4 Materials and methods

4.1 Materials

4.1.1 Hydrogel fabrication

Dextran (Mr ~ 70,000), Chitosan (medium molecular weight), L-cysteine, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC), D₂O and dialysis tubing (MWCO ~ 12,000 Da) were purchased from Sigma-Aldrich, USA. Hydroxylamine hydrochloride, methyl orange solution, Glutaraldehyde was purchased from S D Fine-Chem Ltd., India. Ellman’s reagent, Sodium periodate, Diethylene glycol (DEG), 5, 5’-dithio-bis-(2-nitrobenzoic acid) (DTNB) were procured from SRL chemicals, India. BSA standard protein and Bicinchoninic acid assay kit were procured from Thermo Fisher Scientific Inc.

4.1.2 Biological studies

Lung Adenocarcinoma A549 cells was obtained from NCCS, India. Fetal bovine serum (FBS) was procured from Gibco, USA. RPMI-1640 and other media supplements were purchased from Himedia, India unless mentioned otherwise. Resazurin, Triton-X 100, 2’, 7’– dichlorofluorescein diacetate (DCFDA), Phalloidin-FITC, DAPI stain, PVA-DABCO® antifadding agent, paraformaldehyde, and Doxorubicin hydrochloride were purchased from Sigma-Aldrich. Anti-collagen mouse mAb and cy5 tagged anti-mouse secondary antibody were purchased from Cell Signalling Technology, Inc. LIVE/DEAD™ assays standard kit was purchased from Thermo Fisher Scientific Inc.
4.2 Methods

4.2.1 Modification of polymers and fabrication of scaffolds

4.2.1.1 Oxidation of dextran

10 gm dextran was dissolved in 800 ml of deionized water (dI H₂O). Sodium periodate was added in 200 ml of water and added during stirring. The reaction performed under dark conditions at room temperature. 4 different type of reaction conditions were performed to optimise the Oxidation degree (Table 2). The molar concentration of sodium periodate was taken with respect to molar concentration of dextran monomer i.e. one glucose unit. An equimolar amount of DEG to Sodium periodate was added to the reaction mixture to stop the oxidation reaction. The reaction mixture dialyzed for 3 days. For dialysis, entire solution was filled in dialysis tubing with MW cut-offs ~ 12 kDa and kept in 20 L mili Q water in dark at Room Temperature (RT). The water was stirred continuously with very slow speed and replaced with fresh dI H₂O at every 24 h. finally, the modified dextran was freeze dried and stored at 4 °C in dark for further use.

Table 2: The process parameters for oxidation of dextran reaction.

<table>
<thead>
<tr>
<th>Odex formulations</th>
<th>Reaction time (h)</th>
<th>Amount of sodium periodate (ratio of moles of SP to Dextran monomer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1:1</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>1:1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1:2</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>1:2</td>
</tr>
</tbody>
</table>

4.2.1.2 Thiolation of chitosan

Thiolation of chitosan was achieved by grafting cysteine amino acid to chitosan side chain with EDAC as a crosslinker. Briefly, 1 gm of chitosan was dissolved in 100 ml of 1 % v/v acetic acid solution. 30 mMoles of L-cysteine was added to chitosan solution and allowed
to dissolve. The reaction was started by adding EDAC (5 mMole) to the reaction mixture and carried out for 3 h in dark. The modified polymer was dialyzed to get purified product. For that, the entire solutions were filled in dialysis tubing (Mw cut-offs ~ 12000 Da) and kept in 20 L of solutions with mild stirring in dark. The dialysis was done with 5 mM HCl for first day, followed by 5 mM HCl- 1% NaCl for next day and finally with 1mM HCl for a day. The purified product has was collected, freeze dried and kept at 4 °C in dark for further use.

4.2.1.3 Hydrogel fabrication

Two types of hydrogel was formulated viz, from pure polymers and from modified polymers.

4.12.1.3.1 Pure polymer based hydrogels

Chitosan hydrogel (CH) with different viscosity and different concentration is been used for the hydrogel formulation. Glutaraldehyde was used as cross linker. All the parameters like polymer concentration, polymer molecular weight and cross linker concentration has been used in different combination for the formulation to get desired result. The concentration of cross linker and polymer has been optimised by using different combinations.

Next, Gelatin is been blended with chitosan to form chitosan – gelatine hydrogel (CGH). The gelatine was blended with chitosan in 1:1 ratio and made the final concentration of 2 % of total polymer solution for hydrogel fabrication. The crosslinker concentration and process parameters were kept constant as pure chitosan hydrogel.

4.12.1.3.2 Modified polymer based hydrogel

A solution of Odex with 3, 4, and 5 % w/v and 4 % w/v TC was prepared. An equal volume of both solutions was mixed and poured into cylindrical molds to make hydrogel. For control scaffold, TC solutions cross-linked with 1 % w/v solution of Glutaraldehyde,
added with 1:10 ratio of TC solution, make final scaffold having 4% w/v TC. Both the solutions were allowed to cross-link at RT for 2 h. Following to that, the gel was frozen at -20°C and -80°C for 24 h and then lyophilized to form porous hydrogel scaffolds.

4.2.2 Characterization of polymers and hydrogel

4.2.2.1 Determination of degree of oxidation of dextran

The amount of aldehyde in the oxidised dextran was determined by hydroxylamine hydrochloride method described by Zhao et al [144]. Briefly, 0.1 gm of purified product was dissolved in 0.25 M NH₄OH.HCl- methyl orange solution and allowed to solubilize for 2 h at room temperature. Followed that was titrated with NaOH solution to bring out to the original color solution. The degree of oxidation was determined as moles of aldehyde produced per moles of dextran monomers in the sample by calculation given below.

\[
\text{% Oxidation} = \left( \frac{\text{volume in ml} \times 10^{-3} \times M_{\text{NaOH}} \times \text{Molecular weight}_{\text{dextran monomer}}}{\text{weight in gm}_{\text{Odex}}} \right) \times 100
\]

4.2.2.2 Molecular weight of Odex

Gel Permission Chromatography (GPC) was performed to analyse the molecular weight of degraded dextran during oxidation reaction with infinity 1260 system (Agilent Technologies). The pure dextran and Odex were dissolved in dI H₂O with a concentration of 0.5mg/ml and analysed with PLAquagel-OH mixed column (8 µm - 7.5 x 300 mm) at a flow rate of 1 ml/min.

4.2.2.3 Determination of amount of thiol in thiolated chitosan

The amount of thiol group in the chitosan polymer was determined by Ellman’s assay. The reaction buffer was prepared having 0.1M sodium phosphate, 1mM EDTA with pH 8. The ellman’s reagent was prepared as 4mg/ml in reaction buffer. In a series of tubes, 2.5 ml of reaction buffer and 50 µl of ellman’s reagent were taken. 250 µl of sample was added to this tubes and incubated for 15 min at RT. after that, the absorbance was measured at 412
nm using Uv-Vis spectrophotometer. Different concentration of cysteine was used to as a standard solution of thiol group and from that standard curve was prepared. The modified chitosan was taken as a sample and amount of thiol group in the chitosan polymer was determined from standard curve as moles of thiol grafted per gram of chitosan monomer.

4.2.2.4 Scanning Electron Microscopy (SEM)

Lyophilized hydrogels discs (9 x 1 mm, d X h) were sputter coated with gold. Surface morphologies were recorded with a scanning electron microscope (Evo® 18, Carl Zeiss GmBH, Germany) at 10-20 kV. The pore size of the hydrogels was evaluated from SEM images.

4.2.2.5 Nuclear Magnetic Resonance (NMR)

$^1$H NMR spectra of dextran and Odex was carried out to determine the modification of dextran. 10 mg of each dried sample was dissolved in 1ml of D$_2$O by sonication. The solutions were then poured into NMR tube and spectra were acquired with a 500 MHz Fourier-Transform Nuclear Magnetic Resonance spectrometer (Bruker, Germany) at room temperature.

4.2.2.6 FTIR

The dried powder of dextran, Odex, chitosan, thiolated chitosan, gelatine, cysteine and dried hydrogels were used for FTIR analysis using a Thermo Scientific, USA, instrument with attenuated total reflectance (ATR). All Spectra were acquired in the 4000–400 cm$^{-1}$ range with a resolution of 0.4 cm$^{-1}$ for 20 scans.

4.2.2.7 Pore size distribution

The pore size of the hydrogels was evaluated from SEM images, where Pores were determined by their longest dimension. The pore size was measured in ImageJ software. Total 50 pores per image were selected randomly recording the pore size. The pore size
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distribution was calculated with n=3 images and mean pore size was also calculated from it using ImageJ software.

4.2.2.8 Hydrophobicity index

The hydrophobicity of hydrogel sample with different Odex concentrations was determined by previously reported method[145]. Briefly, freeze-dried samples of each formulation were cut into discs and their corresponding dry weights (Wd) measured. The scaffolds were consequently immersed in dI H2O (W) or 70 % isopropanol (IP) for 36 h and their respective swollen weight were recorded as WS. The swelling ratio (q) for each solvent was calculated as,

\[ q = \frac{W_S}{W_d} \]

The hydrophobicity was calculated as H-index by following equation,

\[ H \text{- index} = \frac{q^{IP}}{q^W} \]

4.2.2.9 Protein adsorption study

Protein adsorption was determined by indirect measurement of BSA protein concentration. Dried hydrogels were cut into pieces and their dry weight was measured. Each scaffold was then saturated in 1X PBS for 24h. BSA protein solution was made with 50 µg/ml concentration and each pre-wet sample was submerged in 1 ml of protein solution for 1 hr. after that, the protein concentration of the remaining solution was determined by Bicinchoninic acid assay. The amount of adsorbed protein was calculated by subtracting the remaining amount of protein from an initial concentration of BSA protein solution.

4.2.2.10 Mechanical analysis

Mechanical testing studies were conducted using a Tinus olsen H5KT system with a 50 N load cell and Herison software. Cylindrical hydrogels were cut into 5 mm height and
pre-wetted in 1X PBS for 30 min. Uniaxial compression at 5 mm/min was applied and compressive modulus was measured by calculating the ratio of stress vs strain.

4.2.2.1 Swelling kinetics study

The dried MDC scaffolds were taken with pre-determined weight (W_d) and were immersed in the dI H2O. At specified time point, the scaffolds were removed, placed on filter paper to remove excess water followed by measuring the weight of swollen hydrogel (W_s). The Equilibrium Water Content for each time point was calculated by the following equation.

\[
\text{Equilibrium Water Content (EWC \%)} = \frac{\text{Swollen Weight (W_s) } - \text{ Dry Weight(W_d)}}{\text{Swollen Weight (W_s)}} \times 100
\]

4.2.3 Generation of 3D tumor and biological characterization

4.2.3.1 Cell revival

A549 Cells were taken out from liquid nitrogen tank and immediately thawed at 37 °C in water bath. 10 ml complete media was pre-warmed in 15 ml centrifuge, thawed cell suspension was added in it and centrifuged at 1200 rpm for 5 min to remove DMSO. Supernatant was discarded and pellet was resuspended in fresh media. Cells suspension was transferred to specific culture vessel and observed under inverted microscope. After that, cells were incubated at 37 °C incubator with 5 % CO2 with regular inspection and media changing at alternate day.

4.2.3.2 Subculture of cells

Cells were sub-cultured when it reached to 70-80% confluency. For that, media was discarded and sufficient amount of 0.25% trypsin-EDTA solution was added to completely cover cell monolayer and kept at 37 °C in incubator for 4-5 min. After that, cells were observed under inverted microscope to confirm detachment of cells by visualizing floating
cells. Equivalent amount of fresh media was added to inactivate the trypsin in culture flask and entire suspension was taken in 15 ml conical tube and centrifuged at 1200 rpm for 5 min. Supernatant was removed and pellet was re-suspended in appropriate amount of fresh media. The cells were counted in haemocytometer and seeded in new culture flask. Cells were then kept in a humidified incubator at 37 °C and 5% CO₂.

4.2.3.3 Cell preservation

For cryopreservation, confluent cells were trypsinized and pelleted down as described in previous section and collected. Resuspended the cell pellet in cryopreservation media having cell culture media with 20% FBS and 10% DMSO. Cells were maintained at 1 X 10⁵ to 10 X 10⁵ cells/ml/vial and immediately transferred to -20° C for 2 h, followed by overnight freezing at -80° C and then preserve in liquid nitrogen for long term storage.

4.2.3.4 Cell seeding on scaffolds

For cell culture, each scaffold was pre-sterilized before cell seeding. Hydrogels were cut into disc having 1 mm thickness. Each disc were submerged in 70% ethanol for 1 h. after that, scaffolds were washed three times with sterile 1X PBS (5 min each time) in the hood. Further, each disc was kept under UV for 30 min for surface sterilization. The sterile scaffolds were pre-conditioned in fresh media for 16-18 h. The 24 well plate were selected for cell culture and was coated with sterile 1 % w/v agarose solution to block the attachment site on the plate surface. After that, single pre-swollen scaffold disc was places at each coated well.

A549 cells cultured in 2D monolayer with RPMI 1640 media supplemented with 10 % foetal bovine serum. After confluency, cells were trypsinized and resuspended in fresh media with ~ 0.5 X 10⁵ cells/ 20 µl density. 0.5 X 10⁵ cells has been seeded in MDC hydrogel and allowed to adhere on the scaffold for 3 h. Subsequently, sufficient media was added very
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slowly and incubated at 37°C in humidified incubator with 5% CO₂. The used media was replaced every day with pre-warmed fresh media.

4.2.3.5 Cell growth

Cell proliferation was quantitatively determined by Alamar blue assay. Cell permeable resazurin is the active component of Alamar blue assay. It is nontoxic compound having blue color and non-fluorescent. At inside the cell, resazurin is converted to resorufin, reduced form of resazurin that gives highly fluorescent red color. Viable cells continuously convert resazurin by metabolic activity, which produce fluorescence increasingly in the media and can be detected to give cell quantities present in media.

Cells were seeded on the scaffolds and allowed to grow for specific time period. At each time point, used media was removed and sterile resazurin solution (0.15 mg/ml in 1x PBS) was added to each well with 1:10 ratio to fresh media and incubated for 1 h at 37 °C. Following to that, 200 µl of suspension was collected in 96 well plate and fluorescence was measured by multi-mode micro-plate reader (BioTek Instruments, Inc.) at 560 nm excitation, 590 nm emission. The excess alamar reagent from scaffold was removed by gentle PBS wash, after that, fresh media was added and incubated again for further proliferation. The fluorescent value was converted to cell number by taking precise number of cells and preparing standard curve from it. The fluorescent value from each scaffolds at each time point were determined by previously calculated standard curve for the cells.

4.2.3.6 Live dead assay

The cell viability in the hydrogel were analysed by LIVE/DEAD™ assays in confocal microscope. It is a fluorescence based two-color assay to define viability of cell by plasma membrane integrity and esterase activity of cells in a population. Briefly, cells were cultured on MDC scaffolds for 2, 4 and 6 days. After each time point, cell laden scaffolds were taken out from the cell culture plates washed with 1X PBS and treated with assay solution
containing 2 μM calcein AM and 4 μM ethidium homodimer-1 (EthD-1) and incubated for 30 min at 37 °C. After that cells were directly observed in confocal laser scanning microscope with z-stacking (LSM780, Zeiss, Germany).

4.2.3.7 Cellular morphology by SEM

The cellular morphology and tumor formation was determined by SEM. The SEM required completely dehydrated samples for analysis. For cell-laden hydrogels, cells were first fixed with 2% glutaraldehyde in cacodylate buffer (0.1 M) by immersing the scaffolds in the solution for 1 h at RT. After that, trace of glutaraldehyde was removed by keeping it in the cacodylate buffer for 30 min. The hydrogels were proceed for dehydration with submerged in gradually increasing concentrations of alcohol sequentially i.e. 30 %, 70 %, 80%, 90 %, 100 % for 10 min each. The samples were then completely dried in desiccator for 2 h. The dried scaffolds were mounted on stub with carbon tape, sputter coated with gold–palladium and analysed under SEM.

4.2.3.8 ImageJ analysis

The quantitative measurement of cellular morphological features, ImageJ software was used. The SEM images were utilised to calculate the cell structures. The cellular boundaries in the images were drawn manually by free hand drawing tool in the software and measurement command was applied to record the dimensions of that shape. Total 12 shape were drawn from 3 different images for analysing shape features. The cell morphological features were calculated in ImageJ using following formula,

\[ \text{Circularity} = 4\pi \times \left( \frac{\text{Area}}{\text{Perimeter}^2} \right) \]

The value of 1.0 indicating a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated shape. The solidity and aspect ratio were calculated as,

\[ \text{Solidity} = \frac{\text{Area}}{\text{Convex area}} \]
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Aspect ratio = (Major axes) / (Minor axes)

4.2.3.9 Cellular morphology by confocal microscopy

The cytoskeleton arrangement in cells was visualized to determine cellular shape, size and nuclear organization. For that, 6 days cultured microtumor were utilized. After the growth, media was removed and cells were gently washed with 1X PBS followed by fixed with 2 % paraformaldehyde for 1 h at RT. The fixative was removed by washed with 1X PBS. Cells were treated with 0.1 % triton X-100 solution for 15 min to permeabilize the cell membrane. Again washed with washed with 1X PBS and treated with phalloidin-FITC for 15 min at RT in dark. After that, the nucleus was counterstained with 50 ng/ml of DAPI solution for 7 min in dark. The scaffolds were then washed three times with 1X PBS in dark to remove excess stain. Cell laden hydrogel were mounted on glass slides with anti-fading agent and observed under confocal microscope.

4.2.3.10 Immunostaining

For collagen immunostaining, sectioning was done with cell-laden hydrogel. For that, cells were fixed with 2 % paraformaldehyde for 1 h, washed with 1X PBS and then embedded in paraffin. The sections were cut at 3–5 μm on microtome, mounted on glass slides and dried overnight at 37°C. After that, sections were dewaxed by immersing in xylene two times for 10 min each. The scaffolds were rehydrated by keeping in graded alcohol series i.e. 100 %, 95 %, 70 %, 50 % and dI H2O for 10 min each. For staining, cells were washed with 1X PBS and permeabilized with 0.1 % Triton-X 100 for 10 min. Followed by washed with 1X PBS, slides were incubated with blocking buffer for 30 min at RT. The processed sections were treated with primary anti-collagen IV mouse antibody (1:1000) prepared in blocking buffer at 4 °C overnight. After removal of primary antibody, cells were washed three times with chilled 1X PBS. Then, the cells were incubated with fluorescence tagged secondary anti-
mouse antibody (1:500 dilution) prepared in 1% BSA for 1 h at RT in the dark. The secondary antibody was removed and washed with chilled 1X PBS three times in dark. The hydrogel sections were then counterstained with 50 ng/ml of DAPI solution for 7 min in dark for nucleus. The sections were then washed three times with 1X PBS in dark. The cell laden hydrogels were mounted with anti-fading agent, concealed with coverslip and observed under confocal microscope.

4.2.3.11 Detection of ROS generation

The total ROS production in the microtumors was determined by 2’, 7’-dichlorofluorescein diacetate (DCFDA) assay and compared with cells grown on the 2D surface. The DCFDA is a non-fluorescent compound oxidized by ROS present in the cell, into highly fluorescent molecule 2’, 7’-dichlorofluorescein (DCF). The cells were grown for 2, 4, or 6 days on the scaffolds and for the 2D control, cells were grown for 2 days on TCPS. At each time point, the media from the well was removed and cells were washed with 400 µl of sterile KRB buffer. 50 µM DCFDA was prepared in sterile KRB buffer and was added to each well. The cells were transferred to incubate for 4 h at 37°C in dark. For cell-laden scaffolds, DCF was extracted by incubating the scaffolds in 400 µl of sterile cell lysis buffer for 30 min with adequate homogenization at every 10 min. For 2D conditions, after DCFDA treatment, cells were incubated with cell lysis buffer for 30 min at room temperature and after that cells were scrap out with scrapper. Finally, the suspension was collected in amber color centrifuge tubes and spin down at 2000 rpm for 5 min. 200 µl supernatant was taken in to 96 well plate and read in multi-mode micro-plate reader at 495/529 nm.

4.2.3.12 Drug sensitivity of cells on MDC scaffold

0.5 x 10⁵ cells were grown on 2D monolayer and 3D scaffolds. The cells were treated with doxorubicin (dissolved in 4 µl DMSO) as 25 µM, 50 µM, and 100 µM concentration in total media (1 ml) after one day (60-70 % confluence) for 2D. The similar dosage were
applied to the cells after 2, 4 or 6 days of growth for 3D scaffolds. After 24 h of treatment, the viability of cells were determined by the Alamar blue assay and normalized with respect to untreated DMSO control. The percent cell viability w.r.t. respective control were plotted for each dosage at 2D or 3D conditions. Each dosage was compared in 2D vs 3D conditions and in both conditions the difference between cell viability at different dosage was also analysed.

**4.2.4 Statistical analysis**

All quantitative results were performed with n=3 and results were expressed as mean ± standard error of mean. Statistical analysis was performed using student’s t-test for comparing control with treatment or 2D with 3D. All values of p < 0.05 were considered as statistically significant.