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ARTICLE

Microwave non-thermal effect reduces the ELISA timing to less than 5 minutes

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This report demonstrates that the microwave-mediated ELISA that occurs in less than 5 minutes is due to microwave non-thermal or microwave specific effect rather than microwave heating effect. To decipher the non-thermal effect, we have designed a system that mimics time dependent temperature rise of a reaction mixture or buffer in the wells of a polystyrene microtiter plate similar to that of microwave exposure. This system is used as an alternative to microwave thermal effect (microwave- thermal- alternate or MTA). We have carried out ELISA for the detection of human IgG in a time dependent manner under microwave irradiation in a microwave oven and by thermal incubation by a specially designed MTA. ELISA results carried out by microwave exposure in 4 min 40 s is akin to 18 h conventional ELISA whereas no significant ELISA values are obtained by microwave-thermal-alternate illustrating predominance of microwave non-thermal effect over microwave thermal effect in microwave-mediated ELISA. We postulate that microwave specific effect is a microwave catalytic effect acting by lowering the activation energy of reactants.

Introduction

Implementation of microwave energy in chemical transformations has yielded vast success to organic and peptide synthesis, polymer chemistry, material sciences, nanotechnology, proteomics and biochemical processes.¹⁻⁸ Over the years, microwave-mediated synthesis has resulted in reduced processing time, enhanced product yield and purity compared to conventional methods.¹⁻⁸ In general, microwave radiations produced by magnetron in a microwave oven get absorbed by the reaction mixtures, depending upon their dielectric properties, and then a part of this electromagnetic radiation is converted to heat energy, producing rapid localized heating of the molecules present in the reaction mixture.^{9,10} A large number of chemists believes that microwave-assisted acceleration of reaction is nothing but the consequence of rapid rise in temperature of the reactants by microwave irradiation.^{1,11} However, there are reports which demonstrate the existence of "specific" or "non-thermal" microwave effect.¹²⁻¹⁴ Besides polarity of reactants and solvents, microwave effect depends on factors such as power (specific absorption rate), wavelength/frequency, overall duration and intermittence of exposure (continuous or

interrupted). Specific absorption rate, also called power density is a main determinate for the thermal effect of MW, whereas duration and intermittence of exposure may play role in the non-thermal biological effects.^{15,16} A similar study conducted on thermophilic and thermostable enzymes revealed the non-thermal effect of MW as the driving force for temperature-independent denaturation of these enzymes.¹⁴ Indeed, microwave assisted DNA hybridization; biocatalysis and enzymatic modification of oligonucleotides imprints the non-thermal picture of microwaves.¹⁵⁻²⁰ Nevertheless, there is not enough experimental studies that can demonstrate the existence of non-thermal effect.

Thus, in order to address whether the specific microwave effect or the localized heating in microwave is responsible for the enhanced rate of enzymatic biological-reactions, herein we have utilized the enzyme-linked immunosorbent assay (ELISA) as a platform of choice in the present study. ELISA is a very useful technique for the detection of minute quantities of antigen or antibody in clinical diagnostics and biomedical research. Conventionally, ELISA procedure is carried out by immobilizing the capture molecule (antigen or antibody) on a polystyrene microtiter plate through adsorption at 37°C or overnight incubation at 4°C. The remaining steps, including the blocking, target (antibody or antigen) binding and detection molecule binding are carried out stepwise, making the assay almost an 18 h protocol. Van Dorp et al., have used microwave to reduce the ELISA incubation time.²¹ They used different power setting and different combination of microwave and conventional steps to achieve an ELISA protocol of 2 h duration. However, in this method the time gain was not attractive. In order to shorten the assay timing, Nahar et al.,

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have used different forms of energies such as the heat, pressure and ultrasound, to reduce the timing of ELISA protocol to less than an hour.²²⁻²⁴ We recently published a preliminary report on microwave-mediated ELISA (MELISA) procedure, in which all the steps of ELISA were carried out in less than 5 minutes.²⁵ In another recent report, Garber et al. has also employed microwave to reduce the ELISA timing to about 2 h for the detection of two different plant toxin ricin and gliadin. However, the authors were inconclusive on the benefit of microwave irradiation and could not differentiate between thermal and non-thermal effects of MW.²⁶

In the present study, we focused mainly on the thermal and non-thermal effects of microwaves. For this, we have made a microwave- thermal- alternate (MTA) where we replicated the heating effect of MW outside the MW oven. Efficient and precision IR thermal imaging device was used to capture the temperature differences during the course of ELISA. Thermal ELISA was carried out by MTA system mimicking microwave thermal effect. To our surprise, we found that microwave thermal effect alone could not produce ELISA results emphasizing that microwave specific effect is prerequisite for MELISA in such a short time.

Experimental

Reagents

Anti-human IgG, bovine serum albumin (BSA), human IgG, anti-human IgG–HRP conjugate, and o-phenylenediamine dihydrochloride (OPD) were purchased from Sigma (USA). Polystyrene microtiter plates (Greiner Bio-one, Germany) were activated by coating the surface with 1-fluoro-2-nitro-4-azidobenzene (FNAB) and exposing them to UV radiation at 365 nm for 10 min as reported earlier.²⁷ All immunoassays were carried out on these activated polystyrene microtiter plates. Microwave irradiation was carried out in a domestic microwave oven (BPL-Sanyo, India) operating at a frequency of 2.45 GHz and a maximum output power of 700 W. Temperature of the reaction mixture and buffer was measured after microwave exposure for each immunoassay step using Guide EasIR-9 Infrared Thermal Imaging Camera having a precision of $\pm 0.2^\circ\text{C}$. Sodium phosphate buffer saline (PBS: 0.01M PB, pH 7.2, 0.85% NaCl) was used as the diluent buffer in ELISA procedure. Washing buffer was prepared by adding 0.1% Tween-20 to PBS. Concentrations of anti-human IgG, human IgG and anti-human IgG-HRP conjugate were optimized by checker board titration. All the experiments were performed in triplicate.

Detection of human IgG by conventional ELISA

Conventional sandwich ELISA was carried out by (i) binding goat anti-human IgG (1 μg /100 μL of PBS/well) in the FNAB activated wells of a microtiter plate in 11 h (overnight incubation) at 4°C , (ii) blocking the unbound surface with 100 μL of 2% (w/v) BSA solution in 1 h at 37°C , (iii) binding human IgG (0.5 μg /100 μL of PBS) at 37°C for 3 h and, iv) binding 100 μL of anti-human IgG-horseradish peroxidase conjugate

(1:4000 dilution) at 37°C for 3 hour. Negative control experiments were conducted by taking rabbit IgG as an antigen. The wells were washed thoroughly with washing buffer after each step. Colour development was carried out by adding 100 μL of substrate solution (6 mg o-phenylenediamine and 8 μL of H_2O_2 in 6 mL of 0.1 M citrate buffer, pH 5.2) to each well, followed by 5 min incubation at room temperature. The reaction was stopped by adding 20 μL of 5% H_2SO_4 solution. Absorbance was recorded at 490 nm in an ELISA Reader (Spectramax 190 microplate Reader, Molecular Devices USA).

Temperature profile of PBS after microwave exposure for different time

PBS (100 μL) was added into triplicate wells of polystyrene microtiter plates and exposed to microwave irradiation at an output power of 700 W for 10, 20, 30, 40, 50, 60, 70 80, 90 and 100 s, respectively. The initial temperature of the buffer in the wells was recorded using the infrared thermal imaging camera. After exposure to each time period, plate was taken out of the oven and the temperatures of the buffer were recorded immediately. Similarly, temperature profiles of PBS before and after microwave exposure at 155 W were recorded for 25- 150 s using the infrared camera. The initial temperatures of PBS (before microwave exposure) during all the experiments were kept constant to minimize temperature errors.

Designing MTA system mimicking thermal effect of microwave

Temperature rise of PBS (100 μL) in the wells of microtiter plate was recorded for 10-100 s in water bath preheated to 62°C , 72°C , 76°C , and 80°C respectively. Initial temperatures of PBS for all the experiments were made constant. All temperatures were measured by IR thermal imaging camera. MTA system is prepared by mimicking the time dependent microwave-induced rise in temperature of PBS with the temperature rise as obtained in preheated-water bath. Different water bath temperatures were used to attain similar temperature rise in the same time as that obtained by microwaves.

Thus, temperature rise of PBS by microwaves in 10-50 s is equivalent to temperature rise in the same time by incubation on water bath preheated at 62°C . Similarly, microwave - induced temperature rise in 60-80 s, 90 s and 100 s is achieved by incubation on water bath preheated at 72°C , 76°C and 80°C respectively. In subsequent experiments all ELISAs by thermal incubation were carried out by this MTA system.

Time dependent immobilization of goat anti-human IgG by microwave irradiation and MTA system

Goat anti-human IgG was loaded into activated wells of microtiter plate and subjected to microwave irradiation for 10, 20, 30, 40, 50, 60, 70 80, 90 and 100 s respectively, at an output power of 700 W. Also, goat anti-human IgG was immobilized into activated wells of microtiter plates by placing

them on water bath maintained at 62°C, 72°C, 76°C and 80°C for 10-50 s, 60-80 s, 90 s and 100 s of incubation periods, respectively. The MW and MTA immobilized wells were thoroughly washed with washing buffer so as to remove the unbound antigen. Subsequent ELISA steps such as blocking, antibody and conjugate binding were carried out by conventional procedure as described in the previous section.

Time dependent blocking by microwave and MTA system

Goat anti-human IgG was immobilized in the wells of microtiter plates by optimized microwave irradiation at 700 W in 70 seconds. After thorough washing with washing buffer, blocking solution was added to the wells and exposed to microwaves at 700 W for 10, 20, 30, 40, 50, 60 and 70 s, respectively. MTA experiments were carried out by immobilizing anti-human IgG to activated wells for 70 s in water bath preheated to 72°C. Blocking was carried out on water bath maintained at 62°C and 72°C for 10-50 s and 60-80 s, respectively. Subsequent steps of antibody and conjugate binding were done conventionally at 37°C.

Antibody binding by microwave irradiation at high and low output power and MTA system

Goat anti-human IgG was immobilized into the wells of microtiter plates by microwaves at 700 W in 70 s followed by blocking at 700 W in 10 seconds. Human IgG was loaded into the wells and exposed to microwaves at output power of 700 W for 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 s, and at power output of 155 W for 25, 50, 75, 100, 125 and 150 s respectively, in two different sets of experiments. MTA experiments were carried out by immobilizing anti-human IgG in 70 s having water bath temperature (WBT) of 72°C. This was followed by blocking in 10 s (WBT 62°C). Human IgG was loaded into the wells and bound thermally on water bath preheated at 62°C for 10-50 s, 72°C for 60-80 s, 76°C for 90 s and 80°C for 100 seconds. In another set of experiment, human IgG binding was performed thermally by MTA system in 25-50 s (WBT 42°C), in 75-100 s (WBT 45°C), 125 s (WBT 48°C) and 150 s (WBT 50°C) respectively. In MW and MTA experiments rabbit IgG was used as negative control sera and binding of conjugate and subsequent colour development were carried out conventionally.

Conjugate binding by thermal incubation and microwave irradiation at high and low output power

Immobilization of goat anti-human IgG and blocking were carried out at 700 W in 70 s and 10 s respectively, followed by human-IgG binding at 155 W in 100 seconds. Binding of anti-human IgG-peroxidase conjugate was done by microwave irradiation at power output of 700 W in 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 s, respectively and at 155 W in 25, 50, 75, 100, 125 and 150 s respectively, in two different sets of experiments. MTA experiments were carried out by immobilizing anti-human IgG for 70 s (WBT 72°C), followed by blocking in 10 s (WBT 62°C) and human IgG binding in 100 s

(WBT 45°C). Goat anti-human IgG peroxidase binding was carried out thermally in 10-50 s (WBT 62°C), 60-80 s (WBT 72°C), 90 s (76°C) and 100 s (80°C), respectively. In another experiment (to mimic MW 155 W), conjugate binding by MTA was performed in 25-50 s (42°C), 75-100 s (45°C), 125 s (48°C) and 150 s (50°C) respectively. Colour development was performed conventionally.

Effect of thermal incubation on MW-assisted ELISA

The effect of thermal incubation on MW-assisted ELISA was studied by carrying out the one step of each ELISA experiment in water-bath (MTA), followed by remaining steps by microwave irradiation using the optimized conditions. The following four experiments were performed to study the effect of thermal incubation on MW ELISA: -

- Immobilization of capture antibody in 70 s (WBT 72°C), followed by MW-assisted blocking at 700 W in 10 s, antigen binding at 155 W in 100 s, and conjugate binding at 155 W in 100 seconds.
- Immobilization of capture antibody (700 W) in 70 s, blocking (MTA) in 10 s (WBT 62°C); antigen binding (155 W) in 100 s and conjugate binding (155 W) in 100 seconds.
- Immobilization (700 W) in 70 s, blocking (700 W) in 10 s, antigen binding (MTA) in 100 s (WBT 45°C) and conjugate binding (155 W) in 100 seconds.
- Immobilization (700 W) in 70 s, blocking (700 W) in 10 s, antigen binding (155 W) in 100 s, and conjugate binding (MTA) in 100 (WBT 45°C)

Control ELISA: Immobilization (700 W) in 70 s, blocking (700 W) in 10 s, Ag binding (155 W) in 100 s, and conjugate binding (155 W) in 100 seconds.

Detection of human IgG by MELISA, MTA-ELISA, ELISA at room temperature and conventional ELISA

Microwave-mediated ELISA (MELISA) was carried out by immobilizing anti-human IgG, blocking, human IgG and antibody-conjugate binding by microwave irradiation at 700 W in 70 s, at 700 W in 10 s, at 155 W for 100 s and at 155 W for 100 s, respectively. Corresponding MTA experiments were done in 70 s (WBT 72°C), 10 s (WBT 62°C), 100 s (WBT 45°C), and 100 s (WBT 45°C) respectively. Corresponding control experiments were done in 70 s, 10 s, 100 s and 100 s respectively at 37°C. Conventional ELISA was carried out by overnight incubation of anti-human IgG at 4°C, 1 h blocking, 3 h binding of human-IgG at 37°C and 3 h binding of anti-human IgG-HRP-conjugate at 37°C. Similar experiments were conducted with negative control sera.

Results

Temperature profile of PBS after microwave exposure for different time

We observed similar rise in temperature for PBS and the reaction mixture on microwave exposure as all the reagents used in ELISA are diluted in PBS. Therefore, for simplicity we have used temperature profile of PBS after microwave

exposure at 700 W for 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 s, and at 155 W for 25, 50, 75, 100, 125 and 150 s, respectively. In each experiment, temperature of the reaction mixture was measured before and after the exposure. Since we are dealing with very small volume of reaction mixture, temperature measurement via mercury thermometer often becomes inaccurate, so we used an external IR thermal camera for capturing several images of reaction mixtures by a single click and analyzed them using Guide analyzer software for temperature measurement. Temperature profile after each microwave exposure time was recorded in Table 1.

Designing MTA system mimicking thermal effect of microwave

Temperature rise of PBS (100 μ L) in the wells of microtiter plate was recorded for 10-100 s in water bath preheated to 62°C, 72°C, 76°C, and 80°C respectively. As seen from table 1, temperature rise of PBS by MW in 10-100 s does not matched the temperature rise by a water bath at a particular temperature. From this table we designed a MTA system that corresponds to temperature rise of PBS by MW in 10-100 s. Accordingly, temperature rise for 10- 50 s irradiation under microwave are similar to temperature rise by water bath preheated at 62°C. Similarly, microwave irradiation for 60-80 s, 90 s and 100 s are similar to temperature rise by water bath preheated at 72°C, 76°C and 80°C respectively. So, for mimicking thermal effect of microwave, MTA is used which is a water bath preheated at different temperature. Table 2 shows MTA for each time of microwave exposure.

Time dependent covalent immobilization of goat anti-human IgG by microwave irradiation and MTA system

When the photoactivated wells of a microtiter plate containing goat anti-human IgG solution were exposed to microwaves (10-100 s, 700 W), they rapidly immobilized the protein molecule following displacement of the fluoro group of the activated microtiter plate by the amino group of the protein.²⁸ The anti-human IgG immobilized- solid phases were tested for their ability to detect human IgG by a conventional ELISA procedure. Even in 10 s of irradiation, appreciable amount of binding of capture antibody was observed which increased with increase in time of irradiation. Maximum immobilization was observed at 70 s of microwave exposure. On the other hand, an insignificant immobilization was obtained even at 70 s of thermal heating by MTA system (Fig.1).

Time dependent blocking by microwave and MTA system

Antibody immobilized wells loaded with BSA solution were exposed to microwaves (700 W) for 10, 20, 30, 40, 50, 60 and 70 s, respectively. The extent of blocking in each case was verified by doing the subsequent step by conventional ELISA. It took only 10 s for blocking by microwave irradiation without nonspecific binding. However, further increase in microwave exposure time caused a sharp increase in the nonspecific

binding (Fig. 2). In contrast, the MTA experiment carried out by 10 s blocking showed insignificant ELISA values.

Human IgG binding by microwave irradiation at high and low output power and its comparison with MTA system

Initially, antibody-antigen binding was carried out at a microwave power output of 700 W in 10, 30, 50, 70 and 90 s, respectively. Nevertheless, the results were discouraging because the nonspecific binding started beyond 10 s (Fig. 3a). In view of this, we have performed this step at lowest output power of the microwave oven. Thus, binding of antigen to antibody was carried out at 155 W of microwave exposure for 25, 50, 75, 100, 125 and 150 s respectively, in six separate experiments. To our surprise binding was maximum in 100 s with negligible nonspecific binding of control sera. Beyond 100 s ELISA value for positive sera decreased and control sera increased (Fig. 3 b).

Conjugate binding by microwave irradiation at high and low output power and thermal incubation

Anti-human IgG-HRP conjugate binding under microwaves at 700 W was carried out in 10, 20, 30, 40, 50, 60 and 70 s, respectively. The results were very much akin to human IgG binding where nonspecific binding increased sharply beyond 10 s (Fig.4a). In contrast, low power (155 W) microwave irradiation for 100 s was found to give excellent result without any nonspecific binding (Fig.4b). In control sera there was no nonspecific binding at least up to 120 s, whereas in positive control sera there was sharp decline in ELISA value beyond 120 s, may be because of the inactivation of the secondary antibody conjugate.

Effect of thermal incubation on microwave mediated ELISA

Thermal incubation for single step of ELISA in each experiment was carried out by MTA and rests of the steps were done by microwave irradiation using same amount of reagents.

No significant ELISA values were obtained when antigen, antibody or conjugate binding were carried by MTA-ELISA. However, blocking step by MTA showed similar value akin to that of MELISA value except that negative control reading was little higher (Fig.6).

Detection of human IgG by MELISA, MTA-ELISA, ELISA at 37°C and conventional ELISA

Figure 6 shows that results of MELISA experiment carried out in 4 min 40 s are akin to that of conventional ELISA carried out in 18 hour. However, ELISA values are insignificant in case of ELISA carried out in 4 min 40 s either by MTA or at 37°C.

Discussion

Microwaves are the radiations that span the 300 MHz to 300 GHz energy spectrum of the electromagnetic radiations and exhibit the classical properties of reflection, absorption and transmission akin to that of light waves. Unlike the photochemical transformations or microwave spectroscopy, microwave-enhanced chemistry is not a quantum chemical phenomenon, rather depends upon the efficiency of interaction and the ability of target materials to absorb microwaves. The microwave-mediated reactions are presumed to result from thermal heating and electrostatic polar effects that took place when microwave interacts and get absorbed by target molecules. Therefore, controlled microwaves can be considered as an efficient tool in chemistry. With vast success in chemical transformations, the application of microwaves in biochemical and enzymatic reactions has resulted in a similar enhancing effect.

Majority of researchers believe that the microwave assisted acceleration of a chemical or biochemical transformation is a result of quick heating, whereas others claim the existence of non-thermal effect or microwave-specific effect. Researchers in the former category are mostly chemists who disprove the microwave-specific effect citing the flaws in the experimental designing, particularly the temperature measurement. In fact, it is challenging to find a system that can raise temperature similar to microwave. One of our aims is to mimic the microwave thermal effect and see if biological reaction can be done by it.

Recently, we have published a preliminary report on microwave-mediated enzyme-linked immunosorbent assay (MELISA) technique which occurs in less than 5 minutes. This is fastest ELISA so far reported.²⁵ As a model, we have preferred MELISA experiment to find out if microwave-specific effect indeed exists, because i) in this ELISA procedure, both covalent as well as non-covalent binding reaction occur, ii) reactions are in aqueous solution; hence conducive for microwave reaction and iii) reactions are carried out in seconds employing small volume (micro litre) of reagents.

For comparison of microwave assisted and thermally conducted ELISA results with utmost nicety, we designed a system that raise the temperature of a reaction mixture kept in the wells of a microtiter plate by same value in same time as by microwave irradiation. Since, all the reagents used in ELISA are diluted in PBS we observed similar rise in temperature of PBS and reagents upon microwave exposure and heating on water bath for different times. For simplicity, we have used temperature rise of PBS for making MTA system. In each experiment, temperature of the reaction mixture was measured before and after the reaction. Since we are dealing with very small volume of reaction mixture, temperature measurement by conventional thermometer seems to be not very much feasible. So we used an external IR thermal imaging camera for capturing the images of the reaction mixture and analyzed them using Guide analyzer software for temperature measurement. So, for mimicking thermal effect of microwave we design microwave thermal alternate (MTA), where reactions for 10-50 s MW irradiation were carried out on water bath preheated to 62°C. Similarly, the reactions for 60-80 s

(MW) were carried out on water bath preheated to 72°C, and reactions of 90, 100 s were performed in water bath preheated to 76°C and 80°C, respectively. Water bath temperatures were determined after a lot of trial and error experiments with different water bath temperature (Table 1).

Earlier we reported heat-mediated ELISA (HELISA) technique where goat anti-human IgG was immobilized at 50°C in 45 minutes; blocking, human IgG and antibody–enzyme conjugate binding were performed at 40°C in 40 min, 50 °C in 45 min and 50 °C in 40 min, respectively.²² Therefore, it is not surprising that thermal heating by MTA system show insignificant results when carried out for each step of ELISA in such a short period (in seconds). Needless to say, temperature of microwave ELISA in optimized conditions does not cross 50°C in any step of MELISA (Figs. 1- 4). In another experiment, one step at a time of MELISA experiment is replaced by MTA-ELISA. Here, also MTA experiments show insignificant results except blocking step as thermal blocking has no effect on positive result. Finally, when all the steps of ELISA are carried out by a particular method, only MELISA results are akin to conventional ELISA but MTA-ELISA and ELISA at 37°C showed insignificant result. All these experiments suggest that the rapid occurrence of ELISA in microwave is a result of MW-specific effect and not the MW-thermal effect.

In MW, the thermal effects are mainly caused by dipolar polarization and ionic conductions, whereas the electrostatic polar effects or the non-thermal microwave effects arises upon direct interaction of electric field with target molecules in reaction medium. The existence of non-thermal effect of MW has remained a debatable topic among researchers.¹¹ From our experiments, it becomes clear that apart from thermal effect, microwave also has non-thermal or specific effect. What is this specific effect that makes microwave reactions super fast? Like catalyst or enzyme microwave reactions are ultra fast even without its thermal effect; then can microwave be considered as a new type of catalyst? In other words, can microwave non-thermal or specific effect be termed as microwave catalytic effect? Based on our results, we are postulating a mechanism where microwave catalytic effect is accelerating the reaction (positive catalyst) as well as destroying the reaction by non-specific binding (negative catalyst). Accordingly, biopolymers having a large number of polar functional groups try to align themselves with applied microwave field. At this stage the protein is unfolded; excess energy at this stage may denature the protein or not aligned properly which was evidenced by non-specific antigen-antibody binding at higher microwave output power that is at 700 W. Low power in domestic microwave oven is achieved by on and off mechanism. In this MW oven, maximum power output is 700 W and in a cycle of 25 s, it is 25 s on at 700 W and in a cycle of 25 s, it is 7 s on and 18 s off at 155 W. We get best results at 155 W where in first 7 s the proteins (antigen and antibody) are forced to align themselves with applied microwave field. In next 18 s, in the absence of any external force they fold themselves naturally; and in presence of their partner they prefer to bind and get stabilized. Best results are obtained in four cycles of 25 s each that is in 100 s. First step of

ELISA is the immobilization of capture molecule which is carried out by covalent binding; this makes surface bound goat anti-human IgG more stable. In blocking step, after 10 s both positive as well as negative control start increasing which may be possible if BSA is leached away and subsequent molecule is non-specifically bound to polystyrene surface.

The right orientations of biomolecules that are prerequisite in transformation or formations of complex biopolymers are exceptionally fast by microwaves as seen from the non-covalent binding steps in MELISA. Here, entropy effect (alignment of molecules), enthalpy effect (increase in vibrational energy of functional group/s) and microwave thermal effect are acting together for microwave reactions. While microwave thermal effect are more important in organic reactions; alignment of molecules by microwave non-thermal effect are prerequisite in biochemical reaction. The non-thermal effect of microwaves can be described best in terms of increase in the pre-exponential factor A [$k = A(-\Delta G/RT)$] and decrease in the activation energy. The transition state stabilizing effect of microwave radiations can also be presumed to attribute in rapidity of microwave-catalyzed reactions through non-thermal specific microwave effect. Besides, positive results, microwave non-thermal effect, may impart damaging results if microwave energy crosses a certain limit. These results are more prominent in case of negative control sera where non-specific binding predominates. In other words at higher energy (either power or time) beyond optimal condition ELISA test lost its specificity.

Conclusions

Our work clearly demonstrates that non-thermal effect of microwave is reality and not an experimental flaw. Microwave non-thermal or specific effect may be termed as microwave catalytic effect.

Further, regardless of the mechanism of action of microwaves on biomolecules, we believe that the microwave-mediated high speed ELISA could be of enormous importance in diagnostics and related laboratory investigations. Since non-covalent binding of a biomolecule is a basic requirement in biology, microwave could be a potentially useful tool in many biological reactions. Also, microwave irradiation from mobile phones or mobile towers need to be studied afresh with respect to microwave non-thermal effect to see its impact, if any on human health.

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Tables

Table 1a. Rise in temperature of PBS by microwave irradiation at 700 W in 10-90 s and thermal incubation on water bath preheated at 62°C, 72°C, 76°C and 80°C respectively. 0 time indicates initial temperature..

Time (s)	T (°C) after MW irradiation	T (°C) after incubation on water bath preheated at			
		60 °C	72 °C	76 °C	80 °C
0	24 ± 1	23 ± 1	23 ± 1	23 ± 1	24 ± 1
10	26	27.6	32.2	35	36.2
20	29.9	29.4	36.6	40	39
30	33.8	33.7	39.8	44	46.5
40	37.2	36.5	43.2	46.8	51.1
50	42	42.1	46.5	49	53.9
60	46.4	45.2	47.3	53.3	57.6
70	49.1	46.8	49.4	56.2	59
80	54.6	47.3	54.3	58.4	61
90	60.9	48.3	55.9	61.3	63.9
100	66.6	49.2	56.2	63.2	66.1

Table 1b. Rise in temperature of PBS by microwave irradiation at 155 W in 25-150 seconds. **Time (s)**

	T (°C) of PBS
25	25.8
50	28.1
75	31.9
100	34.7
125	38.2
150	44.3

Table 2. Designing MTA system mimicking time dependent temperature rise by microwaves.

MW 700 W time (s)	WBT (°C)	MW irradiation time (155 W)	WBT (°C)
10-50	62	25-50	42
60-80	72	75-100	45
90	76	125	48
100	80	150	50

Figures

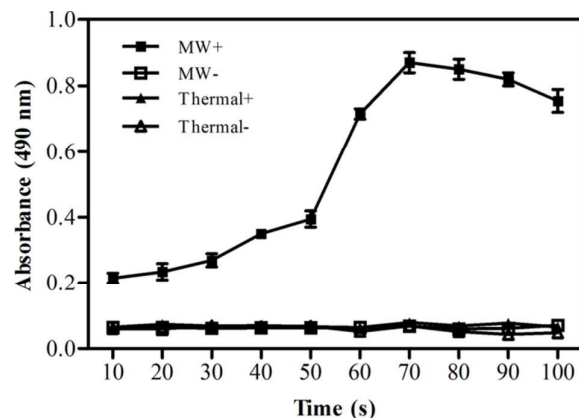


Figure 1. Immobilization of goat anti-human IgG to the activated wells of a microtiter plates in 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 s respectively by microwave irradiation at 700 W and thermally on water bath. Solid symbols (■, ▲) represent ELISA values of positive sera and hollow symbols (□, △) represent ELISA values of negative control.

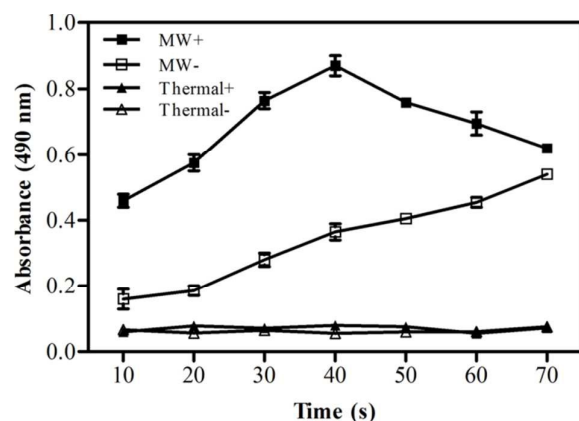


Figure 2. Optimization of blocking steps by microwave irradiation at 700 W and thermally on water bath. Solid symbols (■, ▲) represent ELISA values of positive sera and hollow symbols (□, △) represent ELISA values of negative control.

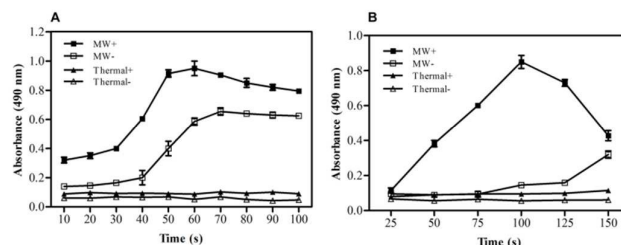


Figure 3. Optimization of antibody binding by thermal (▲, △) and microwave exposure (■, □) at high (A; 700 W) and low output power (B; 155W) in different times. Solid phase was prepared by immobilizing goat anti-human IgG in 70 s followed by 10 s blocking with BSA. Solid and hollow symbols represent ELISA values for positive and negative control respectively.

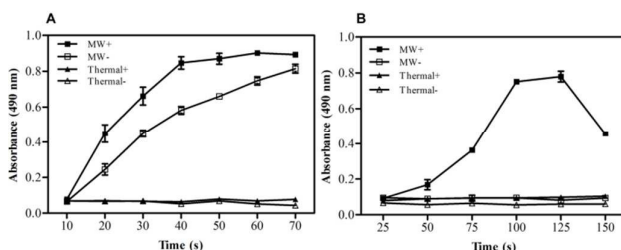


Figure 4. Optimization of conjugate binding by thermal (▲, △) and microwave exposure (■, □) at a) high (700 W) and b) low output power (155W) in different times. Solid phase was prepared by immobilizing goat anti-human IgG in 70 s followed by blocking with BSA by 10 s and antibody binding at 100s (155 W). Solid and hollow symbols represent ELISA values for positive and negative control respectively.

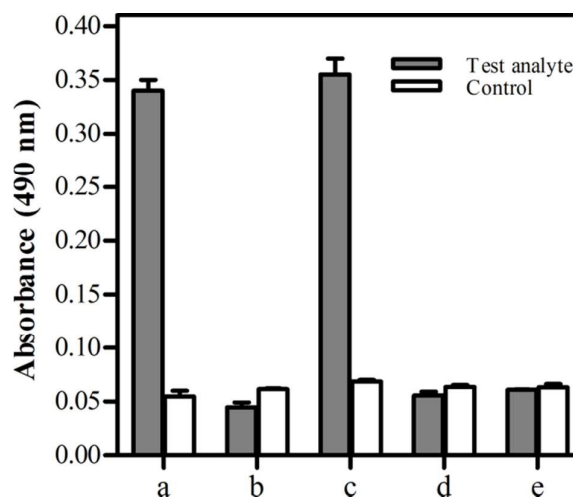


Figure 5. Effect of thermal incubation on microwave mediated ELISA. (a) all the steps were carried out in microwave; (b) Immobilisation of anti human IgG in MTA for 70 s at 72°C, rest of the steps were performed in microwave; (c) immobilisation of anti human IgG in microwave, followed by blocking in MTA for 10 s at 70°C, rest of steps in microwave; (d) first and second step in microwave, third step in MTA in 100 s at 45°C,

last step microwave; (e) first three steps in microwave, conjugate binding in MTA for 100 s at 45°C.

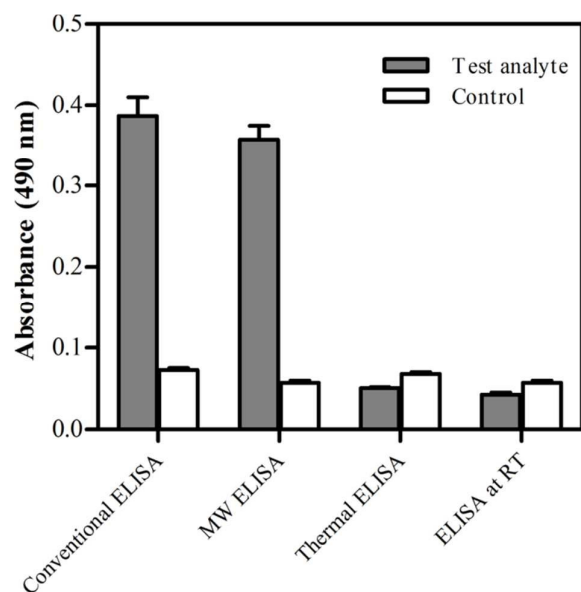
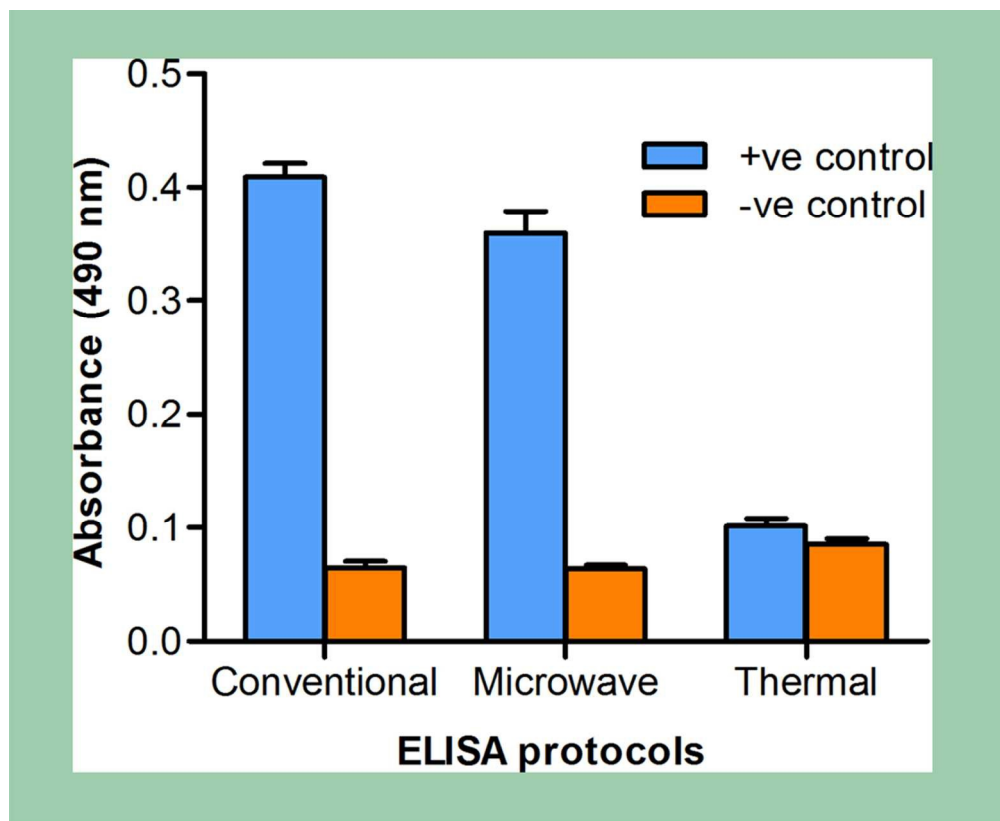


Figure 6. Comparison of ELISA experiments carried out by conventional, MW and water-bath (MTA) and RT procedures.



86x70mm (300 x 300 DPI)