Methylene blue aggregation in the presence of human saliva

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ABSTRACT

Photodymanic antimicrobial therapy (PAT) has been proposed to treat oral infections and the phenothiazinic compound Methylene Blue (MB) has been considered as a suitable photosensitizer for this application. MB is a known methachromatic compound and the dimerization process may lead to different photochemical reactions, into the oral cavity, where the complete isolation of the saliva may not be possible. The aim of this study is to monitor the dimerization process of MB in the presence of human saliva through absorption spectroscopy. Absorption spectra of 30μM MB solutions in water and in human saliva were recorded in the wavelengths ranging from λ=400nm to λ=700nm. The spectra were recorded immediately after mixture and 1min, and 5 minutes after blending. The results were evaluated by spectral analyses and through the calculus of the dimer/monomer ratio. The results demonstrated that immediately after mixture a hypochromic effect characterized by the diminishing on the total absorption in the visible range of the spectrum (λ= 400nm -700nm) is observed, but the aggregation process is not detectable. After 1 minute the ratio between dimer and monomer absorption increase and this increase became higher upon increasing the contact time. The results indicate that the addition of saliva into the mixture leads to and hypochromic effect follow by the dye aggregation. Aggregation is probably an important variable to be analyzed when choosing the pre-irradiation time in oral cavity application, because it may lead to different photochemical routes.

Keywords: Photodinamic therapy, methylene blue, dye aggregation, antimicrobial therapy, hypochromic effect, methachromasia.

1. INTRODUCTION

Photodynamic antimicrobial therapy (PAT) has been proposed as an alternative antimicrobial therapy, particularly, for the treatment of localized and superficial infections. Superficial and localized infections have been the main targets so far, due to the particularity of this therapy, which requires the association of a photosensitizer (Ps) agent or dye and radiation with the appropriate wavelength, delivered directly into the infected area. Therefore, localized infections are easier to treat than spread infections since the Ps can be locate directly into the target and precise illumination is straightforwardly performed[1].

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Oral cavity infections as periodontal disease, root canal infections and fungal infestation as candidiasis have been objects of study in PAT investigations, primordially due to microbial resistance and the use of systemic antibiotics to cure these pathologies, in view of the fact that they are localized, the systemic medication would not be necessary if an appropriate local strategy were available[2, 3, 4]. Furthermore even the currently used local strategies with antimicrobial agents can lead to microbial resistance, if inappropriate or prolonged uses are required[5].

PAT works through two distinct mechanisms known as Type I and Type II reactions. In the Type I mechanism the photosensitizer upon absorption of the radiation suffers an electron transfer reaction with the substrate generating reactive oxygen species (ROS) as superoxide, hydrogen peroxide and hydroxyl radicals. In a Type II reaction the Ps triplet state reacts with molecular oxygen to form a highly reactive ROS, the singlet oxygen, via energy transfer process. The quantum yield for Type I or Type II mechanisms depends on the competition between electron or energy transfer reactions. The prevalent mechanism will be dependent upon the dye photochemistry and its environment[6].

Methylene blue (MB) is a well-know photosensitizer and its photochemistry is mainly by a type II reaction[6]. MB absorption spectrum is concentration-dependent; dye molecules often form dimers or higher aggregates in solution. The dimerization increases with the amplification of the ionic strength and may increase or decrease due to the presence of charged interfaces, depending on the ratio between dye and interface. The different photochemical behavior of MB dimers and monomers has been reported and it may be an important factor that would determine the clinical effectiveness of this therapy[7]. Precedent *in vitro* study had showed that the presence of organic fluids (blood and saliva) decreased the PAT efficiency[8].

The oral environment is constantly bathing with fluids and the presence of saliva and blood may be unavoidable, thus one should understand the dye behavior in this environment, hence this study aimed to monitor the MB absorption characteristics in the presence of human saliva to provide further leads that may contribute to PAT development in oral cavity applications.

2. MATERIAL AND METHODS

The optical characteristics of the dye in the presence or absence of human saliva were obtained through absorbance spectra in a computer-interfaced double-beam spectrophotometer (Cary – 17D Spectrophotometer Conversion, On-Line Instrument System Inc, USA) in 1.0 cm optical path length quartz cuvettes.

Commercially available dye MB (Sigma-Aldrich, USA) was dissolved in commercially available deionized water in the concentration of 30μM. The tested sample was dissolved in deionized water mixed with 10μl of fresh collected human saliva obtained from healthy donors.

The donors were instructed to perform a complete oral cavity cleaning two hours prior to the sample collection. The cleaning consisted in a protocol involving flossing of all teeth, brush tong and teeth, and the use of an alcohol free mouth rinse.

After these procedures the volunteers were instructed to remain two hours without alimentation, only water was allowed.

Absorption spectra were obtained in the visible range from λ=400nm to λ=800nm, immediately after the mixture, 1min, and 5min after blend to evaluate the contact time influence on the optical characteristics of the dye-mixture. Control samples were obtained in the same timeline with MB solution in pure water. All the blanks were performed with the appropriated background solution to avoid the surroundings interference with the results.

The results were analyzed with appropriated software (Origin® Microcal Software Inc, USA). The spectrums were analyzed regarding dimer/monomer ratio according with the following formula for MB:

$$R[\frac{DA}{MA}] = A_{610nm} / A_{660nm} \qquad (1)$$

were R means the ratio; DA, dimer absorption; and MA monomer absorption, calculated in two different wavelengths, since the absorption of the MB dimer is around λ=610nm and monomer λ=660nm[9]. The spectral data was also directly compared.

3. RESULTS AND DISCUSSION

Figure 1 represents the absorption spectra of the dye in water and in water/saliva mixture. Figure 2 represents the calculated ration of $\frac{DA}{MA}$ in the MB solution in the presence or absence of human saliva.

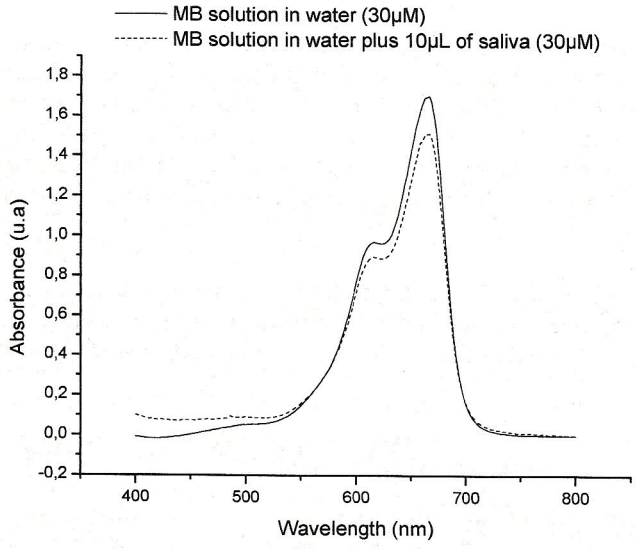

Figure 1. Absorption spectra of MB solution in water and water mixed with human saliva.

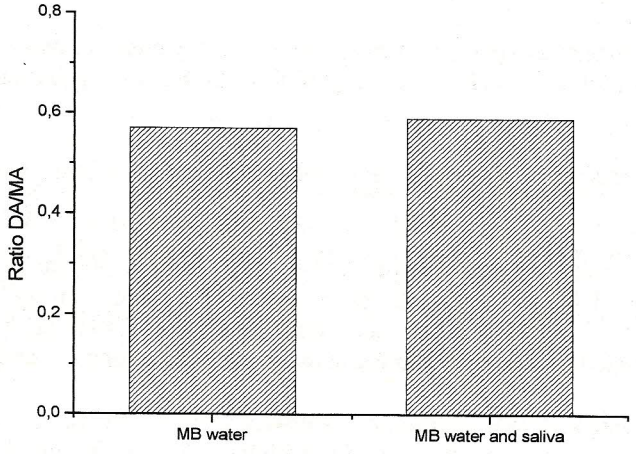

Figure 2. Dimer/Monomer ratio of MB solution in water and water mixed with human saliva immediately after mixture.

It can be observed in Figure 1 an immediate hypochromic effect after mixture. This effect may happen due to the interaction with membranes or other biopolymers [6,7,10] present in a saliva sample owing to the presence of bacteria, and epithelial cells and also it may be an optical effect caused by a higher scattering presented by the saliva sample, although all the blank samples were performed with the appropriated solution, it is not possible to certify that all the samples presented the same amount of cells and bacteria.

In Figure 2 it may be observed that combined with the hypochromic effect the dimerization process also takes place immediately after mixture. As the ionic strength is higher in a solution with saliva the increased dimerization would be expected[7].

Figure 3 represents the absorption spectrum of the samples containing saliva in 3 different timelines. It can be observed an increased hypochromic effect in the sample occurring with the time. This effect, most likely, is due to the formation of MB reduced form leuco-methylene blue (LMB)[10]. Upon intercalation with DNA especially with guanine bases the LMB formation has been reported[10].

The appearance of LMB has many possible routes. As aforementioned the DNA may takes its part, but also the effect may be attributed to the action of reduced co-enzymes as NADH and NADPH[11]. In addition to the observed effect may also be a result of the interaction with numerous proteins such as secretory IgA, lactoferrin, agglutinin, mucins, along with lysozymes and several peptides[12].

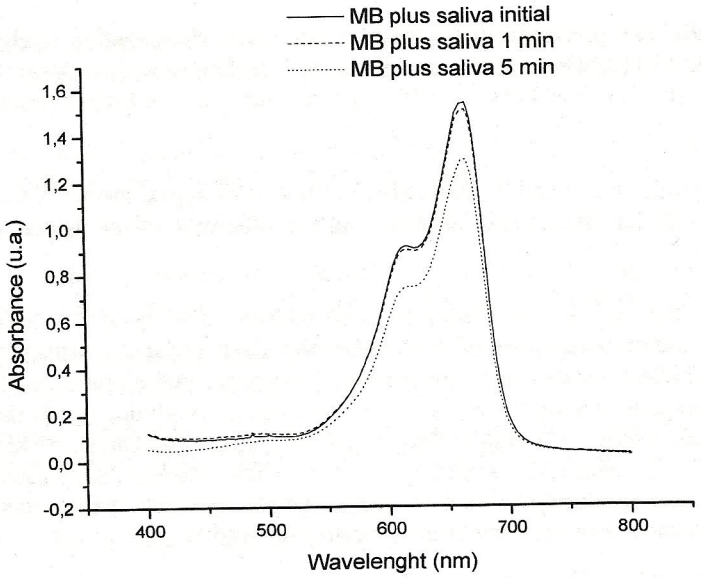

Figure 3. – Methylene Blue Solution in the presence of human saliva in three different timelines.

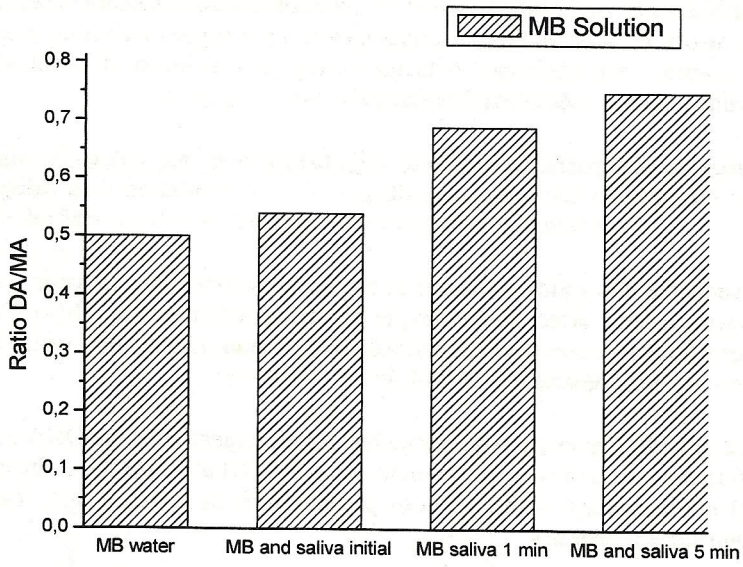

Figure 4. – Calculated ratio of DA/MA in a MB solution in water and in the presence of human saliva in different timelines.

According to the data presented in Figure 4, an increased dimerization is also observed upon increasing the contact time between the MB solution and human saliva. As MB dimers tend to have a higher quantum yield for Type I reaction the longer the contact time between MB solution and saliva a higher probability for Type I reaction will prevail[13].

As singlet oxygen is considered the most efficient reactive oxygen species[14], these optical alterations of the MB solution may be responsible for the decrease in photodynamic efficiency of the dyes in the presence of human saliva as reported previously[8].

The antioxidant function of saliva has also to be considered in an *in vivo* application. Uric acid (UA) that is present in saliva is the major component of the total antioxidant system, constituting 70% of the total antioxidant capacity[15]. Salivary peroxidases catalyze the oxidation of thiocyanate in the presence of hydrogen peroxide (H_2O_2) into hypothiocyanate, therefore deactivating the H_2O_2[12], The function of hypothiocynate in the oral cavity has been discussed in relation to antimicrobial activity. The role of thiocyanate in hydroxyl radical deactivation under acid conditions is also a matter of investigation[12]. Therefore, along with dye photochemistry changes, the presence of saliva may act as a barrier to the action of the ROS formed during the photodynamic process. By now, the isolation of the target area, avoiding as much as possible, the presence of saliva, may be an apposite alternative.

This knowledge may represent a new parameter to be taken into account when choosing clinical parameters to test PAT, because the pre-irradiation time (PIT) used in "*in vitro*" studies varies from periods as short as 1mim[3] to longer periods that may be as long as 30min to one hour[14].

In the presence of an organic fluid as saliva a shorter PIT may represent an increased chance of having a more efficient photosensitizer when using MB.

4. CONCLUSION:

The results indicate that the addition of saliva into the MB solution leads to and hypochromic effect follow by the dye aggregation. It may be an important variable to be analyzed when choosing the pre-irradiation time in oral cavity application.

5. ACKNOWLEDGMENT:

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