

pH Dependent Delivery of Chlorhexidine From PLGA Grafted Mesoporous Silica Nanoparticles at Resin-Dentin Interface

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Research

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Abstract

Background: A low pH environment is created due to the production of acids by oral biofilms that further leads to the dissolution of hydroxyapatite crystal in the tooth structure significantly altering the equilibrium. Although the overall bacterial counts may not be eradicated completely from the oral cavity, however, synthesis of engineered anti-bacterial materials are warranted in order to reduce the pathogenic impact of the oral biofilms. The purpose of this study was to synthesize and characterize chlorhexidine-loaded mesoporous silica nanoparticles (MSN) grafted with poly-L-glycolic acid (PLGA) and to test the *in vitro* drug release in various pH environments, cytotoxicity, and antimicrobial capacity. In addition, to investigate the delivery of CHX-loaded/MSN-PLGA through dentin tubules of demineralized dentin substrates and the interaction of these nanoparticles with commercial dentin adhesive after applying it on demineralized substrates for potential clinical application.

Results: Characterization using SEM/TEM and EDX confirmed the synthesis of CHX-loaded/MSN-PLGA. An increase in the percentage of drug encapsulation efficiency from 81% to 85% in CHX loaded/MSN and 92% to 95% in CHX loaded/MSN-PLGA proportionately increased with increasing the amount of CHX during the fabrication of nanoparticles. For both time-periods (24 h or 30 Days), the relative microbial viability significantly decreased by increasing the CHX content ($P < 0.001$). Generally, the cell viability percentage of DPSCs exposed to MSN-PLGA/Blank, CHX-loaded/MSN, and CHX-loaded/MSN-PLGA, respectively was $>80\%$ indicating low cytotoxicity profiles of experimental nanoparticles. After 9 months in artificial saliva (pH 7.4), significantly highest micro-tensile bond strength value was recorded for 25:50 CHX/MSN and 25:50:50 CHX/MSN-PLGA. A homogenous and widely distributed 50:50:50 CHX-loaded/MSN-PLGA nanoparticles exhibited excellent bonding with the application of commercially available dentin adhesive.

Conclusions: A pH sensitive CHX release response was noted when loaded in MSN grafted PLGA nanoparticles. The formulated drug loaded nanocarrier demonstrated excellent physicochemical, spectral, and biological characteristics. Showing considerable capacity to penetrate effectively inside dentinal tubules and having high antibacterial efficacy, this system could be potentially used in adhesive and restorative dentistry.

Background

Effective dentin bonding is by far the most debated topic and gaining more attention in restorative dentistry. The use of contemporary adhesive restorations is widespread, and their long-term clinical success are dependent on their handling characteristics [1, 2]. The resin-dentin interface is the most critical factor and is presumably considered a fragile link between composite and dentin. Two of the most common unwanted failures that are associated with contemporary adhesive restorations that negatively affect clinical success are 'lack of durability' and 'microleakage' [3].

Bacterial activity at the tooth-restoration interface leads to the development of secondary caries. This is facilitated by the gap formation at the perimeter of the interface that promotes bacterial colonization, thereby facilitating demineralization of the tooth structure [3]. Simultaneously, a low pH environment is created due to the production of acids by biofilms that further leads to the dissolution of hydroxyapatite crystal in the dentin substrate significantly altering the equilibrium [4, 5]. Although the bacterial counts from micro-niche may not be eradicated completely from the oral cavity, however, synthesis of engineered anti-bacterial materials are warranted in order to reduce the pathogenic impact of the oral biofilms [6, 7].

In the recent years, porous materials have offered a promising therapeutic solution to a wide variety of fields [8]. Of these, mesoporous silica nanoparticles (MSNs) are a category of porous materials with exceptional surface area and significant number of pores that load various quantities of drugs within for improved efficacy [9, 10]. Moreover, these synthesized structures can undergo surface modifications, possess excellent biocompatibility and high pore volume. Numerous research reports have indicated that MSNs can also be modified to be stimuli-responsive controlled release systems, which rapidly release drugs in response to changes in microenvironments of pathological sites or intracellular stimuli [11]. Among these stimuli-responsive systems, the pH-responsive system is of particular interest [10]. Poly (L-glycolic acid) (PLGA) is another of the widely studied synthetic polypeptides due to its modifiable carboxyl side group and pH-responsive property [12].

Herein, we reported a facile strategy to graft PLGA on the surface of pH-responsive MSN through the N-carboxyanhydride (NCA) ring-opening polymerization of γ -benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) [10]. Chlorhexidine (CHX), a biguanide antimicrobial drug was chosen as a model drug to evaluate the drug loading/releasing behavior of MSN-PLGA for potential application in adhesive dentistry. We hypothesized that the resultant MSN-PLGA would demonstrate superior properties in terms of loading and delivering more CHX compared to the unmodified MSN carrier. The purpose of this study was to synthesize and characterize CHX-loaded/MSN-PLGA and to test the *in vitro* drug release in various pH environments, cytotoxicity, and antimicrobial capacity. In addition, to investigate the delivery of CHX-loaded/MSN-PLGA through dentin tubules of demineralized dentin substrates and the interaction of these nanoparticles with commercial dentin adhesive after applying it on demineralized substrates for potential clinical application.

Methods

Chlorhexidine base ($\geq 99.5\%$) and other chemicals including L-glutamic acid γ -benzyl ester ($\geq 99.9\%$), poly (lactic-co-glycolic acid – PLGA; 50/50, mol wt 30,000-60,000), triphosgene reagent grade, 3-aminopropyltriethoxysilane (APS), N-cetyltri-methylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), phosphate buffered saline (PBS), MTT assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Scotchbond™ Universal Adhesive, adhesive microbrush applicator, and Filtek™ Supreme XTE composite universal restorative material was purchased from 3M ESPE, St Paul, MN, USA.

Fabrication of nanoparticles

Fabrication of BLG-NCA

A suspension of 20 mL of ethyl acetate mixed with 1.2 gm of triphosgene (0.0042 mol) and 2.0 gm of L-glutamic acid γ -benzyl ester (BLG) (0.0084 mol) was prepared, respectively. Both mixtures were stirred for 2 h at 75 °C to achieve a clear solution that was later evaporated under reduced pressure and crystallized three times using ethyl acetate and n-hexane. This mixture was subsequently dried at room temperature under vacuum and yielded a total of 1.7 g (80%) of BLG-NCA.

Preparation of MSN and MSN-APS

Nanoparticles were prepared according to the procedure as previously described [10]. In this study, MSN-PLGA/CHX at two different concentrations of CHX (25 and 50 mg) were fabricated. As a control, MSN-PLGA-Blank and MSN-CHX (25 and 50 mg) were fabricated. CTAB (1 g) was added into a solution containing 400 mL of distilled water and 2.5 mL of 2 N NaOH and vigorously stirred at 75 °C for 3 h. Later, 2.5 mL of TEOS was rapidly added into the solution and incubated for 2 h. The subsequent white precipitate was filtered and rinsed with ethanol and dried overnight under vacuum at 40 °C that produced a white powder (MSN-CTAB). MSN-CTAB (0.1 g) was dispersed in 20 mL of ethanol and heated to 85 °C. To functionalize MSN with glutamic acid, 0.1 mL APS was added into the dispersion. The obtained mixture was centrifuged, washed again with ethanol for several times, and dried overnight under vacuum at 45 °C for 12 h to produce another white powder of MSN-CTAB-APS. CTAB was separated from MSN-CTAB and MSN-CTAB-APS by refluxing in ethanol solution of ammonium nitrate ($\text{NH}_4\text{NO}_3/\text{C}_2\text{H}_5\text{OH}$, 10 mg/mL) for 6 h at 80 °C. The CTAB-removed product was extracted and dried to produce MSN and MSN-APS as white powder, respectively.

Synthesis of poly(γ -benzyl-L-glutamate) grafted over MSN (MSN-PBLG)

Synthesized BLG-NCA of 0.8 g was added in 10 mL of dry DMF and mixed with a solution of 0.1 g MSN-APS in 20 mL of dry DMF. Continuous stirring of the mixture was carried out over the period of 3 days at 40 °C. The mixture was centrifuged, washed with ethanol, and dried overnight under vacuum for 12 h at 45 °C, to form a white powder of MSN-PBLG.

Preparation of MSN-PLGA and chlorhexidine loading

MSN-PBLG of 0.1 g was dispersed in 10 mL trifluoroacetic acid (TFA) in an ice bath and HBr (1 mL, 33 wt% in acetic acid) was added dropwise within 10 min and stirred for 2 h. The reaction mixture was poured into 40 mL ice-cold dry ether, followed by centrifugation, washing with distilled water, and drying overnight under vacuum at 45 °C for 12 h, to form white powder of MSN-PLGA. The incorporation of CHX into MSN and MSN-PLGA was adopted and modified from previous protocols [9, 13]. In brief, 25 and 50 mg of CHX was dissolved in 5 mL of dichloromethane added with 50 mg of MSN or MSN-PLGA, respectively, and sonicated for 12 min and slowly shaken at room temperature for 24 h, followed by centrifugation and vacuum drying.

Percentage of drug (CHX) encapsulation/loading

The obtained free CHX was separated from encapsulated CHX by centrifuging the mixture at 25°C at 10,000 rpm for 10 min. The supernatant was conserved for evaluating the drug loading content. The quantity of free CHX was estimated using a spectrophotometer (UV-1900i UV-VIS, Shimadzu, Japan) at 289 nm wavelength. All sampling with their measurements were performed in triplicate. The drug encapsulation-efficiency (DEE) and drug loading (DL) were calculated using the respective formulas:

$$\text{Percentage of DL} = \text{Weight of CHX} / \text{Weight of CHX-loaded/MSN-PLGA} \times 100\% \quad (1)$$

$$\text{Percentage of DEE} = \text{Weight of CHX} / \text{Weight of CHX used for encapsulation} \times 100\% \quad (1)$$

Nanoparticles were synthesized where CHX was loaded within MSN and PLGA to produce five different types of groups according to the percentage of the CHX as:

MSN-PLGA/Blank; 25:50 CHX-loaded/MSN; 50:50 CHX-loaded/MSN; 25:50:50 CHX-loaded/MSN-PLGA and 50:50:50 CHX-loaded/MSN-PLGA

Dynamic light scattering

The control (MSN-PLGA/Blank) and experimental nanoparticles (25:50 and 50:50 CHX-loaded/MSN and CHX-loaded/MSN-PLGA at ratios of 25:50:50 and 50:50:50) were subjected to dynamic light scattering (DLS) (Malvern Mastersizer Nano ZS, UK) for determination of *z*-average particle-diameter, zeta-potential, and size-distribution. Nanoparticles diluted in distilled water (1:100 wt/v) were analyzed at 37°C with a scattering angle of 90° (n = 10/group). All sampling with their measurements were performed in triplicate.

Morphological characterization of the nanoparticles

The morphological features of all types of nanoparticles were examined under scanning electron (Verios, XHR SEM) and transmission electron microscope (TEM, FEI Titan G2 80-200 Tokyo, Japan) coupled to energy-dispersive X-ray spectroscopy (EDS - Oxford Instruments AZtecEnergy software, USA) for elemental analysis. Nanoparticles were cleaned using absolute ethanol for any surfactants and sonicated for 5 minutes. A droplet of aqueous particle dispersion was allowed to evaporate on a round carbon-coated copper mesh grid (Emgrid, Australia) stabilized by the help of Dumont tweezer (ProSciTech, Australia). The samples were imaged at 200 kV under the TEM.

pH directed chlorhexidine release from the nanoparticles

To study the *in-vitro* CHX release, nanoparticles were suspended in 5 mL of PBS solution at room temperature with constant slow stirring. To emulate different biological environments, two PBS solutions with two different pH values: 7.4 and 5.0 were investigated up to 24 h. The volume of the solution was kept constant by collecting 3 mL of the solution and at the same time replacing with 3 mL of fresh PBS at appropriate time intervals. Subsequently, the specimens were centrifuged and the percentage of released

CHX was measured using a spectrophotometer (UV-1900i UV-VIS, Shimadzu, Japan) at 289 nm wavelength. All sampling with their measurements were performed in triplicate.

Spectral analysis of the nanoparticles

Raman signatures were recorded using Raman spectroscopy (WITec Alpha300+, GmbH, Ulm, Germany) to confirm the inclusion of CHX into the nanoparticles. The instrument was calibrated using a silicon wafer at a magnification of 20 x 0.5. Nanoparticles were fixed on the transparent glass slide (spread dimensions: 0.8 cm width and 1 cm length) and placed on the microscope sample stage. After choosing a 100 x 0.5 objective lens and focusing the sample surface, an optical image was taken of the sample area. All Raman analyses were performed after calibration by selecting 50-micron fiber 532 nm laser. The fitting of the Raman spectrum was done using a Voigt line shape function via non-linear least squares (GRAMS32 AI Version 6.00 Peak Fit). The Raman images acquired were transferred into CytoSpec software where the entire data between 400-3200 cm^{-1} were pre-processed and normalized in addition to the removal of cosmic rays. Twenty spectra were recorded per specimen to establish reproducibility of the wavelength of at least $\pm 1.5 \text{ cm}^{-1}$. The resolution within the order of the system was averaged to 5 cm^{-1} while keeping the spectrometer slit at 100 μm width measuring the Raman at 520 cm^{-1} of the silicon spectrum.

To further confirm the spectral characteristics, Fourier transformed infrared (FTIR) analysis using attenuated total reflection (ATR) was used with the spectrum range between 400 and 4000 cm^{-1} at a resolution of 4 cm^{-1} . For the analysis, 10 mg of each nanoparticles was placed onto the diamond crystal on potassium bromide slab. The powder was pressed against the diamond crystal with a flat pressure anvil connected to a pressure device and spectrum were recorded for all the powders. Each sample was run in triplicate to view if there was any difference recorded.

Biofilm characterization and MTT assay

The MTT solution totaling 0.5 mg/ml from 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide kit] (MTT, Sigma-Aldrich, St. Louis, MO, USA) was prepared. The bacterial suspensions of *Streptococcus mutans* (*S. mutans*) (ATCC UA159) were prepared from the inoculum of overnight cultures and adjusted to OD_{600} of 0.5 McFarland turbidity ($\sim 10^8$ bacteria/mL). *S. mutans* were incubated anaerobically for 24 h at 37°C in Brain-Heart infusion (BHI) and suspensions adjusted to 1×10^8 CFU/ml. All suspensions were transferred into 24-well plates. Later, 10 μL of the bacterial suspension was transferred into each well containing 2 mL of BHI and 1% sucrose and incubated for 24 h at 37°C. The non-adherent bacterial cells were washed away by PBS solution. For antibacterial evaluation, sterile filter paper-disks impregnated with 25 μL of MSN-PLGA, 25:50 CHX-loaded/MSN, 50:50 CHX-loaded/MSN, 25:50:50 CHX-loaded/MSN-PLGA, and 50:50:50 CHX-loaded/MSN-PLGA nanoparticles ($n=9$), were placed inside 12-well plates followed by incorporation of 2 mL of MTT solution and incubated at 37°C for 24 h. The MTT solution was pipetted out and exchanged with 2 mL of dimethyl sulfoxide (DMSO). The well-plates were gently

shaken for 15 min and the absorbance read in spectrophotometer (UV-1900i UV-VIS, Shimadzu, Japan) at 560 nm wavelength.

Cytotoxicity evaluation

The cytotoxicity of the nanoparticles was investigated as described in a previous protocol ($n = 9$) [13] using dental pulp stem cells (DPSCs) (Alameda, California, USA). Cells were seeded at 1×10^4 cells/well (passage 4) in a 96-well plate, incubated overnight and exposed to 25, 50 and 75 $\mu\text{g}/\text{mL}$ of MSN-PLGA, 25:50 CHX-loaded/MSN, 50:50 CHX-loaded/MSN, 25:50:50 CHX-loaded/MSN-PLGA, and 50:50:50 CHX-loaded/MSN-PLGA nanoparticles for 24, 48 and 72 h ($n=7$ /group at each concentration and incubation time). Untreated DPSCs without any treatment were used as control. The MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] reagent (CellTiter 96 AQueous One Solution Assay, Promega, USA) prepared in DMEM was added to each well followed by a 2 h incubation in 37°C with 5% CO_2 . The microplates were read at 560 nm in a spectrophotometer (UV-1900i: UV-Vis Shimadzu, Kyoto, Japan) and the cell viability (expressed in percentage) was assessed after 0 h, 24 h, 48 h, and 72 h [14]. All tests were performed in triplicates.

Delivery of nanoparticles to demineralized dentin specimens

Sound human molars (21–35 years) were used for investigating delivery of nanoparticles to demineralized dentin substrates through micron-sized dentinal-tubules. Following extraction, teeth were stored in 0.2% sodium azide at 4°C to inhibit microbial growth and were used within 2 months from the time of extraction. Dentin specimens were prepared for nanoparticle treatment as described previously [15]. Following preparation, specimens were randomly grouped to be treated with MSN-PLGA, CHX-loaded/MSN and CHX-loaded/MSN-PLGA (at CHX/MSN-PLGA ratios of 25:50:50 and 50:50:50) carried on distilled water at a nanoparticles/carrier ratio of 2/1 (w/v) for further investigations. The exposed outer dentin-surfaces were etched with 35% phosphoric acid gel for 15 s, rinsed with distilled water for 15 s and dried with air-syringe for 2 s leaving the dentin surface slightly moist. A drop-wise application of 25 μL of nanoparticles/carrier suspension to each dentin specimen for 60 s was followed by surface rubbing for 5 s with a microbrush applicator. Following nanoparticles application, the dentin surface was left undisturbed for 15 s, gently air-blown for 3 s and blot-dried by absorbent paper to remove excess water. The dentin specimens were then prepared for SEM examination (Ziess 1555 VP-FESEM, Japan) and resin/dentin bonding procedure.

Resin/Dentin bonding and SEM investigation

Following nanoparticles delivery to demineralized dentin-substrates, a two-step etch-and-rinse dentin bonding system was applied according to manufacturer's instructions. Subsequently, each tooth specimen was restored to a 4-mm resin composite restoration in equal increments with each increment light-cured for 20 s (Curing Light 2500; 3M ESPE, MN, USA). The restored teeth specimens were stored in distilled water for 24 h at 37°C to accelerate polymerization reaction followed by occluso-gingival sectioning into 1 mm slabs using a low-speed diamond saw under running water. Each obtained resin-

dentin slab was polished with increasingly fine diamond pastes (3 μm and 1 μm) and cleaned ultrasonically for 10 min. Further, the slabs were air-dried for 48 h, gold sputter-coated and viewed by FESEM (Zeiss 1555 VP-FESEM, Japan) and for respective EDX analysis (EDS - Oxford Instruments AZtecEnergy software, USA).

Micro-tensile bond strength

To test micro-tensile bond strength ($\mu\text{-TBS}$), 5 wt.% of all the nanoparticles were added separately in the commercial adhesive (ScotchbondTM bond, 3M ESPE, USA) [9]. The restored teeth were sectioned using a low-speed diamond saw (Buehler, Lake Bluff, IL, USA) under water coolant into resin–dentin beams (0.9 x 0.9 mm) and stored in artificial saliva (pH 7.4) for one week. The artificial saliva was used as the testing medium and prepared according to the protocol described by Levallois et al. [16] that involves the dissolving of reagents (0.125M NaCl, 0.964M KCl, 0.189M KSCN, 0.655M KH_2PO_4 , 0.2M Urea, 0.229M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.76M $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$, 0.178M NH_4Cl and 0.631M NaHCO_3) in distilled water (pH=7.4) to produce a total volume of 1.0 L. The beams in each group ($n=75$) were then randomly divided into 5 subgroups ($n=15$ in each subgroup). The samples were tested for $\mu\text{-TBS}$ immediately following the one-week and 9 months storage in the artificial saliva (pH 7.4). The artificial saliva solution was replenished every 7 days and the pH was re-checked using pH meter (Orion 818 pH meter, Thermo Fisher Scientific, USA). For bond strength testing, each beam was mounted on a metallic jig fixed to a universal testing machine (Instron E3000, Microtester, Instron Corp., Canton, MA) using cyanoacrylate adhesive (Zapit; Dental Ventures of America, Corona, CA, USA). A tensile load was applied at a crosshead-speed of 0.5 mm/min⁻¹ until failure. For determining μTBS in MPa, the de-bonded beams were removed, and the cross-sectional area was measured at the site of fracture to the nearest 0.01 mm by the help of a digital caliper (Model 500-196-20, Mitutoyo Digimatic Caliper).

MMP-8 and Cathepsin profilometry

The dentin was cut from the extracted teeth ($n = 16$) and segments pulverized using liquid nitrogen to obtain powder using a mortar and pestle (Reimiller, Reggio Emilia, Italy). Five 1 g aliquots of dentin powder were demineralized with 10wt% H_3PO_4 for 24 h at 27°C and later thoroughly rinsed in deionized water with constant stirring at 4°C for 1 h. The groups were further treated with all nanoparticles by introducing a slurry made from PBS (0.1 mM). The dentin powder was suspended in an extraction buffer for 24 h to extract the proteases. Supernatants were collected after centrifugation at 25,000 rpm for 25 min at room temperature. The supernatants were dialyzed in bags with 30-kDa molecular cut-off overnight, lyophilized and frozen at -20°C until they were analyzed for MMP-8 and cysteine cathepsin (CTX) using enzyme-linked immunosorbent assay (ELISA) (Human MMP-8 ELISA Kit – Lot #5619 for MMP-8; Human CTSK/Cathepsin K ELISA Kit – Lot #5614 for cathepsin K, both from Lifespan Biosciences, Seattle, WA, USA) according to manufacturer's instructions.

Statistical analysis

Data were presented in means and their standard deviations. Normality testing was performed before running any statistical test. A specialized statistical software was used to analyze data using one-way ANOVA followed by Tukey-Kramer *post-hoc* test. Significance level was set at $p < 0.05$.

Results

Characterization of the fabricated nanoparticles

Representative SEM and TEM micrographs with the associated EDX mapping of the nanoparticles is demonstrated in Figure 2. CHX-loaded/MSN nanoparticles exhibited a more uniform morphology with relatively larger particle size compared to CHX-loaded/MSN-PLGA which showed more irregular particle morphology. EDX analysis revealed the existence of silicon, oxygen, chlorine, and nitrogen in all CHX-loaded nanoparticles. Carbon was associated with PLGA modification of MSN (CHX/MSN-PLGA).

Table 1 shows the results of the physicochemical characteristics of the nanoparticles. The unloaded MSN-PLGA/Blank nanoparticles were ~ 107 nm in diameter with size larger than CHX-loaded/MSN-PLGA nanoparticles. A significant reduction in z-average diameter from ~ 98 nm to ~ 84 nm was exhibited upon increasing the CHX content of MSN nanoparticles. The z-average diameter reduced further in CHX-loaded/MSN-PLGA compared to CHX-loaded/MSN. Similarly, a decrease in particle size also showed a decrease in PDI values, suggesting an acceptable homogeneity level for all fabricated nanoparticles. An overall negative charge of -15.42 mV was recorded for MSN-PLGA/Blank nanoparticles, whereas CHX inclusion in MSN significantly shifted the surface charge to positive values ranging from 23.67 mV to 29.51 mV. The CHX-loaded/MSN-PLGA demonstrated an even higher positive surface charge compared to CHX loaded/MSN.

Chlorhexidine content

The DEE and DL of CHX-loaded/MSN, MSN-PLGA/Blank, and CHX-loaded/MSN-PLGA are reported in Table 1. It is observed that an increase in the percentage of DEE from 81% to 85% in CHX loaded/MSN and 92% to 95% in CHX loaded/MSN-PLGA proportionately increased with increasing the amount of CHX during the fabrication of nanoparticles. Therefore, DEE of 81% produced a DL of 15%, substantially increasing to 19% upon further adding of CHX to 50 mg. The trend was even significantly higher upon grafting PLGA over MSN with DEE of 96% that produced a DL of 24%. The *in vitro* drug release behaviors of MSN-PLGA/Blank, CHX-loaded/MSN, and CHX-loaded/MSN-PLGA nanoparticles at pH 7.4 and 5.0 were experimented and shown in Figure 3A-B. The drug release rate of CHX-loaded/MSN-PLGA was more pH-dependent and increased with the decrease of pH. The cumulative release of CHX amount from CHX-loaded/MSN-PLGA reached up to 70% after 24 h at pH 5.0, much higher than that at pH 7.4, which was 49% only. At lower pH, the trend of higher drug release was seen in 25:50:50 CHX-loaded/MSN-PLGA. Nanoparticles 50:50:50 CHX-loaded/MSN-PLGA and 50:50 CHX-loaded/MSN showed a close pattern, however 50:50:50 CHX-loaded/MSN-PLGA showing slightly higher drug release than 50:50 CHX-loaded/MSN indicating that the drug release was similar and the difference in release amount between different pH values was not significant. The drug release of CHX-loaded/MSN was also slightly pH-

dependent. However, CHX-loaded/MSN-PLGA exhibited a more significantly pH-dependent drug release behavior than CHX-loaded/MSN.

Spectral analysis

Raman spectra of all synthesized nanoparticles MSN, PLGA and pure chlorhexidine powder are shown in Figure 4(A-B). When using excitation wavelength of 532 nm laser, the synthesized mesoporous silica powder (red spectrum) shows a Raman spectrum having prominent signals at 638, 802 and 1300 cm^{-1} [17]. The band around 1300 is attributed to the lactic units. The peaks between 400 to 1350 cm^{-1} are assigned to pure CHX powder. CHX-loaded/MSN exhibited a characteristic peak of Raman scattering at 638, 802, 1011, 1035, 1067 and 1300 cm^{-1} (Figure 4A). The Raman spectrum at 1446 cm^{-1} for CHX-loaded/MSN-PLGA is suggestive of anti-symmetric vibration from the lactic unit corresponded as CH_2 deformation which is confined to a lower intensity in CHX-loaded/MSN groups. The assignments at 1040 cm^{-1} and 1127 cm^{-1} corresponds to CH_3 rocking and CH_2 wagging [18] (Figure 4B).

The FTIR spectra of pure CHX, synthesized MSN and PLGA has been shown in Figure 5 for reference. Pure CHX exhibiting characteristic peaks at 1093 and 2947 cm^{-1} that indicates C-N bending and C-H vibration [19]. The two bands that appeared at 1080 and 810 cm^{-1} belonged to the characteristic peaks of Si-O-Si on the SiO_2 framework of MSNs [20]. PLGA displays characteristic absorption bands at 1100–1250 and 1750–1760 cm^{-1} which represent the esters and carbonyl groups [21]. The above-mentioned fingerprint peaks specific to CHX can be observed in all the CHX-loaded/MSN and CHX-loaded/MSN-PLGA.

Antibacterial and cytotoxicity testing

Figure 6(A-B) shows the results of the antimicrobial activity and cytotoxicity of all the nanoparticles. For both time-periods (24 h or 30 Days), the relative microbial viability significantly decreased by increasing the CHX content ($P < 0.001$). After 24 h, the 50:50 CHX-loaded/MSN and 25:50:50 CHX-loaded/MSN-PLGA showed equal antimicrobial efficacies ($P > 0.05$). The highest antimicrobial capacity at 24 h was demonstrated by 50:50:50 CHX-loaded/MSN-LGA. On day 30, the bacterial viability of the group 25:50:50 CHX-loaded/MSN-PLGA remained low under 30% (Figure 6A). For cytotoxicity, the results showed significant differences in DPSCs viability between drug loaded and unloaded nanoparticles only (Figure 6B). Generally, the cell viability percentage of DPSCs exposed to MSN-PLGA/Blank, CHX-loaded/MSN, and CHX-loaded/MSN-PLGA, respectively was $>80\%$ indicating low cytotoxicity profiles of experimental nanoparticles. In addition, none of the CHX concentrations showed statistical significance over time.

μ TBS

The outcomes of μ TBS are summarized in Table 2. After storage in one week of neutral artificial saliva, groups containing 5 wt.% 25:50 CHX/MSN, 50:50 CHX/MSN, and 25:50:50 CHX/MSN-PLGA showed no significant difference in μ TBS compared with the commercial adhesive ($p > 0.05$). However, after 9 months

in artificial saliva (pH 7.4) significantly highest μ TBS value was recorded for 25:50 CHX/MSN and 25:50:50 CHX/MSN-PLGA with no significant difference compared with the 1-week value.

MMP-8 and cathepsin K concentrations

The results of MMP-8 and cathepsin K concentrations are depicted in Figure 6C. The MMP-8 levels were significantly low in CHX-loaded nanoparticles as compared to unloaded-MSN-PLGA ($p < 0.05$). The concentrations of active cathepsin-K in 25:50:50 CHX-loaded/MSN-PLGA were the lowest among all groups ($p < 0.05$) (Figure 6C). The PLGA coating had a significant effect on the MMP-8 and cathepsin K activity.

Characterization of nanoparticles-treated demineralized dentin substrates and adhesive-dentin interface

The nanoparticles delivered using aqueous-carrier showed the ability to penetrate considerable depths inside dentinal-tubules (Figure 7). The nanoparticles appeared to be evenly distributed, confined to dentinal-tubule structure, and attached to tubular walls after air-blowing and blot-drying steps (Figure 7B-D). A representative overall low magnification SEM imaging of the resin-dentin interfaces of specimens treated with the 50:50:50 CHX-loaded/MSN-PLGA and 50:50 CHX-MSN nanoparticles followed by the application of commercially available dentin adhesive system revealed formation of intact resin/dentin hybrid-layer along with numerous well-developed resin-tags (Figure 7E&F). The high magnification of dentin-bonded specimens sectioned vertically indicated a homogenous and widely distributed 50:50:50 CHX-loaded/MSN-PLGA nanoparticles and exhibiting excellent bonding. Some areas of the hybrid layer showed tight agglomerations of the nanoparticles (Figure 7G-I).

The line EDX performed on specimens bonded with MSN-PLGA/Blank and 50:50:50 CHX-loaded/MSN-PLGA exhibited high percentage of amorphous carbon and silica that represents MSN-PLGA with no evidence of the drug CHX. The presence of nitrogen and chlorine depicts the presence of CHX along the line of resin-dentin interface (Figure 8 A&B). EDX mapping of resin matrix within hybrid layer from 50:50:50 CHX-loaded/MSN-PLGA showing nanoparticles distribution and the presence of carbon (C) and silica (Si) for MSN and PLGA within the resin matrix and distribution of nitrogen (N) and chlorine (Cl) as an evidence for CHX (Figure 8C).

Discussion

To investigate the pH-responsive behavior and controlled release from the CHX loaded nanocarrier delivery system, a mesoporous silica nanoparticle modified with PLGA was fabricated where the CHX component served as a cationic template incorporated and co-condensing with PLGA (Figure 1). The study aimed to investigate and characterize the feasibility of CHX-loaded/MSN-PLGA as nanocarriers as potential modifiers of experimental dentin adhesives. The preparation process of MSN-CTAB involved the sol-gel technique which was followed by the functionalization of amino groups on the outer surface of MSN through APS [10, 22]. Raman and FTIR analyses (Figure 4 and 5) provided evidence for the successful preparation of these nanocarrier systems; MSN displaying strong peaks at 638, 802 and 1300

cm^{-1} while peaks between $400\text{-}1350\text{ cm}^{-1}$ were assigned to CHX. In addition, FTIR further confirmed the signals that appeared at 1093 cm^{-1} for CHX, 810 for MSN and peaks $1100\text{-}1250\text{ cm}^{-1}$ associated with CHX-loaded/MSN-PLGA. Observing high shift frequency and significant intensity at 810 cm^{-1} , proved the nanocrystalline silicon overlapped by surrounding signals. The Raman shift to lower confinements would be best explained by the incorporation of CHX causing a change in spectral efficiency of Raman scattering (from 802 to 810 cm^{-1}). This may be due to the scattering of optical photons from the silicon [23, 24]. The maximum intensities observed are very inherent to the nanocrystalline silicone, a predominant fraction seen in the MSNs. When MSN were examined under SEM and TEM/EDX (Figure 2), the nanoparticles appeared smooth with regular mesoporosity. The mesoporosity became irregular and less clearly defined after loading of CHX signifying the adsorption of the drug onto the external surface of MSN and inside the mesoporous cavities [9]. EDS elemental mapping indicated strong intra-particulate silicon, carbon, and chlorine in CHX-loaded/MSN-PLGA nanoparticles. Several studies have proved that the amount of CHX loading from CHX-loaded/MSN exceeds $>40\text{ wt.}\%$ [13, 25], and this relatively high loading complements MSN as a superior nanocarrier for the CHX.

The in-vitro drug release behaviors from the nanoparticles (Figure 3 A-B) were investigated in PBS at two different pH values (7.4 and 5.0). The idea was to design a study in such a way as to mimic the low pH environment which is generally found at the bacterial insult due to acidogenesis [26]. Following this concept, the deterioration of bonds at the adhesive-dentin interface has been widely studied both *in vivo* [27, 28] and *in vitro* [29-31]. Although the degradation mechanism remains unclear, microleakage and collagen degradation are probably the two primary factors that are implicated in the impairment of resin-dentin bonding [32]. It is well-established that microleakage is caused by the residual niche of *S. mutans* at the resin-dentin interface, and these living microbial remnants maintain reduced metabolic capability and low sensitivity to antimicrobial agents [33]. Thus, it is of utmost importance to develop a drug-loaded nanocarrier system that possesses antibacterial efficacy and suppress biofilm formation. CHX has been extensively studied in several *in vitro* studies due to its wide antibacterial effectiveness, inhibition of dental biofilm formation, and is non-toxic to human cells [34, 35]. Previous studies have indicated that MSN is capable of loading drugs and releasing at considerably low pH [10, 36]. Priyadarshini et al. [13] reported that the percentage of encapsulation efficiency of PLGA nanocarriers was proportional to increasing the amount of CHX addition. Thus, we hypothesized that PLGA grafted over MSN would carry more CHX and show a sustained release of CHX in low pH for a long time. For this reason, these nanoparticles system after incorporating in a dentin-adhesive system would surmount the bacterial growth and show high antibacterial efficacy. The aforementioned tests in our study backed our assumptions.

Regarding the antimicrobial activity (Figure 6A), the metabolic activity of *S. mutans* remarkably reduced by the addition of a higher percentage of CHX-loaded MSN with or without PLGA even after 30 days of storage. This indicates that the antimicrobial efficiency of these groups can be maintained for a longer time. This long-term antibacterial efficacy is in association and depends significantly on the CHX release (Figure 3 A-B). All CHX-loaded nanoparticles did show an initial burst release than showing a nearly

constant release with similar slopes. However, the low pH due to bacterial biofilms might trigger higher CHX release from the CHX-loaded/MSN-PLGA modified adhesives with the subsequent enhancing of the antimicrobial effect. Therefore, this pH sensitive CHX release could be potential significance against cariogenic biofilms related to dentin substrate. Various research studies document the antibacterial substantivity of CHX ranging from 48 h up to 12 weeks [37, 38]. Therefore, it is arduous to give recommendations on its clinical applicability, and further research is warranted to evaluate the antimicrobial susceptibility of CHX-loaded/MSN-PLGA for clinical use.

Substantial amounts of penetrated nanoparticles were revealed by SEM, verifying the capability of 60 s application of 10% nanoparticles for deep infiltration within dentinal-tubules structure. The use of water as an experimental nanoparticles' dispersion phase/carrier was efficient to facilitate the delivery and infiltration of Nano-PLGA/CHX nanoparticles through dentinal-tubules within 60 s application-time, which is considered clinically realistic and acceptable. The ability of water to infiltrate inside slightly dried demineralized dentin-substrates carrying the dentin bonding resin is a well-known phenomenon in adhesive dentistry [39]. The close association of spherical nanoparticles across the resin-tag morphology further confirmed nanoparticle penetration, their presence inside dentinal-tubules and successful retention even after resin-infiltration. However, further advances are mandatory to estimate the nanoparticles penetration-depth. An ethanol-containing 2-step etch-and-rinse dentin adhesive system was used to further synchronize the nanoparticles penetration-phenomena with resin-infiltration inside dentinal-tubules to form hybridized resin-tags. The prominence of air-blowing and blot-drying steps on promoting nanoparticles confinement inside dentinal-tubules is of clinical importance to conserve and protect the intact morphology of resin-dentin hybrid-layer specifically after nanoparticles degradation. However, recommendation on the use of CHX-loaded/MSN-PLGA nanoparticles after acid-etching, at this point of our study, is limited to the adhesive system based on etch-and-rinse principle. Further studies should investigate the spatial correlation of nanoparticles around and/or inside the resin tags within the structure of dentinal tubules. Moreover, to scrutinize the effect of resin infiltration on drug release characteristics of delivered nanoparticles.

With regards to the μ -TBS, it is noted that the bond strength was significantly reduced in the groups of nanoparticles containing the highest amount. Hence, it could be postulated that the μ -TBS is significantly affected by the incorporation of nanoparticles and may not be affected by the drug itself.

There lies a universally stated notion that S1' pocket of MMPs can accommodate residues and can catalytically cleaved but are not structurally open. Moreover, CHX tends to stabilize genolytic activities, and because of MMPs not being entirely free and to be attached only at the fibronectin and hemopexin sites [40]. This limitation of cleavage is speculated to have been controlled by the CHX present within the nanoparticles. This becomes a pivotal reason why enzymatic activity is lowered as seen in specimens with a higher concentration of CHX and stable release. CHX is cationic at physiological pH and can remove calcium and zinc ions which are important for MMP activities. It also shuts down the essential cysteine and/or sulfhydryl groups on the catalytic site of the MMP enzymes [41].

This study introduced the significance of pH-responsive CHX nanocarrier delivery system formed of MSN modified with PLGA as a potential modifier of resin-based dentin adhesives in terms of improving the antimicrobial activities, proteases inhibition, and resin-dentin bonding integrity and durability. This reported pH-sensitive CHX release response could be of crucial advantage for resin-dentin bonding application due to the associated decrease in pH in the surrounding microenvironment resulting from biofilm formation, acid etching, and acidic content of bonding monomers.

Conclusion

A pH sensitive CHX release response was noted when loaded in MSN grafted PLGA nanoparticles. The formulated drug loaded nanocarrier demonstrated excellent physicochemical, spectral, and biological characteristics. Showing considerable capacity to penetrate effectively inside dentinal tubules and having high antibacterial efficacy, this system could be potentially used in adhesive and restorative dentistry.

Abbreviations

μ -TBS: micro-tensile bond strength; ANOVA: one-way analysis of variance; APS: aminopropyltriethoxysilane; ATR: attenuated total reflectance; BLG-NCA: γ -benzyl-L-glutamate N-carboxyanhydride; BHI: Brain-Heart infusion; CHX: chlorhexidine; CTAB: N-cetyltri-methylammonium bromide; DEE: drug encapsulation efficiency; DL: drug loading; DPSCs: dental pulp stem cells; EDS: energy dispersive X-ray analysis; ELISA: enzyme-linked immunosorbent assay; FTIR: Fourier transformed infra-red spectroscopy; MMP: matrix metalloproteinase; MPa: megapascal; MSNs: mesoporous silica nanoparticles; MTS: [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)]; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS: phosphate buffer saline; PLGA: poly-L-glycolic acid; SEM: scanning electron microscopy; *S. mutans*: *Streptococcus mutans*; TEM: transmission electron microscopy; TFA: trifluoroacetic acid; TEOS: tetraethyl orthosilicate;

Declarations

Ethics approval and consent to participate: All experiments were performed on extracted teeth obtained from the tooth bank in the University of Western Australia. The study was approved by the Human Ethics Committee of this university (permit: RA/4/20/6344) and carried out in accordance with the 'Checklist for Reporting In-vitro Studies' (CRIS) guidelines.

Consent for publication: Not applicable

Availability of data and material: The analyzed datasets generated during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Author contributions: **ZA:** Data curation, Formal analysis, Investigation; Methodology, Writing - original draft, Writing - review & editing, **SA:** Methodology, Software, Formal analysis, Writing - review & editing, Writing - original draft, **HN:** Validation, Formal analysis, Writing - review & editing, Writing - original draft, **AF:** Conceptualization, Supervision, Project administration, Writing - original draft, Resources, Writing - review & editing, Funding acquisition

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Tables

Table 1. Mean and standard deviation of the percentage of drug encapsulation efficiency (DEE), drug loading (DL), nanoparticle recovery, nanoparticle size (z-average diameter), zeta potential (ζ) and polydispersity index of MSN-PLGA/Blank, CHX-loaded/MSN and CHX-loaded/MSN-PLGA at 25 mg and 50 mg of CHX incorporation, respectively.

Groups	DEE (%)	DL (%)	Nanoparticle recovery (%)	z-Average diameter (nm)	Zeta potential (mV)	Polydispersity index
MSN-PLGA/Blank	-	-	57.1 ± 11.6 ^A	107.4 ± 7.4 ^A	-15.4 ± 1.9 ^A	0.27 ± 0.02 ^A
25:50 CHX/MSN	81.4 ± 5.9 ^A	15.3 ± 2.3 ^A	65.5 ± 13.9 ^B	97.9 ± 4.1 ^B	23.6 ± 9.9 ^B	0.18 ± 0.04 ^B
50:50 CHX/MSN	85.4 ± 3.7 ^A	19.7 ± 1.9 ^{AB}	66.8 ± 12.8 ^B	84.2 ± 5.9 ^C	29.5 ± 9.4 ^B	0.15 ± 0.01 ^B
25:50:50 CHX/MSN-PLGA	92.7 ± 6.6 ^B	20.4 ± 2.5 ^B	67.6 ± 11.4 ^B	81.2 ± 3.4 ^C	41.3 ± 8.1 ^C	0.083 ± 0.01 ^C
50:50:50 CHX/MSN-PLGA	95.8 ± 5.6 ^C	24.1 ± 3.1 ^B	64.1 ± 15.2 ^B	74.9 ± 4.6 ^D	42.9 ± 6.7 ^C	0.065 ± 0.01 ^D

* Dissimilar uppercase letters show statistical significance at $p < 0.05$ within each column

Table 2. Mean and standard deviation of the micro-tensile bond strength (μ TBS in MPa) for the modified experimental adhesives, and the commercial adhesive after one week of storage in artificial saliva and 9 months of storage in artificial saliva.

Groups	One week in AS (pH 7.4)	9 months in AS (pH 7.4)
25:50 CHX/MSN	38.2 ± 5.9 A (a)	29.2 ± 7.8 A (a)
50:50 CHX/MSN	36.4 ± 7.2 A (a)	22.8 ± 6.5 B (b)
25:50:50 CHX/MSN-PLGA	39.7 ± 7.4 A (a)	28.7 ± 5.0 A (b)
50:50:50 CHX/MSN-PLGA	31.8 ± 6.3 B (a)	19.4 ± 7.6 B (b)
MSN-PLGA/Blank	32.5 ± 6.0 B (a)	18.1 ± 9.8 B (b)
Commercial adhesive (Scotchbond™ bond, 3M ESPE)	42.3 ± 5.8 A (a)	28.5 ± 7.7 A (b)

*Dissimilar uppercase letters (A-E) show statistical significance at $p < 0.05$ within each column

**Dissimilar lowercase letters (a-c) show statistical significance at $p < 0.05$ within each row.

Figures

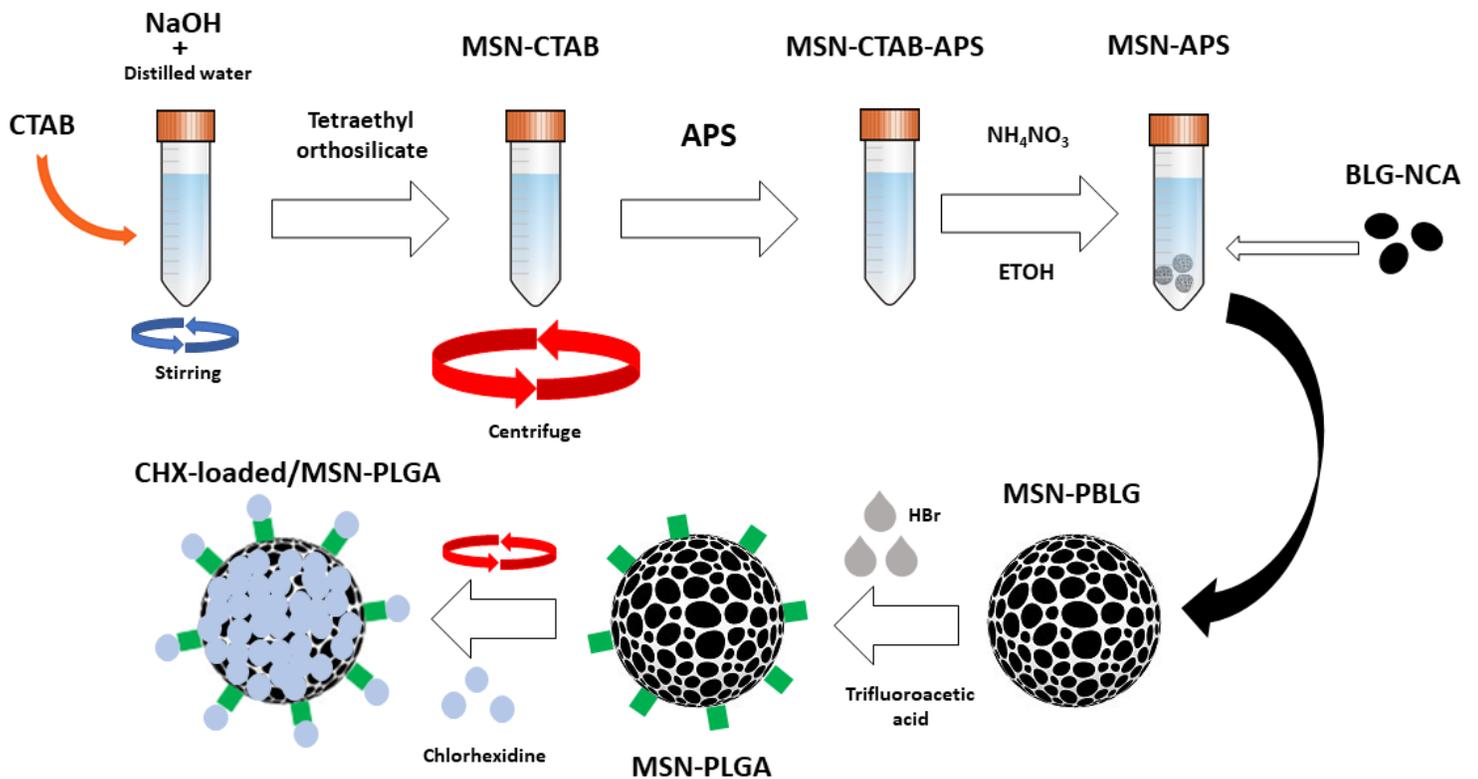


Figure 1

Schematic diagram showing fabrication technique of CHX-loaded MSN-PLGA.

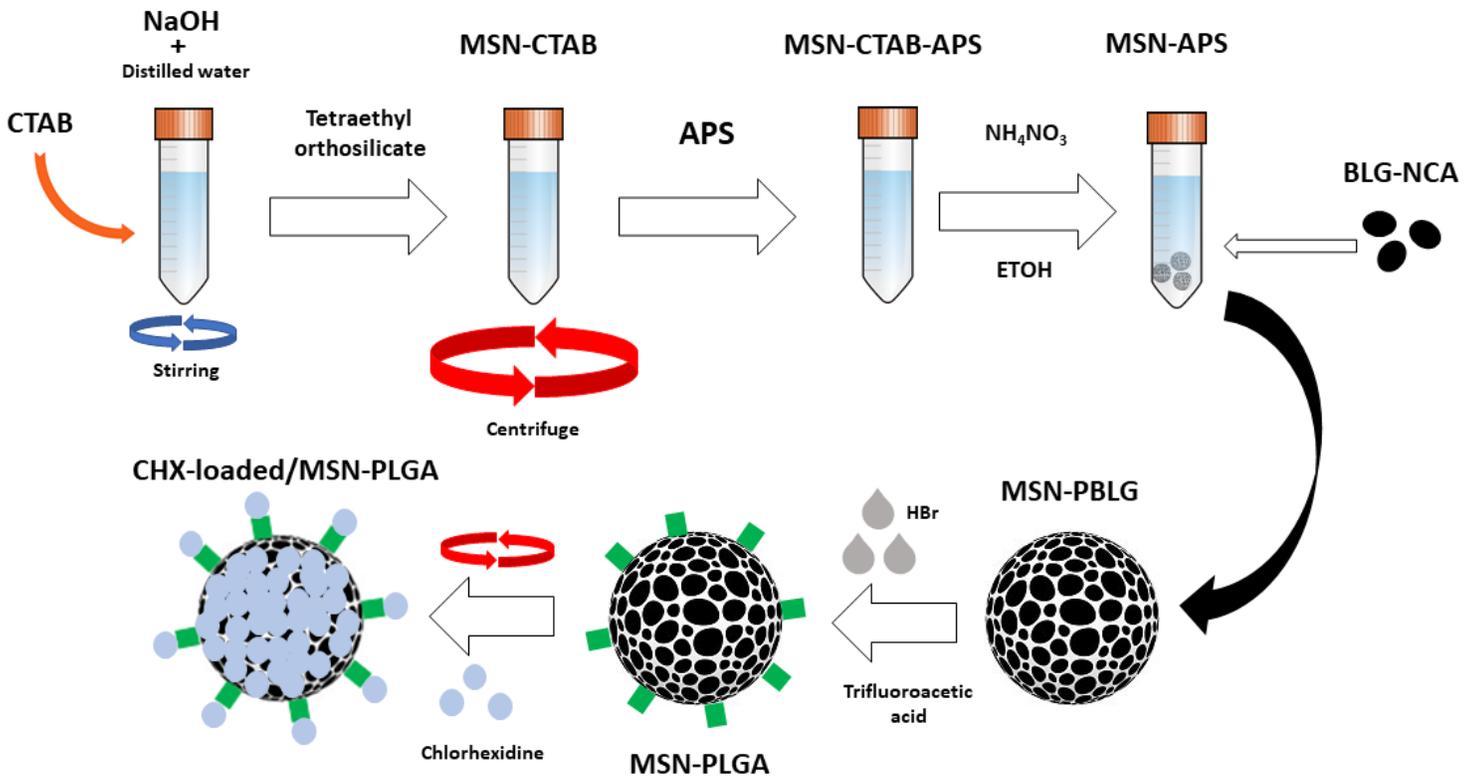


Figure 1

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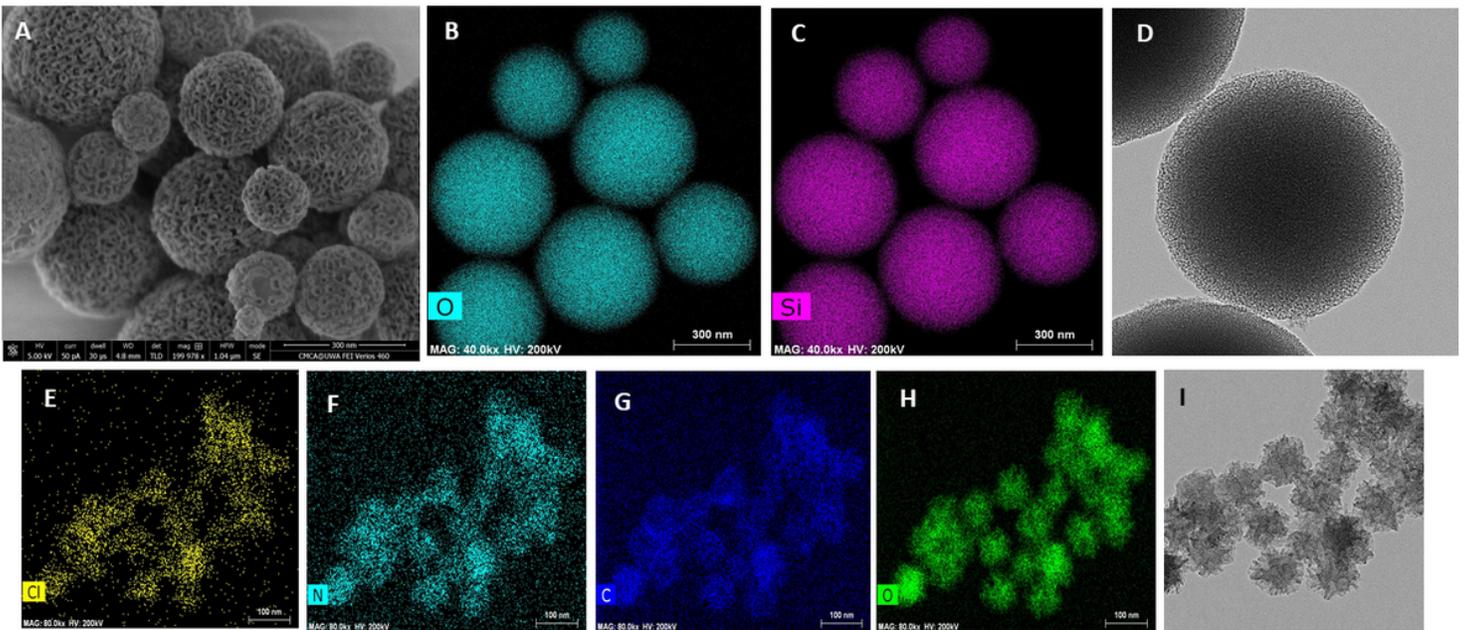


Figure 2

Selected SEM and TEM images at low and high magnification for (A & D) CHX-loaded/MSN; (E & H) CHX-loaded/MSN-PLGA nanoparticles and the associated EDS elemental analysis confirming the existence of

oxygen (B) and silica (C) [CHX-loaded/MSN], the carbon (G) is associated with PLGA modification and chlorine (E) nitrogen (F) from CHX loading in CHX-loaded/MSN-PLGA. CHX-loaded/MSN nanoparticles exhibited a more uniform morphology with relatively larger particle size compared to CHX-loaded/MSN-PLGA which showed more irregular particle morphology.

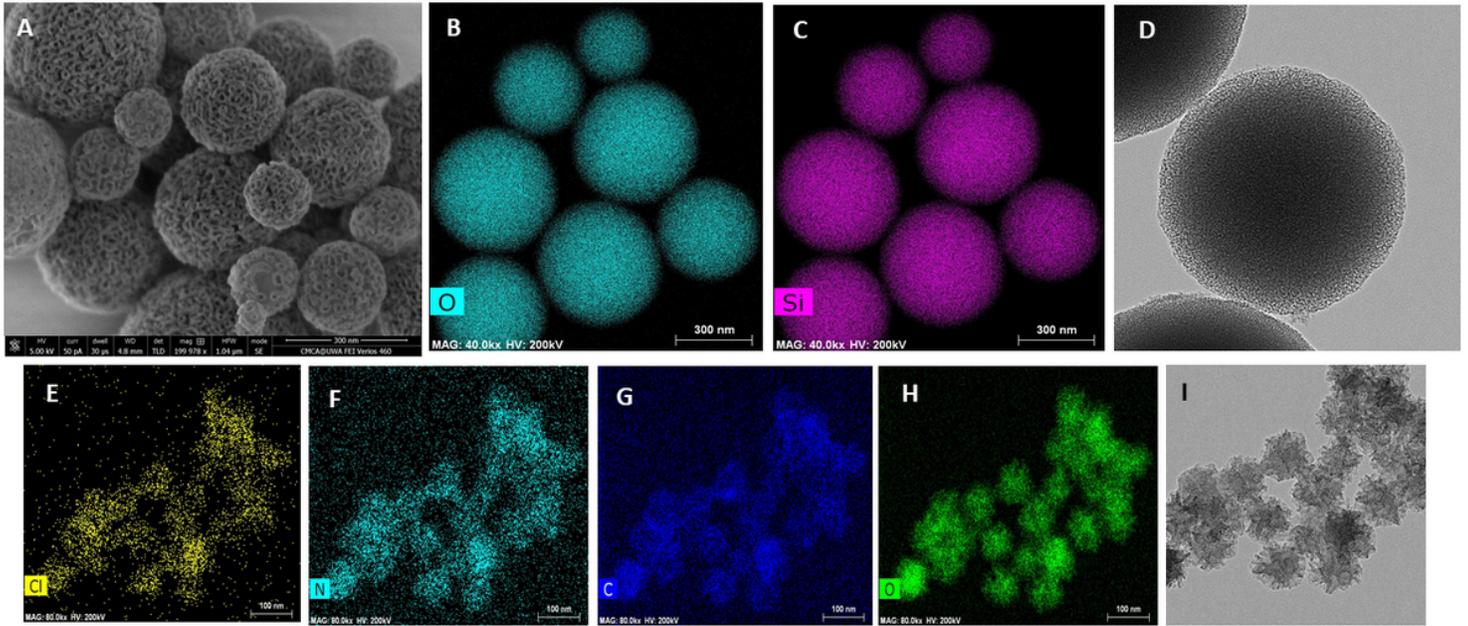


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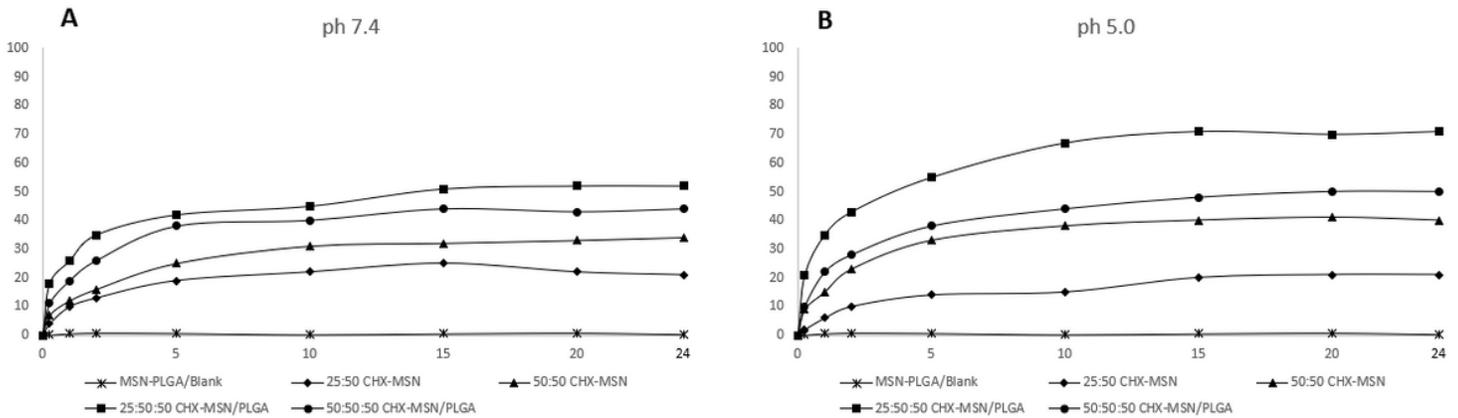


Figure 3

In-vitro CHIX release profiles of 25:50 CHX-loaded/MSN, 50:50 CHX-loaded/MSN, 25-50-50 CHIX lrp,d;d2M/14,SN-PLGA, 50:50:50 CHX-loaded/MSN-PLGA and MSN-PLGA/Blank at pH (A) 7.4 and (B) 5.0

up to 24 h.

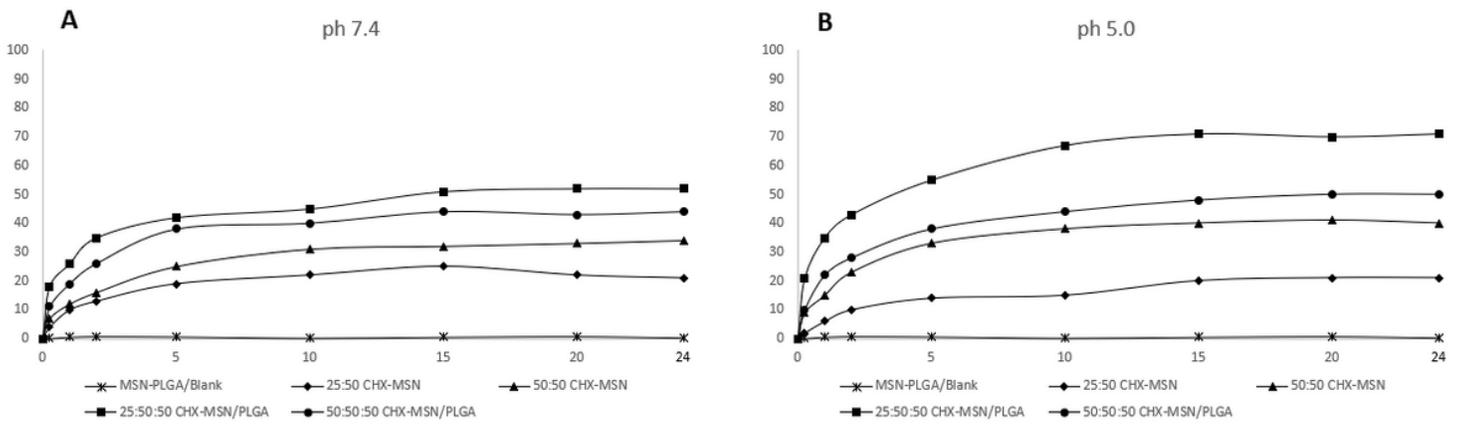


Figure 3

In-vitro CHX release profiles of 25:50 CHX-loaded/MSN, 50:50 CHX-loaded/MSN, 25-50-50 CHX-loaded/MSN-PLGA, 50:50:50 CHX-loaded/MSN-PLGA and MSN-PLGA/Blank at pH (A) 7.4 and (B) 5.0 up to 24 h.

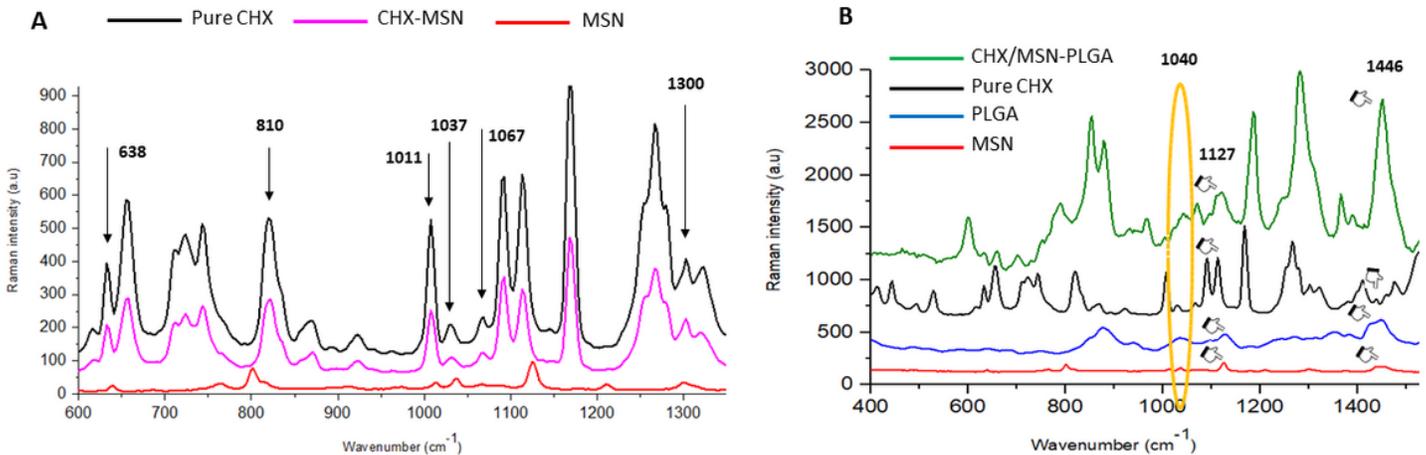


Figure 4

Raman spectra of all synthesized nanoparticles MSN, PLGA and pure chlorhexidine powder are shown. (A) When using excitation wavelength of 532 nm laser, the synthesized mesoporous silica powder (red spectrum) shows a Raman spectrum having prominent signals at 638, 802 and 1300 cm⁻¹. The band around 1300 is attributed to the lactic units. The peaks between 400 to 1350 cm⁻¹ are assigned to pure CRX powder. CHX-loaded/MSN exhibited a characteristic peak of Raman scattering at 638, 802, 1011, 1035, 1067 and 1300 cm⁻¹. (B) The Raman spectrum at 1446 cm⁻¹ for CHX-loaded/MSN-PLGA is suggestive of anti-symmetric vibration from the lactic unit corresponded as CH₂ deformation which is confined to a lower intensity in CHX-loaded/MSN formulations. The assignments at 1040 cm⁻¹ and 1127 cm⁻¹ corresponds to CH₃ rocking and CH₂ wagging.

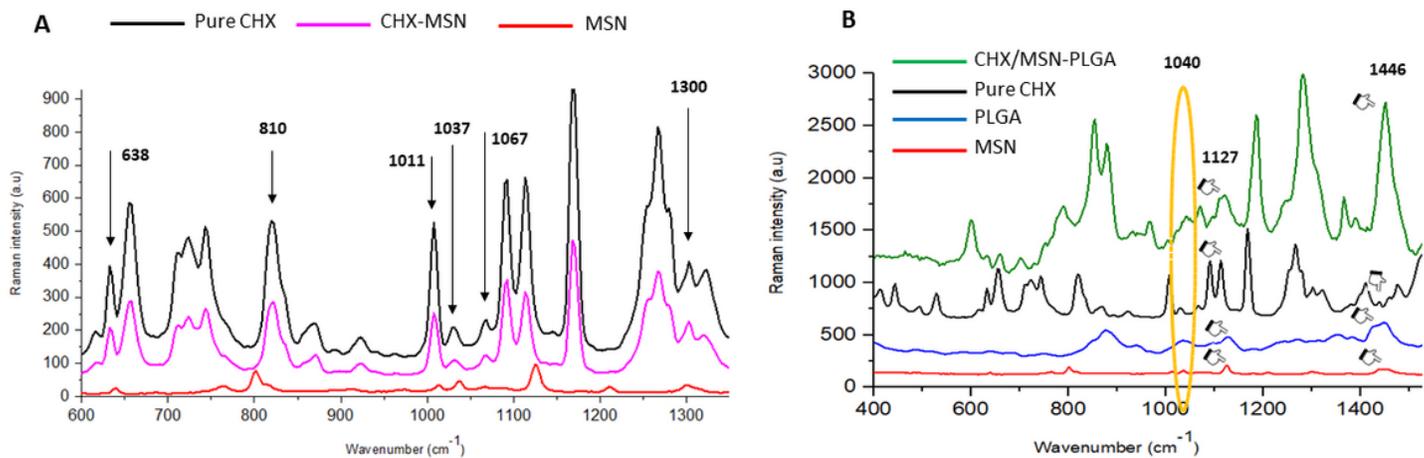


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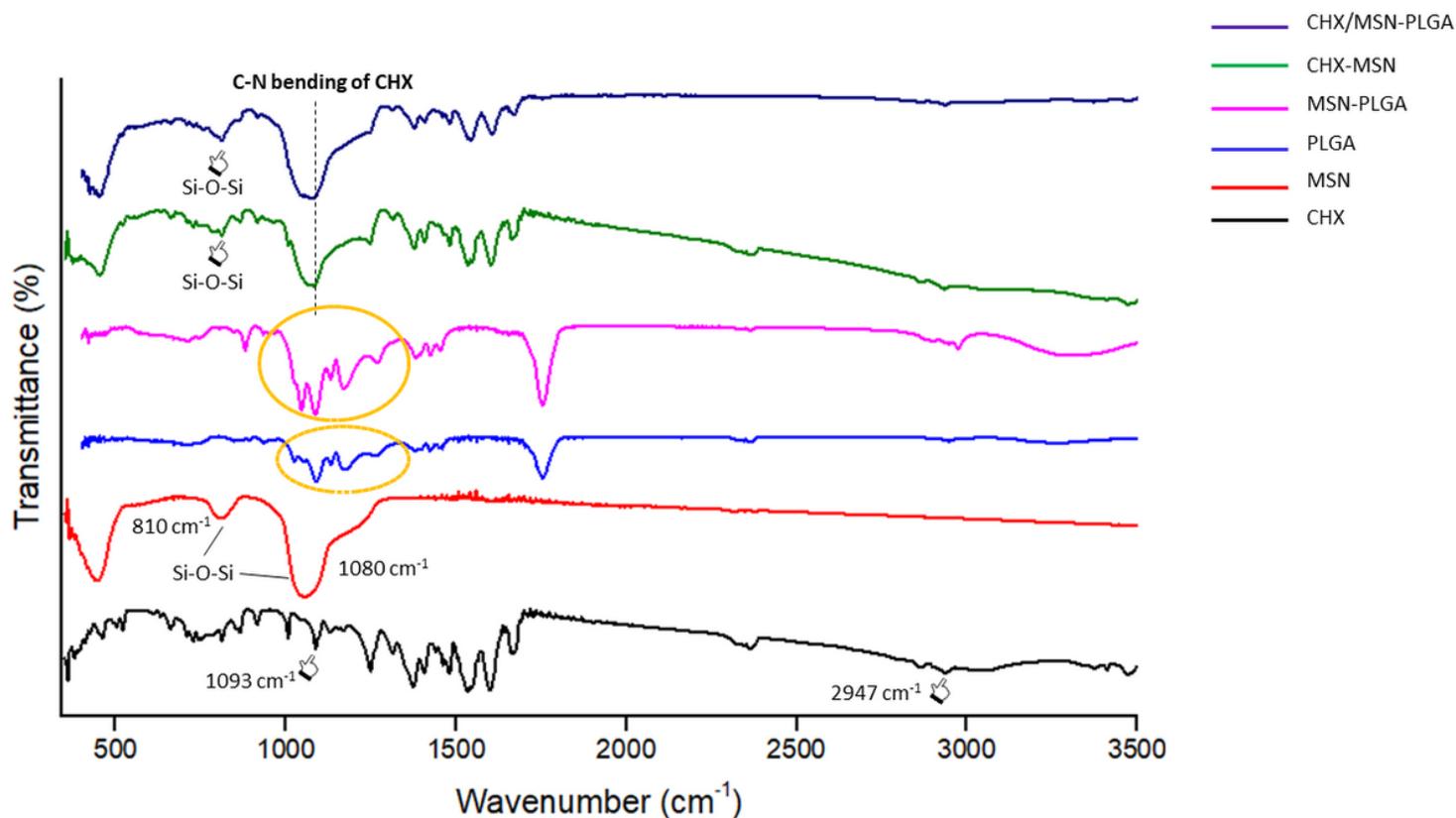


Figure 5

The FTIR spectra of pure CHX, synthesized MSN and PLGA has been shown for reference. Pure CHX exhibiting characteristic peaks at 1093 and 2947 cm^{-1} (pointers) that indicates C-N bending and C-H vibration. The two bands that appeared at 1080 and 810 cm^{-1} belonged to the characteristic peaks of Si-O-Si on the SiO₂ framework of MSNs. PLGA displays characteristic absorption bands at 1100-1250 and 1750-1760 cm^{-1} which represent the esters and carbonyl groups (dotted circle). The above-mentioned fingerprint peaks specific to CHX can be observed in all the 'CHX-loaded/MSN and CHX-loaded/MSN-PLGA.

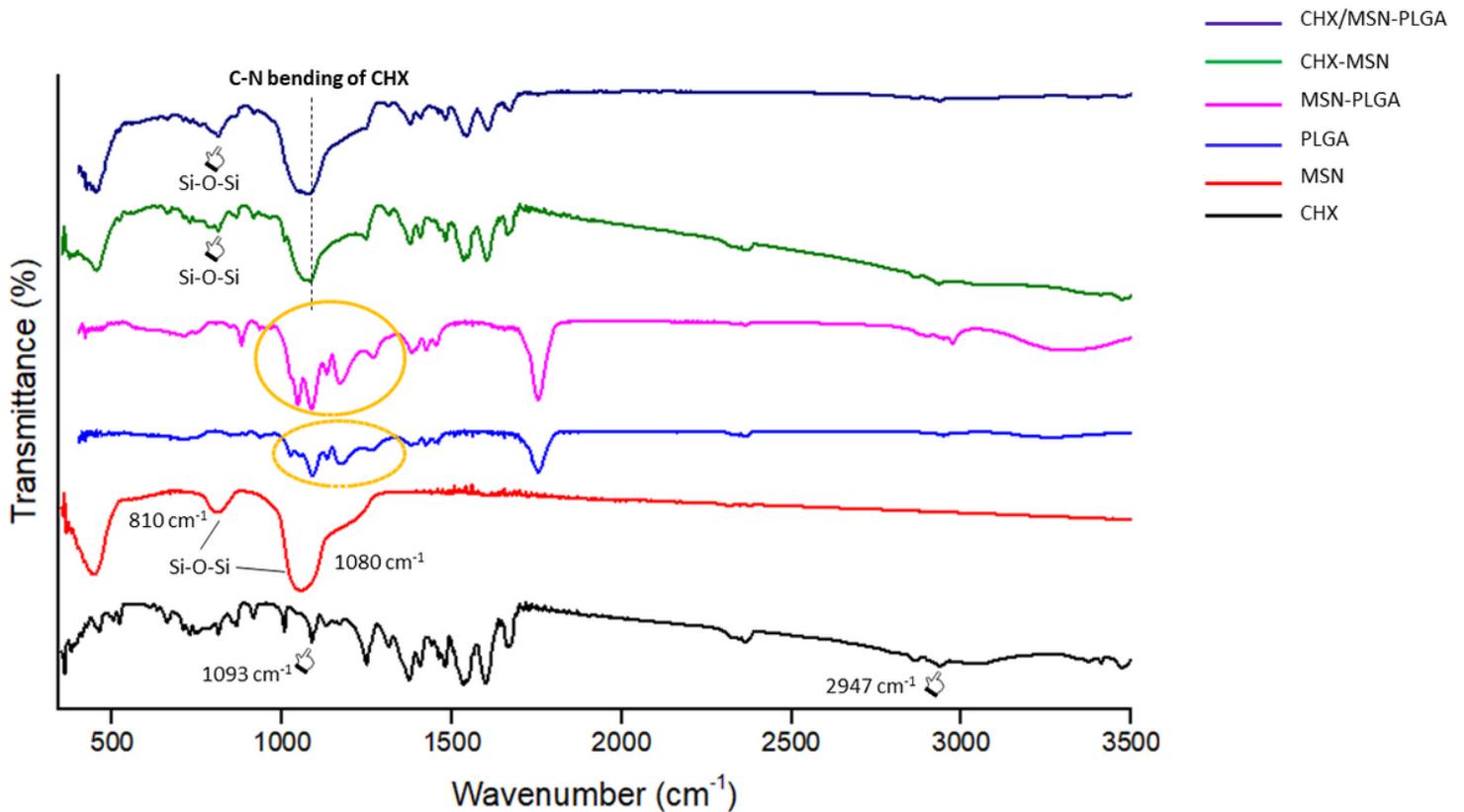


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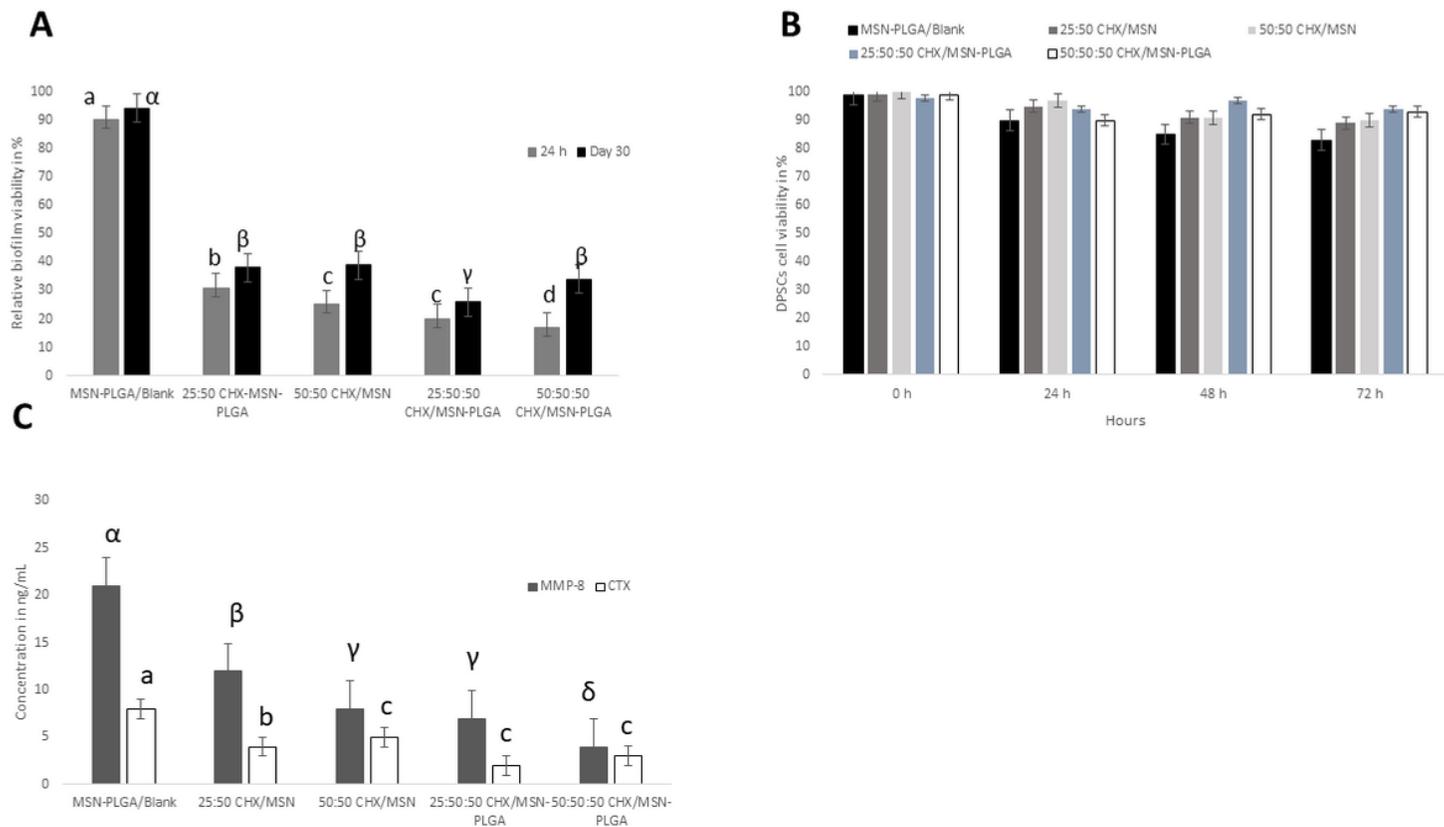


Figure 6

(A) MTT assay showing relative percentage of *S. mutans* biofilm viability at 24 h and 30 days; (B) percentage of DPSCs viability. (C) the result of MMP-8 and cathepsin K concentrations.

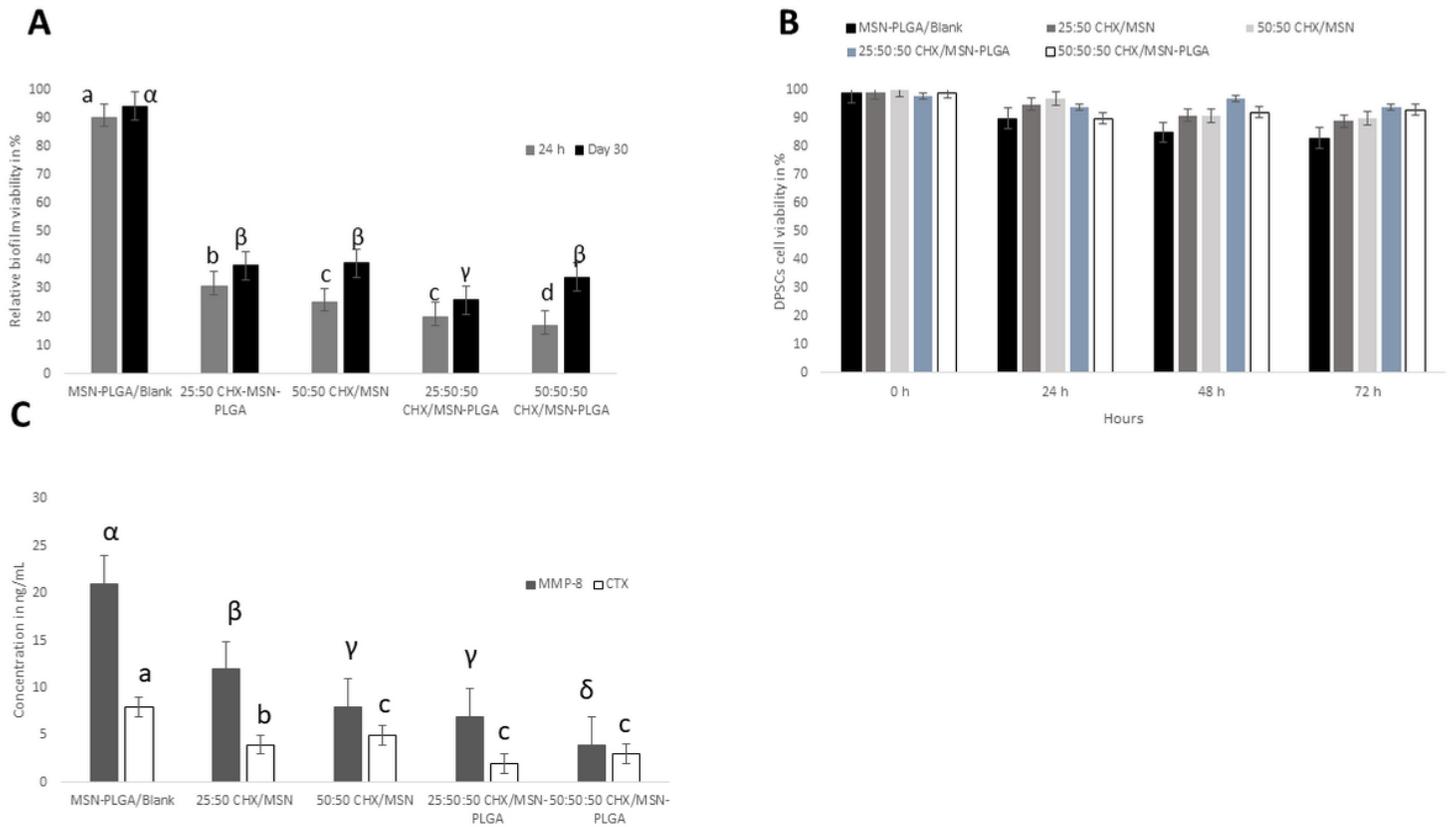


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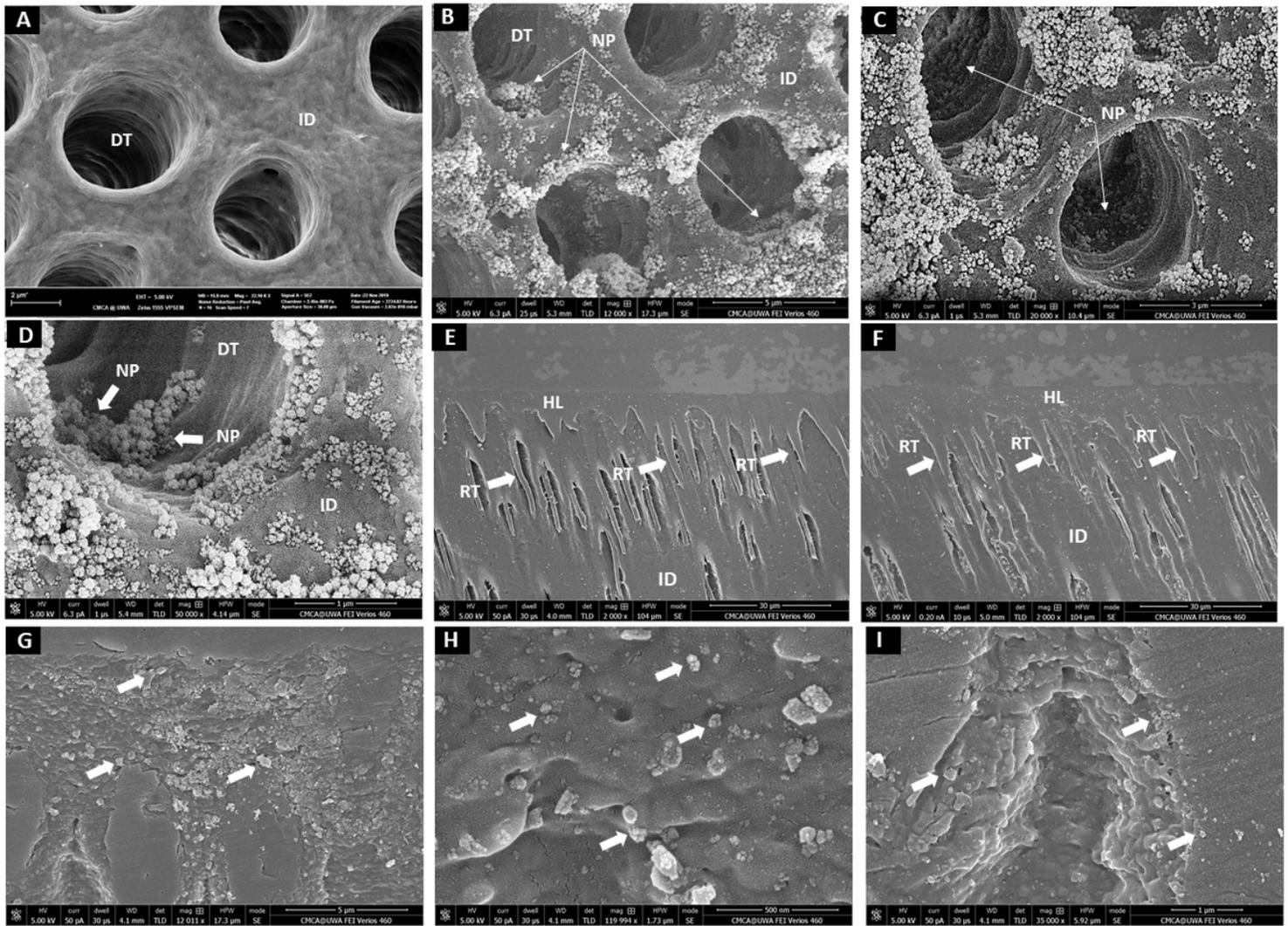


Figure 7

(A) Representative SEM image showing the widely open dentinal tubules after acid-demineralization. (B-D) Selected SEM images showing the ability of the CHX-loaded/MSN-PLGA nanoparticles (NP) delivered through the aqueous-carrier to penetrate and infiltrate inside the dentinal tubules. The nanoparticles appear to be evenly distributed, confined in dentinal tubule structure and attached to tubular wall after the air-blown and blot-drying steps. The nanoparticles are also seen to penetrate considerable depths within the dentinal tubules. (E) Representative SEM image showing the overall homogenous intact resin-dentine hybrid layer (HL) along with well-developed resin tags of the 50:50:50 CHX-loaded/MSN-PLGA and (F) 50:50 CHX-MSN nanoparticles treated dentin specimens after the application of the commercially available dentine bonding system (Scotchbond™ Universal adhesive). DT: dentinal tubules, ID: intertubular dentine, NP: nanoparticles, RT: resin tags. (G-H) Representative high magnification SEM image showing the hybrid layer with widely distributed CHX-loaded/MSN-PLGA nanoparticles (white arrowheads) and exhibiting excellent bonding. Note some areas show tight agglomerations of the nanoparticles. A higher magnification area between two resin tags showing well-penetrated nanoparticles within the deep dentinal space.

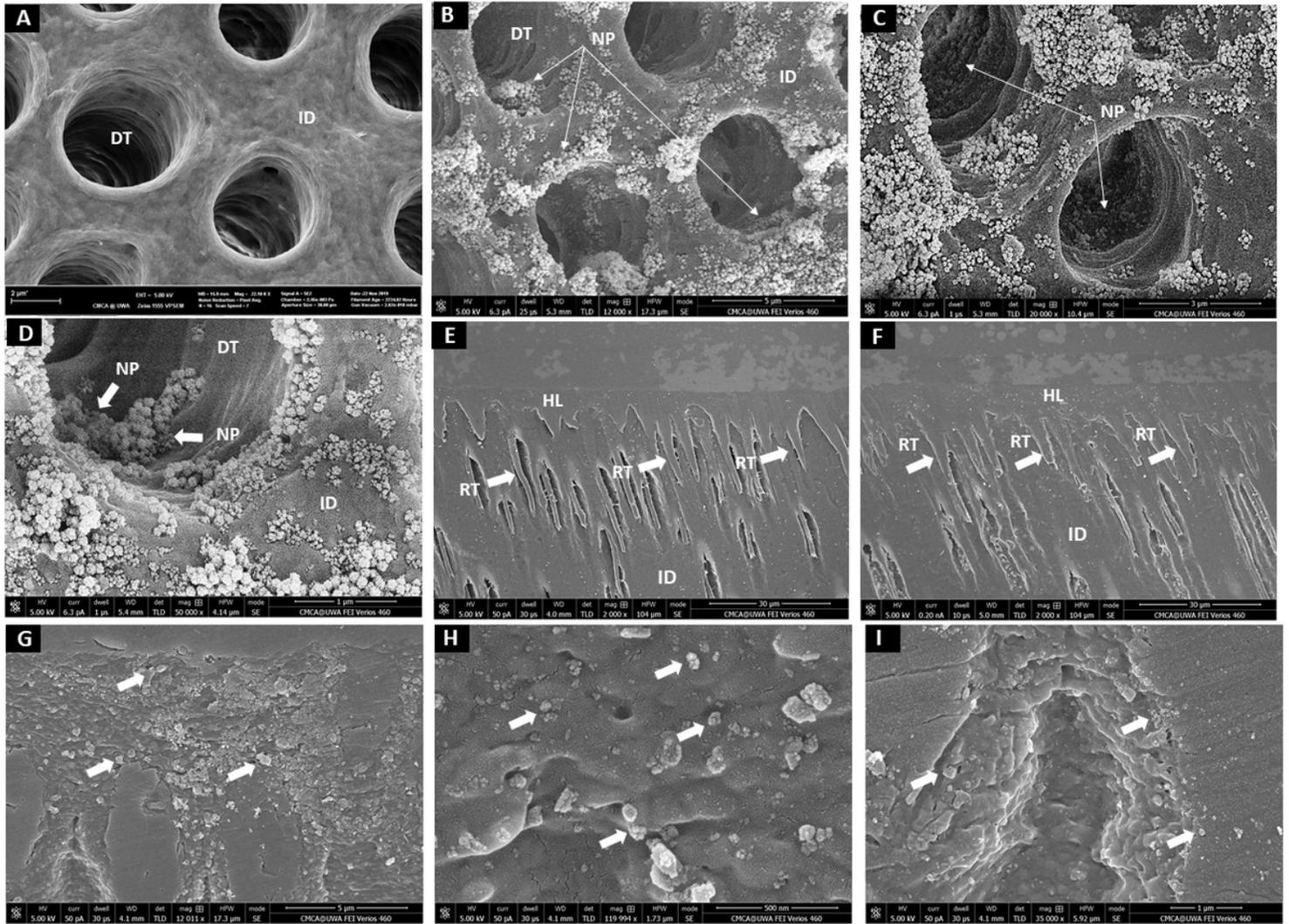


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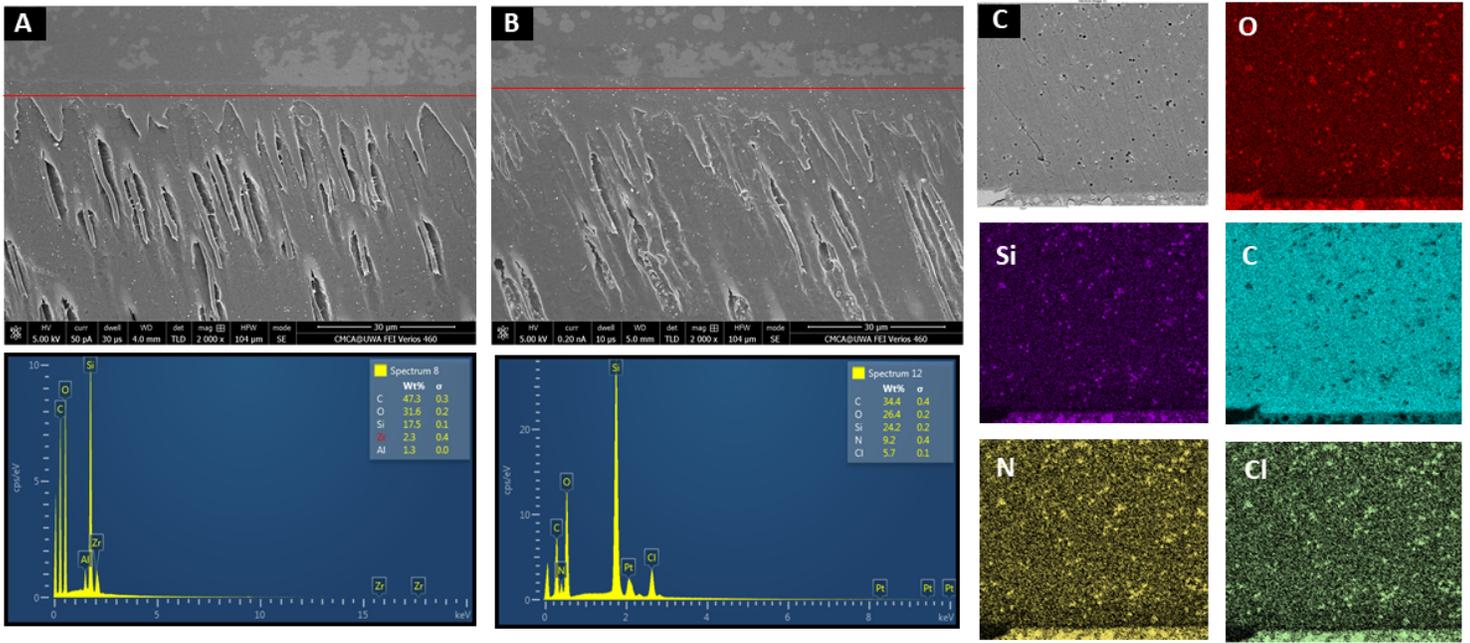


Figure 8

(A-B) Representative SEM images of resin-dentin interface bonded using MSN-PLGA/Blank and 50:50:50 CHX-loaded/MSN-PLGA with their respective line EDX analyses exhibiting high percentage of amorphous carbon and silica that represents MSN-PLGA with no evidence of the drug CHX. The presence of nitrogen and chlorine depicts the presence of CHX along the line of resin-dentin interface. (C) EDX mapping of resin matrix within hybrid layer from 50:50:50 CHX-loaded/MSN-PLGA showing nanoparticles distribution and the presence of carbon (C) and silica (Si) indicating for MSN and PLGA within the resin matrix and distribution of nitrogen (N) and chlorine (Cl) as an evidence for CHX.

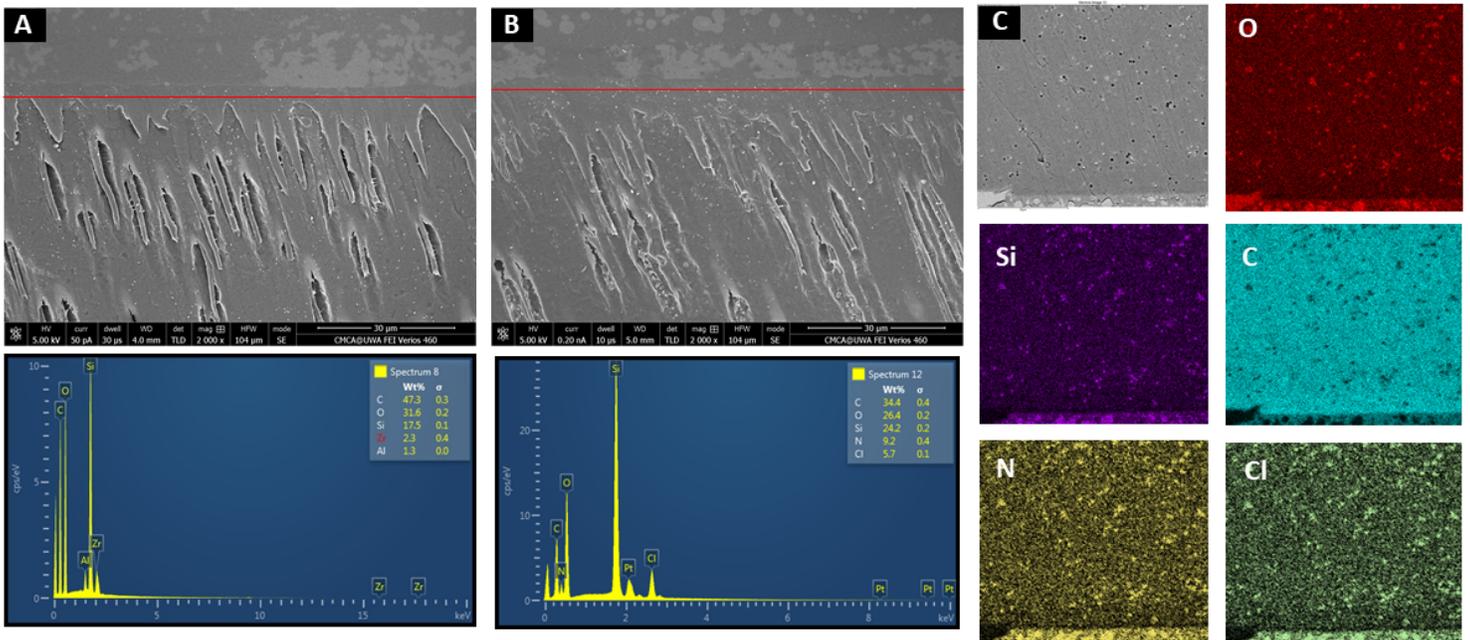


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