

## Chapter 3

# Extracellular-Matrix-Mimicking Peptide Hydrogel as a Cytocompatible Biomaterial Platform

### 3.1 ABSTRACT

Self-Assembled short peptide-based hydrogel platform has become widely applicable biomedical therapeutic maneuver for their soft, tunable architecture which can influence cellular behavior and morphology to an inordinate extent. Here in this piece of work, a short supramolecular hydrogelator peptide has been designed and synthesized using a fusion approach: from the C-terminus conserved “FFGLM” section of biologically abundant neuropeptide: Substance P. In addition to this to incorporate a good hydrophobic-hydrophilic balance, the truncated pentapeptide segment was further C-terminally modified by the incorporation of an integrin-binding “RGD” motif to it. This octapeptide ensemble “FFGLMRGD” by virtue of its N-terminus Fmoc group undergoes rapid self-assembly to give rise to an injectable, pH responsive, hydrogel-based self-supporting platform which exhibited good cytocompatibility to the cultured mammalian cells both in 2D as well as 3D culture condition without exerting any potent cytotoxic effect in LIVE/DEAD experiment. The rheological experiment demonstrated its hydrogel-like mechanical properties including thixotropicity. The atomic force microscopic as well as FESEM image of the fabricated hydrogel shows a tangled fibrous surface topography owing to the presence of N-terminus Fmoc-FF residue within the sequence of the designed peptide. Further *in vitro* scratch assay performed on fibroblast cell lines confirmed the wound-ameliorating potency of this designed hydrogel which substantiates its future therapeutic prospects.

### 3.2 INTRODUCTION

Molecular self-assembly is a spontaneous physiochemical process to develop novel biomaterials with diversified biomedical applicability.<sup>1-6</sup> It's nature's own “Lego bricks” to fabricate custom-made biological structures by mere utilization of simple building blocks with characteristic functional role.<sup>7,8</sup> Deciphering the self-assembling pathways rendering the formation of higher-order supramolecular structures will impart a better perception of physiologically relevant macromolecules. In this milieu chief objective of peptide, nanotechnology is to find shorter peptide sequences to achieve a comparable functional effectivity of biologically pivotal peptide-protein based macromolecules.<sup>9-12</sup> Such a bottom-up design approach paves an effective blueprint to construct an extracellular matrix mimicking cytocompatible biomaterials which can be further implemented as an applicative material for tissue engineering as well as therapeutic purpose.<sup>13-17</sup>

In this context, N-terminus Aromatic capping groups bestow an important minimal structural functionalization to introduce self-assembling properties in short peptide motifs.<sup>18-20</sup> Effectual  $\pi$ - $\pi$  interactions between juxtaposed aromatic capping groups facilitate the self-assembly of shorter peptide sequences.<sup>21</sup> Both ionic as well as hydrophobic modulation of the designed peptide sequence is crucial for the induction of the hydrogelation through self-assembly. Shreds of evidence suggest apart from aromatic capping groups like Nap, Fmoc, etc. existence of various natural amyloidogenic and beta aggregation susceptible peptide segments like “FF”, “KLVFFA” could also effectively act as a promoter of hydrogelation through self-assembly.<sup>22-29</sup> The presence of such a minimal aggregation-prone sequence has been proven to be a reason for aggregation in disease-forming amyloids also.<sup>10,13</sup>

Notably, Tachykinin peptides comprised a crucial genre of neuropeptides that are abundantly found in amphibians and mammals.<sup>30,31</sup> The Tachykinin family is known for its conserved C-terminal sequence, Phe-X-Gly-Leu-Met-NH<sub>2</sub>, where “X” could be an aromatic or an aliphatic hydrophobic amino acid. In this regard, tachykinins bear an unusual similarity with amyloid beta-42<sup>25-35</sup> and its hydrophobic beta aggregation-prone sequence.<sup>32</sup> Both neurotoxic A $\beta$ <sub>25-35</sub> and tachykinins possess conserved “XXGLM” residues at their C-terminus but interact differently with neurons. Surprisingly tachykinins like Substance P, and Neurokinin are found to rescue neurons from A $\beta$ <sub>25-35</sub> mediated toxicity. Substance P is one of the members of the tachykinin family with an 11 amino acid sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH<sub>2</sub> and plays various important roles as a mediator of inflammation, vasodilation and pain sensitization and neurogenesis processes. However, its emerging role as a cellular growth, proliferation, adhesion, and angiogenesis is worthy to note.<sup>33</sup> Substance P has been known to stimulate cell growth in primary as well as immortalized cell line cultures, and previous study substantiate the fact that substance P could promote wound healing in humans.<sup>34,35</sup> Externally applied SP has been proven to effectively heal skin wounds in a rat full-thickness wound model.<sup>36-37</sup> Earlier research results demonstrated that externally applied SP via its upregulated cytokines could promote the proliferation of cells which is a prerequisite factor for tissue repair or replacement, and stimulate the neogenesis of blood vessels. Substance P (SP) was recognized to stimulate cell growth however, the mechanisms of growth control by SP are still unknown. In addition to this, it is also observed that substance P can induce the *in vivo* expansion of mesenchymal stem cells and can effectively amplify their migration from the bone marrow to the peripheral regions.<sup>38</sup> Considering all these beneficiary effectivities of SP in both *in vitro* and *in vivo* experiments for the first time we have designed and synthesized a novel hydrogelator peptide sequence from the C-terminus truncated part of Substance P containing the beta aggregation susceptible “FFGLM” motif which due to its characteristic hydrophobic nature undergoes aggregation.<sup>32-39</sup> Earlier Dharmadana *et al.* extensively studied the self-assembly pattern of full length substance p peptide and identified their huge potential in development of futuristic biologically inspired nanomaterials.<sup>39</sup> Motivated by their findings and considering the importance of highly conserved C-terminus region of tachykinin peptides we have further minimizes this undecapeptide sequence to obtain an abridged penta-peptide sequence “FFGLM”. Here it is important to note that this truncated C-terminus of substance p peptide itself contains an important “FF” residue within its sequence. To balance the hydrophobic-hydrophilic interaction which is crucial to make cytocompatible hydrogel formulation we have further introduced an integrin binding “RGD” motif to its C-terminal domain.<sup>40-42</sup> Such functionalization increases its solubility and made the hydrogel much more biocompatible one.<sup>40,43</sup> Such peptide-based hydrogel facilitates cellular adhesion upon immobilization on the surface, and upon biodegradation does not cause any cytotoxic side effects.<sup>44-47</sup> From earlier experimental results we further observed that FF dipeptide can give rise to robust self-assembled nanotube structures but in absence of N-terminus capping group its unable to give

rise to hydrogel like supramolecular assembly.<sup>13</sup> The incorporation of the fluorenylmethyloxycarbonyl (Fmoc) group to the N-terminus of the diphenylalanine sequence (Fmoc-FF), as first reported by Gazit *et al.*, resulted in the production of a self-supporting hydrogel, which was generated by diluting Fmoc-FF dissolved in hexafluoroisopropanol with water. Since this initial study, considerable effort has been expended in elucidating different ways to initiate gelation, minimising variability in resultant hydrogel networks, and developing applications for these nanostructured scaffolds in tissue engineering and drug delivery. However, due to the leaching effect, the use of cytotoxic solvents for hydrogelation, and the simultaneous oxidation of Fmoc functionality, such N-terminus Fmoc functionalized peptides have some controversial harmful consequences.<sup>48</sup> Despite of all these finding several recent cytocompatible peptide based supramolecular assemblies reported by Nishbet *et al.* were established with the incorporation of Fmoc in their sequences.<sup>49-52</sup> Inspired by those finding and to incorporate further mechanical strength to the designed biomaterial scaffold we have introduced an N terminus Fmoc group in our sequence too. Apart from the N-terminus Fmoc aromatic capping group the presence of successive two aromatic amino acids (FF) in the “FFGLM” truncated peptide segment also intensifies the quick self-assembly process and strengthens the mechanical properties of the generated hydrogel.<sup>13,53</sup> Furthermore, this Fmoc-protected octapeptide undergoes hydrogelation at physiologically relevant pH which enables us to apply this hydrogel-coated surface for mammalian cell culture and provide similar cytocompatibility as observed previously for full-length SP. As per our experimental result, this self-assembled peptide-based hydrogel was found to provide optimal biocompatibility with an array of mammalian cell lines starting from fibroblast to hMSC, thus widening their scope to be utilized as a biomaterial for drug delivery and tissue-engineering applications.<sup>54</sup> The wound-attenuating therapeutic prospect of the designed composition was further checked by using an *in vitro* scratch recovery assay. This represented rational fusion approach utilizes the combination of a hydrophobic cytocompatible domain truncated from SP along with an integrin-binding “RGD” motif to materialize a unique peptide-based supramolecular hydrogelator imparting comparable cytocompatible and therapeutic benefits similar to its mother sequence (SP).

### 3.3 EXPERIMENTAL SECTION

**3.3.1 Peptide Synthesis.** All Fmoc-protected amino acids and the required solid phase support: Rink Amide resin were purchased from Novabiochem (Merck Millipore). Diisopropylethylamine (DIPEA), and cleavage solution reagents: Trifluoroacetic acid (TFA), 1,2 Ethane dithiol, Thioanisole, Phenol, and HPLC water were purchased from Spectrochem. All the peptides were synthesized using a microwave-assisted solid phase peptide synthesis process using a microwave-assisted peptide synthesizer procured from CEM Liberty. During each coupling stem 5 equivalent of each amino acid was taken together with 5 equivalent of HBTU and solubilized in 0.3(M) DIPEA solution prepared in Dimethylformamide (DMF) solvent. Coupling reactions were performed at a 40-watt microwave power for 5 minutes under nitrogen bubbling conditions. 20% piperidine solution made in DMF was used as a deprotection reagent to remove the Fmoc group after each coupling step except after the last amino acid coupling. All the deprotection reactions were performed at 40-watt microwave power for 3 minutes. DMF-DCM solvents were used simultaneously for the washing of solid phase support. Complete cleavage of the synthesized peptide from rink amide solid support was done by using a cleavage cocktail solution comprising 92% TFA, 2% H<sub>2</sub>O, and 2% Thioanisole, 2% EDT, 2% Phenol to the synthesized peptide containing dried resin and stirred for 4 hours in room temperature. After this excess TFA was removed from the mixture by passing nitrogen gas through it (inside a hood). Finally, the synthesized peptide was precipitated by adding this residual reduced volume mixture to an ice-cold diethyl ether

solvent and kept inside a -20°C refrigerator to ensure complete precipitation. The next day after centrifugation the curdy white precipitation of the peptide was recovered and dissolved in 1:1 H<sub>2</sub>O: acetonitrile solvent and freeze-dried by lyophilization of the same. Further purification of the synthesized peptide was done by using preparative HPLC (Shimadzu) and an HPLC chromatogram was obtained. Characterization of the synthesized peptide was done by ESI-MS.

**3.3.2 Fabrication of Peptide Hydrogel.** Post lyophilized HPLC purified peptide was taken in a dry glass vial starting from low weight. To achieve hydrogelation 1X PBS (Phosphate buffer saline) was used as the solvent. Gentle sonication was performed at 37°C temperature to ensure solubility. At 20mg/ml concentration, the hydrogelation was achieved upon gentle heating cooling cycle within the 60°C to 37°C range resulting in a 2wt.% hydrogel in 1X-PBS. For the cell-biological work, the hydrogel was made using an isotonic condition with 300 mM sucrose in 1X-PBS. The hydrogelation was further confirmed by an inverted vial experimental analysis.

**3.3.3 Atomic Force Microscopic Analysis.** 2 wt.% Peptide hydrogel was prepared as described above and kept for 24h incubation at room temperature. On the next day 10µL of the vortexed hydrogel sample was evenly smeared over the surface of freshly cleaved mica foil with the help of a spin coater. The sample-coated mica foil was dried overnight under a desiccator. AFM images were taken by scanning the hydrogel-coated region of the mica surface in a vibration-proof enclosed box using a Park XE70 scanning probe microscope operated in the tapping mode settings.

**3.3.4 Field Emission Scanning Electron Microscopic Analysis of Hydrogel.** Similarly, as described above the hydrogel sample was drop-casted on the surface of clean silicon wafers and dried overnight at ambient temperature. Before the electron microscopic scan, the sample was evenly sputter coated with gold to make the surface electronically conductive. Images were taken using ThermoFisher FESEM (Quattro ESEM) at 5000x and 10,000x magnification operating at HV=5kV

**3.3.5 Thioflavin T Binding Experiment.** 1mM Thioflavin T stock solution was prepared in filtered 1x PBS by dissolving the required amount of it. The resultant solution was sonicated for a few minutes to get a homogenous working solution. A 96-well black/clear bottom plate was used to measure the fluorescence emission spectrum of each experimental solution. 10µL of ThT stock solution was mixed homogeneously with 200µL of the hydrogel and added to each well. To perform a control experiment instead of hydrogel 200µL of 1x PBS was used. The microplate was kept in incubation in dark for 15 minutes. Subsequent fluorescence emission spectra were measured by using a multimode plate reader (Thermofisher Scientific; Varioskan LUX) within the 460-500nm range with an excitation at 450nm wavelength.

**3.3.6 Congo Red Binding Analysis.** Similarly, Congo Red absorbance shift upon addition to hydrogel sample was analysed. A stock solution of Congo Red (300µM) was prepared in filtered 1x PBS. 10µL of stock solution was taken and mixed homogeneously with 90 µL of vortexed hydrogel solution and kept in a 96-well black/clear bottom plate. In order to perform the control experiment 1x phosphate buffer saline was used instead of a hydrogel sample. The absorbance spectrum was recorded within the 300nm to 700nm region for both control and hydrogel samples by using a multimode plate reader (Thermofisher Scientific; Varioskan LUX).

**3.3.7 Attenuated total Reflection-Fourier transform infrared spectroscopy (ATR-FTIR).** The ATR-FTIR sample was made by dissolving the peptide powder in HPLC grade water at a final concentration of 2% (w/v). The spectrum was captured on a Bruker fitted with a trough plate

made of a ZnSe crystal with a 45° angle of incidence (Model: Vertex, 70V+PMA50). 500L of the hydrogel was applied directly to the trough plate's surface. At ambient temperature, the spectra ranged from 4000 cm<sup>-1</sup> to 1500 cm<sup>-1</sup>. HPLC grade water spectrum was used as background and was subtracted from the sample spectrum

**3.3.8 Circular Dichroism Spectroscopy.** Circular dichroism (CD) spectra of peptide hydrogels were evaluated using a Quartz Cell with Stopper (for CD polarimeter), Path length: 1mm, Exterior: 12.5(W) x 2.5(L) x 48(H) mm, Volume: 350 µL (Starna Scientific UK). All the measurements were carried out at 37°C within the 190nm to 250nm wavelength range. A background correction was done by subtraction of 1X PBS spectra at 37°C using JASCO 2821 PLC-230 LP circular dichroism polarimeter. The spectra were plotted using Graph Pad prism 8.0.2 software.

**3.3.9 Determination of Mechanical and Thermal Properties of Hydrogel.** All the rheological experimental analysis was executed by using a temperature-controlled Anton Par MCR102 rheometer. All the measurements were carried out at a physiological temperature of 37°C which is physiological temperature. A stainless steel-made 40mm rotating plate with parallel-plate geometry was employed to carry out the experiments. The gel-to-sol transition temperature (T<sub>gel</sub>) of the hydrogel (2 wt%) was investigated using a multicell differential scanning calorimetric device from TA Instruments. In the sample cell, the gel was inserted, and in the reference cell, solvent was applied. The heat change was measured at 1 second intervals in pans with lids closed. DSC scans began with a 4 °C isotherm for 5 minutes, followed by a 1 °C min<sup>-1</sup> ramp up to 290 °C.

**3.3.10 Analysis of pH Responsiveness.** PBS buffer with a pH of 7.4 was used to make the peptide hydrogel. Acetate buffer solutions correspond to pH 5.5, and di-Sodium hydrogen phosphate buffer of pH 12.0 were prepared by respective protocol. In order to avoid the effect of ionic strength, 0.15 M sodium chloride solution was used, and the pH values were further confirmed by using a pH meter. For the release assay 0.3mM Calcein solution was used to make 2wt.% hydrogel sample.

**3.3.11 Cell Culture.** Human lung fibroblast cells: WI-38, Mouse muscle myoblast cell: C2C12, Human breast adenocarcinoma cells: MCF7 were procured from NCCS Pune and cultured in DMEM culture medium supplemented with 10% FBS, 1% penicillin/streptomycin in a humidified incubator with 5% CO<sub>2</sub> at 37° C. Human Wharton's jelly mesenchymal stem cells were procured from Himedia and cultured using mesenchymal stem cell expansion medium (Himedia AL512) supplemented with 10% FBS, 1% antimycotic antibiotic solution (Himedia A002). Cells were grown in T25 cell culture flasks and the medium was changed. upon 24h interval. Upon reaching 80% confluency cells were trypsinized by using 0.05% trypsin-EDTA and centrifuged at 2500rpm for 4 minutes and plated for the experiment.

**3.3.12 Cytotoxicity Assessment of Designed Peptide and its Self-Assembled Hydrogel Form (MTT Assay).** An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] based colorimetric assay was performed to check the initial cytotoxic effect of this designed hydrogelator peptide towards WI-38, C2C12, MCF7, and hMSC cells. To carry out this experiment cells were seeded in a flat bottom 96-well plate up to 60% confluency and treated with a different concentration of hydrogelator peptide (100 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL) followed by an incubation period of 24h. Fresh MTT solution was prepared in filtered PBS at 5mg/ml concentration and vortexed for 1 min for complete dissolution. 100µL of this solution was added to each well and incubated in dark for 4h at 37°C temperature. After the end of the incubation interval solution from each well was pipetted out gently and 50µL of 1:1 DMSO: MeOH solution was added to solubilize purple formazan precipitate

developed due to MTT reduction. After 10-15 min incubation in dark, the absorbance was measured at 550nm wavelength using a microplate reader (Thermofisher Scientific; Varioskan LUX).

**3.3.13 LIVE/DEAD Assay.** To check the cytocompatible effectivity of the designed hydrogel one Live/Dead cellular assay was performed on 2D hydrogel cultured cells. Before the experiment glass bottom, confocal dishes were covered with a pre-hydrogel solution of peptide and incubated at 37°C temperature in presence of a culture medium. Upon hydrogelation trypsinized cell suspension was added to its surface and incubated for 1h to ensure minimum cellular adhesion. For the control experiment cells were cultured for the same time duration upon tissue culture plate (TCP). After this, additional media was added gently from the corner of the glass bottom plate and incubated for the next 24-48h. To carry out this experiment a LIVE/DEAD assay reagent containing 2µM Calcein AM and 1µM Propidium iodide was used. The non-fluorescent Calcein AM upon entering into live cells gets converted to green fluorescent Calcein due to the hydrolysis of acetoxymethyl ester functionality induced by intracellular esterase. Propidium Iodide being a DNA intercalating agent exhibits its red fluorescent signal upon entering dead cells with a mangled cell membrane. After the addition of 200µL of Live/Dead assay solution prepared in colorless DMEM media, to each glass bottom flask, an incubation period of 30min was maintained. Cells were washed gently with 1x PBS and imaged at 10X magnification by using an inverted fluorescence microscope (Olympus IX83) simultaneously at FITC/TRITC channels.

**3.3.14 Actin Staining Analysis.** To check the cytoskeleton arrangement of the cultured cells upon hydrogel coated as well as only TCP surface an actin staining-based assay was done. Cells were cultured on TCP for control and hydrogel-coated surface as described previously. Upon reaching 70% confluency at the end of the 48h incubation period cells were fixed by using 4% paraformaldehyde solution for 1 h and permeabilized using 0.1% Triton X-100 containing 1% BSA in filtered phosphate buffer saline for 30 min. Alexa Fluor-488 conjugated Phalloidin was used to visualize the actin filament present inside the cells. Hoechst 33258 was used to counterstain the nucleus. 200µL of 5µM solution of Alexa Fluor-488 conjugated Phalloidin was added to each glass bottom flask and incubated in dark for 30 minutes. After gentle washing with 1x PBS, 200 µL nucleus staining dye Hoechst was added at 2µM concentration and incubated in dark for the next 15 min. After three times washing with 1x PBS cells were imaged at 10X magnification by using an inverted fluorescence microscope (Olympus IX83) simultaneously at FITC/DAPI channels.

**3.3.15 Cell Migration Assay to Check Wound Healing Activity of Hydrogel.** Approximately 3000 cells /well, WI38 cells were cultured in a 12-well plate using high glucose DMEM media containing 10% FBS supplemented with 1% penicillin/streptomycin in a humidified incubator with 5% CO<sub>2</sub> at 37° C. Upon reaching 80% confluency a scratch was made perpendicularly by using a sterile 200µL pipet. Next dislodged cells were washed by using 1x PBS and cells were incubated with serum-free low glucose DMEM for control. In the treated wells a 2wt.% hydrogel conditioned low glucose, serum-free DMEM media was administrated similarly. Cells were imaged at 10X magnification at 0h,12h, 24h, and 48h time intervals to monitor cellular migration. The covered area was calculated by using ImageJ software and plotted against time duration.

**3.3.16 Statistical Analysis.** All the experimental analysis was statistically quantified by using Graph Pad Prism 8.0.2, and the results were expressed as mean ± standard deviation (SD). Statistical differences were estimated by one-way ANOVA and student-t-test for grouped and column analysis. In all analyses,  $p < 0.05$  was taken as an indicator of a significant difference. [\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ]

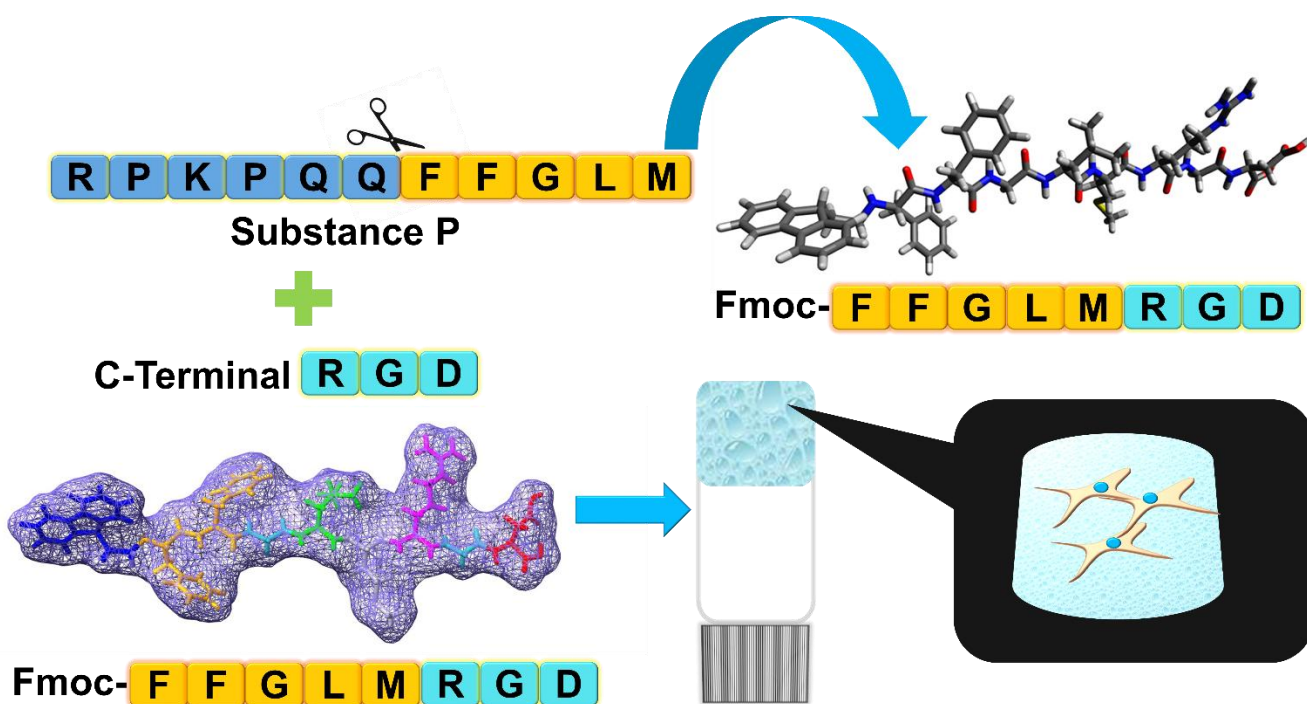


**Figure 3.1** Schematic representation of the designed hydrogel forming peptide by fusing truncated Substance P sequence “FFGLM” along with a C-terminus RGD epitope.

### 3.4 RESULTS AND DISCUSSION

**3.4.1 Design and Synthesis of Hydrogelator peptide and Characterization.** In an attempt to make an ECM mimicking biomaterial with cytocompatible wound healing therapeutic prospect similar to biologically abundant neuropeptide Substance P, we have designed and synthesized a fused multi-domain peptide containing both Substance P truncated motif and integrin binding segments. We herein report the design of a novel peptide sequence comprising with C-terminal fragmented peptide sequence “FFGLM” from Substance P followed by a crucial integrin interacting RGD motif [Figure 3.1]. Such incorporation of polar “RGD” epitope containing both basic and acidic amino acids not only affects its overall biocompatibility but balances the hydrophobicity of the entire peptide chain without changing the isoelectric pH of the overall sequence to a larger extent. The resultant “FFGLMRGD” octapeptide along with its N-terminus Fmoc (fluorenylmethoxycarbonyl) functionality has been synthesized using solid phase peptide synthesis and its respective molecular mass, purity, and  $^1\text{H}$  NMR spectrum were checked before further analysis [Figure 3.2(a)-(b)], [Figure 3.3].

The N-terminus Fmoc group was kept protected strategically to intensify the desired  $\pi$ - $\pi$  interaction among the aromatic Fmoc and phenylalanine residues which further facilitated the formation of the soft hydrogel at lower peptide concentration. Here the designed peptide (20mg/mL) in an attempt to solubilize in phosphate buffer saline formed a homogenous self-supporting hydrogel material with 10-15 min gentle sonication in a 60°C water bath. The successful formation of self-supporting hydrogel was confirmed by a vial inversion study. [Figure 3.4(a)] The hydrogel inner nanostructures were further explored simultaneously by Atomic Force Microscopic and Field emission scanning electron microscope. The designed Fmoc-FFGLMRGD peptide, due to the presence of Fmoc-FF at its N-terminus exhibited the formation of entangled fibrous structures in AFM as well as in FESEM analysis which indicate an enhanced intermolecular interaction between assembled nanofibers [Figure 3.4(b)-(d)].

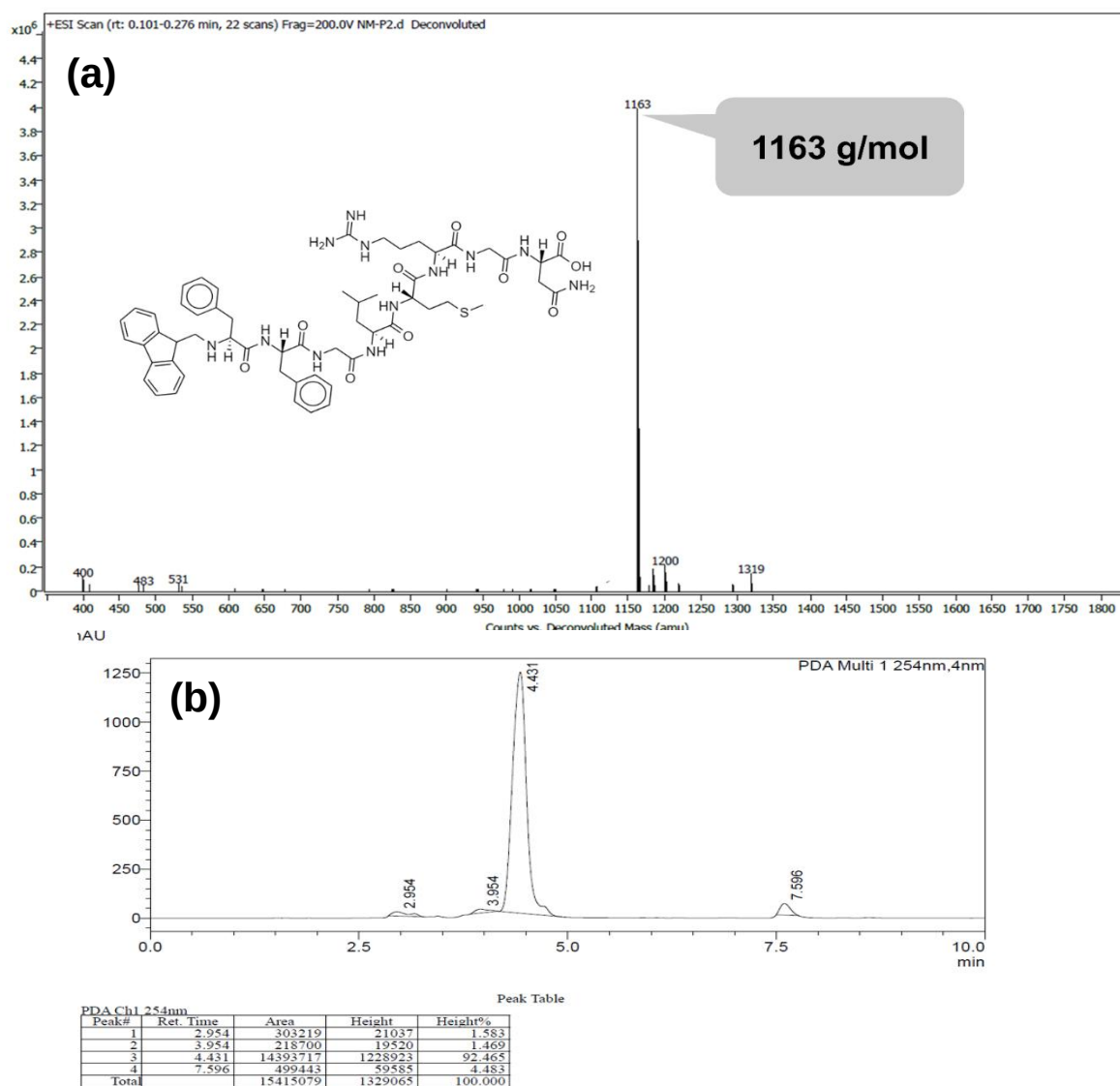


**Figure 3.2** (a) ESI-MS of synthesized hydrogelator peptide. (Expected Mass:1163.35g/mol) (b) HPLC Chromatogram of synthesized hydrogelator peptide.

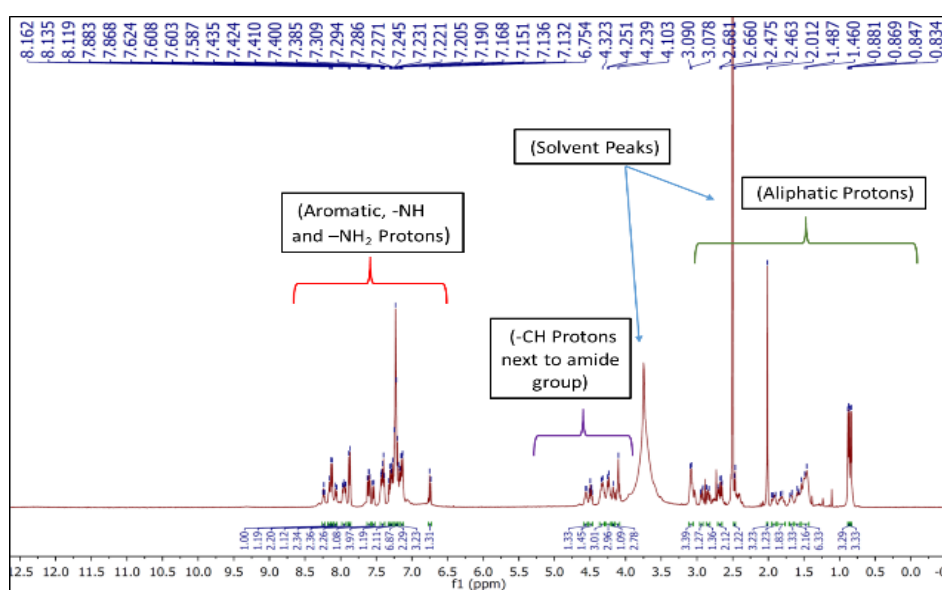


**Figure 3.3** <sup>1</sup>H NMR spectra of the designed hydrogelator peptide in d<sub>6</sub> DMSO

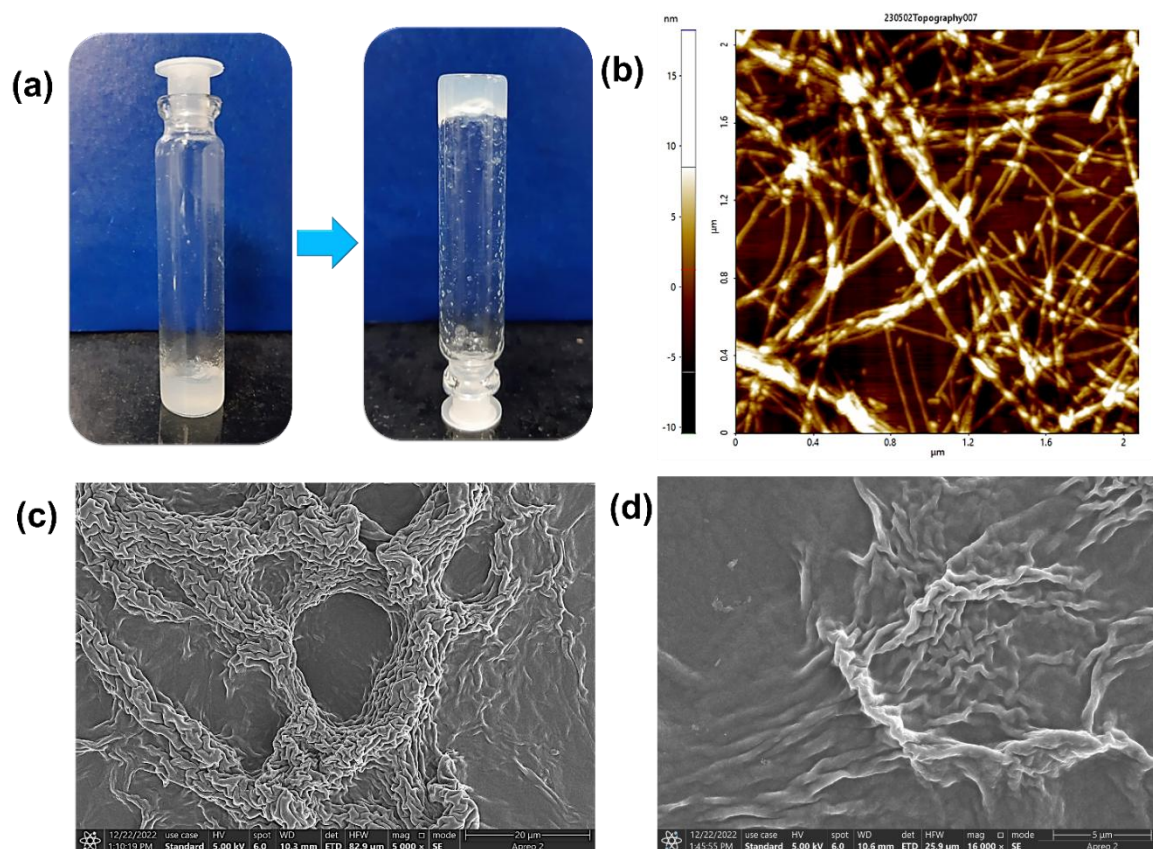


**Figure 3.4** (a) Inverted vial shows the formation of self-supporting designed peptide hydrogel (2wt.%). (b) Atomic Force Microscopic image of designed peptide hydrogel. FESEM image of hydrogel at (c) 5000x and (d) 16,000x showing entangled fibrous structural features.

**3.4.2 Spectroscopic Analysis of Secondary Structure.** As we have mentioned earlier that both tachykinins and amyloid beta 42 have similar C-terminus hydrophobic residues crucial for their self-assembly mediated aggregation, so here we are interested to check whether this substance p truncated peptide motif containing hydrogel possessed such kind of amyloidogenic nature or not. To get an insight into this we have performed Thioflavin T and Congo Red binding experimental analysis for the hydrogel. The binding of ThT with cross- $\beta$  structures formed due to amyloid assembly is a traditional approach to detecting the presence of amyloidogenic aggregation. Thioflavin T exhibits extremely sensitive fluorescent activity vastly controlled by the viscosity of the immediate environment. In this experiment, incubation of ThT along with the hydrogel solution caused a sharp increase in its fluorescence emission intensity indicating the presence of a higher extent of cross- $\beta$  structures in the hydrogel state as compared to the control [Figure 3.5 (a)]. This spectroscopic determination was further confirmed by ThT fluorescence microscopic imaging of the peptide hydrogel shown in Figure 3.5(b) where an intense green fluorescence was observed from hydrogel fibrils after incubation with ThT. A Congo Red binding spectrophotometric assay was further performed to assess the amyloid  $\beta$  aggregated structures during hydrogel formation. Generally, the absorbance maxima of CR upon binding to amyloids exhibit a significant red shift from its characteristics 498nm region to 520 to 550nm. Here in our experiment, we found that compared to the control (only PBS and CR) upon incubation with hydrogel the intensity

of the CR absorption maxima increased and exhibited a red shift from 498 to 515–522 nm [Figure 3.5 (c)].

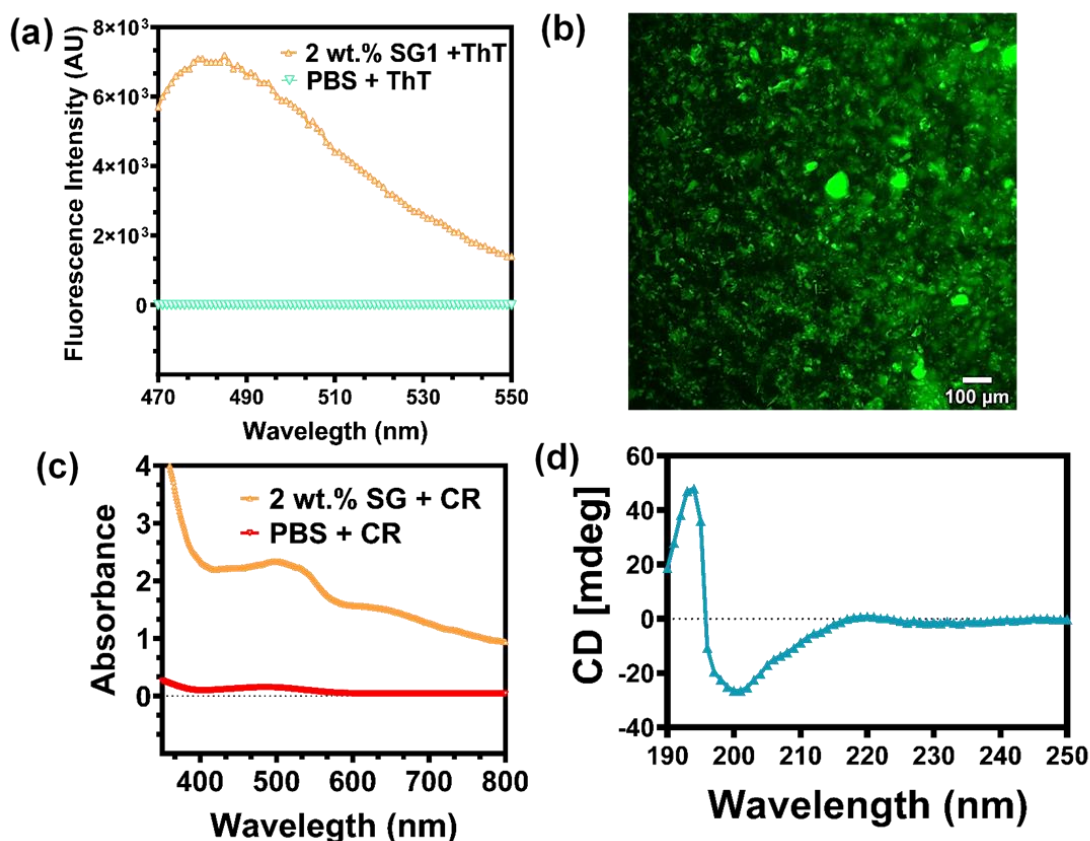


**Figure 3.5** (a) Thioflavin T fluorescence emission spectra obtained for the designed hydrogel showing an enhanced fluorescence intensity at 485nm. (b) Fluorescence microscopic image of ThT bound hydrogel fibrils exhibiting enhanced fluorescence emission at FITC channel. (c) Congo Red absorbance spectra showing an enhanced as well as red shifted characteristic absorbance upon co-incubation with hydrogel. (d) Circular Dichroism spectral analysis for designed peptide hydrogel showing significant beta-sheeted secondary structure.

The presence of a  $\beta$ -sheet structure within the hydrogel was confirmed by ATR-FTIR spectroscopy [Figure 3.7(a)]. The spectra revealed prominent peak at 1616  $\text{cm}^{-1}$ , indicating the existence of aggregated  $\beta$ -sheeted structure along with a short peak at 1745  $\text{cm}^{-1}$  probably coming from the  $-\text{COOH}$  side chain functionality of Aspartic acid. To corroborate these above observations, the nature of secondary structures aroused during the self-assembling of the peptide was further analyzed through CD spectroscopic analysis of the formed hydrogel. Interestingly, the hydrogel showed a conspicuous negative band at 200nm and a positive peak at 195nm which again suggests the emergence of a secondary structure containing both random as well as  $\beta$ -sheet-like arrangement upon self-assembly [Figure 3.5(d)].

**3.4.3 Analysis of Mechanical and Thermal Properties of the Hydrogel Ensemble.** The stiffness, as well as mechanical strength of the designed hydrogel, plays a vital role in cytocompatibility analysis cellular fate determination, and the therapeutic development process. In this regard, the mechanical strength of the fabricated hydrogel was analyzed by using oscillatory rheology. A significantly higher storage modulus ( $G'$ ) as compared to loss modulus ( $G''$ ), data obtained from a frequency sweep experiment shown in Figure 3.6(a) confirms the overall formation of the hydrogel network. Interestingly from the experimental data we found that the designed hydrogel scaffold exhibited an intermediate mechanical

property in terms of  $G'$  and  $G''$  when compared with the earlier reported observation with the hydrogel comprised both individual Fmoc-FF and Fmoc-RGD peptide.<sup>55</sup> In our next experiment, a strain sweep analysis was done to evaluate the linear viscoelastic region of the hydrogel. In this experiment an increasing strain was applied to the hydrogel sample, keeping the applied frequency constant. Results shown in **Figure 3.6(b)** revealed that for up to a 1% increase in shear strain, the storage ( $G'$ ) and loss modulus ( $G''$ ) values sustain their respective hydrogel-like differences and did not change with an increase in strain %. However, upon a larger applied strain % the storage modulus ( $G'$ ) value drop under the loss modulus ( $G''$ ) indicating a gel-to-sol conversion. Hydrogels are often found to have thixotropicity due to the presence of nonbonding interactions, which can ultimately be restored upon removal of the deformation causing strain. Such shear strain-sensitive property is pivotal for its cytocompatibility as well as its therapeutic implementation. Finally, the thixotropicity of the designed hydrogel was determined by implying a step strain rheology study where from **Figure 3.6(c)** we can find that the synthesized hydrogel can recover its hydrogel form even after withdrawal of 50% increased applied strain. An injectability test was also performed with 2wt.% hydrogel which demonstrated the sheer thinning property of it [**Figure 3.6(d)**]. All the experiments were performed in a triplicated manner.

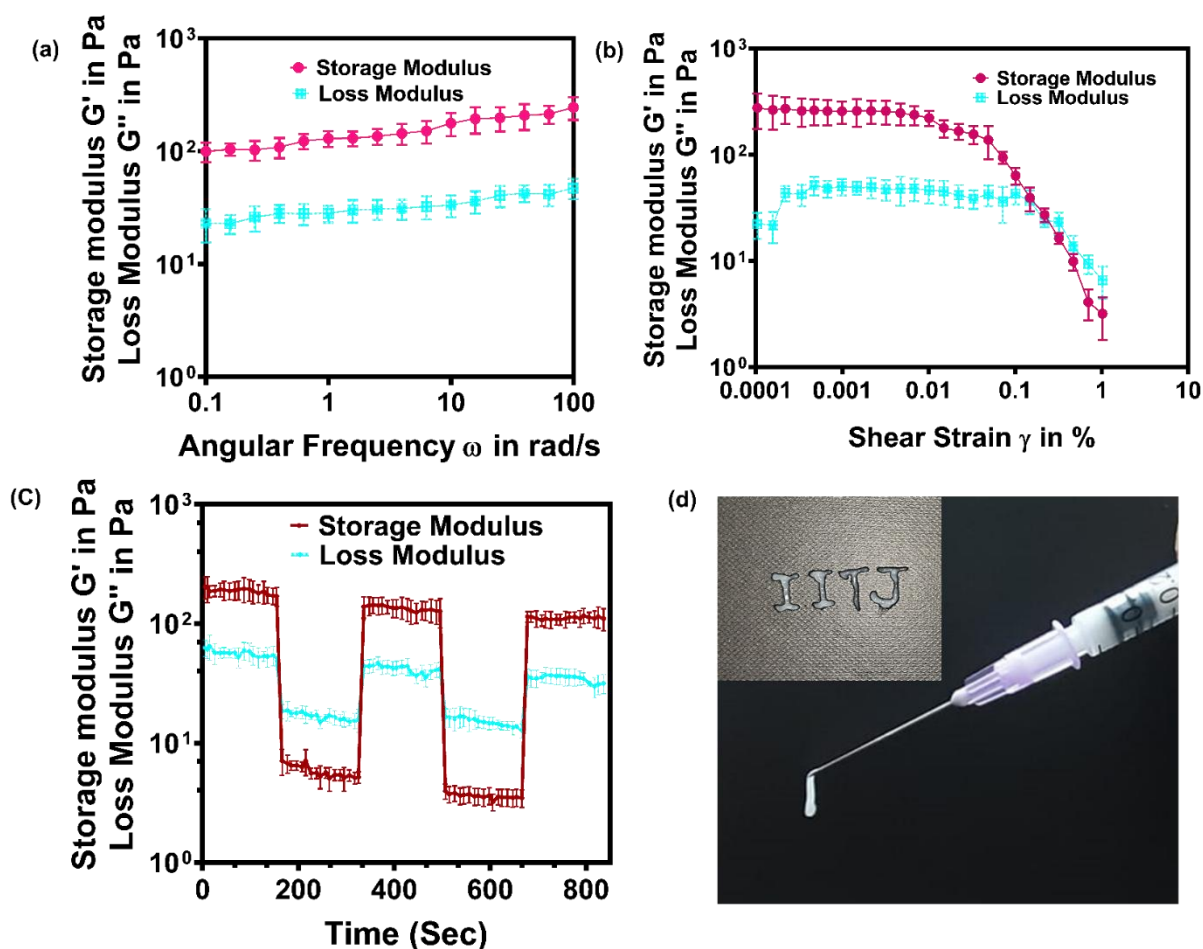


**Figure 3.6** (a) Frequency sweep study of the synthesized hydrogel to check its characteristic hydrogel like mechanical property. (b) Strain sweep study to unravel the linear viscoelastic region of the fabricated peptide hydrogel. (c) Thixotropicity measurement for the hydrogel showing its sheer thinning property. (d) Analysis of the injectable property of the designed 2wt.% hydrogel.

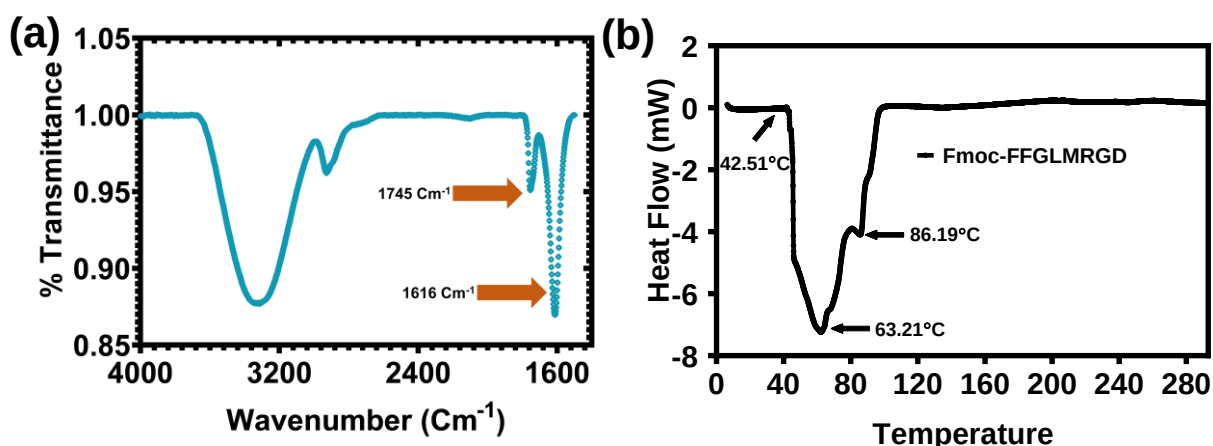


**Figure 3.7** (a) ATR-FTIR spectra of the designed peptide hydrogel. (b) DSC thermogram (heating) of the hydrogel.

Further corresponding gel-to-sol transition ( $T_{gel}$ ) of the 2.0 wt.% hydrogel prepared in phosphate buffer saline was studied using differential scanning calorimetry (DSC) [Figure 3.7(b)]. DSC data revealed the initiation of the disruption of gel phase from 42.51°C temperature which can be attributed to the loss of 3D hydrogel framework with further increase in temperature. However, a sharp endothermic peak at 63.21 °C along with a small endothermic peak at 86.19 °C indicate the melting of fibrillary aggregates. The width of the DSC transition can be attributed to the presence of differently organised populations causing heterogeneous thermal property. The corresponding DSC plot indicates that the above sample possessed a significant supramolecular organisation, with homogeneous and ordered gel states.

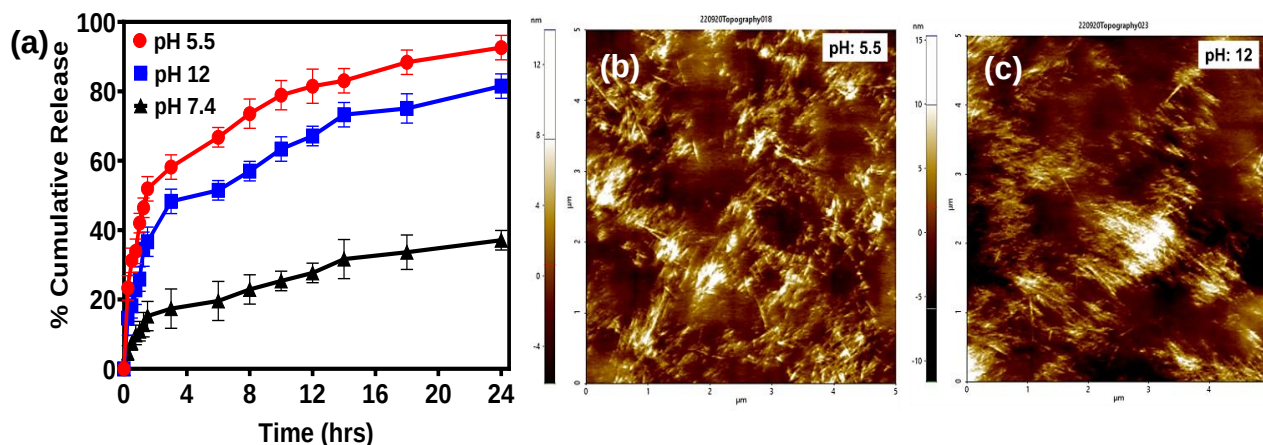


**Figure 3.8** (a) pH responsive cumulative calcein dye release study from the hydrogel network by fluorescence spectroscopy. (b) Atomic Force microscopic images of 2wt.% fabricated hydrogel incubated at different pH condition.

**3.4.4 Effect of pH on Integrity of the Designed Hydrogel.** As our designed substance P inspired hydrogelator peptide contains an RGD motif so we were interested to check whether this fabricated hydrogel composite have any pH responsive property or not. In order to check so we have performed a pH responsive dye release assay at different three pH levels. In this regard we have found that the design Fmoc capped octapeptide can only form mechanically

stable hydrogel at neutral pH (pH: 7.4). Any attempt to make self-supporting hydrogel using buffer solutions of pH 5.5 and 12 were lead to unsuccessful hydrogelation. Further to check the effect of pH on the stability of hydrogel integrity we have performed a dye release study with calcein loaded hydrogel matrix formed in neutral condition. pH-responsive dye release efficacy was analysed by gently incorporating 500 $\mu$ L of pH 5.5, 12 buffer on the top of 500  $\mu$ L dye loaded hydrogel surface. Time-dependent release was monitored by gently aspirating 100 $\mu$ L of supernatant from the top of the preformed hydrogel surface and subsequent fluorescence intensity measurement using a multimodal plate reader with an excitation at 485 nm and an emission wavelength of 520 nm. In a control experiment, the same amount of PBS buffer with a pH of 7.4 was added, and the release was monitored in the similar fashion. From the experimental data shown in **Figure 3.8(a)** it is apparent that the fabricated hydrogel at neutral pH exhibited slowest calcein release with 37% cumulative release from its supramolecular assembly even after the 24hr of incubation period, indicating its superior stability at pH 7.4 at 25°C temperature. However, the hydrogel degraded very quickly at both acidic (pH 5.5) as well as in basic pH causing a higher cumulative release % of loaded calcein which can be attributed to the rapid disarranging of the supramolecular assembly. At pH 5.5 probably the rapid protonation of the guanidine side chain of arginine residue caused the most deformation on the hydrogel network due to enhanced electrostatic repulsion and subsequent higher release of loaded calcein took place. In a similar way deprotonation of aspartic acid side chain at pH 12 can give rise to electrostatic repulsion causing deformation of fibrous hydrogel network. Further to check the morphology of this disintegrated hydrogel matrix after an incubation period of 24hr we have performed an AFM analysis for each incubated hydrogel sample at pH 5.5 and 12 respectively. The corresponding AFM images of the hydrogel sample at these pH values demonstrated a shorter disintegrated fibrillary aggregated morphology indicating the deformation of earlier entangled long fibrous structure of the hydrogel formed at neutral pH [**Figure 3.8(b)-(c)**]. From the above analysis we concluded that the designed hydrogel possesses significant pH sensing property which can eventually potentiate its future prospect as wound healing biomaterial with simultaneous pH triggered drug delivery platform.

**3.4.5 Assessment of Biocompatibility of the Designed Hydrogel Assembly.** Biomaterial surfaces functionalized with integrin binding epitopes like “RGD” are known to promote cellular adhesion by enhancing the requisite cell-matrix interactions. However, a contentious cytocompatibility of Fmoc capped peptide based hydrogel materials cannot be overruled completely. In this regard evaluation of the cytotoxicity of the designed hydrogel material upon a diverse array of cell lines is crucial for its futuristic application in tissue engineering. Generally, peptide-based biomaterials exhibit minimum cytotoxicity upon enzyme-mediated degradation in the biological system. In the present study, initial screening of the synthesized peptide was made by an MTT-based colorimetric assay where the cellular metabolic activity is measured after a 24h incubation with varying concentrations of peptide. Viability was analyzed for different types of cell lines including human lung fibroblast cells: WI-38 [**Figure 3.9 (a)**], Mouse muscle myoblast cell: C2C12 [**Figure 3.9(b)**], Human breast adenocarcinoma cells: MCF7 [**Figure 3.9(c)**] and hMSC cells [**Figure 3.9(d)**].

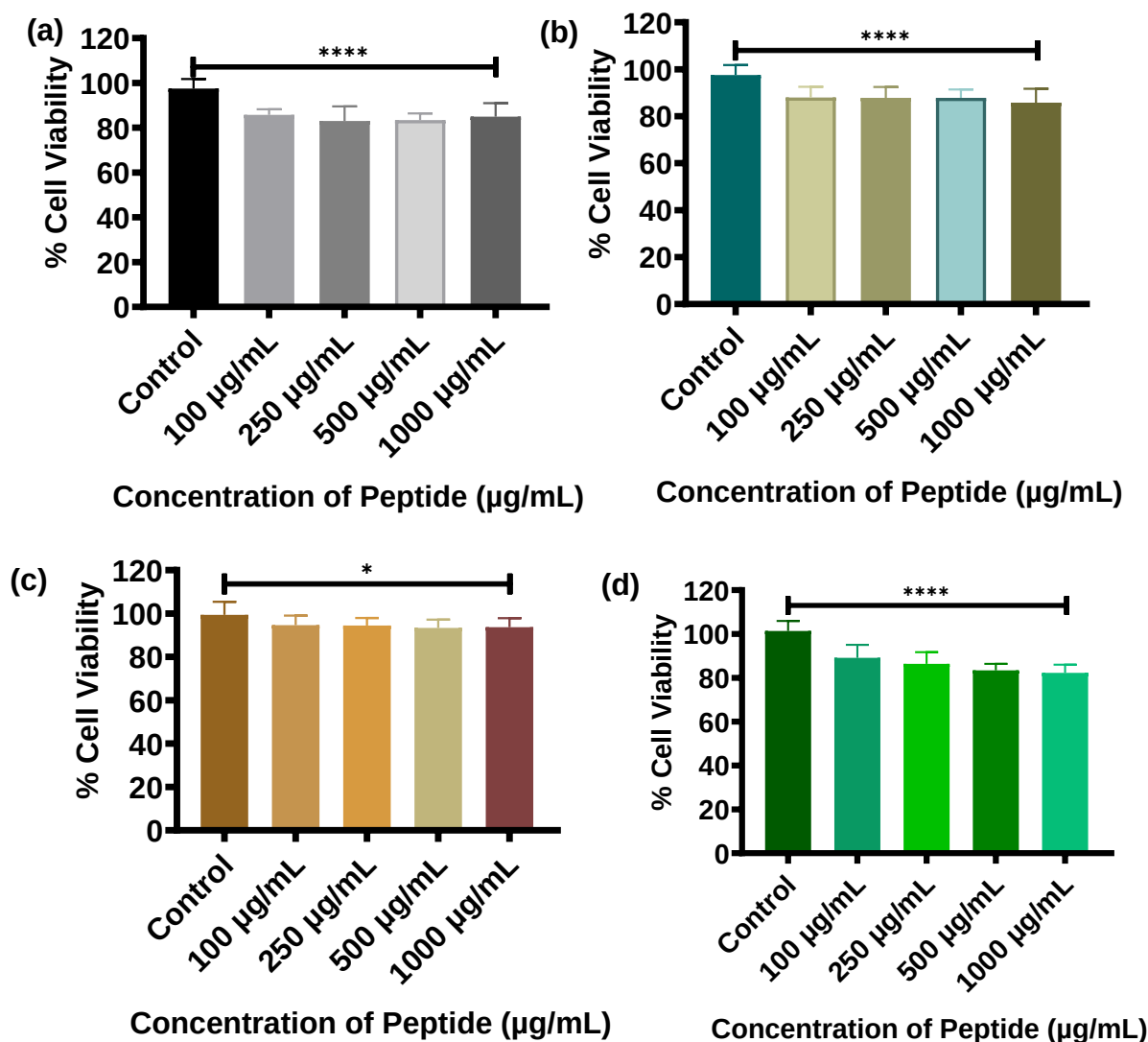


**Figure 3.9** Cell Viability assay result performed with the synthesized hydrogelator peptide on (a) WI-38, (b) C2C12, (c) MCF7 and (d) hMSC cell line.

The metabolically active viable cells caused the reduction of yellow-colored tetrazolium MTT (3-(4,5-dimethylthizole-2)-2,5-diphenyltetrazolium bromide) into a purple formazan which upon solubilization in 1:1 DMSO: MeOH gives absorbance at 550nm. In our experimental analysis, the synthesized peptide demonstrated up to 90-95% viability upon incubation with WI-38 [Figure 3.9 (a)] and C2C12 cells, [Figure 3.9(b)] and the viability % remains almost 98% for adenocarcinoma cell lines.[Figure 3.9(c)] Motivated by this result and considering the therapeutic effectivity of mesenchymal stem cells in various tissue engineering scaffolds we further checked the biocompatibility of the designed peptide with human Warton's jelly-derived mesenchymal stem cells. A 90% viability even at 1000µg/mL concentration of peptide was observed for hMSC upon 24h treatment incubation [Figure 3.9(d)]. This cytotoxicity assessment data indicates the non-cytotoxic nature of the synthesized peptide towards both cancerous and normal cell lines. Now to check the biocompatibility of the fabricated hydrogel in culture condition a Live/Dead staining was made after a 48 h culture duration on hydrogel coated surface. In this experiment, we were curious to explore the stability of the coated hydrogel matrix in cell culture conditions to provide cellular adhesion and proliferation. To

get an insight into the mechanical stability of fabricated hydrogel in cell culture conditions, a thin coating of synthesized hydrogel was prepared on the glass bottom surface of the confocal dish and kept in incubation with culture media for 2 h. Upon hydrogelation, the culture media was aspirated out gently and 200 $\mu$ L of each cell suspension containing approximately  $3 \times 10^3$  cells was added onto the top of the hydrogel layer. To perform the control experiment, cells were plated on the bare confocal dish. After 24 h of incubation both the control and hydrogel-coated surface exhibited substantial cellular adherence.

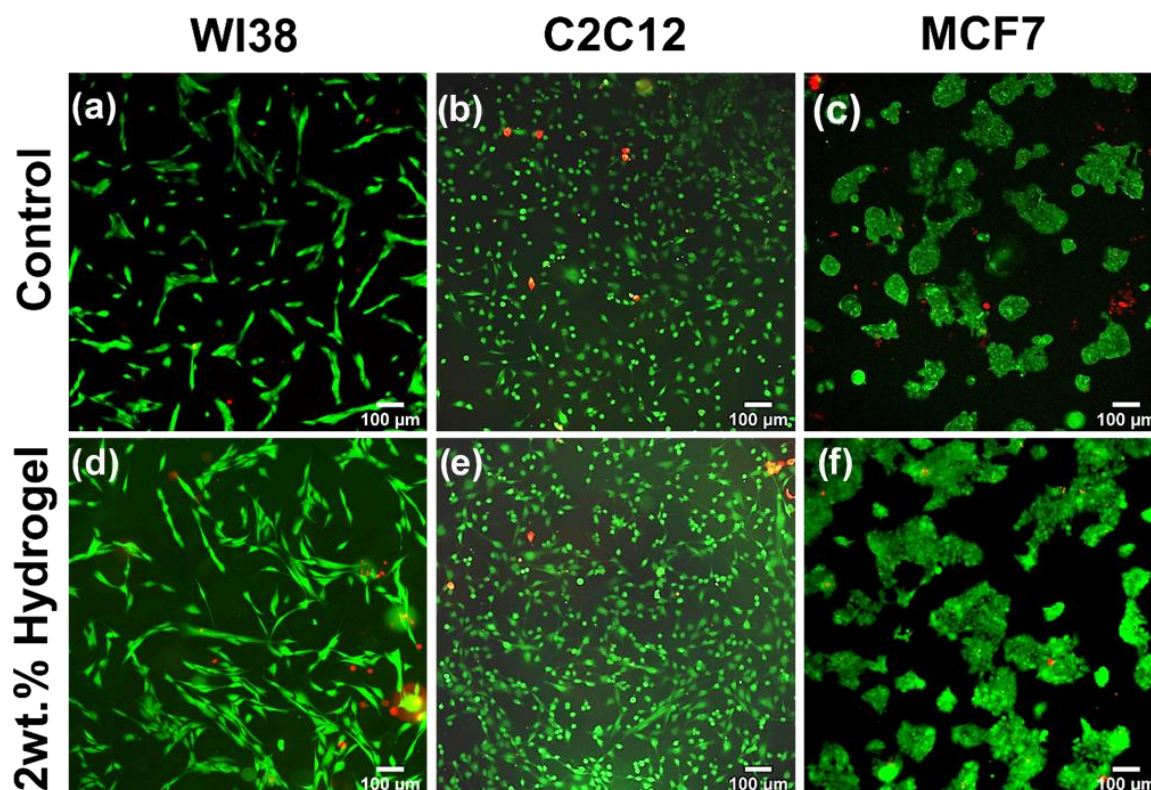


**Figure 3.10** LIVE/DEAD microscopic image for (a) WI-38, (b) C2C12, (c) MCF7 cell lines, cultured over TCP for control (upper panel) and hydrogel coated surface (lower panel) (d) WI-38, (e) C2C12, (f) MCF7 for 72h. (Scale bar corresponds to 100 $\mu$ m)

A LIVE/DEAD staining experiment using Calcein AM/Propidium Iodide cocktail was performed after a 72 h culture period to monitor the amount of metabolically active viable cells present both on the hydrogel coated and control surface. From the fluorescence microscopic images taken at 10 $\times$  magnification, after 48 h of culture duration a substantial number of live cells were observed to be present on both hydrogel-coated and control surfaces indicating the good cellular adherence provided by the hydrogel matrix. Along with this an elongated spread morphology of the WI-38 and C2C12 cells were observed on hydrogel cultured cell as compared to the control one which might have resulted from the RGD functionalization of the designed peptide hydrogel [Figure 3.10 (a)-(e)]. For adenocarcinoma cells (MCF7) similar to the control cells cultured over the TCP, the hydrogel-coated cells also exhibited cluster growth phenomenon which is a key characteristic of MCF7 cells [Figure 3.10 (c)-(f)]. However, for all these three different cells an increase in cell population compared to control is noteworthy to mention which can be ascribed to both the “FFGLM” truncated motif of substance P peptide as well as integrin-binding C-terminus “RGD” motif. Such an increase in cellular population was also observed during an actin staining assay performed after 72h of culture duration where the actin cytoskeleton of cultured cells was found to retain a healthy morphology similar to the control cells. From the microscopic images shown in Figure 3.11(a)-

(f), it can be concluded that the designed hydrogel can promote cellular proliferation and viability by providing improved cellular adhesion. Moreover, the hydrogel matrix provides an overall conducive 2D environment that fosters cell proliferation and growth.

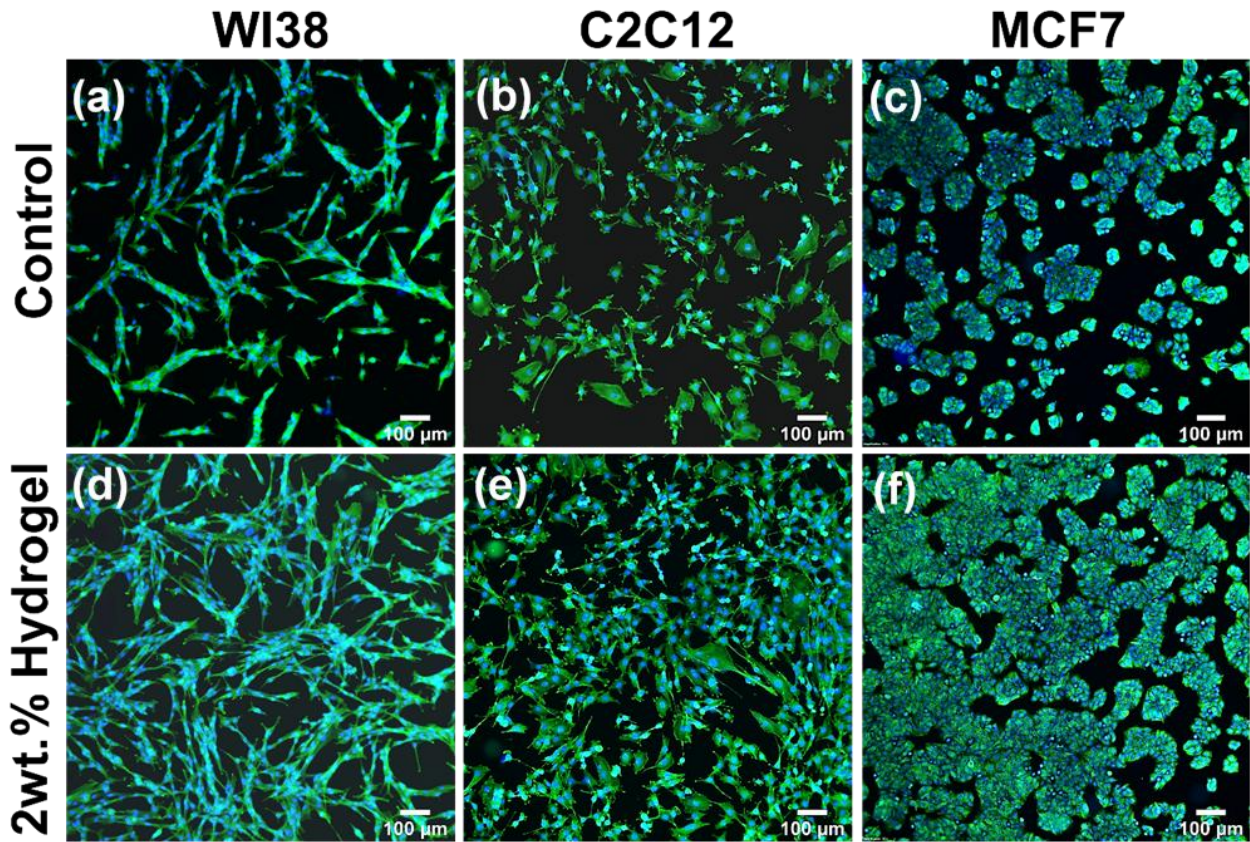


**Figure 3.11** Fluorescence microscopic images of Alexa Fluor-488 conjugated phalloidin stained actin cytoskeleton network of (a)WI-38, (b) C2C12, (c) MCF7 cells cultured for 72h on TCP for control (upper panel) and on hydrogel coated surface (d)WI-38, (e) C2C12, (f) MCF7.

Fascinated by the above result and considering the huge therapeutic potential of hMSC we further checked the effectivity of this hydrogel in 2D and 3D cultures of hMSC cells. Interestingly, hMSC cells cultured on 2D hydrogel coated surface were found to have an overall good morphology similar to the control one which is also per our earlier cell viability data [Figure 3.12 (a), (b)]. (lower panel). Nucleus are stained by Hoechst 33258. (Scale bar corresponds to 100μm) Notably, similar to fibroblast, myoblast, and adenocarcinoma cells, hMSC cultured over hydrogel surface also increased in number more than the control group indicating the proliferative activity of the designed Substance P-derived hydrogel.

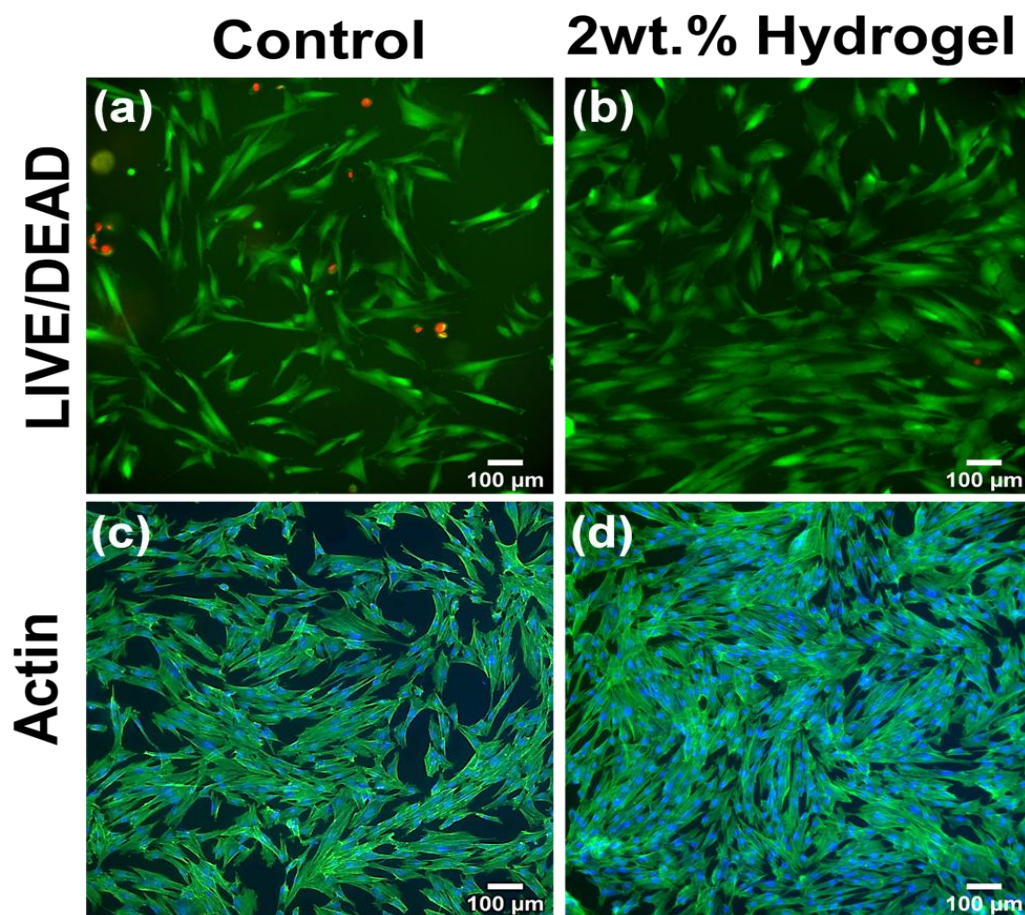


**Figure 3.12** Fluorescence microscopic images representing LIVE/DEAD experimental analysis performed on hMSC cultured on both (a) TCP (control) and on (b) 2wt.% synthesized hydrogel for 72h. lower panel fluorescence microscopic images reveal the Alexa Fluor 488 conjugated phalloidin stained actin filaments of hMSC cultured on (c) TCP and (d) 2wt.% hydrogel coated surface. Nucleus were stained by Hoechst 33258 (Scale bar corresponds to 100µm).

The actin cytoskeleton of cultured hMSC on both control [Figures 3.12 (c)] and hydrogel coated surface [Figure 3.12 (d)] were found to exhibit healthy morphology after an incubation period of 72h. Such excellent compatibility with mesenchymal cells further broadens the future therapeutic and tissue engineering prospect of the designed hydrogel. During the 3D culturing process, we have considered collagen as our control 3D matrix and for the treated one we have made an isotonic hydrogel formulation as described earlier in the materials and method section. The cultured cells were finally fixed and stained with AlexaFluor 488 labeled phalloidin to visualize their cytoskeleton network inside the 3D matrix. Z- stack images were taken at 40x magnification to check the cells [Figure 3.13(a)-(b)]. As per our observation, the synthesized peptide hydrogel was able to retain its 3D coverage around the cells for up to 72 h in culture condition and cells inside of peptide hydrogel matrix were in their healthy morphology similar to those observed in the collagen matrix. After 72h the peptide slowly turned into its sol form and lost its integrity. However, in control, the collagen 3D matrix was still in its place.

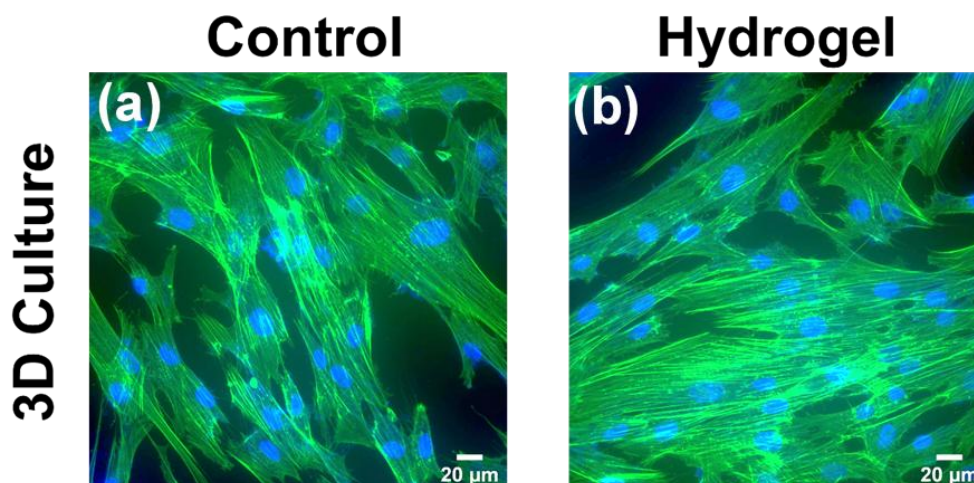


**Figure 3.13** Fluorescence microscopic images of Alexa Fluor 488 conjugated phalloidin stained actin skeleton of hMSC cultured for 72h duration inside 3D culture condition maintained by collagen for (a) control and (b) Treated (2wt.% synthesized hydrogel). Nucleus were stained by Hoechst 33258. (Scale bar corresponds to 20µm)

**3.4.6 *In vitro* Assessment of Wound Healing Therapeutic Efficacy.** Post-injury tissue reconstruction is an important aspect of the healing process which require the migration of cells to the injury site. Substance P has been observed to induce cellular migration toward the wound vicinity and speed up the wound-healing process. An *in vitro* migration-based scratch healing assay was eventually performed to analyze the healing potential of the designed substance p-derived fusion peptide sequence in inducing cellular migration. WI38 cells were allowed to adhere on the 12-well plate to grow in a single monolayer and a fine scratch had been made with a 200µL tip followed by peptide treatment in serum-free media. The scratch region was monitored consistently by using an inverted microscope at different time intervals starting from 0 to 72 hr. Images were taken in greyscale to quantify the healed area by the migrated cells. From the microscopic images [Figure 3.14(a)] as well as further quantification results shown in Figure 3.14(b) it appeared to us that the hydrogel-conditioned media induce a substantial migratory effectivity on the cultured serum-starved cells from 24h interval duration. After 48 hr the treatment media was changed with another and the difference in scratch healing extent remains quite significant as compared to the control. At the end of 72 h, the scratch in the hydrogel-treated group was found to heal uniformly whereas in the control it remained incompletely covered. From the overall study, we got an insight regarding the hydrogel and its overall cytocompatible nature exhibited in 2D culture conditions, which substantializes its applicability as a futuristic therapeutic biomaterial having tissue repairing potency.

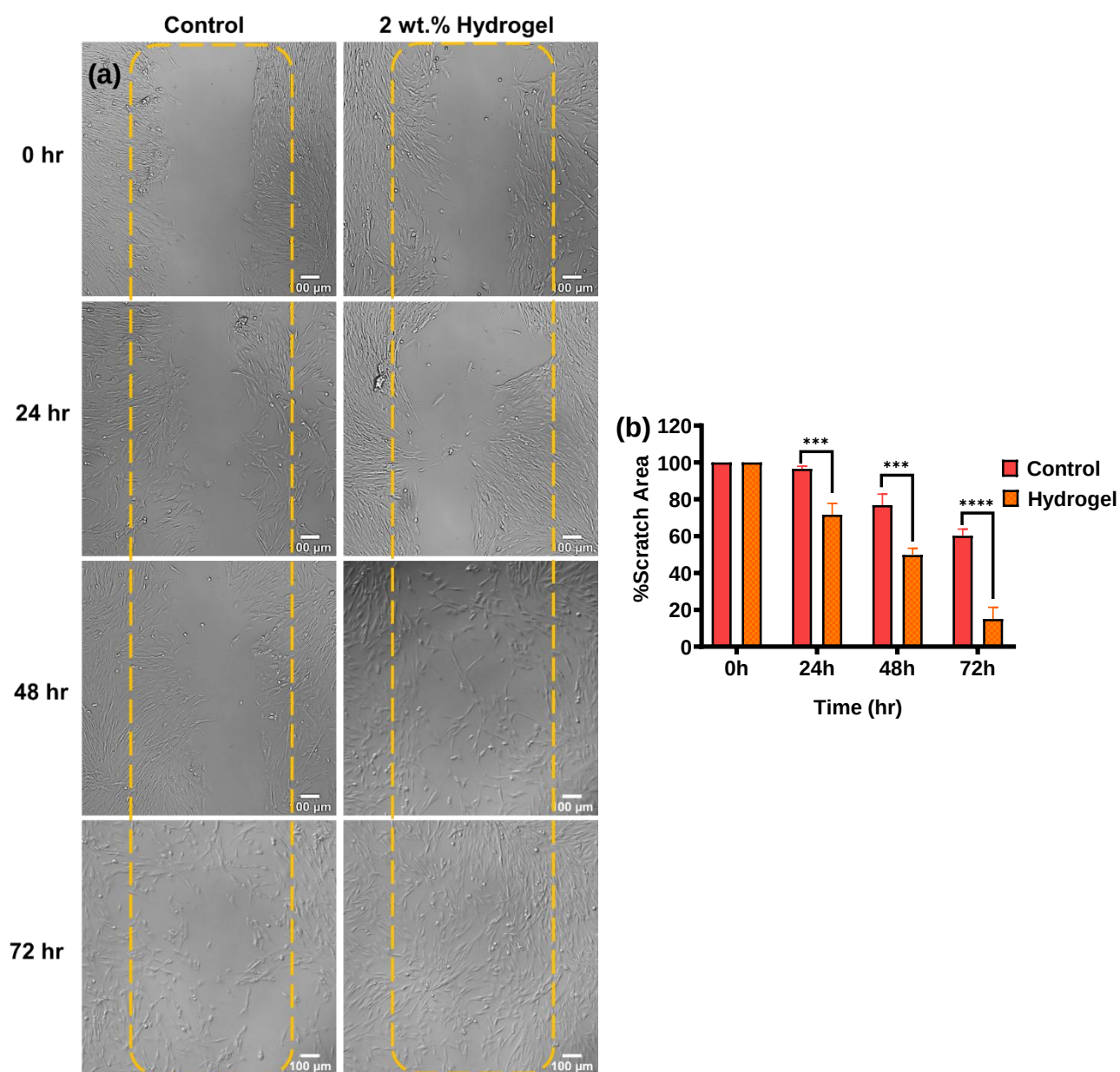


**Figure 3.14** (a) DIC images of scratch healing assessment performed on WI-38 cells for both control (left panel) and synthesized hydrogel (20mg/mL) containing media treated cells (right panel). (Scale bar corresponds 100μm). (b) Quantified result obtained for scratch repairing assay performed on WI38 cells in presence of hydrogel conditioned media.

### 3.5 CONCLUSION

Cytocompatible ECM impersonating engineered peptide-based hydrogels provides bodacious biomaterial-based therapeutic effectivity in tissue engineering and drug delivery sectors. Though an 11 amino acid comprising tachykinin neuropeptide Substance P exhibited excellent wound healing and cell proliferating aptitude in earlier research results its therapeutic application as a biomaterial-based platform was not well explored. Here in this reported work, we have constructed a Substance P mimicking supramolecular hydrogelator

octapeptide by fusing truncated C-terminus peptide sequence “FFGLM” derived from Substance P along with an integrin-binding “RGD” motif. This designed octapeptide under its N-terminus aromatic capping group and its uniquely balanced hydrophobic and hydrophilic residues undergoes quick self-assembly at biological pH (pH 7.4) to give rise to a soft yet thixotropic hydrogel matrix with superior thermal and pH responsive property. From the scanning electron microscopic data, we further explored its tangled fibrous structural features. Cell viability results demonstrated its noncytotoxic effectivity towards a diverse array of cell lines which further inspired us to check its effectivity in hydrogel form. This fabricated hydrogel network due to its encapsulated high % of water content and presence of integrin binding RGD epitope provides an excellent cytocompatible environment for the *in vitro* cell culture. Occurrence of “RGD” epitope together with aggregation prone “FFGLM” region in a single peptide molecule facilitates the formation of a biocompatible hydrogel platform with more simplicity and effectivity than the previously reported work.<sup>55</sup> In this work, we have used four different types of cell lines to check the biocompatibility of this hydrogel with them and used both LIVE/DEAD and actin staining assessment to check its cytocompatible nature. The extent of calcein AM stained live cell population was at par with the control TCP plates even after 48h of culture duration which was further supported by the healthy actin skeleton network of the cultured cells found in Alexa fluor-488 phalloidin staining of the intracellular actin network. Further scratch assay results indicated that this fusion peptide hydrogel can provide a comparable wound healing effectivity as provided by full-length Substance P peptide which was not reported earlier with Fmoc FF or even with FmocFF/FmocRGD hydrogel composites. From the above results and experimental analysis, we envisioned this designed hydrogel as a futuristic cytocompatible matrix having both wound healing and pH responsive drug releasing therapeutic effectivity.

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