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Fabrication temperature modulates bulk properties of polymeric gels synthesized by different crosslinking methods

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Effect of natural matrix like extracellular matrix (ECM) on cell growth, proliferation and induction of mechanotransduction signals are well documented. Mimicking mechanical and rheological character of ECM to repair, reconstruct and regenerate injured or impaired tissue using the concepts of tissue engineering is of significant importance. We intended to study the effect of "synthesis temperature" on mechanical and rheological properties of monomeric and polymeric scaffolds fabricated from either monomer based polyacrylamide gels or by physical gelation (agarose gels) or chemical crosslinking (chitosan, chitosan-gelatin and chitosan-agarose-gelatin (CAG) gels) at -15 °C, -5 °C or 25 °C. Our results indicated that in absence of porogens in the form of ice crystals, conventional hydrogels had higher mechanical strength and low porosity as compared to cryogels synthesized at -15 °C and -5 °C. Furthermore, rheological analysis performed on these gels indicated temperature dependence of rheology. CAG cryogels had higher storage modulus as compared to freeze dried (FD) hydrogels or conventional hydrogels in the wet state making them amiable for cartilage tissue regeneration. Out of all synthesized gels, CAG showed better mechanical and rheological properties and were further studied to analyze their interaction with primary chicken chondrocytes. *In vitro* experiments on CAG scaffolds revealed significant amount of ECM production along with a good cell proliferation as indicated by MTT assay and SEM analysis.

1. Introduction

The main objective of tissue engineering is to regenerate, reconstruct, and/or replace damaged tissue/organ by mimicking native tissue as closely as possible. To accomplish these goals it is imperative to combine cells within natural or synthetic scaffolds that help cells to exist in their native environment. Cells secrete various molecules that make a variety of natural scaffolds. These scaffolds govern the attachment, organization, and proliferation of cells to fabricate biologically functional tissue(s). Extracellular matrix (ECM) acts as a scaffold since it is a natural, three dimensional (3D) environment that provide cells an attachment platform in order to maintain tissue's structural integrity and its biological functions. ECM also acts as an instruction manual for cells guiding them during different chemical, electrochemical and physical stimuli to control cellular growth, differentiation and migration, thus exhibiting mechanotransductive behaviour [1-3].

Cell-ECM interactions are of paramount importance and have been the research focus of various scientific communities across different fields including material science, regenerative medicine and tissue engineering. Researchers for long have tried to harness and mimic these interactions using various natural and synthetic scaffolds [4, 5]. A key component of tissue engineering is the fabrication of 3D scaffold that not only provide a 3D environment to support cellular growth and proliferation, but also mimics many other functions of ECM including differential and dynamic mechanical behaviour based on tissue type [6].

In vivo differential and dynamic mechanical property of ECM across tissues at different stages of life has shown to influence cell proliferation, migration, and differentiation [7-9]. Furthermore, the role of mechnotransduction in stem cell lineage specification, cell migration and disease in tissues or organs like bone, cartilage, skin, muscle, brain, eye, etc. is also well documented [3, 10-14]. Recently, independent of its chemical nature, the mechanical properties of ECM have been recognized as vital in regulating cellular functions through physical couplings that associate the ECM to the cell cytoskeleton. These characteristics allow for direct translation of force into remodelling of cellular gene expression and phenotype [15-17]. Conversely, the mechanical properties of ECM can thus be modulated by a variety of covalent and physical interactions between the individual ECM constituent molecules. One of the frequently used parameter for the measurement for mechanical strength or rigidity of tissues is the elastic (Young's) modulus. Elastic modulus of various tissues like brain, lungs, breast, muscle, fat, and bone lie in the range of 0.1 kPa to 28,900,000 kPa. Recent studies have used various tissue engineering scaffolds including hydrogels, Matrigel™, crosslinked gelatin, and other synthetic substrates with elastic moduli in the same range that of native tissue to mimic native environment and thus influence cellular outcomes [18]. Thus, a major challenge for researchers is to incorporate identical or at least similar mechanical properties to their scaffolds just like the native tissue [19].

Considerable advances in designing of artificial matrices, those can mimic properties of ECM paved a way for evolution of much more dynamic scaffolds. An enhanced attention is paid for the matrices those not only support cell growth and proliferation, but also exhibit efficient nutrient transfer, gas exchange (i.e., O_2 and CO_2) and metabolic waste removal together with efficient signal transduction. Scaffolds in various forms have been synthesized, studied and employed for different tissue regeneration purposes [20]. Hydrogels have been one of the earliest and well studied forms of biocompatible tissue engineering gels that have proven their worth to mimic ECM like properties [21]. Hydrogels have excellent water uptake and retention property, which allows free diffusion of solute molecules. Various forms of hydrogels have thus far been synthesized from various sources (natural/synthetic polymers) or/ and with varied crosslinking methods (monomer based, chemically cross linked and physically cross linked) for tissue engineering or biomedical use [22]. The use of free-radical cross-linking copolymerization of a hydrophilic, non-ionic monomer has been used as the most common way to produce

these gels. Thus, the hydrogel's structure and its property including its mechanical strength are function of concentration of crosslinker, chemical nature and initial concentration of the monomer units. However, despite of its wide application range, its use has been limited in medical and biological field by a number of limitations including inhomogeneity and poor/ imbalanced mechanical strength [23-25]. Thus, designing of polymeric gels with a balanced mechanical strength is vital for certain existing as well as developing applications. One of the techniques to obtain gels with fast response time and good mechanical strength is *cryogelation* [26]. This technique produces highly interconnected, macroporous structures due to phase separation at cryo-conditions. The phenomenon of phase separation imparts cryogels with fast responsive time and a good mechanical strength as compared to macroporous hydrogels [27-30]. Cryogels thus far have been used in varied biological applications like cell separation, neo-cartilage formation, cell proliferation and stimulation [31-34].

In the present work, we have performed an in-depth analysis of rheological and mechanical properties of monomer based, chemically crosslinked, and physically crosslinked, porous scaffolds fabricated at -15 °C, -5 °C and 25 °C. Furthermore, we also evaluated proliferative differences of chicken derived chondrocytes on chitosan, chitosan-gelatin, and chitosan-agarose-gelatin cryogels by varying polymeric component thus influencing mechanical strength, elasticity and porosity.

2. Materials and Methods

2.1 Materials

Acrylamide, ammonium persulfate (APS) and glutaraldehyde (25%) were purchased from MERCK India Pvt. Ltd., Mumbai, India. *N,N'*-Methylenebisacrylamide and tetramethylethylene-diamine (TEMED) were purchased from SD fine chemicals, India. Gelatin, from coldwater fish, low viscosity chitosan, Dulbecco's modified eagle's medium (DMEM) high glucose, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), trypsin solution, and collagenase type II were purchased from Sigma Aldrich, U.S.A. Fetal bovine serum (FBS) from Gibco Life Technologies, U.S.A, while low eeo agarose was purchased from SRL labs, India**.**

2.2 Methods

2.2.1 Preparation of monomer based polyacrylamide cryogels and hydrogels

Polyacrylamide (pAAM) cryogels were prepared by dissolving 600mg of acrylamide (6% w/v) in 10 ml of degassed water, followed by addition of 200mg (2% w/v) of N,N'-methylenebisacrylamide as a crosslinker. This monomeric solution was precooled at 4 °C after thorough vortexing. Precooling of the monomeric solution is recommended as free radical reactions occur instantaneously and prechilling reduces the rate of reaction. The initiator ammonium persulfate (APS) 10mg (0.1% w/v) and catalyst TEMED 10µl (0.1% v/v) was later added to above solution with intermittent vortexing for 10-15 s. Resultant solution was then poured immediately into precooled 2.5ml syringe moulds (8mm diameter) with their nozzle closed with parafilm. The moulds containing the reaction mixture were immediately transferred into a circulating liquid cryostat maintained either at-5 °C or -15 °C for a period of 12-16 h. After the incubation period, gels were thawed in de-ionized water at room temperature for 2-3 h. Thawed gels were then re-frozen at -80 °C for a period of 6h and dried by lyophilization for 24-36h. The dried gels were stored in moisture free bags for further use. Polyacrylamide hydrogels were fabricated by following the same protocol and by maintaining same monomeric concentration as above with only variation in synthesis temperature. Hydrogels were incubated at 25 °C in a hot air circulating incubator for 12-16 h rather than in a circulating liquid cryostat. Also, fabricated hydrogels were either air-dried or were lyophilized for 24-36 h by first freezing samples at -80 °C for 6 h and then transferring frozen samples into the lyophilizer for drying. The hydrogels subjected to air-drying will hereafter be referred to as conventional hydrogels, while the lyophilized hydrogels will be called as freeze dried (FD) hydrogels. All hydrogels either conventional or FD in the following sections have been fabricated in the similar manner.

2.2.2 Preparation of chemically crosslinked chitosan, chitosan – gelatin, chitosan-agarose-gelatin cryogels and hydrogels

a) Preparation of chitosan cryogels and hydrogels:

Chitosan cryogels were prepared by dissolving 200mg (2% w/v) of low viscosity chitosan in 8ml of 1% acetic acid. The polymeric solution was left overnight on rocker to ensure complete dissolution of the polymer. The solution was then precooled to 4 °C for 30min. Meanwhile, 0.2% (v/v) of the crosslinker glutaraldehyde (25%) was made in 2ml of 1% acetic acid and added to the chilled polymeric solution to make up the final volume to 10ml. This viscous polymeric solution was homogenized by vortex mixing for 15 s. The solution was then poured into precooled 2.5ml syringe moulds and incubated in the circulating liquid cryostat maintained at either -5 °C or -15 °C for 12-16 h. The gels were then thawed in de- ionized water for 2-3 h and dried in a similar manner as specified above. Chitosan FD hydrogels (2%) and conventional chitosan hydrogels (4%) were fabricated by following the same protocol as mentioned in section 2.2.1. The concentration of conventional hydrogels has been optimized in order to ensure better handling during different analysis techniques.

b) Preparation of chitosan- gelatin cryogels and hydrogels:

Chitosan 200mg (2% w/v) was dissolved in 9ml of 1% acetic acid followed by the addition of 600mg (6% w/v) gelatin. The polymers were left overnight on rocker to homogenize completely. The polymer solution was then precooled at 4 °C. Glutaraldehyde 0.2% (v/v) was mixed with 1ml of 1% acetic acid and added to the polymeric solution to make up the total reaction mixture volume to 10ml. The solution was then vortexed thoroughly followed by pouring into precooled 2.5ml syringe moulds. The moulds were incubated in liquid circulating cryostat maintained either at-5 °C or -15 °C for 12-16 h. Gels were thawed and dried as mentioned earlier. Chitosan-gelatin hydrogels (both conventional and FD) were fabricated at 25 °C by following above-mentioned protocol and by maintaining same polymeric concentration as above. Air-dried hydrogels were dried at room temperature while FD hydrogels were dried using lyophilization.

c) *Preparation of chitosan-agarose-gelatin cryogels and hydrogels:*

Chitosan-agarose–gelatin (CAG) cryogels having 13% (w/v) of total gel strength were prepared by dissolving 200mg (2% w/v) chitosan in 5ml of 1% acetic acid. Upon complete dissolution of chitosan, gelatin 600mg (6% w/v) was added to the chitosan solution and kept on rocker for complete dissolution. Additionally, agarose 500mg (5%) was dissolved in 4ml of de-ionized water by heating it to 80 °C for half an hour. After complete dissolution, chitosan-gelatin solution was added to the agarose solution and the mixture was allowed to homogenize by heating at 60 °C for 30 min. Once all the polymers were mixed thoroughly, the mixture was allowed to cool to 40 °C followed by the addition of 0.2% (v/v) glutaraldehyde (25%). The resultant mixture was vortexed vigorously for 10s and transferred into plastic moulds. The moulds were immediately transferred into a liquid cryostat maintained at either -5 °C or -15 °C. The drying procedure was not changed and freeze-drying was used to dry the cryogels. CAG hydrogels were also synthesized exactly the same way with variation only in the temperature. The reaction was allowed to occur at room temperature (25 °C), rather than at sub-zero temperatures. However, the hydrogels were dried either using freeze-drying or air-drying methods as mentioned earlier.

2.2.3 Preparation of physically crosslinked agarose cryogels and hydrogels

Agarose cryogels were prepared by dissolving 500mg (5% w/v) agarose in 10ml of de- ionized water. The solution was heated to around 80-90 °C for 30min or until it became clear. The solution was allowed to cool to around 45 °C at room temperature and then poured into 2.5ml plastic syringe moulds. The moulds were placed in a liquid circulating cryostat at -15 °C or -5 ºC for around 12-16 h after which they were thawed in de- ionized water for 2-3 h. The thawed gels were frozen again at -80 °C for 6 h and shifted to a lyophilizer in order to ensure complete drying. Temperature was maintained at 25 °C to synthesize the hydrogels while all other parameters remained unchanged. Both conventional and FD agarose hydrogels were synthesized as mentioned earlier.

2.3 Characterization of synthesized cryogels and hydrogels

2.3.1 Structural and morphological characterization using scanning electron microscopy (SEM)

Scanning electron microscopy was used as a tool to visualize the internal pore architecture and surface properties of the fabricated cryogels and hydrogels. Samples of 3mm height and 7mm diameter were prepared and sterilized with 20 to 100% ethanol gradient followed by three subsequent washes with sterile phosphate buffer saline (PBS), pH 7.4. All scaffolds were then dried in a desiccator to remove excessive moisture. An hour before the experiment, the samples were coated with gold using a Cressington sputter coater. The current of the coating unit was kept constant at 40mA and coating was performed for a period of 75 s. Coated samples were then fixed to SEM stubs using double sided conducting carbon tape. The samples were then placed in the vacuum chamber of the equipment and extremely low-pressure condition was generated. SEM was conducted using Zeiss SEM machine. A voltage of 10kV was maintained during the experiment.

2.3.2 Rheological analysis for the determination of viscoelastic properties

The rheological analysis was performed on both dry and wet samples using Anton-Paar compact table top rheometer MCR 102. Each sample was cut to a dimension of 5mm height and 7mm diameter. Analysis on wet sample was performed by placing samples in de-ionized water for 1 h before the experiment. Care was taken while placing the samples under the load cell so as to avoid the slipping of the samples under load. Oscillatory logarithmic sweep was used to calculate the values at a frequency of 1Hz with an initial load of 1N. The readings were recorded at 37 °C with intervals of 15 s for a total of 900 s.

2.3.3 Evaluation of compressive strength using mechanical analyser

Bose electroforce mechanical analyser was used to evaluate the compressive strength. The sample dimensions for both dry and wet state were carefully recorded before placing the samples under the load cell. All experiments were conducted in triplicates with the ramp speed of 0.5mm/s. The failure length in case of each sample was set to be 50% of the initial sample length. Load and displacement values obtained from the machine were used to calculate the stress and strain. To obtain the stress values, the load values were divided by the circular area of each sample while the strain was calculated by dividing the displacement by original length of the sample. The results were plotted and compressive strength was analyzed for each sample.

2.3.4 Swelling kinetics

Sections of 2mm height and 7mm diameter were cut from each sample for swelling experiment. Swelling and de-swelling kinetics was performed in a cyclic manner on three days as reported earlier [32]. At the start of each cycle, the dry weight of scaffolds was recorded for water retention capacity. For cycle 1, dry weight of gels was recorded and then gels were placed in 24-well plates filled with de-ionized water under static conditions. Different readings of weight were recorded at every 30s for 5 min, followed by an interval of 5 min and 10 min. After cycle 1, swollen gels were air dried and processed further for cycle 2 and 3 by repeating steps mentioned in cycle1.

The % water retention capacity of the gels was calculated using the following formula:

$$WR(\%) = (Wtp - Wdry)/Wwet \text{ X } 100$$

Where, *WR (%)* is the % water retention capacity of the gels, *Wtp* is the weight at a specific time point, *Wdry* is the dry weight of the gels and *Wwet* is the wet weight of the gel. Conventional hydrogels were not used in this experiment mainly due to the tediousness involved in handling these gels in the dry state.

2.3.5 Estimation of percentage pore volume by cyclo-hexane method

The average pore volume (%) of different gels was computed using the cyclo-hexane method [38]. Gels of same length (3mm height, 7mm diameter) were cut and their dry weights were recorded. Later, the gels were immersed in cyclo-hexane for a period of 1 h and then the weight of the swollen gel was recorded. Average pore volume was computed by the following formula:

$$\text{Average Pore Volume} = (Wwet- Wdry)/Wwet \text{ X } 100$$

Where, *Wdry* is the dry weight of the gel while *Wwet* is the wet weight of the gel after 1h. The experiment was performed in triplicate and average values were considered. Only cryogels and FD hydrogels were considered in this experiment mainly due to the difficulties that arise in handling conventional hydrogels in the dry state.

2.4 Cell- material interaction

2.4.1 MTT assay for accessing *in vitro* biocompatibility

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was used for determining the *in vitro* biocompatibility of the synthesized scaffolds. Chitosan cryogels 2% (w/v), chitosan-gelatin gels 8% (w/v) and chitosan-agarose-gelatin 5% (w/v) were used for this assay. The scaffolds were cut into 3mm height and 7mm diameter followed by the sterilization process as mentioned in section 2.3.1. The scaffolds were seeded with primary chondrocytes isolated from the knee joint of a three week old chicken. The cartilage was removed aseptically and cut into 0.5- 1mm pieces and washed with sterile phosphate buffered saline (PBS). PBS washed chicken cartilage pieces were trypsinized and further incubated in collagenase type II (prepared in plain DMEM media) enzyme solution for 12-14 h. After enzymatic digestion, the resultant cell suspension was mildly spun and re-suspended in complete DMEM media supplemented with 10% foetal bovine serum (FBS) in a T-75 flask. The cells were allowed to proliferate for a period of 7-10 days with intermittent media change after every 48 h. After reaching 80% confluence, the cells were given a passage to increase the cell number to a desired level. A cell density of 10^6 cells/ml was used to perform the experiment and a total of 250µl of cell suspension was used per scaffold. These seeded scaffolds were incubated for a period of three weeks, which included three time points at week 1, week 2 and week 3. All the scaffolds were incubated in complete DMEM media supplemented with 10% FBS under aseptic conditions in a humid CO_2 incubator at 37 °C with 90% relative humidity. The experiment was also carried out on 2D tissue culture treated plates that acted as a control. However, the polymeric scaffolds of each type were placed separately, in triplicates, in 12 well, non-treated tissue culture plates.

The assay was performed in triplicates to reduce the error. On the day of the assay, culture media was removed from each well, followed by the addition of MTT reagent (0.5mg/ml in plain DMEM). MTT treated samples were then incubated for 4-5h at 37 °C. The MTT reagent was aspirated from each well followed by the addition of 1.5 ml dimethylsulfoxide (DMSO) per well. The plates with DMSO were incubated for 15-20 min. After incubation, DMSO was carefully pipetted out into 2ml micro-centrifuge tubes and absorbance was read at 570nm against DMSO as a blank. Absorbance vs. time histogram was plotted for both control and test samples in order to access the cell proliferation and biocompatibility of the synthesized matrices.

2.4.2 SEM analysis to access cell- material interaction

SEM was used to investigate the behaviour of seeded chicken chondrocytes on the surface of fabricated scaffolds. The ECM production and cell morphology was examined *via* SEM in order to analyze the biocompatibility of the synthesized scaffold. Sterile ethanol gradient (20- 100%) treated scaffolds of 3mm height and 7mm diameter were exposed to UV light for 30 m in a biosafety hood. The dehydrated sterile scaffolds were then seeded with chicken chondrocytes and incubated for a maximum of two weeks. At every week, the cell-seeded scaffolds were fixed using 2.5% glutaraldehyde for 4-6 h at 4 °C, followed by a wash with PBS. The fixed scaffolds were prepared for SEM analysis in a similar fashion as mentioned in section 2.3.1.

2.4.3 Mechanical and rheological changes due to cell adhesion and proliferation

CAG scaffolds were chosen to further investigate mechanical and rheological changes caused due to proliferation and adhesion of chondrocytes on the CAG scaffolds. In order to analyze differential mechanical and rheological properties between seeded *vs.* non seeded scaffolds, non seeded CAG scaffold incubated with DMEM complete media supplemented with 10% foetal bovine serum for a period of 24 h was used as a control. Moreover, chondrocyte seeded CAG scaffold (cell density of 10^6cells/ml) incubated with complete DMEM media for a period of four weeks was taken as the test sample and the experiment was done in triplicates to minimize error. These samples were analyzed to check the compressive strength and elasticity after cells were seeded and all the parameters remain the same as mentioned in sections 2.3.2 and 2.3.3.

3. Results and Discussions

In our current work we have primarily focused on two different types of gels namely, cryogels and hydrogels. Cryogelation is a relatively new technique in which the polymeric solution is incubated under sub-zero temperatures and rapid phase separation takes place which leads to the formation of ice crystals in frozen liquid phase. During this process polymer concentrates in the unfrozen liquid microphase by the phenomenon of cryoconcentration. Presence of a crosslinker in the mixture then leads to crosslinking of the polymer, which results in the formation of a highly interconnected porous network. The ice crystals upon thawing give rise to a porous and interconnected 3D macroporous structure known as *cryogels*. On the contrary, in case of hydrogels, the polymeric solution and the crosslinker react at room temperature to form a polymeric network, which is comparatively brittle, less porous and poorly interconnected due to lack of phase separation process. We have characterized these gels by different techniques that are discussed in detail in the following sections. In the latter part of this work, we choose to evaluate the potential of natural polymer based cryogel scaffold in supporting cell growth. However, these scaffolds varied from each other in terms of polymeric components, while all other parameters remained unchanged, which led to varied rheological and mechanical behaviour of the materials due to a change in the precursors. We primarily focused on chitosan, chitosan-gelatin and CAG cryogel scaffolds for analysing the proliferation of chicken chondrocytes. CAG scaffolds have already been known to support the proliferation of chondrocytes and aid in the development of neo- cartilage [34]. However, our aim was to compare the effect of addition of polymeric components to basic chitosan cryogels to achieve effective cell proliferation capability of CAG cryogel scaffolds. Chitosan and gelatin mimic the protein component of the ECM while agarose adds to the mechanical strength and elasticity of the scaffolds [32]. The motivation of doing this study was to investigate the importance of mechanical and rheological properties of a 3D scaffold on cell proliferation. In

the cell-material interaction section, these properties have been tailored by majorly varying the temperature and polymeric components of cryogels thus emphasizing the novelty of this research work.

3.1 Synthesis of cryogels and hydrogels

Figure 1 represents digital images of some of the polymeric gels described earlier at different temperatures and by using different crosslinking methods. The monomer based, 6% pAAM gels were synthesized by free radical polymerization [33]. While as, three different types of chemically crosslinked gels i.e. 2% and 4% chitosan, 8% chitosan-gelatin and 13% CAG gels were synthesized utilizing schiff base reaction. Schiff base reaction crosslinks aldehyde groups of glutaraldehyde and free amino groups of chitosan and gelatin. As agarose (low EEO) exhibits self gelation property below 40 °C, 5% agarose gels were synthesized without the use of a crosslinker . All the gels were

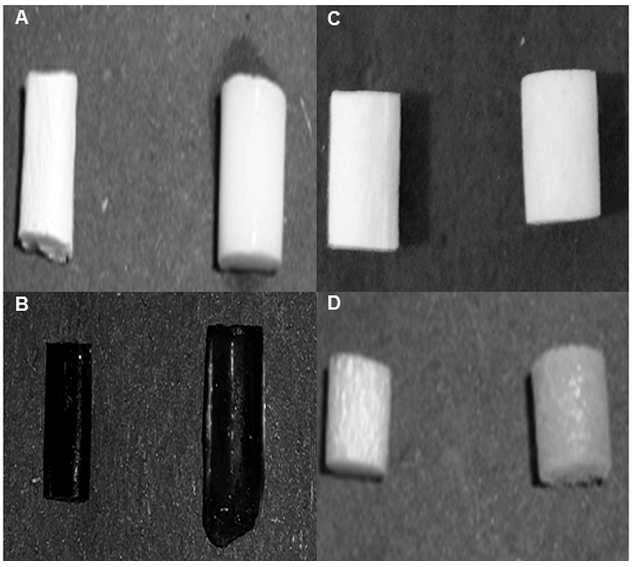

Figure 1: Representative digital images of polymeric gels synthesized at different temperatures. Panel A, B, C and D represents FD polyacrylamide hydrogels (6%) fabricated at 25 °C, conventional chitosan hydrogels (4%) made at 25°C, CAG cryogels (13%) made at -15°C and agarose cryogels (5%) synthesized at -5 °C. Gels in panel A were freeze-dried while panel B gels were air dried at room temperature.

synthesized by varying the temperature. Three temperatures, -15 °C, -5 °C and 25 °C were used to observe the effect of temperature on the bulk properties of cryogels. Chitosan hydrogels have been synthesized at two different concentrations owing to their structural stability at higher concentration thus making handling easier. We used 2% FD chitosan hydrogels and 4% conventional chitosan hydrogels. While cryogelation imparts well-defined pore morphology, high interconnectivity, high elasticity and mechanical stability owing to cryo-structuring, we focused on studying the difference in bulk properties of cryogels and hydrogels synthesized with same monomer/polymer concentration. However, the only difference that makes these fabrication techniques contrasting is the variation in the synthesis temperature, which is one of the motivations behind this work.

3.2 Scanning Electron Microscopy (SEM)

In order to study the effect of temperature on the pore morphology of the gels, SEM analysis was performed (Figure 2). A common trend was observed among all gel types fabricated at different temperatures. The gels synthesized at -15 °C had open and homogenously distributed pore morphology (Figure 2A) as compared to the gels synthesized at -5 °C and 25 °C (Figure 2B, C and D). Moreover, the pore size of the gels made at -15 °C was bigger than the gels synthesized at other temperature which ensures convective fluid flow through cryogel monoliths. This can be attributed to the fact that the phase separation i.e. the formation of ice crystals and unfrozen liquid microphase takes place very fast. The ice crystals are formed rapidly in the frozen phase while polymerization and/or crosslinking take place in unfrozen liquid microphase. After phase separation, the solute molecules begin migrating leading to the formation of a cryo-concentrate.

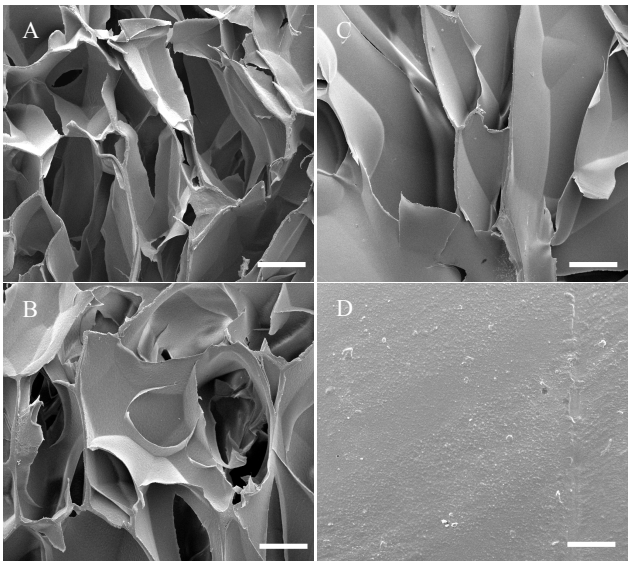

Figure 2: Representative scanning electron microscopy (SEM) images (magnification 200X) of agarose gels synthesized at different temperatures. Panel A, B, C and D represents agarose gels (5%) synthesized at -15 °C (A), -5 °C (B) and freeze-dried agarose hydrogel made at 25°C (C) and air-dried agarose hydrogel (D), respectively.

Moreover, under sub-zero temperatures, due to the cryo-concentration the rate of reaction also accelerates allowing the monomers/polymers to react rapidly. Meanwhile, the ice crystals keep growing from the periphery of the mould towards the center till they meet the facet of another crystal [28]. This phenomenon of cryo-structuring ensures large pore size in case of cryogels. Additionally, the pore size in case of cryogels also depends on a number of factors like, concentration of the precursors, amount of crosslinker, freezing temperature, etc. thus cryogels can be tailored as per the desired application. Even though, the SEM analysis reveals that the pore morphology of cryogels synthesized at -15 °C is more prominent and well defined, it should be noted that the size of ice crystals formed at -5 °C is generally larger than the crystals formed at -15 °C and the pore size is bigger at higher freezing temperatures when compared with low freezing temperature [35]. However, the crystals are not able to branch or propagate in an efficient manner due to uncontrolled nucleation and also due to depression in freezing point leading to rough and closed morphology of pores. However, in our experiments, gels

synthesized are more porous and well defined at -15 °C because of the significant depression in the freezing point that happens at -5 °C. This freezing point depression results in the formation of a new phase known as the vitreous or glass phase, which leads to the formation of smaller pores.

In case of conventional hydrogels, lack of ice crystals leads to the absence of a physical barrier between polymeric chains, causing them to come closer thus resulting in the formation of significantly smaller pores (Figure 2D). However, a comparison between the pore morphology of conventional and FD hydrogels indicates that FD gels possess some pores (Figure 2C). This difference in pore morphology is caused by the freezing of the free water in FD gels, which leads to formation of pores upon lyophilization. The transport of solute molecules in conventional hydrogels mainly takes place because of the space that exists between the polymeric chains and there exist a number of theories that govern the movement of molecules [36].

Thus, it can be concluded from the SEM results that the cryogels synthesized at -15 °C has larger, well-defined and interconnected pores because of proper crystallization under cryo-conditions throughout the gel. The gels synthesized at -5 °C and 25 °C have smaller pores due to the depression in freezing point and lack of ice crystal formation, respectively. The conventional hydrogels, being dried in a vacuum desiccator rather than lyophilizing leads to the formation of very small and less interconnected pores as observed in the SEM analysis.

3.3 Rheological analysis

Rheology can be defined as the measure of viscoelastic properties of a material or the study that analyses the flow or deformation of materials when there is an impending stress. The main objective of studying rheology in this work is to explain how various parameters like temperature, type of monomeric/polymeric precursors, source of the precursors i.e. natural or synthetic, state of the material i.e. dry or wet affect the rheological properties of a material. The storage modulus (G') of a material is basically the measure of the energy that a material can store upon application of certain force. This energy is however, transformed into elasticity of the material. The loss modulus (G") of a material is the viscous behaviour of the material when a force is applied. The force applied causes the material to flow and thus there is loss of energy while the material flows. This energy lost is quantified by the loss modulus. The phase angle (δ) is also another important parameter defining the rheological properties of the material. The phase angle is basically the difference in waveform shift that is recorded when a force is applied and a response to the force is generated. A phase angle value closer to 0° indicates that a material is more elastic while as the phase angle value closer to 90° indicates viscous nature of a material. In our study, we have compared different types of gels synthesized at different temperatures to observe the effect of different crucial parameters like temperature, concentration etc. on the rheological properties of polymeric gels.

3.3.1 Rheological analysis of pAAM cryogels and hydrogels

Among all synthesized gels, pAAM cryogels synthesized at -15 °C shows the maximum value of storage modulus both in dry (930 kPa) and wet state (14.2 kPa) (Figure 3) indicating towards maximum elasticity. Moreover, a low value of phase angle also confirms to that fact. These gels also exhibit constant elastic behaviour throughout the test, which means that there are no molecular rearrangements in the structure due to the application of constant load. However, small fluctuations in elasticity were observed in the gels synthesized at -5 °C, which may be attributed to the freezing point depression. Freezing point depression occurs due to the migration of the solute molecules from the free water towards the bound water [37]. This depression in freezing point also causes a change in the stiffness of the material as indicated by the fluctuating phase angle in both dry and wet states of -5 °C cryogels. Even though the FD hydrogels exhibit some resistance to the initial load, its structural integrity was compromised at later time points where the gel starts showing a pseudo elastic behaviour due to the continuous application of load. This phenomenon is also indicated by the increase in the storage modulus and a decrease in phase angle value. Conventional pAAM hydrogels exhibits least elastic behaviour in the wet state and that might be because of the inherent structure of the polymer (Figure 4). Thus, among all monomer based gels synthesized at different temperatures, cryogels synthesized at -15 °C posses maximum elasticity.

3.3.2 Rheological analysis of C, CG, CAG cryogels and hydrogels

It is a well-documented fact that water in case of polymeric solutions generally occurs in three forms, *viz;* free water, intermediate water and bound water. In case of cryogelation, the free water as the name specifies, is free from any solute molecules and freezes immediately under sub-zero temperatures. As a consequence of this, the solute molecules run towards the left water i.e. the intermediate and the bound water. The concentration of the solute in the bound water is the highest and it does not freeze easily because of the depression in freezing point. This bound water results in the formation of unfrozen liquid microphase. The depression in the freezing point is a colligative property and depends on numerous factors like the concentration, nature of precursors i.e. hydrophilic or hydrophobic. The viscosity of the polymeric solution is also known to be a potential cause in delaying or retarding the growth of ice crystals. We have observed a uniform trend in the storage modulus of all chemically crosslinked gels in the dry state. The storage modulus value is the highest in case of chemically crosslinked gels when fabricated at 25 ºC, which from our current experiments depends on the stiffness of these materials in their dry states. The values of storage modulus have constantly increased by the addition of another polymer. Chitosan FD hydrogels (Figure 5) have the least storage modulus value followed by FD chitosan-gelatin (Figure 6) hydrogels and chitosan-agarose-gelatin (Figure 7) cryogels possess a maximum value of storage modulus in the dry state. Additionally the viscosity modulus also behaves in a similar manner indicating that FD hydrogels are also more viscous when compared with cryogels. In the wet state, a different behaviour of polymeric gels is observed. Chitosan and chitosan-gelatin FD hydrogels and cryogels synthesized at -5 °C tend to possess almost similar values of storage modulus.One of the possible reasons is that cryogels made at -5 ºC do not tend to form a cryoconcentration and they also possess similarities to FD hydrogel and thus their rheological properties resemble more to

conventional hydrogels in the wet state than to the cryogels fabricated at -15 ºC. However, the addition of gelatin did not affect the storage modulus values of chitosan and chitosan-gelatin cryogels made at -15 ºC and the storage modulus values are nearly equal in both cases. When gelatin is added to either cryogels made at -5 ºC or FD hydrogels, there is a significant increase in the storage modulus values and this clearly indicates the effect of lack of cryoconcentration formation in both hydrogels and cryogels.

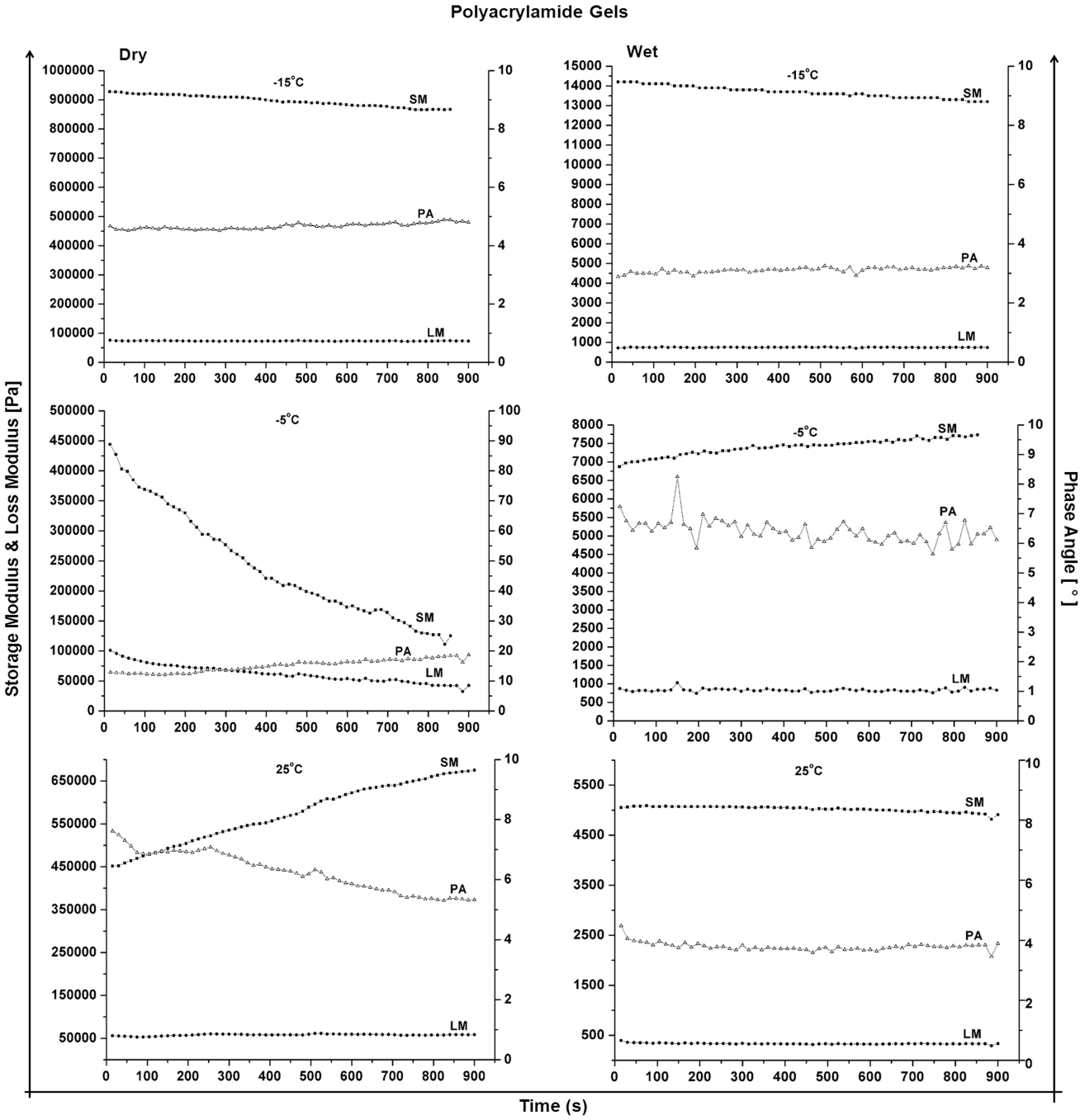

Figure 3: Rheological behaviour of polyacrylamide cryogels and FD hydrogels at different temperatures. Rheological analysis of polyacrylamide gels fabricated at -15 °C, -5 °C and 25 °C was performed in both dry and wet state at 37 °C. SM Indicates storage modulus, LM Indicates loss modulus and PA Indicates phase angle.

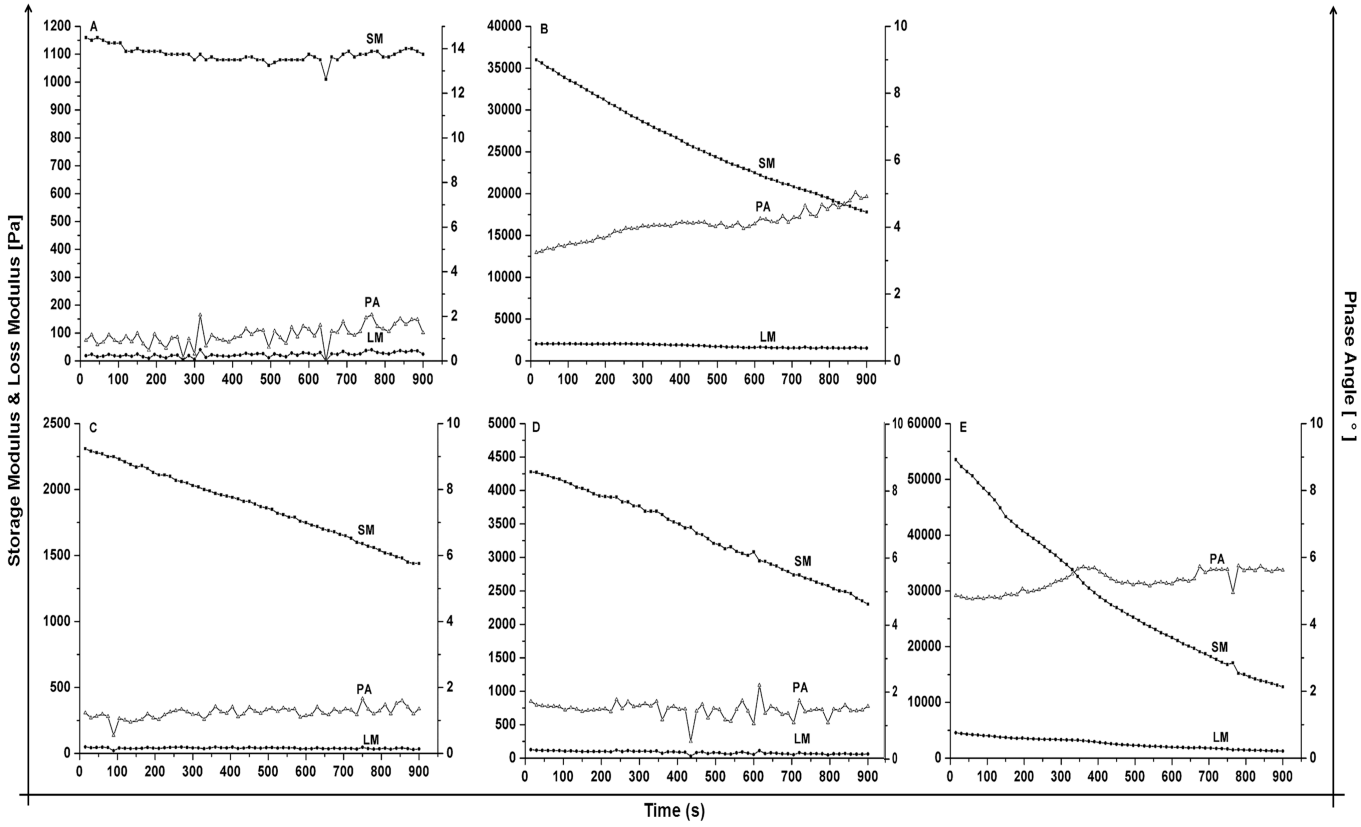

Figure 4: Rheological behaviour of conventional hydrogels. The rheological analysis of conventional hydrogels of polyacrylamide (A), agarose (B), chitosan (C), chitosan-gelatin (D) and CAG (E) in wet state synthesized at 25 °C. The analysis was performed at 37 °C. SM Indicates storage modulus, LM Indicates loss modulus and PA Indicates phase angle.

Since the water uptake of both the gels was different, it might be possible that this unequal uptake of water balances their elastic properties in the wet state, which yields nearly similar storage modulus in these gels. In case of CAG cryogels (Figure 6), the effect of adding agarose is clearly visible. Unlike chitosan and chitosan-gelatin gels, we observed a difference in the storage moduli of both cryogels and FD hydrogel. The storage modulus is least in case of CAG FD hydrogels due to a very high concentration. CAG cryogels made at -15 ºC are the most elastic of the lot in the wet state. This is one of the most important reasons of choosing CAG cryogels for regenerating the elastic cartilage tissue and also studying the effect of ECM production on viscoelastic and mechanical properties of CAG cryogels. All chemically crosslinked conventional hydrogels (Figure 4) in the wet state exhibit an increasing trend of storage modulus with addition of another polymer with CAG hydrogel with maximum storage modulus values and chitosan the least.

3.3.3 Rheological analysis of agarose cryogels and hydrogels

Agarose cryogels fabricated at -15 °C possess highest value of storage modulus in the dry state. However, over time because of the impending force, the gel succumbs to the load and its elastic modulus reduces to more than half to a value of 220 kPa, which may be because the gel is physically crosslinked and the bonds are not very strong (Figure 8). It may be possible that molecular rearrangements might take place due to the application of load. The phase angle also supports this hypothesis. Elasticity modulus of agarose gels synthesized at -5 °C lies between the cryogels synthesized -15 °C and FD hydrogels. Agarose gels synthesized at -15 °C seem to be the least viscous as well, this can be attributed to their strength once again. In the wet state, the FD hydrogels of agarose are the most elastic while the cryogels at -15 °C is the least. One of the possible reasons is that, agarose being a highly hydrophilic polymer, its cryogels synthesized at -15 °C absorbs more water due to higher porosity that causes a decrease in the elastic nature of the polymer. Due to the interaction of polymer with water molecules, more porous gels exhibit least elasticity.

3.4 Mechanical analysis of cryogels and hydrogels

Figures 9,10 & 11 indicate the mechanical behaviour of different polymeric gels made at varying temperatures. The mechanical properties of the gels were tested on a uniaxial Bose Electroforce mechanical analyser. The ratio of height and diameter was kept same in all the gels. The gels were placed on the load cell of the machine while uniaxial force was applied from the top. The ramp moved at a speed of 0.5 mm/s. The output generated from the tests was then reconverted in terms of stress and strain. The ultimate compressive strength of each gel was extracted after plotting the stress- strain curve for each sample. The ultimate compressive strength of a material is a bulk property of a material and the porosity of a scaffold directly tells about its strength.

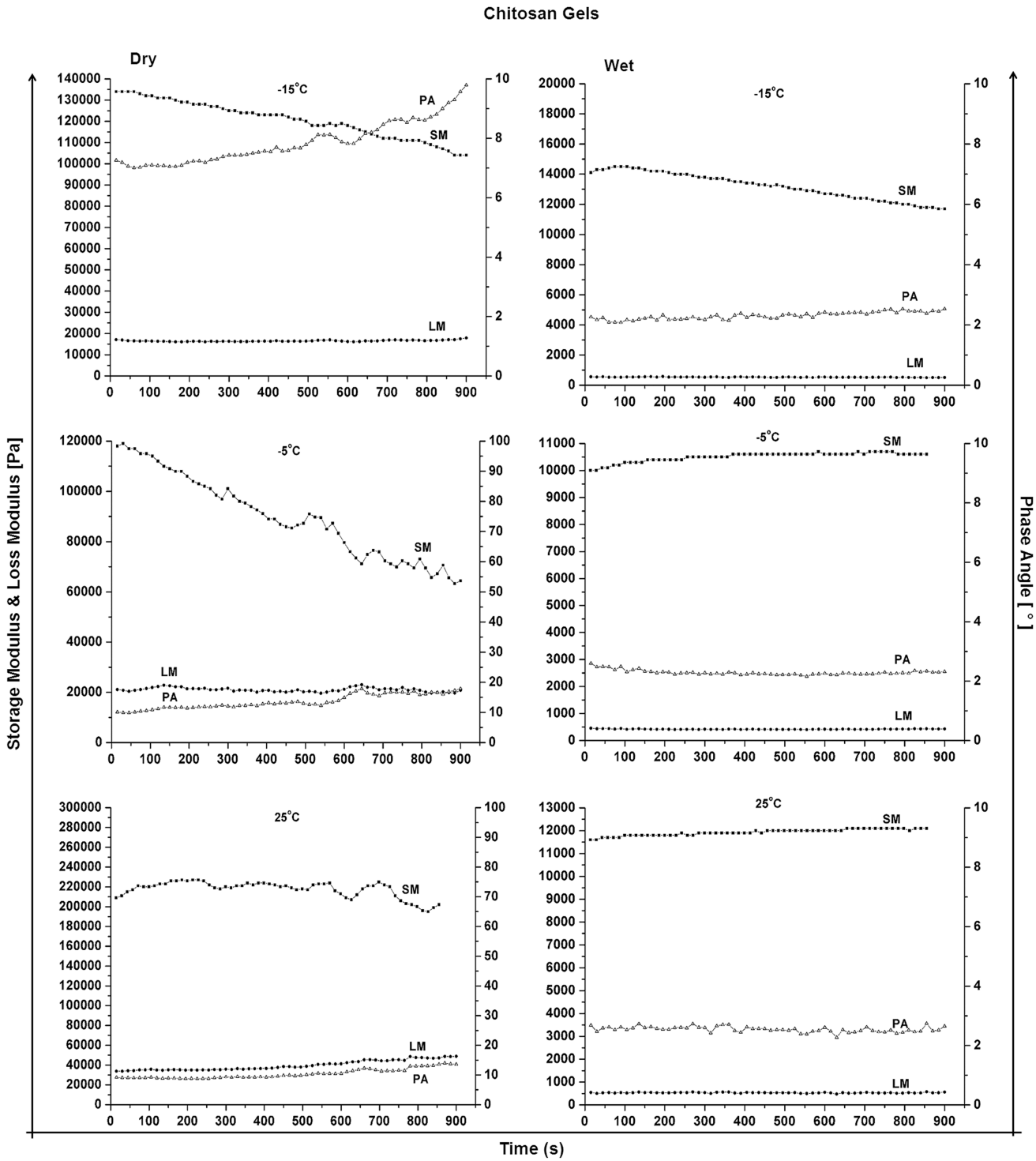

Figure 5: Rheological behaviour of chitosan based polymeric gels at different temperatures. Graphical representation of rheological analysis of chitosan gels synthesized at -15 °C, -5 °C and 25 °C was performed in both dry and wet state at 37 °C. SM Indicates storage modulus, LM Indicates loss modulus and PA Indicates phase angle.

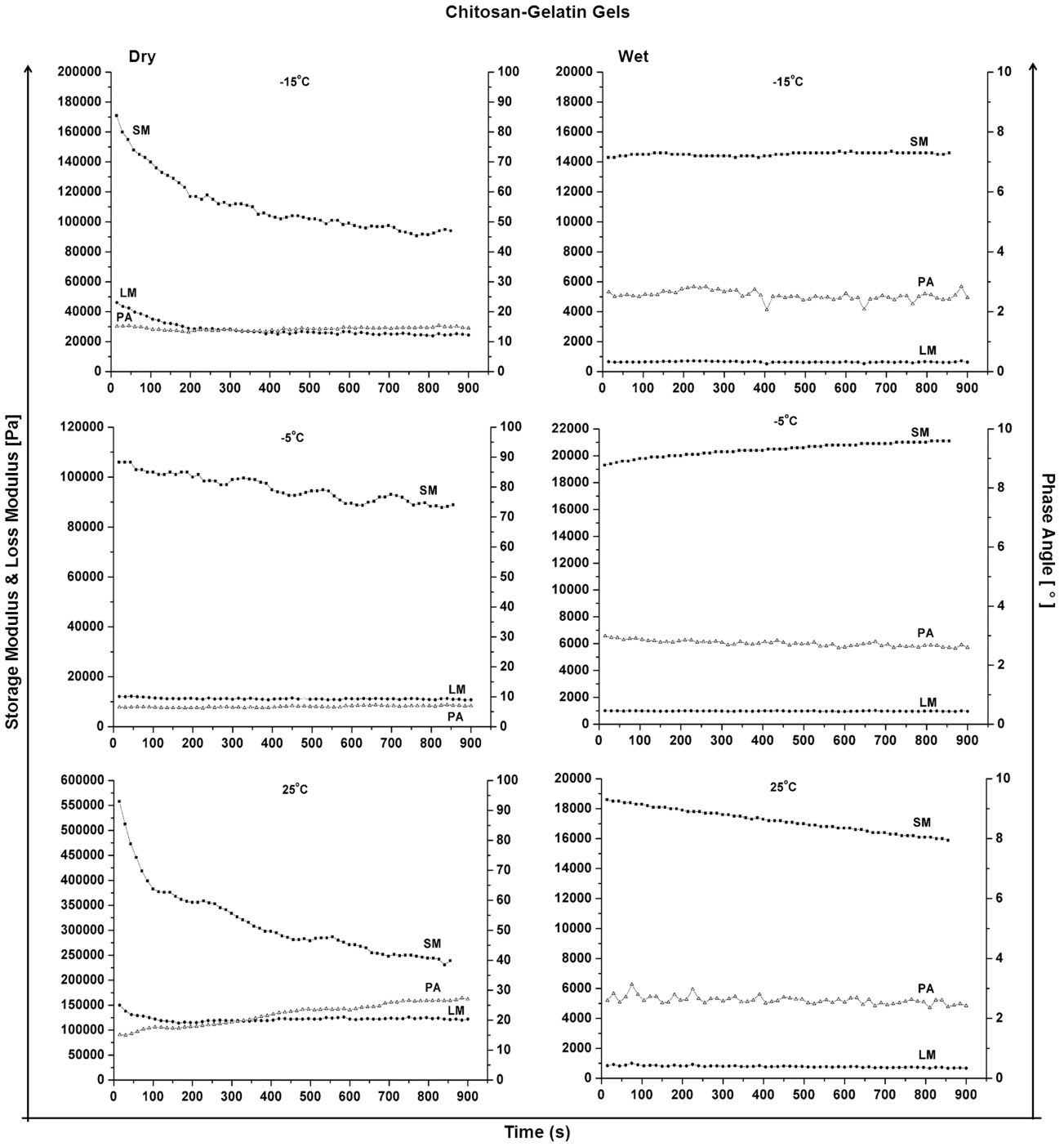

Figure 6: Rheological behaviour of chitosan-gelatin based polymeric gels at different temperatures. Graphical representation of rheological analysis of chitosan gels synthesized at -15 °C, -5 °C and 25 °C was performed in both dry and wet state at 37 °C. SM Indicates storage modulus, LM Indicates loss modulus and PA Indicates phase angle.

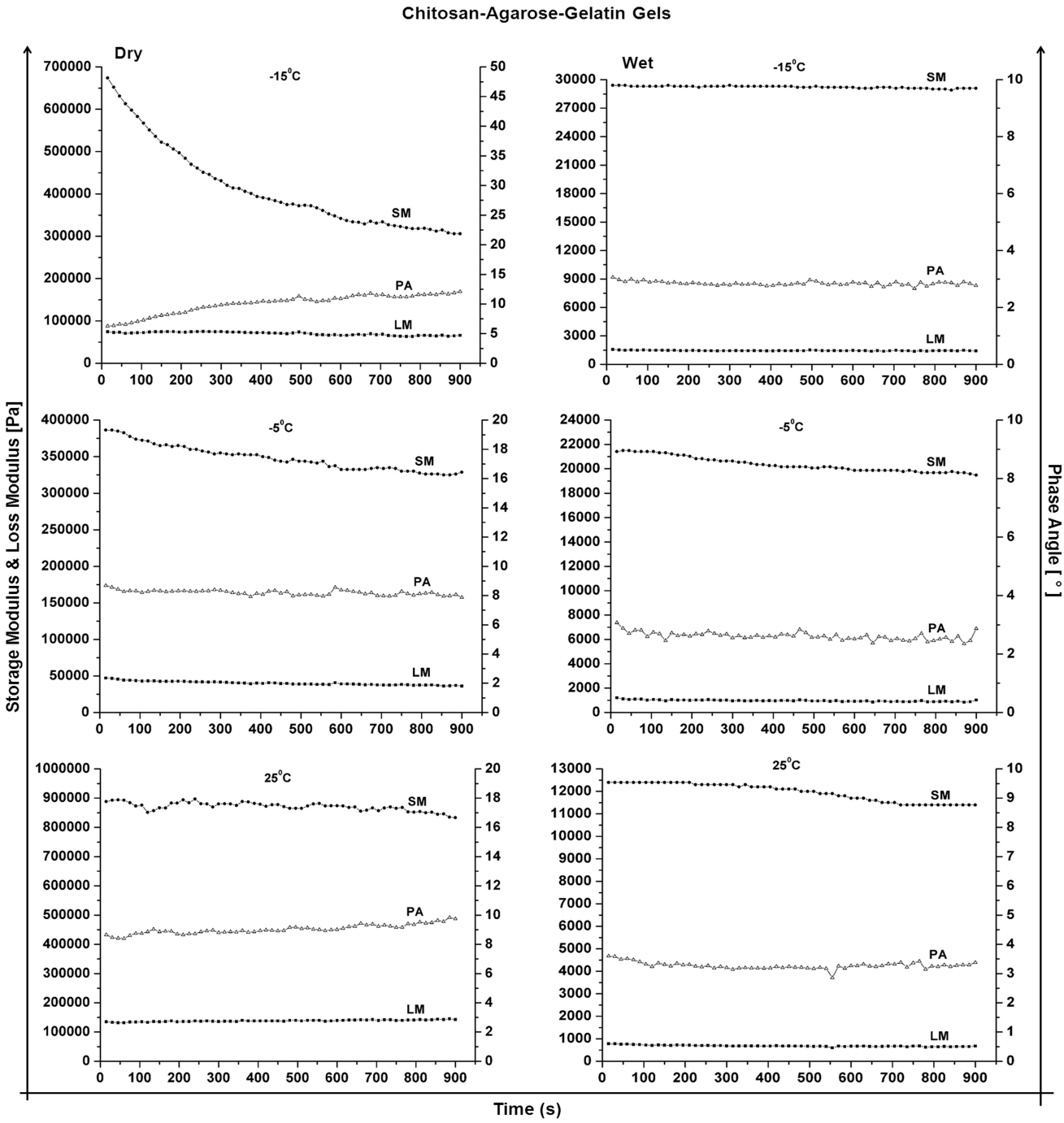

Figure 7: Rheological behaviour of CAG based polymeric gels at different temperatures. Rheological analysis of CAG gels fabricated at -15 °C, -5 °C and 25 °C was performed in both dry and wet state at 37 °C. SM Indicates storage modulus, LM Indicates loss modulus and PA Indicates phase angle.

A number of factors like the inherent property of the monomer/polymer precursors, concentration of the precursors, concentration of the crosslinker, degree of crosslinking, type of crosslinking, synthesis temperature, etc. decide the mechanical fate of synthesized scaffolds. Thus, it becomes imperative to maintain equilibrium between the pore size and mechanical strength in order to achieve an ideal scaffold. Pore size and thickness of the pore walls together formulate the pore volume, which plays an important role in mechanical property of the scaffold[38]. From the synthesis perspective, cryogels are flexible since they allow some degree of freedom to the developer in terms of porosity and mechanical strength. Often a scaffold cannot be used for a specific application due to its lacuna of pore size or the mechanical strength. Development of application specific matrices is easier with cryogels compared to other gels.

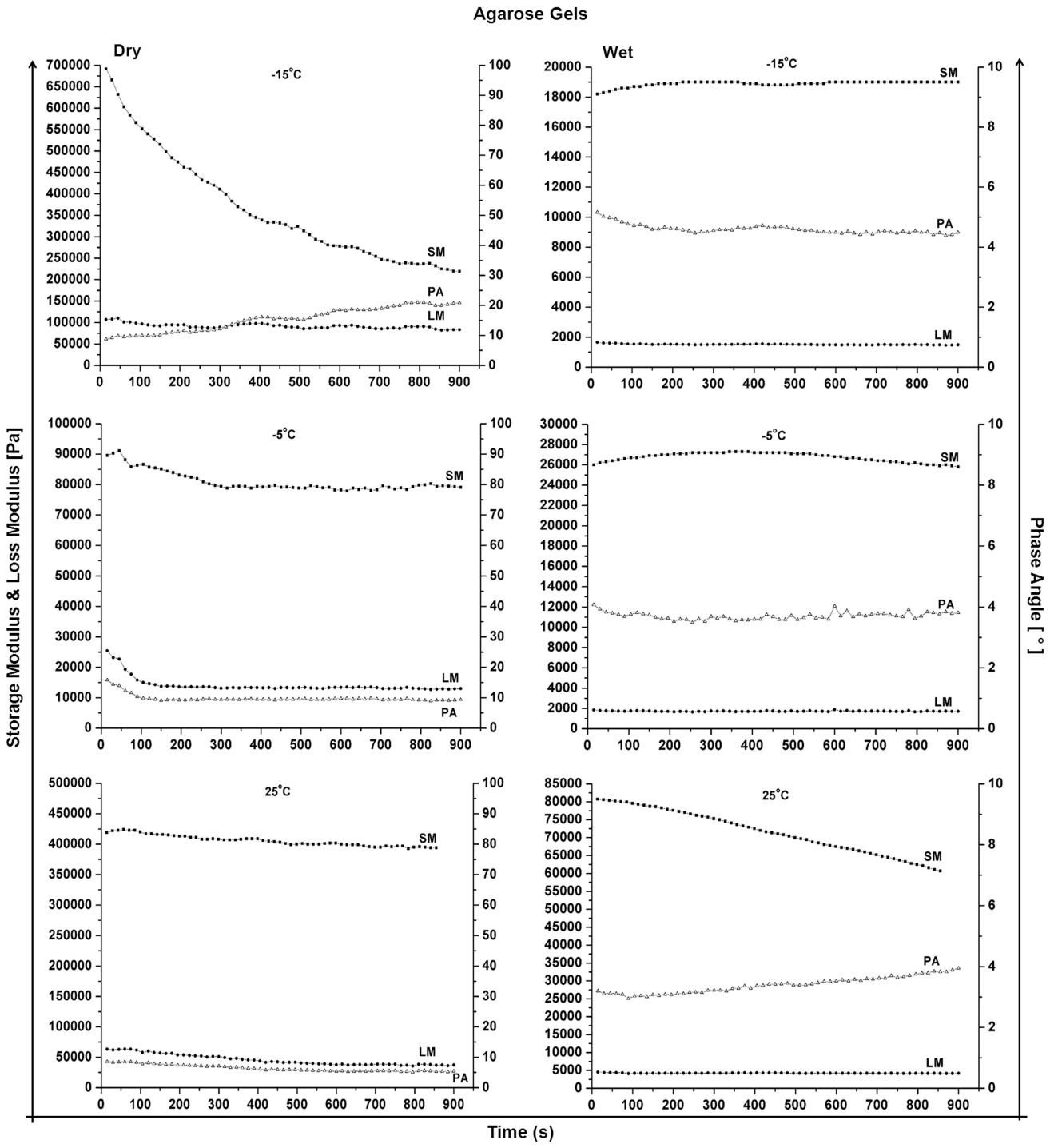

Figure 8: Rheological behaviour of agarose cryogels and FD hydrogels at different temperatures. Rheological analysis of agarose gels fabricated at -15 °C, -5 °C and 25 °C was performed in both dry and wet state at 37 °C. SM Indicates storage modulus, LM Indicates loss modulus and PA Indicates phase angle.

3.4.1 Monomer based pAAM cryogels and hydrogels

In both dry and wet state, pAAM cryogels synthesized at -15 °C are the weakest with an ultimate compressive strength of 145 kPa and 12 kPa, respectively (Figure 9). The gels synthesized at -15 °C possess low strength because they have large pores as compared to the hydrogels and hence possess less strength. Cryostructuring causes larger pores and high porosity and thus less mechanical strength of the cryogels in the dry state. The cryogels synthesized at -5 ºC are stronger than -15 °C gels but possess an almost similar value of ultimate compressive strength as that of FD hydrogels. They possess an ultimate compressive strength of 225 kPa, which is very close to the ultimate strength of FD hydrogels. Hydrogels and cryogels made at -5 ºC are stronger in the dry state. They form a mesh like structure with minimal pores and hence are stronger than cryogels in this case.

The strength in the gels synthesized at -5 °C can be attributed to the depression of freezing point that increases the size of the unfrozen liquid microphase and thus, polymer walls are thicker and smaller pores are formed. In the dry state, cryogels synthesized at -5 ºC and 25 ºC exhibit a brittle behaviour by rupturing immediately after maximum compressive strength is applied. Conventional pAAm hydrogels are strongest in the wet state (Figure 10).

3.4.2 Chemically crosslinked chitosan, chitosan-gelatin, chitosan- agarose-gelatin cryogels and hydrogels

Among all the chemically crosslinked gels, the FD hydrogels are the strongest with maximum ultimate compressive strengths of 180, 350 and 700 kPa respectively for chitosan, chitosan-gelatin and CAG gels (Figure 11). In case of FD hydrogels, with the addition on an extra polymer, the compressive strength almost doubles in the dry state. Due to a highly porous nature of polymeric cryogels fabricated at -15 ºC, their mechanical strength in the dry state is the weakest, which increases with the addition of other polymers. The ultimate compressive strength of cryogels made at -5 ºC ranges between the cryogels and FD hydrogels due to their structural resemblance to FD hydrogels. A comparison within the group, for example between chitosan gels fabricated at different temperatures, we have observed that hydrogels possess the maximum ultimate compressive strength (180 kPa) followed by cryogels made at -5 ºC (70 kPa). The least value of compressive strength is seen in the cryogels made at -15 ºC (<60 kPa). In the dry state, we have seen a very similar trend in the ultimate compressive strength of all chemically crosslinked gels. In the wet state, chitosan and chitosan-gelatin gels responded mechanically similar as compared to their respective dry behaviours. More porous cryogels fabricated at -15 ºC were weaker followed by gels fabricated at -5 ºC. On the contrary, CAG cryogels fabricated at -15 ºC possess maximum compressive strength (70 kPa) in the wet state while both CAG cryogels made at -5 °C and CAG FD hydrogels possess nearly equal (35 kPa) ultimate compressive strength. An optimum synthesis temperature and a smart balance of polymeric precursors can be responsible for such behaviour. This mechanical stability of CAG cryogels in the wet state makes them more attractive for tissue engineering of cartilage when compared with scaffolds made by other fabrication techniques. In case of chemically crosslinked conventional hydrogels (Figure 10) in the wet state, they all possess significantly higher values of ultimate compressive strength when compared to chemically crosslinked cryogels or FD hydrogels. Chitosan and chitosan-gelatin cryogels both yield soon before they behave in a plastic manner. However, CAG conventional hydrogels obeys Hooke's law till the end following which it loses its elasticity.

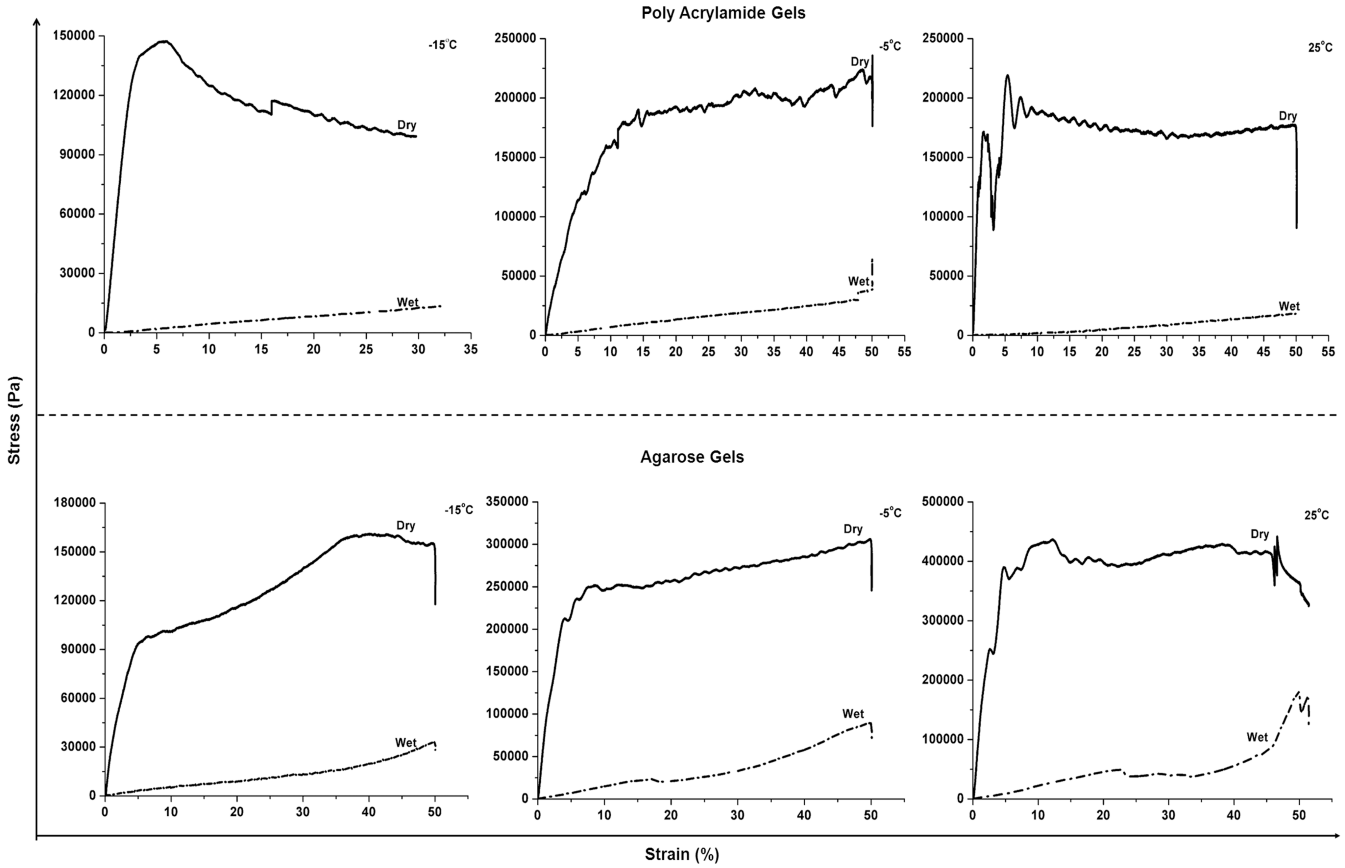

Figure 9: Stress vs. strain curve of monomer based polyacrylamide and physically crosslinked agarose cryogels and FD hydrogel. The mechanical analysis of monomer based polyacrylamide gels (top panel) and agarose gels (bottom panel) made at three different temperatures viz; -15 °C, -5 °C and 25°C. Dry Indicates dry state and Wet indicates wet state.

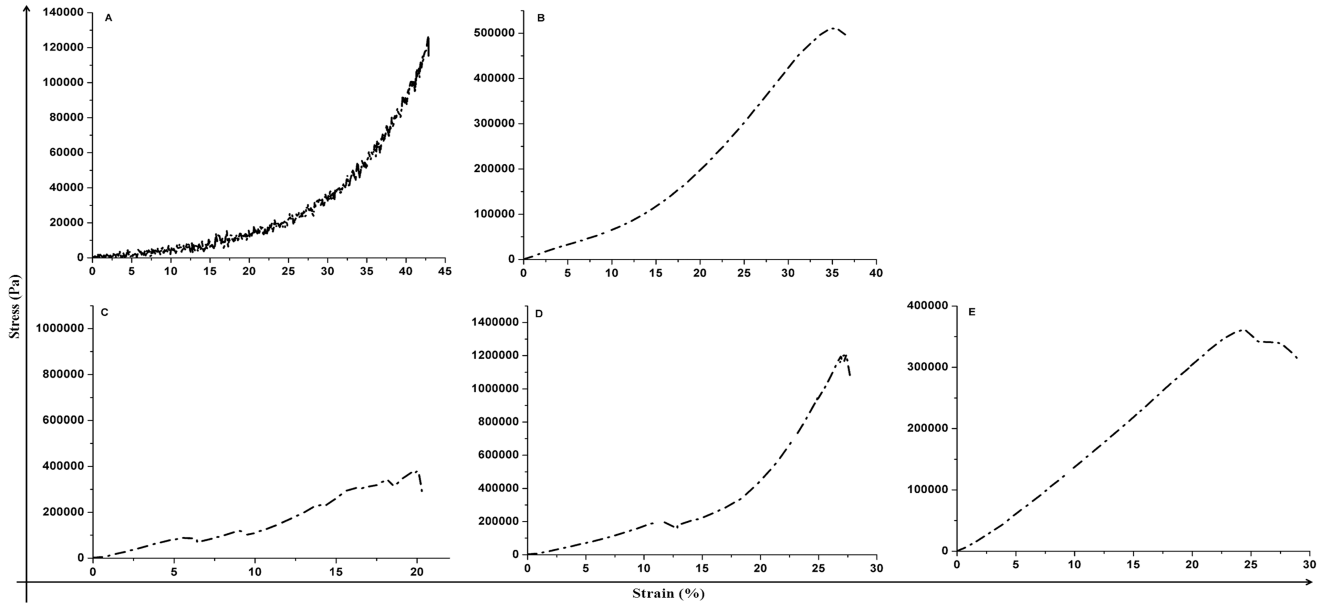

Figure 10: Stress vs. strain curve of conventional polymeric hydrogels. Mechanical analysis of conventional hydrogels of polyacrylamide (A), Agarose (B), Chitosan (C), Chitosan-Gelatin (D), CAG (E) in wet state, synthesized at 25 °C.

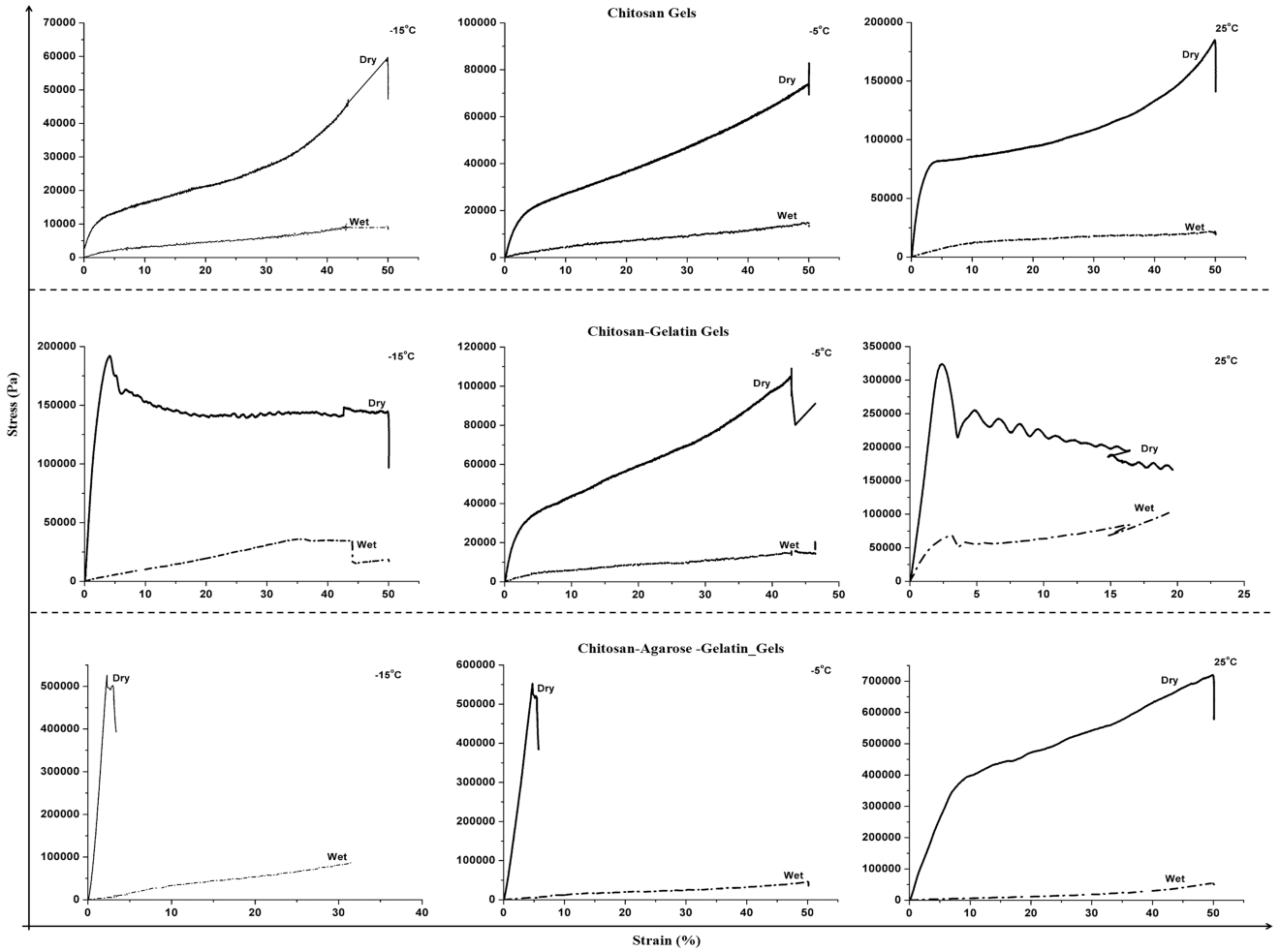

Figure 11: Stress vs. strain curve of chemically crosslinked polymeric cryogels and FD hydrogels. The mechanical analysis of chemically crosslinked chitosan (top), chitosan-gelatin (middle) and CAG (bottom) gels synthesized at -15 °C, -5 °C and 25 °C in dry and wet states.

3.4.3 Physically crosslinked agarose cryogels and hydrogels

The most porous agarose gels synthesized at -15 °C are the weakest of the lot possessing an ultimate compressive strength value of 165 kPa (dry) and 20 kPa in the wet state (Figure 9). This can be because of the larger pore size formed in the gels at -15 °C. With the increase in temperature there is a significant increase in the mechanical strength of the agarose gels. Apart from the pore size, the inherent strength and flexibility of the inert agarose polymer also adds to the increasing strength at higher temperatures. Moreover, at higher temperatures the polymeric chains in agarose come very close to each other or in other sense an entangled network is formed, leading to the formation of a very stable polymeric network [39]. FD agarose hydrogels possess the highest compressive strength values of 440 and 170 kPa in dry and wet states, respectively. Conventional hydrogels of agarose are far stronger than FD hydrogels or cryogels in the wet state with a whooping 500 kPa ultimate compressive strength (Figure 10).

3.5 Swelling kinetics

The distribution of pores and degree of interconnectivity plays a pivotal role in the swelling kinetics of the gels. It can be clearly seen that cryogels swell instantaneously to their maximum limits (Figure 12). This is an indirect indication towards how well the pores are organized in cryogels. This characteristic feature of cryogels can be beneficial in situations wherein retention of large volumes of nutrients is required in a shorter span of time. One of the possible reasons is the presence of highly interconnected capillary pores in cryogels [40]. However, swelling also depends on concentration of the precursors and the pore size. Another important reason that might be responsible for a high swelling degree of cryogels is the presence of large pores. Hydrogels take more time to swell mainly due to the absence of capillary pores and that is the reason hydrogels swell at later time points. Moreover, due to low degree of interconnectivity of pores in hydrogels, there is a slow response shown by hydrogels when it comes to uptake of fluids. Due to well-defined, larger pores, all cryogels synthesized at -15 °C swell to their maximum value in a short span of time. However, pAAm cryogels, swell maximum out of all the gels synthesized at -15 °C, which may be attributed to their synthetic precursors. Cryogels synthesized at -5 °C follow the cryogels synthesized at -15 °C which is due to the cryo structuring when compared with both types of hydrogels.

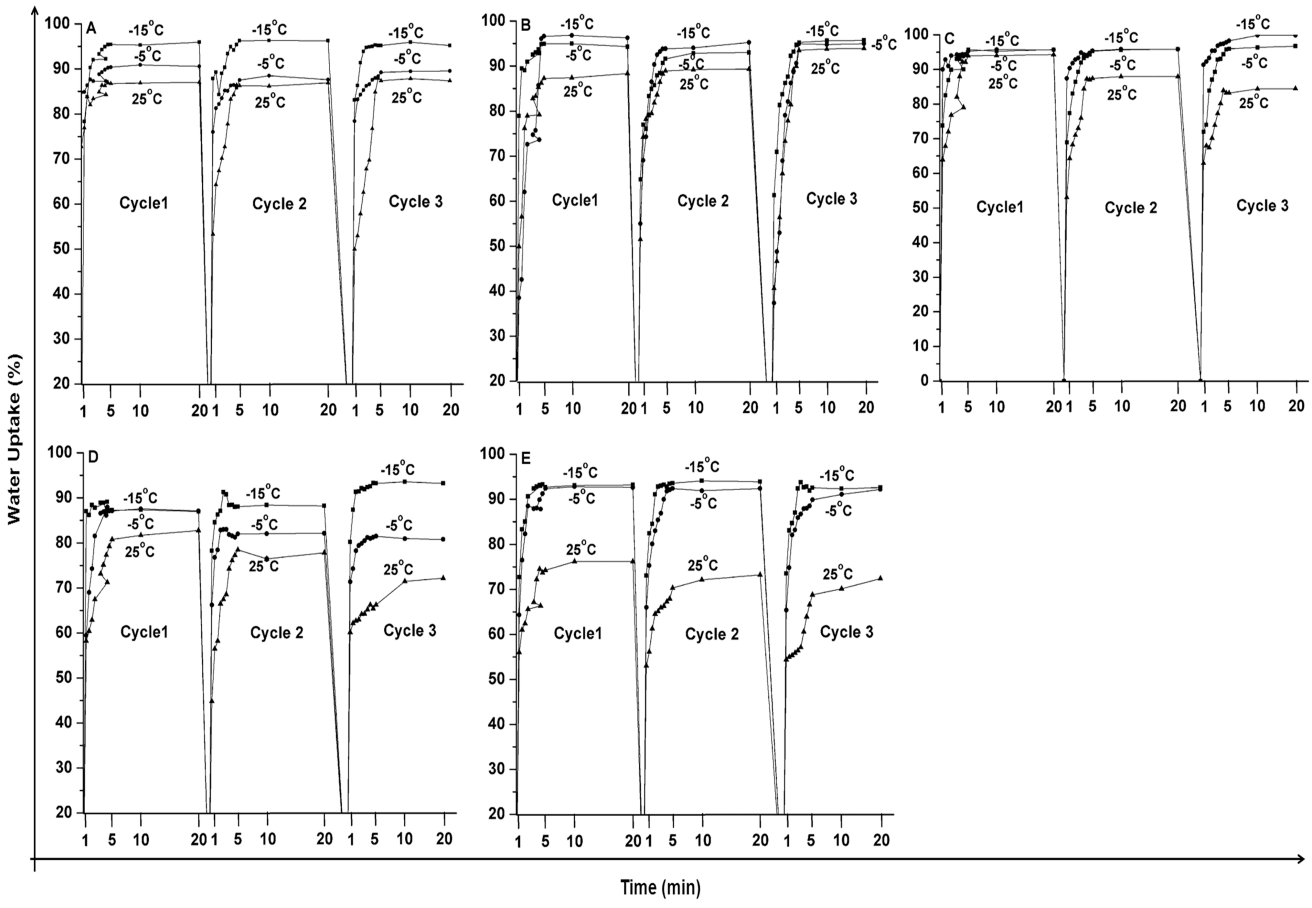

Figure 12: Swelling-deswelling behaviour of polymeric gels synthesized at different temperatures. Panel A, B, C, D and E represent polyacrylamide, agarose, chitosan, chitosan-gelatin and CAG cryogels and FD hydrogels synthesized at -15 °C, -5 °C and 25 °C. [-□-] Indicates cryogel at -15°C, [-○-] indicates cryogel at -5°C and [-△-] indicates FD hydrogels synthesized at 25 °C.

3.6 Percentage pore volume

The percentage pore volume of 72%-92% was observed among fabricated gels using cyclo-hexane method. All the cryogels synthesized at -15 °C exhibit maximum pore volume that can be attributed to larger pores caused by the cryo-structuring. FD hydrogels seem to possess closed pores, which have also been seen in SEM images and because of this the hydrogels have less percentage pore volume. Also the pore volume in case of FD hydrogels is less while the thickness of the walls is more, this can also be another potent reason of less pore volume in hydrogels. The gels fabricated at -5 °C possess larger pores when compared to the hydrogels. Again cryo-treatment may be responsible for the apparent results. We also observed a decrease in the percentage porosity with the increase in concentration of the precursors. This decrease may be attributed to the comparatively smaller pores those are formed with the increasing concentration.

3.7 Cell- material interaction

Significant differences in the mechanical and rheological properties of gels with variation in temperature motivated us to study the cell behaviour on them. We studied the behaviour of chicken chondrocytes on different cryogel matrices synthesized at -12 °C Moreover, natural polymeric precursors were preferred over synthetic polymers in the cell material interaction section mainly due to higher affinity of cells to natural polymers than synthetic polymers. As CAG cryogels have already been used in our laboratory for cartilage tissue engineering applications we furthered our research on these matrices. We tried to evaluate the effect of mechanical and rheological behaviour of different materials on the growth and proliferation of chicken chondrocytes. CAG cryogels were chosen as a standard for chondrocyte proliferation while chitosan and chitosan-gelatin cryogels were used as controls to understand how cells responded on other material surfaces.

3.7.1 MTT assay for accessing *in vitro* biocompatibility

The assay is performed in order to evaluate the biocompatibility of the synthesized matrices by growing the cells onto them. It is a colorimetric analysis for checking cellular activity and it works by reducing the tetrazolium dye into its insoluble form leading to the formation of formazan crystals when it comes in contact with live cells. The crystals are then dissolved using dimethyl sulfoxide, an organic solvent and the solution immediately discharges a purple colour. The absorbance from this purple colour is then read spectrophotometrically at 570nm.The MTT assay was performed on the cryogel scaffolds of chitosan, chitosan-gelatin and chitosan-agarose-gelatin synthesized at -12 °C. It was observed that cryogels synthesized at -12 °C or -15 °C did not show any significant deviation with respect to their bulk properties due to a minimal temperature variation.

The cell viability analysis results using the MTT assay were quite fascinating and unravelled expected results. While the chondrocytes proliferated at a faster rate on the 2D i.e. treated well plates, the proliferation rate reached its maxima at the second week after which the cells could not proliferate much and the cell number decreased to a lower value on the last time point. This can be due to the presence of lesser surface area available for the cells. So initially when the cells proliferate they occupy the available surface after which they die due to lack of surface and build up of the toxins (Figure 13). Among the polymeric 3D scaffolds, chitosan cryogel scaffold exhibits maximum cell growth at the two-week time point. However, there is a sudden reduction in the proliferation that is possibly caused by the incompetence of the scaffold in terms of supporting chondrocytes due to abominable mechanical and elastic properties. Due to a balance of elastic and mechanical properties and optimum surface area, CAG scaffolds were expected to exude better results. The scaffolds show a gradual growth initially which maybe because the cells require some time to acclimatize to the 3D environment and a sustained cell growth is seen even on the later (3rd week) time point also. CAG scaffolds among all other scaffolds show maximum sustained growth and thus it can be concluded from the biocompatibility assay that all the scaffolds support the growth and proliferation of chicken chondrocytes. However, CAG scaffolds are the best among the group for the proliferation of chondrocytes due to the balance of pore size and rheological properties, which are pre-requisites for a tissue-engineering scaffold.

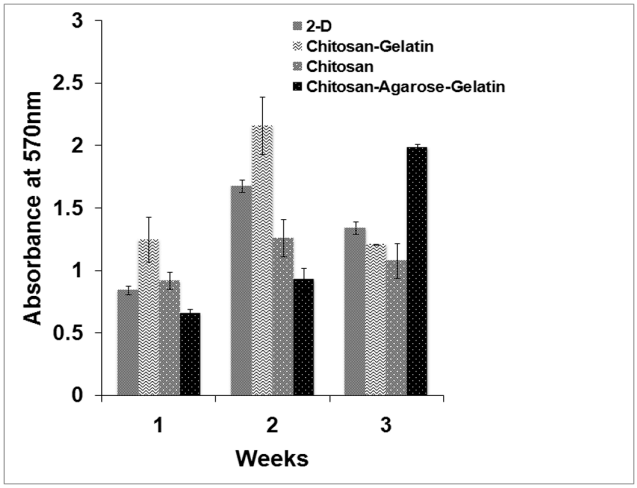

Figure 13: *In vitro* biocompatibility analysis using MTT assay. The MTT assay results performed on chemically crosslinked cryogels of chitosan, chitosan-gelatin and CAG synthesized at -12 °C in order to access the cell viability.

3.7.2 SEM analysis to access cell- material interaction

Scanning electron microscopy was also used as a tool to analyze the cell-material interactions on CAG cryogel scaffolds synthesized at -12 °C. The sterile CAG scaffolds were seeded with isolated chicken chondrocytes at a cell density of 2 x 10^5 cells per scaffold. SEM analysis of seeded scaffolds for each time point indicated good ECM production and cell proliferation. Chondrocyte ECM secretions could be observed from as early as one week while a healthy cell growth with high ECM production and cell number was observed at the end of the second week (Figure 14). In conclusion, it can be conferred that chondrocytes acclimatized well to the microenvironment provided by the CAG cryogel scaffold by mimicking the native tissue habitat, which is very well reflected by the significant amount of ECM secreted with higher cell proliferation rate.

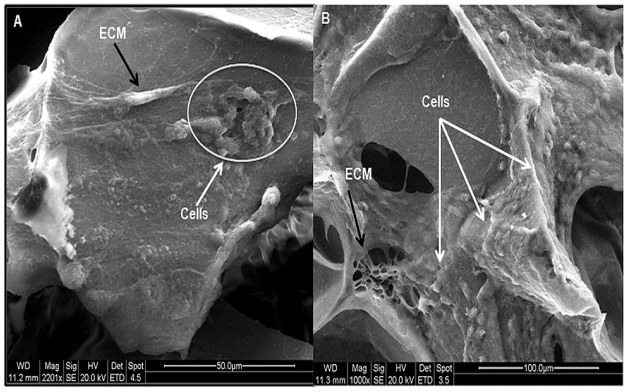

Figure 14: Scanning electron microscopy (SEM) images of CAG cryogel scaffolds fabricated at -12 °C seeded with chicken chondrocytes. (A) represents CAG scaffold seeded with chondrocytes at one week time point while, (B) represents cell seeded CAG scaffolds at two weeks. Scale bar (200μm).

3.7.3 Mechanical and rheological changes due to cell adhesion and proliferation

Though the emphasis of this work has majorly been on determining how temperature variations and changes in the monomeric/polymeric precursors affect the bulk properties like mechanical strength and rheological characteristics of different polymeric gels, and also how these parameters effect cell adhesion and proliferation. A more logical extension of the current work would also be to analyze the *vice versa,* i.e. the impact of cell adhesion and proliferation on the mechanical and rheological properties of these polymeric gels.

Once the chondrocyte viability and proliferation results were compared and analyzed, we chose to characterize the better scaffold in terms of mechanical and rheological properties and thus cell seeded CAG scaffolds were chosen. The mechanical study clearly indicates that there is a huge increase in the compressive strength of the scaffolds seeded with cells after four weeks. The ultimate compressive strength increases from a value of 50 kPa to a value of approximately 250 kPa (Figure 15; bottom panel). The probable reason of this increase can be due to the secretion of extra cellular matrix (ECM) by the cells, which mainly comprises of strong collagen fibers. ECM secreted by the chondrocytes occupies the space between the pores of these cryogel scaffolds and may also integrate closely with the scaffold to increase their mechanical strength. On the contrary, the rheological results suggest that the overall elasticity of the material reduces after four weeks of cell growth when compared to the control sample and there is a minimal reduction in the viscous property of the seeded samples, which obviously happens due to the ECM secretions (Figure 15; top panel). These results hold significant importance since these parameters are very important for a scaffold to qualify for cartilage tissue engineering applications. The above results along with the cell viability results show the potential of CAG scaffolds as the most appropriate scaffolds for cartilage tissue engineering.

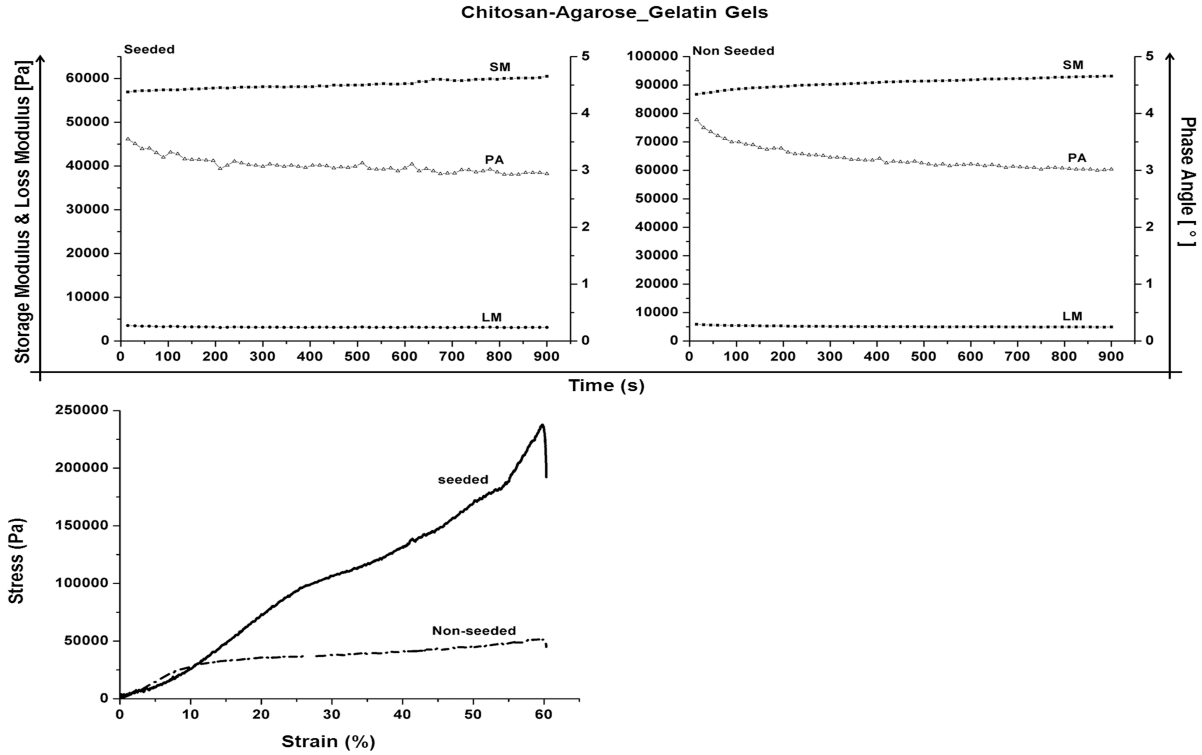

Figure 15: Rheological and mechanical analysis of chondrocyte seeded and non-seeded CAG cryogel scaffolds after 4 weeks. Rheological analysis of CAG cryogels synthesized at -12 °C both in dry and wet states before and after cell seeding is represented in top panels. SM Indicates storage modulus, LM indicates loss modulus and PA indicates phase angle (PA). While bottom panel represents the mechanical analysis of wet CAG scaffolds before and after cell seeding. Scaffolds were seeded with chondrocytes and incubated for 4 weeks in complete DMEM in case of seeded scaffolds while as non-seeded scaffolds were incubated in complete media for 24h before analysis could be performed.

4. Conclusion

Rheological and mechanical analysis performed on synthesized polymeric gels indicated influence of "fabrication temperature" on mechanical as well as visco-elastic behaviour of the material. Our results revealed effect of cryo-structuring on distribution of pores and pore morphology with respect to all crosslinking methods used for synthesis of polymeric gels. This was also evident from comparative analysis of FD hydrogels and conventional hydrogels against cryogels. Comparative study of hydrogels against cryogels reflected more pronounced effect of temperature on mechanical fate of scaffold. A decrease in temperature leads to the formation of smother and larger pores as a result of fast nucleation of ice crystals. Moreover, this decrease in synthesis temperature can also cause a decrease in compressive strength of a polymeric material owing to larger pores. Our results indicated a direct correlation of porosity with visco-elasticity. The effect of visco-elastic and mechanical properties on cell material interaction was also studied. Our study suggested that scaffolds demonstrating a right balance of bulk properties are thus good candidates for supporting cell proliferation and growth.

5. Notes and references

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