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Photocatalytic inactivation of spores of *Bacillus anthracis* using titania nanomaterials

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ABSTRACT

Studies on photocatalytic inactivation of spores of *Bacillus anthracis* have been carried out using nanosized titania materials and UVA light or sun light. Results demonstrated pseudo first order behaviour of spore inactivation kinetics. The value of kinetic rate constant increased from 0.4 h^{-1} to 1.4 h^{-1} indicating photocatalysis facilitated by addition of nanosized titania. Nanosized titania exhibited superior inactivation kinetics on par with large sized titania. The value of kinetic rate constant increased from 0.02 h^{-1} to 0.26 h^{-1} on reduction of size from 1000 nm to 16 nm depicting the enhanced rate of inactivation of *Bacillus anthracis* Sterne spores on the decrease of particle size.

1. Introduction

Bacillus anthracis is one of the most pathogenic bacteria and etiological agent of deadly disease anthrax. In humans this disease occurs in three forms namely cutaneous, gastrointestinal and pulmonary. It causes illness differently in each type of the above with serious complications and symptoms, however, only in pulmonary anthrax 90% mortality rate was observed in the untreated cases. LD_{50} of anthrax was found to be as low as 4000 spores [1–3].

B. anthracis exists in two different forms, vegetative cells and spores. Spores of *Bacillus* species are more resistant to harsh environmental conditions than vegetative cells [1–3]. They were also found to be more resistant to a wide variety of chemical and physical treatments as per the reported results [1–3]. The spore's structure and chemical composition play major roles in its resistance to these extreme treatments. It's structure includes the exosporium, coats, outer membrane, cortex, germ wall, inner membrane, and central cell. Chemically, these layers are constructed with unique proteins, glycoproteins and other macromolecules that give protection to its DNA and other important enzymes encapsulated inside the spore. In relation to deadly anthrax disease, spores have been regarded as messengers of infection and hence the paramount importance has been given to their inactivation and destruction [4–9].

After World Trade Centre attack in United States, *B. anthracis* has attracted renewed attention. Consequently, researchers have started to explore the ways and means of decontamination of its deadly spores [10–18].

Chlorination has been a general method for decontamination of deadly bacteria either in the form of vegetative cells or in the form of spores since few decades. However, *B. anthracis* Sterne, *Bacillus cereus* and *Bacillus thuringiensis* exhibited noticeable resistance towards chlorination disinfection treatment [10]. Chemical disinfectants like calcium hypochlorite [11], free available chlorine [11], sodium hypochlorite [11], hydrogen peroxide [11,12], peracetic acid [12], formaldehyde [11], glutaraldehyde, sodium hydroxide, propylene oxide, cupric ascorbate and phenol and gases like ethylene oxide, chlorine dioxide, hydrogen peroxide plasma, methylene bromide [17] and ozone showed promising sporicidal activities [11–17]. Of these, hypochlorite, peracetic acid showed 99.9% inactivation efficiency against spores of *Bacillus subtilis* and this bacteria was reported as one of the surrogates of *B. anthracis* [11–18]. Heat treatment, UV, and γ radiation also exhibited promising decontamination properties against spores of *Bacillus* species. These reports have also indicated that, *B. thuringiensis* and *B. anthracis* Sterne along with *B. cereus* form a better group of surrogates for virulent *B. anthracis* [19–23].

Although above decontaminants exhibited superior decontamination properties, certain disadvantages were also observed. The active chlorine content decreases with storage time, and bleach is corrosive to many surfaces. The chlorination treatment was observed to produce toxic by-products. Organic decontaminants leave large amount of non-biodegradable waste. Hydrogen perox-

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ide and oxone based decontaminants lack stability. So, it is required to look for other options that are user friendly and stable.

Inactivation of spores by photocatalytic oxidation method is one of the promising approaches, which eludes the production of toxic by-products as observed in the case of chemical disinfectants. Moreover, application of sun light along with nano-titania is environmentally benign method for decontamination of biological warfare agents. Recently, Prasad et al. have reported photocatalytic inactivation of *B. anthracis* vegetative cells by using titania nanomaterials and UVA light [24]. Zhao et al. [25] have studied the photocatalytic inactivation of *B. cereus* spores using UVA radiation and titanium dioxide catalysts. However, studies on *B. anthracis* Sterne would be more appropriate than *B. cereus* due to its genetic similarity with virulent *B. anthracis* spores. The virulence of *B. anthracis* is based on the presence of two virulence plasmids, pXO1 (181.7 kbp) and pXO2 (94.8 kbp) which are not found in potential surrogates *B. cereus*, *B. thuringiensis* and *B. subtilis*. The plasmid pXO1 encodes three toxic factors: the protective antigen (PA), the lethal factor (LF) and the oedema factor (EF). These components associate into two bipartite exotoxins, PA-LF and PA-EF. The plasmid pXO2 encodes a poly D glutamic acid capsule enabling the bacterium to withstand phagocytosis during infection. The loss of pXO2 renders the cells incapable of establishing an infection. Sterne strain resembles the characters of pathogenic Ames strain, without any virulence. This makes Sterne as an ideal strain to mimic the pathogenic strains of *B. anthracis* in lab studies [26].

On the other hand, direct illumination of contaminated water with UV light centred at 254 nm was established as a possible method for photolytic decontamination of pathogens. This type of radiation is injurious to health and involves occupational risks. Nevertheless, natural light, sun light and UVA light centred at 320–400 nm have been found to be the better alternatives than UVC light for decontamination of deadly pathogens. However, UVA exposure must be less than 10 W/m^2 for periods lasting 1000 s or more for safety indoor applications.

Inspired by the above work, we have studied the kinetics of inactivation of spores of *B. anthracis* Sterne by using nano-sized titania and UVA radiation (in mW/cm^2 which is safe) or sun light. Synthesized as well as commercial samples have been used for the above experiments.

2. Materials and methods

2.1. *Materials*

Titania of anatase phase with different sizes 1000 nm, 200 nm, 70 nm and 35 nm and that of rutile phase with size 40 nm, titanium tetrachloride, ethanol were procured from Sigma–Aldrich chemicals, USA and Alfa-Aeser, UK. Titania of anatase phase with crystallite size 16 nm was prepared in our laboratory by a reported method [27].

2.2. Preparation of *B. anthracis* Sterne spores

Single colony of *B. anthracis* Sterne strain was taken from brain heart infusion (BHI) agar plate and inoculated into BHI broth and was grown overnight at 37 °C. 50 μl of this overnight growth was spread plated onto modified germination (G) medium plates (0.2% yeast extract, 0.2% $(NH_4)_2SO_4$, 1.5% Bacto agar, 0.0025% $CaCl_2{\cdot}2H_2O$, 0.05% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.005% $MnSO_4{\cdot}4H_2O$, 0.0005% $ZnSO_4{\cdot}2H_2O$, 0.0005% $CuSO_4{\cdot}5H_2O$, 0.00005% $FeSO_4{\cdot}7H_2O$) (Kim and Goepfret 1974). The plates were incubated at 37 °C in dark until >95% of the material observed was spores. Sporulation was monitored regularly by malachite green staining and light microscopy. Colonies scraped from the surface of the agar were re-suspended in distilled water, washed twice in distilled water at 4000 × *g* for 5 min and heat-inactivated at 65 °C for 30 min to kill any viable vegetative cells and to activate germination. The heat activated spores were washed 10 times with sterile double distilled water at 4000 × *g* for 5 min. After final sedimentation, spores were re-suspended in distilled water to yield a final concentration of 10^7 colony forming units (CFU)/mL, as determined by vegetative outgrowth on nutrient agar (Difco) plates [28].

2.3. Photocatalytic experiments and analysis

Aqueous solutions containing 10^4 CFU/mL of *B. anthracis* were magnetically stirred with and without nanosized and bulk sized titanium dioxide at room temperature (30 ± 1 °C) under the illumination of UV-A light (320–400 nm) or sun light for investigating the photolytic (without photocatalyst) and photocatalytic inactivation of *B. anthracis*. LD_{50} of anthrax was found to be as low as 4000 spores, hence, we have used the concentration more than LD_{50}, i.e., 10^4 CFU/mL. 100 μL of sample was withdrawn at regular intervals of time for plate count experiments. Subsequently, the plates were incubated at 37 °C and colony counts were taken after overnight incubation. Data was taken in 3–5 replicates to ensure the reproducibility. UVA light irradiation experiments were performed in a photoreactor obtained from M/s. Luzchem, Canada of LZC 4V model. The intensity of light was varied by glowing more number of lights. Intensity of light was measured by digital light meter (SLM 110 model) of A.W. Sperry Instruments, USA with the help of the adopters provided at the place where the samples were exposed with radiation. Turbidity measurements were done on Perkin Elmer spectrophotometer using formazin solution as a primary standard.

2.4. Particle size analysis by X-ray diffractometer

XRD patterns were obtained by X Pert Pro Diffractometer, Panalytical, Netherlands, using Cu Kα radiation. The Scherer formula was used to calculate the crystallite size of the titania materials. The same is given in the following:

$$\text{Crystallite size (Å)} = \frac{0.9\lambda}{B\cos\theta}$$

where $B = \sqrt{\text{FWHM}^2 - 0.3^2}$ and λ is X-ray wave length (1.54 Å). FWHM is full width half maxima of the peak and θ is Bragg angle. FWHM was calculated from the peak having highest intensity in all the samples using X pert high score plus LTU software.

2.5. Characterization of treated *B. anthracis* spores by scanning electron microscopy

SEM measurements were carried out on a FEI instrument. Water dispersions of treated and untreated spores were placed on stubs prior to gold coating. Subsequently, they were completely dried at room temperature to remove water from the samples. Dried stubs containing the treated and untreated samples were coated with gold and then SEM images were recorded.

3. Results and discussion

Prior to inactivation studies on *B. anthracis* spores, synthesized and purchased nano-sized titania materials were characterized by X-ray diffraction technique and the data is represented in Fig. 1. Peaks at 2θ = 25.275° (1 0 1), 2θ = 37.82° (0 0 4), 2θ = 48.075 (2 0 0), 2θ = 53.875 (1 0 6), 2θ = 62.725 (2 1 5) are all attributable to the anatase phase. Crystallite sizes of these nano-sized titania materials were calculated by Scherer equation. Data revealed the sizes of

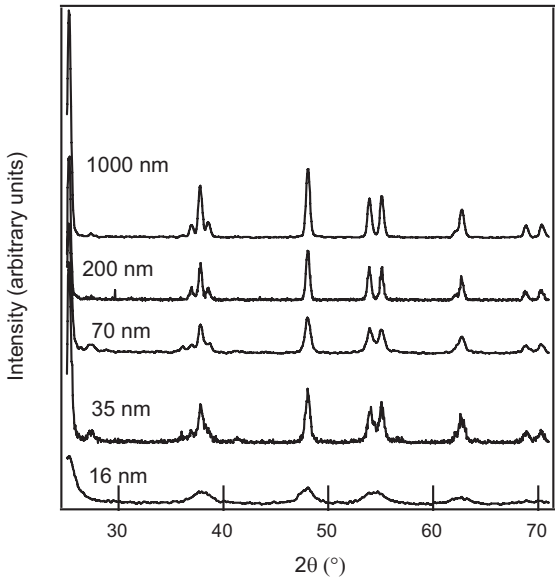

Fig. 1. X-ray diffraction data of titania materials used for inactivation studies against *Bacillus anthracis* Sterne spores.

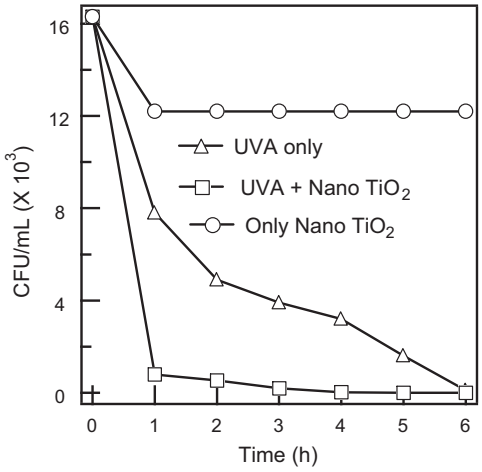

Fig. 2. Effect of time on photocatalytic inactivation of *Bacillus anthracis* Sterne spores of 10^4 CFU/mL concentration under sole treatment of UVA light, sole treatment of 10 mg of nano titania and treatment with 10 mg of nano titania and UVA light of 2.2585 mW/cm^2.

titania materials to be 1000 nm, 200 nm, 70 nm, 35 nm and 16 nm. All these materials were found to be of anatase phase.

Our recent studies [24] on inactivation of *B. anthracis* have indicated 100% decontamination of vegetative cells within 1 h. However, as per the present study on inactivation of *B. anthracis* Sterne spores, only 10–20% of spores were observed to be inactivated within 1 h. These observations clearly demonstrated that photocatalysis assisted inactivation of vegetative cells took lesser time than inactivation of spores of same organism. Reason is spores have complicated structure and chemical composition; hence have enhanced resistance towards various disinfectants that promote their inactivation. When we look from outside to inside, a spore contain layers of exosporium, coats, outer membrane, cortex, germ cell wall, inner membrane and central core. All these form a dense shield structure of spore formed by proteins. Because of this, inactivation time would be influenced by complexity, chemical composition and thickness of the spore wall structure.

In order to understand the effect of time on inactivation under sole treatment of UVA light treatment and synchronized photocatalysis treatment by UVA light and titania nanomaterials on *B. anthracis* Sterne spores, we have conducted experiments with 10 mg nano-titania, 10^4 CFU/mL of *B. anthracis* Sterne spores and 2.2585 mW/cm^2 of UVA light and the results are illustrated in Fig. 2. Apparently, 100% of *B. anthracis* Sterne spores were found to be inactivated in 5 h due to synchronized treatment of UVA light and titania nanomaterials. Only, 89% of spores were found to get inactivated in 5 h, showing the additive effect of titania and superiority of photocatalysis over sole UVA light treatment. However, in the case of sole treatment of nano-titania in the absence of light only 24% of inactivation of spores was observed.

During the inactivation, concentration of *B. anthracis* Sterne spores decreased exponentially with progression of time indicating the pseudo first order kinetics. Logarithmic plots of concentration of (*B. anthracis*) spores (CFU/mL) and time illustrated linear curves for both UVA and synchronized photocatalysis treatment of UVA and nano-titania confirming the pseudo first order behaviour of photolytic and photocatalytic inactivation of *B. anthracis* Sterne spores (Fig. 3.). The values of kinetic rate constant and half life of inactivation were calculated to be 0.4 h^{-1} and 1.733 h for sole UVA light treatment, 1.4 h^{-1} and 0.48 h for synchronized treatment of UVA light cum nano-titania and 0.03 h^{-1} and 23.1 h for only nano-titania in the absence of light. Above data clearly indicated the enhanced rate of inactivation of *B. anthracis* Sterne spores due to photocatalysis treatment when compared to photolytic treatment made possible by using only UVA light. In the presence of UVA light, titania nanoparticles participated in oxidation reactions which aided the inactivation of bacteria existing either in the spore form or in the form of vegetative cells. In addition to this, *B. anthracis* Sterne spores were found to be more resistant to UVA radiation than growing cells. This observation can be attributed to rapid repair of spore photoproduct formed while exposing to UVA radiation. Whereas in the case of photocatalysis the repair mechanism did not work as they were overpowered by oxidation destruction of the enzymes which aided the repair of the damaged organism.

Sporicidal activity of *B. anthracis* by UVA light treatment can be attributed to DNA damage and this observation is consistent with the reported results [4]. In addition to this, core of the spore seemed to contain little amount of water [4]. UVA light facilitated the generation of hydroxyl radicals in the above which killed the bacteria. Moreover, its inactivation efficiency was observed to get

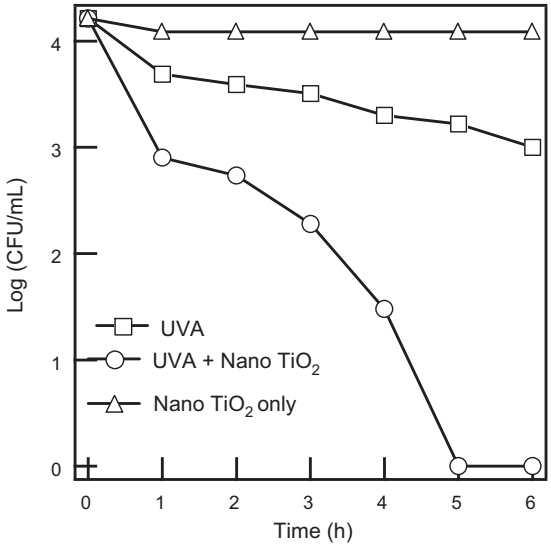

Fig. 3. Kinetic of inactivation of *Bacillus anthracis* Sterne spores of 10^4 CFU/mL concentration under sole treatment of UVA light, sole treatment of 10 mg of nano titania and 10 mg of nano titania and UVA light of 2.2585 mW/cm^2.

enhanced by the presence of nanosized titania. Titanium dioxide in the anatase form inherently behaves as semiconductor. Upon illumination of TiO_2 dispersed in water with UVA light, excess electrons seemed to be generated in the conduction band and positive holes seemed to be generated in the valance band. Subsequently, the photon generated electrons and holes observed to have migrated to the surface of titania and served for redox reactions on the surface. On the surface of titania, the photo generated hole sites in the valance band of titania seemed to react with water or surface hydroxide groups to form hydroxyl radicals ($OH^{\bullet}$), whereas excess electrons promoted to the conduction band seemed to react with molecular oxygen to form superoxide ions which further reacted with water to form additional amount of hydroxyl radicals. These $OH^{\bullet}$ along with superoxide ion radical seemed to have destroyed the spore and became responsible for the above synergistic inactivation of *B. anthracis* Sterne spores in the presence of titania nanoparticles by photocatalytic oxidation method.

To confirm the above observations and to understand the reasons of death of spores we have studied the morphology of treated and untreated spores with scanning electron microscopy (SEM). SEM is proved to be a suitable method for investigation of cell morphology and structures. Any changes occurring in the spore structure can easily be observed by SEM. Fig. 4(a–c) illustrates the SEM images of *B. anthracis* Sterne spores (a) untreated, (b) treated with UVA light or sun light and (c) treated with UVA or sun light and nano titania. After irradiation of spores with UVA light or sun light for 6 h, the cell morphology and its structure changed significantly. The spores were observed to coalesce and shrunken and these morphology changes can be attributed to evaporation of cellular fluids due to the heat build up within the spores upon irradiation either by UVA light or by sun light. In addition to this, DNA damage or photochemical reactions may have lead spores to coalesce and further their death. Moreover, spore contains some quantity of water or cellular fluids within. UVA or sun light may have promoted formation of hydroxide and super oxide radical out of the water and caused internal damage to the spore. This could have lead to changed physical structure or morphology (Fig. 4b). In the presence of titania, spore death could have occurred due to many reasons. One could be DNA damage or evaporation of cellular fluids or internal damage due to generation of radicals within the core of the spore. Another could be the decomposition of spores outer structure and complete disruption of complete spore. The outer layers like exosporium, coat, inner and outer membranes, cortex and core of the spore were seemed to get damaged physically due to the extreme oxidation treatment. This extreme treatment caused the formation of holes and damaged outer layers, and then converted to spore to organic debris as depicted in Fig. 4(c).

In the case of photocatalytic inactivation of *B. anthracis* assisted by nano titania, amount of the same is very important as it influences the rate of inactivation. In order to investigate how amount of nano-titania (anatase) influences the rate of inactivation, it was varied and the spore count was recorded. The concentration of spores (10^4 CFU/mL) and intensity of UVA light (2.2653 mW/cm^2) were kept constant and the results are presented in Table 1. Results indicated that, as the amount of nano-titania (anatase) was increased

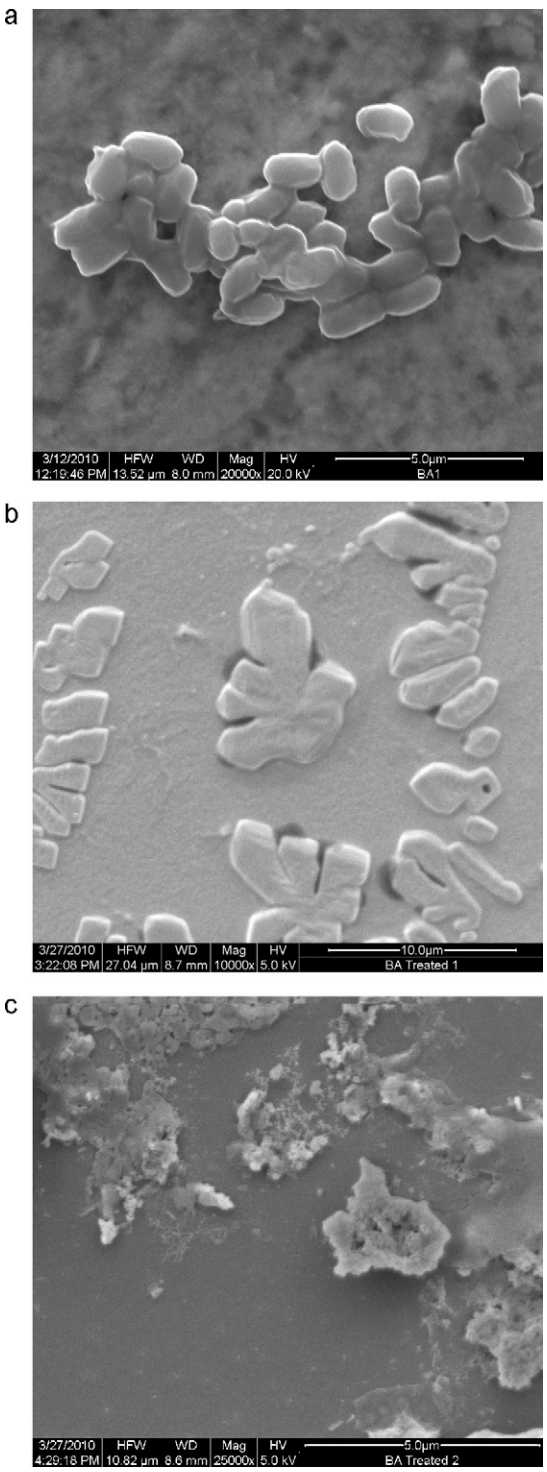

Fig. 4. SEM images of *Bacillus anthracis* Sterne spores (a) untreated, (b) treated with UVA light (2.2585 mW/cm^2)/sun light and (c) treated with UVA (2.2585 mW/cm^2)/sun light and 10 mg nano titania.

Table 1
Effect of amount of nano titania (35 nm size) on the kinetics of inactivation of *Bacillus anthracis* Sterne spores of 10^4 CFU/mL at 2.2653 mW/cm^2.

Amount of catalyst (mg)	Turbidity (NTU)	Rate constant (h^{-1})	Half life (h)
0.1	20	1.33	0.52
0.6	110	1.43	0.48
1.0	200	2.74	0.25
1.25	230	2.9	0.24
2.5	500	1.27	0.54
10	2000	1.21	0.57

from 0.1 mg to 1.25 mg, value of kinetic rate constant of inactivation of *B. anthracis* Sterne spores was found to increase from 1.33 h^{-1} to 2.90 h^{-1} while the value of half life decreased from 0.52 h to 0.24 h as the turbidity value increased from 20 NTU to 230 NTU. On further increase from 1.25 mg to 10 mg, the turbidity value increased to 2000 NTU and value of rate constant decreased to 1.21 h^{-1} indicating the reduced rate of inactivation of *B. anthracis* Sterne spores at higher amounts of nanotitania due to increased turbidity. The increase of rate of inactivation of spores with the increase in quan-

Table 2
Effect of intensity on kinetics of inactivation of *B. anthracis* Sterne spores of 10^4 CFU/mL using 1.0 mg titania (35 nm) of anatase phase.

Intensity of UVA (mW/cm^2)	Only UV rate constant (h^{-1})	UV + nano TiO_2 rate constant (h^{-1})
0.3692	0.10	1.05
0.5873	0.43	2.00
2.2653	1.15	2.74

tity of nano-titania (0.1–1.25 mg) can be attributed to increased photocatalytic activity. As quantity of catalyst increased more amounts of hydroxyl and superoxide radical species were seemed to be formed due to decreased activation energy and facilitated the enhancement of rate of inactivation by destroying the spore. Due to high surface-area-to volume ratio of nano-photocatalyst ($nanoTiO_2$), it adsorbed more number of spores or it was adsorbed to spore rapidly owing to the operation of van der Waals forces. It was also expected that, size reduction also influenced the formation of charge carriers thereby promoting inactivation rate. However, on further increase of quantity of catalyst (up to 10 mg) the turbidity of solution increased to 2000 NTU and caused depletion in the amount UVA light reaching the spore or catalyst materials due to back scattering thereby adversely affecting the rate of inactivation.

After this, effect of intensity of UVA light on photolytic and photocatalytic inactivation of *B. anthracis* Sterne spores was studied and the results are included in Table 2. The concentration of spores was 10^4 CFU/mL and amount of titania (70 nm) was 1.0 mg. Results demonstrated that, when intensity of light was increased from 0.3692 mW/cm^2 to 2.2653 mW/cm^2, the value of rate constant of inactivation under the sole treatment of UVA light increased from 0.1 h^{-1} to 1.15 h^{-1} depicting the enhancement of rate of inactivation. In the presence of nano-titania also, the value of rate constant increased from 1.05 h^{-1} to 2.9 h^{-1}, however, rate of inactivation is even more better than in sole UVA treatment. Analysis of the kinetic results indicated that, in the mentioned range of intensity of UVA light, rate of inactivation in the presence and absence of nano-titania was observed to be increased with the increase in intensity. In all the values of intensity studied, photocatalysis treatment exhibited better inactivation rates as reflected by better values of rate constants than the values of rate constant without photocatalyst, i.e., nano-titania (only UVA light). As the intensity of UVA light increased more number of electrons that were present in the valance band of nano-titania (anatase) were seemed to get excited to conduction band thereby increasing number of electrons and holes on the surface. Owing to this, concentration of number of radicals $OH^{\bullet}$ and superoxide increased thereby positively influencing the rates of *B. anthracis* Sterne spore inactivation.

It is a well known fact that, as the particle size changes electrical properties, magnetic properties and catalytic properties of the materials change. To probe the role of particle size of nano-titania (anatase) on the photocatalytic inactivation of *B. anthracis* Sterne spores, titania materials of size 16 nm to 1 μm were studied and the results are shown in Table 3. It is apparent from the results that, as the size of the particles increased from 16 nm to 1 μm, value of rate constant of inactivation of *B. anthracis* Sterne spores decreased from 0.26 h^{-1} to 0.02 h^{-1}. Increase of size of the anatase titania particles from 16 nm to 1 μm decelerated the rate of inactivation of spores by 13 folds approximately. Evidently, as the size of nano-catalyst decreased, the surface area-to-volume ratio increased along with number of defect sites. They promoted the adsorption of spores and photocatalysis aided inactivation of the same. In addition to these, as the size decreased, surface charge carrier transfer rate seemed to get influenced and enhanced, there by affecting the rate of inactivation of *B. anthracis* Sterne spores.

Table 3
Role of particle size of nano-titania (anatase) on the photocatalytic inactivation of *B. anthracis* Sterne spores 10^4 CFU/mL and 1.3424 mW/cm^2.

Avg. diameter of the particles of titania (nm)	Rate constant (h^{-1})	Half life (h)
16	0.262	2.65
35	0.227	3.05
200	0.074	4.08
1000	0.020	34.6

After this, we also have studied the effect of phase of titania on the inactivation of *B. anthracis* Sterne spores by using the nanoparticles of titania existing in anatase and rutile phases. Value of rate constant of inactivation in the case of nano-titania (anatase) was found to be 0.92 h^{-1}, where it was found to be 0.4 h^{-1} in the case of rutile phase titania. It is apparent from the above data that, anatase phase titania exhibited superior inactivation properties. Anatase phase of titania possesses better photoactivity when compared to rutile phase due to larger Fermi level in the former as per the previously reported literature [24].

Sun light has wide spectrum of radiation starting from ultra violet to visible. It is also harmless when compared to UVC or UVB light radiations. It is also well known that, sun light checks the microorganism and saves mankind. However, it is not known whether it kills spores of *B. anthracis*. Hence, we have investigated the combined effect of sun light and nano-titania (anatase) on the inactivation of *B. anthracis* Sterne. The value of rate constant of inactivation of spores in the presence of only sun light was determined to be 0.9 h^{-1}, whereas, in the presence of nano-titania along with sun light it was found to be 1.3 h^{-1}. Interestingly sun light inactivated the *B. anthracis* Sterne spores when used alone or along with nano-titania (anatase). However, in the presence of nano-titania along with sun light the additive effect of titania was predominant in inactivation of spores thus promising battle field applications. Similar studies were conducted with *B. cereus*, a different surrogate by Zhao et al. [25]. An inactivation rate coefficient of 4.4 d^{-1} was achieved on titania coated membrane in the presence of 30 W/cm^2 UVA radiation. However, our present studies with *B. anthracis* Sterne spores were found to be more suitable for simulating the inactivation of virulent agent because of genetic similarity. Due to established sporicidal mechanism, this method not only improved safety level by reducing the possibility for micro-organism re-growth but also facilitated the complete decontamination.

4. Conclusions

Summarizing the above mentioned data, spores of *B. anthracis* Sterne have been efficiently inactivated by using titania nanomaterials and UVA light or sun light. The inactivation kinetics demonstrated pseudo first order behaviour. Spore death can be attributed to the leakage of cellular fluids due to damage of spore as observed by scanning electron microscopy data and DNA damage. Sun light also exhibited superior decontamination properties against *B. anthracis* Sterne spores.

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