Al-Azhar Journal of Dentistry

Volume 10 | Issue 2 Article 18

Restorative Dentistry Issue (Removable Prosthodontics, Fixed Prosthodontics, Endodontics, Dental Biomaterials, Operative Dentistry)

4-1-2023

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How to Cite This Article

Abdel Hameed, Passant; Motawea, Inas; Essawy, Hisham; and Hosny, Mohamed (2023) "Effect of Protein Repellent Addition to Self-Healing Experimental Resin Composite on Microcosm Biofilm and Cytotoxicity," *Al-Azhar Journal of Dentistry*: Vol. 10: Iss. 2, Article 18.
DOI: https://doi.org/10.58675/2974-4164.1505

AL-AZHAR
Journal of Dentistry

The Official Publication
of The Faculty of Dental
Medicine For Girls,
Al-Azhar University
Cairo, Egypt.

Print ISSN 2974-4156 • Online ISSN 2974-4164

AZJD, Vol. 10, No. 2, April (2023) — PP. 325:334

Effect of Protein Repellent Addition to Self-Healing Experimental Resin Composite on Microcosm Biofilm and Cytotoxicity

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Codex : 1-06/23.04

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http://adjg.journals.ekb.eg

https://doi.org/10.58675/2974-4164.1505

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ABSTRACT

Purpose: To investigate the effect of addition of MPC protein repellent agent to self-healing experimental resin composite on the self-healing efficacy, microcosm biofilm and the cytoxicity. **Material and Methods:** Polyurea formaldehyde microcapsules were synthesized by Insitu Polymerization Technique. MPC and the microcapsules are added to the prepared experimental resin composite with different MPC concentrations. Protein repellence essay was examined by the Micro bicinchoninic Acid method. Biofilm models formed of streptococcus mutans growth was prepared and visualized by confocal laser electron microscope to investigate the live \ dead assay over the studied specimens. The cytotoxicity of the specimens was examined via SRB assay. Fracture toughness was examined using universal testing machine till fracture. Self-healing efficacy is evaluated. **Results:** In protein deposition, it was found that the 0%MPC has the highest value while the least value was found in the 10% group. In live/dead bacterial assay, it was found that the 0%MPC has the highest living bacterial value and the lowest dead bacterial value while the 7.5% group has the lowest living bacterial value and the highest dead bacterial value. In the mechanical evaluation, the virgin fracture toughness, the highest facture toughness was found in the control specimens while the least fracture toughness was found in 10% group, the self-healing efficacy results showed that the highest self-healing value was found in the 0%MPC while the least self-healing efficacy value was found in the control group. **Conclusion:** Innovative self-healing composite with protein repellence give rise for a great variety of dental biomaterials with more promising results and long clinical serviceability

KEYWORDS

Self-healing, Protein repellent, Biofilm models, confocal electron scanning, cytotoxicity

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INTRODUCTION

Dental resin composite is considered to be a multi component material which composed of organic and inorganic elements, both elements play an important role in achieving and enhancing the composite resin properties. Among all restorative materials, the resin composites are commonly used as dental filling for carious teeth because of their superior esthetics, besides their uncomplicated direct manipulative technique [1,2].

Since fracture is considered to be one of the main reasons of failure, which was reported to be probably due to masticatory forces and thermal stresses that occur inside the oral cavity during function causing the accumulation of minute cracks which will finally lead to the fracture of the whole restoration, moreover the replacement of this fracture restoration will highly scarify the remaining tooth structure [3].

Besides the absence of convenient mechanical properties in dental resin composite there is high rate of failure due to biological complications such as secondary caries which causes major technical complication in the functioning restoration, therefore new rational materials are designed to overcome these drawbacks [4]

In order to stop crack progression and avoid the catastrophic failure of the restoration, the development of polymer having self-healing capability becomes mandatory. The idea of self-healing property is achieved by the inserting of capsules with intact shell holding healing liquids in their core. These capsules will rupture when facing a propagating crack, releasing the flowable healing liquid which in turn will polymerize under the action of the catalyst present in the surrounding matrix[5,6,7]. The selection of the suitable monomer to be used as healing agent is based on its flowability to flow and fill the crack, besides its biocompatibility and its chemical affinity towards the accelerator in the presence of peroxide initiator to trigger the free radical polymerization reaction [8,9].

In the process of salivary pellicle formation, it was proved that protein adsorption on the teeth surface is the primary step which facilitate further bacterial attachment and then biofilm formation, therefore antibacterial mechanism will be mediated primarily with the inhibition of protein adsorption. There are a lot of antibacterial approaches working on designing new formulations overcoming protein adsorption, through these approaches polymer MPC showed extensively promising results [4].

MATERIALS AND METHODS

Research Ethics Committee approval was obtained for this study (Final Code: REC-MA-21-03), Synthesis of self- healing microcapsules was done by insitu polymerization technique [11]. The prepared microcapsules were examined by optical microscope (TE2000-S, Nikon, Japan) to examine the morphological structure of the capsule and to confirm the microencapsulation process proceeded appropriately.

Characterization of microcapsules: scanning electron microscope was used to evaluate the microcapsule surface and shell wall thickness, thermogravimetric analysis was used to examine the thermal stability of microcapsules, finally the Fourier Transmission Infrared Spectroscope (FTIR) was used to evaluate the ability of encapsulated TEGDMA to polymerize.

Fabrication of experimental dental composite resin: Bisphenol A glycerolate dimethacrylate (BisGMA) and triethylene glycol dimethacrylate (TEGDMA) were mixed at 3:1 mass ratio to form the resin matrix [10]. The monomer mixture was rendered light-curable by adding 1% of photo-initiator Phenyl-bis 2,4,6-trimethylbenzoyl Phosphine oxide (BAPO). Then 0.5% by mass of benzoyl peroxide (BPO) was dissolved in the mixture. BPO serves as an initiator to react with the N,N-Bis(2-hydroxyethyl)-p-toluidine (DHEPT) in the healing liquid to trigger the polymerization. The microcapsules were added by 7.5% concentration to the

prepared photoactivated resin matrix then the MPC powder was mixed with the prepared resin matrix at mass ratios (0%, 5%, 7.5%, 10%) [11] . Silanated fillers were added to the resin matrix in order to reach a cohesive mass reliable for packing inside the mold. Barium boro aluminosilicate filler with a median particle size 1.45μm was added into the resin at a filler loading 70% by mass, the optimum amount of filler added was adjusted by using a sensitive balance (WPS2100\C\1, Max2100gm, Min500mgm) and standardized to the rest of the specimens. Each specimen with MPC mass ratio was placed inside a labeled bottle to be ready for testing procedure.

Sample size of 5 in each group has 80% power to detect a difference between means of 8.96 with a significance level (alpha) of 0.05 (two-tailed) with 95% confidence intervals. In 80% (the power) of those experiments, the P value will be less than 0.05 (two-tailed) so the results will be deemed "statistically significant". In the remaining 20% of the experiments, the difference between means will be deemed "not statistically significant". Report was created by GraphPad StatMate 2.00.

Specimens' grouping: 150 specimens were prepared for laboratory study. The specimens were divided into control group without microcapsules or MPC mass ratio (n=30) and four experimental groups (n=120) each group has 7.5% microcapsule mass ratio with different MPC concentration, according to MPC concentration the four groups were: group 1: 0% MPC +7.5% microcapsule mass fraction, group 2: 5% MPC+ 7.5% microcapsule mass fraction, group 3: 7.5% MPC + 7.5% microcapsule mass fraction and group 4: 10%MPC + 7.5% microcapsule mass fraction. Each group was splitted into 5 sub-groups (n=6) regarding the testing procedure (fracture toughness, self-healing efficacy, protein repellence essay, live/dead staining of biofilm and cytotoxicity)

Scanning Electron Microscopic examination: Scanning was carried out for the composite specimens to examine the uniform distribution of the filler and the microcapsule through the matrix using Scanning Electron Microscope (Model Quanta 250 FEG (Field Emission Gun) attached with EDX Unit (Energy Dispersive X-ray Analyses), with accelerating voltage 30 K.V. and magnification 14x up to 1000000.

Fracture toughness measurement: 6 specimens for each group were prepared using the single edge notched design (ASTM 399-83) [12]. A rectangular copper split mold with internal dimensions (25mm in length, 5mm in width and 2.5mm in thickness) was used, four molds were fabricated for different microcapsule concentrations. The mold was designed with a razor blade insert fixed into a slit in the middle of its long side and projecting in the mold with a length of 2.5mm throughout the total thickness of the mold. This resulted in a 0.5 notch/depth ratio of the specimen. Each prepared specimen was loaded up to failure in a 3point bending test using universal testing machine. The KIC -virgin (in Mpa.$m^{1/2}$) was calculated according to the equation: [12,13]

$$\mathbf{KIC= [3Pla^{1/2}/2bw^2] \times f\ (a/w)}$$

Where, KIC: is the fracture toughness in Mpa.$m^{1/2}$, P: is the load at fracture in Newton, L: is the distance between the supports in mm, a: is the crack length in mm, w: is the width of the specimen in mm, b: is the thickness of the specimen in mm.

Self-healing efficacy measurement: examining the self -healing efficacy was done by reinserting the two fractured halves back into the original mold as soon as possible after fracture to achieve intimate contact between the two fractured halves, and this fracture toughness evaluated is said to be the healed fracture toughness. The self-healing efficacy (η) was calculated according to the underlying equation: [9,14]

$$\eta = \frac{\text{KIC-healed}}{\text{KIC-virgin.}} \times 100$$

Specimen preparation for protein repellence essay: Teflon disc molds of 9mm in diameter and

2mm in thickness were used, in which the uncured composite pastes were inserted. After light curing, the specimens were incubated in distilled water 37°C, 24hr.

Protein repellence essay: Amount of protein adsorption was calculated via protein analysis kit [16]. Each composite disc specimen was placed inside a sterile Eppendorf tube then phosphate buffered saline (PBS) was added on it, the discs were immersed in the PBS for 2hrs. After 2hrs the PBS was removed from the tubes then bovine serum albumin (BSA) 4gm/L was added for 2hrs at 37°c, this BSA act as the standard source of protein supplied by the protein analysis kit. Rinsing and agitation of discs in PBS at 300rpm was done for 5 min. A solution of 1% sodium dodecyl sulphate (SDS) in PBS was prepared then added to the discs inside the Eppendorf tubes. The discs were immersed in the previously mentioned solution and sonicated at room temperature for 20min to release the bovine serum albumin present on the disc. The protein analysis kit was used to determine the BSA conc. in the SDS solution. Each prepared sample was compared with the standard through the spectrophotometer [15].

Dental plaque biofilm model and live/ dead assay: Discs corresponding to each MPC conc were placed inside Eppendorf tubes, discs are sterilized using diethylether solution for 24hrs at 37°C. Streptococcus mutans (RCMB017 (1) ATCC 25175) was sub cultured from old strain St. mutans and incubated for 24hrs at 37°C, Sterilized discs within Eppendorf tubes were opened in a sterile air laminar flow cabinet. Each Eppendorf tube was filled with 2ml of previously prepared St. mutans suspension. The samples were inspected to confirm presence or absence of St. mutans biofilm by using scanning electron microscope SEM.

Streptococcus mutans biofilm models were examined by confocal laser scanning microscope (CLSM). The disc specimens were investigated to evaluate the presence of living bacterial cells in the formed biofilm via live / dead viability stain. The used dyes in this study include Acridine Orange (AO) and Propidium Iodide (PI) which were used for staining of the dentin specimens to detect live/ dead bacterial cells. In order to monitor the green fluorescence (live cells) and red fluorescence (dead cells), dual channel imaging was used after the staining of the biofilm. A confocal laser scanning microscope was used to view the fluorescence emitted from the stained bacterial cells [15] .

Cytotoxicity assay: Oral epithelial cells were supplied from Nawah Scientific Inc., (Mokatam, Cairo, Egypt). Cells were preserved in DMEM culture media enriched with 100 mg/mL streptomycin, 100 units/mL penicillin and 10% fetal bovine serum in humidified, 5% (v/v) CO2atmosphere at 37 °C. SRB test was assessed in the examination of cell survivance of the five groups of resins by the evaluation of cell viability and its proliferation. Six discs (8mm in diameter, o.5 mm thickness) corresponding to each group were submerged in 10ml of culture media inside the 96well plate, then it was stirred for 24hrs at 37°C to acquire resin eluent. Original extract solution samples were diluted to 128-fold dilution, this dilution was chosen as it represents the average individual salivary flow, both the 128-fold besides the original extract solution was examined by SRB test [16].

Samples composed of 100μl of cell suspension (5 x 103 cells) were inserted into the wells of 96well plates and incubated for 24hrs, then the culture media was removed and replaced by the previously prepared resin eluent extract solution. After 72hrs of exposing the cells to the resin eluent specimens,150μl of 10% TCA (trichloroacetic acid) was added for cell fixation and incubated at 4°C for 1 hour, then washing of cells was done 5 times by distilled water. The 96 well plates were filled with 70μl of SRB solution (0.4% w/ v), incubated in dark place at room temperature for 10 minutes. Before the addition of 150ml of TRIs (tris-base solution) (10mM) to dissolve protein bound SRB stain, the plates were washed three times with 1%

acetic acid and left to dry overnight. Finally, the absorbance was measured by spectrophotometer at 540 nm using a BMG LABTECH®- FLUOstar Omega microplate reader (Ortenberg, Germany). Pure culture media without resin eluent extract was considered as control to culture oral epithelial cells and its absorbance was set as 100 %.

Cell survivance was calculated following the underlying equation:

A-resin / A-control, where A-resin was the absorbance of cells cultured with resin eluents extract, and A-control was the absorbance of cells cultured in the culture media without resin eluents extract [16].

Statistical analysis:

The mean and standard deviation values were calculated for each group in each test. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests, data showed parametric (normal) distribution. One-way ANOVA followed by Tukey post hoc test was used to compare between more than two groups in non-related samples. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

Ethics code:

Final Code: REC-MA-21-03

RESULTS

Fracture toughness: Mean Fracture toughness values of the virgin and healed composite specimens among different concentrations are represented in table (1) and plotted in figure (1). The mean virgin fracture toughness at concentration 10% was significantly lower than the other groups. Increasing the MPC concentration to 10% reduces the mean virgin fracture toughness values from (3.23 ± 0.32) for the control group to (0.67 ± 0.04) for the Concentration 10%, while The highest mean Healed Fracture Toughness value was found with group 1 (0%MPC) (1.94 ± 0.31), followed by Concentration (5% MPC) (1.084 ± 0.187) and (Concentration 7.5%) (0.99 ± 0.15), followed by concentration (10% MPC) (0.51± 0.066) while the lowest Healed Fracture Toughness value was in the control group (0% microcapsule, 0% MPC) (0.00 ± 0.00). The healed fracture toughness significantly increased from no healing for control group to maximum healing for group1 (0% MPC)

Table (1) *Mean Fracture toughness values of the virgin and healed composite specimens among different concentrations.*

Fracture Toughness / MPC Concentrations	Virgin		Healed		*p-value*
	Mean	**SD**	**Mean**	**SD**	
Control group (no MPC, no microcapsule)	3.234	0.329	0.00	0.00	**<0.001***
0%MPC	2.412	0.338	1.944	0.308	**<0.001***
5% MPC	1.984	0.166	1.084	0.187	**<0.001***
7.5% MPC	1.722	0.061	0.992	0.158	**<0.001***
10% MPC	0.670	0.042	0.510	0.066	**<0.001***
p-value	**<0.001***		**<0.001***		

**; significant (p≤ 0.05).*

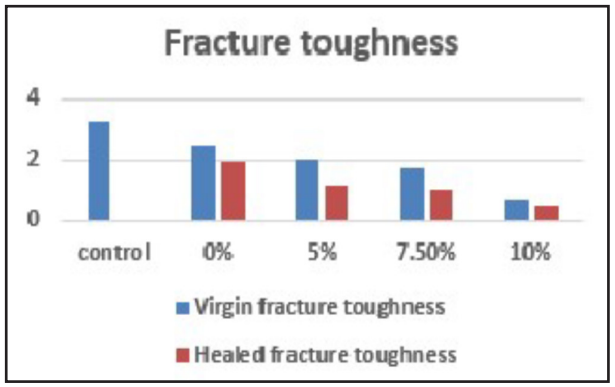

Figure (1) Bar chart representing the virgin and healed fracture toughness among different MPC concentrations

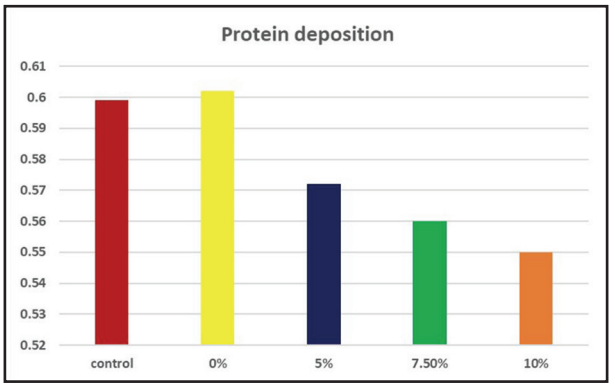

Figure (2) Bar chart representing protein deposition values among different MPC concentrations

Self-healing efficacy: The highest value of healing was present in the (0% MPC) (76.262% ± 10.513). However, no healing was found in the control group (0% microcapsule+ 0% MPC) (0.00 ± 0.00).

Protein Deposition results: Mean protein deposition values of composite resin specimens with different MPC concentration are represented in table (2) and plotted in Fig.2. The highest value was presented in the 0% group but the least value was presented in 10% group, increasing the MPC concentration to 10% reduce the amount of protein deposition from (0.602 ± 0.014) for the 0% group to (0.550 ±0.028) for 10%group.

Table (2) *Mean protein deposition values of composite resin specimens with different MPC concentration.*

Protein deposition / MPC Concentration	Mean	SD
Control group	0.599	0.016
0% MPC	0.602	0.014
5% MPC	0.572	0.014
7.5% MPC	0.560	0.013
10% MPC	0.550	0.028
p-value	**<0.003***	

Live / dead bacterial assay: Mean living bacterial values and dead bacterial values of composite resin specimens with different MPC concentrations are represented in table (3) and plotted in Fig (3). The highest mean live bacterial value was found in 0% MPC (90.85 ±4.734), while the least mean live bacterial value was found in 7.5% MPC (61.3356 ±12.284). Regarding the dead bacterial mean values, the highest dead bacterial mean value was found in 7.5% MPC (9.0238±4.6594), Fig (4) shows representative CLSM images of biofilms on 0% MPC and 7.5% MPC.

Table (3) *Mean living bacterial values and dead bacterial values of composite resin specimens with different MPC concentrations.*

Cell viability percentage / MPC Concentrations	Living bacteria%		Dead bacteria%	
	Mean	SD	Mean	SD
Control group (no MPC, no microcapsule)	86.83	10.6619	13.1363	1.6658
0%MPC	90.8523	4.7348	9.0238	4.6594
5% MPC	79.5363	8.7608	19.655	7.6233
7.5% MPC	61.3356	12.2849	37.3733	11.9746
10% MPC	68.2867	9.8920	31.7133	9.89205
p-value	<0.001*		<0.001*	

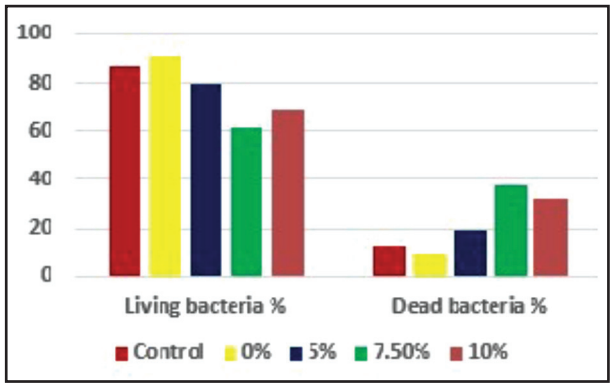

Figure (3) Bar chart representing living bacteria % and dead bacteria % values among different MPC concentrations

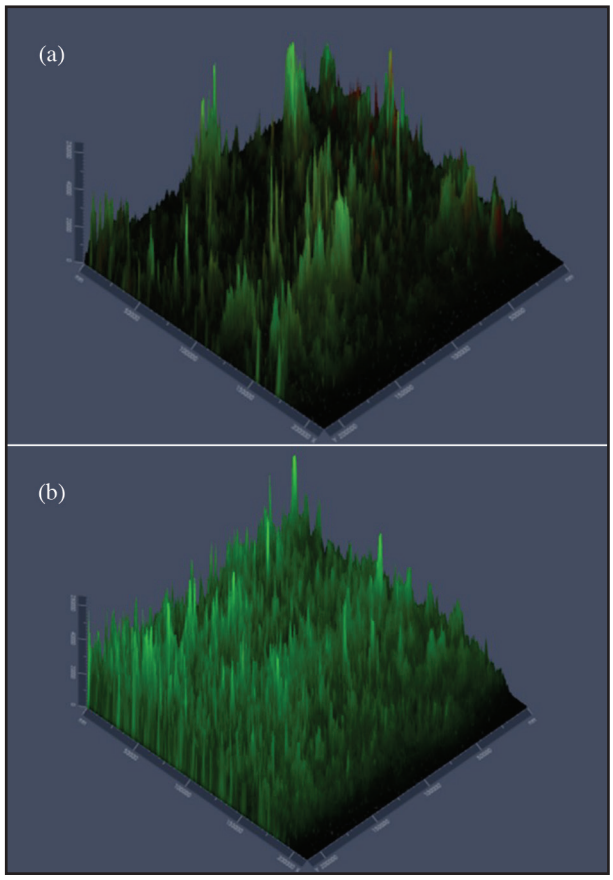

Figure (4) Representative 2.5D CLSM image of biofilm on resin composite specimen, (a) CLSM image for biofilm on 7.5% MPC showing low value of live bacteria, (b) CLSM image for biofilm on 0% MPC showing high value of live bacteria

Cytotoxicity assay: Mean cell viability % values of composite resin specimens with different MPC concentration are represented in table (5). Regarding the cell survivance results, it revealed the absence of significant difference among the 5 groups at both 128 folds and eluent solutions. The cell viability percentage of the 5 groups are similar to the fresh culture media control without any resin eluent extract, therefore it was concluded that the addition of microcapsules with the MPC did not affect the cell survivance remarkably.

Table (5) *Mean cell viability % values of composite resin specimens with different MPC concentration*

Cell viability percentage / MPC Concentrations	128-fold		eluent	
	Mean	**SD**	**Mean**	**SD**
Control group (no MPC, no microcapsule)	89.937	0.242	99.230	0.375
0%MPC	92.093	1.411	97.433	0.249
5% MPC	89.857	0.095	97.133	1.039
7.5% MPC	89.257	0.508	97.253	3.306
10% MPC	90.373	1.590	96.757	0.071
p-value	**0.050ns**		**0.391ns**	

ns; non-significant (p>0.05)

DISCUSSION

The current study represents an effective fusion between highly chosen mechanism, in which both will work together on overcoming two major causative factors which are considered to be from the most common reasons for composite resin failure, this would be achieved by the development of advanced dental composite having both protein repellence capability besides crack healing ability.

The prepared capsules are in the micro scale ranging from 24-70μm, these microcapsules successfully achieve the purpose of their addition to the resin composite by undergoing insitu rupture

leading to the efficient delivery of healing agent at crack site. Moreover, it must be mentioned that increasing the size of the capsule by using super enlarged macro-capsule will become weak spots into the composite as it will affect the resin matrix cross linking thus reducing its mechanical properties. [10,16-18].

In the current study, microcapsules were added by 7.5% mass ratio as this ratio was shown in previous study to achieve an optimal healing efficiency besides preserving the resin composite mechanical properties, also crack healing is spontaneously induced without external intervention due to the flowability and reactivity of the healing agent which stands for the chemical resemblance between the healing agent and the polymer matrix [11]

Previous studies were carried out to develop restorations with surfaces having bacterial repellent ability, this was done by surface coating with highly hydrophilic material, this hydrophilicity causes the inhibition of bacterial adhesion by the repulsion of protein adsorption [4].

In the present study, the incorporation of highly hydrophilic MPC into resin composite provides antibiofilm potential resistance modulated by the inhibition of protein adsorption, as the attachment of bacteria on the material surface is mediated by adsorbed protein, hence MPC plays an important role in eliminating bacterial adhesion [4]. This finding was in agreement with the current study as increasing the MPC mass fraction reaching 10% allowed more protein repellent potency.

Live dead assay was performed on biofilm model holding cultured streptococcus mutans bacteria to evaluate the effect of MPC incorporation on providing the composite resin by antibacterial potential and its effect on eliminating biofilm formation .Since the streptococcus mutans bacteria is one of the dominant bacteria in the oral cavity and considered to be the pioneers in causing dental caries so eliminating their attachment and decreasing their bacterial count in the dental plaque would highly help in avoiding the occurrence of recurrent caries in dental composite restorations.

Live dead assay was investigated by confocal laser scanning microscopy CLSM as it provides higher resolution by using short wavelength and greater contrast compared to conventional microscopes besides the ability of obtaining optical sections and 3D reconstruction of the images , CLSM provides biofilm images characterized by red color showing dead bacteria volume, green color showing live bacteria volume moreover dead and live bacterial coverage on every two dimensional x-y segment in the formed biofilm [21].

In the present study, CLSM examination revealed that the specimens with 0%MPC concentration showed the least dead bacterial volume and the highest live bacterial volume while the specimens with 7.5% MPC concentration showed the highest dead bacterial volume and the lowest live bacterial volume. Moreover, this finding would strongly support the role of MPC in eliminating the bacterial attachment in dental biofilm as due to the hydrophilicity of MPC which highly decreases the protein deposition on the surface of the composite resin therefore prevent the formation of any conditioning layer that may act as anchorage for bacteria on the surface [4].

Assessment of the cytotoxic behavior of the self-healing dental composite holding MPC as a protein repellent agent was performed to evaluate its biocompatibility after the incorporation of microcapsules and the MPC, in our present study cytotoxicity screening was done by SRB colorimetric method to evaluate the cytotoxic effect of the experimental resin composite on the oral epithelial cells. SRB colorimetric method is characterized by simplicity, offering reliable and reproducible data, with high reflectivity in which the culture cell protein levels are proportional with the cell number [20]. SRB is an aminoxanthene dye having bright pink

color, the dye under acidic condition binds to the basic amino acids presents in the cellular protein, then it undergoes dissociation in basic condition, the amount of SRB dye extracted from the stained cells is indicating the total protein mass thus correlated to the cell count. In the current study, SRB colorimetric method revealed that the cell viability was not endangered by the addition of either MPC or microcapsules, the results showed that 7.5% microcapsules was enough to cause repair of damage without affecting the biocompatibility of the resin composite. Moreover, these results were consistent with previous studies that clamed the good biocompatibility of the MPC as it was employed in the fabrication of various medical devices under the acceptance of the food and drug administration of the United States [4,15]

In stress-bearing areas, the mechanical properties of resin composite will play an important role affecting the longevity of the restoration, therefore in order to achieve a novel resin composite having both self- healing ability and protein repellency this modulation must not reduce the mechanical properties of the composite.

In this study, the incorporation of microcapsules with mass ratio 7.5% into the dental resin composite did not alter the mechanical properties, and this percentage was chosen according to the previous study[11] which revealed that increasing the microcapsule mass ratio more than 7.5% causes adverse effect on the mechanical properties of resin composite due to lack of interaction in terms of bonding between the microcapsules and the continuous matrix phase. In addition, the present study demonstrated that the incorporation of MPC powder to mass ratio reaching 10% causes the reduction of the virgin fracture toughness from (3.23 ± 0.32) at control group to (0.67 ± 0.04) at for 10% MPC. The reduction in mechanical properties of resin composite holding 10% MPC can be attributed to the effect of the presence of different phases like the MPC and the microcapsules in the cross-linked resin matrix[4,19]

Self-healing efficacy results showed that the highest value of healing was found with (0% MPC) (76.262% ± 10.513), while no healing was found with (Control group (0% microcapsule+ 0% MPC) (0.00 ± 0.00). The results of the present study were consistent with other studies which showed the higher microcapsule concentration, the higher the healing effect.

CONCLUSION

Within the limitations of the present study, it was concluded that the incorporation of microcapsules and MPC protein repellent agent would provide a dual strategy in overcoming the composite resin failure, without affecting the composite resin biocompatibility. Moreover, it was concluded that the incorporation of 7.5% MPC mass ratio into the experimental composite resin achieve high protein repulsion and elevated dead bacterial value in the biofilm models without compromising the healing effect of the microcapsules or altering the mechanical properties of the resin composite if it is compared to the conventional resin composite.

RECOMMENDATIONS

Further investigations are recommended to empower the antibacterial property of the resin composite by adding antibacterial agents with the protein repellent agent, in addition to maintaining the mechanical properties of composite to allow more serviceability.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in this study

FUND DECLARATIONS

The authors declare the lack of any fund provided.

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