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Inhibition of *Candida albicans* biofilm by pure selenium nanoparticles synthesized by pulsed laser ablation in liquids

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Abstract

Selenoproteins play an important role in the human body by accomplishing essential biological functions like oxido-reductions, antioxidant defense, thyroid hormone metabolism and immune response; therefore, the possibility to synthesize selenium nanoparticles free of any contaminants is exciting for future nano-medical applications. This paper reports the first synthesis of selenium nanoparticles by femtosecond pulsed laser ablation in de-ionized water. Those pure nanoparticles have been successfully used to inhibit the formation of *Candida albicans* biofilms. Advanced electron microscopy images showed that selenium nanoparticles easily adhere on the biofilm, then penetrate into the pathogen, and consequently damage the cell structure by substituting with sulfur. 50% inhibition of *Candida albicans* biofilm was obtained at only 25 ppm. Finally, the two physical parameters proved to affect strongly the viability of *Candida albicans* are the crystallinity and particle size.

Graphical abstract text

Sequence of a three steps mechanism displaying the interaction between pure selenium nanoparticles and *Candida albicans* biofilm.

Keywords

nosocomial diseases; hospital-acquired infections; femtosecond laser; laser-induced bubbles; structural phase transition; size effect

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://>

Background

Selenium (Se) was discovered accidentally in 1817 by Berzelius, a Swedish chemist, after analyzing a contamination of sulfuric acid which was responsible of the reddish coloration of the solution. Due to similar chemical properties with the element tellurium and because tellurium had been named for *Tellus*, the Roman goddess of the earth, Berzelius strove for balance by calling its sister element - selenium - for the Greek goddess of the moon, called *Selene*(1, 2). Geologically, selenium is not found abundantly in mineralized deposits from where it can be mined economically; but, either found naturally as impurities in copper sulfide ores, from where it is recovered as a byproduct during copper refining(3). Therefore, selenium has been classified by the American Physical Society and the Materials Research Society as a critical element that might encounter supply disruptions(4, 5). Selenium is then a trace element that is also essential for life (adult dietary requirement ~40 µg Se/day) while at the same time being toxic at high levels (>3200–6700 µg Se/day) (6, 7). Selenium enters the food chain through the plants consumed by humans and animals. The plants absorb the selenium directly from the soil therefore selenium intake among human populations is highly dependent on the geographical area(8, 9). In human beings, selenium is incorporated into at least 25 selenoproteins that have a wide range of effects e.g. antioxidant activity, chemopreventive, anti-inflammatory and antiviral properties(10–14). It has been shown that selenium and selenoproteins have the potential to reduce the progression of pathology in several neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, Huntington's disease(15). Moreover, selenium nanoparticles (Se NPs) can also be used as inhibitors of protein glycation(16). Furthermore, it has been shown that Se NPs can also affect other organisms. In particular they prevent the growth and proliferation of *E. coli* and *S. aureus*, the two bacteria that are mainly responsible of nosocomial diseases(6, 17), also called hospital-acquired infections (HAI), i.e. those occurring within 48 hours of hospital admission, 3 days of discharge or 30 days after an operation. Infections due to those bacteria are difficult to treat as they are drug-resistant to a large group of antibiotics(18, 19). In the United States of America, two million people are infected each year by nosocomial diseases, of whom, 5% die(20). Another important pathogen responsible of nosocomial disease is a fungus named *Candida albicans* that grows as yeast and filamentous cells causing oral and genital infections in humans(21). For those reasons, it is extremely urgent to develop new drugs to fight those infections. The solution may come from nanotechnology where nanoparticles being less than one hundred nanometers in size are especially adapted to interact with bacteria(22) and fungus(23) due to their large surface to volume ratio. Therefore, there is a specific interest in synthesizing pure Se NPs for nano-medical applications(24, 25).

Methods

Materials and synthesis

One requirement for nanomaterials to be used in medical applications is to have the surface of the nanomaterial free of any contamination, including residual chemicals from the synthesis process. This is the main advantage of synthesizing nanoparticles by Pulsed Laser Ablation in Liquids (PLAL). It is known that selenium can have different allotropic

structures: trigonal, monoclinic (α -, β -), cubic (α -, β -), rhombohedral, ortho-rhombic and amorphous(26). However, the most common allotropic phases of selenium are trigonal, α -monoclinic and amorphous. Each of these phases is the result of specific synthesis conditions and, therefore, can be used to determine the mechanism through which the nanoparticles have been generated. The synthesis of Se NPs by PLAL using nanosecond lasers has already been reported and characterized by different groups (27–31). The primary mechanism of nanoparticle generation in those studies is melting. This paper reports the first synthesis of Se NPs by PLAL using a femtosecond laser (Supplementary data available, Figure S1). The irradiations were performed using a Ti:sapphire femtosecond laser (Mai-Tai HP from Spectra Physics) with a pulse duration of less than 100 fs and a repetition rate of 80 ± 1 MHz. The wavelength used for irradiations was 800 nm (near infrared) with a power of 3 W. The laser beam has a diameter of less than 1.2 mm at 800 nm. It was directed to enter from the top into a square cuvette containing several bulk selenium pellets (size <5 mm, purity 99.999%, Sigma Aldrich – 204307-5G) placed at the bottom of the cuvette in 2 ml of de-ionized water (Fischer Scientific).

Characterization of selenium target and nanoparticles

The bulk selenium pellets used as a target during the synthesis were characterized by Raman spectroscopy. The Raman spectra were taken on a X-Plora-PLUS from Horiba-Yvon Jobin at different temperatures within a controlled heating stage (THMS600, Linkham). The used wavelength was 785 nm.

The selenium nanoparticles synthesized by pulsed laser ablation in liquids were characterized by Dynamic Light Scattering (DLS), UV-visible spectroscopy, atomic emission spectroscopy (AES) and electron microscopy. The size distribution spectra and zeta potential were measured using the Zetasizer Nano ZS from Malvern Instruments. The temperature was set at 25°C. The absorption spectra were taken on a Cary VARIAN UV-vis spectrometer. The baseline was taken on a solution containing only DI water. For the AES, the spectrum was acquired with an Atomic Absorption Flame Emission Spectrophotometer from Shimadzu, AA-6200. The equipment was calibrated using 5 selenium references of known concentration. For the electron microscopy analysis, a drop of the Se colloidal solution was deposited onto a commercial copper TEM grid and dried in air. High Resolution Transmission Electron Microscopy (HRTEM) experiments were carried out on a JEOL 2010F microscope operating at 200 kV. Scanning electron microscopy (SEM) was carried out on a Hitachi STEM 5500 operating at 30 kV.

Culture conditions of *C. albicans*

Candida albicans strain SC5314 (32) was used in this study as in Ref. (33). Cells from frozen stocks stored with 15% glycerol at -80°C were streaked onto a Yeast-peptone-dextrose (YPD) agar plate, and incubated at 30°C overnight. A loopful of the cells growth was inoculated into flasks of YPD broth in an orbital shaker (150–180 rpm) and grown for 14 to 16 h at 30°C . These are ideal conditions for *C. albicans* to grow as budding yeast. Biofilms are quantified using well established phenotypic assay previously reported by our group (23). Briefly, after cells are harvested from overnight YPD broth cultures they were washed and grown in RPMI-1640 (Corning-Cellgro), with biofilms formed on sterile flat-

bottom microtiter plate (Costar, Corning Inc. USA) following incubation for 24 h. The biofilm and sessile cells strongly attached to the flat-bottom wells were gently washed twice with sterile phosphate-buffered saline (PBS) to ensure removal of non-adherent yeast cells, and the amount of biofilm inhibition quantified using a colorimetric assay based on the reduction of a tetrazolium salt, the XTT reduction assay (Sigma Chemical Co., St. Louis, Mo.), absorbance at 492 nm was recorded using an automated plate reader (Benchmark Microplate Reader; Bio-Rad Laboratories, Hercules, CA).

Results

Synthesis of Se NPs and laser-induced bubbles

Shortly after the irradiation begins, a “screening” bubble appears at the surface of the pellet centered at the location where the laser beam is incident to the surface of the target, (Figure 1). In PLAL, the process through which matter is ejected from the target directly depends on the pulse duration, irradiance and target properties. For a nanosecond pulse, it occurs via electron-phonon relaxation whereas for a femtosecond pulse it occurs mainly via photo-fragmentation(34). However, due to the high repetition rate and low irradiance, thermal effect occurs. For femtosecond pulses, the laser energy is absorbed by the electrons of the target through photon-electron interactions. The energy is transferred to the lattice electron-phonon interactions on a picosecond time scale(34–36). This energy transfer results in the creation of vapor and plasma phases in the surrounding liquid, however ablation takes place before the onset of plasma formation by Coulomb repulsion(37). The expansion of the plasma generates a shock wave that occurs in tens of nanoseconds and lasts a few microseconds, under the confinement of liquid(36, 38). Therefore, due to pressure and temperature variations, PLAL is generally associated with the generation of bubbles either in the liquid or at the solid-liquid interface. Two kind of bubbles exist namely boiling bubbles (temperature variation) and cavitation ones (pressure variation). The first ones are originated by the overheating of the liquid which leads to its vaporization. The second ones are initiated by a heterogeneous nucleation at the solid-liquid interface due to a pressure variation. In our experiment, the laser beam is unfocused and the irradiance is around $2.65 \times 10^2 \text{ W/cm}^2$, well below the irradiance threshold required for 800 nm light to induce the dielectric breakdown of water(39) which ranges between $\sim 10^9 \text{ W/cm}^2$ and 10^{11} W/cm^2 . Therefore, it is unlikely that the “screening” bubble is a cavitation bubble originating from the expansion of the plasma associated with the dielectric breakdown of the liquid. The other possibility is that the “screening” bubble is a boiling bubble originating from the vaporization of water due to a rapid heat transfer from the Se pellet to the liquid. This bubble is sustained throughout the duration of the irradiation thus keeping the produced nanoparticles at the bubble interface which then obstructs disruption of the interface(40). Furthermore, at irradiances well below the optical breakdown of the target, $\sim 10^{13} \text{ W/cm}^2$, both thermal and non-thermal mechanisms lead to ablation of the target(40) while above this threshold only the non-thermal mechanism survives.

In order to understand the origin and dynamics of the “screening” bubble, two videos were recorded through a visible (Sony High Definition Flash Memory Handycam® Camcorder model HDR-CX150) and thermal infrared camera (FLIR E40), respectively. Both videos

show the birth and growth of the laser-induced bubble (Supplementary Information available). As the irradiation continues, the “screening” bubble grows symmetrically in both the horizontal and vertical directions. After three to four minutes, it stops growing horizontally and elongates vertically. After one to two minutes, it resumes its horizontal growth while elongating vertically. Soon after it reaches its maximal horizontal extension, the “screening” bubble contracts horizontally, leading to the detachment from the target and floating up to the surface. After this occurs, another “screening” bubble appears exactly where the beam is hitting the surface of the target. The lifetime of the “screening” bubble approximates 10 min but can sometimes reach up to 20 min. This long lifetime is obtained by the coalescence of all the individual boiling bubbles appearing at each laser pulse which are formed by the vaporization of de-ionized water in the vicinity of the laser impinging on the selenium pellet. Using the images from the video camera, the horizontal and vertical dimensions of the bubble were measured and used to determine the growth rate. The growth rate was linear with time except during the first 30 s of irradiation (Figure 1b).

To characterize the formation of this “screening” bubble, the temperature of the solvent was measured with a thermal camera and a thermocouple (Figures 1 c and 1 d). The data recorded with the thermocouple are reported in Figure 1d when the solution was irradiated without and with the Se pellet. The temperature recorded in the irradiated solution without the Se pellet is lower than the one irradiated with the Se pellet. The temperature reaches a plateau in both cases, at 37°C and 69°C for the solution without and with the Se pellet, respectively. The data can be fitted with the following function:

$$T(t) = T_{\text{plateau}} + \frac{b}{t+a} \quad (1)$$

T_{plateau} is the temperature when the time tends to infinite. The ratio b/a is physically related to the difference between the room temperature and the plateau temperature. The a and b parameters are 6.3399 min and -112.2109 °C.min when the solution does not contain the Se pellet and 3.1576 min and -156.3503 °C.min when the solution contains the Se pellet. Using those parameters, we can confirm the consistency of both measurements (thermal camera and thermocouple) and the validity of Eq. 1. Indeed, at $t = 30$ s, $T(0.5\text{min}) = 26 \pm 1$ °C which is in very good agreement with the temperature measured by the thermal camera, $T(0.5\text{min}) = 25$ °C, because the thermocouple measurement was done at the top of the cuvette.

From studying the images obtained with the video cameras, it can be observed that the surface of the “screening” bubble has a reddish appearance. We attribute this color to the presence of Se NPs generated by laser ablation of the pellet prior to the bubble formation. A very interesting observation is the fact that once the bubble is formed, it appears to prevent any further ablation of the pellet, screening it from the effects of the laser beam. Therefore, in order to have a steady generation of nanoparticles via a sustained ablation of the target, it is necessary to continuously move the cuvette to prevent the formation and growth of the “screening” bubble. While irradiating the pellet with the laser beam, there is a non-continuous acoustic signal (a rapid sequence of acoustic sounds) that appears to correspond with the generation of nanoparticles. We recorded this audible signal with a stereophonic

microphone and found a distinct spectral signature for the time periods when the acoustic signal occurs (Supplementary Information available, Figure S2). The signal has frequency components between 10 and 17 kHz which is presumed to be related with the photoacoustic wave generated by the laser beam impinging on the selenium pellet, thus leading to rapid heating and expansion at the surface which propagates to the surrounding fluid as an acoustic ultrasound wave.

Crystallinity of Se NPs

Preliminary to the irradiations, the bulk selenium pellet that constitutes our target has been characterized by Raman spectroscopy. The temperature has been varied from 25°C to 200°C by step of 5–10°C. It is clear from Figure 2 that the bulk selenium pellet is amorphous at room temperature and then undergoes a structural phase transition (amorphous-trigonal) around 90°C. It is well known that melting and structural phase transitions are size-dependent(41, 42). Therefore, Eq. 2 can be used to calculate the size-dependency of those phase transitions(43, 44):

$$\frac{T_m}{T_{m,\infty}} = 1 - \frac{\alpha_{\text{shape}}}{D} \quad (2)$$

T_m is the size-dependent melting temperature. $T_{m,\infty}$ is the bulk melting temperature. D is the diameter of the nanoparticle. $\alpha_{\text{shape}} = [D(\gamma_s - \gamma_l) / H_{m,\infty}] [A/V]$ is the parameter quantifying the size effect on the transition temperature and depending on the nanostructure shape. A/V is the surface to volume ratio. $H_{m,\infty}$ is the bulk melting enthalpy, equal to $4.06 \times 10^8 \text{ J/m}^3$ (45). $\gamma_{s(l)}$ is the surface energy in the solid (liquid) phase, equal to 0.291 and 0.106 J/m², respectively(45–47). In the case of a spherical selenium nanoparticle, $\alpha_{\text{sphere}} = 2.73 \text{ nm}$. As a first approximation, the size effect on both phase transitions can be considered to be the same this is justified by the fact that all the properties are function of $1/D$ (43, 44). Figure 3a illustrates the nano-phase diagram of selenium where the solid-liquid and amorphous-trigonal phase transitions have been calculated using Eq. 2. It has already been noted by Guisbiers *et al.*(28) that the crystallinity of the Se NPs synthesized with a nanosecond laser was size-dependent i.e. the largest nanoparticles were amorphous and the smallest ones were crystalline with a transition at room temperature found to be around ~80 nm. Here, with the femtosecond laser, the largest nanoparticles observed by TEM (Figure 3b) are amorphous while the smallest ones are crystalline (trigonal phase(48)), in excellent agreement with the phase diagram shown on Figure 3a (Supplementary Information available, Figure S3). The difference between those two types of laser irradiations is that the irradiation with the nanosecond laser was done on a crystalline powder (trigonal phase) of selenium while the irradiation here with the femtosecond was done on a bulk amorphous pellet. And in both cases; the target was immobile. Another important point of comparison is the temperature of the solution during the synthesis that stays at room temperature (~20°C) for the nanosecond laser and that reaches 69°C with the femtosecond laser.

Morphology and stability analysis

From TEM images (Figure 3b), it is clear that the shape of the particles synthesized by a femtosecond laser is mostly spherical. Generally, the particles produced by femtosecond pulsed laser are non-spherical as already observed by Ref.(34), however, the ablated material, selenium in our case, is a low melting point material, 494 K. Therefore, this low melting temperature combined with a high repetition rate, 80 MHz, allows the birth of thermal effects. EDX spectra on different Se NPs confirmed the purity of the nanoparticles produced, no oxides have been formed (Supplementary Information available, Figures S4 and S5). The size distribution has been analyzed by Dynamic Light Scattering (DLS). The DLS spectra of the different colloidal solutions are presented on Figure 4a. The size range of the synthesized nanoparticles is between 50–400 nm. We can see that as the irradiation time increases the solution becomes more mono-dispersed. At 60 min, the average size of the nanoparticles is around 128 ± 30 nm. The smallest nanoparticles were not detected by using the DLS technique because the size measured by DLS (called hydrodynamic diameter) is representative of the size of a hypothetical sphere that diffuses at the same rate as the particles being measured and the scattered intensity is proportional to d^6 where d is the particle diameter. Therefore, the light scattered by the largest particles may hide the light scattered by the smallest ones. As an example, if we consider two particles, one with a diameter 10 times larger than the other one, the d^6 factor tell us that the largest particle will scatter 10^6 more light than the smallest one. Finally, UV-visible spectroscopy has been carried out and the spectra is shown on Figure 4b for each selenium colloidal solution obtained after different irradiation time (5 min, 10 min, 15 min, 30 min and 60 min). The absorption increases with time and is mainly occurring below ~ 600 nm, explaining the ruby red color of the colloidal solution.

Inhibition of *C. albicans*

Figure 5 shows the inhibition of *C. albicans* biofilm in a dose response manner as a function of selenium nanoparticle concentration for different batches. Sample 1 represents the Se NPs just after their synthesis, the second sample is the one containing the smallest Se NPs (i.e. they have been separated from the largest ones by centrifugation at 15000 rpm) and the third one is the one having only crystalline Se NPs (i.e. the synthesized nanoparticles have been heated at 90°C during 2 hours to ensure the phase transition occurs from amorphous to trigonal). Obviously, from Figure 5, the 2nd sample is the most effective indicating that the size is a key parameter during the inhibition process of *C. albicans*. However, by comparing samples 1 and 3 in Figure 5, it is clear that crystallinity is also contributing to the inhibition of *C. albicans* (49). The concentration of Se NPs obtained after 60 min irradiation was measured by Atomic Emission Spectroscopy (AES) to be around 26 ± 1 ppm. The concentration can be upgraded by constantly moving the target during the irradiation and by reducing the repetition rate of the femtosecond laser. By doing this, the influence of the “screening” bubble will then be consequently reduced.

Discussion

Microbial colonization of medical devices is a widespread problem responsible of nosocomial infections(50). Once in use, the colonized medical devices can be difficult to

shown that the size and crystallinity of the produced Se NPs are the key parameters in the inhibition of *C. albicans* biofilm. Work is going on to improve the production rate by moving automatically the target to consequently reduce the influence of these boiling bubbles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a) Sketch of the synthesis methodology: Pulsed Laser Ablation in Liquids (PLAL) b)

Growth rate of the induced bubble versus time of irradiation. Inset: Photo of the screening bubble after several minutes of irradiation. The side length of the plastic cuvette is 12 mm and the diameter of the bubble is measured to be around $\sim 1.7 \pm 0.1$ mm. c) Series of infrared photos showing the cross-side view of the cuvette taken with the infrared camera every 10 s during the first 50 s of irradiation. d) Temperature of the solvent measured with a thermocouple versus the irradiation time when the irradiation is conducted without and with the Se pellet.

Figure 2.

Raman spectra of one bulk selenium pellet recorded at different temperatures, from 25°C to 200°C. A structural phase transition occurs around 90°C (amorphous to trigonal).

Figure 3.

a) Transition temperatures (melting and structural phase transition, trigonal→amorphous) versus size for a spherical selenium nanoparticle. The dashed area indicates the water temperature during the selenium nanoparticles synthesis. The numbers indicate the synthesized selenium nanoparticles observed by TEM. b) and d) TEM images of selenium nanoparticles synthesized by a pulsed femtosecond laser in de-ionized water (irradiation time = 1 h). c) and e) Their corresponding diffraction pattern. (Supplementary Information available)

Figure 4.

UV-visible spectra of the selenium colloidal solution at different irradiation times. The absorbance occurs mainly below ~600 nm and increases with irradiation time. b) Size distribution of the selenium colloidal solution obtained by Dynamic Light Scattering for different irradiation times. Inset: mean size versus the irradiation time. Inset: Photo of the colloidal solution after 1h irradiation.

Figure 5.

a) Biofilm-related phenotypic assay inhibition by selenium nanoparticles. Two-fold serial dilutions of selenium nanoparticles (26 to 0.03 ppm) were added to *Candida albicans* preformed biofilm. Sample 1 - selenium nanoparticles, Sample 2 - small selenium nanoparticles, Sample 3 - heated selenium nanoparticles. All biofilm assays were carried out in duplicate on at least three separate occasions and the results are presented as the mean $\pm$ standard deviation. b) Cytotoxicity of ARPE cells line after 24 h exposure to selenium nanoparticles. The ARPE cells were exposed for 24 hours to different amounts of selenium nanoparticles ranging from 1 to 50 ppm for dose-dependent cytotoxicity. Cell viability was measured by the Cell-Titer 96 assay and compared to untreated cells (control), whose viability was set at 100%. Each result represents the mean $\pm$ standard deviation of four independent experiments; each of these was performed at least in triplicates.

Figure 6. SEM images of a) Control *Candida albicans* biofilm, b) Selenium nanoparticles adhering on the outer cell membrane of *Candida albicans* in biofilm at 26 ppm of selenium nanoparticles, c) Selenium nanoparticle (26 ppm) on the outer cell membrane of *Candida albicans* and entering into it, d) Selenium nanoparticles (26 ppm) inducing shrinking and folding of the outer cell membrane of *Candida albicans* in biofilm (indicated by the red circles).