ADHESION OF *Streptococcus mutans* ON TOOTH COLOURED RESTORATIVE MATERIALS

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UNIVERSITI SAINS MALAYSIA

2016

ADHESION OF *Streptococcus mutans* ON TOOTH COLOURED RESTORATIVE MATERIALS

by

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Thesis submitted in fulfilment of the requirements

for the degree of

Master of Science

September 2016

ACKNOWLEDGEMENTS

First and foremost, all praise and thanks be to Allah who had given blessings and strength in completing my research work and for providing me a meaningful opportunity to step into the enjoyable of research world.

My special thanks to my supervisor, Assoc. Prof. Dr. Dasmawati Mohamad for being able to allocate her precious time in continuous guidance, endless support, and immense knowledge. My sincere thanks to my co-supervisor, Dr. Nurul Asma Abdullah who always there to give me a positive advice and excellent supervision. Another big thanks to my co-supervisor, Dr. Zuryati Ab Ghani for her untiring encouragement and giving me a very constructive criticism on my research work.

Deepest gratitude to the respectful laboratory technologists especially Ms. Nora, Ms. Fadilah and Mr Hairie for their great assistance, and not to forget to Ms. Asiah, Mr. Marzuki, Mr. Yusof, Ms. Eda, Ms. Khairiena, Ms. Khadijah and Mr Hisham for their time and valuable efforts. Special thanks to research officer, Ms. Liyana and R&D staff for their timeless help.

I am deeply pleased and grateful to my beloved colleagues, Siti Nurshazwani, Siti Suraya, Siti Nurnasihah, Nur Izyan, Fatimah Suhaily, Ain Fatihah, Nurul Hidayat, Mr Manaf and Shaminea whom help me a lot by exchanging idea, giving a moral support to each other and overcoming the ups and downs together. Huge wishes of good luck to these lovable people in completing their research work.

My deepest and special thanks to my pillars and my only backbone which is my beloved family who keeps praying continuously, infinite support for my success and never giving up on me. Big thanks to all who directly or indirectly help me in completing this research work.

Last but not least, I would like to express my appreciation to the Ministry of Higher Education (MyBrain), Yayasan Pelajaran MARA (YPM) and Fundamental Research Grant Scheme (FRGS) from Ministry of Higher Education Malaysia (203/PPSG/6171146) for the financial support.

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cm^2	Centimeter squared
dl	Deciliter
g	Gram
kV	Kilovolt
M	Molar
ml	Millilitre
mg	Milligram
mM	Millimolar
mm	Millimetre
mm^2	Millimetre squared
ng	Nanogram
nm	Nanometre
OD	Optical density
R_a	Roughness average
sd	Standard deviation
µl	Microlitre
µm	Micrometre
µM	Micromolar
µg	Microgram
%	Percentage
∞	Infinity
ºC	Degree celsius
AC	Tapping mode
AFM	Atomic force microscopy
ATCC	American type cell culture
BHI	Brain heart infusion
Bis-GMA	Bisphenol-glycidyl methacrylate
Bis-EMA	Ethoxylatedbisphenol A glycol dimethacrylate
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CDTA	Trans-1,2-cyclohexylenedinitrilotetraacetic acid
CLSM	Confocal laser scanning microscope
C_t	Threshold cycle
DC	Direct contact
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide-tri phosphate
EDTA	Ethylenediaminetetraacetic acid
F	Forward
F^-	Fluoride ion
FAS	Fluoro-alumino-silicate
GBP	Glucan binding protein
GIC	Glass ionomer cement
gtfB	Glucosyltransferase B
gbpB	Glucan binding protein B
GTF	Glucosyltransferase

H^+	Hydrogen ion
H_2O	Water
HCl	Hydrochloric acid
HEMA	2-hydroxyethyl methacrylate
HF	Hydrogen fluoride
hr	Hour
HS	High salt
ISE	Ion selective electrode
LB buffer	Lithium boric acid buffer
LED	Light-emitting diode
LS	Lower salt
$MgCl_2$	Magnesium chloride
min	Minute
mRNA	Messenger ribonucleic acid
Mw	Molecular weight
NaCL	Sodium chloride
NTC	Non template control
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PEGDMA	Polyethylene glycol dimethacrylate
PI	Propidium iodide
PMCR	Polyacid-modified composite resin
Ppm	Part per million
qPCR	Quantitative real-time polymerase chain reaction
R	Reverse
RMGIC	Resin-modified glass ionomer cement
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RQ	Relative quantitation
rRNA	Ribosomal ribonucleic acid
s	Second
SEM	Scanning electron microscope
S. mutans	*Streptococcus mutans*
SPSS	Stastical Package of Social Sciences
TE-buffer	Tris-EDTA buffer
TEGDMA	Triethylene glycol dimethacrylate
TISAB	Total ionic strength adjustment buffer
UDG	Uracil DNA Glycosylases
UDMA	Urethane dimethacrylate
Uv	Ultraviolet
2D	Two dimensional
3D	Three dimensional

LEKATAN *Streptococcus mutans* PADA BAHAN RESTORATIF BERWARNA GIGI

ABSTRAK

Aplikasi teknologi nano pada masa kini telah berkembang secara meluas di dalam pergigian estetik kerana pengisian zarah bersaiz nano yang menawarkan banyak kelebihan yang hebat seperti mampu mengurangkan lekatan bakteria oleh bakteria oral yang bersifat kariogenik terutamanya koloni oral terawal iaitu *S. mutans*. Lekatan awal oleh *S. mutans* ini pada permukaan bahan telah menyumbang kepada pembentukan biofilem, kemerosotan permukaan bahan dan mungkin menggalakkan karies gigi. Untuk memulihkan karies gigi, permintaan dalam penggunaan resin komposit dan semen ionomer kaca modifikasi resin (RMGIC) dalam bidang pemulihan telah meningkat disebabkan oleh nilai estetiknya. Perbezaan saiz pengisi oleh bahan-bahan seperti pengisian nano, pengisian mikro dan pengisian mikrohibrid digunakan untuk membanding dan menilai lekatan *S. mutans* ke atas bahan-bahan ini pada beberapa tempoh inkubasi. Empat bahan telah digunakan dalam kajian ini seperti RMGIC; Ketac™ N100 (pengisian nano) dan Fuji II™ LC (pengisian mikro) dan resin komposit; Filtek™ Z350 (pengisian nano) dan Filtek™ Z250 (pengisian mikrohibrid). Kajian mikroskop yang melibatkan mikroskop daya atom (AFM) telah dijalankan untuk menilai kekasaran permukaan bahan yang dieram, mikroskop konfokal laser imbasan (CLSM) untuk menilai ketebalan biofilem dan mikroskop elektron imbasan (SEM) untuk pemerhatian taburan *S. mutans* pada bahan. Sukatan pelepasan fluorida telah dilakukan ke atas bahan RMGIC untuk menganalisis pengeluaran fluorida oleh bahan tersebut. Tambahan pula, pertumbuhan bakteria telah dilakukan untuk menilai aktiviti pertumbuhan *S. mutans* pada bahan-bahan

yang diuji. Ekspresi gen juga telah dijalankan untuk menentukan tahap ekspresi gen oleh gen-gen *gtfB* dan *gbpB*. Dapatan data telah di analisis secara statistik sama ada dengan ujian T bebas dan ujian analisis varians satu hala pada aras bererti $p<0.05$. Daripada keputusan tersebut, Fuji II LC telah meningkatkan pengeluaran fluorida secara signifikan berbanding Ketac di dalam kedua-dua media storan ($p\leq0.001$). Kedua-dua bahan pengisian nano telah memberikan nilai yang rendah untuk kekasaran permukaan sementara tiada perbezaan secara signifikan untuk ketebalan biofilm yang telah ditunjukkan kecuali pada hari ke 7. Kumpulan RMGIC menunjukkan pertumbuhan *S. mutans* yang rendah berbanding kumpulan komposit pada semua tempoh inkubasi. Pengisian nano RMGIC memberikan tahap ekspresi yang rendah oleh gen *gtfB* dan *gbpB* secara signifikan berbanding bahan-bahan yang lain ($p<0.05$). Daripada keputusan ini, kekasaran permukaan dan pelepasan fluorida oleh bahan RMGIC telah dikenal pasti sebagai faktor penting yang memberi kesan ke atas lekatan dan pengumpulan bakteria *S. mutans* pada bahan. Secara amnya, kedua-dua bahan pengisian nano mempunyai kebolehan dalam mengurangkan lekatan bakteria oleh *S. mutans* berbanding bahan mikro kerana kebanyakan keputusan membuktikan bahawa pengisian nano memberikan kekasaran permukaan yang rendah, ketebalan biofilem yang rendah dan tahap expresi gen yang rendah. Perbandingan antara kedua-dua kumpulan pengisian nano, Ketac menunjukkan penambahbaikan yang cemerlang dalam mengurangkan lekatan *S. mutans* berbanding Z350 disebabkan kebolehan Ketac dalam pelepasan fluorida. Penemuan-penemuan ini mencadangkan pengisian nano RMGIC sebagai bahan yang ideal dalam mengurangi pengumpulan *S. mutans*, yang mana boleh menghalang lekatan *S. mutans* pada permukaan bahan.

ADHESION OF *Streptococcus mutans* ON TOOTH COLOURED RESTORATIVE MATERIALS

ABSTRACT

Currently, the application of nanotechnology has become broadly developed in aesthetic dentistry due to its nanofiller particles size which offered numerous excellent advantages such as capable in reducing the bacterial adhesion of cariogenic oral bacteria mostly of early oral colonizers of *S. mutans*. This initial adhesion of *S. mutans* on the surface of materials contributed to the biofilm formation, surface deterioration of materials and may cause dental caries. In order to restore a carious tooth, the use of composite resin and resin-modified glass ionomer cement (RMGIC) in the restoration field has been increased due to the demand for aesthetic value. Different filler sized materials such as nanofilled, microfilled and microhybrid were used to compare and evaluate the adhesion of *S. mutans* on these materials at several incubation times. Four materials were used in this study which were RMGICs; Ketac™ N100 (nanofilled) and Fuji II™ LC (microfilled) and composites resins; Filtek™ Z350 (nanofilled) and Filtek™ Z250 (microhybrid). A microscopy study was performed which include atomic force microscopy (AFM) for evaluation of surface roughness of the incubation materials, confocal laser scanning microscopy (CLSM) for evaluation of biofilm thickness and scanning electron microscopy (SEM) for distribution observation of *S. mutans* on materials. Fluoride release measurement was carried out for RMGIC materials to analyse the fluoride release of the materials. In addition, bacteria growth was done to assess the growth activity of *S. mutans* on the tested materials. Gene expression was also performed to determine the gene expression levels of *gtfB* and *gbpB* genes. Data obtained were statistically

analyzed with either Independent T-test or One-way ANOVA at significance level of $p<0.05$. From the result, Fuji II LC gave a significantly higher of fluoride release compared to Ketac in both storage media ($p\leq0.001$). Both nanofilled materials gave a lower value of surface roughness while no significant difference of biofilm thickness between nanofilled and microfilled materials was shown except on day 7. RMGIC groups gave a lower *S. mutans* growth compared to composite resin group at all the incubation times. Nanofilled RMGIC gave significantly lower of expression levels of *gtfB* and *gbpB* gene compared to other materials $p<0.05$. From the results, surface roughness and fluoride release by RMGIC materials were recognized as a significant factor that affected the adhesion and accumulation of *S. mutans* on materials. In general, both nanofilled materials has the capability in reducing the bacterial adhesion of *S. mutans* compared to micron sized materials since most results in this study proved that nanofilled gave lower surface roughness, less biofilm thickness and low level of gene expression. Comparison between both nanofilled groups, Ketac showed an excellent improvement in reducing *S. mutans* adhesion compared to Z350 due to its fluoride release ability. These finding suggested a nanofilled RMGIC as the ideal material in reducing the accumulation of *S. mutans*, which could inhibit the adhesion of *S. mutans* on the surface materials.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

In order to survive and persist in oral environment, bacterial cells are required to adhere and attach on the surfaces and formed a structured cell clusters called biofilm (Lawrence *et al.*, 2007, Johnson, 2008). Accumulation of bacteria does not limit to tooth surfaces only, but also exist in the oral environment, commonly dental materials. The adhesions of the microbial cells to the surface texture play a major role in the accumulation of bacteria on intraoral solid surfaces. This initial adhesion may promote a successful colonization of bacteria on the surfaces of teeth and restorative materials, hence may induce a biofilm formation, surface deterioration of dental materials (Gharechahi *et al.*, 2012) and the pathogenesis of infections related to biomaterials (Liu *et al.*, 2008). This biofilm formation on dental materials may lead to secondary caries and may induce in gingival inflammation (Aykent *et al.*, 2010).

Due to the formation of biofilm, streptococci bacteria are found to be involved in groups of the early colonizing bacteria. Karthikeyan *et al.* (2011) has reported that more than five of *Streptococcus* species were identified as early colonizers to tooth surface in oral biofilm. *S. mutans* was the most prevalence organism and is considered to be the most cariogenic among the oral streptococci (Dong *et al.*, 2012). The adaptation of *S. mutans* and other oral streptococci in bacterial adhesion involved numerous of genes. Previous study by Shemesh *et al.* (2007) has indicated

that several genes are associated with adherence of *S. mutans* biofilm on oral cavity. *GtfB* and *gbpB* genes were believed to be a significant factor in constitute the sucrose-dependent pathway for *S. mutans* to adhere on the tooth surface and are of central significance in biofilm formation and development of caries (Tyagi *et al.*, 2013). Several factors influenced the expression of genes that associated with adhesion of bacteria which were environmental conditions (Li and Burne, 2001), and also genetically regulated (Lee *et al.*, 2004).

Due to aesthetic appearance, tooth coloured restoration are more in demand. Composite resin offered many advantages such as it can adhere to the tooth structure by mechanical bonding and offer an acceptable aesthetic result. However, composite resin is not efficient in restoring large defects in posterior teeth, as well as its technical sensitivity to moisture (Hengtrakool *et al.*, 2011) and tend to be more susceptible to bacterial accumulation (Imazato, 2003). Other than composite resin, Glass Ionomer Cement (GIC) has also been used for restoration. Despite of the advantages of conventional GIC such as fluoride release (Okte *et al.*, 2012) and good biocompatibility, conventional GIC has its disadvantages such as slow rate of setting, low fracture toughness and low wear resistance (Hubel and Mejare, 2003). Resin modified GIC (RMGIC) was developed to improve the mechanical properties of conventional of GIC. RMGIC offered high wear resistance, higher moisture resistance, higher fracture toughness and a longer-working time. Hubel and Mejare (2003) have concluded RMGIC provides improvement over the conventional GIC for restoring approximal caries in primary molars.

One of the major factors in choosing the materials is the surface roughness of the materials. Song *et al.* (2015) has reported that surface properties of materials such as surface charge, surface energy and surface roughness influenced oral bacterial adhesion. A surface with high surface free energy and rough may promote accumulation of bacteria (Renvert *et al.*, 2011). Filler size is one of the determining factors for surface texture of restorative materials (McCabe and Walls, 2009). Nowadays, many choices of restorative materials with different filler size can be used to restore carious tooth such as nanofilled, microfilled, macrofilled and micro-hybrid. Recently, the application of nanotechnology has been introduced to the field of aesthetic dentistry and offered many advantages such as high strength, high polish and high translucency (Dresch *et al.*, 2006). In addition, nanofiller size particle enhanced the smoother surface roughness of composite (Bala *et al.*, 2012) which would inhibit the accumulation of bacteria.

Besides surface roughness, fluoride also influenced the adhesion and accumulation of bacteria since the study by Nakajo *et al.* (2009) reported that fluoride can prevent the growth of caries-related oral bacteria. However, there was also a study by Al-Naimi *et al.* (2008) which reported on the role of fluoride and their uneffectiveness in combating bacteria at early periods of adhesion.

The quality and quantity measurement of bacterial adhesion on the materials surface are important in order to understand the study of bacterial adhesion in oral cavity. Hence, the study regards to bacterial adhesion on the materials have been performed using fluorescence microscopy (Walkowiak-Przybylo *et al.*, 2012), scanning electron microscopy (Kim *et al.*, 2012) and the atomic force microscopy (Dorobantu and

Gray, 2010). In order to determine the progression stage of biofilm formation, the ability of bacteria to adhere to the materials surface need to be understood. Hence, the ability of bacteria adhering of early settlers on the tooth surface can be controlled which may reduce the biofilm formation progression. This study evaluated the capability of nanofilled RMGIC in preventing the bacteria adhesion. In addition, this study also identified the factors that promote bacterial adhesion on materials and would reveal the ideal material that could successfully reduced accumulation of *S. mutans* on different material.

1.2 Problem statement

Colonization of bacteria on tooth surfaces or dental materials, dental implants or prostheses may begin rapidly following the exposure to the oral cavity (Hauser-Gerspach *et al.*, 2007). In addition, Montanaro *et al.* (2004) reported that bacterial adhesion take place on the surface with a different chemical of materials immediately upon placement in oral cavity. Thus, this accumulation of bacteria on the dental materials has resulted in biofilm formation and may led to dental caries as well as may cause the symptom that affect daily lives. Dental caries or tooth decay is a major widespread disease in humans. It causes the symptoms that may affect daily lives such as impaired speech, tooth destruction, psychological problems and others. It was reported approximately of 70-90 % of children in Malaysia suffering dental caries and tends to increase throughout the year (Oo *et al.*, 2011, Ruhaya *et al.*, 2012). The adhesion and accumulation of bacteria in oral cavity also may lead to gingivitis. Gingivitis is the most common occurring gingival disease and was defined as an inflammation of the gingival (Overview, 2016). The bacteria are capable of synthesizing products that cause damage to the epithelial and connective tissue cells

as well as intercellular components such as collagen, ground substance and glycocalyx, which later may promote gingivitis (Carranza and Bulkacz, 1996). Therefore, the comprehensive understanding regarding the adhesion of bacteria on the restorative materials, which may later result in dental caries, need to be clarified in order to control the bacterial accumulation on the restorative materials.

It is well known that nanofilled materials offered many advantages in the field of dental restoration such as well polished, reduced surface roughness, high strength and reduced shrinkage. However, the new nanofilled RMGIC has not been studied comprehensively with regards to its effect of surface on the bacterial adhesion. The adhesion ability of *S. mutans* on the restorative materials is influenced by the genetics of the organism. However, detailed understanding of the interaction of genes that are associated with adhesion of *S. mutans* on materials is still lacking. Hence, there is a need to study the adhesion of *S. mutans* on different type of materials with regards to the surface roughness, fluoride release and genes expression levels.

1.3 Justification of the study

This study was conducted to provide the information of the ideal material that could reduce inhibition and minimize bacterial adhesion on restorative material. In addition, this study also was carried out to identify the factors that promote bacterial adhesion on materials. The end result of this study would emphasize the benefit of nanotechnology in relation to the RMGIC product, in controlling the adhesion and accumulation of *S. mutans* on the nanofilled materials. This study would enhance the fundamental knowledge that could be applied clinically.

1.4 Objectives of the study

1.4.1 General objective

To investigate the adhesion of *S. mutans* on the different surfaces of tooth coloured restorative materials.

1.4.2 Specific objectives

1. To quantify fluoride release from nanofilled and microfilled RMGIC in different storage mediums from day 1 until day 21.
2. To evaluate the surface roughness of different surfaces of the incubation nanofilled materials and micron materials of RMGIC and composite resin after incubation with *S. mutans* at 7 hr, 24 hrs, day 7, 14 and 21.
3. To evaluate the biofilm thickness of different surfaces of nanofilled materials and micron materials of RMGIC and composite resin after incubation with *S. mutans* at 7 hr, 24 hrs, day 7, 14 and 21.
4. To determine the bacterial growth of *S. mutans* on nanofilled materials and micron materials of RMGIC and composite resin at 7 hr, 24 hrs, day 7, 14 and 21.
5. To determine the gene expression levels of genes that are associated with adhesion of *S. mutans* on different surfaces of nanofilled materials and micron materials of RMGIC and composite resin at 6 hr and 12 hr.

1.5 Hypothesis

1. There is no different in fluoride release from nanofilled and microfilled RMGIC in different storage mediums from day 1 until day 21.

2. There is no different in surface roughness of the incubation nanofilled materials and micron materials of RMGIC and composite resin after incubation with *S. mutans* at 7 hr, 24 hrs, day 7, 14 and 21.
3. There is no different in biofilm thickness of nanofilled materials and micron materials of RMGIC and composite resin after incubation with *S. mutans* at 7 hr, 24 hrs, day 7, 14 and 21.
4. There is no different in bacteria growth of *S. mutans* on nanofilled materials and micron materials of RMGIC and composite resin at 7 hr, 24 hrs, day 7, 14 and 21.
5. There is no different in gene expression levels of genes that associated with adhesion of *S. mutans* on different surface of nanofilled materials and micron materials of RMGIC and composite resin at 6 hr and 12 hr.

CHAPTER TWO

LITERATURE REVIEW

2.1 Bacterial adhesion

Oral cavity is a unique environment which consists of variation of solid surfaces of soft, hard, artificial and natural and share the same ecological niche. In order to resist shear forces and stay alive within this 'open growth system' of oral cavity, microorganisms such as bacteria requires to adhere either to soft or hard tissues (Shemesh *et al.*, 2010). The accumulation of bacteria is present on tooth tissue as well as on other surfaces in the oral environment, commonly dental restorative materials (Montanaro *et al.*, 2004). Teughels *et al.* (2006) stated that the restorative materials is the next surface for adhesion of bacteria and formation of biofilm following the introduction of bacteria in the oral cavity. Upon exposure to the oral cavity, accumulation and colonization of bacteria may begin directly on either tooth surfaces or dental materials such as dental implants and dental materials (Hauser-Gerspach *et al.*, 2007). Tazi *et al.* (2012) has stated that the continuous presence of the oral microorganisms is promoted by their adhesion to the variety surfaces including restorative dental materials.

The adhesion of bacteria on dental surfaces is a complex phenomenon which involves a variation of important factors (Guggenheim *et al.*, 2001). Initial step of bacteria colonization involves the adhesion and attachment of a salivary pellicle layer onto the surface of tooth (Li *et al.*, 2004). Then, the bacteria will adhere to the host origin receptor's in the salivary pellicle (Ikeda *et al.*, 2007). Following adhesion, the

bacteria begin to anchor and the colonisation of the bacteria takes place on the adjoining of new surface takes place, as mentioned by Hannig (1999).

Recently, numerous studies by Nascimento *et al.* (2014), Hahnel *et al.* (2015), Ionescu *et al.* (2015) have been explored regarding the adhesion of variety of microorganisms on the different surfaces due to investigate the interaction of the adhesion step on the materials. *In vivo* study has examined the adhesion of *Streptococcus sanguinis* to dental implant and restorative materials (Hauser-Gerspach *et al.*, 2007). Besides that, Oh *et al.* (2009) has carried out *in vitro* study on the attachment of *Pseudomonas aeruginosa* on a variety of substrates. Other than that, many studies were carried out to explore the adhesion of diverges of the microorganisms such as bacteria, yeast and fungi (Busscher *et al.*, 2010, Shemesh *et al.*, 2010, Tazi *et al.*, 2012).

Many factors have been reported to contribute to the bacterial adhesion on the surfaces such as the selective salivary proteins adsorption (Hannig and Hannig, 2009), bacterial forces mediation to adhere to surfaces as well as the present of ubiquitous which attract Van der Waals forces which known as attractive forces between bacterium and the surface, acid-base bonding and electrostatic interactions. According to Quirynen (1994), initial bacterial adhesion on materials was determined by the intrinsic physico-chemical properties of the materials. It was believed that the different materials which consist of different physico-chemical properties may affect the bacterial adhesion differently. Montanaro *et al*. (2004) has found that different materials affect the adhesion of *S. mutans* on materials and bacterial adhesion can be seen on these restorative materials following from least adhesion to the most:

Flowable composite < microhybrid composite < resin modified glass ionomers < compomer < ormocer

Apart from the different type of materials, surface of materials also influenced the bacterial adhesion. Recent study by Song *et al.* (2015) has been reported the relationship between surface roughness, surface free-energy, surface charge and numbers of adhering bacteria were affected the bacterial adhesion. Oh *et al.* (2009) stated that changes in surface structures which are topography and surface roughness on the macroscopic scale is identified to be critical for bacterial adhesion and retention. On the initial stages of the biofilm formation, the rough surfaces promote the bacterial adhesion and retention because it allows anchor points for microorganisms and their nutrients (Whitehead *et al.*, 2006).

This initial adhesion was found to influence to oral diseases that infect in the oral evironment. Shemesh *et al.* (2007) has stated that adhesion of bacteria is the crucial step of biofilm formation and this may contributes to dental plaque formation (Razak *et al.*, 2006). The early adhesion of bacteria is a crucial stage in the formation of biofilm since it may affect the mature of dental plaque composition. Buergers *et al.* (2007) has described that the process of adhesion and accumulation bacteria on dental material may promote a biofilm formation, thus may enhance in gingival inflammation and secondary caries.

2.1.1 Biofilm formation and dental caries

Biofilm formation is recognized to involve a stepwise process that starts with the adhesion and attachment of planktonic bacteria on the surface in the oral cavity either

on natural environment or dental materials (Jain *et al.*, 2007). The primary stage of the biofilm formation begins with the adhesion and attachment of the early colonizing bacteria commonly a *Streptococcus sp*. to both dental and material surfaces in the oral cavity. Early colonization is believed to be the most crucial step in biofilm formation, depending on the host surface nature. Following the adhesion process, the bacteria colonize and growth, thus forming micro-colonies. Next, these micro-colonies proliferate and become confluent, forming a biofilm in which the colonies linked with each other in a matrix of exopolymers of bacterial and salivary origin and biofilm. At this stage, a complex biofilm of the variation of species existed are formed in highly organized and structured communities (Busscher *et al.*, 2010). This process were further progressed by maturation to the detachment of biofilm then spreading of the organisms from the biofilm (Ramage *et al.*, 2009). Figure 2.1 and Table 2.1 show the development of the biofilm formation.

Then, this biofilm may lead to the formation of dental plaque. The accumulation of dental plaque may contribute to the dental caries, then further development may cause gingival inflammation, periodontal diseases and peri-implantitis (Grosner-Schreiber *et al.*, 2009). Dental caries is known as the disease that mainly attack the childhood and it may affect them throughout their lifetime (Pitts, 2004) and it has also been identified as the primary factor of oral pain and tooth loss (General, 2000, Selwitz *et al.*, 2007). Dental caries may relate to gingivitis since the significance of plaque might act as the primary etiological factor towards the gingival inflammation. It was reported that less than 20 % of the gingivitis cases will promote to periodontitis (Alexander, 2011).

Dental caries is a multifactorial disease that begins with microbiological shifts within the complex biofilm and is affected by salivary flow and composition, exposure to fluoride and consumption of dietary sugars (Selwitz *et al.*, 2007). According to Kutsch and Young (2011), dental caries is a complex phenomenon which include multiple of phatogens, systemic effect, diet interactions and physiological risk factors. One of the important factors of dental caries is the adhesion of the acidic microorganism on tooth structure. Selwitz *et al.* (2007) has reported that dental caries is initiated within the bacterial biofilm that surround on tooth surface as the acidic by products from bacterial fermentation of dietary carbohydrates attack the tooth and resulted in localised destruction of dental hard tissues.

The aetiology of dental caries have been numerously discussed regarding the history of the study of how dental caries occurs, and how theories to explain caries over the last 120 years. Bradshaw and Lynch (2013) has described two significant factors in the aetiology of dental caries which includes microbial aetiology of caries and on the dietary factors associated with caries. The critical role and the rise of *S. mutans* has suggested that the acids produced by the fermentations sugars by *S. mutans* as the primary factor in dental caries (Miller, 1890). However, debate raged as to the roles of particular microbial species in caries aetiology for most of the 20th century. Consequently, the researches began to consider the other potential significant factors that contribute in dental caries which include the dietary factors (Bradshaw and Lynch, 2013). There were numerous articles discussing on the aetiology of dental caries such as Simon-Soro and Mira (2015) discussing acidogenic of *S. mutans* in aetiology of dental caries, Kutsch and Young (2011) mentioned the role of of bacteria and saliva in the aetiology of dental caries and Petersen and Lennon (2004)

describes the ionic exchange of calcium and phosphate and the pH level in the dental caries aetiology.

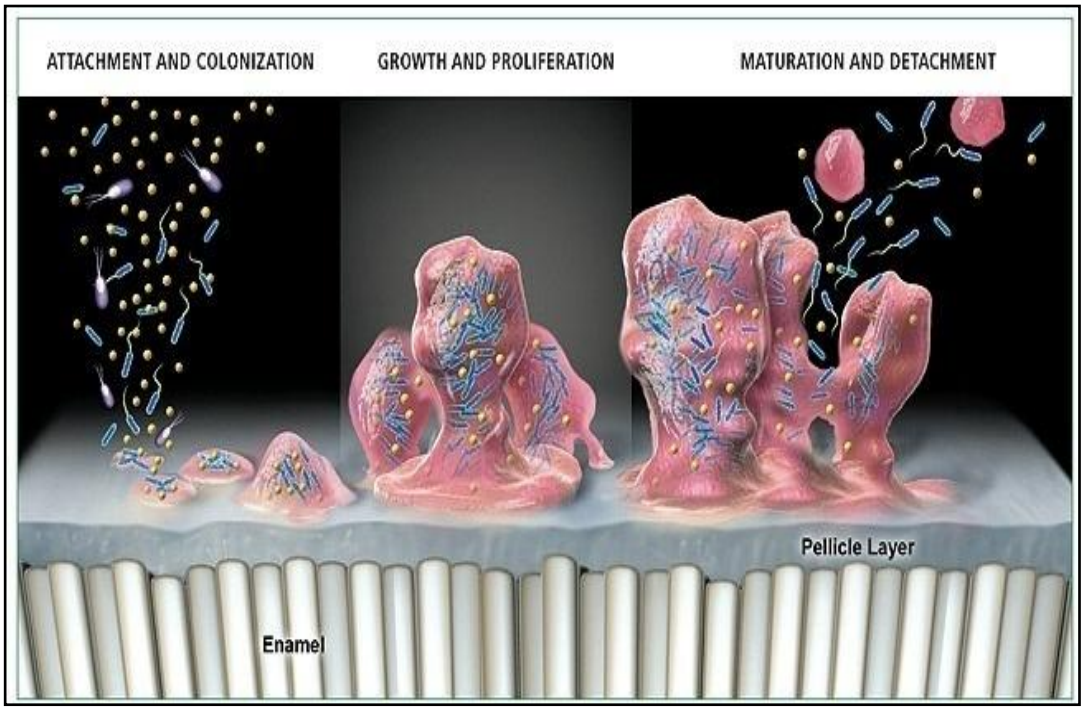

Figure 2.1: Illustrations of the stages in the development of biofilm (Alexander, 2011).

Table 2.1: Summary of sequence of events in biofilm formation (Alexander, 2011).

Stages	**Attachment**		**Succession**		**Proliferation**	**Maturation**
Days	**6-12 hours**	**1-2**	**2-4**	**4-7**	**7-14**	**14-21**
Features	- Initial attachment - Selective colonization of pellicle on tooth surface by salivary / planktonic microorganisms	- Further attachment - Gram-positive Cocci - Mainly Streptococci	- Cocci still dominant - Increasing number of Gram-positive filamentous and rod-shape organisms - Production of extracellular slime layer helping anchor bacteria to tooth surface and provides layer of protection	- Increasing numbers of filamentous organisms - Overall flora more mixed and diverse - Biofilm begins to thicken at gingival margin - Gram-negative vibrious and spirochetes	- Increasing numbers of vibrious and spirochetes - More anaerobes - Increasing virulence factors - Some white blood cells evident - Appearance of mushroom-shaped micro-colonies attached to tooth surface by narrow base - Gingival inflammation observed	- Older biofilms contains vibrious and spirochetes as well as some cocci and filamentous organisms - Some dense packing of filamentous organisms perpendicular to the tooth surface in the palisade layers - Gingivitis evident

2.1.2 *Streptococcus mutans* (*S. mutans*)

2.1.2.1 History of *S. mutans*

J Kilian Clarke discovered and introduced *S. mutans* into research field in 1924. This organism was isolated from carious lesions and was named as *S. mutans*. This organism was called as *S. mutans* due to the appearance of oval-shaped cells which identified streptococci as a mutant species (Clarke, 1924). In the late 1950s, a broader interest of *S. mutans* was received from researchers and was believed as a main cause in the formation of dental caries by the mid of 1960s (Loesche, 1986). In the following two decades, *in vitro* and *in vivo* study of *S. mutans* were developed. According to these pioneer researchers, they found the main virulence features of *S. mutans*: (a) the capability to synthesize abundant amounts of organic acids known as acidogenicity from metabolized carbohydrates; (b) the capability to withstand at low pH known as aciduricity; and (c) the capability to produce extracellular glucan-homopolymers from sucrose, which act as important role in early adhesion, accumulation and growth of biofilms onto the tooth surfaces (Banas and Vickerman, 2003, Bowen and Koo, 2011).

2.1.2.2 Role of *S. mutans* in the cariogenicity

Streptococci bacteria were recognized to be involved in the group of early colonizing of bacteria and recognized as predominant colonizing microorganisms of oral cavity surfaces. *S. mutans* is one of the well-known streptococci bacteria which is recognized to have a major function in the diseases associated with dental caries and pathogenesis of caries (Ikeda *et al.*, 2007). *S. mutans* was found among bacteria proliferating in the dental biofilm and was known as a major phatogen as well as causative agent of dental caries (Islam *et al.*, 2007, Liu *et al.*, 2011). Decades of

research have conclusively revealed that dental pathogen of *S. mutans* as one of the most cariogenic strains in the oral biofilms (Lee *et al.*, 2007) since it is capable of producing acid and glucan which are common extracellular matrices of dental plaque biofilms. At low pH conditions, the biofilm formation in dental plaque by *S. mutans* is said to be more efficient hence resulting in ability of *S. mutans* to out-compete with non- cariogenic commensal (Gross *et al.*, 2012). Endogenous bacteria which are largely consist of *mutans Streptococci*, synthesize weak organic acids as a by-product of metabolism of fermentable carbohydrates. Then, the demineralisation of tooth tissues takes place due to this weak acid production which causes a drop of values of local pH lower than a critical value (Featherstone, 2004). Aykent *et al.* (2010) has reported that *S. mutans* are capable of colonization on tooth surfaces and has strong acidogenity that contribute to demineralization of enamel surfaces. Because of these virulence factors, *S. mutans* mainly participated in the initiation and development of dental caries (Dong *et al.*, 2012).

2.1.2.3 Genes associated with adhesion of *S. mutans*

Sucrose-dependent and sucrose-independent mechanisms was a major important mechanisms which mediated the initial adherence of *S. mutans* to dental surfaces (Koga *et al.*, 1986). For the sucrose-independent adherence, several surface adhesions expressed by *S. mutans,* has the capability to adhere to the salivary pellicles formed on the surface of teeth (Mitchell, 2003) and contribute to the colonizing bacteria on tooth surface by providing them with binding site (Shemesh *et al.*, 2007). Sucrose-dependent is another main mechanism which contributed in *S. mutan's* adherence by produce of homopolymers of glucan from sucrose by glucosyltransferases (GTFs).

The most broadly known virulence factor for most cariogenic bacteria and mostly for *S. mutans* is extracellular polysaccharides and they are the main component in the formation of biofilm (Aires *et al.*, 2010). Li and Burne (2001) mentioned that one of the main virulence factors that initiates formation of caries is the capability of *S. mutans* to synthesize insoluble glucan and extracellular polysaccharides which are necessary for the bacterial accumulation on tooth surfaces. Accumulation of *S. mutans* in the biofilm formation was mediated by extracellular glucan produced from sucrose which is synthesized by GTF. Extracellular glucans which are produced from sucrose by GTFs contributed in adhesion interaction and accumulation of *S. mutans* on the surfaces (Kuramitsu, 1993).

GTF is the enzyme that is known as the virulence factor for *S. mutans*. GTFs enzyme is encoded by *gtf* gene. *S. mutan* was recognized to have at least three of GTFs genes which were *gtfB*, *gtfC* and *gtfD*. The role of *gtfB* was found to produce mostly insoluble polymer (α-1,3-linked) glucan which has been identified to be the reason for the adhesion and accumulation of *S. mutans* on the surface of tooth. This water-insoluble glucan has a rigid structure (Aires *et al.*, 2010) and has high degree of insolubility of their glucan product which cannot be degraded by *S. mutans* enzyme. Hare *et al.* (1978) has stated that the name mutan was given to glucans that consist of abundant of α-1,3-linkages, which allow this glucan to stick to smooth surfaces such as the teeth. Bacteria lacking in glucans have been identified to be far less cariogenic than the wild-type (Munro *et al.*, 1991). While *gtfC* synthesizes mixture of insoluble (α-1.3-linked) and soluble (α-1,6-linked) glucans and *gtfD* produce water-soluble (α-1,6-linked) glucans. This water-soluble glucan of α (1,6)-linkaged serves as extracellular storage (Aires *et al.*, 2010). Glucans provide as short-term storage for

polysaccharides in dental plaque and serve as binding sites for adhesion of oral pathogen to hard surface. When glucans metabolizes, it will create acid that can cause caries. These glucans produced by *S. mutans* are essential and important components of the matrix of cariogenic biofilms (Yousefi *et al.*, 2012).

Among these three genes, *gtfB* was reported as the most important virulence factor of *S. mutans* in initiating the adhesion of *S. mutans* and was observed to have higher expression in biofilm formation (Shemesh *et al.*, 2007). In the previous study, *gtfB* gene was found to be more important for bacterial attachment compared to that of *gtfD* gene (Tsai *et al.*, 2000). *GtfB* gene consisted of major surface protein-antigens of *S. mutans*. This gene was recognized to contribute in the adherence of *S. mutans* to the solid surfaces. *GtfB* gene promotes the coherence of bacteria and adherence to apatic surfaces, providing the formation of dense and highly organized cell clusters which know as microcolonies (Koo *et al.*, 2010, Xiao and Koo, 2010). Numerous previous research have explored the role of *gtfB* which are involved in the virulence factor of *S. mutans* (Napimoga *et al.*, 2005, Shemesh *et al.*, 2010, Yousefi *et al.*, 2012).

Together with GTFs, glucan binding proteins (GBPs) also plays a significant factor in the formation of early adherence and biofilms (Banas and Vickerman, 2003). Sucrose-dependent mechanism of *S. mutans* adherence also mediated by glucan binding proteins, which has found to be involved in the virulence of *S. mutans* (Kuramitsu, 2001). Mattos-Graner *et al.* (2006) has mentioned that the secreted products of *S. mutans* which included of GTFs, their glucan products, and GBPs play a major role in accumulation of bacteria. GBP has been assumed to play a role to

mediate cell-to-surface adhesion cell-to-cell aggregation, and promotes the cohesiveness of plaque. In addition, GBP also act as plaque cohesion, dextranase inhibition, dextran-dependent aggretation, and perhaps cell wall synthesis (Banas and Vickerman, 2003).

GBP was identified to have at least four distinct GBPs which were encoded by *gbpA*, *gbpB*, *gbpC* and *gbpD* respectively. These proteins encourage the adhesion of streptococcal bacteria on teeth and was believed to associated with dental caries (Warren, 1996). Besides of their glucan's similarity, however these proteins were found to have different in function, structure and immunological features (Lynch *et al.*, 2007). *GbpA* was identified to be involved in cellular adherence to the tooth surface and contribute to the virulence of *S. mutans*. Matsumoto *et al.* (2006) has reported that *gbpC* was involved in sucrose-dependent adhesion through adhering to soluble glucan produced by GTFD. Besides that, *gbpD* also consisted of high homology with *gbpA* and was involved in interspecies competition throughout biofilm formation (Shah and Russell, 2004). *GbpB* is the protein that is immunologically different from *gbpA* and was identified to have highly antigenic in humans and rodents. It was believed that *gbpB* is a crucial gene that is positively regulated by the VicRK system under stress condition and *gbpB* was found to be involved in biofilm growth in a select group of clinical isolates (Duque *et al.*, 2011).

Clinical studies have found that most regular antigen that was identified by antibodies in saliva of young children was *gbpB*. This protein also gave a response to the natural immunoglobulin following the early exposure to *S. mutans*, which was possible to modulate infection (Nogueira *et al.*, 2005). *In vitro* study showed that a

systemic or mucosal immunization of rats with *gbpB* encouraged protective immunity to dental caries (Smith *et al.*, 2003), showed that *gbpB* could participate in the cariogenicity of *S. mutans*. In addition, Mattos-Graner *et al.* (2001) has mentioned that *gbpB* shown a positive connection with in vitro biofilm formation and suggested that *gbpB* gene play a roles in the maintenance of cell shape and cell wall of *S. mutans*. This study was supported by (Mattos-Graner *et al.*, 2006) which assumed that *gbpB* gave a function in cell division and synthesis of peptidoglycan. Numerous studies investigated the function of *gbpB* gene in the cariogenicity of *S. mutans* (Matsumoto-Nakano *et al.*, 2007, Duque *et al.*, 2011, Lynch *et al.*, 2013).

2.2 Tooth coloured restorative materials

Restorative materials are used to replace non-functional elements in the oral cavity (Hannig and Hannig, 2009). Decayed primary teeth restoration is very significant and is one of the key factors for the development of healthy and physiological of permanent dentition. For several decades, paediatric dentistry has found amalgam as the standard restorative material with long-proven history and research. Amalgam offered many advantages such as low cost, provides a long shelf life, strong, resistance to wear and easy storage of materials (Yoonis and Kukletova, 2009). Nevertheless, the negative environmental topics of mercury and debates of amalgam on health concern gave the effect on the decreasing use of amalgam in dentistry in the Nordic countries. Since 1995, the use of amalgam as restoration materials has been restricted by the government of Swedish, particularly for children and pregnant woman (Hubel and Mejare, 2003). On top of that, the additional factor that decreased attention on amalgam filling is a silver colour that no longer considered aesthetically acceptable. The effect of dark staining of the tooth and a tattoo of the buccal mucosa

and gingival has been a main reason of the unsatisfactory aesthetics of amalgams. Therefore, there are various alternatives of the restorative materials nowadays. A raise of interest for more aesthetic look has resulted in increasing demand in using tooth coloured restorative materials in dental caries prevention of primary teeth. Tooth coloured restorative materials have been known to offer the aesthetic appearance to the tooth. The improvement and formulation of aesthetic materials particularly on their physical properties has made them acceptable materials in recent years. These aesthetic materials mimic the natural of tooth colour which provide a texture and colour that similar to the patient's teeth to improve the smile. Tooth coloured restorative materials which are composite resin and glass ionomer cement (GIC) are widely used for treating carious teeth (Yoonis and Kukletova, 2009).

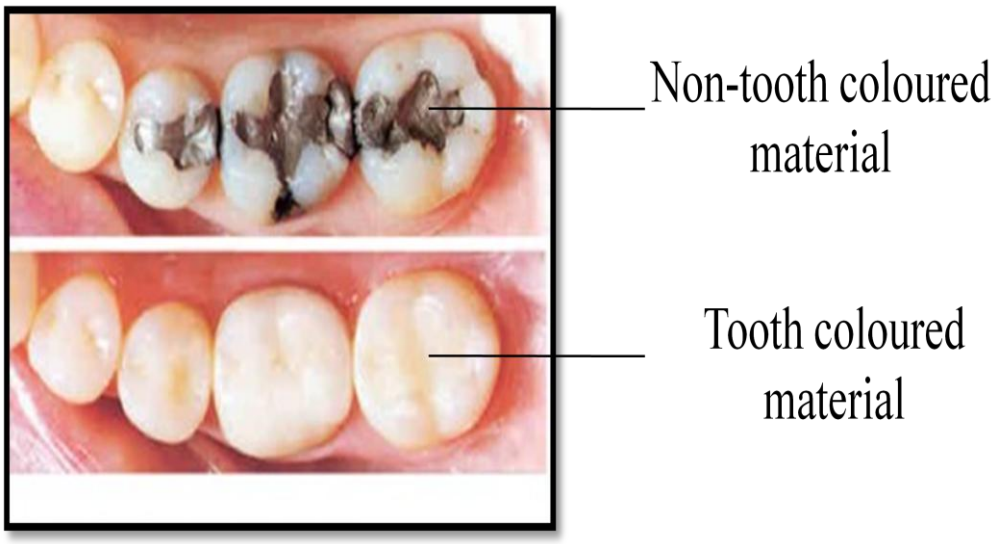

Figure 2.2: Comparison of non-tooth coloured and tooth coloured materials (Restoration, 2016).

2.2.1 Composite Resin

In 1968, composite resin has been used in class II restoration due to its positive development which resulted in decline utilization of the amalgam as the restorations. The improvement of physical and mechanical features of polymerization, resin and bonding systems has made the composite as an important restorative material (Tezvergil *et al.*, 2003). One of the most significant of composite resin is the satisfactory aesthetic appearance which provides a various range of shades that match the enamel, thus offering closely invisible restorations of the teeth. However, composite resin also has a several disadvantages such as polymerization shrinkage, sensitivity to moisture contamination, biocompatibility and limited wear resistance (Hahnel *et al.*, 2010).

Composite resin consists of different type of components: an organic resin polymer matrix, inorganic filler particles, silane coupling agent, initiators/accelerators and pigments. Composite resin consists of several monomers which are urethane dimethacrylates (UDMA), Bisphenol glycidyl methacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA) and ethoxylated bisphenol-A-dimethacrylate (Bis-EMA). Bis-GMA provides many advantages such as lower polymerization shrinkage, high molecular weight (Mw), more rapid hardening and production of stronger and stiffer polymer matrix (Du and Zheng, 2008). On the other hand, its disadvantages are partially negated by a low mobility and relatively high viscosity that might influence to the degree of conversion (Filho *et al.*, 2008). In order to increase the degree of conversion and the filler corporation, TEGDMA which provides a low viscosity diluents monomer was added to thin down the polymer composite (Kim and Shim, 2001). UDMA monomer gave a nearly equal of

molecular weight to Bis-GMA and always applies for the modern composites. However, UDMA gave a relatively low water uptake and less viscous. Each different monomer provides different properties such as polarity, weight, viscosity and polymerization shrinkage. Rahim *et al.* (2012) has reported that Durafill composite resin which contains only monomer of UDMA showed highest solubility compared to Filtek Z350 and Spectrum TPH3 which contain several monomers. This higher solubility was believed to be from 100 % of UDMA monomer which gave higher viscosity of resin matrix. Higher solubility by Durafill composite resin may result in increasing restriction on molecular mobility and hence cause less degree of conversion and degree of cross-linking.

2.2.1.1 Filler particles size of composite

The capability in controlling stress and wear of composite resin depend on the type and the ratio between the organic matrix and the filler particles (Chan *et al.*, 2010). The classification of composite resin is according to the size of filler particle which are macrofill, microfill, microhybrid and nanofill. Macrofilled composite resin consists of crystalline quartz filler. The filler of quartz made up of 8-12 microns of particle size. The quartz filler of macrofilled promotes great optical properties and chemical inertness. However, macrofilled has the possibility to abrade opposing tooth structure, hard to polish due to the large particle size and increase in wear (Ferracane, 1995).

Microfilled composite resin is often used for an anterior restoration which consists of submicroscopic particles of silicon dioxide, ranged approximately 0.04 μm in diameter. Microfilled composite resin was believed to be the first materials to be

wear resistant and sustain the surface quality due to the low filler content and small size of filler. However, major concerns of the microfilled composite resin are low tensile strength, low fracture toughness and increase in polymerization shrinkage (Ferracane, 1995). Microhybrids composite resin contains larger particles and smaller particles of sub-micron sized. The average particle size is smaller than 1.0 µm (Sensi *et al.*, 2007). Most fillers of microhybrid composite have irregular morphology and ground glass particles (Lu *et al.*, 2006). The microhybrid offers high luster, high physical strength, acceptable polymerization shrinkage and the ability to characterize restorations. However, microhybrid tends to exhibit low surface polish retention (Suzuki *et al.*, 1995, Turkun and Turkun, 2004).

Recently, nanofilled composite resin has been introduced to provide the functional need by applying the application of nanotechnology and offer many advantages (Mitra *et al.*, 2003). Nanofilled composite resin contain nano-filler particles in the resin matrix with a size in the range of 0.1-100 nm, which present in two forms which are nanomer and nanoclusters (Moraes *et al.*, 2009). Nanomer particles consist of the individual filler particles of 20-75 nm in dimensions which are mainly spheroidal in shape. While nanoclusters consist of loosely agglomerated collections of the nanoparticles with average size of 1 µm. The function of these clusters are similar to micro-fillers and provides well polished, but also gave the similarities to the large particle which offering a strength and reduced shrinkage. Nanofilled composite resin, are often used for larger, posterior restorations since it provides a strength, polishability and less shrinkage (Chan *et al.*, 2010), improving mechanical properties and allowed for a significant increase in filler volume (Hahnel *et al.*, 2010). Besides that, nanofilled also provides an excellent properties such as wear