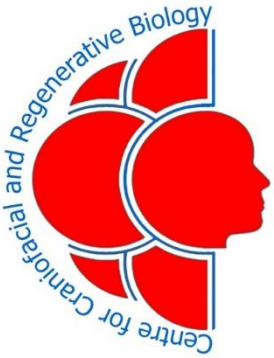

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LAB REPORT

GSK-3 Inhibitors Releasing Nanoparticles for Dentine Repair

Ayesha Siddiqa

King's College University of London

Dental Institute

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SUMMARY:

Tooth decay or caries is a prevalent disease [8] which is the result of poor oral hygiene [1] and unhealthy eating habits [2]. If left untreated, it can result in loss of the entire tooth structure [3.4]. The conventional methods of tooth repair provide a temporary relief [8]. Experiments are being conducted to investigate biological methods for tooth repair and regeneration so that it can provide a permanent relief to the dental patient [10,11,12,13]. In case of loss of the entire tooth, the biological method under trial for tooth repair includes utilizing iPS stem cells to achieve whole tooth regeneration [10]. In case of loss of any of the layers of the tooth, an attempt is made to repair or regenerate that specific layer. In a study conducted in 2017 [14], a GSK-3 inhibitor Tideglusib was loaded in a cavity created in-vivo in a mouse molar by controlled drilling, with the help of a kolspon sponge. It was then sealed with a cement. After 4 weeks, reparative dentine formation was seen. We will use mesoporous silicon nanoparticles (PSi) as a medium to deliver the GSK-3 inhibitors for dentine repair. The rationale for choosing porous silicon nanoparticles as delivery medium is that they have osteogenic potential [18], decreased cytotoxicity [19] and quick degradation in body fluids [20]. Our results showed good biodegradability, lower cytotoxicity, drug-loading and sustained drug release abilities of PSi nanoparticles. Hence, they can be used as the vehicles to deliver GSK-3 inhibitors into the dental cavities for dentine repair. The next steps to this experiment would be to conduct PCR of the target cells to measure Wnt dependent target genes in the presence of pure PSi nanoparticles and drug-loaded PSi nanoparticles, then formulating a delivery system for the cavity and assessment of dentine regeneration.

INTRODUCTION:

Tooth decay or caries is a prevalent disease [8] which is the result of poor oral hygiene [1] and unhealthy eating habits [2]. If left untreated, it can result in loss of the entire tooth structure [3,4]. The basic tooth structure [5,6] consists of three layers: enamel, dentine and pulp. Enamel

is the outermost layer of the tooth. It is not sensitive. Dentine provides base for the enamel and is sensitive as it has nerve endings. It forms bulk of the tooth and protects the pulp. Pulp has blood supply and hence provides nutrition to the tooth.

Enamel is composed of tooth minerals. Tooth decay occurs as a result of loss of these minerals [7]. The minerals are lost when they come into contact with the lactic acid in the mouth. The lactic acid is produced when bacteria in the accumulated plaque come into contact with sugars in the food. The decay begins in the enamel [8], at this point in time, it is not painful. However, as the decay reaches the dentine, it becomes painful. The lost mineral structure must be replaced with a filling before the infection reaches pulp. If the infection reaches pulp, then it is treated with antibiotics and root canal treatment. This treatment drains the infection, by removing the infected pulp. The pulp canals are then filled with an artificial material and sealed with a conventional filling. This tooth is now non-vital or dead. Its weak and is prone to breakage. It needs a cover or crown to prevent breakage from masticatory loads [8]. In case of loss of the entire tooth, conventional methods of repair are bridges, dentures and implants [9].

The conventional methods of tooth repair provide a temporary relief [8]. Experiments are being conducted to investigate biological methods for tooth repair and regeneration so that it can provide a permanent relief to the dental patient [10,11,12,13].

In case of loss of the entire tooth, the biological method under trial for tooth repair includes utilizing iPS stem cells to achieve whole tooth regeneration [10]. In case of loss of any of the layers of the tooth, an attempt is made to repair or regenerate that specific layer.

Enamel is the hardest biological tissue. Its repair has been under consideration for a long time. Its crystalline structure would have to be regenerated in order to repair the enamel. Shao et al. in 2019 [11] used calcium phosphate ion clusters (CPICs) solution layer as a precursor to induce enamel apatite growth. The experiment was performed in-vitro in non-carious human 3rd molars. The teeth were first etched with phosphoric acid. On one half of the tooth, CPICs solution was applied while the other half was left as it is. The teeth were incubated for 48 hours. After washing the teeth with deionized water, they were observed under fluorescent microscope. The side which was covered with CPICs showed repair of enamel whereas no repair was seen on the other half of the tooth (Fig. 1.)

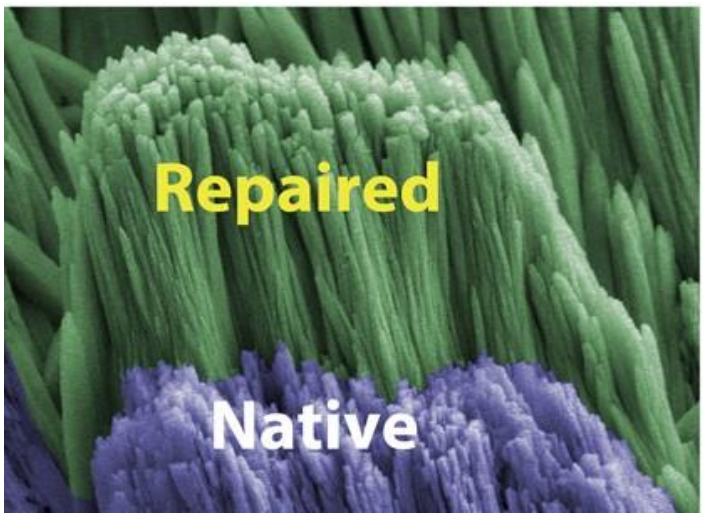


Fig. 1. Replication of the enamel structure. The figure shows the scanning electron microscopic images of both acid-etched enamel and repaired enamel. The figure also shows that enamel rods with varied orientations were repaired. CPICs formed the initial amorphous calcium phosphate layer which evolved into the hydroxyapatite crystalline structure of enamel.

For the repair of pulp, Huang et al. in 2010 [12] demonstrated the use of stem cells isolated from the apical papilla and the pulp of human teeth. These stem cells were seeded onto a scaffold and were inserted into a pulp-less tooth fragment sealed on one side with mineral trioxide aggregate cement. This tooth was then transplanted sub-cutaneously in a mouse. 3-4 months after surgery, the tooth showed development of pulp like tissue with vascularization (Fig. 2.).

Fig. 2. Histological analysis of in-vivo pulp regeneration using stem cells. The figure indicates the beginning of vascularization with blue arrows. rP means the regenerated pulp-like tissue.

The conventional methods of dentine repair use cements which induce formation of dentine [13]. These cements include calcium hydroxide cements and mineral trioxide aggregate cement. These cements induce reactionary dentine formation when are applied to unexposed pulp in the indirect pulp capping technique. And induce reparative dentine formation in the form of a biological seal [26] when applied to exposed pulp in the direct pulp capping technique. The reactionary dentine is formed by the cells of the pulp, the odontoblasts. The reparative dentine on the other hand is formed by odontoblast like cells [13]. These cells are said to differentiate from dental pulp stem cells at the materio-pulpal junction. The reparative dentine formation has recently been observed with GSK-3 inhibitors which cause a stimulation

of canonical Wnt signalling pathway. In a study conducted in 2017 [14], a GSK-3 inhibitor Tideglusib was loaded in a cavity created in-vivo in a mouse by controlled drilling, with the

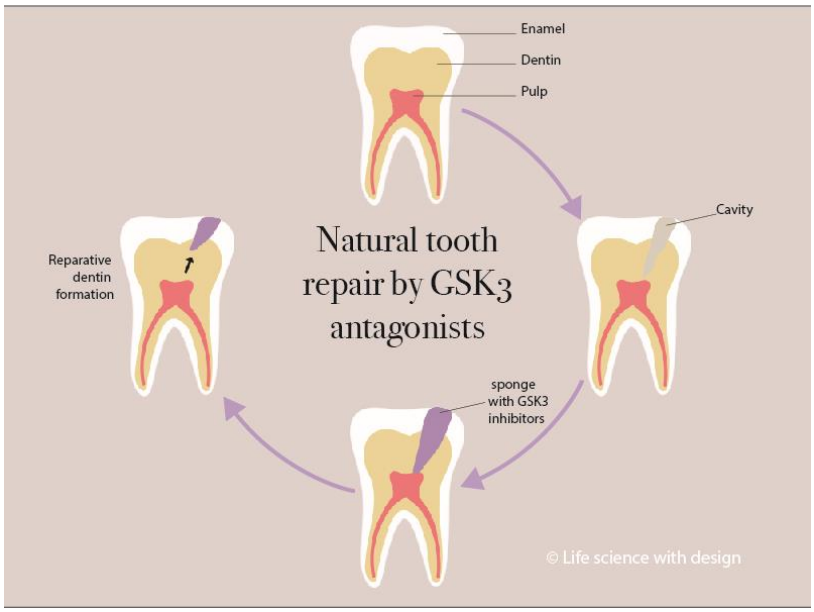


Fig.3. Reparative dentine formation using GSK-3 inhibtor loaded kolspon sponge. A GSK-3 inhibitor Tideglusib was loaded in a cavity created in-vivo in Mouse 1st molar damaged by controlled drilling, with the help of a kolspon sponge. It was then sealed with a cement. After 4 weeks, reparative dentine formation was seen.

help of a kolspon sponge (Fig. 3.). This experiment utilized the phenomenon that the immediate response to pulp injury is the activation of canonical Wnt signalling pathway [13].

GSK-3 is an enzyme which mediates the phosphorylation or addition of phosphate molecules to amino acids [15]. GSK-3 inhibitors are shown to cause dentine repair because of their role in canonical WNT signalling pathway [14]. This pathway has been shown to induce maturation of dental mesenchyme into dentine secreting odontoblasts prenatally. This was demonstrated by an in-vivo immunostaining of X-gal embryos for β-catenin in in lac-Z WNT reporter transgenic mouse. Post-natal effects of WNT in teeth are not fully understood [16].

In the absence of canonical WNT, GSK-3 induces phosphorylation of β-catenin. However, in the presence of canonical WNT signalling, GSK-3 is inhibited which mediates a release of β-catenin from the degradation machinery. B-catenin then enters the nucleus, interacts with

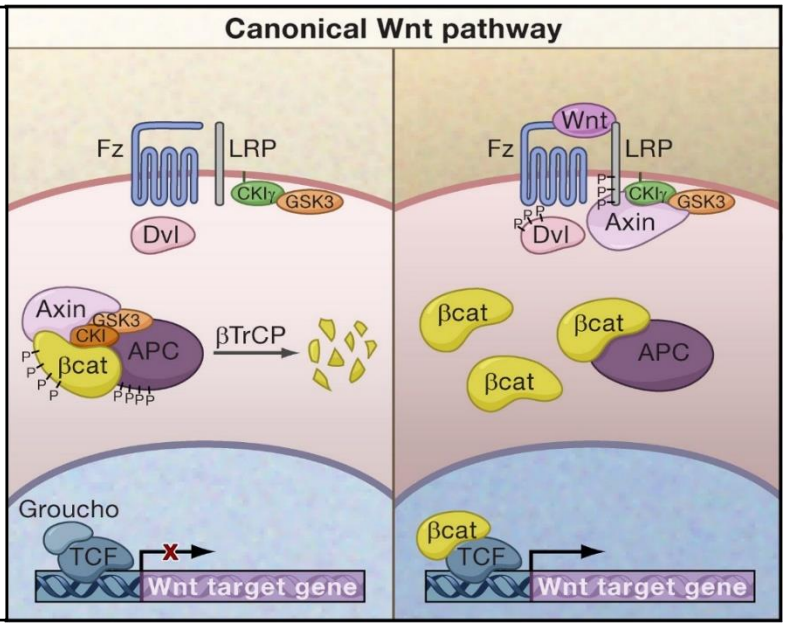


Fig. 4. Canonical Wnt Signalling Pathway. When Wnt ligand is absent, phosphorylation of the complex containing β-catenin occurs which leads to β-catenin degradation (Left). However, when the Wnt lgand is present, hypophosphorylation of the complex containing β-catenin occurs which leads to the release of β-catenin from the degradation machinery. β-catenin now enters the nucleus and takes part in the transcription of target genes (Right) .

Tcf/Lef sites and takes part in the transcription of the target genes (Fig. 4.) [17]. Therefore, as seen in the experiment conducted by Hostiuc et al., 2019, if GSK-3 inhibitors demonstrate formation of reparative dentine, does that indicate that we can enhance reparative dentine formation by over-expressing WNT signalling? The following study aims at utilizing GSK-3 inhibitors to enhance reparative dentine formation. However, instead of kolspon sponge used in the Hostiuc et al., 2019 study, we will use mesoporous silicon nanoparticles (PSi) as a medium to deliver the GSK-3 inhibitors for dentine repair. The rationale for choosing porous silicon nanoparticles as delivery medium is that they have osteogenic potential (Fig. 5.) [18],

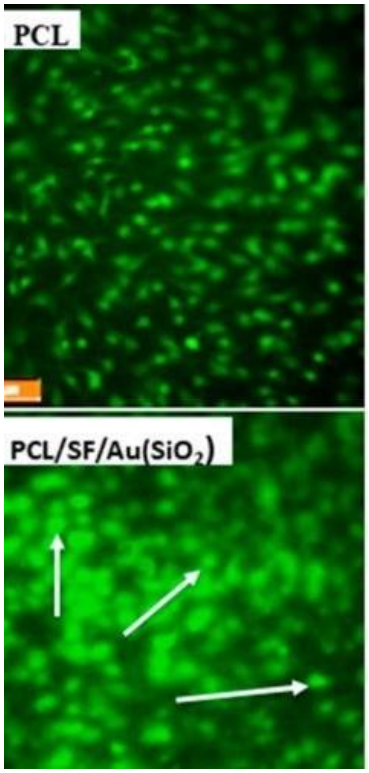


Fig.5. Osteogenic potential of nanoparticles of silica. Polycaprolactone (PCL) has been used for bone tissue engineering for its biocompatibility, however it was inadequate in promoting bone tissue regeneration from mesenchymal stem cells (above). When, however, $nSiO_2$ was added to the scaffold, it enhanced differentiation of human mesenchymal stem cells towards osteogenic lineage (below).

decreased cytotoxicity [19] and quick degradation in body fluids [20]. In terms of drug delivery potential, PSi nanoparticles have been seen to increase the potential of the cells for the drug uptake [21]. These particles have also shown the potential of the targeted drug delivery and release where they target the unhealthy cells [22]. These qualities have led us to propose this study plan where we are going to first collect the nanorods, oxidize them, measure their degradation profile, evaluate their cytotoxicity alone and in combination with the loaded GSK-3 inhibitors and measure their release to the PBS alone and loaded with the drug. The GSK-3 inhibitors that will be used in the study are Bio and Chirr 50nM solutions respectively. PSi nanoparticles have been previously used in drug-delivery systems pertaining to chemotherapy. This study will help us investigate the properties of PSi nanoparticles and whether we can use these particles as a vehicle for delivering GSK-3 inhibitors into the tooth cavity like the Neves et al., 2017 study helped in achieving reparative dentine formation using kolspon sponge [14].

MATERIALS AND METHODS:

1. NANORODS COLLECTION

Silicon wafers [27] were cut into 4 quarters, each quarter was scrubbed into the lid of the wafer container containing IPA solution. These nanorods were collected together into various falcon tubes. Falcon tubes were ultra-sonicated and the preliminary solvent was removed.

2. NANORODS OXIDATION

The IPA suspension of nanorods was placed on hotplate, the supernatant was removed and a compact pellet was obtained. The particles were suspended in 20 ml of H_2O_2. The suspension was sonicated in the ultrasound cleaner for 2 min. 60 ml of sulphuric acid was then added. The suspension was put on the hot plate for 1 hour in order to keep the reaction active.

After the reactant was used up and the reaction was plateaued, distilled water was then added to the solution. The suspension was then spinned in the centrifuge and the supernatant was removed with the pipette. Centrifugation and supernatant removal was then repeated 4 times respectively. The nanorods were then suspended in IPA.

Final solvent was then removed by weighing a glass vial with the cup. The suspension was then put in the glass vial. The capless vial was then placed on a hotplate until the IPA evaporated.

3. Mass Spectrometry

242 ml of nRods were put in 4 eppendorfs each. Eppendorfs were centrifuged to remove the IPA. 200ul of PBS was added to each epindorf with 200 ul pipette. Particles were re-suspended with the help of pipette tip. These were rotated on a rotary wheel for 30 min. PBS was collected in separate eppendorfs. The samples were kept at 4ºC. The addition and collection of PBS was repeated at different time points: 1.5 hrs, 3.5 hrs, 6hrs, 1 d, 2d, 3d, 5d, 7 days. All the samples at different time points were collected in the falcon tubes which were then sent for mass spectrometry. Mass spectrometry helped us find out the degradation profile of the particles in PBS.

4. SEM

Scanning electron microscopy was done to determine particle size which was between 80-100 nm.

5. Cytotoxicity of PSi nanoparticles

This protocol established the cytotoxicity of porous silicon nanoparticles for 17IA4 cells. The cells were suspended according to the protocol. The cell suspension was diluted at $6.5x10^4$ cells per ml in a total of 4 ml. 150ul of cell-rich medium was added in 21 wells of 96 well plate. The cells were given 1-2 days to grow upto >80% confluence. Then under the laminar flow hood, stock A solution was prepared (1 mg/ ml). In an eppendorf, the particle stock solution was diluted to 1mg/ ml for a total of 1ml. 48.5 ul of particle stock solution (concentration 20.6 mg/ ml)+ 951.5 ul of isopropanol were added. In 6 sterile eppendorfs, following amount of particles were added:

202.5 ul sol of stock A + 200 ul isopropanol

205 ul sol of stock A + 200 ul isopropanol

225 ul sol of stock A + 200 ul isopropanol

250 ul sol of stock A + 200 ul isopropanol

250 ul sol of stock A

500 ul sol of stock A

These eppendorfs were centrifuged for 30 min at 14000 rcfs. Isopropanol was removed from all eppendorfs. The particles were allowed to dry for 1 hour under the laminar flow hood. 500 ul of medium was added to each eppendorf. Medium was mixed well by pipetting. Before adding to the wells, the eppendorfs were put in the vortex for 30s. then each Eppendorf was added to the relative wells containing cells. The plate was incubated for 24 hrs. at 24 hrs, cell viability assay was performed.

6. Nanodrop experiment

2 samples were taken in 2 eppendorfs under the fume hood. One eppendorf consisted of particles with the drug and other consists of dry particles. 250 ul of PBS was added to both eppendorfs, down time was marked. The eppendorfs were placed in a sonicator to allow for rapid mixing of samples. These were spinned at 19000 g for 30 min.10 ul of sample was taken carefully from both eppendorfs and was put into clean smaller eppendorfs. Down time was marked again. The remaining liquid was mixed in spinned tubes, the tubes were left alone on the table for 1 hour. After which, these were spinned again and the samples were collected again. 1 ul of sample from each eppendorf was used to conduct the nanodrop experiment.

7. Cytotoxicity for PSi loaded with BIO

This protocol established the cytotoxicity of porous silicon nanoparticles loaded with GSK-3 inhibitor BIO for 17IA4 cells. The cells were suspended according to the protocol. The cell suspension was diluted at $4.0x10^5$ cells per ml in a total of 5.5 ml (total $2.2x10^6$ cells). 150 ul of cell rich medium was added in 33 wells of 96 well plate. Then 3 sterile eppendorfs were taken and were labelled PD1, PD2, PD3, where P stands for particles and D stands for drug. These eppendorfs were filled with PSi solution with volumes 30, 60 and 120 ul respectively. The first 2 eppendorfs were balanced with IPA to make the total volume upto 120 ul for centrifugation. These eppendorfs were then placed in the centrifuge for 20 min at 19000 g. the supernatant was than pipetted and discarded. The eppendorfs were left in opn air to dry until no IPA was left. Now the eppendorfs were topped up with Bio loading solution with volumes 120, 240 and 480 ul. These were mixed with the help of sonicator. These eppendorfs were incubated for 1 hr @RT. Then centrifugation was done for 20 min at 19000 g. All the supernatant was pipetted and discarded, and the eppendorfs were left open to dry until no solution was left. Particle reference was created by taking three eppendorfs, labelled P1, P2, P3, where P stands for particles and D stands for drug. These eppendorfs were filled with PSi solution with volumes 30, 60 and 120 ul respectively. The first 2 eppendorfs were balanced with IPA to make the total volume upto 120 ul for centrifugation. These eppendorfs were then placed in the centrifuge for 20 min at 19000 g. the supernatant was than pipetted and discarded. The eppendorfs were left in opn air to dry until no IPA was left. Then the drug reference was created by taking 3 sterile eppendorfs, which were labelled D1, D2 and D3. These were filled with Chirr solution, which was left to dry until no solution remained. Then the cells were incubated with the particles by adding 500 ul of cell medium to each eppendorf. These were placed in the sonicator for effective mixing. Add 150 ul from each eppendorf to the specified wells. Incubate the well plate for 24 hrs. Cell titer glow assay was started at 24 hrs.

8. Cytotoxicity for PSi loaded with CHIRR

This protocol established the cytotoxicity of porous silicon nanoparticles loaded with GSK-3 inhibitor CHIRR for 17IA4 cells. The cells were suspended according to the protocol. The cell suspension was diluted at $4.0x10^5$ cells per ml in a total of 5.5 ml (total $2.2x10^6$ cells). 150 ul of cell rich medium was added in 33 wells of 96 well plate. Then 3 sterile eppendorfs were taken and were labelled PD1, PD2, PD3, where P stands for particles and D stands for drug. These eppendorfs were filled with PSi solution with volumes 30, 60 and 120 ul respectively. The first 2 eppendorfs were balanced with IPA to make the total volume upto 120 ul for centrifugation. These eppendorfs were then placed in the centrifuge for 20 min at 19000 g. the supernatant was than pipetted and discarded. The eppendorfs were left in opn air to dry until no IPA was left. Now the eppendorfs were topped up with Chirr loading solution with volumes 120, 240 and 480 ul. These were mixed with the help of sonicator. These eppendorfs were incubated for 1 hr @RT. Then centrifugation was done for 20 min at 19000 g. All the supernatant was pipetted and discarded, and the eppendorfs were left open to dry until no solution was left. Particle reference was created by taking three eppendorfs, labelled P1, P2, P3, where P stands for particles and D stands for drug. These eppendorfs were filled with PSi solution with volumes 30, 60 and 120 ul respectively. The first 2 eppendorfs were balanced with IPA to make the total volume upto 120 ul for centrifugation. These eppendorfs were then

placed in the centrifuge for 20 min at 19000 g. the supernatant was than pipetted and discarded. The eppendorfs were left in opn air to dry until no IPA was left. Then the drug reference was created by taking 3 sterile eppendorfs, which were labelled D1, D2 and D3. These were filled with Chirr solution, which was left to dry until no solution remained. Then the cells were incubated with the particles by adding 500 ul of cell medium to each eppendorf. These were placed in the sonicator for effective mixing. Add 150 ul from each eppendorf to the specified wells. Incubate the well plate for 24 hrs. Cell titer glow assay was started at 24 hrs.

RESULTS:

1. MASS SPECTROMETRY

The graph shows the total amount of Si that was released. The degradation profile shows that the maximum degradation occurred at 24 hrs after which it started decreasing. The cumulative release that occurred till day 21 which was approximately 525 ug/ mL.

Fig.6:

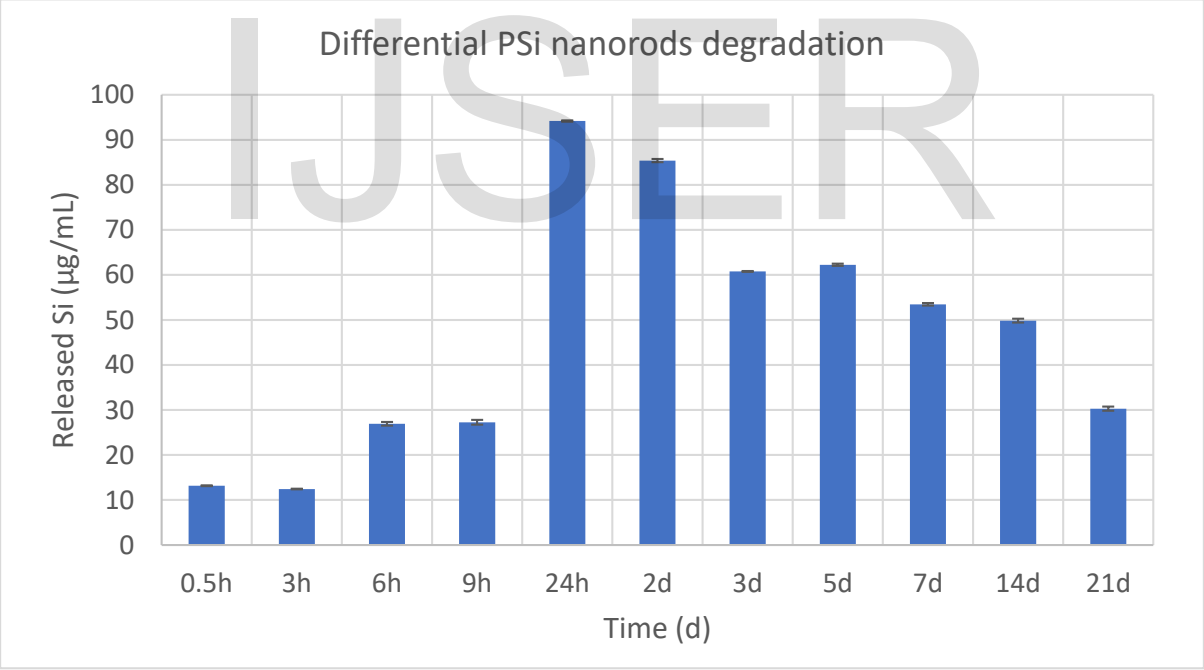
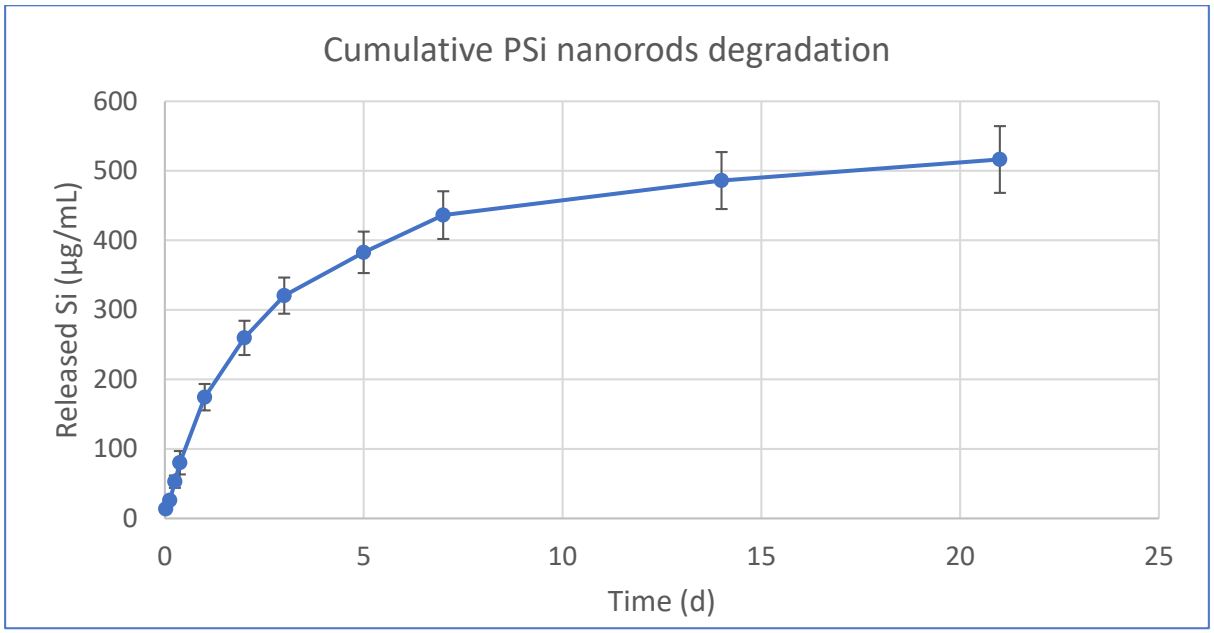


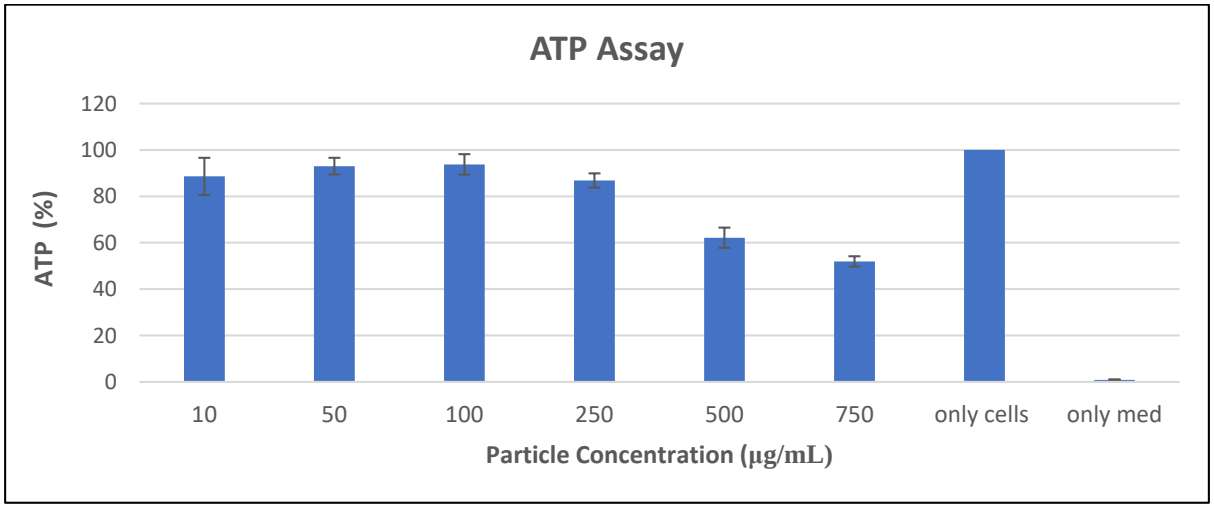
Fig.7:



2. Cytotoxicity of PSi nanoparticles

The graph shows a very slight variance between cytotoxicity of various particle concentrations.

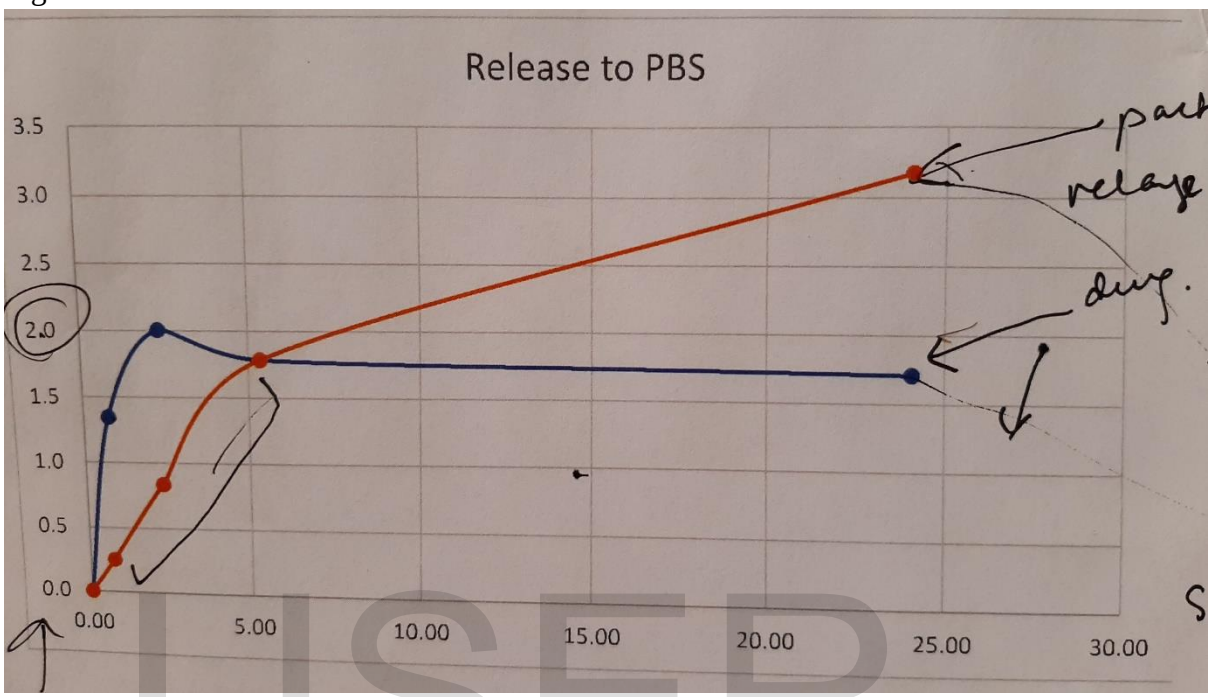
Fig.8:



3. Nanodrop experiment

The graph shows that the release of drug to PBS is slower with drug loaded PSi particles (orange) than drug alone (blue). X-axis represent hours and y-axis represent ug/mL release.

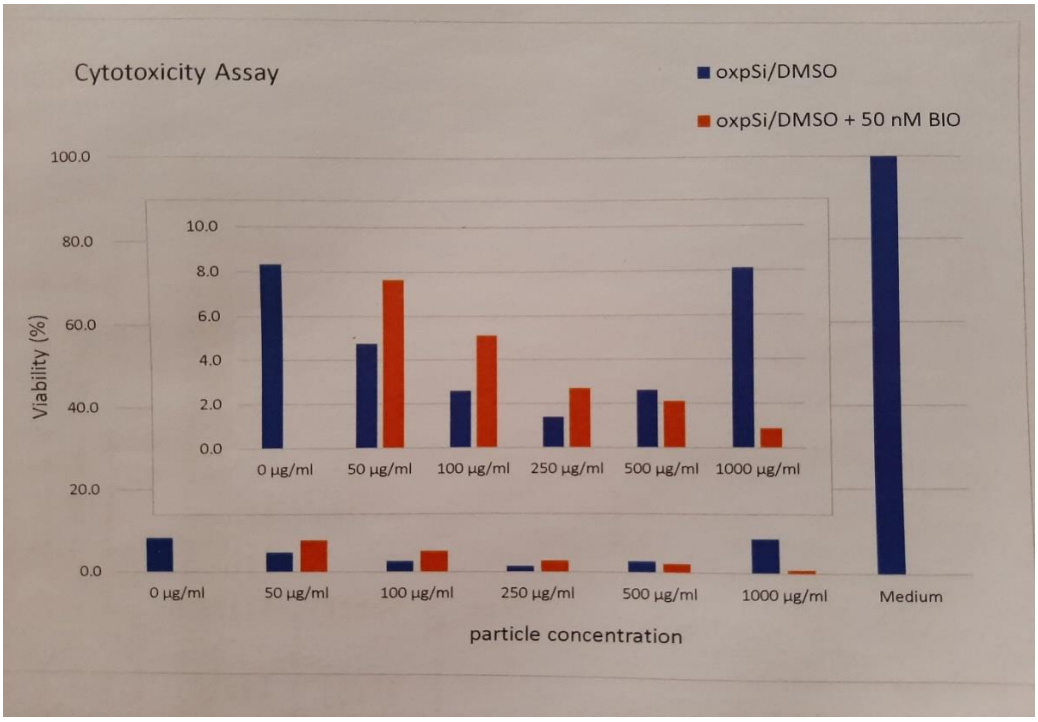
Fig.9:



4. Cytotoxicity for PSi loaded with BIO

The graph shows that cytotoxicity of Bio loaded particles is almost same as pure particles.

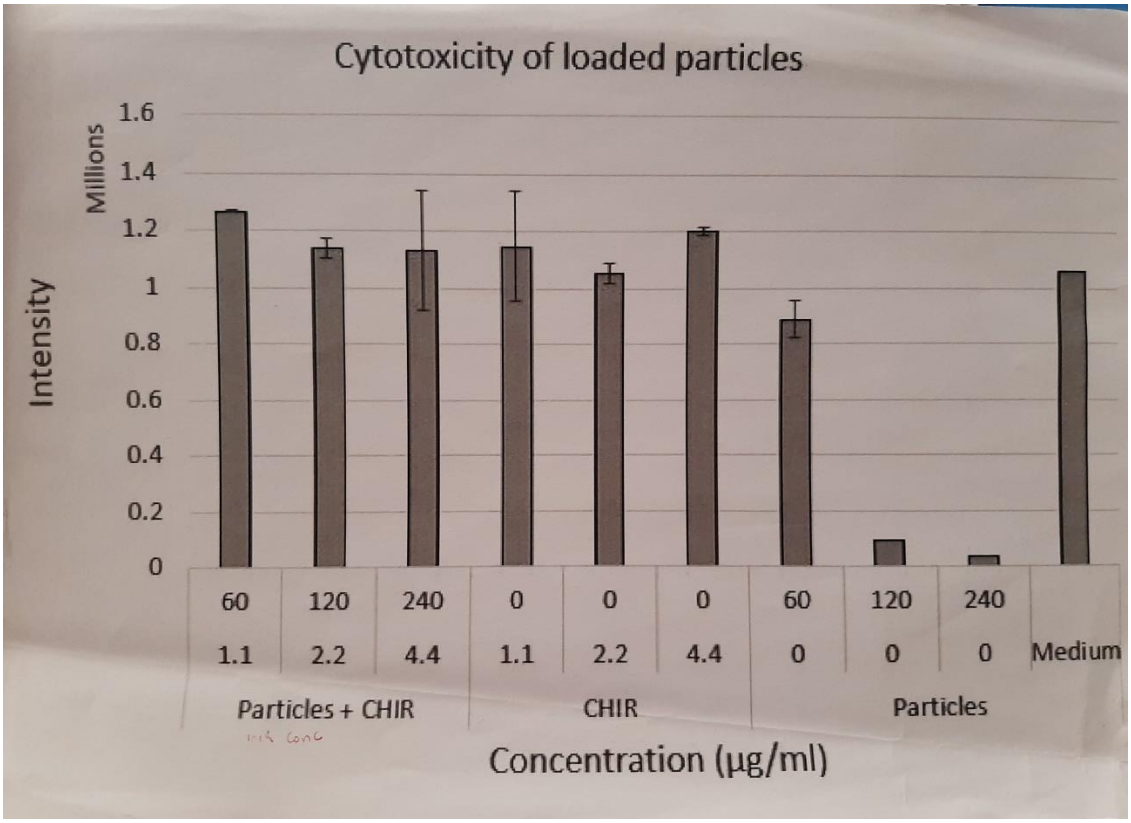
Fig.10:



5. Cytotoxicity for PSi loaded with CHIRR-99021

The graph shows that cytotoxicity of Chirr loaded particles is lower than pure particles.

Fig.11:



DISCUSSION AND CONCLUSION:

The degradation profile of our PSi nanoparticles showed maximum degradation at 24 hrs. According to Hon et al., 2012 [20], the half-life of Si nanoparticles ranges from 5 to 20 hrs depending on the quality of the silica. According to Korhonen et al., 2015 [23] and Sahu et al., 2016 [24], cytotoxicity of PSi nanoparticles is concentration dependent. Our particles, however, showed a very slight variance in cytotoxicity with varying particle concentrations. The nanodrop experiment measured drug release to PBS through drug loaded PSi nanoparticles. It demonstrated a slower release of the drug as compared to the drug only sample, this controlled release of the drug is a desirable quality of the PSi nanoparticles [25]. Then we compared the cytotoxicity of the GSK-3 inhibitors loaded PSi nanoparticles that we were using to the cytotoxicity of pure PSi nanoparticles. It turned out that Bio-loaded particles had the same cytotoxicity as the pure particles. However, the cytotoxicity of the Chirr-loaded particles was lower than the pure particles. These results show that with biodegradability, lower cytotoxicity, drug-loading and sustained drug release abilities, PSi nanoparticles can be used as the vehicles to deliver GSK-3 inhibitors into the dental cavities for dentine repair. The next steps to this experiment would be to conduct PCR of the target cells to measure Wnt dependent target genes in the presence of pure PSi nanoparticles and drug-loaded PSi nanoparticles. Then would be the formulation of a delivery system for the cavity and assessment of dentine regeneration.

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