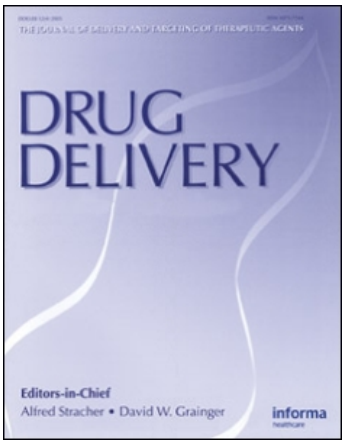

Drug Delivery
Publication details, including instructions for authors and subscription information:
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Evaluation of Chitosan Gel as Antibiotic and Photosensitizer Delivery

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Online Publication Date: 01 September 2008

To cite this Article Fontana, Carla Raquel, dos Santos, David Sotero, Bosco, Joseane Maria, Spolidorio, Denise M. and Chiérici Marcantonio, Rosemary Adriana(2008)'Evaluation of Chitosan Gel as Antibiotic and Photosensitizer Delivery',Drug Delivery,15:7,417 — 422

To link to this Article: DOI: 10.1080/10717540802007433

URL: http://dx.doi.org/10.1080/10717540802007433

Drug Delivery, 15:417–422, 2008

ISSN: 1071-7544 print / 1521-0464 online
DOI: 10.1080/10717540802007433

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This work suggests the use of chitosan gel imbued with the photosensitizer Photogem and with the antibiotic Tetraclin as a possible drug delivery system. The results reveal a decrease in the photosensitizer level of toxicity. Besides, the interaction between Photogem® and chitosan gel causes a red shift in the photosensitizer spectrum, increasing its absorption in the therapeutic window (600–700 nm). These characteristics indicate this compound as a promising natural polymer-based photosensitizer carrier for photodynamic therapy. In summary, our results show that pure and doped chitosan gel may have potential application for antimicrobial action, being an excellent alternative when local control of the drug administration, provided by the gel, is required.

Keywords Antimicrobial, Chitosan/Chitin, Drug Release, Less Toxicity, Photosensitizer, Spectroscopy

1. INTRODUCTION

Chitosan is a hydrophilic biopolymer obtained from chitin, a constituent from the exoskeletons of arthropods, which exhibits several promising biological activities. This bio-polymer presents interesting characteristics, such as nontoxicity, good biocompatibility (Hirano and Noishiki 1985), bioabsorbility (Muzzarelli et al. 1988), and biodegradation, and can be used as a film, gel, or solution (Shin et al. 2005). It has been demonstrated that chitosan presents antimicrobial and antifungal activity against many microorganisms, being commercially used as a bioinsecticide and as an agent for fruit conservation (Choi et al. 2002). In addition, it has been demonstrated to be hemostatic (Rao and Sharma 1997) and bacteriostatic (Tarsi et al. 1997; Je and Kim 2006). This material has been used as a bioadhesive and permeable agent, which allows its application for drug delivery (Takeuchi et al. 2003; Needleman et al. 1998), being specially proposed as an antibiotics releaser bacterial reduction in oral applications (Liu et al. 2004; Decker et al. 2005). Moreover, chitosan polymer has shown to be a promising biomaterial when used for bone repair (Shin et al. 2005; Lee et al. 2002; Muzzarelli and Muzzarelli 2002; Park et al. 2003) and tissue regeneration (Gerentes et al. 2002; Kuo et al. 2006; Pang et al. 2005), prompting recent interest in antibacterial and immunostimulative effect (Moon et al. 2007).

Several controlled drug delivery systems have been developed to enhance the drug efficacy and minimize the side effects (Chen et al. 2005). The application of chitosan gel, for example, can provide a long stay in the target tissues, adequate drug penetration, high efficacy, and acceptability (Ikinci et al. 2002; El-Samaligy et al. 2006). Besides being an interesting option as a local drug delivery system, the antimicrobial action of the chitosan gel and its molecular interaction with the drugs can further enhance the bactericidal effect (Liu et al. 2004). Considering all the interesting characteristics of chitosan previously mentioned, we propose in this study the use of chitosan gel not only to deliver drug for antimicrobial agents (antibiotics), but also to deliver photosensitizers with less dark toxicity for

Received 2 October 2007; accepted 17 December 2007.
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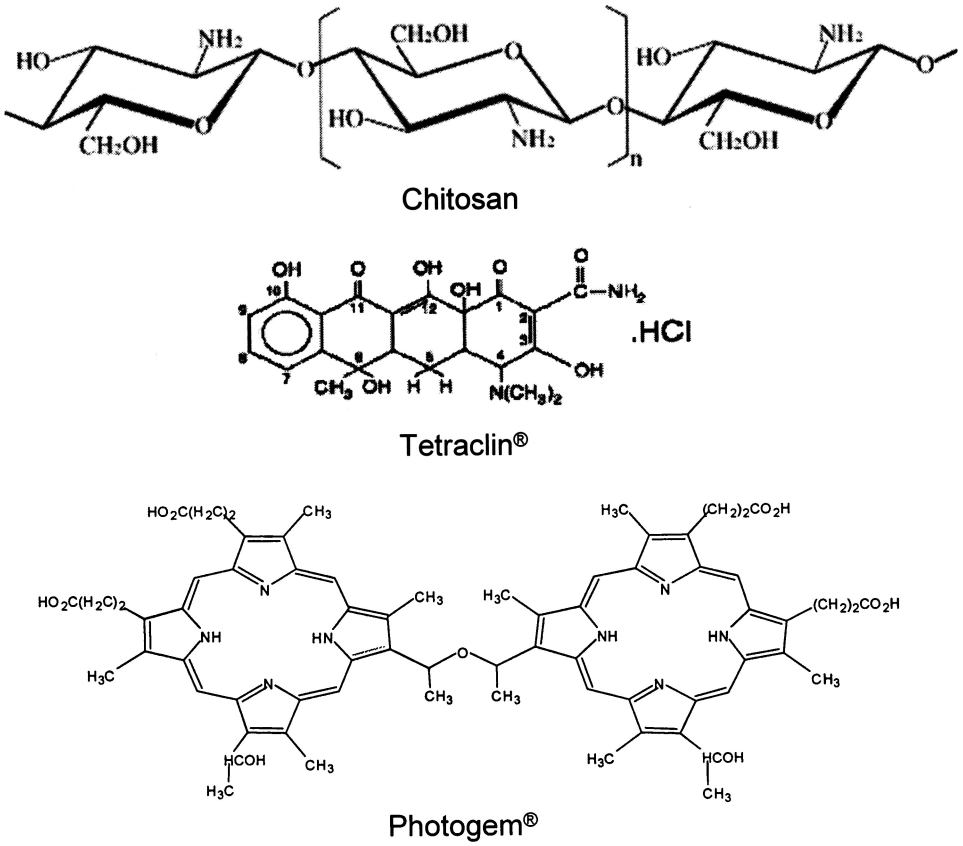

FIG. 1. Molecular structure of molecular compounds studied here.

photodynamic therapy (PDT). We present here the formulation of pure chitosan gel as well as doped with Tetracycline (Tetraclin) and Photogem, drugs commonly and successfully used in antibiotics and PDT treatments, respectively. Tetracycline (Tetraclin) will be used in this study as a control of bacterial reduction action. The antimicrobial activity of those compounds, whose molecular structures are shown in Figure 1, were studied using the disk diffusion method in *Staphylococcus aureus*, an opportunist pathogen found in the microbiota of mucous membrane (buccal, nasal, and aural) and human skin, capable of causing serious infection when penetrated into the human organism. The possible advantages of such approach are discussed in this paper.

2. MATERIALS AND METHODS

2.1. Chitosan

Chitosan, obtained from shrimp chitin (an *N*-acetylglucosamine polymer), was provided by Cyrbe do Brasil Corporation. It has an average molecular weight of 500,000 g/mol, as determined by viscometry, and a 75% degree of deacetylation, as obtained from ^{1}HNMR measurements (Signini and Campana 1999). It was purified twice by solubilization in a 1% aqueous acetic acid solution, filtered with filter paper, precipitated with a 1% aqueous sodium hydroxide solution, and washed extensively with deionized water until neutral pH was reached. Hydrochloroauric acid was purchased from Aldrich and used without further purification. All glassware was thoroughly cleaned using detergent, aqua regia, and copious rinsing with deionized water (Santos et al. 2004).

2.2. Microorganism

The culture medium agar Mueller–Hinton, selective for *S. aureus*, was poured into Petri dishes (100 × 20 mm). After the agar solidified, the plates were wrapped and kept at 4°C for 3 days before the experiments.

S. aureus strain (ATCC 25923) employed in this work was suspended in the tryptic soy broth (Difco) for 24 h at approximately 37°C. For the adjustment of inoculum density, cell numbers were estimated through Mc Farland standard being at approximately 10^8 microorganisms/ml. Bacteria cultivation was carried out in laminar flux, in which 200 μl of *Staphylococcus aureus* broth was added to each Petri dish. This microbial suspension was uniformly spread on the agar with using a sterile Drigalski loop.

2.3. Disk Diffusion Method

The antimicrobial properties of the chitosan gels and solutions studied here were tested with the disk diffusion method,

as standardized by the National Committee for Clinical Laboratory Standards (Odland et al. 2000). This experiment was carried out with the following compounds: (1) chitosan gel (CG), (2) chitosan gel doped with tetracycline (Tetraclin–Teuto) (CG+T), (3) chitosan gel doped with Photogem (CG+P), (4) tetracycline solution (T), and (5) Photogem solution (P). All compounds were prepared previous to each test, according to the subsequent description. Initially, aqueous solutions of hydrochloride tetracycline 100 mg/ml and Photogem 5 mg/l were produced and light protected. These solutions were made using Mili-Q water. A chitosan (Cyrbe do Brasil) slurry was prepared adding 0.25 g of chitosan, purified as previous described by Santos et al. (2004), into 9 ml of 0.16 mol/l ascorbic acid aqueous solution. The chitosan gel doped with the drugs were prepared by adding 9 ml of the solutions mentioned above to the chitosan slurry followed by manually stirring by using a glass stripe. This procedure results in gels with satisfactory aggregation and easy manipulation.

Four sterile absorber paper disks (5 mm diameter) were placed on the agar plates inoculated with the *S. aureus*. These disks were positioned equidistantly from each other, to avoid superposition of the inhibition halos. Using an automatic pipette, 2 μl of compounds previously described were applied on top of each disk. The plates were kept at room temperature for 10 min to allow the material diffusion. Subsequently, samples were grown aerobically at 37°C up to 48 hr. Inhibition zones around the studied compounds were measured after 24 and 48 hr of incubation. A disk without any compound was employed as a control group for our experiment. For each group, 10 repetitions were carried out, in order to provide statistical analyses of the obtained results.

2.4. Spectroscopy

The ultraviolet-visible absorption spectra of Photogem in solution and chitosan gel were recorded from 400 to 800 nm using quartz cuvets on a Cary 17 spectrophotometer.

3. RESULT AND DISCUSSION

The diameter of the inhibition zone was based on lack of bacterial growth, measured after 48 h. However, after 24 h the observed inhibition halo diameters had already reached their final values. The diameters of the halos, representing the bacteria inhibition, were measured using a pachymeter. When halos with no circular symmetry were observed, the diameter was taken as being the average between the largest and smallest axis.

Table 1 presents the statistical study of the inhibition halos' diameters using ANOVA and TUKEY with $p < 0.01$, for the experimental groups C, T, CG + T, and CG.

In Figure 2 it is shown the inhibition halos' average diameters for the compounds T, CG+T, and CG (data from Table 1). The control group (blank sample) exhibited no inhibition halo. In contrast, the chitosan gel (CG) shows a considerable halo, around 12 mm, revealing the efficiency of the chitosan as an antimicrobial agent for *S. aureus*. Previous studies have reported comparable results using different types of bacteria. According to Liu et al. (2004) and Didenko et al. (2005) chitosan possesses reactive positively charged amino groups that interact with the bacterial cell membranes, resulting in the leakage of intracellular constituents and alteration of cell permeability, which leads to the microorganism death (Liu et al. 2004; Je and Kim 2006; Moon et al. 2007). The results for tetracycline in solution (T) and in the chitosan gel (CG + T) present the highest diameters for the inhibition zone, around 32 mm. This result reveals that the association of chitosan with tetracycline did not affect the efficiency of the antibiotic, as demonstrated by similar halo growths observed in Figure 2. The absence of any accumulative effect of the chitosan gel on the tetracycline bacteriostatic effect is probably related to the faster action of tetracycline's, once only the chitosan gel also presented significant bacterial reduction, as shown in Figure 2. Besides providing antimicrobial action, the chitosan gel can also be applied as a tetracycline delivery system for easier administration, with potential application in Periodontology.

TABLE 1
Means inhibition halos' diameters (d), standard deviation (SD), and standard error (SE) of selected groups: C, T, CG + T, and CG

Group	d (mm)	SD	SE
Control	0^a	0	0
T	33.65^b	2.05548	0.65
CG + T	32.05^b	1.34268	0.42459
CG	11.41^c	0.49989	0.15808

(a, b, c) indicate a significant difference between groups (ANOVA, Tukey test, $p < 0.01$).

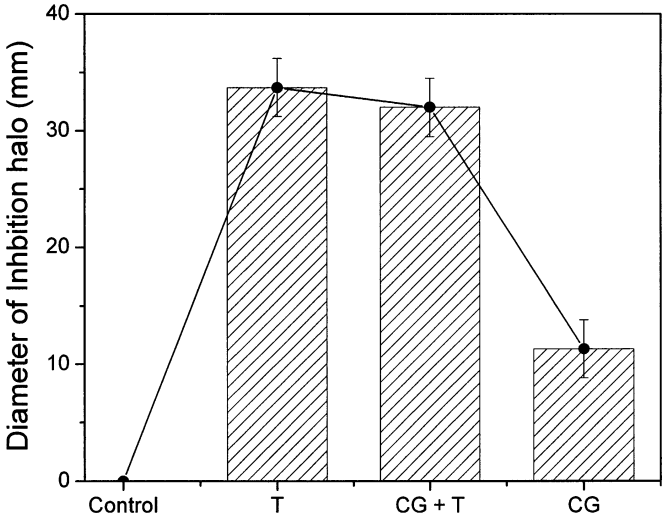

FIG. 2. Average diameter of the inhibition zone for tetracycline in solution (T), chitosan gel doped with tetracycline the (CG + T), and chitosan gel (CG). The first data illustrates the absence of inhibition observed for the control group. The error bars are given by the confidence intervals of statistical test.

TABLE 2
Means inhibition halos' diameters (d), standard deviation (SD), and standard error (SE) of selected groups: C, P, CG + P, and CG

Group	d (mm)	SD	SE
Control	0^a	0	0
P	20.85^b	1.5465	0.48905
CG + P	9.85^c	1.45392	0.45977
CG	11.41^c	0.49989	0.15808

Different letters (a, b, c) indicate a significant difference between groups (ANOVA, Tukey test, $p < 0.01$).

In order to further study the promising use of chitosan as a local drug delivery system, specifically for photodynamic therapy, we produced gels imbued with Photogem. Table 2 presents the statistical study of the inhibition halos' diameters using ANOVA and TUKEY with $p < 0.01$, for the experimental groups C, T, CG + T, and CG.

In Figure 3 we present the results of the chitosan gel as a vehicle for the photoactive drug (Photogem). Figure 3 presents the average diameter of the inhibition zone for Photogem in solution (P), chitosan gel doped with Photogem (CG + P) and chitosan gel (CG). It can be seen that Photogem solution (P), in the concentration employed here, acts against bacteria growth forming an inhibition halo of 20 mm, even in the dark. However, when Photogem® is associated with chitosan (CG + P) the bacterial reduction efficiency decreased, presenting an inhibition halo of 10 mm. This effect can probably be associated with the interaction between chitosan and Photogem, which is a porphyrin whose acid groups are well known to interact with chitosan, hindering the Photogem® toxicity. Furthermore, as can be seen by comparing the results achieved in Figure 3 for chitosan doped with Photogem (CG + P) and chitosan Gel (CG), the bactericidal action of CG is preserved when Photogem is added. The availability of increasing the photosensitizer content in the chitosan gel without raising the dark toxicity level is a desirable feature for PDT applications, in which the main antimicrobial action must happen only when the photosensitizer is activated by proper light irradiation, resulting in a cytotoxic effect that causes damage to the microorganism or target-cell (Wood et al. 1999; Wainwright 1998; Hamblin and Hasan 2004; Pfitzner et al. 2004; Rovaldi et al. 2000).

With the aim of verifying the interaction between chitosan and Photogem, the UV-Vis absorption spectra for the Photogem solution (a) and chitosan gel doped with Photogem (b) were obtained as shown in Figure 4. The two bands observed are due to the Photogem (porphyrin). The band in the ultraviolet region, around 400 nm, corresponds to the B (Soret) band, while the structure around 550 nm is known as the Q band. Both transitions involve $\pi - \pi^*$ from the porphyrin ring (Kalyanasundaram 1992). As expected due to the interaction between the porphyrin acid groups and chitosan, a 50 nm red-shift in the UV-vis absorption spectrum of the doped chitosan gel was observed. Albeit such a shift was observed, it is clear from Figure 4b that the characteristic features (B and Q Band) from the porphyrin are still present, indicating that no compound degradation occurred. Besides decreasing the toxicity as previously mentioned, Figure 4 shows another advantage of using the chitosan

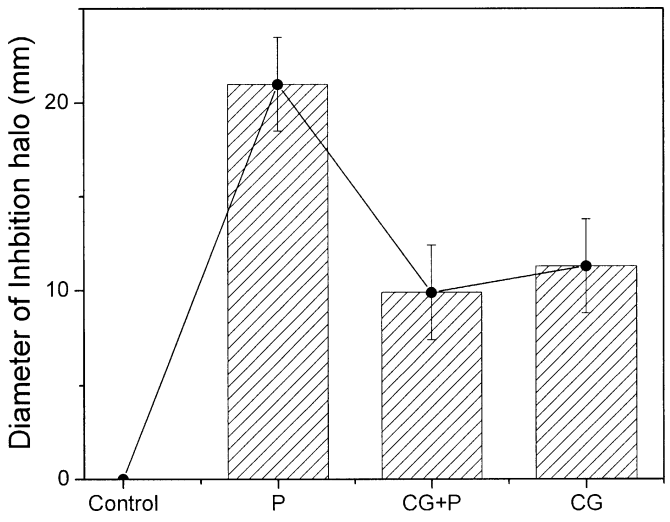

FIG. 3. Average diameter of the inhibition zone for Photogem® in solution (P), chitosan gel doped with Photogem® the (CG + P), and chitosan gel (CG). The first data illustrates the absence of inhibition observed for the control group. The error bars are given by the confidence intervals of statistical test.

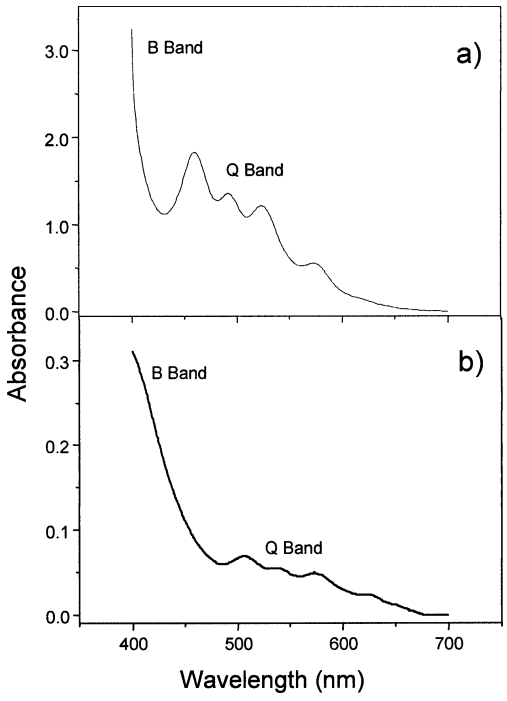

FIG. 4. Absorption spectra for Photogem® solution (a) and chitosan gel doped with Photogem® (b).

gel-Photogem compound. The red-shift caused by the interaction with chitosan increased the Photogem absorption in the 600–700 nm region, in which human tissues are less absorptive. (Rogers et al. 2003; Ion et al. 1998). Therefore, the compound studied here enhances the photoactive drug light absorption in a suitable way for PDT. No changes in these absorbance spectra were observed by increasing the compounds temperature up to 60°C (results not shown), revealing that no thermal degradation will occur upon laser irradiation.

4. CONCLUSIONS

The obtained results demonstrated that chitosan gel pure and prepared with tetracycline present antimicrobial effect. In addition, the chitosan gel/Photogem® association revealed an increase in the photosensitizer absorption in therapeutic window (600–700 nm), allowing the use of light sources with deeper penetration into tissues, which can lead to increasing PDT efficiency. This combination also decreased the photosensitizer toxicity in the dark, another desirable feature for dye release in photodynamic therapy. Furthermore, the gel formulation has additional advantages, when compared with solutions, such as better control and more precision of local drug administration.

ACKNOWLEDGMENTS

Financial support from FAPESP (Fundação de Amparo á Pesquisa do Estado de São Paulo), CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) are gratefully acknowledged. We also thank Prof. Dr. Vanderlei Salvador Bagnato for furnishing Photogem® and Prof. Dr. Cleber R. Mendonca and Dr. Leonardo De Boni for helpful discussion.

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