FUNCTIONALLY GRADED BIOACTIVE COMPOSITES BASED ON POLYVINYL ALCOHOL MADE THROUGH THIOL-ENE CLICK REACTION

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1. MATERIALS AND METHODS

1.1 Synthesis of thiol modified polyvinyl alcohol

Thiol modified polyvinyl alcohol (TPVA) was prepared through acid catalyzed esterification of polyvinyl alcohol and thioglycolic acid (TGA) as reported previously\(^1\). Briefly, 10 g PVA of average molecular weight 98 kDa (Sigma Aldrich) was dissolved in water (5 % m/v) at 80 °C, following which the temperature was lowered to 60°C, and 1.2 mL (13.98 mmol) of HCl and 8.6 mL (123 mmol) of TGA (Sigma Aldrich) were added. The reaction mixture was then stirred for 12 h at 60 °C, after which TPVA was recovered from the solution by precipitating it in excess methanol. The precipitate was washed with methanol several times and dried under vacuum and stored at -20 °C.

1.2 Structural analysis of TPVA

The thiol modification of PVA via esterification reaction with TGA was studied through structural analysis. The samples of PVA and TPVA were subjected to Fourier transform infrared spectroscopy (FTIR), Fourier transform Raman spectroscopy (Raman), proton nuclear magnetic resonance spectroscopy, gel permeation chromatography (GPC) and energy-dispersive X-ray spectroscopy (EDAX) analysis and Ellman assay. FTIR analysis of the PVA and TPVA was done using Thermo-Nicolet 5700 spectrometer in attenuated total internal reflection (ATR) method. The spectra were recorded in the range between 4000 cm\(^{-1}\) - 600 cm\(^{-1}\) at resolution of 4 cm\(^{-1}\). A proton NMR spectrometer (BrukerAdvance DPX-300) was used to record the NMR spectra of PVA and TPVA using deuterated DMSO as solvent. The thiol modification was further analyzed by Raman spectroscopy using confocal Raman microscope (Witec Alpha300R). The samples were used in powdered form and the Raman spectral measurements using frequency doubled Nd:YAG dye laser [output power 40 mW at 532 nm]. The molecular weight of the
TPVA was determined using gel permeation chromatography (GPC) using the Waters GPC system 600 series pump with Waters Ultra hydrogel column.

Free thiol groups immobilized on PVA after thiol modification was determined spectrophotometrically through Ellman assay. Briefly, 2 mM stock Ellman reagent solution [5.5 \textsuperscript{'}-dithiobis (2-nitrobenzoic acid)] (DTNB) (Sigma Aldrich) was prepared in 50 mM sodium acetate (Sigma Aldrich) solution. Tris (Sigma Aldrich) solution of 1 M concentration and pH 8.0 was also prepared. A series of cysteine (Merck) standard solution were prepared starting at 2.5 mM to 1000 mM. 10 μL cysteine solution, 50 μL DTNB solution, 100 μL solutions of Tris, and 860 μL water were mixed to a final volume of 1000 μL. The resulting solution was vortexed and incubated at room temperature for 5 min, and then absorbance was measured at a wavelength of 412 nm. Sample solution was prepared in water at concentrations of 10, 5, 2.5 and 1.5 m/v % of TPVA and 10 μL samples were used in the place of standard and the above procedure was repeated. PVA solution of same concentration was used as blank. Absorbance obtained for standard was plotted against concentration of thiol to obtain the standard curve. Using regression equation, thiol group per gram of the polymer was calculated and based on thiol content, the degree of substitution in the polymer was calculated\textsuperscript{2}. Degree of substitution was further verified by Energy Dispersive Spectroscopy (EDS) using EDAX Genesis XM 4 integrated with the ESEM.

1.3 Synthesis of Hydroxyapatite (HA)

HA was synthesized through a wet chemical precipitation method described in detail elsewhere\textsuperscript{3}. Calcium nitrate tetrahydrate (Ca(NO\textsubscript{3})\textsubscript{4}.4H\textsubscript{2}O) and ammonium dihydrogen orthophosphate (NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4}) in the (Ca/P) molar ratio of 1.67 were made to react at aqueous alkaline condition (pH >10) at 80 °C. The Ca(NO\textsubscript{3})\textsubscript{4}.4H\textsubscript{2}O solution was added drop-wise to NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4} to
precipitate HA. Centrifugation was done to isolate the precipitate and the precipitate was washed several times to remove residual impurities. The final slurry obtained was spray-dried to yield a fine, free-flowing powder of HA with an approximate size of 10 µm.

1.4 Characterization of HA

The in-house synthesized HA was characterized by FTIR and XRD. The finely ground HA was mixed with KBr powder and FTIR spectrum was recorded in diffuse reflectance (drift) mode in the range 4000 cm\(^{-1}\) to 400 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\). The spectrum thus obtained was further converted to absorbance spectrum by performing Kubelka–Munk mathematical transform on the data. The phase analysis of the HA was done using X-ray powder diffraction (XRD) analysis. XRD was done in Bruker D8 Advance X-Ray diffractometer with Cu K\(\alpha\) radiation generated at a voltage of 40 kV and a current 30 mA. The spectra were recorded in the 2θ range of 10–50° at a rate of 4°/min. The diffraction data was then compared with the standard ICDD data to identify the phase of calcium phosphate.

1.5 Preparation of thiol-ene crosslinked gel TPVA gel.

Crosslinked TPVA gel was prepared by mixing TPVA solution with polyethylene glycol diacrylate (PEGDA) (Sigma Aldrich) crosslinker. Briefly to 400 µL 5% (m/v) aqueous solution of TPVA, added 100 µL 6.25 % (v/v) aqueous solution of PEGDA and mixed thoroughly in a 24 well plate. After 1 min, the transparent hydrogels were obtained and were scooped out using a spatula.

1.6 Characterization of the gel

The thiol-ene crosslinking reaction was confirmed by spectroscopic technique. The samples of TPVA, PEGDA and crosslinked polymer were subjected to FTIR and Raman analysis. FTIR analysis of samples in freeze dried form was done using Thermo-Nicolet 5700 spectrometer in
attenuated total internal reflection (ATR) method. The spectra were recorded in the range from 4000 cm$^{-1}$ to 600 cm$^{-1}$ at resolution of 4 cm$^{-1}$. The thiol-ene crosslinking was further analyzed by Raman spectroscopy using confocal Raman microscope (WITec’s alpha300 R). The samples were used in powdered form and the Raman spectral measurements using frequency doubled Nd:YAG dye laser [output power 40 mW at 532 nm].

2. RESULTS AND DISCUSSION

2.1 Structural characterization of thiolated PVA

The FTIR spectrum of thiolated polyvinyl alcohol (TPVA) along with unmodified polyvinyl alcohol is given in figure S1A. The PVA sample gave a broad peak at 3280 cm$^{-1}$ which is produced by the stretching from hydroxyl groups. Peaks at 2940 cm$^{-1}$ and 2908 cm$^{-1}$ on the shoulder of the broad band represents the symmetric and anti-symmetric stretching vibrations of C-H in CH$_2$ part and C-H bending vibrations are observed at 1419 cm$^{-1}$. The peak at 1372 cm$^{-1}$ corresponds to wagging C-H vibrations and peak at 1329 cm$^{-1}$ corresponds to O-H bending vibrations. The peak at 1091 cm$^{-1}$ corresponds to C-O stretching. In TPVA, most of the characteristic vibrations of PVA are present. The notable difference is the appearance of a new peak at 1715 cm$^{-1}$, which due to the C=O stretching vibrations of ester linkage through which the thiol bearing part is immobilized.$^{1,4}$

The Raman spectrum of TPVA along with unmodified PVA is given in figure S1B. In the spectra of PVA a broad spectrum from 3200 cm$^{-1}$ to 3500 cm$^{-1}$ contains the stretching from hydroxyl groups. The peak at 2900 cm$^{-1}$is due to stretching vibrations (symmetric and asymmetric) of CH$_2$. Peaks at 1439 cm$^{-1}$ and 1360 cm$^{-1}$are due to the bending vibrations of CH and -OH. Peak at 1141 cm$^{-1}$ contains C-O stretching and C-C stretching vibrations. Peak at 1088 cm$^{-1}$ contains C-O stretching and -OH bending vibrations and finally 852 cm$^{-1}$ and 915 cm$^{-1}$ is
due to C-C stretching. In TPVA, most of the characteristic vibrations of PVA are present. A new peaks are formed at 1718 cm\(^{-1}\) due to C=O stretching from ester bonds and 470 cm\(^{-1}\) from S-S stretching vibration\(^5,6\). After the reaction followed by purification and storage, the thiol moieties are self-oxidized to form disulfide linkages (S-S), this information is evident from the S-S peak (402 cm\(^{-1}\)) in the Raman spectra of TPVA compared to PVA (figure S1B). We hypothesize that this might be the possible reason why the thiol peak was absent in the Raman spectra. The above spectral data shows the modification of PVA as TPVA and the thiols groups exist in oxidized form (S-S).

![Figure S1](image)

**Figure S1.** The structural characterization data of the TPVA samples. A) FTIR spectrum of polyvinyl alcohol (PVA) and thiolated PVA (TPVA). B) Raman spectrum of PVA and TPVA

The \(^1\)H NMR spectrum of PVA sample represented in figure S2A shows peaks at \(\delta \sim 4.69\) ppm, 4.49 ppm and 4.26 ppm corresponds to the OH group of PVA. A peak at \(\delta \sim 3.84\) ppm is from the proton in CH part, peak at \(\delta \sim 3.40\) ppm and a shoulder at 3.51 ppm corresponds to the H\(_2\)O, a sharp peak at 2.51 ppm is from the residual protons in DMSO solvent. A peak \(\delta \sim 1.99\) ppm is from the methyl proton of residual acetyl part, and peaks at \(\delta \sim 1.44\) ppm, 1.39 ppm and 1.33 ppm are from the protons in CH\(_2\) part. The spectrum of TPVA sample is given in figure S2B. All
the characteristic peaks of polyvinyl alcohol were observed in proton NMR spectra of TPVA along with the addition of some new peaks. A new peak at $\delta = 3.17$ ppm is from the proton present in the carbon adjacent to the thiol group. The S-S oxidation, confirmed from the Raman spectra is the reason for the thiol peak absence in the proton NMR of TPVA which was expected to be formed near 1.5 ppm. Since the proton NMR spectra of TPVA are not free from the peak of OH groups, it can be concluded that the esterification reaction is partial$^{1,4,6}$. The spectral characterization of TPVA polymer via FTIR, Raman and proton NMR confirms the partial substitution of hydroxyl groups by TGA via ester linkages$^{1,4}$.

**Figure S2** Results of the $^1$H NMR analysis. A) Shows the spectrum of Polyvinyl alcohol (PVA), and B) that of the thiolated Polyvinyl alcohol (TPVA)

**Figure S3** Represents molecular weight analysis of PVA and TPVA determined using GPC analysis. PVA showed a number average molecular weight of 90048 Da whereas the TPVA
shows three prominent peaks at 1008558, 107830 and 26466 Da. The 107830 corresponds to the molecular weight of TPVA and the peak at 1008558 is mainly from increase in molecular weight due to the S-S cross-linked TPVA. The presence of this peaks showed that the polymer has undergone disulfide crosslinking via self-oxidation which was also observed in the FT-Raman data in which peak for S-S linkage appeared instead of -SH group. For a thiol-ene reaction to be considered, the number of thiol groups per gram of polymer must be quantified. The thiol content per gram of polymer was calculated colourimetrically by Ellman assay\(^2\). The Ellman’s reagent, commonly known as DTNB, reacts with the free -SH groups to produce a yellow color and the absorbance of this yellow solution is measured spectrophotometrically at 412 nm. The thiol content in TPVA was calculated using the regression equation obtained from the cysteine standard curve (figure S3B). The thiol content per gram of polymer was found to be 1.16 mmol and based on the thiol content calculation, the degree of substitution was found to be 6.2 %. This result was further substantiated with EDAX analysis (figure S3C).
Figure S3 Evidences for thiol substitution. A) GPC curve showing the molecular weight distribution of PVA & TPVA; B) Thiol content estimation from cysteine standard curve; and C) EDAX spectrum of TPVA.

3.2 Characterization of HA

FTIR spectra of HA (figure S4A) showed characteristic -OH groups stretching at 3570 cm\(^{-1}\) and asymmetric C-O stretching in CO\(_3^{2-}\) at 1413 cm\(^{-1}\). The absorption peaks observed at 1099 cm\(^{-1}\) and 1030 cm\(^{-1}\) corresponds to the asymmetric stretching vibrations of the P–O and the peak at 960 cm\(^{-1}\) is assigned to the symmetric stretching mode of the P–O in PO\(_4^{3-}\) groups. The peaks at 605 cm\(^{-1}\) and 563 cm\(^{-1}\) are assigned to the bending vibrations of the O–P–O in PO\(_4^{3-}\) groups. These peaks are very typical of HA. The X-ray diffraction pattern of HA match with the ICDD data of HA (ICDD No.00-009-0432). All the characteristic peaks of HA appeared between the 2θ value of 25 and 50 as shown in figure S4B (marked with bullets).
3.3 Characterization of thiol-ene crosslinked TPVA gel

The formation of crosslinks between thiol and diene during gel formation was confirmed from the FTIR analysis and is shown in figure S5A. In the spectra of PEGDA, a peak at 1635 cm$^{-1}$ corresponds to the C=C and another peak at 1721 cm$^{-1}$ corresponds to the ester C=O. During its crosslinking with TPVA, the C=C will be used up and thus the C=C peak at 1635 cm$^{-1}$ will disappear from the gel and this was evident from the spectra of freeze-dried gel. The crosslinking between PVA thiol and PEGDA during gel formation was confirmed with Raman analysis and is shown in figure S5B. In the spectra of PEGDA cross-linked TPVA, there is small increase in
intensity of C=O stretching at 1718 cm\(^{-1}\) attributed to the formation of the ester bond with PEGDA. Furthermore, the peak at 470 cm\(^{-1}\) is completely absent demonstrating complete utilization of thiol groups by the dialkene for the crosslinking process and that there might not be any residual -SH groups available for oxidation to disulfide bonds. The TPVA synthesis along with the mechanism of crosslinking that results in gel formation is shown as figure S6.

![Figure S5](image)

**Figure S5** Results of Characterization of thiol-ene crosslinked TPVA gel. A) FTIR spectra of PEGDA, TPVA & TPVA-PEGDA cross-linked gel; B) FT-Raman spectra of TPVA and TPVA crosslinked gel.
Figure S6 Reaction scheme of TPVA preparation and mechanism of crosslinking.

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