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Probing of the microwave radiation effect on the green fluorescent protein luminescence in solution

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Abstract

Microwaves have a larger effect on the green fluorescent protein (GFP) fluorescence intensity than is observed by conventional thermal heating. Our measurements show that thermally heating a GFP solution from 7 to 40 °C results in a ∼1% decrease in fluorescence for every 1 °C. On the other hand, under 250 mW of localized microwave irradiation, the fluorescence can decrease by up to 3–10% with an accompanying temperature rise of only 1 °C.
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1. Introduction

The ever increasing number of cellular phones, digital mobile communication systems and other portable devices that operate at microwave frequencies, demand genuine scientific proof that microwaves are harmless at non-thermal levels. As microwave radiation is non-ionizing, the presence or absence of a non-thermal effect upon microwave irradiation is a central issue. One powerful approach of dealing with this is to study the effect of microwave on protein conformation as protein folding is one of the most basic processes necessary for living beings.

Bohr and Bohr have recently compared folding and unfolding of the β-lactoglobulin protein under both thermal heating and microwave irradiation [1]. They have argued for the existence of a non-thermal "microwave effect". de Pomerai et al. claimed different kinetics of green fluorescent protein (GFP) reporter induction in PC161 worms exposed to prolonged mild heat as compared to that of microwaves [2].

In this work, we studied the influence of microwave radiation on GFP fluorescence in solution. The GFP is a stable protein, which fluoresces only in the folded state. Partially folded protein fluoresces less than a fully folded one. Therefore, one can monitor folding of the protein by measuring changes in fluorescence. We choose a mutant form of the GFP called enhanced GFP or EGFP, which fluoresces 35-fold more intensely than wild type (wt) GFP when excited at 488 nm [3].

In contrast to Ref. [2], we performed our experiments on EGFP in a buffer solution and monitored changes in its photoluminescence *under* microwave irradiation. This is possible since we use a special near-field applicator operating at 8.5 GHz, which allows for localized irradiation where the size of the irradiated spot is not limited by the wavelength [4,5]. This applicator allows for easier access to the sample by the laser beam and does not require the use of a microwave cavity or waveguide.

2. Sample preparation

In our experiments we used an enhanced form of GFP (EGFP) (BD Clontech) which was cloned into pDest17 vector (Invitrogen). The EGFP gene was cloned downstream to a 6×His affinity tag, so that the expressed protein will contain six histidins at the N-terminal of the protein. The vector was expressed in E-Coli BL21 pLysS cells (Novagen), using standard induction condition procedures. In short: Cells were grown to OD600 = 0.6 and induced with 0.4 mM isopropyl-β-D-thiogalactopyranosid (IPTG). The cells were then grown at 37 °C and harvested after 16 h. The cells pellet was dissolved in lysis buffer (50 mM Tris–HCl, pH 8.0, 0.3 M NaCl, 10 mM

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$MgSO_4$, 10 mM imidazol, 10% glycerol, 1 mM PMSF) and lysed using M-110EHI microfluidizer processor (Microfluidics Int.) The protein was affinity purified on nickel-NTA beads (Qiagen) columns, using AKTA Explorer FPLC (Amersham Pharmacia). The protein was eluted in 50 mM Tris–HCl, pH 8.0, 0.3 M NaCl containing 250 mM imidazol, and dialysed in 20 mM Tris–HCl, pH 8.0, 50 mM NaCl at 4 °C. The final concentration of the protein was 0.84 mg/ml.

3. Experimental set-up

The fluorescence of the EGFP was excited by an Argon laser at a wavelength $\lambda = 488$ nm (Fig. 1). The EGFP solution was contained in a transparent thin wall glass tube. The glass tube is sealed from one side and open from another to allow introduction of a small thermocouple to measure the temperature during microwave irradiation. We carefully checked that the thermocouple does not affect the results of the microwave irradiation. The photoluminescence is collected at 45° by a condenser lens and an optical fiber. The output of the fiber goes to a monochromator, which is coupled to a photomultiplier tube and a photon counter. The fluorescent signal is corrected for the fluctuations in the intensity of the excitation illumination.

Our microwave applicator is a resonant slot antenna microfabricated on the convex surface of the quarter-wavelength dielectric resonator. The slot length is 8 mm while the slot width can vary from 1 to 500 μm. The irradiated spot at near-field is a replica of the slot. Typically, the effective part of the applicator is its apex, which means that the active part of the slot length is only 0.5 mm long. A 100 μm wide slot, using only 250 mW of microwave power will have an average power density of at least 5×10^5 W/m^2 (the corresponding electric field is $\sim 10^4$ V/m). Therefore, a highly focused microwave beam is obtained. Since this is a near-field applicator, the power density strongly decays with distance even in the absence of the sample, such that at the distance of ∼1 mm from the applicator the field magnitude is very small. As the penetration depth at a frequency of 8.5 GHz, in water is ∼1.5 mm, our applicator affects only a small part of the water-based sample. In part of our experiments we used another applicator, a coaxial tip protruding from the central wire of a coaxial cable (a short monopole antenna).

The fluorescence is excited by a focused laser beam. Our microwave applicator is brought very close to the glass tube with solution to irradiate the laser-illuminated region. The thin glass tube is mostly transparent for the microwave; hence, most of the microwave energy goes directly into laser-illuminated spot. Therefore, although the incident microwave power is rather small, it is absorbed in a small volume. However, this huge power density does not lead to an appreciable temperature change as this small volume is very well cooled by the nearby non-irradiated solution.

4. Results

Fig. 2 shows the photoluminescence spectrum of EGFP. The figure also shows the spectrum under an applied microwave power of 250 mW. In the latter case, the microwave beam was turned on 2 min before the measurement. It is seen that under microwave irradiation the fluorescence intensity is reduced and the spectrum shifts slightly to the red. Similar effects on the fluorescence spectrum (not shown here) were observed by thermal heating of the sample.

We also studied the kinetics of the microwave effect on EGFP fluorescence. At first we turned the CW microwave power on and observed a decrease in fluorescence, which reached a plateau after few minutes of irradiation. We then turned the microwave off and observed the fluorescence to reach its pre-exposed level. This sequence was done at different microwave power levels as shown in Fig. 3. The fall and rise time (taken at half height of the fluorescence decrease) is $t_{1/2}$ ∼22 s. This is very similar to the characteristic time of folding/unfolding of GFP and its chromophore formation. (A 24 s time constant for folding/unfolding was also reported by others where acid denaturated GFP was diluted into buffer at neutral pH [6].)

The decrease of fluorescence as a function of microwave power is linear (Fig. 4).

The effect of microwave irradiation on EGFP fluorescence should be compared to the effect of temperature. To measure the temperature dependence of EGFP fluorescence, we placed the glass tube with the sample in contact with a water bath and varied its temperature so that the heat can spread throughout the

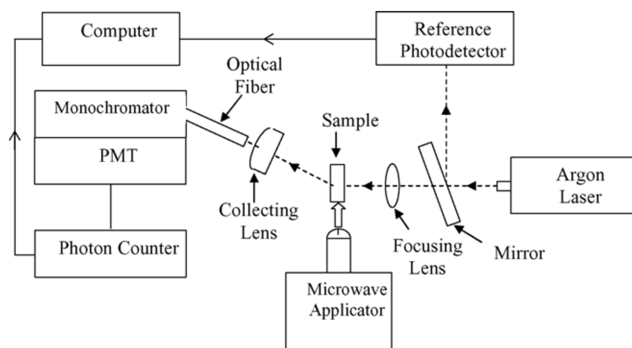

Fig. 1. Experimental set-up. Microwave applicator irradiates only the laser-illuminated volume of the fluorescent solution. The photoluminescence under microwave irradiation is analyzed.

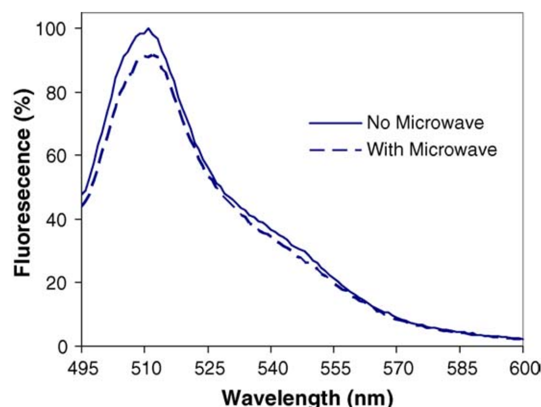

Fig. 2. EGFP fluorescence spectrum under 488 nm excitation at room temperature.

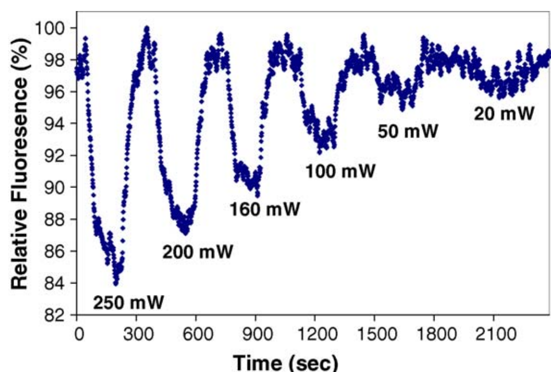

Fig. 3. Fluorescence decreases upon switching microwave irradiation on/off at different applied powers. Note that the fall and rise time $t_{1/2}$ ∼22 s is almost identical at all powers.

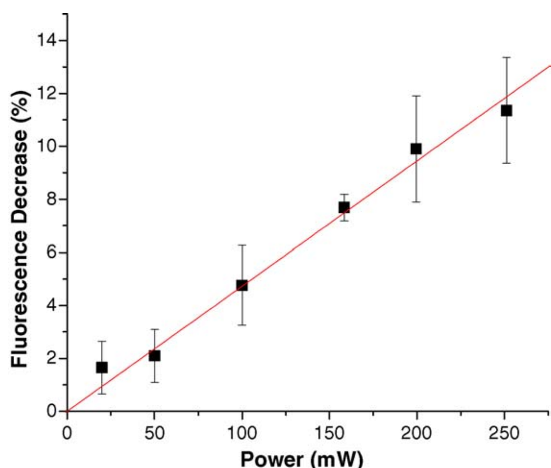

Fig. 4. The decrease in fluorescence in Fig. 3 is linearly dependent on microwave power.

sample. A thermocouple was placed at the laser-irradiated spot in the solution. Fig. 5 shows that the fluorescence decreases with increasing temperature almost linearly whereby heating/cooling by 1 °C results in a ∼1% decrease/increase in fluorescence.

Our next step is to study the effect of microwave irradiation at different power levels on the EGFP fluorescence. We varied the microwave power of the applicator and observed the changes

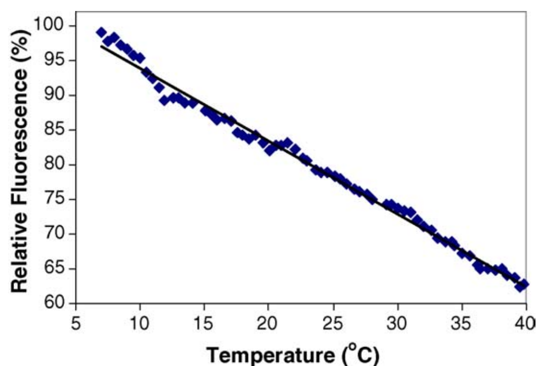

Fig. 5. The temperature effect on fluorescence. Solid line shows a linear approximation. Approximately 1 °C temperature change corresponds to 1% change in fluorescence.

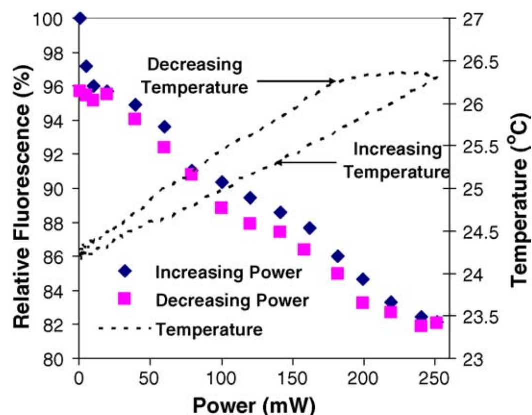

Fig. 6. EGFP fluorescence as a function of CW microwave power. A 250 mW of microwave power corresponds to at least ∼14% decrease in fluorescence. Temperature of the EGFP solution in the vicinity of the applicator as a function of microwave power is also shown. Only 2.2 °C temperature change in total is observed.

in CW fluorescence (Fig. 6). The power was varied in steps of 0.1 dBm every 2 s. Under 250 mW of CW microwave irradiation at 8.5 GHz, the fluorescence is decreased by at least 14%. Placing a miniature thermocouple inside the EGFP solution in the close vicinity of the microwave-irradiated spot and measuring the temperature change as a function of microwave power, we observed a maximum of only 2.2 °C change in temperature (Fig. 6). This would result in 2.2% fluorescence decrease in a pure thermal heating. The result presented here is a clear indication that microwaves can influence fluorescence in a manner different than that of direct heating of the solution.

5. Discussion

Several research papers have attempted to explain the effect of electromagnetic radiation on protein conformation. One possible explanation is that the microwave radiation can be at resonance with small groups of amino acids within the proteins [7] and is capable of exciting the entire molecule into a vibratory mode. Bohr et al. have proposed that the effect of microwave irradiation on protein folding could be the collective excitation of intrinsic modes in the protein, which has a resonance frequency on the order of a few GHz [8]. Others have argued that the existence of such resonances is unlikely to cause a protein conformational change as the protein is bound by its surrounding environment [9]. Instead they proposed that pulsed microwave radiation, even when athermal, can alter protein conformation through a transient heating of the protein in its close environment.

We have conducted a broadband measurement using a coaxial applicator (0.4–20 GHz at 0.01 GHz intervals and 20 dBm of power). We also observed the effect of MW irradiation on fluorescence (although smaller than with our near-field slot applicator). However, this effect did not demonstrate strong resonant features. Therefore, we do not believe that in our experiments there are any excited molecular resonances.

It was shown by Cameron et al. [10] that water molecule pentagons are formed in solution and can enclose a purely hydrophobic molecule, such as a protein. An increase in water

temperature can break the hydrogen bonds of the pentagonal structure and change this water layer to its bulk state. The hydrophobic groups on the surface of the enclosed protein will now be able to interact more freely with each other, and therefore change their conformational state. This hypothesis will require further testing but is very much plausible. A similar idea but formulated differently is that of bound water which corresponds to a layer around the protein molecule. The microwave absorption in this layer is different from that in bulk water. While pure water has peak absorption around 19.2 GHz at room temperature, bound water absorbs at lower frequencies [11]. As our operational frequency is 8–9 GHz, we are closer to the absorption of bound water than to the absorption of bulk water. Therefore, we may well be locally heating the protein molecule bound water layer more intensely while at the same time measuring a small temperature change from the bulk water heating confirming that a heating mechanism is involved in the process albeit not the only mechanism. Very recently, bound water was used as a reporter for conformational changes of biological activities in the low GHz region [12].

Porcelli et al. studied the non-thermal effect upon exposure of 10.4 GHz microwave radiation on two thermophilic and thermostable enzymes and showed an irreversible inactivation of the two enzymes [13]. Irreversible conformational changes were observed after exposure in one enzyme using circular dichroism (CD) and fluorescence techniques pointing to a microwave induced protein structural arrangements. In our particular case, the exposed EGFP returns to its pre-microwave exposed state as observed by fluorescence (Figs. 3 and 6), i.e. our results are reversible.

6. Conclusion

We have demonstrated a genuine microwave effect on EGFP, which most probably arises from the reversible conformational changes of EGFP. This effect is observed by measuring the decrease in fluorescence under microwave irradiation. Thermal effects on fluorescence amounts to 1% decrease per 1 °C temperature rise. However, when a microwave beam is applied, the fluorescence decreases by 3–10% while the bulk temperature increases only by a 1 °C. Therefore, the microwave has a larger effect on conformational changes of the EGFP rather than conventional heating. The understanding of the mechanisms of the interaction of microwaves with macromolecules is still in its early stages. However, the behaviour of these macromolecules under various stimuli is fundamental to biological processes and further studies at the theoretical and experimental levels are required.

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