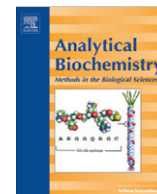


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Notes & Tips

Microwave-mediated enzyme-linked immunosorbent assay procedure

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ABSTRACT

Here we demonstrate a novel microwave-mediated enzyme-linked immunosorbent assay (MELISA) method that has dramatically reduced the enzyme-linked immunosorbent assay (ELISA) timing to less than 5 min with a result comparable to that obtained by 18-h conventional ELISA. Efficacy of the MELISA procedure is demonstrated by detecting human immunoglobulin G (IgG), rabbit IgG, human immunoglobulin E (IgE), human interleukin 1 β (IL-1 β), *Entamoeba histolytica* antibody, and *Aspergillus fumigatus* antibody. MELISA could be an excellent substitute for time-consuming conventional ELISA for rapid diagnosis of diseases in cases of medical urgency, outbreak of infectious diseases, and screening of samples in blood banks or emigration counters.

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The enzyme-linked immunosorbent assay (ELISA)² technique is an important tool for diagnosis of infectious and allergic diseases [1–3], detection of biothreat agents [4], and high-throughput screening of drugs and drug targets [5,6]. Conventionally, ELISA is a time-consuming procedure. It has been found that ELISA timing can be reduced using an activated solid support for covalent binding of an antigen or antibody [3,7]. Several authors have shortened the ELISA procedure to 3 h by performing heat-mediated ELISA (HELISA), where ELISA was carried out at 50 °C on an activated polystyrene microtiter plate [8–10]. Kannoujia and Nahar [11] used pressure energy instead of thermal incubation to reduce the ELISA timing to 1 h, whereas Sharma and Nahar [12] further reduced the ELISA timing to 40 min by employing ultrasound energy. In all of these processes, the ELISA procedure was shortened without using any additional or expensive reagent such as monoclonal antibody or sensitive tags. van Dorp and coworkers attempted to shorten the ELISA timing by microwave irradiation; nevertheless, the time gain for such ELISA was not attractive given that they did it in 2 h [13].

In this study, we demonstrate an ultrafast ELISA procedure in which the steps were carried out by microwave irradiation in a domestic microwave oven (BPL–Sanyo, India) operating at a frequency of 2450 MHz. Efficacy of the microwave-mediated ELISA (MELISA) procedure was demonstrated by detecting human immunoglobulin G (IgG), rabbit IgG, human immunoglobulin E (IgE), hu-

man interleukin 1 β (IL-1 β), *Entamoeba histolytica* antibody, and *Aspergillus fumigatus* antibody. The amoebic antigen and *A. fumigatus* antigen were obtained from the culture as per the published procedure [14]. *E. histolytica* antibody was raised in New Zealand white rabbits, and consent from an animal ethics committee was obtained before the experiment. *A. fumigatus* antibody was obtained from the sera of patients ($n = 9$) suffering from allergic bronchopulmonary aspergillosis (ABPA) and used as positive test sera, whereas pooled sera samples from healthy volunteers ($n = 9$) was used as a negative control. Patients were from the V.P. Chest Institute outpatient center in Delhi, India. Informed consent was obtained from each patient and normal individual. Human IgG, rabbit IgG, and their antibodies and antibody conjugates were purchased from Sigma (USA). ELISA kits of human IgE and human IL-1 β were purchased from Bethyl Laboratories (USA) and BD Biosciences (USA), respectively. For coating a well, 1 μ g of antigen or antibody (1 μ l for anti-human IL-1 β) was diluted in 100 μ l of sodium phosphate buffer saline (pH 7.2, 0.01 M). Antibody and conjugates were used with the optimum titer value or according to the supplier's protocol. MELISA was performed in the wells of a polystyrene module (Dynatech, USA) or a polystyrene microtiter plate (Greiner Bio One, Germany), each of which was activated by coating the wells with 1-fluoro-2-nitro-4-azidobenzene (FNAB) and exposing them to ultraviolet (UV) radiation at 365 nm according to the published procedure [15].

Conventional ELISA was carried out by a procedure involving the steps of (i) antigen or antibody coating into the wells by overnight incubation (12 h) at 4 °C, (ii) blocking the unbound surface with 200 μ l of 2% (w/v) bovine serum albumin (BSA) solution in 2 h at 37 °C, (iii) binding of human IgG, rabbit IgG, human IgE, human IL-1 β , *E. histolytica* antibody, and *A. fumigatus* antibody in 2 h

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E-mail address: pnahar@igib.res.in (P. Nahar).¹ Current address: Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781 039, India.² Abbreviations used: ELISA, enzyme-linked immunosorbent assay; MELISA, microwave-mediated ELISA; IgG, immunoglobulin G; IgE, immunoglobulin E; IL-1 β , interleukin 1 β ; BSA, bovine serum albumin; HRP, horseradish peroxidase.

at 37 °C, and (iv) peroxidase-conjugated secondary antibody (biotin-labeled antibody for human IL-1 β) binding in 2 h at 37 °C. In the case of human IL-1 β detection, streptavidin–horseradish peroxidase (HRP) conjugate binding was performed in 30 min at room temperature. Negative control experiments were conducted by taking antigen or antibody other than test sample. After each step, the wells were washed thoroughly with washing buffer. Color development was carried out by adding 100 μ l of substrate solution (0.067% of *o*-phenylenediamine and 0.043% of H₂O₂ in 0.1 M phosphate citrate buffer, pH 4.5) at room temperature, and the reaction was stopped by 20 μ l of 5% H₂SO₄. Absorbance was recorded at 490 nm in the ELISA reader (SpectraMax 190 Microplate Reader, Molecular Devices, USA).

We carried out MELISA experiments in a manner identical to that of conventional ELISA procedure in terms of amounts of

reagents and buffers, washing condition, color development, and recording of absorbance except that the MELISA steps were carried out in a microwave oven instead of a common laboratory incubator. This made the MELISA procedure more compatible with common laboratory practice for such an assay procedure. It also gave a better comparison of the current and conventional procedure. To optimize the conditions for MELISA, each ELISA step was carried out by irradiating microwaves for a different time period at a fixed output power followed by subsequent ELISA step(s) by the conventional procedure. The preceding ELISA step(s) was carried out by optimized microwave power and time. The results (Fig. 1A) showed that the maximum *E. histolytica* antigen immobilization occurred at 700 W in 70 s of microwave exposure, beyond which no appreciable increase in absorbance value was found. In addition, in the negative control test, no nonspecific binding was

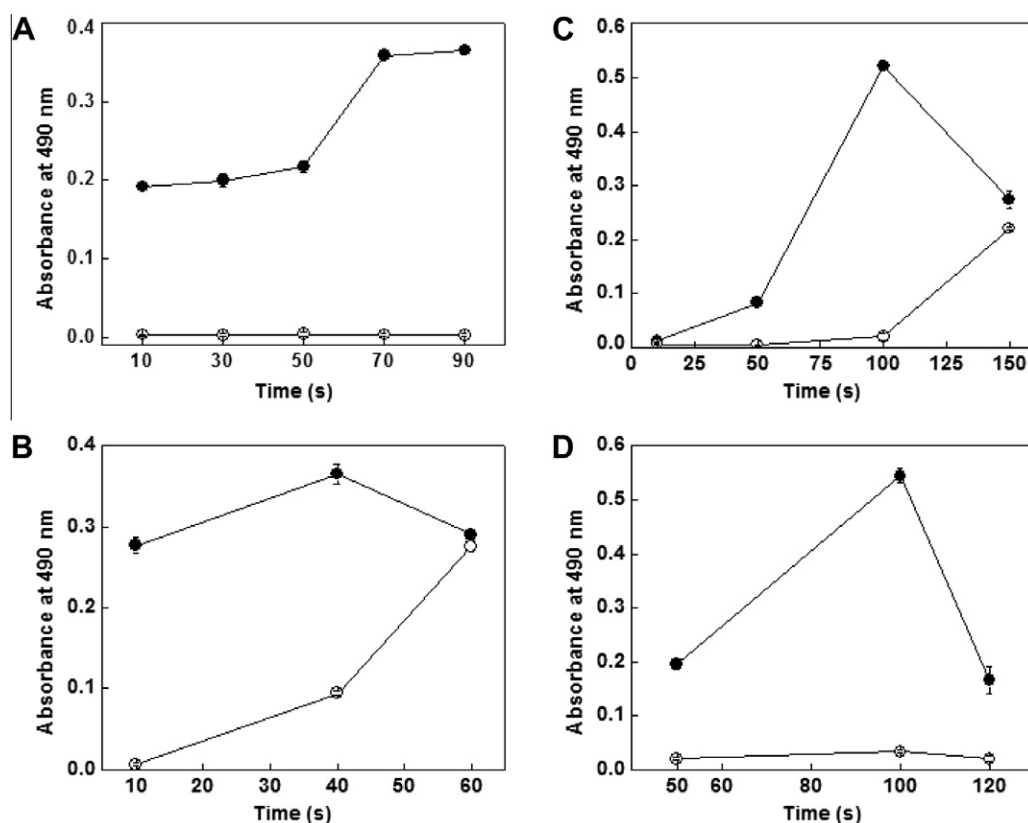


Fig.1. Optimization of MELISA steps. (A) Immobilization of *E. histolytica* antigen in the wells of an activated polystyrene strip occurred by microwave irradiation at a fixed output power of 700 W for 10, 30, 50, 70, and 90 s, respectively. (B) Blocking of antigen-immobilized wells was performed at 700 W for 10, 40, and 60 s, respectively. (C) Binding of *E. histolytica* antibody was carried out by microwave irradiation at 155 W for 50, 100, and 150 s, respectively. (D) Binding of anti-rabbit IgG–HRP was optimized by performing it at 155 W for 50, 100, and 120 s, respectively. In each of the optimization experiments, absorbance was recorded after performing the subsequent ELISA steps by the conventional method. *E. histolytica* antibody was used as positive test sera (solid), and rabbit serum was used as a negative control (hollow). All experiments were carried out in triplicates. Error bars indicate standard deviations.

Table 1

Comparison between conventional ELISA and MELISA procedures.

	ELISA		MELISA	
	Positive control	Negative control	Positive control	Negative control
Human IgG	0.743 $\pm$ 0.074	0.107 $\pm$ 0.040	0.435 $\pm$ 0.054	0.058 $\pm$ 0.027
Rabbit IgG	0.956 $\pm$ 0.072	0.138 $\pm$ 0.041	0.736 $\pm$ 0.060	0.071 $\pm$ 0.039
Human IgE	0.892 $\pm$ 0.070	0.114 $\pm$ 0.043	0.661 $\pm$ 0.059	0.069 $\pm$ 0.033
Human IL-1 β	0.428 $\pm$ 0.061	0.078 $\pm$ 0.030	0.321 $\pm$ 0.053	0.062 $\pm$ 0.026
<i>E. histolytica</i>	0.548 $\pm$ 0.051	0.084 $\pm$ 0.038	0.438 $\pm$ 0.051	0.061 $\pm$ 0.029
<i>A. fumigatus</i>	0.409 $\pm$ 0.071	0.102 $\pm$ 0.031	0.345 $\pm$ 0.048	0.068 $\pm$ 0.042

Note: Incubation conditions for conventional ELISA steps: 12 h at 4 °C, 2 h at 37 °C, 2 h at 37 °C, and 2 h at 37 °C. Microwave exposure conditions for MELISA steps: 70 s at 700 W, 10 s at 700 W, 100 s at 155 W, and 100 s at 155 W.

observed. In contrast, untreated wells of the polystyrene module failed to immobilize antigen even after 90 s of microwave exposure. Hence, activated surface that strongly binds antigen or antibody is a prerequisite for MELISA. The covalently bound antigen or antibody was stable enough to withstand subsequent ELISA steps by microwaves. This may be one of the reasons for the failure of rapid ELISA on untreated surface by microwave irradiation carried out by van Dorp and coworkers [13]. Furthermore, it took only 10 s at 700 W for microwave-mediated blocking without any non-specific binding (Fig. 1B). An increase in microwave exposure time resulted in a sharp increase in assay signal in positive sera as well as in negative control sera. This may be due to detachment of weak BSA binding beyond 10 s leading to higher nonspecific binding. As shown in Fig. 1C, maximum antibody binding occurred at an output power of 155 W in 100 s without any nonspecific binding. However, beyond 100 s, the assay value of positive sera declined, whereas that of negative control sera increased. Thus, in the case of antibody binding, harsh conditions, whether in the form of high microwave output power (results not shown) or higher timing, led to an undesirable outcome. The maximum binding of antibody conjugate was observed in 100 s at 155 W (Fig. 1D). In negative control sera there was no nonspecific binding at least up to 120 s, whereas in positive control sera there was a sharp decline in ELISA value beyond 100 s, perhaps due to inactivation of the enzyme conjugate by harsh microwave exposure.

Efficacy of the MELISA procedure was further validated by detecting six different antigens/antibodies by performing ELISA steps under optimized microwave output power and exposure time and comparing the procedure with the conventional ELISA procedure. Each MELISA experiment was carried out nine times in triplicates. MELISA needed only 280 s to perform all of the steps with results akin to those of the conventional ELISA carried out in 18 h. Besides a dramatic reduction in time, MELISA also gives less nonspecific binding and, thus, provides better a positive-to-negative ratio (Table 1). MELISA showed less than 10% day-to-day variation and gave typical linearity of standards as checked by human IgG. However, fine-tuning of output power and time may be required for performing MELISA in a microwave oven with different output power or of a different make or model.

The mechanism of the microwave effect, whether thermal or nonthermal, on rapid acceleration of the ELISA procedure, like other microwave-induced reactions, is still debatable [16]. Nevertheless, results (Fig. 1A) showed appreciable antigen binding by 10 s of microwave exposure, even though the temperature of the well after 10 s was only 28 °C. On the contrary, antigen failed to bind on activated surface in a thermal incubator in 70 s at 37 °C. Furthermore, no 10-s blocking was obtained by thermal incubation

at any temperature (results not shown). Lastly, Bora and coworkers [8] carried out ELISA at 50 °C in a thermocycler (for quick heating) and were able to reduce ELISA to only 3 h, much longer than the 5-min MELISA. These all pinpoint that the mechanism of MELISA is a combination of microwave thermal and nonthermal effects. Regardless of mechanism, the MELISA procedure could be a potentially useful substitute for the time-consuming conventional ELISA procedure widely used in pathological and research laboratories. Detailed studies of the MELISA procedure with more and versatile clinical samples are in progress.

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