A bioinspired bioactive multi-component polymerizable material for dental restorative applications; effects of calcium content on physico mechanical properties

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Abstract

Novel inorganic-organic hybrid reisns [IOHRs] containing mixture of alkoxides of calcium/magnesium/zinc with polymerizable dimethacrylate groups were synthesised using a simple single-pot modified sol-gel method. Objective of the present study is to investigate the impact of calcium content over bioactivity, polymerization shrinkage, and physico mechanical properties of bioactive IOHR containing mixture of alkoxides of calcium/magnesium/zinc having polymerizable methacrylate groups, along with various fillers they can be used as a dental restorative material. Various formulations with varying concentration of inorganic content were used during the synthesis. The concentration of inorganic content was optimised as 0.1% by weight of with respect to the silane. This optimised formulation based photocured composite [CMZ2] showed better DTS (35-40 MPa), FS (65-70 MPa), VHN (140-146 kg/mm^2), possess low shrinkage, non-cytotoxic in nature, bioactive with good cell adhesion and cell proliferation properties. CMZ2 was found to be a potential novel dental composite.

Keywords: IOHRs, single-pot modified sol-gel, dental composite, polymerization shrinkage, bioactivity.

Introduction

Unlike bone and other biomineralised tissues, enamel cannot be renewed via biological processes once degraded [**1**] due to the acidity in the mouth. Enamel predominantly consists of apatite crystals containing calcium and phosphate [**2**]. Research is going on to develop contemporary biomaterials to relieve from pain, dental caries, contagion and tooth loss. Damaged teeth are now commonly restored or replaced by synthetic materials. Strength and toughness of natural structural materials are challenging to mimic synthetically [**3-4**]. However attempts were taken to develop synthetic structural materials similar to natural one, by the use of structural and mechanical design principles to get a suitable substitute to some extent.

Photocured composites based on poly dimethacrylate resin reinforced with silane coated x particles were extensively used as dental restorative materials [**5-6**]. Some of the commercially available dimethacrylate resisns for the preparation of visible light cured composites were Bis GMA (bisphenol A glycidyl methacrylate), urethane dimethacrylate (UDMA), combination of Bis GMA and UDMA etc. [**7**]. Bis-GMA based visible light cure composite materials are vital in dentistry due to their reported aesthetic quality and good mechanical properties. The viscous Bis GMA is usually blended with triethyleneglycol dimethacrylate (TEGDMA, dimethacylate resin with lower molecular weight) for the clinical purpose. TEGDMA is a good diluent but it enhances polymerization shrinkage in the visibly light cured composites. Ineffective bonding with the inorganic filler and organic matrix is one of the main causes for polymerization shrinkage. Leaching out of organic monomer due to low monomer conversion results in toxicity of Bis GMA based material. Polymerization shrinkage, marginal leakage and non-bioactivity beset with Bis GMA limit its application and switch the dental research to look forward for other monomers. Among the numerous efforts made on improving performance of the dental restoratives, by introducing novel monomer, fillers, dental cements the most significant developments of the dental research was focused on filler part. Most of the studies reported that polymerization shrinkage combined with cyclic mechanical loading lead to interface failure between the prepared tooth cavity and the composite leads to marginal gaps which can serve as suitable anchorage sites for bacterial colonization [**8**]. Possible approaches

for increasing the longevity of restorations is to reduce polymerization shrinkage and to promote remineralization of tooth structure [**9**].

Previous studies [**5**] reported that use of calcium containing IOHRs has the ability to enhance surface hardness by diminishing polymerization shrinkage in dental restoratives. Even though the material possesses low shrinkage, better mechanical properties and found to be non-cytotoxic, it lack bioactivity, cell adhesion and cell proliferation properties. The exploitation of bioactivity of resin is not been yet reported. The objective of the present study is to synthesize novel bioactive IOHR containing mixture of alkoxides of calcium/magnesium/ zinc with polymerisable dimethacrylate groups through a patented [**10**] modified sol gel single pot two-step process. Calcium containing composites are reported to have osteoconductive properties similar to native bone apatite minerals. Wang et al reported that prealinged Ca^{2+} ions commence the nucleation and growth of Hydroxy apatite (HAP) in well aligned manner [**11**]. Few studies proved that combination of magnesium with calcium phosphate nanoparticles in HAP was found to enhance bone forming potential [**12-14**]. Magnesium along with carbonated substituted calcium hydroxyapatite crystals purvey the hardness of healthy tissue in dentin. [**15**]. Recent evidence indicates soft enamel formed as a result of magnesium deficiency which lack sufficient resistance to acid decay. The difference in the alignment of hydroxyapatite microcrystals in the tooth enamel is responsible for the resistance of teeth to caries which is highly specific to individuals [**16**]. Zinc with other metal ions reduces enamel solubility and can modify crystal-growth of the calcium phosphates which results in remineralization [**17-18**]. The previous literature [**12-18**] showed the importance of calcium, magnesium and zinc for the proper functioning and remineralization of tooth. This inspired us to incorporate mixture of alkoxides of calcium, magnesium and zinc through chemical reactions to the resin part which contains polymerizable methacrylate groups and can undergo in situ polymerization. Our previous studies showed that incorporation of alkoxides of calcium, magnesium and zinc enhanced thermal stability and antimicrobial properties to the novel IOHRs [**19**].

Here we prepared dental composites based on the novel IOHRs containing mixture of alkoxides of calcium/magnesiu/zinc having polymerizable di-methacrylate groups and evaluated its physico-mechanical properties comprising diametral tensile strength (DTS), flexural strength (FS), polymerization shrinkage and surface hardness (VHN), bioactivity, in vitro cytotoxicity and cell adhesion properties.

Experimental

Materials

The precursor 3-trimethoxy silyl propyl methcarylate for the synthesis of resin and triethylene glycol dimethacrylate (TEGDMA) used were of Aldrich Chem. Co. Milwaukee, LR grade calcium hydroxide, magnesium chloride, and zinc acetate, AR grade sodium hydroxide, LR grade diethyl ether were of S.D. Fine Chemicals, Mumbai, India. Diphenyl (2, 4, 6-trimethylbenzoyl) phosphine oxide (TPO), 2-hydroxy-4-methoxybenzophenone, 4-(dimethylamino) phenethyl alcohol, 4-methoxy phenol, phenyl salicylate, 2,6 di-tert-butyl-4-methyl phenol were purchased from Sigma-Aldrich, US.

Methods

Synthesis of resin

The inorganic – organic hybrid resins with polymerizable dimethacrylate groups were synthesized as per the patented procedure [**10**] modified sol-gel technique. Resins containing mixtures of alkoxides of calcium /magnesium/zinc were synthesized by using 3-trimethoxy silyl propyl methacrylate as the precursor. Due to proprietary reason formulation and detailed procedure of the synthesis of resin cannot be revealed at this stage. Calcium hydroxide/zinc acetate/magnesium chloride was incorporated in the precursor and the mixture was stirred for 8h. The synthesized resins were extracted with diethyl ether, washed with distilled water and dried. After drying 200ppm 4-methoxy phenol was added to avoid self-polymerisation. Different concentrations of inorganic contents were incorporated into the resin according to the weight percentage of the silane precursor (0-0.3%) in presence of diethyl ether. The feeding ratios of magnesium chloride and zinc acetate were same for all the formulations except for CMZ0. Various formulations with varying inorganic content were used during synthesis for the optimisation of resin (**Table 1)**.

Table 1. Formulations of varying concentrations of inorganic contents used for the synthesis of IOHRs.

S.No	Concentration of inorganic contents	Sample code	Feeding ratio of $Ca(OH)_2$	Molar concentration of calcium content (milli moles)
1	Ca0Mg0Zn0	CMZ0	0%	0
2	Ca0Mg0.1Zn0.1	CMZ1	0%	0
3	Ca0.1Mg0.1Zn0.1	CMZ2	0.1%	0.134
4	Ca0.2Mg0.1Zn0.1	CMZ3	0.2%	0.268
5	Ca0.3Mg0.1Zn0.1	CMZ4	0.3%	0.402

Characterisation of synthesized resins

FTIR spectroscopic technique was used for the characterization of the synthesized resins. Fourier Transform Infrared Spectrometer (JASCO 6300 FTIR Model, Japan) was used for recording the spectra by NaCl method.

Development of VLCs

The visible light cure dental composites were prepared through a patented procedure [**20**]. Due to proprietary reason, formulation and detailed procedure of the development of VLCs cannot be revealed at this stage. The uniform composite paste was obtained by masticating resin matrix, filler, diluents, photoinitiator, accelerator and UV absorbers in an agate mortar. The paste was

packed into the mold and exposed to visible light (Prolite Caulk/Dentsply, 300mw/cm^2) for duration of 60s on both sides.

Diametral tensile strength (DTS)

The specimens prepared for DTS evaluation were done as per ADA specification [**21**]. 6mm diameter x 3mm depth stainless steel mold was used for packing the composite paste and each side was exposed to visible light for one minute [**22**]. DTS was determined using the Equation (1)

$$DTS\ (MPa) = 2P/\pi DL \quad (1)$$

where, P the load in Newtons, D the diameter and L the thickness of the specimen in mm.

Polymerisation shrinkage

Sample preparation is same as that for DTS as per ADA specification [**21, 22**]. The percentage shrinkage can be calculated from the Equation (2) given below.

$$\text{Polymerization shrinkage} = (\text{diameter of ring-diameter of composite/diameter of ring}) \times 100 \quad (2)$$

Vickers hardness number (VHN)

VLCs prepared for the evaluation of Vickers hardness number (VHN) as per ADA specification [**21**]. Vickers Micro hardness tester (Model HMV 2, Shimadzu, Japan) was used for measuring VHN and calculated from the following equation (3).

$$Hv = 0.1891\ F/d^2 \quad (3)$$

where, Hv the hardness number, F the test load in Newtons and d the mean length of the indention diagonal length in mm.

Flexural strength (FS)

FS test specimens were prepared as per ISO specification No. 4049-2009(E) [**23**] and determined using the UTM with a crosshead spead of 1 mm/min. The flexural strength was determined using the equation (4).

$$FS = 3FL/2bd^2 \quad (4)$$

where, F the load at break in Newtons, L the length of the specimen between two metal rods at the base plate in mm, b the width of the specimen in mm, d the depth of the specimen in mm

Depth of cure

Depth of cure of the specimens was measures as per ISO specification No. 4049-200 (E) [**23**].

Bioactivity analysis

Bioactivity of CMZ2 and Bis GMA based VLCs were analysed as per ISO 23317:2012 [**24**]. Surface morphology of the composite samples was studied using JOEL-JSM-6390 Scanning Electron Microscope equipped with Oxford Swift EDS. The photo cured samples were stored in Simulated Body Fluid (SBF) for a period of 0, 1, 7 and 14 days at 37°C. After the time period, bioactivity of samples was evaluated by taking Scanning Electron Microscopic (SEM) images. A magnification of X 10,000 was used in this study.

In vitro cytotoxicity test

In vitro cytotoxicity test was performed as per ISO 10993-5 [**25**] using L929 mouse fibroblast cells. Ultra High Molecular weight Poly Ethylene was used as negative control and stabilised PVC Disc was used as positive control. Test samples were sterilized by steam at 121° C for 20 minutes. Test samples, negative controls and positive control in triplicate were placed on the cells. After incubation at 37±1 °C for 24 to 26 hours, cell monolayer was examined microscopically for the response around the test samples.

In vitro MTT assay

An *in vitro* cytotoxicity testing using direct contact method was performed on two sets, each having six samples of the test material as per ISO 10993-5. The cell culture medium for the L-929 monolaer was replaced with fresh medium. The cell culture medium for the L-929 monolayer was replaced with fresh medium. Ultra High Molecular weight Poly Ethylene was used as negative control and stabilised PVC Disc was used as positive control. Test samples were sterilized by steam at 121°C for 20 minutes. Test samples, negative controls and positive control in triplicate were placed on the cells. MTT assay was done at the end of test procedure to measure the metabolic activity of cells to reduce yellow colored tetrazolium salt 3-(4, 5-Dimethytl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide to purple colored formation. At the end of direct contact test for 24 hours, the test samples and controls were removed and the culture medium was replaced with 400 µl MTT solution (1mg/ml in medium without supplements, wrapped with aluminium foil) and was incubated at 37±1 °C for 2 hour. After discarding the MTT solution 800 µl of isopropanol was added to all wells and swayed the plates. The color developed was quantified by measuring absorbance at 570 nm using a spectrophotometer. The data obtained for test sample were compared with negative control.

In vitro cell adhesion study

An *in-vitro* cell adhesion study was performed using L929 mouse fibro blast cells in Minimal Essential supplemented with 10% Foetal bovine serum. L929 cells were sub cultured and seeded on test materials and control glass cover slip at density of 1x10^{4}cells/cm^2 and incubated for 48 hours at 37±1 °C under humidified atmosphere containing 5% CO_2. After 48 hours cell seeded test material and glass cover slips were fixed in 4 % paraformaldehyde for 48 hours. The samples were rinsed thrice with 0.1M phosphate buffered saline followed by permeabilisation with 0.1 % Triton X-100 in PBS for 1 minute. The samples were rinsed 3 times with PBS and treated with Rhodamine Phallodin (1:100) for 15

minutes. The samples and controls were observed under fluorescence microscope Leica N2.1 filter cubeDMI6000, (ExBp515-560) EmiLP590 and Scanning Electron Microscopy (SEM, Hitachi S2400 Japan).

Statistical analysis

Statistical evaluation was done by means of one-way analysis of variance (ANOVA). $p < 0.05$ was considered as significant.

Results and discussion

FT-IR spectrum of precursor (TSPM) was compared with the synthesized reins (**Fig. 1a**). The three spectra displayed the characteristic peaks for –CH3 stretching, C=O stretching, C=C stretching and Si-O stretching at 2927 cm^{-1}, 1716 cm^{-1}, 1640 cm^{-1} and 1013 cm^{-1} respectively. One peak detected at 983 cm^{-1} is attributable to Si-O symmetric stretching [**26**]. An intense peak for –OH around 3550 cm^{-1} in bare resin as well as in CMZ2 confirmed that the precursor (3-trimethoxy silyl propyl methacrylate) endured hydrolysis (**Fig. 1 (a)**). The free Si-OH groups are expected to enhance bioactivity of the resin which was further confirmed from bioactivity studies [**27**]. **Fig. 1(b)** showed the schematic representation of the synthesized IOHR.

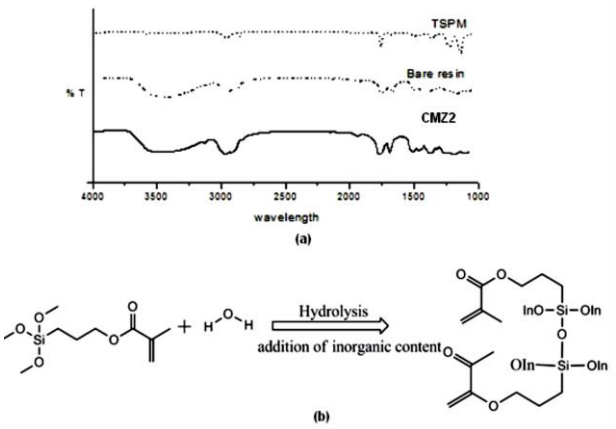

Fig.1. (a) Overlay of FTIR spectra of synthesized resins (b) Schematic representation for the synthesis of IOHRs.

To investigate the effect of inorganic content of newly synthesized inorganic-organic hybrid reisns on physic-mechanical properties comprising of DTS, FS, VHN, depth of cure and polymerisation shrinkage of VLCs based on these resins were measured and the results were shown in **Figs. 2-3**. The effect of calcium ion concentration on physico-mechanical properties of VLCs were investigated by changing the concentration of calcium content from 0 to 0.3 % in the synthesized resins. The mechanical properties of the resin composite should be strong enough for its enduring clinical application in dental restorations. Flexural strength of the composite designates the quantity of flaws within the material which are prospective to cause catastrophic failure when subjected to loading [**28**]. Another material property for characterizing dental composite is diametral tensile strength, because low tensile strength leads to early let-down the performance of the materials [**29**]. FS and VHN of VLCs substantially increased from CMZ0 to CMZ2 ($p < 0.05$) and further decreases to CMZ4 ($p < 0.05$) **(Fig. 2-3)**. DTS values of CMZ2 resin based VLCs was significantly high ($p < 0.05$) compared to CMZ0, CMZ1, CMZ3 and CMZ4 resin based VLCs [**Fig. 2**]. During the synthesis of IOHRs, the combination of the organic and the inorganic components occurred at nanoscopic or molecular scale. [**30**]. Out of the five formulations of VLCs , CMZ2 had the highest FS, DTS and VHN, which may be due to better integrated interfaces between the quartz filler and resin matrix (**Fig. 2**), compared to others.

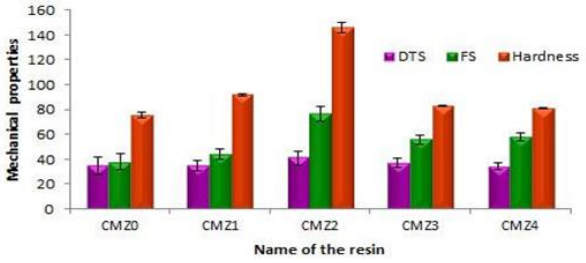

Fig. 2. DTS, FS and hardness of VLCs prepared from IOHRs containing mixture of alkoxides of calcium/magnesium/zinc: effect of variation of calcium content from 0 to 0.3%.

Fig. 3 illustrates that the polymerisation shrinkage of all synthesized resin based VLCs was significantly lower than the reported polymerisation shrinkage value of Bis GMA [31]. The polymerisation shrinkage of VLCs based on CMZ2 is significantly low compared to CMZ0, CMZ1 and CMZ4 resin based VLCs ($p < 0.05$). The depth of cure of CMZ2 based VLCs was 1.6 mm which satisfy the value recommended as per ISO 4049. In IOHRs, alkoxysilyl undergo hydrolysis and polycondensation reaction to form Si-O-Si network followed by organic polymerization [**32**]. These reactions can reduce polymerization stress in IOHR based VLCs compared to conventional [**33-34**].

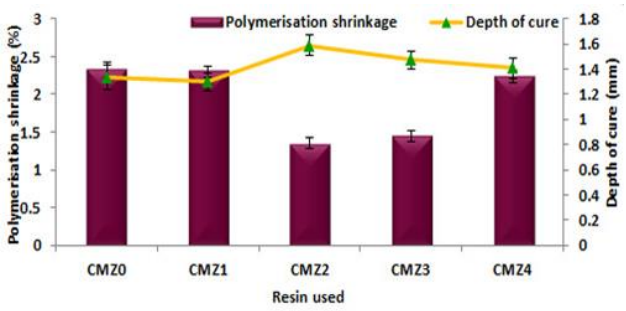

Fig. 3. Polymerisation shrinkage and depth of cure of composites prepared from from IOHRs containing mixture of alkoxides of calcium/magnesium/zinc: effect of variation of calcium content from 0 to 0.3%.

Tagtekin *et al.* reported that ormocer matrix formulation [**35**] (inorganic-organic hybrid matrix) improved microhardness compared to other methacrylate VLCs. Surface hardness is an indirect measurement of monomer conversion [**36**]. So it can be inferred that the IOHR based composites have good monomer conversion and effective bonding between the resin matrix and

inorganic filler. Multifunctional monomers have effective bonding with the filler which in turn reduces polymerisation shrinkage during photopolymerization. It may be the reason for low polymerisation shrinkage and high depth of cure (**Fig. 3**) in these systems. In IOHRs, the covalent bond connects the inorganic and organic components and they interact at the nanoscale during the sol-gel process. Calcium atom can easily dissociate in to a strongly electropositive Ca^{2+} ion. The bond so formed by Ca^{2+} is purely electrostatic so it can excellently crosslinked with biopolymer that have oxygen ligands Ca^{2+} ion can accommodated 8 oxygen ligands in square antiprism configuration with a Ca-O bond distance of 2.4 Å and O-O distance of 2.77 Å. It was reported by Hevish et al., in 1982 that as the concentration of calcium increases the co-ordination number decreases. Thus after the optimum concentration of 0.1% of $Ca(OH)_2$, the co-ordination number may decreases as a result stability of the system may reduce which leads to the deterioration in properties.

From the study (**Fig. 2-3**), it can be observed that the inorganic content has a direct influence in imparting better properties to the VLCs. Better performance obtained for CMZ2 based VLCs is may be due to the availability of multifunctional groups. The synergetic effect of the inorganic content within the resin of this particular composition (0.1% calcium chloride, 0.1% magnesium, 0.1% zinc acetate) supplements better properties for the composites. The absence of reaction site and lack of synergism between the alkoxides incorporated within the resin hinder the performance of other formulations. The addition of inorganic content after 0.1% (CMZ2) may disrupt the synergistic effect in the resin and the addendum can act as an impurity which will deteriorate the properties of the VLCs.

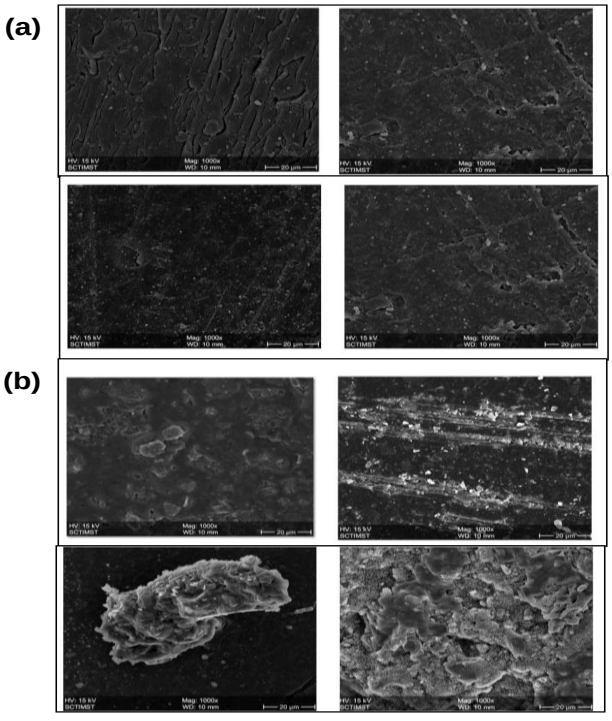

Fig. 4. SEM images of remineralisation ability of (a) Control Bis GMA and (b) Test CMZ2 based VLCs after storing in SBF for 0, 1, 7 and 14 day.

CMZ2 based visible light cured composites with better properties were selected for bioactivity and in vitro studies. **Fig. 4 (b)** demonstrated gradual apatite formation on photo cured samples based on CMZ2 hybrid resin from 0 to 14 days in SBF. No indication of apatite formation was shown by visible light cured composites based on Bis GMA (**Fig. 4 (a)**). It evidenced that the IOHR enhances bioactivity and inorganic content with in the resin strongly influence the structural variation of these resins. The bioactivity of the composites containing non-bioactive quartz point out that novel IOHRs imparts bioactivity to the composite. The stages of apatite formation from SBF at 37°C on CMZ2 VLC (**Fig. 4b**) were in a comparable manner as reported earlier [**2**]. The formation of apatite crystals from SBF proceeds through a multistage process (**Fig. 4b**). On CMZ2 VLC's surface, small agglomerates were observed after 1 day in SBF, while larger agglomerates were formed after 7 days (**Fig. 4b**). The sample had a surface layer of apatite after 14 days. Apatite formation on CMZ2 based VLCs involves the formation and aggregation of prenucleation clusters. But no sign of apatite formation was observed on Bis GMA based VLCs even after 14 days (**Fig. 4a**).

Numerous studies reported that Bis GMA based VLCs were cytotoxic in nature [**37-38**]. A recent study reported the Bis GMA has the ability to induce cytotoxicity and pulpal inflammation [**37-38**]. The test material CMZ2 resin based VLCs was found to be non-cytotoxic to fibroblast cells as per the cytotoxic test conducted (ISO 10993-5) (**Fig. 5 (a)**). MTT assay of test material CMZ2 on day 1 showed 96.5% and on day 3 showed 99.49% metabolic activity respectively. In vitro cell adhesion study showed that L929 cells adhered and spread well on test material and comparable with control cells on glass cover slip (**Fig. 5 (c)**).

So the test material CMZ2 resin based VLCs was found to be non-cytotoxic to fibroblast cells, good cell proliferation and cell adhesion (**Fig. 5**) which indicates that no monomer was leached out from the sample. It yet again confirmed good monomer conversion and effective bonding between the resin matrix and inorganic filler.

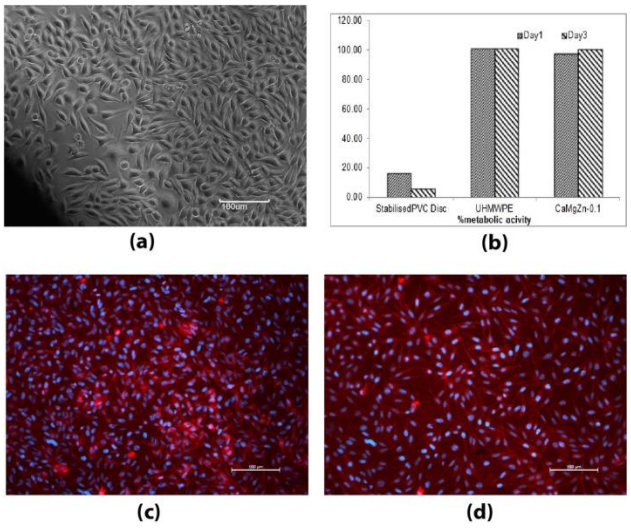

Fig. 5. In vitro studies using CMZ2 resin based visible light cure dental composites (a) in vitro cytotoxicity study, (b) MTT assay, Cell adhesion studies (c) control coverslip and (d) Test material CMZ2.

Conclusion

Novel bioactive IOHRs containing mixture of alkoxides of calcium/magnesium/zinc were synthesized using modified sol-gel method. Visible light cure dental composites with varied concentration of inorganic content in these synthesized resins were fabricated and optimised the concentration of inorganic content in the resin by evaluating its physico-mechanical properties. CMZ2 based VLCs display low polymerisation shrinkage, good hardness, depth of cure, DTS and FS values are as par with the international standards. Thus, CMZ2 based VLCs with optimum physico-mechanical properties was selected for bioactivity studies in comparison with commercially available Bis GMA based composites. The good physico-mechanical properties of CMZ2 based composite are mainly due to effective bonding with the inorganic filler and resin matrix. The synergetic effect of the inorganic content within the resin of this particular composition supplements better properties for the composites. So the bioactivity of CMZ2 based composite was investigated through in vitro studies. These composites are non-cytotoxic with good cell adhesion and cell viability. By integrating the characterization of synthesized resins and its comparison study with the commercially available resin it can be say that, in future these novel resins can solve all the problems of existing materials used for the treatment of damaged tooth. The application of this novel bioactive resin is not limited in the area of dentistry; it can extend to orthopaedic and bioactive coating applications as it contains polymerizable methacrylate groups which can undergo in situ polymerization to get bioactive polymers.

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Author's contributions

Conceived the plan: Lizymol P P; Performed the expeirments: Vibha C; Data analysis: Lizymol P P, Vibha C; Wrote the paper: Lizymol P P, Vibha C. Authors have no competing financial interests.

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