5 Results and discussions

5.1 Fabrication of hydrogel from pure polymer

For the synthesis of uniform hydrogel scaffolds, first the fabrication process parameters were optimized. The prepared solutions were poured in cylindrical moulds and allowed to cross link for 2 h at RT. The hollow cylindrical moulds were covered with paraffin at both ends and pinched with needle to allow uniform drying from both sides (figure 12).

![Figure 12: Cylindrical molds for hydrogel fabrication.](image)

To get information of network structure formation of hydrogel formed by crosslinking of polymer chains, first the hydrogels were fabricated with pure polymer samples. To begin, chitosan with different viscosity and different concentration was used for the hydrogel formulation. Next, gelatine was blended with chitosan to form chitosan–gelatine hydrogel. Glutaraldehyde was used as crosslinker. The parameters like polymer concentration, polymer molecular weight and cross linker concentration was optimised for the formulation to get desired result.

5.1.1 Polymer and crosslinker concentration

To determine the minimum amount of polymer and crosslinker for desired hydrogel formulation, different combination as given in table 3 were used. In 0.5 and 1 % chitosan, all
the hydrogels were squeezed after drying. This could be due to lower amount of polymer in the solution that were unable form strong physical structure (A1, B1, C1 and A2, B2, C2). The polymer density was not enough to create stable structure. At 2% chitosan solutions, the hydrogel were formed with good morphology, however they were collapsed after swelling. With 0.5% glutaraldehyde (C1, C2, and C3), the hardness and mechanical strength of hydrogels were observed to be highest amongst other concentration. Overall, in all of these combinations, hydrogel shape was not properly maintained. This could be due to lower concentration of cross linker added in the solution. 1% glutaraldehyde was required for proper cross linking.

Table 3: combination of polymer and crosslinker concentrations for hydrogel fabrication.

<table>
<thead>
<tr>
<th>Chitosan M.V. (%) w/v</th>
<th>Glutaraldehyde (% v/v)</th>
<th>0.1</th>
<th>0.2</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>A1</td>
<td>B1</td>
<td>C1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A2</td>
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<tr>
<td>2</td>
<td>A3</td>
<td>B3</td>
<td>C3</td>
<td></td>
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</tbody>
</table>

Previous studies discovered the minimum required polymer and crosslinker concentration for the desired hydrogel fabrication. Further, the polymer molecular weight and amount of crosslinker was optimised. For that, 1% w/v solution with lower, middle and higher viscosity chitosan was used with 1% glutaraldehyde with different ratio of 1:10, 1:20, 1:50 and 1:100 to polymer concentration. The gels were dried in centrivap vacuum drier at 4° C. In these entire study, quality of gels were not desirable as shape was completely lost after drying in all the formulation (figure 13). The polymer concentration might not adequate
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in the system to sufficiently crosslink functional moieties and form packed network formation. Therefore, insufficient crosslinking density could not maintain the structural integrity, which resulted in collapse of structure during the drying process. It was also revealed that, at this concentration, the polymer molecular weight did not affect the hydrogel formulations.

Figure 13: chitosan hydrogel of 1% low viscosity chitosan with 1% glutaraldehyde (1:100 ratio), dried in centrivap at 4 °C. The structure was not retained after drying. The scaffolds were squeezed and cracks were observed several places in dried hydrogels.

Moreover, in few samples except 1:10 ratio of glutaraldehyde, they were ruptured at several places after processing. This might be due to unavailability of crosslinker uniformly in the solution. The amount of glutaraldehyde solution should be enough to evenly blend with entire polymer solution. Therefore, despite of having sufficient amount of
glutaraldehyde molecules, because of less amount of its solution, it cross-linked only those moieties, where it was having in proximity to glutaraldehyde. In course of time, the cross-linked network was formed at various places which restrict the polymer and crosslinker movement. As a result, some polymer functional moieties were remain uncross-linked and those portions were ruptured after drying (figure 14).

Figure 14: chitosan hydrogel with 1 % medium viscosity chitosan and 1 % glutaraldehyde (1:20 ratio), dried in centrivap at 4 °C. Due to unequal distribution and unavailability of crosslinker throughout the scaffolds, the crosslinking was not followed and the structures were collapsed at the top after drying process.

These studies pointed out the requirement of polymer concentration and amount of crosslinker for the successful fabrication of desired hydrogels. These results indicated that minimum 2 % polymer with 1 % crosslinker is required for appropriate and sustained cross-linked network structure of hydrogel. Moreover, the crosslinker should be evenly distributed and mixed throughout the polymer to obtain uniformly cross-linked hydrogel structure.

5.1.2 Process parameter

The ice crystals can be form during freezing, which successively crate open pores after drying. Next, effect of freezing on hydrogel synthesis was determined and freezing parameter were optimised. The solution of 2 % chitosan were prepared with middle and high viscosity for hydrogel synthesis. The solutions were cross linked with 1 % glutaraldehyde with 1: 10
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ratio and vacuum dried in centrivap vacuum drier at 4°C without freezing. In this formulation, the gels were formed with improved physical integrity. The gels, were elastic in nature and maintained the network structure after swelling also. However, they were little squeezed, might be due to high temperature drying. Therefore, after addition of cross linker, the solution was poured in mould and kept for freezing at -20°C and -80°C successively, then dried. This methodology has improved the shape of hydrogel (figure 15 A). Similar polymer solution with crosslinker was used to synthesise hydrogel in lyophilizer. Consequently, after freezing step, moulds were kept in lyophilizer at -80°C for drying, which produced hydrogel with improved morphology and shape (figure 15 B).

Figure 15: Hydrogel made up of 2 % (w/v) Medium viscosity chitosan solution cross linked with 1 % glutaraldehyde. A) Dried in vacuum drier, B) dried in lyophilizer at -80°C.

The freezing was done as -20°C for overnight and then in -80°C for 24 h. The freeze drying was done with Labconco FreeZone 6 Liter Console Freeze Dry Systems with Stoppering Tray Dryers chamber. The pre-frozen gel were vacuum dried in chamber with the following programme (table 4).

Table 4: The program cycle for freeze drying. The cycles involves steps and parameters used for drying.

<table>
<thead>
<tr>
<th>Sr no.</th>
<th>Temperature</th>
<th>Cycle</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle 1</td>
<td>-20 °C</td>
<td>Pre-freezing</td>
<td>2-4 h</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>-20 °C</td>
<td>Primary drying</td>
<td>24 h</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>4 °C</td>
<td>Secondary drying</td>
<td>36 h</td>
</tr>
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</table>
All the hydrogels were freeze dried with this set up to maintain the uniformity in structure during drying process. Finally, the optimised parameters for hydrogel synthesis are, 2% w/v solution of medium viscosity chitosan blended with 1% v/v glutaraldehyde (1:10 ratio), poured in moulds and allowed to crosslink for 2 h. Followed to that, the gels were frozen and lyophilized. The final hydrogels with optimized parameters are imagined as figure 15 B.

Similarly, gelatine was blended with chitosan in 1:1 ratio having final concentration of 2% polymer solution and cross-linked with 1% glutaraldehyde to form hydrogels. These hydrogels have more uniform physical structure. It was found more elastic than pure chitosan hydrogel and can be easily compressed by physical force and retain back after removing the force.

Gelatine is a hydrolysed product of natural protein collagen. It has a several attributes including biocompatibility, biodegradability, increased cell adhesion, proliferation, migration makes it suitable biomaterial. Blending gelatine with chitosan can improve the adhesiveness of scaffold due to presence of cell adhesive Arg-Gly-Asp (RGD) peptides. Furthermore, gelatine has free carboxyl groups in its backbone, enabling it to form polyelectrolyte complex with cationic chitosan [146]. This bonding can increase the mechanical strength of scaffold. Moreover, the other physical properties including swelling, porosity would also be affected by presence of gelatine.

5.2 Characterization of pure polymer based hydrogel

5.2.1 FTIR

The FTIR spectra of CH with chitosan powder is shown in figure 16 respectively. The broad peak observed at 3351 cm\(^{-1}\) in both the spectra represented to the characteristic peak of hydroxyl group. The peak at 1592 cm\(^{-1}\) attributed to amino group of chitosan side chain
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A peak at 1640 cm\(^{-1}\) was due to stretching vibration of acetamide in chitosan [148]. A significant peak exclusively appeared at 1540 cm\(^{-1}\) in CH and CGH spectra was of imine bond (C=N) formation by crosslinking reaction between aldehyde group in glutaraldehyde with amino groups in the chitosan [148]. Moreover, the ether group peak at 1074 cm\(^{-1}\) was shifted marginally to 1064 cm\(^{-1}\) and becomes stronger suggesting establishment of a new ether linkage in hydrogel after crosslinking by glutaraldehyde. This representative peak confirms the Schiff base reaction between chitosan and glutaraldehyde forming polymer network structure.

![FTIR spectra of Chitosan hydrogel and chitosan powder. The arrow shows peak of imine bond formation in hydrogel, confirming crosslinking reaction with glutaraldehyde.](image)

**Figure 16:** FTIR spectra of Chitosan hydrogel and chitosan powder. The arrow shows peak of imine bond formation in hydrogel, confirming crosslinking reaction with glutaraldehyde.

The figure 17 shows FTIR spectra of CGH along with chitosan and gelatine powder. The gelatin was considered by its carbonyl and amide peaks at 1639 cm\(^{-1}\) and 1534 cm\(^{-1}\), respectively. The strong absorption at bands at 3270 was due to –N–H stretching in gelatin spectra. Moreover, 1234 cm\(^{-1}\) peake denoted –C–O torsion. FTIR spectra of CGH represented
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similar representative peaks of the parent molecules. The intensity of the peaks at 897 cm$^{-1}$ was decreased in CGH as compare to chitsan powder due to prescence of gelatin. These characteristic peaks were absent in pure gelatin.

![Figure 17: FTIR spectra of Chitosan-gelatine hydrogel.](image)

The CGH spectra represents similarity in peaks of parental compounds. The arrow shows significant peaks for Schiff base formation between chitosan and gelatine with glutaraldehyde to form polymeric chin network structure.

The presence of gelatine was led to changes such as shifting of characteristic bands. The amino and carboxyl peaks at 1654 cm$^{-1}$ and 1580 cm$^{-1}$ in the spectrum of chitosan shifted to a lower wave number as gelatine was incorporated in CGH. These slight but evident changes suggest polyelectrolyte complex formation between chitosan and gelatine polymer chains by intermolecular hydrogen bonding. These property is also depicting the formation of an interpenetrating polymeric chain network of chitosan and gelatine.
5.2.2 Porosity

The porosity and surface morphology of chitosan and chitosan-gelatine hydrogel were analysed by SEM. The images (figure 18) showed interconnected pores in both hydrogels. The scaffolds were highly porous with a mean pore size greater than 140.93 µm and 118.90 µm for CH and CGH respectively. The structure of pores in CGH was different from CH. The pore membrane around each pores in CGH was thick and wide compare to CH. Moreover, the pores in CGH were comparatively spherical related with CH. Scaffolds exhibit size distribution of pores given in the graph. The graph depicted that the CH having pore size range of 80 -240 µm, while in CGH it was 80-160 µm only. Moreover, CGH have 90 % of pores were having 100-140 µm pore size. These data indicated that the CGH have very uniform pores compare to CH. The porosity of the CH and CGH scaffold was measured to be 92.64 and 96.31 in water and 84.25 and 92.21 in N- Hexane respectively.

![Figure 18: Pore size distribution of CH and CGH hydrogel.](image)

The graph prepared from SEM images. The upper raw shows pore size distribution graph for hydrogel formulations of chitosan and chitosan-gelatine hydrogel. The lower panel shows SEM images of respective hydrogels. Total 50 random pores was selected per image. Scale bar: 300 µm.
The porosity is an essential parameter to regulate physico-chemical properties of scaffolds. Importantly, the permeable hydrogel could allow the water to enter inside the scaffolds that determine the swelling properties. Moreover, the pore morphology could also affect the mechanical properties of scaffolds, as increasing the distance between scaffold membranes could imbalance the mechanical stress and reduce the strength of scaffolds. The uniform spherical pores are helpful to maintain the structural integrity of scaffold and mechanical strength. Moreover, the porous structure is required for cells to provide 3D support for adhesion and growth to form tumor sphere. The porous structure allows nutrient and gaseous exchange required for cells vital process. 100-150 µm size pores were reported as optimum pore size for most of the cells that was sufficient enough for cells to communicate with each other and form spheroids [149]. Furthermore, present pores was thought to provide adequate permeability for nutrient transport and formation of spheroids having more than 100 µm size and create hypoxic condition. In this study, it was showed that incorporation of gelatine have improvised the properties of scaffold required for 3D tumor cell culture.

5.2.3 Swelling study

Swelling studies displayed that both the hydrogel swelled more than 90% within 1 min. The CGH demonstrated more than 96 % EWC while in CH it was 93 %. This data determined that the CGH hydrogel having quick water absorption capacity than CH. Incorporating gelatine with chitosan could increase not only the water absorption time, but total water holding capacity also.
Figure 19: Swelling study of chitosan hydrogel and chitosan-gelatine hydrogel in water. Study carried out for the duration of for 24 h, showing equilibrium water content of hydrogel w.r.t. time. All data points were generated with n=3.

The swelling behaviour of hydrogel is crucial parameter for cell culture applications. Water absorption is essential process as the cell culture studies are carried out in aqueous medium only. The quicker and greater water holding capacity of a materials and scaffolds are beneficial for nutrient transport inside the scaffolds. Appropriate design of swelling parameters permits hydrogels to regulate the diffusion of bioactive molecules and cell migration through the complex network structure.

5.2.4 Cells growth and morphology

The A549 lung adenocarcinoma cells were grown on 9 X 1 mm (D x H) CH and CGH scaffolds. The light microscopy images displayed that the cells were adhered on the both the scaffold walls (figure 20). The morphology of cells were different than 2D monolayer. Generally, the cells have spindle shape, elongated morphology in 2D tissue culture
polystyrene surface (TCPS). While in CH and CGH hydrogels, cells were having globular in shape, aggregated with each other. The cells were formed clumps inside the pores and attached on the wall of hydrogels. Moreover, visualization of cells at different focal planes confirmed their infiltration inside the scaffolds and spread across the depth in three dimensions.

![Figure 20: Light microscopic image of A549 cells grown on hydrogel scaffolds. A) Cells on chitosan hydrogel, B) cells on the chitosan-gelatine hydrogel. The images taken at 10X magnification.](image)

To further investigate the cellular morphology, the scanning electron microscopy was done with cell laden hydrogels of CH and CGH. The images were exhibited cells attachment on scaffolds (figure 21). It was visualized that cells were adhered in 3D, however, the cells were very much spread out so the total area was increase. The hydrogel provides 3D architecture for cells to adhere. However, cells could not grow as 3D structure as the dimensionality and aggregation of cells were not increased. This phenomena might be due to greater adhesion of cell surface to the scaffold wall. Chitosan is cationic polymer which supports cell adhesion to its surface. Moreover, gelatine also supports the cell adhesion. Therefore, this scaffolds only provide adhesive surface which allow increased attachment of cell membrane. Due to that, cell membrane exposed to wider area for attachment and eventually spread the cell structure. Therefore, despite of providing 3D structure by the hydrogel, the cells could not form physical 3D structure at individual cell level.
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These initial experiments have established proof of principle for chitosan based hydrogel. Several key outcomes were brought out from these initial experiments. These experiments showed that the hydrogels were successfully prepared based on chitosan polymer. The polymer concentration and molecular weight was determined for desirable fabrication. The hydrogels were characterized physico-chemically defining that the hydrogel have provide suitable architecture and surface for cell adhesion and growth. The cell culture studies confirmed the biocompatibility of scaffolds and showed cellular adhesion and growth on the scaffolds. However, the porosity and surface chemistry properties have only provided the structural features to cells. They have not contributed in encouraging tumor cells to form three dimensional spheroid structure. The cell adhesive nature of chitosan facilitates cell spreading on its surface rather agglomeration. This observations was the inspiration for modifying the surface chemistry of scaffold that limit the cells adhesive property of scaffold.

5.3 Polymer selections and modifications

A balance between fouling and non-fouling surface is most critical criteria for cell assembly and adhesion on scaffold. Therefore, selection of polymer and crosslinker were
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significantly important for successful completion of proposed work. Furthermore, usage of
diluted acetic acid to solubilize chitosan and requirement of glutaraldehyde for crosslinking
was undesirable which needs to be modified to avoid cytotoxicity. Therefore, it was decided
to introduce biocompatible polymer dextran which not only imparts hydrophobicity to
surface but also crosslinks the polymer as in oxidised form. Therefore, both the polymers
were modified. The chitosan was modified to make it water soluble whereas dextran was
modified to provide aldehyde group.

5.3.1 Oxidation of dextran

The dextran oxidation was carried out by sodium periodate in single step reaction
which yields a purified product with a simple dialysis step. The purified product was
lyophilized to obtain dry Odex polymer. The Odex appeared as a white, odourless, fluffy
fibrous structure after lyophilisation. The sodium periodate attacks between C3-C4 carbon
and C3-C2, cleave it and generate aldehyde groups. The open carbons can be further reduced
on a second and independent oxidation reaction by releasing formic acid (figure 22). The
Odex The modification was analysed quantitatively and qualitatively.
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Figure 22: Schematic representation of reaction of oxidation of dextran by Sodium periodate. Sodium peroidate can oxidise the dextran at two positions and can attack twice to make doubly oxidised molecule.

5.3.1.1 FTIR

The Functional groups of dextran and Odex were identified by FTIR spectra (Figure 23). The broad peak at the region of 3500 cm\(^{-1}\) corresponded to the OH stretching vibration of the polysaccharide in both the spectra [150]. However, a decrease in the intensity of OH in the Odex spectra might be attributed to the conversion of OH group into CHO group. Similarly, the peak at 2920 cm\(^{-1}\) was assigned to C–H stretching vibrations of dextran and Odex [150, 151]. The sharp peak appears at 1003 cm\(^{-1}\) and shoulder peak at 1143 cm\(^{-1}\) were the characteristic band of asymmetrical C-O-C vibrations [152]. Small shoulder peaks at 816 cm\(^{-1}\) and 920 cm\(^{-1}\) confirmed the presence of (1→3)-α-D-glucan [153]. The peak at 1636
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$cm^{-1}$ indicated for aldehyde group formation in Odex which was completely absent in pure dextran spectra [154, 155]. These characteristic peaks gives evident that the oxidation of dextran was effectively completed and successfully created multiple aldehyde groups in the polymer backbone. These aldehyde groups would be utilized to crosslink amine groups present in chitosan.

![FTIR spectra of Dextran and Oxidised dextran.](image)

**Figure 23:** FTIR spectra of Dextran and Oxidised dextran. The arrow shows signature peak of modification.

### 5.3.1.2 NMR

The $^1$H-NMR spectra of dextran and Odex are shown in **figure 24**. The spectrum shows multiple peaks at 3.1~3.8 corresponding to protons of the glucopyranosyl ring of dextran monomer. Aldehyde proton was expected between 9.0 to 9.5 ppm in NMR spectrograph of Odex, which was not observed in this data [135, 138]. The absence of aldehyde proton might be due to the formation of hemiacetals or hemialdals by reaction of an aldehyde with
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neighboring hydroxyl groups. The presence of multiple peaks between 4.0 to 5.5 ppm could be the indirect evidence of the occurrence of hemiacetal groups during the reactions [135]. These observations confirmed the oxidation of dextran that produced aldehyde groups on the dextran chain. The utility of Odex as crosslinker and potential to form network structure was further confirmed during the fabrication of hydrogel.

![NMR spectra of dextran and Odex recorded in D2O](image)

**Figure 24:** $^1$H NMR spectra of dextran and Odex recorded in D$_2$O. The insert image on each spectra shows chemical structure of respective polymer.

5.3.1.3 **Titration**

The oxidation degree of dextran i.e. amount of aldehyde group was quantified by reaction with hydroxylamine hydrochloride. The reaction product released hydrochloride molecule which was quantified by titrating the solution with standard NaOH with methyl red indicator. The results showed that the amount of aldehyde was not changed from 1 h reaction to 24 h. These data indicated that the reaction was completed in 1 h and no further oxidation was observed at 24 h reaction (figure 25 A). The oxidation with equimolar and 50 %
equimolar sodium periodate was 55.85 and 27.62 % respectively (figure 25 B). The production of lower concentration of aldehyde as compare to theoretical value was anticipated because of the double oxidation reaction and/or hemiacetal formation in the Odex chain. The hemiacetal could be formed by reaction of aldehyde group with vicinal OH group, which was also detected in \(^1\)H NMR.

![Figure 25: Amount of aldehyde produced per dextran monomer, determined by titration with ammonium hydroxide hydrochloride. A) Effect of time of reaction on oxidation of dextran by sodium periodate, B) Effect of amount of sodium periodate on the dextran oxidation. All experiments were done with n=3; *** denoting P<0.001 significance difference.](image)

It was reported that Odex with approximately 25 % oxidation have least protein absorption [156]. Dextran is a polymer of branched glucan comprising several –OH groups in the structure. The oxidation reaction replace the –OH groups to –CHO groups. Importantly, –OH groups serves as both hydrogen bond acceptors and donors molecules, thus can also react with each other. While –CHO groups are only hydrogen bond acceptors therefore they can only make hydrogen bond with surrounding molecules of water. Oxidation makes relative conversion of –OH to –CHO, which maintain the amount of hydrogen bond donor and acceptor molecules. This balance of donor/acceptor molecules encourage the interactions of polymer with surrounding water molecules and perturb the interactions with proteins [156]. Therefore, Odex with 27.62 % oxidation was selected for the hydrogel formation.
5.3.1.4 GPC

The molecular weight of Odex was determined by GPC. After the oxidation reaction, the molecular weight was reduced to 44416 Da from 70000 Da with 1.4 polydispersity index. This study demonstrated that with 50% equimolar NaIO$_4$ reaction, the Odex chain length was reduced to 37%. This reduction in chain length resulted in contributing factor for 1) the rate of crosslinking, 2) homogeneous distribution of polymeric chain network, which may be attributed to semi IPN network.

5.3.2 Thiolation of chitosan

To perform entire polymerization reaction at physical pH, it was essential to modify the chitosan which usually solubilizes in an acidic environment. The cysteine was grafted on chitosan side chain by zero length crosslinker EDAC. The carboxylic acid group of cysteine was activated to O-acylisourea intermediate by EDAC. The amino groups present in chitosan have delivered nucleophilic attack to this active group. As a result, primary amine present at chitosan formed an amide bond with carboxyl group and EDAC by-product was released (figure 26). The soluble urea derivative by-product was removed from the system during dialysis. The purified product after lyophilisation was having white color fluffy network structure. The modified chitosan was dissolved in diH$_2$O at neutral pH giving indirect evidence of thiolation of chitosan. The thiolation of chitosan was characterized qualitatively and quantitatively.
Figure 26: Schematic representation of reaction of cysteine grafting to chitosan by EDAC crosslinker. The cysteine attach via carboxyl group with amine moiety on the chitosan. The EDAC release as urea by-product after the reaction.

5.3.2.1 FTIR

The grafting of cysteine was characterized qualitatively by FTIR. The FTIR peaks showed at 1646 cm\(^{-1}\) for amide I, 1574 cm\(^{-1}\) determined -NH2 bending, and 1368 cm\(^{-1}\) for amide III supports the presence of chitosan structure in both the spectra (figure 27). The absorption bands at 1061, and 1018 cm\(^{-1}\) was attributed to skeletal C-O-O stretching vibration. The peak at 2364 cm\(^{-1}\) in modified chitosan spectra showed the presence of thiol group on chitosan [157]. These data confirmed that the grafting of cysteine was successfully accomplished on the chitosan back bone which gives aqueous solubility to chitosan.
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Figure 27: FTIR spectra of chitosan and thiolated chitosan. The arrow shows signature peak for thiol group confirming the modification of chitosan.

5.3.2.2 Ellman’s assay

The amount of thiol grafted on chitosan was quantified by Ellman's assay. DTNB reacts with a sulfhydryl group to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB), which was quantified at 412 nm. The amount of cysteine quantified in thiolated chitosan was 6 µM/gm of chitosan. The experimental amount was less as compare to estimated value. The lower amount of cysteine might be due to polymerization of cysteine molecules by EDAC. However, in the current study, the purpose of cysteine grafting was to increase the solubility of chitosan in aqueous solution at neutral pH. The grafting of 6 µM/gm cysteine in chitosan had solubilized the chitosan at pH 7. In addition to that, cysteine provided the thiol group to chitosan chain, which can interact with other thiol groups to form disulfide linkages network in the hydrogel. Importantly, the present amount of cysteine was considered to be safer for cellular studies. The cysteine with more than 100 µm concentration in the cell culture media
was reported to be toxic to the cells by producing hydrogen peroxide and free radicals [158-160].

5.4 Hydrogel fabrication from modified polymer

Three different feeding concentration of Odex (3%, 4%, 5% w/v) were blended with equal volume of 4% w/v TC to form hydrogels. These formulations viz. HB 3/4, HB 4/4, HB 5/4 were fabricated for optimizing hydrophobicity, pore size, and morphology. The control scaffold for Odex named as HB C/4 was having 4% TC cross-linked with glutaraldehyde. The dried hydrogels were having ivory in color with spongy structure. The Cylindrical hydrogel was having diameter of 9 mm and 20 mm height which was cut into the height of 1 mm and used for remaining studies (figure 28).

**Figure 28: Digital Image of hydrogel.** Left; pre-fabricated dry cylindrical hydrogel, centre: sliced 1 mm dry disc and right: wet disc swollen in dI H_{2}O. The disc turn into semi-transparent after swelling.
5.5 Physio-chemical Characterization of scaffolds

5.5.1 FTIR

The FTIR analysis was done to characterize crosslinking of polymers in hydrogel. The presence of peak at 862 cm⁻¹ in Odex and hydrogel represented the (1→3)-α-D-glucan structure [153]. While the peaks at 1060, and 1018 cm⁻¹ in hydrogel and TC spectra was indicated C-O-O stretching vibration, which depicted the structure of chitosan.

![FTIR spectra of MDC hydrogel with Odex and thiolated chitosan. The arrow in the inset spectra shows signature peak of imine bond in the hydrogel.](image)

A Peak observed at 1548 cm⁻¹ in hydrogel spectra, indicated the presence of imine bond (C=N) (figure 29). The formation of imine bond confirmed the crosslinking of amine group of chitosan with aldehyde of Odex. Moreover, the small peak at 550 cm⁻¹ corresponds to the disulfide linkage in the hydrogel. However, this peak was also visualized in TC spectra,
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which indicated that during thiol modification, some thiol groups were formed disulfide bridges.

Overall, these observations confirmed that the cross-linked polymer network structure has been formed in the hydrogel. The Schiff base formation and disulfide linkage has been formed that created binary cross-linked interpenetrating network structure of hydrogel (figure 30).

![Diagram of reaction of cross-linked binary network formation in hydrogel](image)

**Figure 30**: Schematic representation of reaction of cross-linked binary network formation in hydrogel. The aldehyde group of Odex covalently bind with carboxyl group of chitosan establishing interchange imine bond. The thiols of modified chitosan chains make inter or intra molecular disulphide bridges.
5.5.2 *Morphology and porosity analysis by SEM*

The pore size was measured from SEM images by ImageJ software (figure 31). The pore size distribution graph shows that the maximum number of pores in 3% Odex hydrogel were between 190-200 µm, while in 4% and 5% Odex hydrogel, the maximum pores were between 160-170 µm and 150-160 µm respectively. The pore size distribution in 3% and 4% Odex hydrogel was in the range of 120 to 280 µm whereas in 5% Odex hydrogel, the pore size extended from 90 µm to 280 µm. The pore distribution pattern of increasing Odex concentration indicated the increase in cross-linking density which was resulted in the reduction of pore size. Moreover, this distribution also revealed that the 4% Odex hydrogels had more than 70% of pores positioned between 140-180 µm (40 µm range), while in the case of 3% and 5%, it was 160-220 µm (60 µm range) and 120-190 µm (70 µm range), respectively. The narrow distribution of pore size for 4% Odex hydrogel represented very uniform pores throughout the hydrogel. The HB 4/4 could be appropriate as it was having uniform pores throughout scaffold. It was having sufficient void space for cells to grow and form microtumors with size of greater than 100 µm, which is necessary for creating hypoxic condition. The mimicking of *in vivo* hypoxic characteristics is not possible with microtumors having 99 ± 20 µm size [161, 162].
Figure 31: Pore size distribution of MDC hydrogel with different Odex feeding concentration calculated from SEM images. The upper raw shows pore size distribution graph for hydrogel formulations having 3, 4 & 5 % Odex. The lower panel shows SEM images of respective hydrogels. Total 50 random pores was selected per image with n=3 images.

Porosity have a significant impact in regulating cellular adhesion and migration. Higher porosity could enhance the cell adhesion [163]. High porosity with interconnected pores increases the total surface area for cell attachment and accelerates cell proliferation in the scaffolds. High porosity also provides more surface area for cell-matrix interactions, space for ECM deposition, and cell aggregation [164, 165]. Therefore, it is desirable to fabricate highly porous scaffolds for generating 3D tumors. The pore size influences cell adhesion, morphology, spheroid formation and phenotypic expression [166]. The specific pore size provides structural advantages to cells to grown and from spheroids. The cells adhere inside the pores at different locations and “bridges” from adjacent cells. There is an optimal pore size range for endorsing cell ingrowth. Above this range, cells unable to spread and associate to other cells to form networks. The optimum pore size is subjected to the scaffold materials and cell types. Majority of mature cells are unable to colonize on scaffolds having >300 μm pore size because of the inability to connecting large bridging spaces [87, 167-169]. Reversely, smaller pore size may hinder the cellular infiltrations as well as
adequate nutrient and waste exchange. It was showed that chitosan-gelatine scaffolds having 50–80 μm pore size reduced the cell viability of fibroblasts and endothelial cells, relative to 100 to 150 μm [146]. Not only cellular functions are affected by porosity also have an impact on scaffolds properties including elasticity and stiffness. For instance, the elasticity of scaffolds rises as the number of pores in the scaffold increases [170]. Therefore, careful fabrication and selection of microporous hydrogel which balance the overall properties of hydrogel and provide optimised feature for creating 3D tumor is desirable.

5.5.3 Hydrophobicity index

The hydrophobicity index (H-index) was calculated to determine the role of Odex and its concentration on hydrophobicity to the hydrogel. All three concentration of Odex hydrogel and glutaraldehyde cross-linked hydrogel were used for measuring H-index (Table 5). The H-Index was decreased in Odex based hydrogel. However, a very small decrease was observed by increasing the concentration of Odex from 3 % to 5 %. The decrease in H-index from 0.35 to 0.30 of Odex hydrogel as compared to non-Odex hydrogel demonstrated the role of Odex in hydrophobicity of scaffold. The presence of –CHO from Odex and –OH from unmodified dextran bound to water molecules stronger than chitosan.

Table 5: Hydrophobicity index (H-index) of hydrogel formulations.

<table>
<thead>
<tr>
<th>Odex : TC concentration (%) w/v : % w/v</th>
<th>H-index</th>
</tr>
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<tbody>
<tr>
<td>HB C/4</td>
<td>0.3528 ± 0.0169</td>
</tr>
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<td>HB 3/4</td>
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</tr>
<tr>
<td>HB 4/4</td>
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</tr>
<tr>
<td>HB 5/4</td>
<td>0.3019 ± 0.0101</td>
</tr>
</tbody>
</table>

The dextran –OH groups were converted to –CHO groups during oxidation reactions. Addition of Hydrogen bond acceptor molecule –CHO along with –OH groups balances the
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ratio of donor/acceptor molecules of the polymer which binds more strongly to surrounding water molecules [156]. Therefore, incorporating Odex have greatly reduced hydrophobicity to the scaffolds.

It was assumed that the hydrophobicity of a materials is strongly correlated with non-fouling ability of materials [171, 172]. In a hydrophilic material like dextran, the water molecules tightly bound and create a physical and energetic interface to inhibit surface protein adsorption. Removal of water molecules from both, material surface and protein is the essential step to facilitate protein adsorption. The disruption of highly energetic hydrogen bonds between water and material surface produces dehydration entropic effects and reducing free energy barrier for protein adsorption to material [173]. This is an essential basis for non-fouling materials [174]. The surface hydrophobicity of a scaffold is an important factor to influence cell response. The surface property regulate the cell adhesion and spreading on the scaffold, which determine the cell fate.

These studies have demonstrated the successful fabrication of scaffold by varying concentration of modified dextran, however, for further studies 4% Odex-4% TC hydrogel (named as MDC now onwards) had been selected due to their uniform pores compare to other scaffolds. Moreover, the hydrogel having 70 % of pores in the range of 140-180 µm, which is reported as very much suitable for the majority of cells and their fate process [87, 149, 166]. Furthermore, the hydrophobicity index was also lower compared to 3% hydrogel, however, not much difference with 5 % Odex scaffolds.

5.5.4 Protein adsorption

Protein adsorption study were performed to analyse the relation between Odex based hydrophobicity and antifouling nature of hydrogel. The study demonstrated that hydrogel cross-linked with glutaraldehyde had adsorbed BSA protein 5.15 µg/gm on the surface while
in MDC hydrogel it reduced to 2.24 µg/gm. Almost 50 % reduction in BSA protein adsorption evident the anti-fouling property of Odex on the hydrogel surface.

It is important to understand that the protein adsorption was rather reduced however not completely hindered, which could be due to the presence of thiolated chitosan in the hydrogel. The protein adsorption is depend upon surface properties including surface charge, hydrophobicity and polarity. Chitosan is cationic polysaccharide widely used as biomaterials for cell adhesive properties. Overall, blending Odex with modified chitosan compensate the protein adsorption on the surface. The fine balance between fouling and anti-fouling surface confine the protein adsorption and hence cell adhesion to the surface and possibly encourage cells to form aggregates.

5.5.5 Swelling studies

The swelling study was done in dI H₂O for 24 h at 37 °C. The equilibrium water content was more than 98 % in five minutes. In the course of 24 hours, no significant change had been observed afterward the hydrophilized scaffold surface and porous network allowed speedy water adsorption (figure 32). The scaffold contains 98.5% porosity which increases the surface to volume ratio significantly as demonstrated by change in diameter of the scaffold from 9 mm to 11mm after swelling (figure 32 inset).
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Figure 32: Swelling study of MDC scaffold. The experiment of 24 h, showing equilibrium water content of hydrogel w.r.t. time. All data points were generated with n=3. The inset image was the digital picture of dry hydrogel and wet hydrogel after swelling. The swelling have increased the size and transparency of hydrogel.

The swelling characteristics of hydrogel determine that how and what extent the scaffold can hold the water. The swelling parameter is an important feature of hydrogel as the diffusion of bioactive molecules occurs in aqueous media. The present scaffold showed very quick swelling behaviour in water that might be attributed to higher affinity towards water molecules of Odex. The greater EWC of scaffolds indicated strong bonding to water, which would be contributing factor towards non-fouling property of present hydrogel scaffold.

5.5.6 Mechanical properties

The rise in water adsorption and its affinity for water molecule also affected the mechanical properties of scaffold. The results showed young modulus (YM) of swelled MDC hydrogel was $33.1 \pm 2.6514$ kPa while in glutaraldehyde cross-linked hydrogel was $24.767 \pm 3.4847$ kPa. The increase in stiffness could be due to the presence of Odex based cross-
linking and increased water affinity in the gel. It has been well studied that tumor has stiff microenvironment. The normal lung have YM of 4~10 kPa which increases to 25~35 kPa in the diseased state [175]. It is apparent that each tissue has a distinctive stiffness and each call in a tissue has a characteristic rheology. The stiffness profile can change during development (branching morphogenesis in lung) or in pathological conditions (fibrotic tissue formation in tumors) [176]. Indeed, the mechanical properties of ECM and cellular rheology can strongly affect the cellular including cell differentiation, tissue organization and cell migration. Interestingly, data shows that MDC scaffold closely resembles this property of lung tumor compare to tissue culture polystyrene surface (TCPS) having 2~4 GPa stiffness [1].

Tumors are commonly identified by physical palpation as a rigid structure of a compliant tissue [91]. Tumor rigidity reflects a raise in interstitial tissue pressure and solid tension due to a disturbed vasculature and abnormal tumor growth [177]. An elevation in the elastic modulus of neoplastic cells driven by an altered cytoplasmic arrangement and matrix stiffening associated to fibrosis [49, 178]. The growing tumor cells produces compressive stress on the surrounding ECM, vasculature and interstitial space. This is accompanied by alterations in composition, integrity and topology of extra cellular matrix [179]. The modification of ECM includes increased expression and deposition of fibronectin, proteoglycans and collagen types I, III, IV [180, 181]. The elevated level of collagen and elastin is accompanied by higher expression of LOX enzyme, which crosslink both of this ECM proteins. In the stiff cancerous tissue, higher density and crosslinking of collagen alter the focal adhesion kinase (FAK) assembly. This in turn exposed the cells to FAK and induce the integrin mediated signalling [182]. Moreover, it provides three dimensional adhesions and a persistently elevated signalling of bidirectional FAK–Rho loop, which is essential to create and maintain the invasive phenotype. These studies evident that matrix stiffness plays significant role in tumor imitation, progression, migration and metastasis. Therefore, it is
crucial to maintain matrix stiffness for precisely recapitulating the native tumor phenotype, which is appropriately present in MDC hydrogel.

5.6 3D tumor formation and biological characterization

5.6.1 Cellular growth on hydrogel

To mimic the 3D tumor formations for drug toxicity assay, the biocompatibility and cell growth on the scaffold was accessed by cell proliferation assay (figure 33). The result demonstrated that the number of cells were $3.66 \pm 2.29 \times 10^5$ at day two as compare to seeding population of $0.5 \times 10^5$. This steep cell proliferation rate was reduced to 1.5 fold on successive days. The initial four-fold increase in cell number can attributed due to availability of cell adhesive surface and nutrient to majority of cells, which makes all the cells in actively growing state. However as cell were keep growing, the unavailability of adhesive surfaces forced cells to form tumor spheroids inside the scaffolds. In this closely packed tumor spheres, nutrient, oxygen and waste gradient could be generated. Because of that, unlike in monolayer culture, it would form differential zones including active zone of proliferative cells, quiescent zone of viable non-proliferating cells and necrotic core [183, 184]. Therefore, overall proliferation rate was compensated, as like in vivo solid tumors [185, 186].
5.6.2 Cellular viability

To evaluate the viability of cells grown on MDC scaffolds, confocal microscopy with live-dead staining was performed (figure 34). The result demonstrated that the cells were grown as aggregates with negligible number of dead cells. The size of microtumors were $60 \pm 23 \mu m$ on day 2 (figure 32, A-C), $146 \pm 29 \mu m$ on day 4 (figure 34, D-F) and $178 \pm 24 \mu m$ on day 6 (figure 34, G-I). Moreover, the number of microtumors per scaffold were also increased from day 4 to day 6. Z-stacking image of 100 $\mu m$ range was implemented on day 6 for the cell-laden scaffolds. The microtumors were not only expanded in x, y axes but the z-axes also showed the size more than 100 $\mu m$. 

Figure 33: Alamar blue assay for cell numbers of A549 cells on MDC hydrogel as a function of time. * $P < 0.01$ significance between day 2 & day 4; day 4 & day 6; n=3.
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Figure 34: Confocal images of A549 cells on MDC hydrogel stained with live Dead stain. The green color cells represent live cells and red for dead cells. A-C) 2 days, D-F) 4 days, G-I) 6 days of culture. Scale bar 200 µm.

These results demonstrated that MDC scaffold is highly supportive of tumor cell viability and growth. In addition to that, these results confirms 3D distribution of cells inside the scaffold establishing 3D microtumors consistent with previous results.
5.6.3 **Morphology of cells and 3D tumor formation on scaffolds**

5.6.3.1 **Cellular shape**

To study the surface encouraged cell behavior, SEM analysis of cell-laden hydrogel was performed (figure 35). The SEM images confirmed that cells were adhere selectively to the walls of Odex scaffolds and indicating for initiation of cellular clump on day 2 (figure 35 A, B). To confirm the role of Odex based surface chemistry, hydrogel having TC cross-linked by Glutaraldehyde was used as a control for cell adhesion and tumor formation analysis. On control scaffolds, cells were attached consistently with fibroblastic morphology (figure 35 C, D). The cellular clumps on MDC hydrogel were grown in size from 44.63 ± 5.87 µm at day 2 to 68.38 ± 16.92 at day 4. While the control surfaces continued to represent as cell adhesive substrate (figure 35 E-L). The size of microtumor was not grown significantly from day 4 to 6 on MDC scaffold, however an increase in number of microtumors per scaffold. Moreover, the SEM images displays tumor spheroids at day 4 had distinct cell periphery (figure 35 f). This indicates that the cellular clump has been formed. Interestingly, at day 6, the images are showing compact microtumor structure where cells were entirely covered by ECM molecules (figure 35 J). The microtumors were visualised as a single sphere without having any cellular boundary. These results indicate ECM production in microtumors at day 6. The SEM images revealed that the morphology and topography of control scaffold and MDC scaffolds were similar. Interestingly, A549 cells when grown on control were proliferating but were flattened, homogeneously spread as monolayer. Whereas the tumor cells were having spherical morphology and multilayer growth on MDC scaffold. These studies indicated that MDC scaffolds are biocompatible for the lung cancer cells to proliferate, along with that it encourage the cells to initiate the 3D tumor formation at multiple sites from day two onwards.
Further, to quantify the cellular morphological features, the structural measurement of cells in SEM images was accomplished with ImageJ software. The result showed that area of cell was 101.084 ± 29.72 µm in MDC hydrogel while in control it was 156.152 ± 36.367 µm (figure 36 A, B). These data confirmed flatten and spread morphology of cells in control scaffold. Moreover, the perimeter, major axes and minor axes of cells in MDC hydrogel were smaller than the control. This could be due to increase in geometry of cellular structure in MDC hydrogel, which decreased the cell spreading and therefore two-dimensional surface area. In support to that, the circularity of cells, which indicates the shape of cell, was 0.879 ± 0.09 µm for MDC hydrogel while in control it was 0.387 ± 0.055 µm (figure 36 C). These data confirm that the cells in MDC hydrogel had round shape compare to control, which had more elongated structure. The solidity of cell positively associated with matrix stiffness. The increased solidity is represent to more smoother and compact cellular shape and less cell deformability [187, 188]. The cells in MDC hydrogel showed little higher solidity than
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control which demonstrated that the stiffer substrate provide higher contractile forces to cell resulting in formation of smoother cell membrane.

Figure 36: Cellular morphology of the microtumors generated on MDC hydrogel and control. A) Cell morphology on MDC hydrogel. B) Cell morphology on control scaffolds. C) Table showing the morphological characteristics of cells on respective hydrogel calculated from respective SEM images. Red line in the SEM images shows peripheral area of individual cell. For each calculation n=3 images were selected and the structural parameters were determined by imageJ software.

The surface chemistry of MDC scaffolds have restricted the cell adhesion to scaffold. The cells adhere to adjacent cells and ECM molecules via adhesion receptors. These adhesion molecules such as integrin, selectin and cadherin bridge over the external ECM molecules to intracellular cytoskeleton and transpose the external signals to nucleus and determine cell
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fate. Consequently, cell adapt the structural features depending upon the chemical clues from external environment. The change in structure alter the exposure of signalling proteins, which ultimately results in changing the cell fate. It was well studied that the cell’s association to its surrounding environment directs the cellular morphology, proliferation, apoptosis and gene expressions [189-192]. For instance, breast tumor cells endogenously express β4 integrin and are sensitive to apoptosis in both 2D and 3D condition. Nevertheless, when these cells were induced to form polarized structure by biochemical signaling in 3D reconstituted basement membrane (rBM), they acquired an apoptosis-resistant phenotype with endogenously activated NFκB p65 [81]. This resistance highlights a connection between cellular polarity and intracellular signalling directing cell fate. In another study, the effect of surface property on cancer stem cell phenotype was studied. It was observed that, human glioblastoma cell lines U-87 MG and U-118 MG were formed spheroids on chitosan-alginate scaffolds which was having 62 % CD 133+ stem cell population. Conversely, when these cells cultured on PCL scaffolds or PCL coated chitosan-alginate scaffolds having similar 3D structure to chitosan-alginate scaffolds, they were grown as clusters [193]. Moreover, there was a 1-2 % CD 133+ cells were observed on these scaffolds. This study suggested that it was probably a combination of the 3D environment and chemical property of the scaffolds that promoted formation of the tumor spheroids. The development of tumor spheroid was encouraging the growth of cancer stem cells. These studies proposed the relation between surface chemistry based cell morphology and their effect on tumor cell phenotype. It supports the microtumor formation on MDC hydrogel in present study was having different phenotypic expression and resemblance of in vivo solid tumors.

5.6.3.2 Cytoskeleton organization

The cellular shape observed as spherical with encompassing nucleus occupying majority of area and surrounded by actin fibers(figure 35 A). The globular shape in these
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Images corroborated with previous observations of SEM. Because of expanding the size, nucleus have taken up the cytoplasmic area. Expansion in gross nuclear size and disturbance of nuclear cytoplasmic ratio are often associated with cancerous tissue [194]. It was discovered that increased nuclear size was associated with elevated expression of p53 while reduced expression of p16INK4A, a regulator of Rb in lung adenocarcinomas [195]. The nuclear-cytoplasmic ratio is vital for maintaining cell integrity and cell cycle progression [196, 197]. The cell can perceive nuclear size to measure the appropriate time to enter the cell cycle [198]. Moreover, association between nuclear size and RNA transcription levels was reported in different studies, suggesting larger nucleus facilitate increased transcription [199]. The enlarged nuclear area associated with higher cellular proliferation rates, aneuploidy [200]. This study showed cells with having extended nucleus area, that indicates microtumors with aggressive phenotype and proliferative cells.

![Confocal images of A549 cells on MDC hydrogel stained for stress fiber arrangement and nucleus size on microtumor. A) Z-stacking and 3D reconstructed image of microtumor formation in scaffolds having cells-cytoskeleton arrangement in microtumors. Scale bar 100 μm. B-C) 2D image of microtumors on MDC hydrogel at B) 40X, C) 100X. Scale bar 20 μm.](image)

Figure 37: Confocal images of A549 cells on MDC hydrogel stained for stress fiber arrangement and nucleus size on microtumor. A) Z-stacking and 3D reconstructed image of microtumor formation in scaffolds having cells-cytoskeleton arrangement in microtumors. Scale bar 100 μm. B-C) 2D image of microtumors on MDC hydrogel at B) 40X, C) 100X. Scale bar 20 μm.

It has been also reported that majority of animal cells exhibit intense stress fibers aligned with their major axes when exposed to rigid substrates, like plastic or glass. On the other hand, cells grown on compliant substrates shows very thin and randomly arranged stress fiber structures [182, 201]. Since MDC scaffold stiffness is closer to native tumor ECM, similar arrangement of randomly oriented stress fiber was observed (figure 35 B, C).
5.6.4 **Expression of ECM proteins**

To evaluate whether cancer cells in MDC hydrogel produce ECM proteins, collagen IV expression was determined by immunstaining. The **figure 38** showed the expression of collagen throughout microtumor. Increased ECM proteins deposition is associated with cancer progression. Cancer cell-derived ECM proteins increases matrix density and provides cell-stromal crosstalk. Collagen is a major component of ECM. The increased collagen deposition in tumor stroma directly associated with matrix stiffness, which enhance the integrin signaling and elevate cell survival and proliferation [3, 28, 202, 203]. Microtumor formation in the MDC scaffolds was having not only tumor cell aggregates, but having ECM based complex microenvironment like *in vivo* tumor. All over, these observations suggested a well representative microtumor formation in the MDC scaffolds.

![Image of immunostaining](image)

**Figure 38:** Immunostaining of paraffin sectioned cell laden scaffold showing collagen expression. A) Nucleus stained by DAPI, B) collagen expression stained by Alexa Fluor® 568 tagged antibody, C) Merged image. Scale bar 50 µm.

5.6.5 **Generation of ROS**

As tumor expand beyond oxygen diffusion limit, it generate oxygen gradient towards the core. Reduction of oxygen inside the tumor create tissue hypoxia. Hypoxia is directly or indirectly generate reactive oxygen species[204]. ROS was determined as preliminary indicator of hypoxia in the microtumor generated on MDC scaffolds and compared with cells grown on 2D TCPS. The each absorbance from DCFDA assay value was divided by cell
numbers from respective scaffolds indicating arbitrary units per cell. The graph showed more than 15 fold increase in ROS generation in MDC scaffolds compared to 2D TCPS culture (figure 39) at every time-points. These data corroborate with morphology analysis where microtumor formation was observed from day two. The ROS was increased to 19.61 fold at 6 days grown microtumors. Cells proliferated on flat tissue culture plate had monolayer morphology. Therefore, the oxygen diffusion was homogeneous throughout the area in 2D culture. On the other hand, oxygen diffusion was decreased in the core of microtumor, which produced a gradient of oxygen concentration.

![ROS generation graph](image)

**Figure 39:** DCFDA assay showing ROS production in the microtumors at specific time point. The values in the bar represent fold change W.R.T. 2D control. All experiments were done with n=3, p<0.01.

In present study, the cells were grown on scaffolds as 3D and on TCPS as 2D control with having similar nutrient, cell passage, cell numbers and external conditions. The
dimensionality was the major difference between the samples which could induce ROS by hypoxic pathways. It was studied that ROS can be produced by hypoxic conditions and ROS level was reported as indirect evidence of hypoxia in 3D models [205, 206]. Therefore, in the study it was anticipated that the increased ROS could be due to hypoxic conditions in 3D. However, the ROS would be the indirect indicator of hypoxic condition, additional study would be performed to confirm this finding. The hypoxic environment is the characteristic feature of solid tumor having poor oxygen and nutrient tension leading to produce hypoxia [207, 208]. Hypoxia, in turn, activate HIF-1α which modulate cell survival, proliferation, metastasis, and drug response [50, 209-211]. Therefore, mimicking hypoxic condition could increase the consistency of this model for drug toxicity assays.

5.6.6 Drug response of cells grown on hydrogel

To determine the utility of MDC scaffold as a model for drug screening assays, Doxorubicin was used as a model anticancer drug to evaluate drug toxicity. Doxorubicin is a well-recognized chemotherapeutic drug known to stabilize DNA-winding enzymes, intercalate with DNA and target several downstream molecules to exhibit various cytotoxic effects. For 2D condition, cells were grown for 24 h and treated with 25, 50 and 100 µM drug for 24 h. For 3D scaffolds, cells were allowed to grow for 2, 4 or 6 days and after each time point, similar dosage was applied to 2D condition. The figure 40 represents percent cell viability with respect to control for each time point. The graph showed that at 25 µM concentration, 50 % cells were viable in the case of the 2D surface while in case of all 3D condition, the cell viability was more than 39 % after 2 days, while at 4 and 6 days grown microtumors, 57 % and 83 % cells were survived. The microtumors size was increased when grown for a longer duration, which might restricted the drug diffusion towards the centre. Contrariwise in 2D, equivalent drug was available throughout the culture. Therefore, overall effect of drug was reduced in 3D culture compare to monolayer culture. At higher dosage of
50 µM and 100 µM, the cell survival was continued to decrease to 46 % and 19 % respectively in TCPS culture. Yet the cell viability was 55 % and 51 % on 4 days and 78 % and 77 % on 6 days grown microtumors respectively. This data indicated that increasing dosage from two-fold to four-fold did not show significant effect on cell viability as the microtumor size increases. The cells might have less susceptibility to drugs in 3D tumor compare to monolayer [186, 212]. Hence dose-dependent toxicity was minimized in expanded microtumors.

**Doxorubicin toxicity**

![Graph showing cell viability of A549 cells after treatment with doxorubicin drug.](image)

*Figure 40: cell viability of A549 cells after treatment with doxorubicin drug. All experiments were done with n=3; *, # signifying P < 0.05, **,## representing P < 0.01 and *** denoting P < 0.001 significance difference.*

These higher drug resistance of cells, when grown as 3D micro tumor, might be due to the following aspects. The primarily difference in physiological condition of cells in 2D and 3D may attribute to alter the drug response. 2D cultures were having cells with stretched and monolayer morphology. While in present 3D tumor model cells were having rounded and clustered morphology which represents *in vivo* tumors [213, 214]. Next, Due to morphological changes, 3D distributions and spatial organizations of surface receptors would
be differently expressed in 3D tumors [215]. Thus drugs which target these specific receptors would change the drug efficacy in 3D tumors. Moreover, the Cells growing in 3D microenvironment have different gene expression than cells in monolayer having an unnatural condition [59]. The collagen expression could increase the cell-ECM interaction which gives rise to complex microenvironment. Overall, the three dimensional tumor sphere surrounding with ECM proteins could subjugate the drug diffusion and penetration. Therefore, the drug toxicity could be reduced. Further, the larger microtumors had oxygen and nutrient gradient throughout the sphere forming heterogeneous populations, having dormant cells in the core while actively proliferating cells in the outer region. These might evolve stem cell population which are more resistant to drugs. However, this possibility should be further investigated by p-gp expression in the cells to validate multi drug resistance in the cells. Furthermore, hypoxic conditions and higher glycolysis may change intracellular pH which reduces the efficacy of weakly basic drug like doxorubicin by reducing drug uptake, developing drug resistance [216, 217].