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Mechanical Properties of Chitosan Incorporated in Maxillofacial Silicone and its Anti Candidal Activity *In Vitro*

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ABSTRACT

Background: One of the major problem associated with the use of maxillofacial silicone material is microorganisms and fungal growth especially Candida albicans, which can result in chronic Infection, inflammation and degradation of silicone material that's why the development of antimicrobial silicone elastomer became so important. So a multiple studies are performed trying to improve this property, this study concentrated on incorporating chitosan micro particles into the silicone matrix.

Aim of the Study: The aim of this study was to evaluate the effect of adding different concentrations of chitosan micro particles into silicone matrix on antifungal activity and some mechanical properties as tear and tensile strength.

Materials and Methods: Chitosan particles were added in different concentrations (1.5%, 2.5%, and 3.5% by weight) into room temperature vulcanized silicone elastomer material. 220 specimens were made and divided according to the test to be performed into four groups. Viable count test and disk diffusion test was performed to evaluate the antifungal properties of silicone material incorporated by chitosan. Tear and tensile strength of the maxillofacial silicone was also tested.

Results: The results of viable count showed a highly significant decrease in colony forming unit of C. albicans in experimental groups contrast with control group. In disk diffusion test the measurement of inhibition zone was concentration dependent so the inhibition zone increased as the concentration increased. There was a highly significant increase in the mean value of tear strength while there was significant decrease in the tensile strength of silicone after chitosan addition.

Conclusion: The addition of chitosan micro particles into RTV silicone result in developing anti-candidal activity for maxillofacial material. Also this addition resulted in increased tear strength while the tensile strength decreased.

Key words: Chitosan, Maxillofacial silicone, *Candida albicans*, Mechanical properties

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INTRODUCTION

Head and neck malformations may result from Congenital or developmental anomaly, trauma or cancer resection [1].

These anomalies can be managed either surgically by plastic repair conducted on living tissues or artificially by alloplastic repair. In the favourable circumstances, the treatment of choice is the plastic repair. It is more desirable than the artificial repair with a maxillofacial prosthesis [2].

However, when functional and esthetic demands cannot be surgically fulfilled because of insufficient residual hard and soft tissue, reduction in vascularization and possibility of unwanted effects of treatment modalities, limitation of the surgical treatment may appear; thus, practical and attractive alternative by fabrication a facial prosthesis considered to be the best solution [3].

Taking in mind social and psychological pressures of patients with facial defects, the use of maxillofacial prosthetic materials is rapidly increased to enhance both esthetic and functional deficiencies found in the present materials [4].

The maxillofacial prostheses surfaces are made of silicone exposed to soft tissues, saliva and nasal secretion. These body fluids may cause colonization of different microorganisms on their surfaces leading to silicone elastomer degradation or infection. One of the most common colonizing microorganisms is *Candida albicans*. Plaque which contains high *C. albicans* levels becomes acidic as a result of candidal metabolism which may later produce inflammation of mucosal surfaces or microbial colonization on the hard tissues [5]. There is a continuous increase in the percentage of drug consumption because of the development of drug resistance by microbial agents [6].

This opportunistic pathogen causes great problems, it is resistant to most antimicrobial products, namely amphotericin-B, which is regarded the standard choice for

the treatment of mycoses. Despite still being regarded the drug of choice against *C. albicans*, these antifungal agents are being reported as inefficient with many cases of resistances, particularly to fluconazole, being observed. This problem has led to seek for alternative drugs to be used in the treatment of *C. albicans* infections [7].

In this study an attempt was made to fabricate maxillofacial silicone material with antimicrobial effect especially against *C. albicans* by incorporation Chitosan micro-particles. Chitosan, a natural extract from chitin, is a polysaccharide that has been proven to have a broad spectrum of antimicrobial activity that encompasses effect against fungi, yeast and bacteria. While newest studies have revealed a significant antibiofilm activity upon several microorganisms, including *C. albicans* [7].

MATERIALS AND METHODS

Room temperature vulcanized VST-50F silicone elastomer (Factor II Inc., USA) was used in this study. Chitosan powder (Cheng Du Micxy Chemicai., China) was incorporated into the maxillofacial silicone in different percentages (1.5%, 2.5% and 3.5% by weight). A total of two hundred and twenty (220) specimens were prepared and divided into four groups according to the test to be performed. FTIR analysis was performed to determine if there is any chemical reaction between chitosan and the maxillofacial silicone.

Mold making for specimens' fabrication

Samples dimensions were designed using AutoCAD 2015 then processed by the CNC machine to form the matrix part of the mold into which the material was poured. Matrices were four clear acrylic sheets of 2.2 ± 0.05 mm thickness they were machined according to the sample shape of each test [8].

Chitosan and maxillofacial material mixing and pouring

The chitosan powder was first weighted followed by the addition of accurate weight of silicone (part A) to prevent dispersion of the filler. The silicone with chitosan were mixed by a vacuum mixer for 10 minutes, the vacuum was shut off for the first three minutes to avoid suction of the Chitosan powder and then turned on for the remaining 7 minutes at 360 rpm speed and vacuum value of -10 bar. Then the cross linker (part B) added and mixed again by vacuum mixer for 5 minutes [9].

The silicone then poured into the mold cavity. The cover part was placed over the mold and fixed in place with screws and nuts at the corner and G-clamps at the mold borders (Figure 1). The material was allowed to set at room temperature (23°C ± 2°C) for 2-4 hours according to manufacture instruction, then molds were opened and the silicone sample cleaned and washed under tap water to remove remaining separating medium and then dried with a paper towel [9].

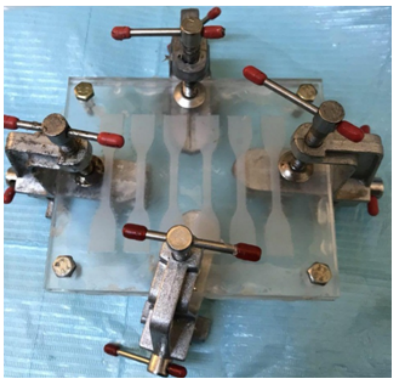

Figure 1: Mold assembly after silicone pouring

Evaluating the antifungal activity of chitosan loaded silicone specimens using viable count test

Specimen fabrication

Mold prepared with Specimens dimensions of 10 × 10 × 2.3 mm (length, width and thickness respectively) [10].

Then mixing and pouring of chitosan loaded silicon to obtain special specimens used to estimate viable count of *C. albicans*.

Isolation of *C. albicans*

C. albicans was gained from the oral cavity of 16 patients with signs of oral thrush and denture stomatitis. Isolation of *Candida* from the oral cavity with the use of a smear was taken by a swab [11].

This includes gentle rubbing of the lesion by a sterile cotton swab, and then inoculating a primary isolation medium like Sabouraud dextrose agar [12].

These swabs were cultured on Sabouraud dextrose agar and then aerobically incubated at 37°C for 24-48 hours after that kept at 4°C for more investigation [13].

Identification of *C. albicans*

Candida was identified by colony morphology on SDA which appear creamy, smooth, pasty convex [13], and by using Gram stain method in microscopical examination [14], also, germ tube formation procedure was utilized [15], and the final confirmation was done by biochemical way by using API Candida system (bioMérieux).

Evaluating viable count of *C. albicans*

To determine the antifungal activity of the chitosan/ silicone composites, *C. albicans* was diluted in 0.9% NaCl, and a candidal suspension of about 107 CFU/ml (0.5 McFarland standards) was made using a McFarland densitometer. Each chitosan/silicone specimen was putted in a tube containing 9.9 ml of Sabouraud dextrose broth, into which were dispensed 100 µl of the fungal suspension. The final cells concentration was 105 CFU/ml [16].

Then incubated for 24 hours at 37°C, after that 100 µl of each mixture was placed in 9.9 ml of NaCl (0.9%) and tenfold dilution was applied. 100 µl was taken from the second dilution and spread on SDA and aerobically incubated for 24 h at 37°C (Figure 2).

This procedure had been repeated after 14 days and 30 days of specimens' storage in artificial saliva at 37°C.

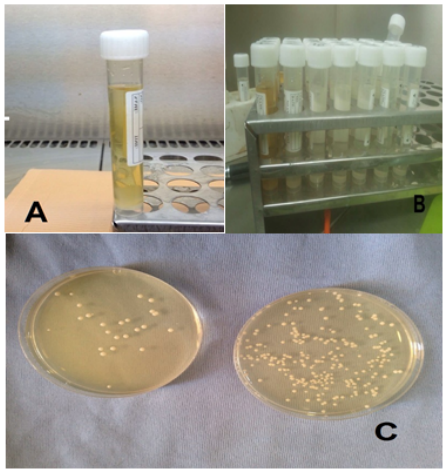

Figure 2: (A) Placement of specimen in the broth, (B) Serial dilution, (C) *C. albicans* growth after 24 hours of spread plate

Evaluating the antimicrobial activity of chitosan loaded silicone specimens using disk diffusion test

Mold prepared with 10 cm width and length and 0.5 mm in thickness. After mixing and pouring of chitosan with silicone result in silicone sheet of 0.5 mm in thickness. Then a metal rod with 6 mm in diameter and sharp edge was used to cut small discs (6 mm) to be used in disk diffusion test. Mueller-Hinton (MH) agar containing 5 µg/ml methylene blue and 2% glucose was used [17].

The media was prepared depending on manufacturer's instructions. Five well isolated colonies of *C. albicans* from Sabouraud dextrose agar taken and suspended in 0.85% normal saline 5 ml to achieve turbidity of 0.5 McFarland. A sterile swab was immersed into the inoculum suspension and excess fluid was pressed out. Glucose Methylene blue-MH agar was swabbed carefully in 3 directions to enhance even growth on the surface of the agar plate [18].

The agar surface has been left for about 5 minutes, then the silicone disks (with and without chitosan) were placed on the agar surface and the plates were left at room temperature for 120 minutes for diffusion of the antifungal agents [19], then plates were incubated aerobically for 24 h. At 37°C, electronic digital caliper was utilized to measure the inhibition zone that appears around the disks.

Tear strength test

An angle test sample without nick was fabricated according to ISO 34-1:2015 specifications [20].

The sample with one apex (right angle) and two tab ends with a thickness of 2 ± 0.2 mm. The un-nicked angle sample is used to measure tear initiation and propagation; the stress was concentrated at the angle until tear was started; followed by more stresses which was responsible for propagation of tear.

Thickness of the sample was determined by a digital caliper in the area where tearing is estimated to occur (at the right angle). The sample was fixed in a computerized universal testing machine. Then the sample was stretched at a speed of 500 mm/min of grips separation until it tears completely. At break the maximum force was recorded. The tear strength, presented in N/mm, was calculated according the following formulation (1):

Tear strength=F/d (1)

Where, F is the maximum force in Newtons; d is the thickness in Millimeters.

Tensile Strength

Type 2 dumb-bell sample was fabricated According to ISO 37:2017 [21].

Sample thickness was determined at the centre and at both end of the testing length (narrow portion) by an electronic digital caliper. The average of them was used to measure the area of the narrow part of the sample. The width and the length of the narrow part were also measured.

The sample was fixed in a computerized universal testing machine. Then the sample was stretched at a speed of 500 mm/min of grips separation until it separated completely. The maximum force and at break were then recorded. The tensile strength, presented in MPa, was measured using the following formula (2):

Tensile strength=Fm/Wt (2)

Where, Fm is the maximum force in Newtons; W is the narrow portion width of sample in Millimetres; t is the sample thickness over the narrow portion in Millimetres.

RESULTS

The results of the study showed that there was no chemical reaction between chitosan micro particles and maxillofacial silicone material as FTIR (Fourier transform infrared spectroscopy) analysis (Figures 3 and 4).

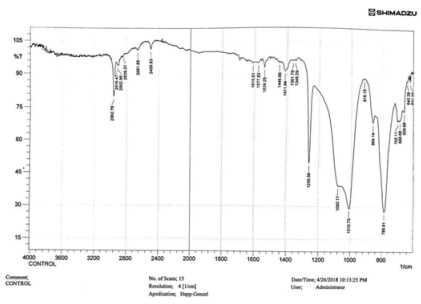

Figure 3: FTIR spectrum of VST-50F platinum RTV silicone elastomer before the addition of chitosan

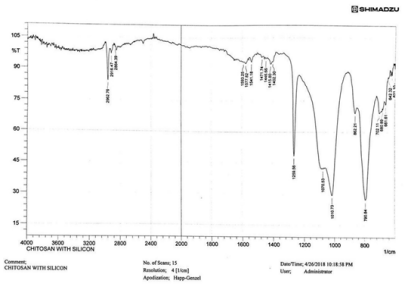

Figure 4: FTIR spectrum of VST-50F platinum RTV silicone elastomer after the addition of chitosan

The results of viable count test showed a highly significant decrease in colony forming unit of *C. albicans* in experimental groups (1.5%, 2.5%, 3.5% chitosan) contrast with control group. The decrease in colony forming unit of *C. albicans* was chitosan concentration dependent with lowest mean value at 3.5% chitosan (Figure 5 and Table 1). In disk diffusion test the result showed highly significant increase in the measurement of inhibition zone as the concentration of chitosan increase (Figure 6 and Table 2).

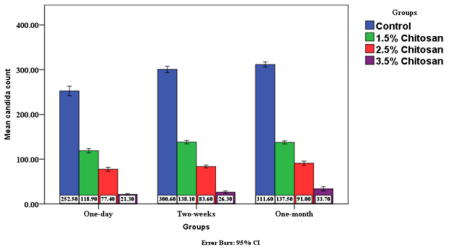

Figure 5: Bar charts showing mean values of CFU/ml at different periods of the study, (A) One day incubation, (B) After 14 days of incubation, (C) After 30 days of incubation

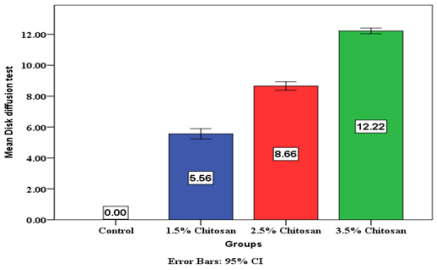

Figure 6: Bar charts showing mean values of disk diffusion test of different groups

Table 1: Comparison of the means of *C. albicans* count-using one way ANOVA-in different groups in each incubation period

Time		Sum of Squares	df	Mean Square	F	Sig.	Effect size (Partial eta square)
1 day	Contrast	290894.1	3	96964.69	1254.8	0	0.991
	Error	2781.9	36	77.275			
14 days	Contrast	418721.3	3	139573.8	3363.674	0	0.996
	Error	1493.8	36	41.494			
3 days	Contrast	431058.9	3	143686.3	3187.127	0	0.996
	Error	1623	36	45.083			

Table 2: Comparison the means of disk diffusion test-using one-way ANOVA-in different groups

Disk diffusion test	Sum of Squares	df	Mean Square	F	Sig.	Effect size
Between Groups	804.416	3	268.139	2513.715	.000 HS	0.998
Within Groups	3.84	36	0.107		-	
Total	808.256	39		-		

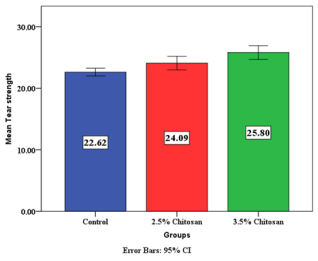

Figure 7: Bar chart of tear strength mean values of all study groups

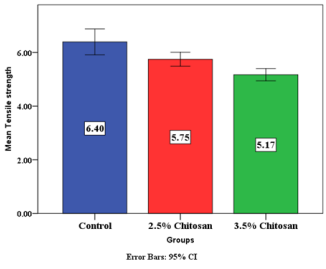

Figure 8: Bar chart of tensile strength mean values of all study groups

The results showed a highly significant increase in the mean value of tear strength after incorporation of chitosan into silicone elastomer for all experimental groups (Figure 7 and Table 3) with a significant decrease in the mean value of tensile strength of silicone after chitosan addition (Figure 8 and Table 4).

Table 3: One-way ANOVA for tear strength test results

Tear strength test	Sum of Squares	df	Mean Square	F	Sig.	Effect size (eta)
Between Groups	50.534	2	25.267	13.44	.000 HS	0.706
Within Groups	50.758	27	1.88			
Total	101.292	29				

Table 4: One-way ANOVA for tensile strength test results

Tensile strength test	Sum of Squares	df	Mean Square	F	Sig.	Effect size
Between Groups	7.524	2	3.762	16.28	.000 HS	0.739
Within Groups	6.239	27	0.231			
Total	13.762	29				

DISCUSSION

The maxillofacial prostheses surfaces made of silicone in contact with soft tissues, saliva and nasal secretion. These fluids may cause colonization of different microorganisms on their surfaces leading to silicone elastomer degradation or infection. One of the most common colonizing microorganisms is *Candida albicans*. Plaque which contains high *C. albicans* levels becomes acidic as a result of candidal metabolism which may later produce inflammation of mucosal surfaces or microbial colonization on the hard tissues [5].

In this study we select VST-50F RTV (room temperature vulcanized) silicone elastomer because of its favourable mechanical properties like tear and tensile strength so it can be removed from the adjacent tissues with little chance of distortion. It's an economical product with low viscosity when compared with other maxillofacial silicone material so it provide availability and ease in manipulation. It has a fast setting time about 4-6 hour at room temperature and there is no need for water bath and other equipment that used with heat vulcanized silicone. All these characteristics made it suitable for this study.

Chitosan, a natural extract from chitin, is a polysaccharide that has been proven to have a broad spectrum of antimicrobial activity that encompasses effect against fungi, yeast and bacteria. While newest studies have revealed a significant anti-biofilm activity upon several microorganisms, including *C. albicans* [7].

The effect of chitosan on candida growth

The results of the study for viable count test revealed a highly significant decrease in colony forming units/ml of *C. albicans* after incorporating the silicone material with chitosan micro particles which indicate the development of a composite with antifungal activity.

Disk diffusion test showed highly significant increase in the inhibition zone measurement as the chitosan concentrations increase with highest result for 3.5% chitosan (12.2 mm).

Multiple explanations have been proposed about chitosan antimicrobial activity; it was suggested that the interference between microbial cells and chitosan could be on the cell surface, which increase permeability and irregularity of cell walls lead to subsequent leakage of intracellular components which may prevent RNA and DNA synthesis and direct cell death. Another proposed action is the chitosan with its positive charge interacting with the negatively charged cell membrane which alters its permeability, allowing release of intracellular material to the media. The poly cationic characteristic of chitosan and its derivatives is fundamental for antifungal efficacy against *Candida* species. The high susceptibility of *C. albicans* to react with chitosan could be attributed to the presence of sialic acid in cell wall components, as terminal residues of glycoprotein glycan. Sialic acid-rich glycoprotein binds selectins (a family of cell adhesion molecules that bind sugar polymers) in humans and other organisms [22].

Also it was suggested that low concentrations of chitosan will cause: [23]

- An efflux of K^+ and stimulation of extracellular acidification.
- An increased transmembrane potential difference of the cells.
- An increased uptake of Ca^{2+}.

These effects are due to a decrease the negative surface charge of the cells. At higher concentrations, in addition to the efflux of K^+, it produced:

- A large efflux of phosphates.
- A decreased uptake of Ca^{2+}.
- An inhibition of respiration and fermentation.
- The inhibition of growth.

Another suggestion proposes that the chitosan acts as a chelating agent and minimize access of the fungi to

nutritional materials found in the surrounding environment. Another mechanism is the capability of chitosan to penetrate the cell wall of fungi and interact with DNA, which prevent mRNA transcription and protein and enzyme synthesis in the cells. Also chitosan can bond to metals, which are toxic products involved in the growth of microorganisms [24]. There for the chitosan considered to be more efficient in reducing the *Candida albicans* growth.

The effect of chitosan on mechanical properties of maxillofacial silicone

Tear strength: After chitosan addition to silicon elastomer (2.5% and 3.5%), the tear strength is increased in comparison to the control group by (6.498% and 14.058%) respectively. This increase may be due to the fact that chitosan may contain 2.5% and 3.5% of the monomer entrapped within the silicone polymer substructure, this monomer may regard as an impurity that could be evaporated easily from the material [25]. Such impurities decrease the curing rate by contaminating the catalyst [26]. The tear strength is higher when the matrix is slightly under-cured [27].

Tensile strength: The results showed significant decrease in tensile strength of silicone elastomer after chitosan addition with least tensile strength at 3.5% chitosan. The explanation suggested that the chitosan particles were distributed into the continuous polymeric phase of the elastomer; therefore, the functional cross-sectional area of the composite was smaller than that of the pure polymeric matrix of elastomer, even if there were no bubbles or cracks between the matrix and the chitosan particles. Tensile stress pulled the chitosan particles off the polymeric matrix with detachment proceeding along the interface of chitosan particle and polymeric matrix. Furthermore, the chitosan impart a certain strengthening property as the micro cracks between the matrix and chitosan can consume energy. When the concentration of chitosan increased to a certain amount, the chitosan particles became close to one another or aggregated and then the micro cracks transform into macro-defects, which resulted in a decrease in tensile strength [28].

CONCLUSION

Taking in consideration the limitations of the present study, it was concluded that the incorporation of chitosan micro particles into VST-50F maxillofacial silicone material helps to produce silicone elastomer with antifungal activity, thus decrease the susceptibility to develop Candidal colonization and *Candida* associated infection. This activity seemed to be concentration dependent. This incorporation also enhanced the tear strength so decrease the chance of distortion when being removed from the tissues but decrease the tensile strength of the material.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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