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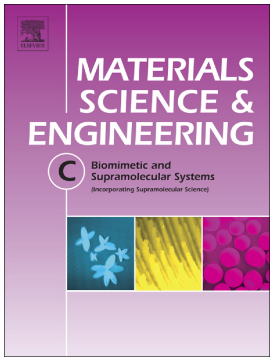

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Injectable natural polymer compound for Tissue engineering of Iintervertebral disc: invitro study

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Abstract:

Intervertebral disc degeneration is recognized to be the leading cause for chronic low-back pain .Injectable hydrogel is one of the great interests for tissue engineering and cell encapsulation specially for intervertebral (IVD) affecting rate of regeneration success, in this study we assessed viscoelastic properties of a Chitosan-β glycerophosphate - hyaluronic acid,Chondroitin-6-sulfate,type 2 of Collagen , gelatin , fibroin silk (Ch-β-GP-HA- CS- Col-Ge-FS) hydrogel which was named as NP hydrogel that is natural extracellular matrix of IVD.

Chitosan-based hydrogel was made in the ratio of 1.5%: 7%: 1%:1%:1%-1.5%-1% (Ch: β-GP: HA-CS-Col-Ge-FS). Gelation time and other rheological properties were studied using amplitude sweep and frequency sweep tests. Also, the cytotoxicity of the hydrogel invitro assessed by MTT and trypan blue tests. Morphology of the hydrogel and attachment of NP cells were evaluated by SEM.

Our result showed that NP hydrogel in 4^{o}C is an injectable transparent solution. It started gelation in 37^{o}C after about 30 min. Gelation temperature of NP hydrogel was 37^{o}c. Storage modulus (G′) of this hydrogel at 37 ^{o}C was almost constant over a wide range of strain.MTT and trypan blue tests showed hydrogel was cytocompatible.The obtained results suggest that this hydrogel would be a natural and cytocompatible choice as an injectable scaffold for using in vivo study of IVD regeneration.

Key Words: Chitosan-β glycerophosphate- hyaluronic acid ,Collagen ,Chondroitin sulfate, gelatin, fibroin silk, intervertebral disc, thermoresponsive, injectable

Introduction:

Low back pain is a worldwide prevalent health care issue nowadays. 60-80% of people in United States of America have experienced low back pain (1) Herniation of intervertebral disc (IVD) and its degeneration are the major reasons of low back pain, which occur because of structural damage of disc (2). Therapeutic strategies for disc degeneration treatment are tissue engineering and gene transferring, which have done in laboratory animals (3).

Use of appropriate scaffold is an important point in tissue engineering and especially for cartilage restoration. Scaffolds prepare a three-dimensional condition for proliferation, production and secretion of extracellular matrix and formation of normal tissue (4-7). The purpose in tissue engineering is to find the proper substances with significant traits for restoration of tissue. These traits are biodegradable (6-8) and biocompatible, which mean don't induce inflammatory reactions and toxic production (6-8). Having proper pores and controlled porosity, scaffold surface must be appropriate for adherence, proliferation and migration of cells (9).

Injectable hydrogel scaffolds are an important category of biomaterials in tissue engineering (9). Softness, inherent flexibility, injectability, easily casting into different shapes and three dimensional structures of hydrogels which mimic the extra cellular matrix of natural tissues make them very useful in this field (10). These hydrogel materials form by the changes in the environmental conditions, such as ionic charge, electrical stimulus, temperature or pH of aqueous polymer solutions (11) .Yan et al. suggested Temperature-sensitive hydrogels are more attractive than other ones because growth factors, cells and other biologically active elements can be loaded more easily inside them (12).

Chitosan as a natural, biocompatible, biodegradable and abundant polymer can form injectable thermoresponsive hydrogels (13). This scaffold has been used in regeneration of bone and cartilage and also in tissue engineering (14 ,15). Injectable thermosetting chitosan based hydrogels at first described by Chenite et al (16). They used chitosan/glycerophosphate salt combination (17). Chitosan/glycerophosphate salt (CH–GP) hydrogels are physically cross linked and don't have toxicity of synthetic hydrogels. So many studies performed about CH-β-GP hydrogels in different concentrations (18 ,19). IVDs are located between spines, which contain 3 parts. The outer part is annulus fibrosis (AF), the middle part is transitional zone (TZ) and the inner part is nucleus pulposus (NP), which produce the nucleus of disc (1, 3). AF and NP formation are mainly from

extracellular matrix. Water, proteoglycans, and Collagen in the extracellular matrix (ECM) of NP tissue provide fluidity and viscoelasticity to the structure, acting as a shock absorber, and maintaining loads in IVDs Some polymers could help chitosan to improve its mechanical and biological virtues. The proportion of each component of GAG in the

Human NP is contain 90% sulfated GAG and 10% hyaluronan (20) .Gelatin is one of them, which improves the biological activity of scaffold because of its specific sequence that increases cell adhesion and migration (21).

In a previous study, it was found that Chondroitin-6-sulfate in combination with other natural scaffolds increases proliferation, NP cell survival and even the strength of the composite scaffolds (22).

Injectable hydrogel is one of the great interests for tissue engineering and cell encapsulation especially for intervertebral (IVD) affecting rate of regeneration success (14, 15).

The goal of this study was to prepare and assess injectable natural compound hydrogel (NP hydrogel) that is similar to ECM structure of IVD with appropriate efficiency .We assessed behavior of NP cells on NP hydrogel in vitro model. To do that, we used chitosan based hydrogel containing chondroitin-6-sulfate and collagen II. We added gelatin and fibroin silk to increase hydrophilicity, stability and strength of compound hydrogel. Compound hydrogel was characterized by

rheometer .We also assessed cytotoxicity of hydrogel by cultured of NP cells on NP hydrogel.

Methods and materials:

Preparation of injectable hydrogel:

Step 1: Preparation of silk fibroin solution:

Mori cocoons were prepared from Iranian silkworm research center (Guilin, Iran) and silk fibroin was extracted following the as described previously (23). Briefly, silk sericin was removed using boiling of mori cocoons in 0.02 M Na2CO3 solution for 30 min. The silk fibers were then rinsed three times with deionised water and dried at 37 °C for 24 h. Subsequently, extracted fibroin were dissolved in 9.3 M LiBr solution for 3h and then dialyzed against distilled water us by a cellulose dialysis membrane with 12 kDa molecular weight cut-off. Water was exchanged several times to remove LiBr salt from the fibroin protein solution .The final concentration of the fibroin silk solution was determined gravimetrically using drying the solution. The fibroin protein had a 9% (w/v) concentration.

Step 2: Preparation of Col/HA solution:

Type II Collagen (Col) dissolved in 0.01 M acetic acid to yield 1% (w/v) aqueous solution. Hyaluronic acid sodium (HA) (Sigma-Aldrich, St. Louis, MO) was

dissolved in phosphate-buffered saline (PBS) separately to yield 1 % (w/v) aqueous solution. Finally, collagen solution mixed in HA solution.

Step 3: preparation of FS / Col-HA solution:

Two solutions were mixed using repeated pipetting at a ratio of 50:50 (vol. %) to obtain homogeneous FS/Col-HA solution (1% w/v FS and 1% w/v Col-HA).

Step 4: preparation of Ch / CS - Ge solution:

Ch (Chitosan) 1.5 % (w/v) obtained using dissolved in acetic acid (0.1 M). CS (Chondroitin-6- sulfate) and Ge (Gelatin) were dissolved in distilled water separately to yield 1% (w/v) and 1.5% (w/v) aqueous solutions, respectively. Then Ch solution were mixed with CS-Ge solution at a ratio of 50:50 (vol. %) (1.5 % w/v Ch , 1 % w/v CS and 1.5 % w/v GE).

Step 5: preparation of FS/Col-HA/Ch-CS-Ge solution: solutions FS, Col-HA and Ch-CS-Ge mixed with equal ratio 1:1:1.

Step 6: β-GP solution: 1.12 g of sterilized β-glycerol phosphate (β-GP) (Sigma, Germany) dissolved in 1.7 ml of sterile deionised water to yield 7 %(w/v) aqueous solution.

Final step: Thermoresponsive hydrogel: Final mixed solution and β-GP solution kept on ice for 15 minutes and then cold β-GP solution added drop wise to the cold

final mixed solution with continuous stirring to form a clear solution .obtained solution were thermoresponsive hydrogel that named NP hydrogel.

Rheological assessments:

All rheological tests that mentioned below performed by a Physica MCR 300 rheometer (Anton-Paar, Ashland, VA, USA) with appropriate tools.

Gelation time determination:

The gelation time of thermo responsive hydrogel was measured by using the rheometer equipped with a cone-cup tool (CC27). To simulate in vivo injection, samples from 4ºC transferred to 37ºC at time zero and gelation time was measured as a function of time at constant temperature of 37ºC.The elastic modulus (G′) and the viscous modulus (G″) were evaluated from the oscillatory measurements at a frequency of 1 Hz. The gelation time was determined as the time past in which G′ and G″ curves intersect each other.

Amplitude sweep:

Amplitude sweep was done using cone-plate tool (CP25-2) at 37ºC. The distance between the plates was 0.05mm. Samples were placed directly between the parallel plates. Beneath plate was heated up to 37ºC prior to measurement. Mineral oil ٥ was used to prevent dehydration. Amplitude sweep records the linear viscoelastic

region (LVER) of a material. Storage (elastic) modulus (G′) is used to define it. The limit of the LVER is where the structure of the material starts to break down. Thus LVER is a direct measurement of structure and indicates the stability of sample. We performed amplitude sweep at a frequency of ω=10 rad/s with a strain range from 0.1% to 100 % (19).

Frequency sweep:

Frequency sweep such as amplitude sweep was done using cone-plate tool (CP25-2) at 37ºC. Based on this output; material can be classified into 3 general categories: viscoelastic solid, viscoelastic liquid and gel materials. Storage (elastic or solid like) modulus (G′), loss (viscous or liquid like) modulus (G″) and complex modulus were drawn versus frequency. LVER should be recorded using amplitude sweep prior to frequency sweep. This test was done in strain (γ) =5 % (in LVER region), and frequency range from 0.1 to 100 rad/s.

Inverted tube test:

This test was used to observe gelation time and gelation temperature of NP hydrogel and observe color of NP hydrogel before and after gelation and too. Prepared NP hydrogel Put on incubator with 37^{0} C. after 30 min, Tube of NP hydrogel inverted then gelation and color of this hydrogel observed.

Fourier Transform Infrared Spectrometer (FTIR):

To confirm the presence of different compounds in the scaffold's formulations, Fourier transform infrared (FTIR) measurements were performed.

The spectra were collected over a range of 1000-4000 cm-1 to monitor any changes in the amide groups.

Scanning Electron Microscopy (SEM):

SEM was used to observe the Surface morphology of the swollen composite hydrogel and attachment of NP cells. NP cells were seeded on the hydrogel surface in vitro. Three days after seeding, specimens were fixed, washed, dehydrated, mounted, and sputter-coated following standard SEM procedures .Samples viewed under at an accelerating voltage of 15 kV.

Isolation of NP cells:

NP tissue harvested from lumbar disc of scarified rabbit. Harvested tissue minced in Hanks balanced salt solution (HBSS) (Gibco BRL, Grand Island, NY) along with antibiotics. The NP cells were then isolated from these slices in an enzymatic solution (0.2% collagenase and 0.04% pronease, purchased from Sigma) for four hours at 37°C. The cell suspension in the enzyme solution was filtered through a 40‑μm nylon mesh (Falcon, NY), and then, centrifuged at 1800 rpm for 10 minutes and resuspended in Dulbecco's modified

Eagles medium (DMEM/F12) (Gibco BRL), with 10% fetal bovine serum (FBS). After isolation, it was incubated at 37°C in 5% CO2 before the subsequent experiments. The culture medium was changed thrice a week.

Encapsulation of NP cells:

The NP cells were mixed with NP hydrogel at a density of 5×10^5 cell/ml which were then cultured in the Trans well mounted on 24-well plates. Each Trans well has 200 mL of NP hydrogel that contains 1×10^5 NP cells, and 1.3mL of culture medium was added in each well. The medium was refreshed every 3 days.

Cytotoxicity assay:

MTT assay was used to analyze toxicity of the thermo responsive hydrogel. Isolated NP cells after three passages at density of 10^4 cells per well were seeded in 96-well plate. 24 hours after seeding and incubating, 10 µl of the hydrogel was added to each well and incubated for 72 hours. In third day after aspiration of wells, 100 µl of 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml MTT in PBS; Sigma-Aldrich) were added to each well and incubated for 4 hours at 37^o C. For formazon crystal lysis, dimethyl sulfoxide (DMSO) was added in equal volume to each well and shaked for 15 min in a dark place. Spectrometric absorbance was measured at 570 nm with an ELISA plate reader (DYNEX-MRX) (24).

Trypan blue:

Cell number and viability were evaluated via trypan blue exclusion. In compound hydrogel, isolation of NP cells was done by immersion of scaffold in a soluble containing trypsin/EDTA. 10 mL trypan blue was added to almost 10 mL cellular suspension of hydrogel after the suspension was centrifuged. Then, 10 ml of this solution was put on neobar slide to calculate death cells by the inverted microscope.

Results:

Preparation of injectable Chitosan-based hydrogel:

In Preparation of this hydrogel after adding cold final mixed solution into cold β-GP the solution flow ability did not changed. Both β-GP and final mixed solutions were transparent in 4^{o}C and the color of the β-GP-final mixed solution was transparent too. Upon gelation the color change and was opaque (Fig 1). This is a key point in correctly preparation of this gel. Since β-GP solution (before adding final mixed solution) in room temperature and even lower temperature can be gelled and turns to opaque, after preparing, it was kept in low temperature in a refrigerator at about 4^{o} C (25).

Rheological studies:

Time Sweep:

Each test should be conducted at 37 ° C if possible, since in tissue engineering applications the gel will be forming in vivo at or near this temperature.

Figure 1 shows the changes of G′ and G″ versus time measured at 37°C. The gelation point is the time when storage and loss modulus (G′ and G″) become equal. Thus the gelation time of NP hydrogel was about 30 min (Fig 2).

Strain sweep:

To determine the LVER, an amplitude sweep performed over a strain from 0.1 to 10,000. Figure 3 show the results. In the test G″ is almost constant and G′ in strain 100, firstly decrease then increase and constant. The length of the LVER is a measure of stability. The result obtained of this test showed that elastic modulus is higher than storage modulus over the whole strain range indicating that this hydrogel has a stable structure after setting in 37°C(Fig 3).

Frequency Sweep:

The results of the frequency sweep using the appropriate strain (as determined in the preceding section) allow us to rigorously choose a frequency to ensure that the

time sweeps we perform reflect the formation of the gel network and not some other structural change.

After determination of LVER by using amplitude sweep, frequency sweep was performed in the LVER region. As shown in Figure 3, the system was frequency independent and elastic modulus of NP hydrogel is dominant over the entire frequency range. It means that after gelation, there were little changes in viscoelastic characteristics of the hydrogel within these frequencies (Fig 4).

Gelation Temperature:

The temperature was varied by 1 C/min and the sweep extended from 20-40 ^{0} C at a frequency of 1 hertz.NP gel displayed a thermo gelation temperature of 37 C, evidenced by an increase in the storage modulus at that temperature (Fig 5).

Inverted tube test

The prepared NP hydrogel was transparent and it was running after inverting the tube and flowed out of the tube and then incubated for 30 minutes at 37 ° C Within 30 minutes, once every ten minutes, its gelation was studied by inverting the container and observation of color. After 30 minutes, it was observed that our solution became gel and it was not running. Its color had become opaque (Fig 1).

FTIR:

FTIR spectra of NP hydrogel showed a peak at 1658cm−1, which corresponds to the primary amide groups of chitosan. The peak at 1101cm−1, which is attributed to phosphate groups, was present in NP hydrogel was assigned to C–O stretching of chitosan and HA, which corresponds to C-O-C bond stretching vibration of symmetric ester band and too.

Bands characteristic for phosphate υ4 and υ3 stretching of B-GP were seen in the regions 600–500 cm−1 and 1200–800 cm−1.

The ampholyte gelatin is characterized by its amino band at 1577 cm1 and carbonyl peak at 1658 cm -1. The peak at 3428 cm−1 (–OH group), 1658 cm−1 (–C=O stretching), 1577 cm−1 (N–H bending vibration) was assigned to Chondroitin Sulphate. The peak at 3428 cm−1 also was assigned to Collagen.

The absorption bands at ∼1658 cm−1 (amide I), ∼1577 cm−1 (amide II), ∼1232 cm−1 (amide III), and ∼ 782 cm−1 (amide V) were assigned to fibroin silk.

Isolation and culture of NP cells:

Figure 5 shows the morphology of isolated and cultured cells after three passages. NP cells had Spindle shaped and fibroblast like morphology (Fig 7). These cells grow rapidly like other fibroblast cells.

Cytotoxicity assay:

MTT was performed to test the impact of NP hydrogel on cell viability in vitro. OD of wells in which cells cultured with hydrogel, increased insignificantly. It means that the survival of NP cells cultured beside NP hydrogel at day 0-21 increased, and this increase was statistically significant. The survival of NP cells in NP hydrogel compared to the control group was significant too (Fig8).

Trypan Blue:

Results of cell count showed an increased insignificantly. It means that the Cell number and viability of NP cells in sided compound hydrogel at day 0-21 increased and this increase was statistically significant .The viability of NP cells in NP hydrogel compared to the control group was significant and too (Fig 9).

SEM:

The surfaces of the NP hydrogels (Fig 10-a) contain many pores, which vary in size, although most were smaller than 200 m in diameter. This hydrogel had high porosity that is suitable for tissue engineering (Fig 10-a).NP cells cultured 3 days on scaffold. Attached NP cells had fibroblastic morphology (Fig 10-b).

Discussion:

Our thermoresponsive hydrogel is a biocompatible chitosan based hydrogel that can be used in engineering of some tissues with injection into tissue especially for IVD regeneration. In this study we analyzed the NP hydrogel by rheometry tests as appropriated candidate for IVD tissue engineering .In rheometry tests we observed gelation time was about 30 min (Fig 2). This time seems to be enough for cell based therapy in IVD. Dr Nader -Meshkin et al. showed this time to be about 20 min for CH-GP-HEC solution (26). Kedong Song In et al fabricated chitosan/glycerol phosphate / Collagen .They observed the gelation time of the prepared solution at 37^{o}C was found to be of around 12 min (27) . Other studies showed the gelation time of CH/GP and C/G/GP systems at 37^{o}C (28) .In our study showed gelation temperature was about 37 ^{o} C. This temperature is necessary for invivo tissue engineering. Reported the gelation temperature of the CH/GP system was35.84^{o}C.The gelation temperature decreased to 33.88^{o}C, 33.61^{0}C, and 31.13^{0}C for the C/G/GP system with addition of 1%, 1.5%, and 2% of gelatin, respectively (28). We added gelatin to NP hydrogel to increase hydrophilicity of NP hydrogel.

Cytotoxicity of NP hydrogel evaluated by MTT and trypan blue tests .MTT and trypan blue results showed NP hydrogel was cytocompatible and safe complex biomaterial that maintains viability and proliferation of NP cells and can use for many tissues. Yung-Hsin Cheng et al. cultured rabbit NP cells in gelatin/ the

thermosensitive chitosan/ β -glycerol phosphate. The results of cell activity, cytotoxicity and cell proliferation assays showed NP cells cultured in CH/Ge/GP hydrogel had normal cell viability and cell proliferation that indicated the hydrogel was no cytotoxicity (28).In other study Adipose derived stem cells cultured in CH/GP/COL .displayed a typical adherent cell morphology and good proliferation with very high cellular viability in this hydrogel(27).The used materials In this hydrogel are current materials in tissue engineering of types of tissues .Chitosan, collagen ,chondroitin sulfate, fibroin silk, gelatin and hyaluronic acid are suitable materials in IVD tissue engineering. Recently the development of a promising thermosensitive hydrogel prepared from mixtures of glycerol phosphate salt (GP) and chitosan for application in cartilage tissue engineering has been reported .Addition of GP to scaffolds is one of several methods have been tried. We used GP in our hydrogel to increased mechanical strength too (29). Chitosan-GP hydrogel is injectable and we used this hydrogel to increase stability and durability of NP hydrogel and synthesis injectable scaffold. In tissue engineering of IVD for obtain of appropriate result is better that use materials as scaffold similar to ECM of IVD and we added collagen, chondroitin sulfate, and hyaluronic acid to chitosan-GP hydrogel. Gelatin added to NP hydrogel to increase hydrophilicity of NP hydrogel. Fibroin silk added to increase stability and durability of compound. According to we used excellent candidate biomaterials with described properties.

In preparation of hydrogel we observed that if final mixed solution (based chitosan solution) rapidly added to β-GP solution or mixed inhomogeneously, after heating and gelation, hydrogel gains a non continuous structure and becomes opaque. Reported that CH-β-GP hydrogel has an opaque color (11). Chenite et al and Li et al (16, 19) described, Chitosan and β-GP have electrostatic attractions due to their positive and negative charges. Firstly, our NP hydrogel was transparent, after 30 minutes it had become opaque (Fig 1).

Frequency test often used method to measure the viscous and elastic properties of a hydrogel. Two parameters are most often reported storage (elastic) modulus and viscous (Loss) modulus which represent the relative degrees of the material changes in hydrogel. A typical response for a polymer melt is to exhibit elastic dominated behavior at high frequencies and viscous dominated behavior at low poses are equal. This is obviously a well-defined point and conveniently this "cross-over "frequency and modulus has been shown to depend on the molecular weight and molecular weight distribution of some linear polymers(32).

Frequency test in our hydrogel showed the system was frequency independent and elastic modulus of NP hydrogel is dominant over the entire frequency range .It means that after gelation, there were little changes in viscoelastic characteristics of the hydrogel within these frequencies (Fig 4).

The result obtained of strain sweep test indicated that elastic modulus is higher than storage modulus over the whole strain range indicating that this hydrogel has a stable structure after setting in 37ºC(Fig 3).

We successfully synthesized natural compound hydrogel that was biocompatible, injectable with high porosity and properties structure similar to ECM of IVD .We have detected Cytotoxicity , Rheological properties and Porosity of natural compound hydrogel in an in vitro model for disc regeneration .

Conclusion:

NP hydrogel is a promising vehicle for injection in cell therapy without the need for open surgery .It is a biocompatible and nontoxic hydrogel that sets at 37ºC in about 30 min with a stable structure throughout the wide range of frequencies .Gelation time, gelation temperature ,stability, porosity and non- cytotoxicity of this hydrogel are suitable factors in tissue engineering . NP hydrogel may be an appropriate choice as an injectable scaffold for using in IVD tissue engineering and also it is similar to ECM structure of IVD that can be suitable scaffold for cells of IVD and regeneration .Representing a new alternative as a scaffold for IVD tissue engineering, with the added advantage of being a gel at the body's temperature that turns liquid at room temperature.

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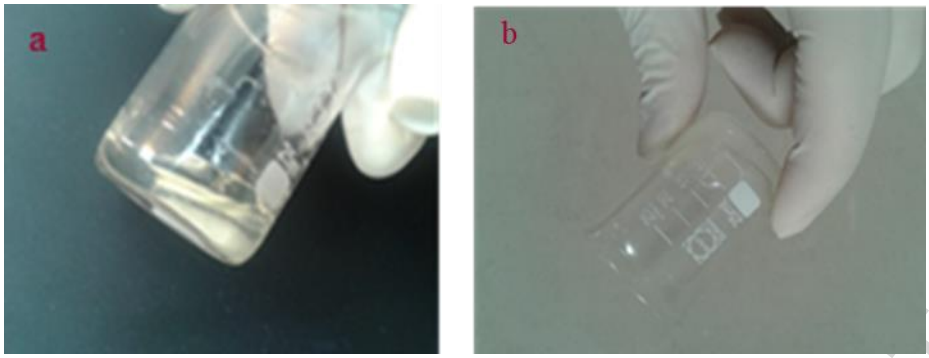
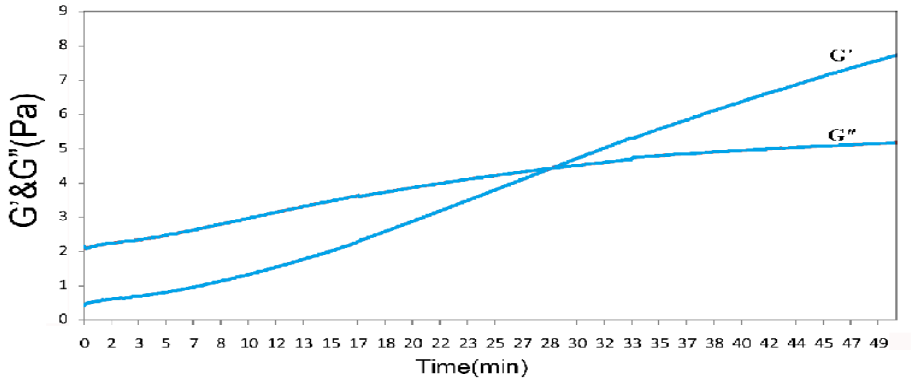


Fig1



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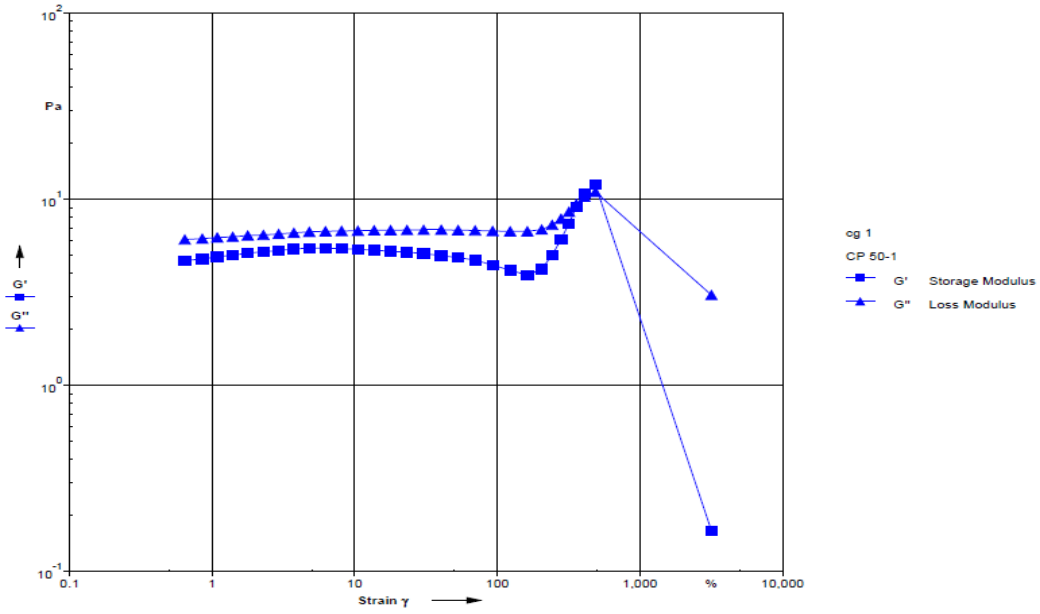

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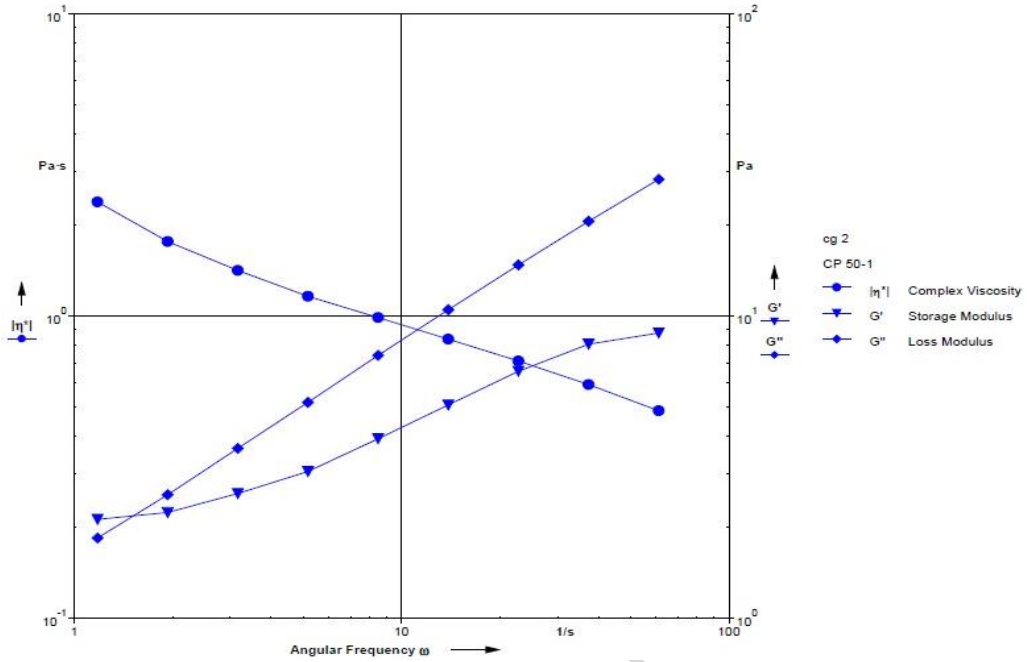

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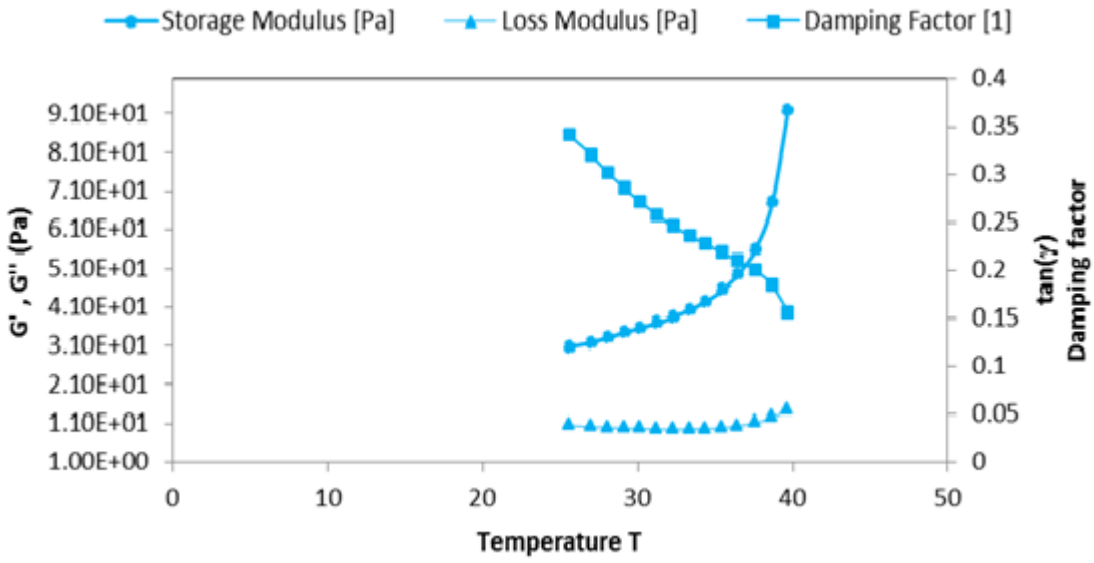

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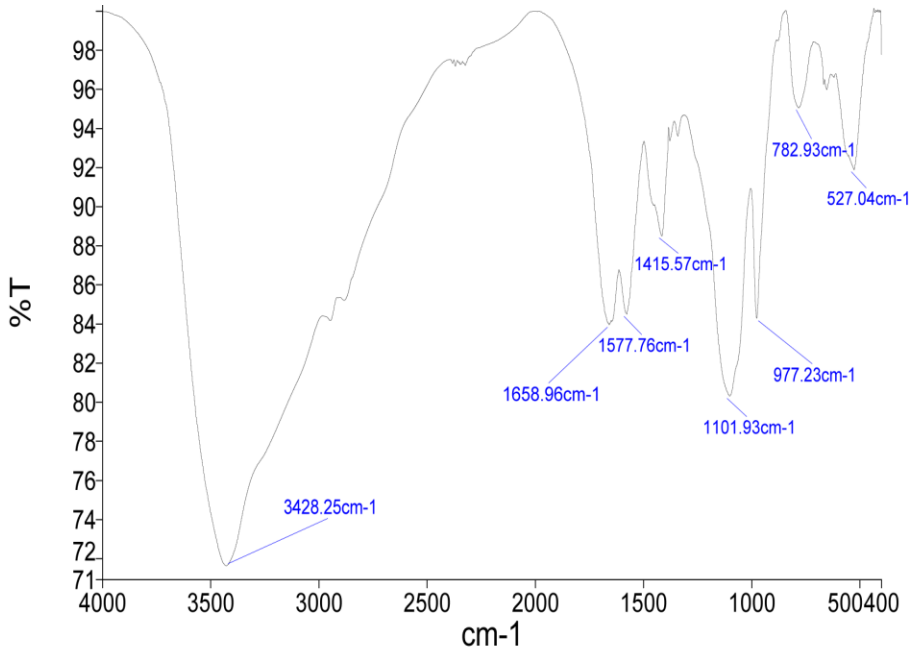

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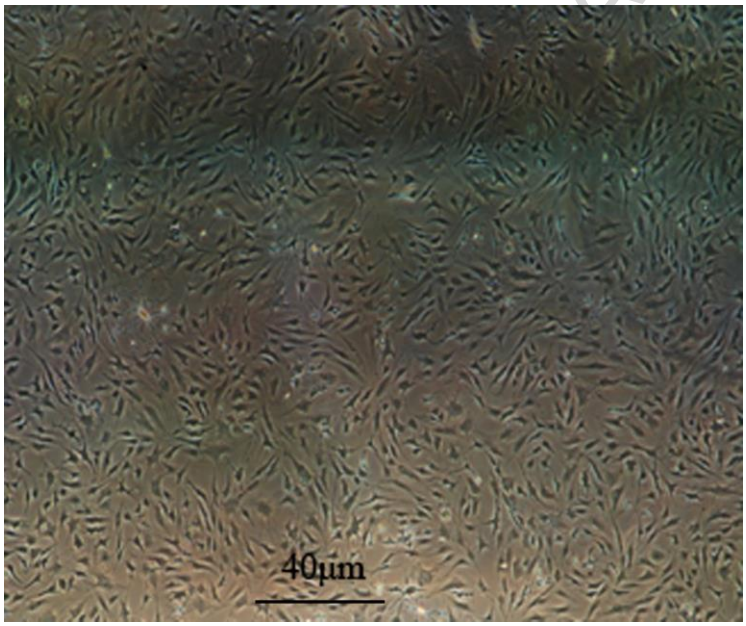

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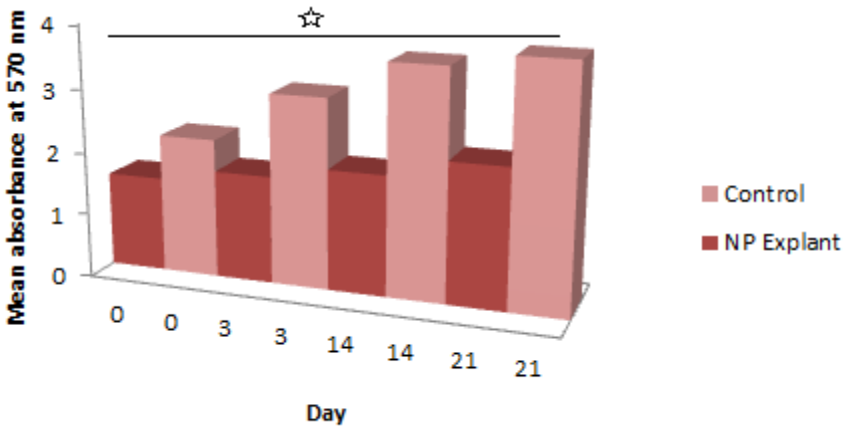

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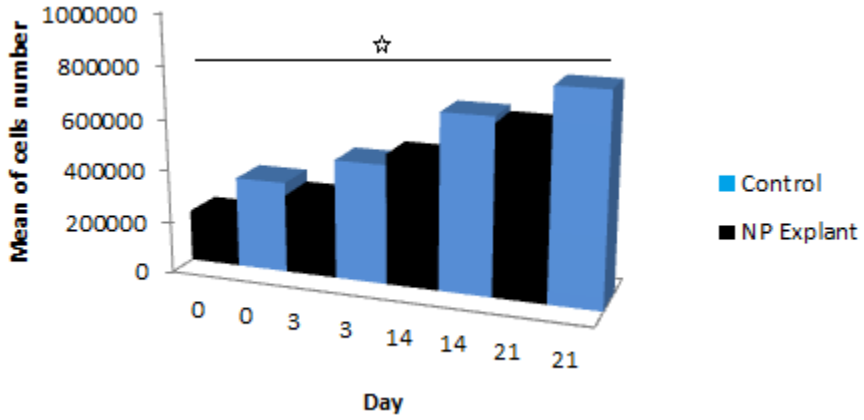

Fig 9

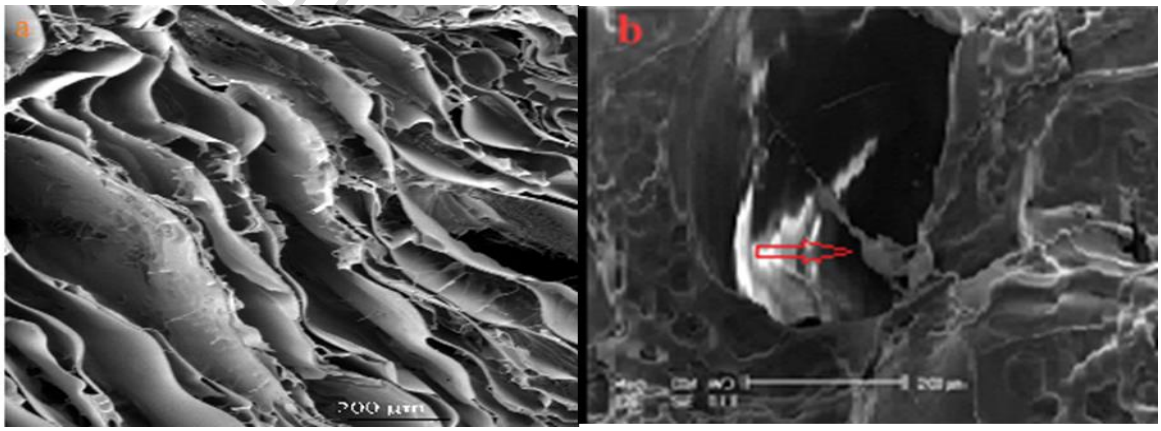

Fig 10

Legends:

Figure1: Final mixed solution: 1-a: in 40C (transparent), 1-b: in 37 ^{0}C (opaque).

Figure2: Changes of storage and loss modulus (G′ and G″) versus time for the gel solution in 37oC for estimation of gelation time.

Figure 3: Storage and loss modulus (G′ and G″) of the prepared NP hydrogel over a strain range at 37oC.

Figure 4: Storage (G′), loss (G″) and complex modulus of the prepared NP hydrogel as a function of angular frequency at 37^{o}C.

Figure 5: NP hydrogel was shown to undergo an increase in the storage modulus G', beginning at 37 0 c.

Figure 6: FTIR spectra of types of used materials in NP hydrogel

Figure 7: Image taken from the human NP cells after three passages using a phase contrast microscope (× 40 magnifications).

Figure 8: Result of MTT assay to investigate survival of NP cells cultured in NP hydrogel and control groups at day 0 to 21 days (*P<0.05)

Figure 9: Comparison percent of alive NP cells in NP hydrogel and control groups (*P<0.05)

Figure 10: SEM micrograph of NP hydrogel surface (a) and cultured NP cells on the scaffold (b).The arrow showing attached NP cell.