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Multifunctional inorganic-organic hybrid resins with polymerizable methacrylate groups for biomedical applications; effects of synthesis parameters on polymerisation shrinkage and molecular weight

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ABSTRACT

Inorganic-organic hybrid resins revolutionarize the biomedical field by virtue of its versatility. In this work, bioactive inorganic-organic hybrid resins containing mixture of alkoxides of calcium/magnesium/zinc with polymerizable tetramethacrylate groups was synthesized using 1,3-bis methacryloxy 2-(trimethoxy silyl propoxy) propane as the precursor. We optimised the processing parameters by investigating the influence of pH of the medium used for the hydrolysis of silane on molecular weight of the resultant resin obtained. Physico-mechanical properties including polymerisation shrinkage of photocured composites prepared from novel inorganic-organic hybrid resins were evaluated and compared. The resin hydrolysed at pH 2 have low molecular weight with high filler loading capacity (325 phr) than the one hydrolysed at pH 10. Photocured polymeric composite fabricated from resin hydrolysed at pH 2 showed lower polymerisation shrinkage, better depth of cure, good diametral tensile strength, non-cytoxic to L929 fibroblasts with good cell viability and cell adhesion. This new biocompatible polymer with low polymerisation shrinkage stands as a potent candidate in biomedical applications, especially in the field of dental, orthopaedic and coating applications. Copyright © 2016 VBRI Press.

Keywords: Biocompatible polymers; polymer composites; mechanical properties; inorganic-organic hybrid resin; polymerisation shrinkage.

Introduction

Inorganic-organic hybrid resins are generally synthesized through sol-gel method. Inorganic-organic hybrid resins have improved thermal and physico-mechanical properties due to the mixing of organic and inorganic components at molecular/nano level. The hydrolysis and condensation reactions through sol-gel process are influenced by different parameters including pH, temperature, water to alkoxide proportion, type and quantity of catalyst, reactant's concentration, etc. [**1-4**]. The interaction of organic-inorganic sol-gel composites was reported to be either through hydrogen bonding or weaker interaction between organic and inorganic parts [**5**]. The significant application of these resins is in field of coatings, electrochemical devices, biomaterials, etc. [**6**]. Silicones have been employed in various biomedical applications due to their good biocompatibility, elasticity, thermal and oxidative constancy, however it exhibit extreme hydrophobicity [**7**]. Inorganic–organic hybrid resins with silica base are reported to have highly controlled ionic and hydrophilic surface with excellent durability and adhesion onto various substrates [**8**] and found to be promising materials for biomedical applications including biomedical devices and biosensors. Inorganic-organic hybrid resins have application in dental composites, bone cement and denture based materials for its low polymerisation shrinkage compared to the existing resin matrix, since polymerisation shrinkage is a significant concern in various dental and orthopaedic applications [**9**].

The process of composite resin polymerization occurs through the conversion of the monomer molecules into a polymer network, pursued by the contraction of the gap between the molecules which cause polymerization shrinkage in the composite [**9-10**]. Shrinkage is the property of resin matrix and the rate of polymerization of the cured resin was directly influenced by the cross-linking density of the resin. Decline in cross-linking density of the resin will lead to decrease in glass transition temperature, strength and solvent resistance of polymerized resin. If insufficient monomer conversion occurred during polymerization, amount of leachable residual monomer increases. This leachable uncured monomers lead to cytotoxicity of the resin matrix [**11-12**]. Polymerisation shrinkage, biocompatibility and promotion of surface hydrophilicity are the major challenges encountered in the biomedical fields. In dental biomaterial research, recent works targets on the development of bioactive restorations by developing photo polymerized curative resin-based restorative materials, novel dental cements, to promote mineral precipitation in mineral-depleted dental hard tissues at the bonding interface etc. by compromising the physico-mechanical properties [**13-18**].

To the best of our knowledge, development of a bioactive resin which can impart bioactivity in photocured composite is not yet studied. Thus, the development of a bioactive, biocompatible resin with low polymerisation shrinkage and good mechanical properties will be expected to be a boon in biomedical research, particularly in the areas of orthopaedic, dental, and coating applications. Our previous studies reported that use of inorganic–organic hybrid resins containing alkoxides of calcium in dental restoratives can diminish polymerization shrinkage and improve wear resistance [**19**]. Calcium containing composites are reported to have osteoconductive properties similar to native bone apatite minerals. Few studies proved that blend of magnesium with calcium phosphate nanoparticle in Hydroxy apatite (HAP) was found to enhance bone forming ability [**20-22**]. In dentine, magnesium and carbonate substituted calcium hydroxyapatite crystals are consolidated in a crystal lattice, which offers hardness to the healthy tissue [**22, 23**]. It was earlier reported that soft enamel formed as a result of magnesium deficiency which lack resistance to acid decay. Disparity in the arrangement of hydroxyapatite microcrystals in the tooth enamel is responsible for the resistance of teeth to caries which is highly specific to individuals [**24**]. Zinc with other metal ions reduces enamel solubility and can modify crystal-growth of the calcium phosphates which results in remineralization [**25, 26**]. The importance of calcium, magnesium and zinc for the proper functioning and remineralzation of tooth has been reported by several groups [**20-26**]. This inspired us to synthesize novel inorganic-organic hybrid resins containing alkoxides of calcium, magnesium and zinc with polymerizable tetramethacrylate groups. The objective of the present work is to investigate the effect of hydrolysing pH on polymerisation shrinkage and molecular weight by evaluating the physico-mechanical properties of photocured composites based on these synthesized resins.

Experimental

Materials

The dimethacrylate resin 1,3-bis methacryloxy 2-(trimethoxy silyl propoxy) propane, triethylene glycol dimethacrylate (TEGDMA) used were of Aldrich Chem. Co. Milwaukee, Laboratory Rasayan (LR) grade magnesium chloride, calcium hydroxide and zinc acetate, Sodium hydroxide and Hydrochloric acid (Analytical grade), LR grade diethyl ether employed 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 inorganic–organic hybrid resin

Inorganic-organic hybrid resins with polymerizable tetramethacrylate group were synthesized through modified sol-gel technique [**27**], using 1, 3-bis methacryloxy 2-(trimethoxy silyl propoxy) propane as the starting material. 1, 3-bis methacryloxy 2-(trimethoxy silyl propoxy) propane was synthesized by reacting 1.1 moles of glycerol dimethacrylate with 1 mole of chloropropyl trimethoxy silane in presence of benzyl triethyl ammonium chloride catalyst [**28**]. Resins containing mixtures of alkoxides of calcium /magnesium / zinc were synthesized by reacting, 1, 3-bis methacryloxy 2-(trimethoxy silyl propoxy) propane and deionised water in the molar ratio (1:3). The resins were synthesized by the hydrolysis of the silane at two different pH conditions (pH 2 [CMZR2] and pH 10 [CMZR10] and compared. The silane-water mixture was subjected to vigorous stirring for 30 minutes. To the stirred mixture added 1 ml 6N HCl/NaOH followed by the addition of calcium hydroxide, zinc acetate and magnesium chloride (0.1% weight of silane) and kept stirred for 6 hours. The hydrolysed silane was kept at room temperature overnight for post condensation. The product obtained was then extracted with ether, washed with distilled water, till alkali/acid free and dried. To the dried sample about 200 ppm 4-methoxy phenols was added in order to avoid self-polymerisation.

Characterisation of synthesized resins

The synthesized resins were characterised by using Refractive index (RI), Fourier transform infrared spectroscopy (FT-IR) and Gel permeation chromatography (GPC).

Refractive Index (RI): Refractive indices of the resins were measured using an Abbey refractometer (ATAGO 3T, Japan).

Fourier Transform Infrared spectroscopy (FT-IR): FT-IR spectra of CMZR2 and CMZR10 were obtained at room temperature by applying a thin film on NaCl cell with a FT-IR spectrometer (Jasco FT-IR 6300, Japan). The scanned wave number range was 4000 – 600 cm^{-1}, at 4 cm^{-1} of spectral resolution.

Gel Permeation Chromatography (GPC): The molecular weight determination of the synthesized resins was carried out by using GPC (Prominence Gel Permeation Chromatography System, Shimadzu). 20 μL of sample solution in THF was used. Experimental details are as follows:

Flow rate: 1ml/ minute.

Detector: refractive index detector

Preparation of photocured composites

Photocured composites were prepared from CMZR2 and CMZR10 by the previously patented procedure [**29**]. Inorganic-organic hybrid resins [CMZR2/CMZR10] (60 parts) diluted with TEGDMA (40 parts) was used as the resin matrix along with Diphenyl (2, 4,6-trimethylbenzoyl) phosphine oxide (TPO) as the photoinitiator. The filler used for the preparation of the paste consists of 285-325 phr of silanated quartz and 12% fumed silica. During the preparation of paste, 325 phr filler was mixed with CMZR2 resin while 280 phr with CMZR10 resin. Other chemicals used for the preparation of resin mixture were 4(dimethyl amino) phenethyl alcohol, 4-methoxy phenol, phenyl salicylate, 2-hydroxy-4 methoxy benzophenone and 2, 6 di-tert-butyl -4-methyl phenol (which act as inhibitors, activators and UV stabilizers).

The resin matrix, filler, diluent, initiator, accelerator and UV absorbers were masticated in a agate mortar to get a uniform paste which was packed into the mold and exposed to visible light for duration of 60 s on both sides. Prolite (Caulk/ Dentsply) was used to polymerize visible-light-activated materials with an intensity not less than 300 mw/cm^2.

Evaluation of photocured composites

Vickers hardness number (VHN): Photocured samples prepared as per ADA specification [**30**] were used for evaluation of Vickers hardness number (VHN). VHN was measured for each side of the sample using a Vickers Micro hardness Tester (Model HMV 2, Shimadzu, Japan) using a previously reported procedure [**31**]. VHN was calculated using Equation (1):

$$Hv = 0.1891\ F/\ d^2 \qquad (1)$$

where, Hv = hardness number, F = test load (N) and d = mean length of the indentation diagonal length (mm).

Polymerisation shrinkage: 6mm diameter and 3 mm depth stainless steel mold was used for the sample preparation. The internal diameter of the mold was calculated accurately by a digital venier caliper (Mitutoyo, Japan). Six measurements were taken in all directions and the mean value was calculated. The composite paste was packed in the mold and exposed to visible light for one minute on both sides. The samples were then taken out from the mold and the diameter of the cured sample was measured at six points and the mean value was calculated. The percentage shrinkage was calculated using the Equation (2):

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

Diametral Tensile Strength (DTS): Specimens for the measurement of DTS were prepared as per ADA specification [**30**]. The composite paste was packed in stainless steel mold (6 mm diameter and 3 mm depth) and each side is exposed to visible light for one minute. The DTS was determined using the Universal Testing Machine (UTM) (Instron, Model 3363, UK). The load at which break occurs was noted, and DTS was calculated using the following Equation (3):

$$DTS\ (MPa) = 2P/\pi DL \qquad (3)$$

where, P- the load in Newtons, D -the diameter, and L- the thickness of the specimen in mm. Mean and standard deviation of six samples were calculated.

Depth of cure: Depth of cure of the specimens was measured as per ISO specification No. 4049–2000 (E) [**32**]. The composite paste was packed in brass mold (3 mm diameter and 6 mm depth) and exposed to visible light on one side for one minute. The sample was then removed from the mold and the uncured part was removed. The depth of the cured part was measured accurately to the nearest millimeter using the digimatic vernier caliper of accuracy 0.01 mm. Divide the value by 2 to get the depth of cure. Mean and standard deviation were calculated. Statistical analysis was carried out using Anova (Single factor).

Flexural strength: Flexural strength test specimens were prepared as per ISO specification No. 4049-2009(E) [**32**] (25 mm length, 2 mm depth and 2mm thickness). The paste was packed into the mold and exposed to visible light for duration of 4 min on both sides. The flexural strength was determined using the UTM with a crosshead speed of 1 mm/min. The flexural strength was determined using the Equation (4).

$$FS(MPa) = 3FL/2bd2 \qquad (4)$$

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

In vitro cytotoxicity test: *In vitro* cytotoxicity test using direct contact method was performed as per ISO 10993-5 [**33**]. 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 sterilised by steam at 121 °C for 20 minutes. Test samples, negative controls and positive controls 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 test 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 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 sterilised by steam at 121 °C for 20 minutes. Test samples, negative controls and positive controls in six replicate were placed on the cells. MTT assay was performed at the end of the test procedure to measure the metabolic activity of cells to reduce yellow colored tetrazolium salt 3-(4, 5-Dimethyl 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 replace with 400 µl MTT solution (1mg/ml in medium without supplements, wrapped with aluminium foil) and were incubated at 37±1 °C for 2hours. After discarding the MTT solution 800 µl of Isopropanol was added to all wells and swayed the plates. The colour 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: Analysis of variance (ANOVA) for physico-mechanical properties were carried out to identify which data groups were significantly different from others ($p < 0.05$).

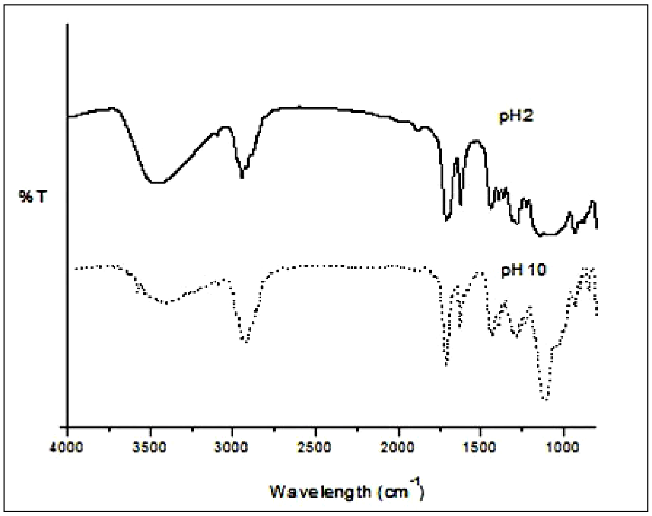

Fig. 1. Overlay of FT-IR spectra of resins synthesized at different pH.

Results and discussion

We triplicate the synthesis of hybrid resins. The reproducibility of chemical synthesis of hybrid resins were checked by using different characterization techniques like Refractive index (RI), Fourier transform infrared spectroscopy (FT-IR) and Gel permeation chromatography (GPC). The same characteristic peaks and values were obtained for all the three sets which confirmed the chemical synthesis of hybrid resins was reproducible.

Fig. 1 showed the overlay of FT-IR spectra of resins synthesized at different pH. Both the spectrum showed strong peaks around 1000-1200 cm^{-1} range indicates the presence of asymmetric stretching vibration of O-Si-O group. Meanwhile the peak around 800-900 cm^{-1} peak showed symmetric stretching vibration of Si-O-Si bond. Characteristics peak of carboxyl group was shown by both spectrum (pH2- 1721 cm^{-1} and pH10- 1724 cm^{-1}). Characteristics peak at 1637 cm^{-1} (pH2), 1636 cm^{-1} (pH10) showed the vibration of $C=CH_2$ group. The peaks around 2800-3000 cm^{-1} corresponds to the presence of C-H stretching (both spectrum). A broad band at 3479 cm^{-1} indicates the presence of Si-OH for resin hydrolysed at lower pH.

Table 1. Molecular weight and refractive indices of resins hydrolysed at pH 2 and pH 10.

Sample code	pH	RI	Mn	Mw	Mn/Mw
CMZR10	pH 10	1.481	2135	2882	1.35
CMZR2	pH 2	1.473	1073	1107	0.9692

The refractive indices of CMZR10, CMZR2 were shown in **Table 1**. The refractive index of quartz is reported to be 1.458 [**34**]. Transmitted amount of visible light was strongly dependent on the magnitude of refractive index difference that existed between the resin and silica filler. The transmitted amount of visible light exhibited a good correlation with the curing depth and hardness of the resin composite. To improve the monomer conversion, it is important to reduce the refractive index difference that exists between the base resin and silica filler. The refractive index difference between CMZR2 and quartz filler is 0.015 which is lower compared to the difference between CMZR10 and quartz filler (0.23). It depicts that CMZR2 based composites is expected to have good monomer conversion and physico-mechanical properties.

Table 1 clearly showed that narrow molecular weight distribution with low polydispersity index of tetramethacrylate resin hydrolysed at lower pH (CMZR2). The synthesis parameters of the hydrolysis of the tetramethacrylate resin at lower pH were in a controlled manner which results the availability of multifunctional monomers compared to the resin hydrolysed at higher pH.

Table 2. Filler content of composites in tetramethacrylate resins hydrolysed at pH 2 and pH 10.

Sample code	pH	Filler content (phr)
CMZR10	pH 10	280
CMZR2	pH 2	325

The higher filler loading capacity of the resin hydrolysed at lower pH (**Table 2**) indicates good wettability for the same. Wettability of resin may be due to more free Si-OH groups in the resin hydrolysed at lower pH. A broad peak around 3479 cm^{-1} in the FT-IR spectra of CMZR2 substantiates the presence of free Si-OH groups in the resin. Multifunctional monomers with low molecular weight are found to have effective bonding with the filler which in turn reduces polymerisation shrinkage during photopolymerization. Intra/inter molecular hydrogen bonding, steric hindrance and decrease in the availability of free Si-OH groups are expected to be the reasons for the high molecular weight of CMZR10 (**Table 1**) compared to CMZR2. The high molecular weight and more viscous nature of CMZR10 is the reason for its low filler loading capacity.

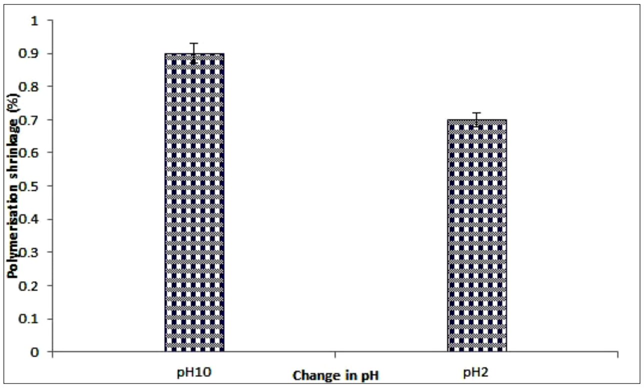

Fig. 2. Effect of pH on polymerisation shrinkage of photocured composites.

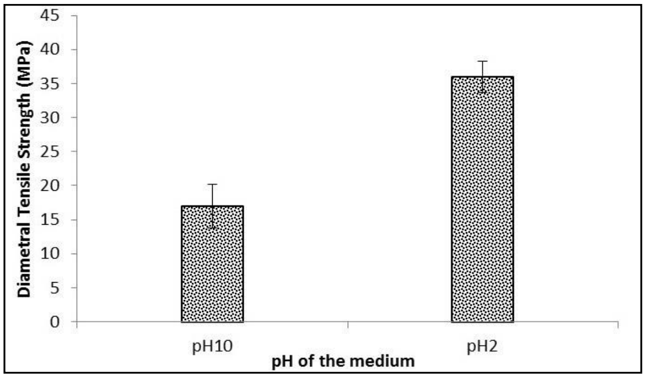

Fig. 3. Effect of pH on diametral tensile strength of photocured composites.

The effect of pH on polymerisation shrinkage and molecular weight of the synthesized resins were studied and evaluated in terms of the physico-mechanical properties of photocured composites prepared from tetramethacrylate inorganic-organic hybrid resins containing alkoxides of calcium/magnesium/zinc [**Fig. 2-5**].

Fig. 2 illustrates the effect of pH on polymerisation shrinkage of photocured composites based on CMZR2 and CMZR10. It shows that the polymerisation shrinkage of CMZR2 based photocured composites was significantly lower ($p=0.08$) compared to photocured composites based on CMZR10. The decrease in polymerisation shrinkage is due to the effective bonding between the quartz filler and the resin matrix.

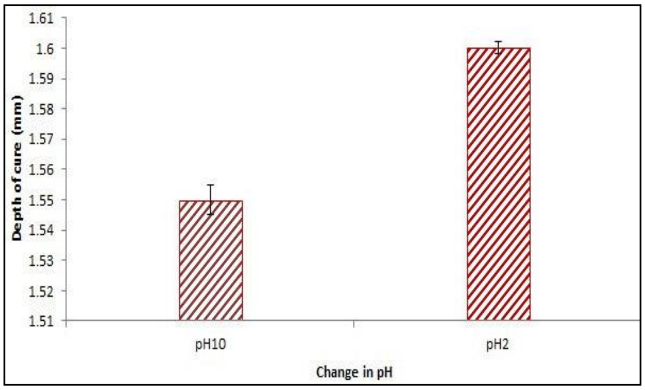

Fig. 4. Effect of pH on depth of cure of photocured composites.

Fig. 3 showed a significant increase ($p < 0.05$) in the DTS values for the composites based on CMZR2. It may be due to the presence of multifunctional monomers in the resin matrix which enables its effective binding with the quartz filler during photopolymerization. The reduction in the difference in refractive index between CMZR2 and quartz filler may enhances polymerisation reaction and diametral tensile strength of the composites. The depth of cure values for photocured composites based on CMZR2 and CMZR10 are summarized in **Fig. 4**. A significant increase ($p < 0.05$) in the curing depth was observed for CMZR2 based composites. The marginal difference in the refractive index between CMZR2 and quartz filler results in the increase in depth of cure of CMZR2 based composites. Usually polymerisation shrinkage increases as the depth of cure increases. Unlike to this usual trend, polymerisation shrinkage of CMZR2 based photocured composites decreases with increase in depth of cure.

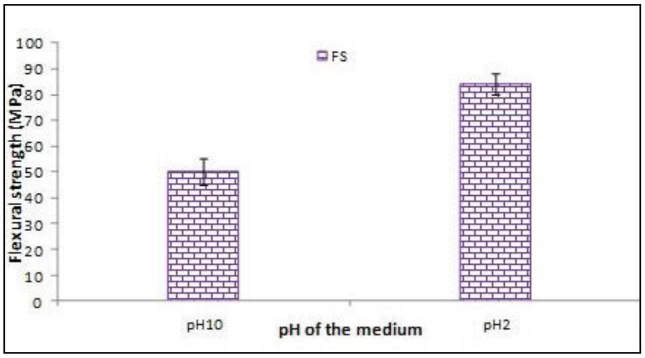

Fig. 5. Effect of pH on flexural strength of photocured composites.

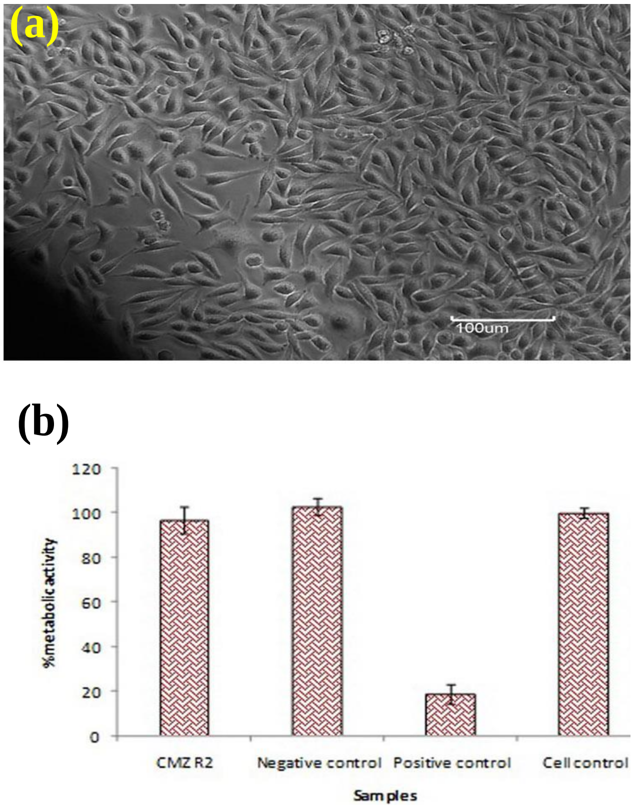

Fig. 6. In vitro studies of CMZR2 based composites (a) in vitro cytotoxicity and (b) cell viability using L929 fibroblasts cells.

The effect of pH on flexural strength of CMZR2 and CMZR10 based photocured composites is illustrated **Fig. 5**. CMZ2 based photocured composites exhibit significantly high ($p < 0.05$) flexural strength compared to the CMZR10 based composites.

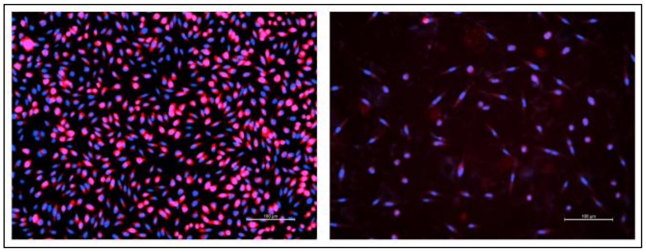

Fig. 7. Microscopic images of CMZR2 based composites on cell adhesion studies (a) control coverslip and (b) test sample.

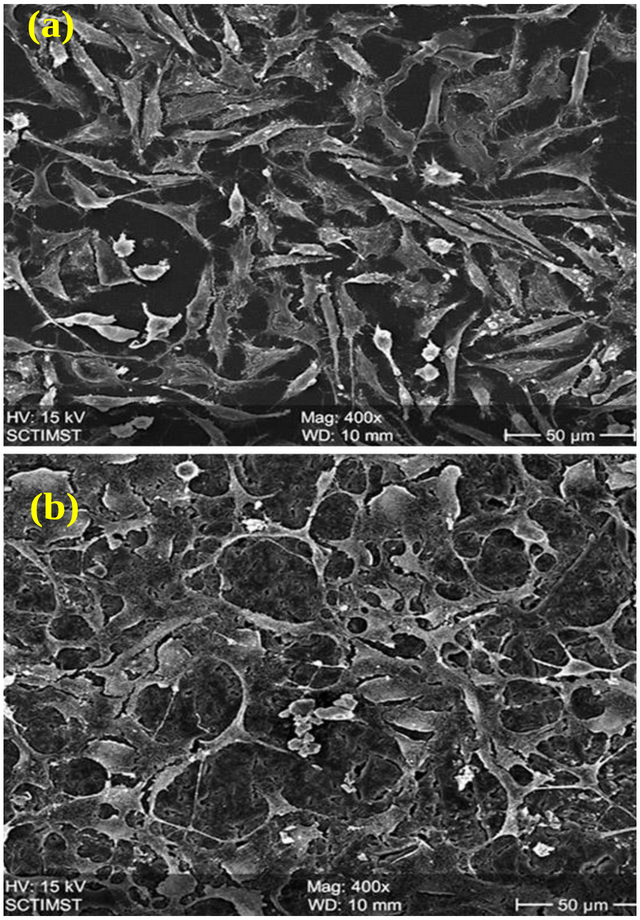

Fig. 8. SEM images of CMZR2 based composites on cell adhesion studies (a) control coverslip and (b) test sample.

The optimised CMZR2 with enhanced properties were selected for *in vitro* analysis including *in vitro* cytotoxicity, MTT assay and cell adhesion studies.

The CMZR2 based composite material showed non-cytotoxic reactivity. The cells around the materials displayed spindle morphology without cell detachment and lysis (**Fig. 6**(**a**)). At the end of direct contact test, possible toxicity of materials exerted on cells were analyzed and determined by MTT assay (**Fig. 6(b)**). Cells exposed to CMZR2 based composites showed 96.4± 3.49 % cell activity compared to control cells. The cells incubated with negative and positive control showed metabolic activity of 101 ± 2.18 and 6.49 ± 5.62 percentages respectively.

Microscopic and SEM images for the cell adhesion of the test sample (CMZR2 based photocured composites) and control coverslip are shown in **Fig. 7, 8** respectively. The cell adhesion studies showed that L929 cells adhered and spread well on test materials and comparable with control cells on glass cover slip [**Fig. 7, 8**]. The non-cytotoxic nature along with good cell viability and cell adhesion properties to L929 cells of the test sample proved that no monomer was leached out from the sample. Good monomer conversion and effective bonding between the resin matrix and inorganic filler is the reason for this biocompatibility of the test sample.

Conclusion

From the above results it can be concluded that hydrolysis of tetramethacrylate resin at lower pH exhibited higher wettability, comparable refractive index with quartz, low molecular weight, narrow molecular weight distribution and free Si-OH groups attributed for its good physico-mechanical properties. The free Si-OH groups in the structure are expected to enhance bioactivity of the resin. The polymerisation reaction was controlled and synthesis parameters were optimized for the resin hydrolysis at lower pH. Photocured composites with optimum physico-mechanical properties were selected for *in-vitro* studies. These composites showed good cell adhesion, cell viability and cell adhesion properties. This novel bioactive resin with good physico-mechanical properties including low polymerisation shrinkage can extend its applications to orthopaedic, dental and bioactive coating applications as it contains polymerizable methacrylate groups which can undergo *in-situ* polymerization to get enhanced bioactive polymers.

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