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FAST TRACK COMMUNICATION

Inactivation of viruses with a very low power visible femtosecond laser

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Abstract

We demonstrate for the first time that, by using a visible femtosecond laser, it is effective to inactivate viruses such as bacteriophage M13 through impulsive stimulated Raman scattering. By using a very low power visible femtosecond laser having a wavelength of 425 nm and a pulse width of 100 fs, we show that M13 phages were inactivated when the laser power density was greater than or equal to 50 MW cm^{-2}. The inactivation of M13 phages was determined by plaque counts and depended on the pulse width as well as power density of the excitation laser.

1. Introduction

Not only are traditional biochemical and pharmaceutical methods for inactivating viruses and other lethal microorganisms partially successful, but also they evoke problems of drug resistance and clinical side effects. Ultraviolet (UV) irradiation can disinfect microorganisms but without selectivity, and it usually leads to the side effect of mutation. It is therefore a considerable challenge to develop new methods which effectively inactivate the unwanted microorganisms such as viruses and bacteria while leaving the sensitive materials like cells unharmed.

One experimental technique toward this goal is microwave absorption. Resonant microwave absorption has been proposed in the literature to excite the vibrational states of

microorganisms in an attempt to destroy them [1, 2]. However, water, which usually coexists with the microorganisms, is a notorious absorber in the microwave spectral ranges. Thus, it is extremely difficult to transfer microwave excitation energy to the vibrational energy of microorganisms. To overcome this difficulty, it is essential to transfer the excitation sources from the microwave range to, say, the visible range in which water is transparent.

Impulsive stimulated Raman scattering (ISRS) has been shown to be a viable way of producing large-amplitude vibrational modes in molecules in liquid solution as well as in solid-state systems [3]. In this paper, we report for the first time the use of a visible femtosecond laser system to excite a coherent acoustic Raman-active vibrational mode (which is associated with vibrations of viral capsids) in M13 phages through ISRS to such a high-energy state as to lead to their inactivation. Our work demonstrates a new method of manipulating, controlling, and inactivating unwanted microorganisms. It suggests that the basic principles of impulsive coherent excitation using a laser optical system can represent a general way to selectively alter the function of or even inactivate viruses and potentially other microorganisms through the property of their mechanical acoustic excitations. In addition, since structural change due to the mutation of microorganisms leads to very minimal variation of the vibrational frequency of their capsids, damage caused to viruses and/or other microorganisms through vibration of their mechanical structures likely would not be immune to simple mutation of cell surface receptors, and the same treatment procedure remains valid; our approach would thus not evoke problems of drug resistance and as a result would be applicable to drug-resistant strains of microorganisms.

2. Sample and experimental set-up

The M13 bacteriophage helper phage samples used in this work were purchased from Stratagene.

The excitation source employed in this work is a diode-pumped continuous-wave (cw) mode-locked Ti–sapphire laser. The laser produces a continuous train of 80 fs pulses at a repetition rate of 80 MHz [4–7]. As shown in figure 1, the output of the second harmonic generation system (SHG) of the Ti–sapphire laser is used to irradiate the sample. The excitation laser is chosen to operate at a wavelength of $\lambda = 425$ nm and with an average power of about 40 mW, unless otherwise specified. The excitation laser provides a nearly transform-limited pulse train having a pulse width with full width at half maximum (FWHM) $\cong$ 100 fs and spectral width with FWHM $\cong$ 60 cm^{-1}. A lens of extra-long focus length ($f = 36$ cm) is used to focus the laser beam into the sample area. The tightest laser-focused volume, which is the most efficient volume for the interaction of the laser with M13 bacteriophage through ISRS, is a cylinder approximately 100 μm in diameter and 500 μm in height. In order to facilitate the interaction of the laser with M13 bacteriophages which are inside a Pyrex cuvette and diluted in 2 ml water, a magnetic stirrer is set up so that the M13 bacteriophages will enter the laser-focused volume as described above and interact with the photons. All the laser-irradiated M13 bacteriophage samples contained 1×10^7 pfu ml^{-1}. The assays were performed on the laser-irradiated samples after proper dilution. The typical exposure time of the sample to laser irradiation was about 10 h. We believe that the amount of time (10 h) required reflects the particular arrangement of our experimental set-up and is not related to the efficiency of the inactivation of M13 bacteriophages by the laser system. A thermocouple is used to monitor the temperature of the sample to ensure that our results are not due to the heating effects. In fact, we have found that the increase of the temperature of the M13 bacteriophage samples is less than 3 °C after 10 h laser irradiation. All the experimental results reported here are obtained at $T = 25$ °C and with single laser beam excitation.

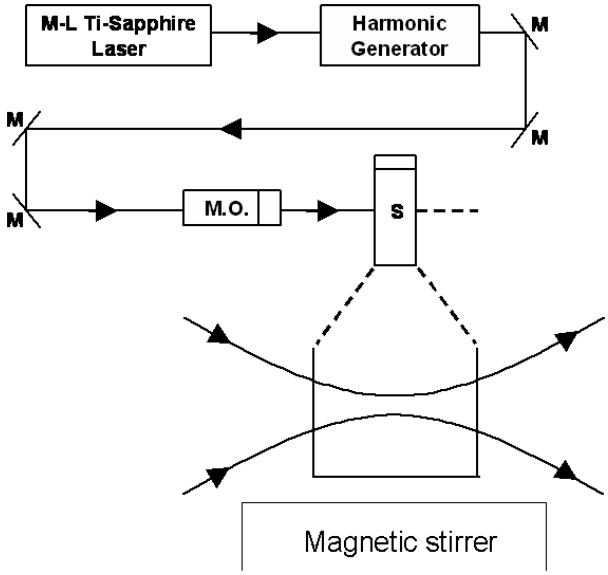

Figure 1. Experimental set-up for the inactivation of M13 bacteriophages. M: mirror; M.O.: focusing lens; S: sample. The magnification shows the sample area where the laser beam is focused. The cylindrical volume where the laser beam focuses most tightly defines the active volume for the inactivation of M13 bacteriophages through the ISRS process.

The activity of M13 bacteriophages was determined by plaque counts. For determining the infectivity of the helper phages from different batches, we diluted the phage to 10^3 pfu in 50 μl of phosphate-buffered saline (PBS) and added the diluted phage to 1 ml of TG-1 *E. coli* growing at an OD 600 of 0.4. The *E. coli* solution was then added into 3 ml of agarose top (10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 1 g $MgCl_2{\cdot}6H_2O$, 7 g agarose in 1 l of water). After brief vortex, the mixture was poured evenly onto TYE plates (15 g Bacto-Agar, 8 g NaCl, 10 g Tryptone, 5 g yeast extract in 1 l of water) and cooled down at room temperature until solidification. The plates were incubated at 37 °C overnight and plaques were counted the next day. Plaque formation assay was performed in triplicate for each batch of phage.

All data are expressed as mean $\pm$ standard deviation (SD). Student's t-test was used for comparison of groups with 5% as a significant level.

3. Experimental results, analysis and discussions

Figure 2(a) shows the number of plaques for a sample with nominally prepared 1×10^3 pfu of M13 bacteriophages without laser irradiation. Here, 'nominally prepared' means that we prepare/dilute the M13 bacteriophage samples based on the pfu concentration specified by the manufacturer upon purchasing. The number of plaques is determined to be 1184 ± 52 counts. Figure 2(b) shows the corresponding runs after laser irradiation. It shows that the number of plaques after laser irradiation is 7 ± 3 counts ($P < 0.0001$ versus sham-treatment; $t = 39.14$, $df = 4$). Similar results for another M13 bacteriophage sample with nominally prepared 5×10^2 pfu are shown in figures 3(a) and (b). The number of plaques in this case is determined to be 521 ± 64 counts for the sample without laser irradiation, and 3 ± 1 counts after laser irradiation ($P < 0.0001$ versus sham-treatment; $t = 14.02$, $df = 4$). The intriguing feature is that there is very minimal amount of plaques for the laser-irradiated samples as

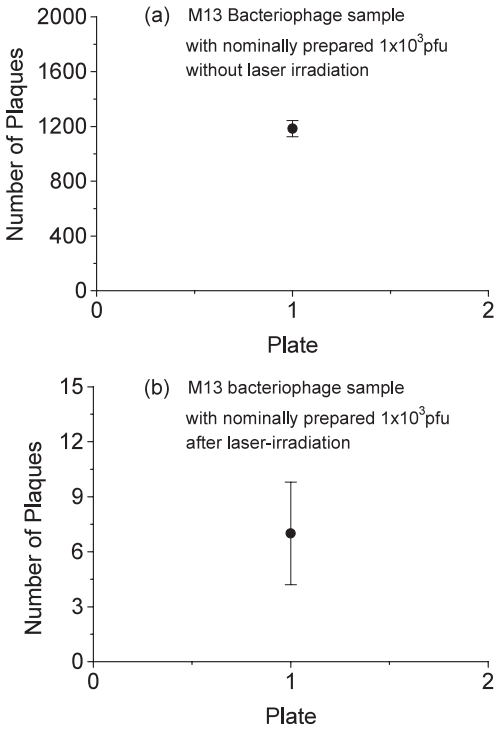

Figure 2. The number of plaque counts for a sample with nominally prepared 1×10^3 pfu of M13 bacteriophages: (a) without laser irradiation; (b) after laser irradiation for about 10 h. The extremely small number of plaques observed in the irradiated sample is a manifestation of almost complete inactivation of the M13 bacteriophages in the sample. The data point is represented by its mean to three replicas and the standard deviation.

compared with the reference samples, indicative of the inactivation of M13 bacteriophages by the laser irradiation. We attribute the observed inactivation of M13 bacteriophages to laser-driven coherent excitations through the ISRS process.

ISRS has been successfully demonstrated to produce large-amplitude coherent vibrations in the molecules in liquids as well as in solid-state systems [8–12]. Yan *et al* [3] predicted that ISRS should occur with no laser intensity threshold even when only one ultrashort laser pulse is passed through many types of media. In this case, ISRS is a forward-scattering process which is stimulated because the Stokes frequency is contained within the spectral width of the excitation pulse. Furthermore, they demonstrated that ISRS was a process through which excitation of a coherent lattice or molecular vibrations would take place whenever a sufficiently short laser pulse passed through a Raman-active solid or molecular liquid or gas. For a single-beam excitation, if the damping is ignored, then the amplitude (R_0) of the displacement away from the equilibrium intermolecular distance caused by ISRS can be shown to be given by [3]

$$R_0 = 4\pi I\,(\delta\alpha/\delta R)_0\,\mathrm{e}^{-\omega_0^2\tau_\mathrm{L}^2/4}/m\omega_0 nc; \tag{1}$$

where I is the intensity of the excitation laser; α is the polarizability of the medium; R is the displacement away from the equilibrium intermolecular distance; $\delta\alpha/\delta R$ is proportional to the Raman scattering cross section; ω_0 is the angular frequency of the coherent vibrational excitation; τ_L is the FWHM of the pulse width of the excitation laser; m is the molecular mass; n is the index of refraction; and c is the speed of light.

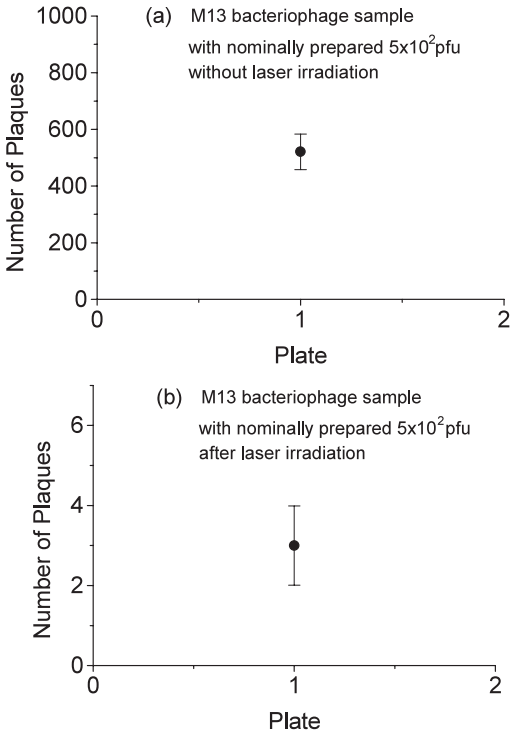

Figure 3. The number of plaque counts for a sample with nominally prepared 5×10^2 pfu of M13 bacteriophages (a) without laser irradiation; (b) after laser irradiation for about 10 h. The extremely small number of plaques observed in the irradiated sample is indicative of almost complete inactivation of the M13 bacteriophages in the sample. The data point is represented by its mean to three replicas and the standard deviation.

From equation (1) it is clear that larger Raman cross sections and higher laser power densities, as well as lower vibrational frequencies, would contribute to larger excited vibrational amplitude. In fact, for a moderate Raman scattering cross section, a sufficiently low vibrational frequency and a reasonable excitation power density, an amplitude of the vibrational displacement in the range 0.01 to 1 Å could be achieved through ISRS.

An intriguing aspect, implied from equation (1), that is worth mentioning is that for the one-beam excitation experiment employed in this work, the primary beam as well as the Stokes beam, whose photon energies are denoted by $\hbar\omega_{\mathrm{L}}$ and $\hbar\omega_{\mathrm{s}}$, respectively, define the excited coherent vibrations with energy $\hbar\omega_0$ such that $\hbar\omega_0 = \hbar\omega_{\mathrm{L}} - \hbar\omega_{\mathrm{s}}$. As a result, the FWHM of the spectral width of the excitation laser has to be larger than the energy of the excited coherent vibrations, which, because of the Gaussian distribution of the excitation laser in both time and space and by using uncertainty principle, gives rise to the factor $\mathrm{e}^{-\omega_0^2\tau_{\mathrm{L}}^2/4}$ in equation (1). This exponential dependence indicates that the product of angular frequency of the excited coherent vibration (ω_0) and the FWHM of the excitation pulse width (τ_{L}) has to be small in order that the amplitude R_0 of the excited coherent vibration can be significant, i.e., $\omega_0\tau_{\mathrm{L}} \leqslant 1$. This explains why the excitation laser should be ultrashort in pulse width for ISRS experiments. It also explains why the longer excitation laser pulse, even with the same laser intensity of $\widetilde{=}6.4 \times 10^{-6}$ j/cm^2, produces less inactivation, as shown in our experimental results, given in table 1.

In our previous cw Raman scattering experiments [13, 14], we have reported the observation of the low-frequency ($\widetilde{=}8.5$ cm^{-1}) Raman-active vibrational mode of M13

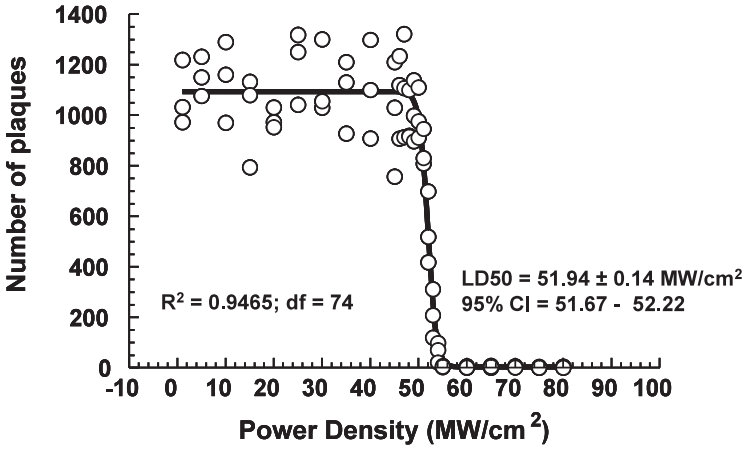

Figure 4. The number of plaques as a function of the excitation laser power density for an M13 bacteriophage sample with nominally prepared 1.1×10^3 pfu. The sharp cut-off for the number of plaques at around 50 MW cm^{-2} is indicative of the onset of the inactivation of the M13 bacteriophages.

Table 1. Status of M13 bacteriophages on the pulse width of excitation laser. The number within the brackets indicates the laser spectral width in cm^{-1}.

	Pulse width (fs)							
Status	100 (60)	250 (25)	500 (12)	600 (10)	700 (8.5)	800 (6.5)	1000 (5)	1500 (3)
Inactivation (Yes or No)	Yes	Yes	Yes	Yes	Yes	No	No	No

bacteriophages. There are several low-frequency Raman-active modes associated with the vibrations of the M13 capsids predicted from the theoretical calculations based on an elastic continuum model and appropriate Raman selection rules derived from a bond polarizability model [13, 14]. The observed Raman-active mode at 8.5 cm^{-1} has been shown to belong to an axial torsion mode of the M13 capsids. The mode has a lifetime of about 1.1 ps as determined from Raman measurement. This particular Raman-active mode was observed in our Raman scattering experiments because it, unlike other Raman-active modes for M13 capsids, is less damped by the surroundings due to its nature of axial vibrations.

Therefore, we believe the most likely scenario is that, under our current ultrashort pulse laser excitation experiments with M13 bacteriophages, the amplitude of this Raman-active mode at 8.5 cm^{-1} has been coherently excited by ISRS to an extent that leads to their inactivation. To further support this explanation, we have carried out similar experiments with M13 bacteriophages by varying the power density as well as pulse width of the excitation laser.

Figure 4 shows the number of plaques as a function of the laser power density for M13 bacteriophage samples with 1.1×10^3 pfu after being irradiated with an excitation laser having 100 fs pulse width and $\lambda = 425$ nm. It is very interesting to observe an abrupt inactivation of the M13 bacteriophages at an excitation laser power density of about 50 MW cm^{-2}. This observation is indicative of the fact that the M13 bacteriophages become inactivated as the amplitude of the vibrations exceeds a certain threshold. The reason why the M13

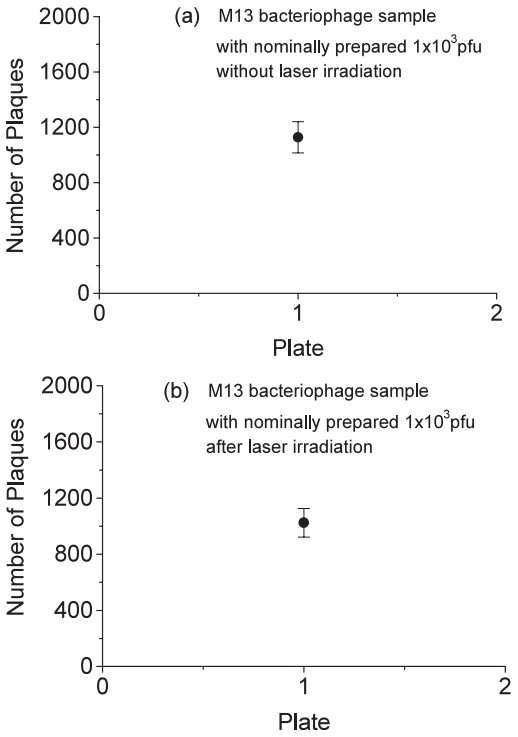

Figure 5. The number of plaque counts for a sample with nominally prepared 1×10^3 pfu of M13 bacteriophages: (a) without laser irradiation; (b) after laser irradiation for about 10 h. The excitation laser was derived from a DCM dye laser synchronously pumped by the second harmonic of a mode-locked cw YAG laser. The data point is represented by its mean to three replicas and the standard deviation.

bacteriophages were inactivated at certain threshold amplitude is not clear at this moment. More work related to this area of research is needed.

We have also found that within the statistical error of the experiments there is almost no observable inactivation of the M13 bacteriophages if the pulse width of the excitation laser is longer than about 800 fs while the intensity of the excitation laser remains constant at $\widetilde{=}64 \times 10^{-6}$ j/cm^2 (this intensity corresponds to an ultrafast laser with 100 fs pulse width and 40 mW of average power). The experimental results are summarized in table 1. According to equation (1), if the laser intensity remains constant, the amplitude of vibrational displacement excited by an ultrashort laser decreases with the increasing laser pulse width. In fact, equation (1) predicts that the amplitude of vibrational displacement excited by an ultrashort laser having a pulse width of 700 fs with a laser intensity of 6.4×10^{-6} j/cm^2 approximately equals that by an excitation laser having 100 fs pulse width but with laser intensity 5.0×10^{-6} j/cm^2. Therefore, the experimental results in table 1 are consistent with the predictions by equation (1) and with the power-density results of figure 4. We also notice that because the laser energy per pulse in the case of 800 fs or longer pulse width is actually the same as that in the case of 700 fs or shorter pulse, the experimental results in table 1 rule out the possibility that our observed inactivation of M13 bacteriophages is due to transient, micro-thermal effects that might develop at the tightest laser focused volume.

Figure 5(a) shows the number of plaques for a sample with nominally prepared 1×10^3 pfu of M13 bacteriophages without laser irradiation. The number of plaques is determined to be

1128 ± 120 counts. Figure 5(b) shows the corresponding runs after irradiation by a DCM dye laser having an average power of 100 mW, a pulse width of 5 ps, a wavelength of 650 nm, a focused spot of 5 μm and a repetition rate of 76 MHz for 10 h. The excitation laser was derived from a DCM dye laser synchronously pumped by the second harmonic of a mode-locked cw yttrium aluminium garnet (YAG) laser. It shows that the number of plaques after laser irradiation is 1024 ± 100 counts. These experimental results indicate that within our experimental uncertainty the M13 bacteriophages remain active; in other words, the laser does not inactivate M13 bacteriophages under these experimental conditions. This result is again consistent with that predicted from ISRS. Although the excitation laser intensity is as high as 6.4×10^{-3} j/cm^2, the pulse width is relatively long. The exponential term in equation (1) predicts a factor of about 10^7 decrease in the amplitude of excited coherent phonons; as a result no inactivation occurs.

We notice that viruses have been known to be inactivated by ultraviolet photons with wavelengths around 250 nm. There is a possibility that our excitation laser might be generating 212.5 nm through the nonlinear property of the sample system; as a result the produced ultraviolet photons inactivate the M13 bacteriophages. However, this possibility can be ruled out. Because we have found that there is no inactivation of M13 bacteriophage when the laser power density is set at 64×10^{-6} j/cm^2 while the laser pulse width is chosen at $\widetilde{=}5$ ps, if the excitation laser with a pulse width of 100 fs and power density of 64 MW cm^{-2} inactivated the M13 bacteriophages by the generated ultraviolet photons, then a laser with longer pulse width but the same power density would have produced more ultraviolet photons and would have been able to inactivate the M13 bacteriophages as well. Therefore, it is unlikely that the nonlinearly generated ultraviolet photons (if there are any) play a significant role in the inactivation of M13 bacteriophages observed in our current experiments.

Finally, we note that, partly because our experimental results on power density and pulse width dependences are consistently explained by IERS based upon the assumption that a single laser pulse inactivates the M13 bacteriophage and partly because the fact the pulse separation produced in the laser system is as long as 13 ns, we believe that one single pulse in our visible femtosecond laser is capable of inactivating the M13 bacteriophage.

4. Conclusion

We have shown that microorganisms such as bacteriophage M13 can be inactivated by a very lower power visible femtosecond laser system through ISRS. Our work demonstrates a new strategy for manipulating, controlling, and inactivating unwanted microorganisms. It suggests that the basic principles of impulsive coherent excitation using a laser optical system can be a general way to selectively alter the function of or even inactivate viruses and potentially other microorganisms through the coherent excitation of sufficiently large amplitude of the Raman-active vibrational mode of their capsids via the ISRS process. Furthermore, since damage caused to viral organisms through vibration of their mechanical structures likely would not trigger an immune response to simple mutation of cell surface receptors and the same treatment procedure remains valid, our approach would thus not evoke the problems of drug resistance. As a result, it would be applicable to drug-resistant strains of microorganisms as well.

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