ANTIBACTERIAL, CYTOTOXIC AND GENOTOXIC EFFECTS OF NANO-HYDROXYAPATITE-SILICA GLASS IONOMER CEMENT AND ITS DENTINE PULP COMPLEX RESPONSE IN A RAT MODEL

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by

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LIST OF SYMBOLS

%	Percentage
~	Around
<	Less than
±	Plus, minus
≤	Less than or equal to
α	Level of significance
μm	Micrometre
Ag^{+}	Silver ion
Al	Aluminium
Al_2O_3	Alumina
Al^{3+}	Aluminium ion
$AlPO_4$	Aluminium phosphate
Ba	Barium
Ca	Calcium
Ca^{2+}	Calcium ion
CaF_2	Calcium fluoride
COOH	Carboxylic acid
CO_2	Carbon dioxide
F	Fluoride
F+	Fluoride ion
G	Gram
H_2O	Water
H_3PO_4	Phosphoric acid

Kg	Kilogram
L	Length
La	Lanthanum
Mg	Magnesium
ml	Millilitre
mm	Millimetre
N	Sample size
Na^{+}	Sodium ion
NaCl	Sodium Chloride
Na_3AlF_6	Cryolite
NaF	Sodium fluoride
Nb_2O_5	Niobium pentoxide
NH_3	Ammonia
Nm	Nanometre
O	Oxygen
°C	Degree centigrade
OH	Hydroxide
P	Phosphorus
SDF	Silver diamine fluoride
Ra	Surface roughness
S	Second
Si	Silicate
Si^{+4}	Silicon ion
SiO_2	Silica
SiO_4	Silicon-oxygen tetrahedron

Sr	Strontium
SrO	Strontium oxide
TiO_2	titanium oxide
V	Volume
Si	Silicate
Wt	Weight
wt %	Weight percentage
Zn	Zinc
ZnO	Zinc oxide
ZrO_2	Zirconia
SiNPs	Silica-based NPs
H_3PO_4	Phosphoric Acid
$Ca(OH)_2$	Calcium Hydroxide
$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite

LIST OF ABBREVIATIONS

4NOPD	4-Nitro-o-phenylenediamine
9AA	9-Aminoacridine
ADA	American dental association
ALS	Alkaline Labile Sites
ANOVA	One-way analysis of variance
ART	Atraumatic restorative technique
ASPA	Alumino-silicate polyacrylic acid
BAG	Bio-active glass
BHI	Brain Heart Infusion
BisGMA	Bisphenol A glycidyl methacrylate
C.albicans	*Candida albicans*
Ca/P	Calcium/phosphorus
CEJ	Cemento-enamel junction
cGIC	Conventional glass ionomer cement
CHX	Chlorhexidine
D	Dentine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPSC	Dental pulp stem cell
DPX	Dibutylphthalate Polystyrene Xylene
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*

EDTA	Ethylenediaminetetraacetic acid
EDX	Energy dispersive x-ray
ELISA	Enzyme-linked immunoassay
FA	Fluorapatite
FBS	Foetal bovine serum
FDA	Food and drug administration
FPG	Formamido-pyrimidine DNA glycosylase
FTIR	Fourier transform infra-red
GIC	Glass ionomer cement
H & E	Haematoxylin and Eosin
H_2O_2	Hydrogen peroxide
HA	Hydroxyapatite
HEMA	2-hydroxyethyl methacrylate
HPLFs	Human periodontal ligament fibroblasts
IC	Inhibitory concentration
ICC	Intra-class coefficient
ISO	International Organization for Standardization
L. acidophilus	*Lactobacillus acidophilus*
LMP	Low-melting point
MBC	Minimum bactericidal concentration
MCB	Molecular Cell Biology
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentration
MMS	Methyl methanesulfonate

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
nano-Ag	Nano-silver
Nano-FA	Nano-fluorapatite
nano-HA	Nano-hydroxyapatite
nano-HA-SiO_2	Nano-hydroxyapatite-silica
nano-HA-SiO_2-GIC	Nano-hydroxyapatite-silica-glass ionomer cement
Nano-SiO_2	Nano-silica
NBF	Neutral buffered formalin
OL	Odontoblast layer
P	Pulp
PAA	Polyacrylic acid
PBS	Phosphate buffered saline
PD	Predentine
PTEE	Polytetrafluoroethylene
RDT	Remaining dentin thickness
RMGIC	Resin modified glass ionomer cement
S typhimurium	*Salmonella typhimurium*
S. aureus	*Staphylococcus aureus*
S. mutans	*Streptococcus mutans*
SA / NaN_3	Sodium azide
SE	Standard error
SEM	Scanning electron microscope
SEM/EDX	Scanning electron microscope/Energy dispersive x-ray

SHED	Stem cells from human exfoliated deciduous teeth
SPSS	Statistical Package for the Social Sciences
SSB	Single strand breaks
TDF	Tertiary dentin formation
TDT	Tertiary dentin thickness
TEGDMA	Tri ethylene glycol di methacrylate
TEOS	Tetraethyl orthosilicate
TSA	Trypticase soy agar
USM	Universiti Sains Malaysia
WHO	World Health Organization

LIST OF APPENDICES

KESAN ANTIBAKTERIA, SITOTOKSIK DAN GENOTOKSIK BAGI SIMEN IONOMER KACA NANO-HYDROXYAPATITE-SILIKA DAN TINDAK BALAS KOMPLEKS PULPA DENTIN DALAM MODEL TIKUS

ABSTRAK

Kajian ini bertujuan untuk menilai kesan antibakteria dan kesan genotoksik simen ionomer kaca nano-hidroksiapatit-silika (nano-HA-SiO_2-GIC) serta menilai tindak balas kompleks dentin-pulpa dalam model haiwan. Kepekatan bakteria minimum (MBC), kepekatan penghambatan minimum (MIC) dan ujian pembunuhan mengikut masa (TKA) dijalankan untuk menilai keberkesanan antibakteria bagi nano-HA-SiO_2-GIC pada kepekatan 10% dan dibandingkan dengan simen ionomer kaca konvensional (cGIC) terhadap tiga bakteria berbeza iaitu *Streptococcus mutans*, *Staphylococcus aureus*, dan *Enterococcus faecalis*. Mutagenisiti dan kerosakan DNA bagi nano-HA-SiO_2-GIC juga dinilai menggunakan ujian Comet dan ujian Ames. Seterusnya, kajian in-vivo dijalankan untuk menilai dan membandingkan tindak balas kompleks dentin–pulpa selepas pemulihan oklusal dan servikal pada geraham tikus yang dipulihkan dengan nano-HA-SiO_2-GIC dan cGIC. Telah didapati bahawa kedua-dua *S. aureus* dan *E. faecalis* menunjukkan rintangan yang lebih tinggi terhadap cGIC dengan MIC sebanyak 30µg/mL. Sebaliknya, MIC bagi cGIC terhadap *S. mutans* adalah 20µg/mL. MIC bagi nano-HA-SiO_2-GIC adalah sama dengan cGIC untuk *E. faecalis* (30µg/mL), manakala MIC sebanyak 10µg/mL bagi *S. mutans* dan *S. aureus* ($p < 0.05$). Ujian pembunuhan mengikut masa menunjukkan nano-HA-SiO_2-GIC membunuh 99% bakteria yang diuji dalam masa 6 jam, manakala cGIC mengambil masa 8 jam untuk membasmi bakteria tersebut. Kebolehhidupan sel tertinggi (159.4%) untuk nano-HA-SiO_2-GIC diperhatikan pada kepekatan 3.125 mg/mL, manakala

kebolehhidupan sel terendah (24.26%) diperhatikan pada kepekatan 200 mg/mL. Nilai IC_{50}, IC_{25} dan IC_{10} masing-masing adalah 95.27, 51.4 dan 20.1 mg/mL untuk cGIC, dan 106.9, 55.8 dan 22.9 mg/mL untuk nano-HA-SiO_2-GIC. IC_{10} bagi kedua-dua bahan ujian tidak menunjukkan kerosakan DNA yang signifikan berbanding kawalan negatif berdasarkan ujian Comet ($p > 0.05$). Walau bagaimanapun, terdapat perbezaan yang signifikan pada nilai *tail moment* antara semua kepekatan kedua-dua jenis kumpulan GIC serta kawalan positif ($p < 0.05$). Ujian Ames menunjukkan nano-HA-SiO_2-GIC menghasilkan kurang daripada dua kali ganda purata koloni revertan berbanding kawalan negatif. Bagi kajian in-vivo, parameter seperti gangguan tisu pulpa, penyusupan sel radang, kehadiran bakteria, dan pemendapan dentin tertier telah diukur bagi setiap kumpulan. Secara keseluruhan, tiada perbezaan antara pemulihan servikal dan oklusal dari segi ketebalan dentin yang tinggal (RDT). Selepas seminggu tikus dikorbankan, lapisan odontoblas terganggu dan kawasan pulpa berhampiran dentin yang terpotong menunjukkan keradangan sederhana dalam kedua-dua jenis pemulihan. Selepas satu bulan, tiada bukti gangguan pada lapisan odontoblas dikesan. Dari segi keradangan, tisu pulpa pulih dalam hampir semua kes kecuali satu kes cGIC, namun beberapa kes dalam kumpulan nano-HA-SiO_2-GIC masih menunjukkan reaksi keradangan yang ringan hingga sederhana, terutamanya pada pemulihan oklusal. Perbezaan yang signifikan pada ketebalan dentin tertier (TDT) diperhatikan pada geraham pertama bagi kedua-dua cGIC (66.21 ± 43.15) dan nano-HA-SiO_2-GIC (96.66 ± 41.2) berbanding geraham kedua (31.97 ± 5.30). Penambahan nano-HA-SiO_2 ke dalam cGIC secara signifikan meningkatkan sifat antibakteria, didapati tidak mutagenik dan tidak menyebabkan kerosakan DNA pada kepekatan terendah (IC10) berdasarkan ujian Comet. Selain itu, ianya menunjukkan tindak balas kompleks dentin-pulpa yang lebih baik berbanding cGIC. Berdasarkan penemuan kajian ini,

nano-HA-SiO_2-GIC menunjukkan hasil yang memberangsangkan dan berpotensi untuk digunakan sebagai bahan pergigian klinikal pada masa hadapan.

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ABSTRACT

The aim of this study was to assess antibacterial and genotoxic effects of nano-hydroxyapatite-silica glass ionomer cement (nano-HA-SiO_2-GIC) and its dentine pulp complex reactions' evaluation in an animal model. Minimal bacterial concentration (MBC), minimal inhibitory concentration (MIC), and time kill assay (TKA) were carried out to assess antibacterial efficacy for 10% nano-HA-SiO_2-GIC and compared with conventional Glass ionomer cement (cGIC) against three different bacteria *Streptococcus mutans*, *Staphylococcus aureus* and *Enterococcus faecalis*. Mutagenicity and DNA damage of nano-HA-SiO_2-GIC using Comet assay and Ames test were also evaluated. Further, an *in-vivo* study was performed to evaluate and compare the dentin–pulp complex response following occlusal and cervical restorations in rat molars restored with nano-HA-SiO_2-GIC and cGIC. It was found that both *S. aureus* and *E. faecalis* exhibited comparatively greater resistance to cGIC with an MIC of 30µg/mL. In contrast, the MIC of cGIC against *S. mutans* was 20µg/mL. The MIC for nano-HA-SiO_2-GIC were the same for *E. faecalis* when compared with cGIC (30µg/mL) whereas it was 10µg/mL for both *S. mutans* and *S. aureus* ($p < 0.05$). Time kill assays revealed that nano-HA-SiO_2-GIC effectively killed 99% of the tested bacteria after 6 hours whereas cGIC was able to eradicate these bacteria in 8 hours. The highest cell viability (159.4%) for nano-HA-SiO_2-GIC was noticed at 3.125 mg/ml, while the lowest (24.26%) was observed at 200 mg per ml. IC_{50}, IC_{25} and IC_{10} values were 95.27, 51.4 and 20.1 mg/ml for cGIC, and 106.9, 55.8

and 22.9 mg/ml for nano-HA-SiO_2-GIC respectively. The IC_{10} of both test materials showed no significant DNA damage compared to that of the negative control based on the Comet assay ($p > 0.05$). Despite this, a significant difference was present in the tail moment between all concentrations of both types of GIC groups as well as the positive control ($p < 0.05$). Nano-HA-SiO_2-GIC showed less than double the average number of revertant colonies compared to that of the negative control when tested using Ames test. For *in-vivo* studies, parameters such as disorganization of the pulp tissue, inflammatory cell infiltration, detection of bacteria, and tertiary dentin deposition were measured for each group. Overall, there was no difference between cervical and occlusal restorations in terms of remaining dentine thickness (RDT). One week after the sacrifice, the odontoblastic layer was disrupted the pulp area close to the cut dentin displayed moderate inflammation in both types of restorations. One month after sacrifice, there was no evidence of disruptions of the odontoblast layer. In terms of inflammation, the pulp tissue recovered in almost all cases except one of c-GIC, but a few cases of the nano-HA-SiO_2-GIC group still displayed mild-to-moderate inflammatory reactions, especially on the occlusal restorations. A significant difference in tertiary dentin thickness (TDT) in first molars was observed for both cGIC (66.21 ± 43.15), and nano-HA-SiO_2-GIC (96.66 ± 41.2) as compared to second molars (31.97 ± 5.30). The addition of nano-HA-SiO_2 to cGIC significantly enhanced the antibacterial properties, found to be non-mutagenic and do not cause DNA damage at the lowest concentration of IC_{10} based on the Comet assay. In addition, it exerted favourable dentine pulp complex response when compared to cGIC. Based on the findings of the current study, nano-HA-SiO_2-GIC produce promising findings and thus can be suggested as a future potential material for use in clinical dentistry

CHAPTER 1

INTRODUCTION

1.1 Background

Glass Polyalkenoate Glass ionomer cement (GIC) is a biocompatible material which encourages both additional remineralization and inhibition of demineralization of tooth structures adjacent to fillings. Fifty-five years ago, Wilson and Kent (1972), developed tooth adhering cement known as GICs. Because of their groundbreaking work giving such advantages like anti-cariogenic due to release of fluoride, and direct adhesion to the tooth structure, GICs were principally used in the dental setup.

Fluoride releasing property of GIC has been documented in the literature (Mickenautsch *et al.,* 2011). After being released from GIC, the fluoride ions take part in the remineralization and demineralization phenomena and may act directly on the carious process (Dowling *et al.,* 2006). Although it has many benefits, the application of GIC as a restorative material remains contentious due to concerns over secondary caries and weak mechanical qualities. However, the application of these materials in clinical settings has been restricted due to their poor mechanical qualities, such as low fracture toughness and flexural strength (Wilson, 1991; Mount *et al.*, 2002). Furthermore, the considerable opacity, vulnerability to moisture during the initial setting process, and rough surface of these cements contribute to their undesirable characteristics.

The physico-mechanical properties of GIC are enhanced by adding gold (Au) and silver (Ag) particles into the glass powder. Though their flexural strength was more than that of cGIC, the resultant Cermet ionomer cements were still insufficiently strong to replace amalgam (McLean *et al.,* 1994). Few attempts have also been made to improve its mechanical properties by incorporating conventional GIC with additives such as hydroxyapatite (HA), zirconia, glass, stainless steel, HA-zirconia, HA-glass etc, but the outcomes were not momentous (Berg and Croll, 2015). For that reason, the addition of nano sized fillers into GIC is becoming prevalent as they have demonstrated better mechanical properties in comparison to conventional GIC (Najeeb *et al.,* 2016). The incorporation of nano-HA alone has been demonstrated to generate a favourable environment for the remineralization of enamel (Moshaverinia *et al.,* 2008a), while the use of nano-silica in GIC enhances its bioactivity, possibly preventing the creation of marginal gaps within the tooth (Mabrouk *et al.,* 2012).

In 2014, for the first time, the amalgamation of nano-HA and nano-silica as a filler for GIC has been reported by Rahman and colleagues. The addition of nano-HA-SiO_2 enhanced the hardness of the tested material relative to cGIC. (Rahman *et al.,* 2014). They suggested that these promising findings could be associated to an increase in packing density because of nano-silica particles filling the gaps between the nano-HA in GIC matrix (Shiekh *et al.,* 2014).

The use of nanoscale materials in dentistry is becoming progressively popular and advantageous due to their superior properties in relation of antibacterial properties, strength, and aesthetic values when equated with commercial materials (Sreenivasalu *et al.,* 2022). Previous literature has extensively described numerous nanoparticles that exhibit significant potential as antibacterial agents for the management of tooth

decay and other dental conditions (Sawarkar *et al.*, 2016; Yudaev *et al.*, 2022). Dental experts also stress the need to prevent tooth decay by regulating biofilm growth, promoting remineralization, and using antibacterial measures to prevent oral cavity infections. However, up till now, there is still no data available in terms of minimum inhibitory concentration, minimum bactericidal concentration or time kill assay related to nano-HA-SiO_2-GIC against the commonly present bacteria related to oral cavity.

Second important criterion for dental materials that are considered optimal is their biocompatibility. This is due to their interaction with dental tissues and potential impact on the tissue response once the material is introduced into the mouth cavity. Therefore, it is imperative to conduct tests to assess the biocompatibility of dental biomaterials in order to ensure their safety for usage. Cytotoxicity is one of the in-*vitro* method used to assess a material's biocompatibility (Luddin, 2019). Genotoxicity tests are also important in biological research because they are closely linked to the initial stage of cancer development. This can be triggered by the stimulation of cell cycle proliferation or errors in the mitotic phase due to DNA damage, which impairs the ability to repair the damage (Bull *et al.*, 2006). Noorani and coworkers (2017) evaluated the cytotoxicity of nano-HA-SiO_2-GIC on human dental pulp stem cells (DPSCs), and it was found to have a positive effect on these cells. A separate investigation conducted by Hii *et al.* (2019) demonstrated that nano-HA-SiO_2-GIC displayed favourable biocompatibility on DPSCs, which was similar to that of cGIC. Furthermore, additional work related to dentinogenic differentiation potential on the same material revealed positive outcomes and supported the actual evidence that nano-HA-SiO_2-GIC contributes to the odontogenic differentiation of DPSCs (Ching *et al.*, 2020).

The International Organization for Standardization (ISO) has established recommendations that outline various procedures for assessing the biocompatibility of biomaterials used in medical devices. These tests, which are conducted both *in-vitro* (outside of a living organism) and *in-vivo* (inside a living organism), aim to determine the compatibility and potential toxicity of the biomaterial. The ISO standard 7405 specifies testing methods for dental materials and the preclinical assessment of the biocompatibility of medical devices used in dentistry (ISO-7405, 2018). Chapters 6.3 and 6.4 of this ISO standard specifically address the examination of dental pulp and dentine utilization tests. The third important criterion is the *in-vivo* investigation of restorative material and its effects on dentine pulp complex. The molar teeth of rats, encompassing the pulp tissue, might be regarded as miniature versions of human molars, exhibiting identical morphological, histological, biological, and physiological characteristics (Dammaschke, 2010).

The above-mentioned convincing results of nano-HA-SiO_2-GIC offer some promise for the newly developed nano-HA-SiO_2-GIC to be used in wider application as a restorative material. However, its dentine pulp complex response in animals has not been investigated which warrants the current study to be investigated.

1.2 Problem statement

GIC is known for its anti-cariogenic properties due to their release of fluoride (Svanberg, 1992; Tyas, 2018). The majority of commercially available GIC meet the basic requirements of ISO standards and are classified as clinical grade GICs (ISO-9917-1, 2007). Nevertheless, some limitations such as susceptibility to dehydration,

low mechanical and physical properties limit their use as a filling material, especially under heavy occlusal load (Pelka *et al.,* 1996). Recently, nano-HA-SiO_2 has been successfully incorporated with noteworthy improvement in hardness and flexural strength (Moheet *et al.,* 2018). Additionally, preliminary data related to fluoride release, colour stability, cell attachment and dentinogenic differentiation potential also revealed favourable findings of this nano-HA-SiO_2-GIC (Moheet *et al.,* 2020; Ching *et al.,* 2020).

Despite the many investigations carried out, up to date, still, there is lack of information on the antibacterial properties and genotoxic potential of this nano-HA-SiO_2-GIC. Besides, no *in-vivo* studies have been attempted to verify the effects of this material on animal models. Consequently, data related to antibacterial, genotoxicity and histopathological evaluation of nano-HA-SiO_2-GIC is unavailable. Therefore, the current study aims to investigate the antibacterial and genotoxic potential of this newly developed material as well as to determine its effects on tooth dentin pulp complex before it can be recommended as an alternative, stronger restorative material for future use in clinical dentistry.

1.3 Justification of the study

To the best of our knowledge the effect of nano-HA-SiO_2-GIC on different bacteria involved in caries initiation and progression has yet to be examined. Moreover, its cytotoxic and genotoxic effects need to be carried out as dental restorations are always in close contact with dental pulp and cells of periodontium. Furthermore, its biological effects on animal models to observe its dentine pulp complex response is not yet performed and documented. It is hoped that the results of

this study will provide a better understanding of the antibacterial efficacy involved in this newly developed nano-HA-SiO_2-GIC and it has the potential to form new dentine in more realistic animal-based study. This can validate an innovative material that chemically binds with dentine in stress-bearing dental applications to provide greater tooth protection while also having strong antibacterial qualities and no cytotoxic or genotoxic effects.

1.4 General objectives

The general objective of the current study is to investigate the antibacterial properties and genotoxic potential of nano hydroxyapatite-silica glass ionomer cement *in-vitro* and evaluate its histological dentine pulp complex response *in-vivo* in an animal model.

1.5 Specific objectives

1. To compare the antibacterial properties of nano-HA-SiO_2-GIC with conventional GIC (cGIC) using minimum bactericidal concentration, minimum inhibitory concentration and time kill assay.
2. To determine the inhibitory concentrations (IC_{50}, IC_{25} and IC_{10}) of nano-HA-SiO_2-GIC on human periodontal ligament fibroblast (HPLFs) using MTT assay.
3. To evaluate the DNA damage of nano-HA-SiO_2-GIC using single cell gel electrophoresis assay (Comet assay) on human periodontal ligament fibroblast (HPLFs).
4. To investigate the mutagenicity of nano-HA-SiO_2-GIC using Ames test
5. To evaluate the dentine pulp complex response after occlusal and cervical restorations of nano-HA-SiO_2-GIC in Wistar rat's molar teeth

as compared to cGIC in terms of pulp tissue disorganization, inflammatory cell infiltration, tertiary dentine formation and stained bacteria.

1.6 Research questions

1. Does nano-HA-SiO_2-GIC possess better antibacterial properties than conventional GIC (cGIC)?
2. Does nano-HA-SiO_2-GIC have any cytotoxic effect based on MTT assay on human periodontal ligament fibroblast (HPLFs)?
3. Does nano-HA-SiO_2-GIC cause any DNA damage on human periodontal ligament fibroblast (HPLFs) as compared to cGIC?
4. Does nano-HA-SiO_2-GIC exhibit any mutagenic effect based on Ames test?
5. Does nano- HA-SiO_2 GIC have better dentine pulp complex response as compared to cGIC in Wistar's molar teeth when used as a restorative material?

1.7 Alternate hypothesis

1. Nano-HA-SiO_2-GIC has better antibacterial properties than cGIC.
2. Nano-HA-SiO_2-GIC does not show any cytotoxic effect based on MTT assay on human periodontal ligament fibroblast (HPLFs).
3. Nano-HA-SiO_2-GIC does not cause any DNA damage on human periodontal ligament fibroblast (HPLFs) as compared to cGIC.
4. Nano-HA-SiO_2-GIC does not exhibit any mutagenic effect based on Ames test.
5. Nano-HA-SiO_2-GIC has better dentine pulp complex response in Wistar's molar teeth as compared to cGIC when used as a restorative material.

1.8 Research summary

The novel material was produced by adding 10% nano-HA-SiO_2 powder to conventional glass ionomer cement (cGIC) powder and mixed with the liquid of cGIC. In the first part, the nano-HA-SiO_2-GIC was then evaluated for its antibacterial effects by performing minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time kill assay on three different types of bacteria (*Streptococcus mutans, Staphylococcus aureus and Enterococcus faecalis*). These tests were carried out in accordance with the International Standard Organization (ISO) standards; ISO-20776-1(2019) (Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices). The newly synthesized nano-HA-SiO_2-GIC was compared with the commonly available conventional type of cGIC; GC Fuji IX (Japan). In the second part, cytotoxicity test was performed by conducting MTT assay to get IC_{50}, IC_{25} and IC_{10} values which were

utilized in genotoxicity study of Comet assay or Single cell electrophoresis test as per ISO-10993-5(2009) recommendations which is concerned with biological evaluation of medical devices - Test for *in-vitro* cytotoxicity. Another genotoxicity study was performed using Ames test with four tester strains of *Salmonella typhimurium (S. typhimurium*) bacteria (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (*E. coli*) (WP2 uvrA). The third part was *in-vivo* study of evaluation of dentine pulp complex's reaction after performing occlusal and cervical restorations in rats' molar and comparing with cGIC. This *in-vivo* study was performed in accordance with ISO-10993-2(2022) (Biological evaluation of medical devices - Part 2: Animal welfare requirements). The overall study design flow chart is shown on the next page as Figure 1.1.

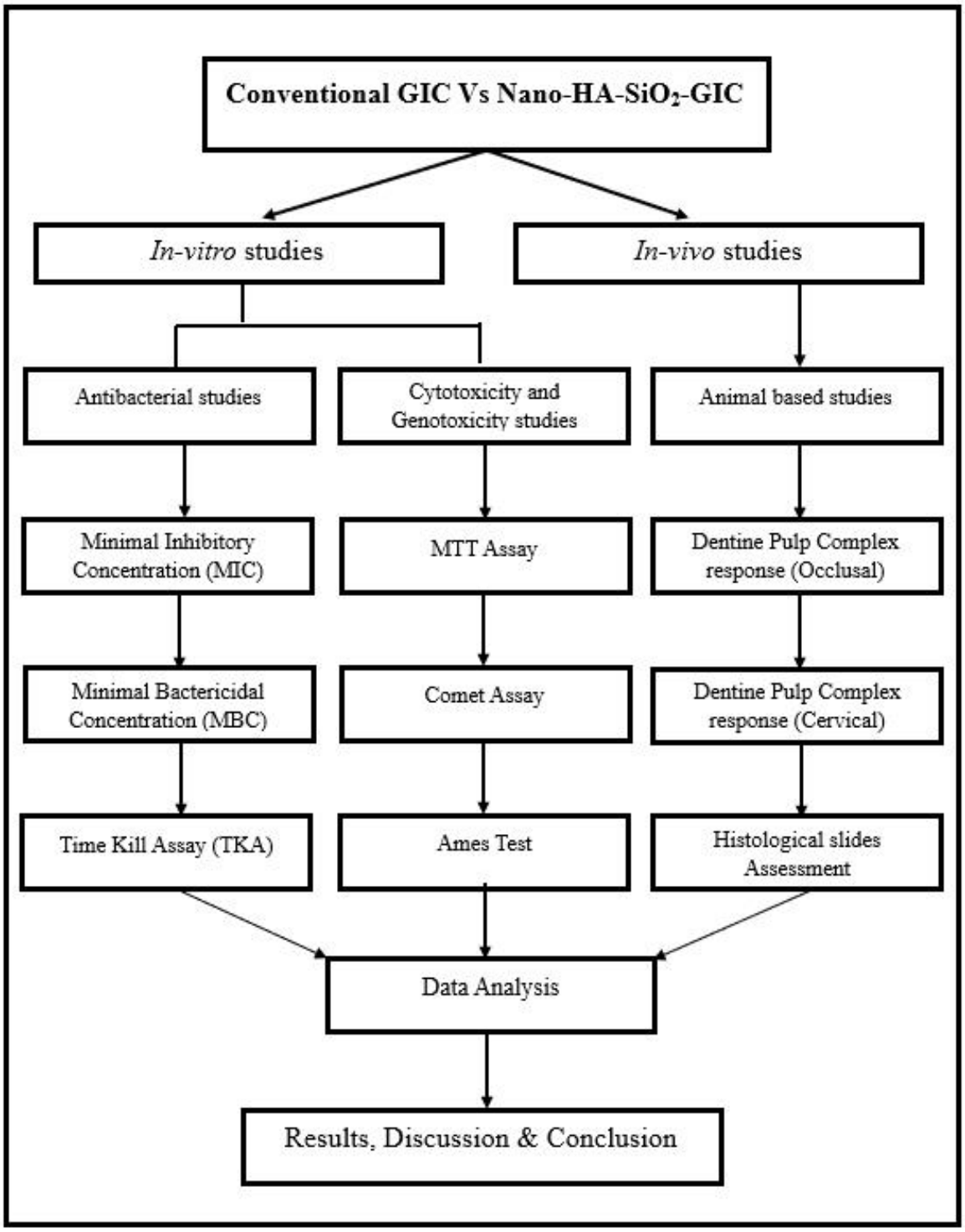

Figure 1.1 Flow chart of Research Methodology

CHAPTER 2

LITERATURE REVIEW

2.1 Dental caries and clinical challenges

Dental caries is defined as "a multi-factorial disease involving many complicated risk and protective variables" (Young *et al.,* 2015). A carious lesion is the clinical presentation of caries; the disease severity for every individual carious lesion is controlled by numerous environmental, biological, behavioural, and individual factors (Young *et al.,* 2015).

As one of the commonest oral infectious diseases, progression of dental caries can lead to pain, reduced quality of life and loss of dentition (Naik *et al.,* 2016). Prevalence of dental caries ranges from 60 to 90 % among children and almost 100 % in adults, with underprivileged individuals suffering most from it (Naik *et al.,* 2016). Dental caries affects over 2.4 billion people worldwide, or 36% of the population (Yadav *et al.,* 2016). Dental caries resulted in the loss of primary teeth in more than 530 million children worldwide (Shitie *et al.,* 2021).

Dental treatments are time consuming, costly and cannot prevent recurrence in the absence of proper oral hygiene maintenance. It is therefore categorized as a public health disease. Further, novel microenvironments developed during cavity preparation or anatomical defects such as marginal gaps can cause postoperative sensitivity, recurrent caries, pulp inflammation and ultimately necrosis in dental tissue. A possible way to prevent this secondary caries development is creating dental materials that are resistant to biofilm development and deposition (Fúcio *et al.,* 2016).

Management of dental caries is through prevention and treatment. Dental restorative materials help fill the gaps created due to carious activity. An excess of material options for dentists is now available for dental restorations. However, all dental materials must ensure quality restoration and provide dental seal to prevent recurrent caries. Unfortunately, this objective remains elusive, and the rate of repeat dental restorations due to secondary caries is common. These repetitive interventions have inspired dentists to develop novel materials that overcome the challenges mentioned above (Naik *et al.,* 2016).

2.2 Overview of Glass ionomer cement (GIC)

Over the years, Glass Ionomer Cement (GIC) has received much attention among dental materials as a restorative cement due to its special chemical bonding characteristics with the tooth structure. Key properties of GIC include adhesion to dental tissue as well as base metals, anticariogenic and fluoride leaching properties, thermal and biocompatibility with the tooth and surrounding tissue, and adequate tooth structure reinforcement and strengthening qualities (Amin *et al.,* 2021).

Water, basic (ion-leachable) glass, and polymeric water-soluble acid are the three main components of GIC (Sidhu and Nicholson, 2016). The polymeric acid liquid form is mixed with powder glass form to create a viscous paste with quick setting time. Hence placement and manipulation of the material is time sensitive in clinical settings. Variants in the compositional ratio of cement and powder may be present. However, post-setting properties are usually similar or correspond to a range of mechanical properties (Sidhu and Nicholson, 2016).

Many drawbacks of the material still exist, which prompted efforts to improve the cement's quality by addition and modification of some materials. Some of these include addition of polyvinyl phosphonic acid, fibre-reinforcement, bioactive apatite with or without zirconia, zinc, strontium oxide, stainless steel, silica, amino acids, and N-vinylpyrrolidone respectively (Najeeb *et al.*, 2016).

Additionally, the use of nanostructures in dentistry has grown into a significant area of exploration. GICs have been improved mechanically by adding a variety of nanomaterials, including ceramic, polymers, and metals (Kerby and Bleiholder, 1991; Rahman *et al.*, 2014; Amin *et al.*, 2021).

2.2.1 Historical background of GICs

The foundations of GICs began when dental silicate and zinc polycarboxylate cements were being researched for their properties and methods to improve their qualities as dental restorative materials (Wilson, 1991). Initial studies on dental silicate cements demonstrated the acid-base setting reaction and development matrix of metal phosphates with unreacted glass fillers. These research efforts helped refine the alumina/silica basicity ratio. This led to the creation of G200, one of the first glass cements with high fluoride content and chelating additives to control setting reactions. It was during these findings that the efficacy of tartaric acid was highlighted (Nicholson, 2016).

Based on these findings, some of the first experiments on developing GICs involved using calcium aluminosilicates with fluoride, with corresponding alterations to its ratios to enable optimal reactions with aqueous polyacrylic acids (Nicholson,

2016). Other added materials included strontium and fluoride to prevent premature gelation and thereby increase setting time of the cement.

2.2.2 Classification of GIC cements

GIC cements are classified based on clinical indications as shown in Table 2.1 (Sidhu and Nicholson, 2016).

Table 2.1 Different classification of GIC cements

Type 1	Luting and bonding cements	• Luting of crowns, inlays, onlays and bridges • Low powder: liquid ratio (1.5:1 to 3.8:1) • Radiopaque • Fast setting • Moderate strength • Good water resistance
Type 2 sub i	Restorative cements	• For anterior aesthetic repairs • High powder: liquid ratio (from 3:1 to 6.8:1) • Excellent colour fidelity and translucency • Moisture isolation for 24 hours with petroleum jelly or varnish • Radiopaque
Type 2 sub ii	Restorative cements	• For posterior restorations • High powder: liquid ratio (3:1 to 4:1) • Early and strong resistance to water absorption • Radiopaque
Type 3	Lining or base cements	• Low powder to liquid ratio (1.5:1) as liners and bases for cavity walls • High powder to liquid ratio (3:1 to 6.8:1) for base • Can serve as substitute of dentine when used with composite resin • Radiopaque
Other uses	Fissure sealants	• Hydrophilic • Dimensionally stable • Superior marginal adaptation and seal • Lower caries rates • Inferior retention rates compared to composite resin sealants

2.2.3 Composition of GIC's

A restorative material based on the interaction of glass powder and polyacrylic acid are together known as GIC cements. GICs demonstrate chemical bonding to tooth structure, binding to both organic and inorganic tooth components. A powder, an aluminosilicate glass that dissolves in acid and a diluted solution of polyacrylic acid (PAA) (or an analogous polyacid) makes up the cement. Calcium (Ca_2+) and aluminium (Al_3+) ions released from the powder reacts with carboxylic acid -COOH groups to form divalent salts, which cross-link and cure the polymeric acid. The carboxylic groups also react with the dental surfaces to affect chemical adhesion to the tooth structure. Further, interdiffusion into the collagen fibrils allows for micromechanical adhesion of the cement to the tooth structure (Anusavice *et al.,* 2012).

GIC consistency depends on the powder composition, particle size and powder liquid ratio. The powder is composed of silica, calcium, alumina, and fluoride as a base formulation. Added to it are barium, strontium, or other higher atomic number metal oxides to increase radio-opacity. Commonly used GICs subclass are the resin modified GIC (RM-GIC). Some metal-reinforced GICs have metal incorporated to increase toughness and stress-bearing capacity. However, addition of metals gives the cement a grayish and more radiopaque, creating an alloy admixture and cermet (Amin *et al.,* 2021). The growing interest of researchers into the area of nanotechnology over the past 10 years has corresponded to a rise in patent applications. Dental biomaterials with nanostructures have improved characteristics especially mechanical (Moheet *et al.,* 2018) and antibacterial properties (Foong *et al.,* 2020).

2.2.4 Properties of GIC

GIC is considered as one of the aesthetic restorative materials. GIC is sensitive to the method utilized in preparing the cement. GIC continues to set in the oral cavity after it has been placed, which plays an vital role in increasing its hardness and improve its performance. Once it fully matured in the oral environment, fluoride released from GICs to the surrounding tissues inhibits secondary caries and has a very high level of abrasion resistance (Carey *et al.,* 2003; Billington *et al.,* 2007). Clinical performance in terms of marginal adaptation, marginal discolouration and anatomical shape is commendably comparable to that of composite resin cervical restorations; in fact, retention rates indicate that GIC restorations may well be superior. (Francisconi *et al.*, 2009).

Compared to other cements, GIC also demonstrates good flexural strength (Sidhu and Nicholson, 2016). GIC adheres to the tooth surface through chemical bonding with the tooth structure. It shows capability to bond with polar structure / materials: bone, enamel, dentine and metallic surfaces through free -COOH groups and progressively stronger ionic bridges of hydrogen bonds (Khoroushi and Keshani, 2013). The growth of an interfacial zone that forms an ion exchange layer improves this even further. Depending on the type of cement, the components may be found with calcium in this layer, thus further strengthening bonding adherence and reducing marginal leakage significantly (Sidhu and Nicholson, 2016). The ion exchange also helps in reducing the pH of the surrounding medium and counter the acidic effects of carious activity (Moheet *et al.,* 2021). Like dentine, its dimensional stability is compromised at temperatures above 50 ℃ (Khoroushi and Keshani, 2013).

2.2.5 Setting of GIC

GICs are made of fluoro-aluminosilicate powder, which may leach ions after reacting with polyacrylic acid (PAA). The components of basic powder include calcium fluoride (CaF_2), aluminium phosphate ($AlPO_4$), cryolite (Na_3AlF_6), alumina (Al_2O_3), sodium fluoride (NaF), and silica (SiO_2) (Berg and Croll, 2015). Now, the liquids are made of maleic, itaconic or tricarboxylic acid (homo and copolymers). PAA (between 40% and 50%) was once used (Moshaverinia *et al.*, 2008a), but its shelf life was short due to its high viscosity and propensity to become gel. This gelation is because of extreme intermolecular hydrogen bonding.

To overcome this problem, tartaric acid was added to the liquid, which regulates the setting qualities (Permana *et al.*, 2016). In the three-dimensional structure of GICs aluminosilicate network, aluminium ions (Al) function as both the network-forming ions and the network-dwelling ions in the glass matrix, whereas silicone ions (Si_{+4}) are found in spaces created by four oxygen anions. When the negative ions from the glass (that are weakly attached) are attacked by carboxylic acid, a continuous reaction begins which causes the disruption of the three-dimensional matrix which is the outcome of a carboxylic acid attack at the sites of the Al ions network.

Aluminium ions and other ions are released after the attack, forming ionic networks with polymers. Al/Si ratio directs the rate of setting reaction. (McLean *et al.*, 1994; Najeeb *et al.*, 2016; Sidhu and Nicholson, 2016). During this process, ionic connections are created between carboxylate groups on the polyacid molecules on one side and calcium ions on the tooth surface on the other side (Sidhu and Nicholson,

2016). For the correct glass network to form before the acidic polymer is mixed into the glass, the counter ion ratio should be similar to the Al/Si ratio. For the cement to have precise reactivity and hydrolytic stability, the ratio of Al_2O_3 to SiO_2 must be at least 1:2 in mass.

Ions from the tooth surface and GIC diffuses into the interfacial region over time to form an ion-exchange layer as shown in Figure 2.1, which strengthens the chemical interaction between the tooth and the cement (Van Noort, 2002). In terms of the setting qualities and characteristics of GICs, the cements usually set through an acid-base reaction in 2-3 min.

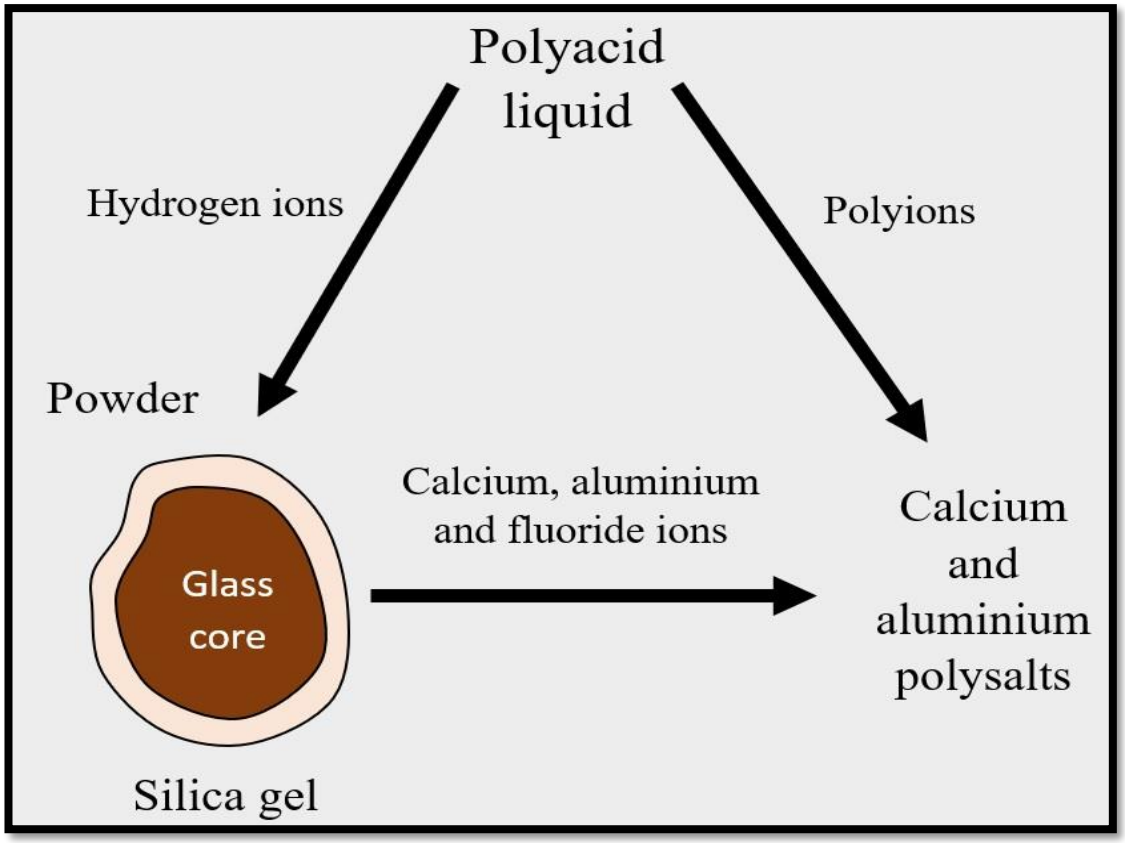

Figure 2.1 Ion leaching phase of GIC setting reaction

In addition to Al and Si, additional ion concentrations have a significant impact on how GICs are set. The diffusion-controlled process is used to set GICs in two processes. The first hardening of the cement is caused by the production of ionic cross connections, which is the first step.

The second step is maturation, which takes place continuously throughout the day as shown in Figure 2.2. Al_2O_3/SiO_2 ratio and CaF_2 content both have been altered in order to improve aesthetics and transparency. Barium (Ba), lanthanum (La), strontium (Sr), or zinc (Zn) are all used to create radiopacity (Najeeb *et al.*, 2016).

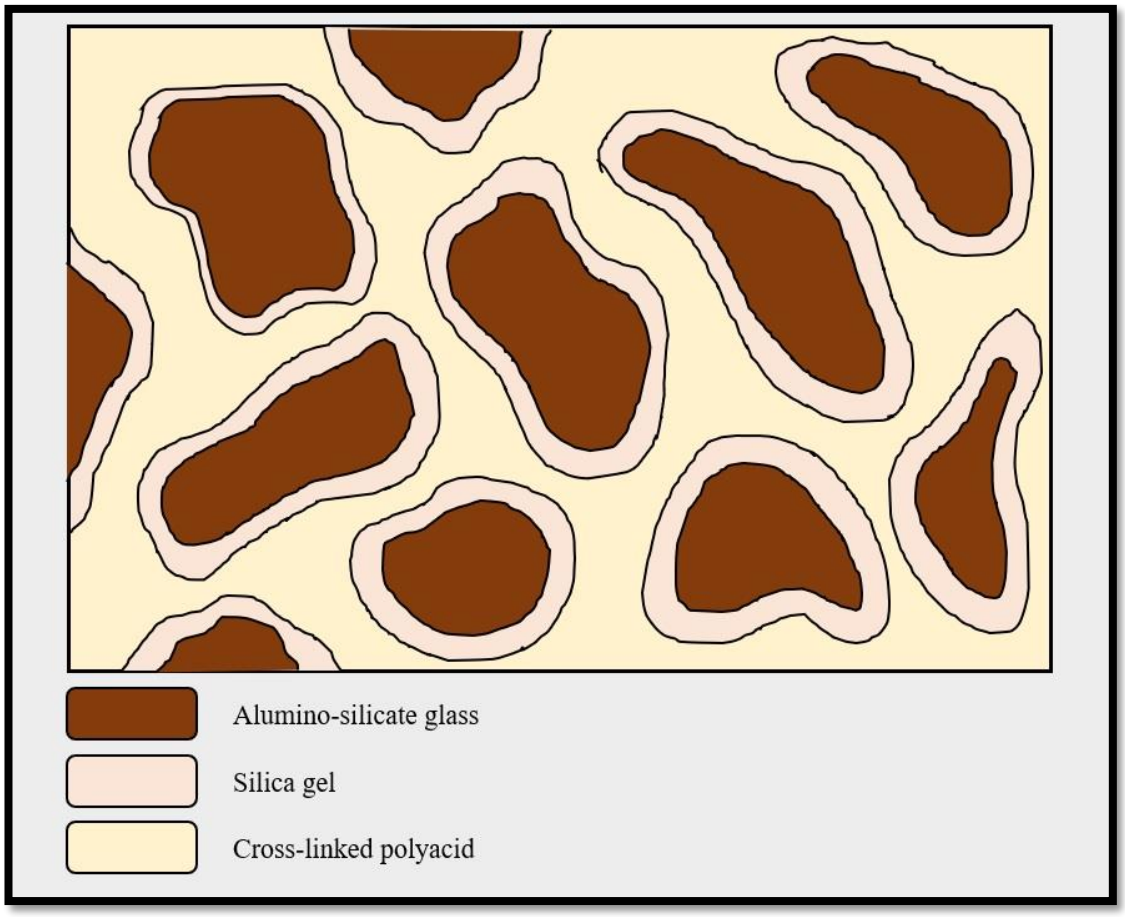

Figure 2.2 Structure of set Glass ionomer cements

2.3 Dental caries and antibacterial effects of GICs

A mineral modification of the dental tissue leading to a loss of the tooth structure is known as dental caries, and it may be caused by a variety of factors (Oong *et al.,* 2008). Microbial biofilm is one biological component that is essential to the development of dental decay. The biofilm, in general, is a complex matrix made up of proteins and exopolysaccharides that operate as a physiological barrier for bacterial cells and shield them from antimicrobial drugs. (Kim *et al.,* 2015; Ikram *et al.,* 2016; Arshia *et al.,* 2017). One of the most well-known oral biofilm-forming bacteria, *Streptococcus mutans* (*S. mutans*), is linked to the development of dental plaque. By producing organic acids from dietary residues, this species causes the oral environment to become more acidic, which demineralizes the dental structure (Seneviratne *et al.,* 2011). Clinical studies report that even in cases where tooth restoration has taken place, bacterial activity can still resume. This leads to one of the premises presented that having restorative materials that help reduce bacterial activity can lead to reduced caries recurrence (Naik *et al.,* 2016).

Caries progression and reinfection can be prevented by utilizing three therapies:

- Isolating carious progression in the oral cavity: This cuts the supply of food and nutrients to caries causing bacteria, thus slowing growth, and stopping further progression of caries.
- Operative treatment: removal of carious enamel and dentine.
- Using biomaterials that help reduce caries activity or inhibit it.

S. mutans and *Staphylococcus aureus* (*S. aureus*) has been suggested as the primary cause of dental caries in people and experimental animals (Naik *et al.*, 2016; Enan *et al.*, 2021). The capacity of these organisms to produce extracellular water-insoluble glucans is closely related to their carcinogenicity. These glucans have a strong bond to many different solid surfaces, including the surface of teeth. These bacteria attach tightly and persistently to the tooth surface as a consequence of this biochemical process, which eventually results in the creation of dental plaque and the development of dental caries. (Koga, 1986). Meanwhile, *Enterococcus faecalis (E. faecalis*) is a potent microorganism that is highly associated with root canal infections (Love *et al.*, 2002).

Depending on the composition of the cement, GIC can release different biologically beneficial ions in its environment, and hence is classified as a bioactive cement (Sidhu and Nicholson, 2016). GIC holds an advantage of slowly and continuously leaching fluoride in the oral cavity after placement, helping strengthen the tooth structure and the filling material. Fluoride release provides protection against secondary caries and abrasion resistance. Acidic conditions increase fluoride release, which is useful in the oral cavity in response to various changes due to eating and drinking, a phenomenon called buffer (Czarnecka *et al.*, 2002). The fluoride release is readily taken up by hydroxyapatite crystals, thus strengthening the tooth structure, reducing temperature sensitivity, and deterring other carious attacks (Sidhu and Nicholson, 2016). Ngo *et al*. (2006) was able to demonstrate the leaching of strontium and fluoride from conventional GIC (cGIC) to demineralized dentin by using electron probe microanalysis (EPMA) technique.

2.4 Nanotechnology and its implications in GIC

Nanotechnology has been defined by Saini *et al* (2010) as "the science and engineering involved in the design, synthesis, characterization, and application of materials and devices whose smallest functional organization, at least in one dimension, is on the nanometre scale or one billionth of a meter". The technology allows for creation of structures at macro levels influenced by the molecular properties of these materials, enabling one to confer desired characteristics. In medicine, it has proven beneficial in creating advanced treatments, medication, drug and gene delivery, tissue engineering, tumour detection and treatment, biologically inert materials, and prosthesis (Gil, 2018). In dentistry, smart materials can be developed using either one or combination of techniques: material synthesis, biomimetic approaches, and tissue engineering (Vasiliu *et al.,* 2021). To achieve these, two approaches are taken: top down and bottom up, indicating the method used to assemble or create materials as mentioned in Figure 2.3.

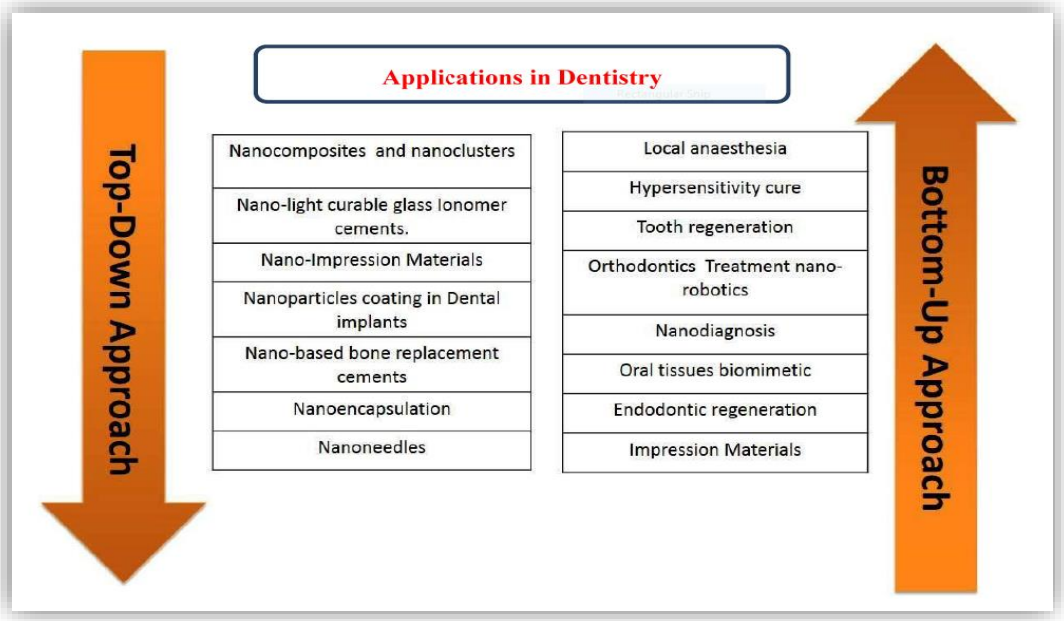

Figure 2.3 Breakthrough approaches of nanotechnology and their applications in dentistry. Adapted from (Khurshid *et al.,* 2015).

Dental nanotechnology promises creating a new kind of materials that will comply with the intrinsic and extrinsic demands of the oral tissues, performance in oral cavity and safety for use in human tissues. Due to its exclusive properties of increased surface area and free carriers, nanoparticles demonstrate quantum effects properties. This makes nanomaterials tougher and stiffer, with improved transparency and scratch resistance, resistant to solvents, heat, and gas permeability (Khurshid *et al.*, 2015).

Dental nanotechnology show promise in dental biomimetic remineralization, caries vaccine, reduced dentinal hypersensitivity through remineralization, better osteointegration of implants, improved prognosis of root canal sealants, improved tissue engineering resulting in better regeneration of pulpal tissue and other mineralized tissues like alveolar bone and dentine (Abou Neel *et al.*, 2015).

2.5 Nanomaterials used in GIC.

There are many categories of nanomaterials which in the last decade by different researchers were incorporated in GIC like nanomaterials based on metals like silver (Singh and Ramarao, 2012; Jowkar *et al.*, 2019), magnesium oxide (Noori and Kareem, 2019), copper (Aguilar-Perez *et al.*, 2020*)*, titanium oxide (Elsaka *et al.*, 2011) and zinc oxide (Dizaj *et al.*, 2014; Panahandeh *et al.*, 2018) while other based on polymers like nanochitosan (Petri *et al.*, 2007; Senthil Kumar *et al.*, 2017), cellulose based nanocrystals (Silva *et al.*, 2016; Menezes-Silva *et al.*, 2019) and nanofibers (Bhaviripudi *et al.*, 2007) etc and nanomaterials based on carbon like graphene oxide (Nair *et al.*, 2010; Chen *et al.*, 2020), carbon nanotubes (Goyal and Sharma, 2021), and nanodiamonds (Mulder and Anderson-Small, 2019). All these inclusions have

positive effects in either antibacterial, mechanical properties, physical or biological properties as shown in Figure 2.4. Since our project is based on nanomaterials based on inorganic structures so it will be explained below in detail.

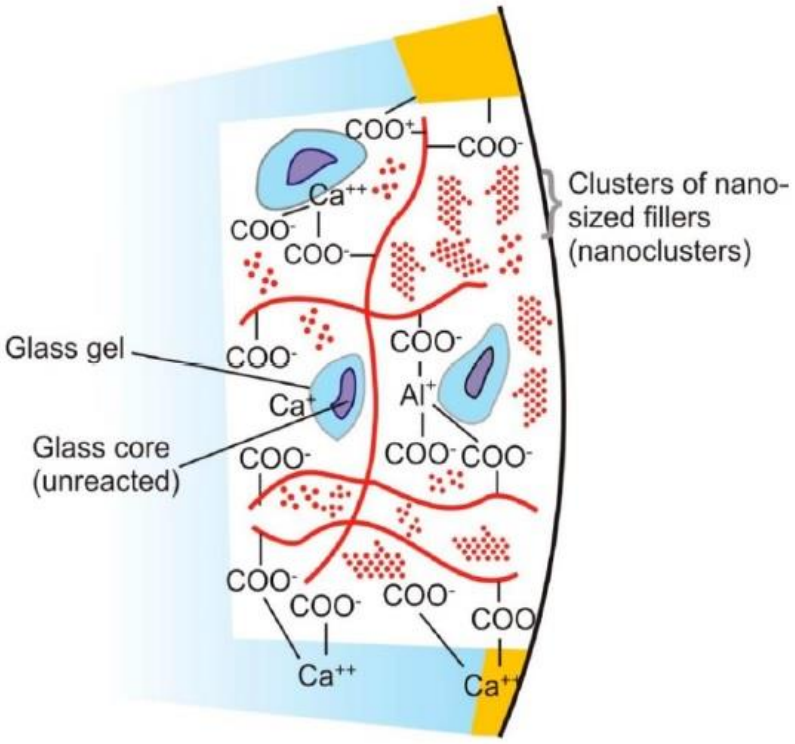

Figure 2.4 A diagram shows the composition of glass ionomer cement and how it interacts chemically with tooth structure. Adapted from (Najeeb *et al.*, 2016).

2.5.1 Nanomaterials based on inorganic structures.

Particles without carbon atoms are known as inorganic NPs. They are silica clay, hydroxyapatite, barium sulphate, zirconia, silica, silica, and silica clay. Inorganic NPs have attracted researchers' interest recently because of their physical characteristics, such as their optical and magnetic properties and their chemical characteristics. (Lohse and Murphy, 2012).