitivity for the detection of DV.

### 4.3. Microballoon mixing calibration

In order to facilitate the future prospect for the implementation of microballoon mixers in centrifugal microfluidics, maximum frequency required in a mixing cycle is theoretically discussed and presented as Eq. (6). **Fig. 5** shows the liquid levels in the mixing chamber corresponding to the rotational frequency measured with Eqs. (5) and (6). Comparing the theoretically predicted values with experimentally recorded angular frequencies indicates that the accuracies of Eqs. (5) and (6) are ~91% and 86%, respectively. According to Eq. (4), the induced centrifugal pressure at the spinning speed of 13.5 Hz is equal to ~3 KPa which can be easily tolerated in our design. However, we have experimentally confirmed the consistency of the system at higher rotational frequencies (up to 108 Hz) which is due the high elasticity and durability of the latex film used. For supplementary use of the same mixing system for the pumping of mixed liquids, Eq. (5) which allows controlling liquid levels via rotational frequency is introduced. Microballoon mixing similar to reciprocating flow enables pumping liquids toward the center of disk. Microballoons are able to move large volume of liquids at lower range of rotational frequency compared to pneumatic based techniques (Aeinhevand et al., 2014). Therefore, 3D reciprocating flow by microballoons is expected to enhance mixing at lower speeds compared with conventional 2D reciprocating flow mixers.

### 5. Conclusions

The work presented in this study offers a new method of mixing liquids on centrifugal microfluidic platforms using latex-made microballoons. Microballoon mixer accelerates the mixing progress by producing a periodical 3D reciprocating flow. The converging–diverging passage produced during the liquid exchange between the microchamber and the microballoon improves the mixing efficiency up to 40% compared to the Stopped-flow mixing method which is mainly based on Euler forces. The small space used on the disc for the control the reciprocating flow and the operation at low rotational frequencies without any external power source makes microballoon mixers an attractive option for a wide range of applications. In this study we have assessed the application of our technique in real biomedical processes and the effect of microballoon mixers for the detection
of dengue virus. Our results indicate that the mixing technique dramatically enhance biomolecules reaction rates and therefore increases the detection sensitivity of dengue virus.

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Appendix A. Supplementary information

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References