J Fluoresc (2012) 22:1633–1638
DOI 10.1007/s10895-012-1107-4

ORIGINAL PAPER

Singlet Oxygen Generation Enhanced by Silver-Pectin Nanoparticles

Luciana S. A. de Melo · Anderson S. L. Gomes · Sybele Saska · Karina Nigoghossian · Younes Messaddeq · Sidney J. L. Ribeiro · Renato E. de Araujo

Received: 16 March 2012 / Accepted: 27 June 2012 / Published online: 28 July 2012

Abstract We demonstrate the potential application of silver-pectin nanoparticles on photodynamic therapy, on a solution-base platform. Photodynamic therapy is a medical technique which uses a combination of photosensitizing drugs and light to induce selective damage on the target tissue, by electronically excited and highly reactive singlet state of oxygen. Metal enhanced singlet oxygen generation in riboflavin water solution with silver-pectin nanoparticles was observed and quantified. Here 13 nm silver nanospheres enclosed by a pectin layer were synthesized and it interaction with riboflavin molecule was analyzed. Pectin, a complex carbohydrate found in plants primary cell walls, was used to increase the biocompatibility of the silver nanoparticles and to improve metal enhanced singlet oxygen generation (28.5 %) and metal-enhanced fluorescence (30.7 %) processes at room temperature. The singlet oxygen sensor fluorescent green reagent was used to quantify the enhancement of the riboflavin singlet oxygen production induced by the silver colloid. We report a 1.7-fold increase of riboflavin emission and a 1.8-fold enhancement of singlet oxygen production.

Keywords Metal nanoparticles · Photodynamic therapy · Plasmonics · Photosensitizer

L. S. A. de Melo
Materials Science Program, Center for Natural and Exact Sciences,
Federal University of Pernambuco,
Av. Professor Luís Barros Freire, Cidade Universitária,
50670-901, Recife, PE, Brazil

A. S. L. Gomes
Department of Physics, Federal University of Pernambuco,
Av. Professor Luiz Freire, Cidade Universitária,
50670-901, Recife, PE, Brazil

S. Saska · K. Nigoghossian · Y. Messaddeq · S. J. L. Ribeiro
Institute of Chemistry, São Paulo State University,
R. Francisco Degni, Quitandinha,
14800-900, Araraquara, SP, Brazil

R. E. de Araujo (✉)
Laboratory of Biomedical Optics and Imaging,
Federal University of Pernambuco,
Av. dos Reitores, Cidade Universitária,
50670-901, Recife, PE, Brazil
e-mail: renato.earaujo@ufpe.br

Introduction

Photodynamic therapy (PDT) is a medical technique which uses a combination of photosensitizing drugs and light to induce selective damage on the target tissue. Applications of the photodynamic effects are mainly explored on cancer treatment as an alternative to chemotherapy or radiotherapy procedure [1–4]. Moreover, photodynamic methods are already in use either routinely or in experimental studies on several medical fields, such as dermatology, ophthalmology, gastroenterology, cardiology, neonatology [5], fungal and bacterial infections [1, 3, 6–9].

In PDT, under suitable illumination photosensitizer can give rise to activated species which are very reactive on biological environment [1, 2, 6, 10, 11]. Following light absorption the photosensitizer molecule is promoted to a singlet excited state, with a short lifetime (ns). The photosensitizer can return to it ground state by emitting a photon (fluorescence) or by internal conversion with energy loss as heat. Intersystem crossing can lead the excited photosensitizer molecule to a triplet state, with a longer lifetime (ms) [12], increasing the probability of energy transfer to other molecules. There are two mechanisms by which the triplet state photosensitizer can react with biomolecules known as the Type I and Type II reactions [13]. Type I reaction involves electron/hydrogen transfer directly from the photosensitiser, producing ions, or electron/hydrogen abstraction from a substrate molecule to form free radicals. The second

one (Type II reaction) produces electronically excited and highly reactive singlet state of oxygen [14, 15].

Many products can behave as PDT photosensitizers: porphyrins, chlorines, bacterioclhorins [16], phtalocyanine, naphtalocyanines, purpurins [10, 17], aminoacridine [1], indocyanine green [11], methilene blue [18], riboflavin [19]. In particular the riboflavin photosensitizer molecule is a water solved vitamin and is also known as B2 vitamin. In human body, riboflavin plays an important rule on several metabolic redox reactions, through the cofactors FMN and FAD, which act as electron carriers [20].

The inefficient production of singlet oxygen ($^1O_2$) and free radicals, to react with biological targets, can limit the use of PDT photosensitizers [21, 22]. Because singlet oxygen plays a very important role in PDT effects, an abundant supply of oxygen is very important [21]. Recently a new phenomenon named Metal enhanced singlet oxygen generation (MEO), by silver island film, was demonstrated [12, 23]. Zhang et al. [12] and Zhang et al. [23] showed that localized plasmons in noncontinuous silver films (2-dimension system) can provide an enhancement of the reactive oxygen production in the evaluated medium.

Metal nanostructures are increasingly receiving attention as important starting points for efficient contrast for biological imaging and spectroscopic applications [24–32], as well as for photothermal therapeutic applications [33–36]. Moreover silver nanoparticles polymer covered have been employed in nanobiomedicine. Shell structures can protect nanoparticles against enzymatic and hydrolytic degradation, improving the delivery of bioactive agents [37].

We demonstrate the potential application of new silver-pectin nanoparticles on PDT. Metal enhanced singlet oxygen generation in riboflavin water solution with silver-pectin nanoparticles was observed and quantified. Here pectin, a complex carbohydrate found in plants primary cell walls, isolates the silver nanospheres increasing the biocompatibility of the colloid and controlling chemical interactions among silver nanoparticles and other structures. The developed silver-pectin nanoparticles consist of 13±4 nm silver nanospheres involved by a layer of pectin. Moreover, as nanoparticle-photosensitizer distance is an important parameter for the enhancement of singlet oxygen production we also analyzed the riboflavin interaction with silver nanospheres without pectin. We report an increase in the riboflavin emission and an enhancement in singlet oxygen production.

Materials

Synthesis of Silver Nanoparticles

The developed nanoparticles consist of silver nanospheres involved by a monolayer of pectin. On the nanoparticles synthesis, commercial pectin (CP Kelco brand) type Genu 105; $AgNO_3$ and sodium citrate (Sigma Aldrich) were used. A 1.0 mL of pectin solution (0.5 %w/v) and 3 mL of $AgNO_3$ solution (10^{-3} M) were added under stirring to 25 mL of distilled water. The mixture was heated to 80 °C and then was added to 1 mL of sodium citrate solution (0.1 M). The system was maintained heated (80 °C to 100 °C) for 30 min.

Pectin molecule has a complex structure mainly composed of (1→4) linked α-D galacturonic acid esterified units [38]. Methoxyl and acid groups in pectin are a favorable factor to stabilize metal colloids by the electrostatic repulsion effect and steric effect.

The synthesis protocol was also performed without the addition of pectin. Therefore, bare silver nanoparticles without pectin were obtained.

Riboflavin Photosensitizer

Baier et al. [39] classified riboflavin as an efficient photosensitizer, with 0.54±0.07 quantum yield of singlet oxygen production [39]. Here 0.35 μM of riboflavin (Sigma Aldrich) water solution were prepared and used as singlet oxygen photosensitizer.

Riboflavin, in water, has four well known absorption bands at the UV-Blue region of the electromagnetic spectrum centered around 220 nm, 265 nm, 375 nm and 447 nm [40]. The two absorption bands with longer wavelengths come from π-π^* type transitions. The riboflavin emission is characterized by a fluorescent peak at 534 nm [41]. A phosphorescence emission of riboflavin, around 600 nm, was reported by Steele and Cusachs [42].

Singlet Oxygen Sensor

The singlet oxygen sensor green reagent (GR), a high selective sensor for singlet oxygen from Molecular [43, 44], was used. A 157 μM of GR water solution was prepared, as describe by Zhang *et al.* [12]. GR absorption band has maximum around 500 nm. It exhibits a faint green fluorescence, which increases in an environment within singlet oxygen. The GR maximum emission is at 525 nm [43, 44].

Methods

All samples were characterized by a UV-visible spectrophotometer from SHIMADZU (UV-3150/3101). Riboflavin photosensitizer and GR fluorescent emission were analyzed using an ISS spectrofluorimeter (PC1).

Silver nanoparticles morphology was determined by transmission electron microscopy (TEM). The Tecnai20 transmission electron microscope from FEI was explored.

The singlet oxygen production by riboflavin water solution (Rb) and by riboflavin-silver colloids, with pectin (Rb, Np, Pec) and without pectin (Rb, Np) were analyzed adding GR solution to 700 μl of the samples.

Blue LEDs system at 415 nm with 31 mW was adapted in the spectrofluorimeter. The riboflavin photosensitizer and silver colloids were excited by the blue LEDs during 2 min period. Immediately after the riboflavin-silver colloid excitation, the fluorescence of the GR sensor was detected by the spectrofluorimeter, using a 502 nm excitation.

Results and Discussion

Figure 1 shows the UV-visible absorption spectra of the silver colloids. A localized plasmon resonance peak around 412 nm is observed for the silver nanoparticles with and without pectin. One can notice that pectin does not introduce significant changes on the absorption spectrum of the silver colloids. A small shift (≤4 nm) of peak band indicates the presence of the pectin. In Fig. 1, the absorption spectrum of the riboflavin water solution is also presented.

The TEM images, in Fig. 2, show the spheroid morphology of the prepared silver nanoparticles. From the analysis of 100 nanoparticles at the TEM images a 13± 4 nm average diameter for the silver nanoparticles was determined. With the TEM images, no difference on the silver particles average size was observed for uncoated or pectin coated nanoparticle. In spite of the high energy of the electronic beam from the TEM can destroy the pectin coating [45] limiting the identification of the nanostructure organic shell, few TEM micrographs allowed pectin layer partner identification.

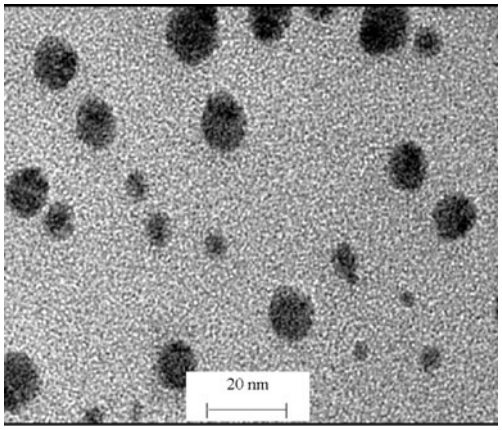

Fig. 2 TEM picture of silver nanoparticles

A zeta potential analysis was also performed indicating that the presence of pectin enhances the colloid stability and slightly reduces the superficial charge of the nanoparticle.

The fluorescence emission spectra of riboflavin (water solution, in silver colloid, in the silver/pectin colloidal solution) are presented in Fig. 3. On the fluorescence emission analysis blue light at 415 nm was used to excite the samples. One can observe, in Fig. 3, an enhancement of the riboflavin fluorescence when silver nanoparticles are present in the solution. A 1.3-fold enhancement was observed on colloids with bared silver nanoparticles. More efficient fluorescence amplification, 1.7-fold enhancement, was measured using silver-pectin nanoparticles.

The measured fluorescent spectra express the average interaction of riboflavin and silver nanoparticles. In the solution, each riboflavin molecule-silver nanoparticle

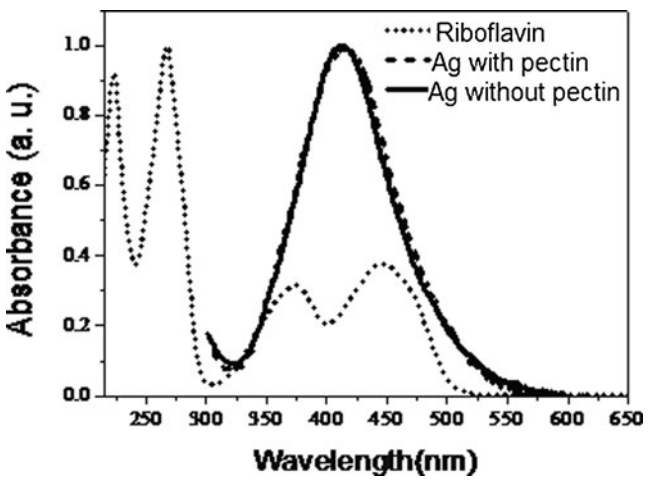

Fig. 1 UV–vis absorption spectra of colloidal solutions of silver nanoparticles with (*dashed line*) and without (*continuous line*) pectin. Absorption spectra of riboflavin (*dotted line*)

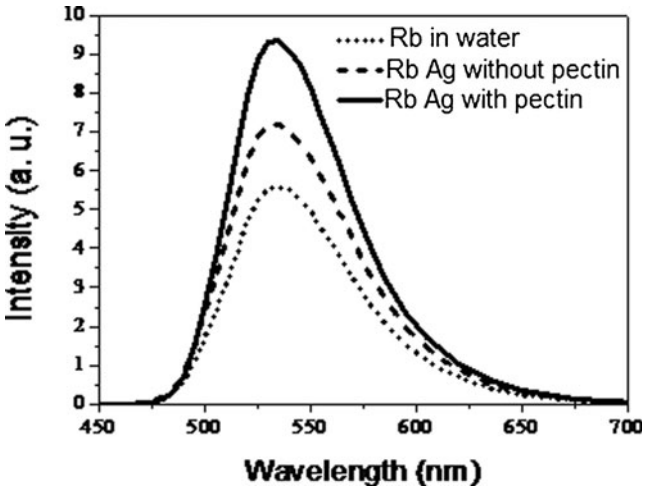

Fig. 3 Fluorescence emission spectra, λ_{ex}=415 nm, of riboflavin in water (*dotted line*); and of riboflavin in silver colloid with (*dashed line*) and without (*continuous line*) pectin

interaction can lead to quenching or to the enhancement of the photosensitizer molecule fluorescence. Quenching processes results from resonant energy transfer which depopulates the molecule excited state with an additional nonradiative decay rate, k_r, given by [30, 46]:

$$k_r = \frac{1}{\tau_0}\left(\frac{R_o}{r}\right)^6. \quad (1)$$

Where, R_o is the Forster distance, τ_o is the fluorophore lifetime in the absence of metal nanoparticles, and r is the riboflavin molecule-silver nanoparticle distance.

Therefore quenching process is expected to be effective for small riboflavin molecule-silver nanoparticle distances (<10 nm). Field enhancement effects (enhanced excitation) are expected to be dominant for longer distances [30, 46].

In a suspension, the photosensitizer molecule-silver nanoparticle distance can be established by the silver nanoparticle and photosensitizer concentrations, by the thickness of the pectin layer and by the molecule-nanoparticle electrostatic interaction. Analyzing the fluorescent behavior of samples with similar concentration of silver nanoparticles (with or without Pectin) and photosensitizers, one can infer that pectin structure play an important role by limiting the distance between silver nanoparticles and riboflavin molecules and establishing a higher amplification of the photosensitizer fluorescence.

The enhanced excitation of riboflavin molecule, as a consequence of the photosensitizer-silver interaction, can also induce an increase on the medium singlet oxygen production [47, 48].

To quantify the enhancement of the riboflavin singlet oxygen production induced by the silver colloids, the GR fluorescence were measured. The emission spectra of the GR solutions were obtained by using 502 nm as excitation wavelength. Although the riboflavin absorption contribution at 502 nm is not strong, a faint emission of the photosensitizer can also be identified.

Figure 4 shows the emission spectra of the GR sensor in both riboflavin water solution and riboflavin-silver/pectin colloid. One can observe a 1.7-fold enhancement of the sample emission induced by the silver/pectin nanoparticles. Quantification of metal enhancement of singlet oxygen ($^1O_{2,MEO}$) generation was obtained by evaluating the areas under the curves, at Fig. 4, and it value is given by Zhang et al. [23]:

$$^1O_{2,MEO} = \frac{\int (GR, Rb, Np)_{after} d\lambda - \int (Rb, Np)_{after} d\lambda}{GR_{MEF}\left(\int (GR, Rb)_{after} d\lambda - \int (Rb)_{after} d\lambda\right)}. \quad (2)$$

Where $\int (GR, Rb, Np)_{after} d\lambda$ and $\int (GR, Rb)_{after} d\lambda$ are the integrated spectra of GR sensor in the riboflavin solution, with and without nanoparticles, after 415 excitation. The

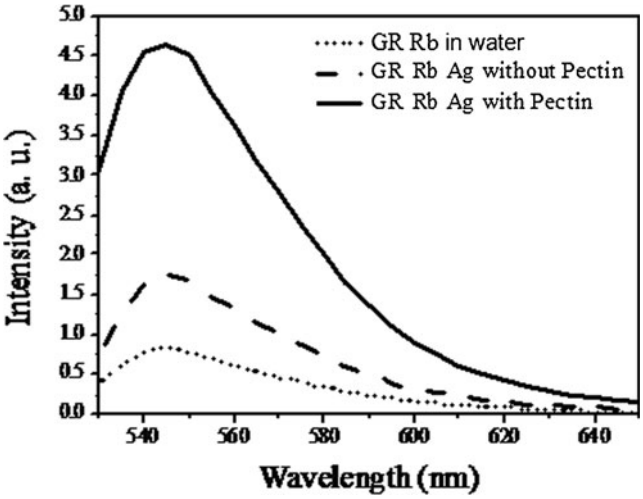

Fig. 4 Fluorescence emission spectra, λ_{ex}=502 nm, of GR in: riboflavin in water (*dotted line*); and of riboflavin in silver colloid with (*dashed line*) and without (*continuous line*) pectin, after exposure to blue light

fluorescence contributions of the riboflavin solution, with and without nanoparticles, $\int (Rb, Np)_{after} d\lambda$ and $\int (Rb)_{after} d\lambda$, after exposure to blue light are subtracted from the acquired spectra in Fig. 4. The fluorescence spectrum of GR can also be affect by interaction of GR sensor and the nanoparticles [29]. This contribution, GR_{MEF}, to the determination of the metal enhancement of singlet oxygen generation was considered in the analysis. Here GR_{MEF} is given by:

$$GR_{MEF} = \frac{\int (GR, Np) d\lambda}{\int (GR) d\lambda}, \quad (3)$$

where $\int (GR, Np)_{after} d\lambda$ and $\int (GR)_{after} d\lambda$ are the integrated spectra of GR sensor in water solution, with and without nanoparticles.

The metal-enhanced singlet oxygen generation was also measured on colloids of silver nanoparticles without pectin layer. Table 1 summarizes the obtained results. The silver – pectin nanoparticles induced a 1.8-fold enhancement of the singlet oxygen production by riboflavin solution. The results show that pectin improves both metal enhanced florescence (30.7 %) and metal-enhanced singlet oxygen generation (28.5 %) processes.

Table 1 Metal Enhanced Florescence (MEF) and Metal Enhanced singlet oxygen generation (MEO) for riboflavin-silver colloids. MEF and MEO values were calculated for riboflavin-silver colloids, with (Rb, Np, pectin) and without (Rb, Np) pectin shell

Sample	(Rb, Np)	(Rb, Np, pectin)
MEF[a]	1.3	1.7
MEO[b]	1.4	1.8

Conclusions

A new metal enhanced singlet oxygen generation solution-base platform was proposed for PDT applications. Here 13 nm silver nanospheres enclosed by a pectin layer were synthesized and it interaction with riboflavin molecule was analyzed. The singlet oxygen fluorescent sensor Green Reagent was used to monitor the singlet oxygen production in the colloid. The presence of pectin increases the biocompatibility of the silver nanoparticles and improves metal enhanced singlet oxygen generation and metal enhanced fluorescence at room temperature. It was reported a 1.7-fold increase in the riboflavin emission and a 1.8-fold enhancement in singlet oxygen production. Moreover, we observed that pectin improves MEF and MEO processes, by 30.7 % and 28.5 % respectively. Our results, exploring Riboflavin-Ag/pectin colloid, can lead to several applications of metal enhanced singlet oxygen generation solution-base platform on PDT, as antimicrobial action on oral cavity.

Acknowledgments We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support through the National Institute of Science and Technology of Photonics (INCT de Fotônica), to the Programa de Núcleos de Excelência (PRONEX-FACEPE/CNPq). We acknowledge the Laboratory of Non Conventional Polymers (UFPE) for the ISS equipment used.

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