Nanofibrous gelatin scaffolds integrated with NGF-loaded alginate microspheres for brain tissue engineering

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ABSTRACT

Neural regeneration research is designed in part to develop strategies for therapy after nerve damage due to injury or disease. In this study, a **new gelatin based biomimetic scaffold was fabricated for brain tissue engineering applications. A** technique combining thermally induced phase separation and porogen leaching was used **to create interconnected macropores and nanofibrous structure.** To promote tissue regeneration process**es**, the scaffolds were integrated with nerve growth factor (NGF) loaded alginate microspheres. The

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results show**ed** that **nanofibrous matrix could only be obtained when** gelatin concentration was at least 7.5% (w/v). The **scaffold with** a modulus value (1.2 kPa) similar to that of brain **tissue (0.5-1 kPa) was** obtained by optimizing the heat treatment time, **macropore size** and gelatin concentration. The encapsulation efficiencies of NGF into **0.1% and 1%** alginate **microspheres** were 85% and 100%, respectively. The release rate of NGF from the **microspheres** was controlled by the alginate concentration and **the** poly (L-lysine) coating. The immobilization of the **microspheres** in the scaffold reduced burst release and significantly extended the release period. The nanofibrous architecture and controlled release of NGF from the **microspheres** induced neurite extension of PC12 cells, demonstrating that the released NGF was in **an** active form. Our results suggest that the scaffolds **prepared in this study** may have potential applications **in brain tissue engineering due to topologic and mechanical properties similar to brain tissue and pore structure suitable** for **cell growth and differentiation.**

Key words: Gelatin Scaffold, Nanofiber, Alginate Microspheres, NGF, Controlled Delivery, Brain Tissue Engineering.

1. Introduction

The regeneration capacity of neurons and axonal regrowth in **the** brain following an injury or disease is limited due to the presence of inhibitory molecules and lack of neurotropic factors at **the** injured site (Schmidt and Leach 2003). To promote tissue regeneration, cell-based therapies have been used **via** transplantation of mesenchymal stromal cells, neural stem/progenitor cells, embryonic stem cells, induced pluripotent stem cells into the injured **and/or** diseased sites. However, this strategy was **not** found **to be** successful due to low cell survival, uncontrolled differentiation and ineffective cell integration into the host tissue (Tam et al. 2014). Scaffolds play a crucial role as an artificial microenvironment to support survival of transplanted cells, facilitate cell regeneration and prevent further damage to adjacent

tissue. **Potential application areas of 3D scaffolds in brain tissue engineering include an *in vitro* nerve tissue stem cell niche (Qu et al. 2009; Qu et al. 2011; Ma et al. 2014; Mahmood et al. 2014), cell culture matrix for the investigation of neurodegenerative disease, treatment mechanism and regeneration of brain tissue (D'Avanzo et al. 2015; Kim et al. 2015; Simão et al. 2015), implantable drug delivery system in brain cancer (Mak et al. 1995; Westphal et al. 2003; Vukelja et al. 2007) and neurodegenerative diseases. Most of these studies are *in vitro* and still in their early stages and more investigation including both *in vitro* and *in vivo* studies are needed before moving onto clinical studies. However, the utilization of scaffolds in repairing damaged brain tissue is proposed as a feasible treatment option (Spires et al. 2004). Hydrogels are a commonly used type of scaffold for brain tissue engineering applications. They can be injected into a lesion site and enable the filling of** irregular cavities with a minimally invasive procedure (Pettikiriarachchi et al. 2010). However, their high water sorption capacity adversely affects **their** mechanical integrity, facilitating fast degradation, **resulting in** collapse of the structure. As a result, cell reorganization in hydrogels occurs in a microenvironment without physical constraints, preventing formation of large-sized neural networks (Tang-Schomer et al. 2014). **A silk-**fibroin based **three dimensional** (3D) scaffold allowed long-term neural tissue viability up to 9 weeks and showed better performance than collagen-based hydrogels (Tang-Schomer et al. 2014) due to the increased degree of neural clustering in the scaffold structure as opposed to **dispersal of** neurons in the hydrogel. Self-assembling peptide nanofiber scaffolds manufactured from oligopeptides or amphiphilic molecules were also applied **to** brain lesions (Pettikiriarachchi et al. 2010). Although they have high porosity, tissue-like water content and enhanced **signalling,** resulting from the presence of bioactive peptide sequences, they suffer from weak mechanical properties due to **their** high water content and are susceptible to **rapid** enzymatic degradation

(Pettikiriarachchi et al. 2010). **Studies over the last two decades have shown that** 3D **macroporous nanofibrous** scaffolds **represent** a promising approach for enhancement of neural network formation and ingrowth (Ma and Choi 2001; Wei et al. 2007**b**).

The idea of combining macroporous nanofibrous scaffold with protein loaded microspheres was first proposed by Ma (2004) and **the advantages of these biomimetic scaffolds were shown in cartilage and bone tissue engineering (Wei et al. 2007a; Wei and Ma 2008)**. In brain tissue engineering, nanofibrous scaffolds were produced from a variety of synthetic polymers using **an electrospinning technique (Pettikiriarachchi et al. 2010), however, 3D scaffolds featuring both nanofibrous, macroporous structure and incorporated with neurotrophic factor loaded microspheres have not yet been investigated. In this study, we therefore aimed to prepare gelatin based nanofibrous scaffolds integrated with NGF loaded alginate microspheres for brain tissue engineering applications. Our major design criteria for optimizing preparation conditions was the elastic modulus of native brain tissue, since colonization, migration, differentiation of neural cells (Leipzig and Shoichet 2009; Saha et al. 2008) and altered neurite formation and trajectory (Balgude et al. 200; Jiang et al. 2008) were found to be influenced by matrix stiffness. In addition to mechanical and structural properties, biodegradation rate of the scaffold is also an important issue for brain tissue engineering applications. It becomes necessary to have a long-term scaffold especially for large lesions. We have selected gelatin as the scaffold material since it is a derivative of collagen and it has integrin binding domain arginine, glycine and aspartic acid sequence (RGD) (Chen et al. 2006) in its structure which enhances adhesion of cells. Its degradation rate can be easily adjusted to match desired rate by adjusting degree of crosslinking. A strong polyanion, alginate, has been chosen for loading NGF through mainly electrostatic interactions. The method developed by Ciofani et al. (2008) has been modified to achieve increased microsphere yield (ratio of**

mass of particles obtained to the mass of polymer used in the emulsion) and consequently high NGF loading into alginate microspheres. The scaffolds were prepared by combining thermally induced phase separation and porogen leaching processes. As an innovative approach, we utilized the same reaction for crosslinking the scaffold and immobilizing alginate **microspheres** to the scaffold using EDC-NHS coupling reagents. The influences of manufacturing conditions on the structure and mechanical properties of the scaffolds were examined. In addition, the *in vitro* performance of the scaffold on the proliferation and differentiation of the model cell line, PC12, was investigated. Overall, our 3D nanofibrous scaffolds, which have tunable mechanical properties and allow for sustained delivery of NGF, can become a potential therapeutic candidate for brain tissue engineering applications, especially in traumatic brain injuries. **To the best of our knowledge, this study is the first which develops biomimetic nanofibrous scaffold for brain tissue engineering applications.**

2. Materials and Methods

2.1. Materials

Paraffin with a melting point of 53-57 ºC, gelatin (type B, from bovine skin), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), (2-(N-morpholino) ethanesulfonicacid) hydrate (MES), alginate (medium viscosity), sodium chloride, sodium citrate, Poly-L-lysine with a molecular weight 30,000-70,000, Tween 80 and Span 80 were **all** supplied by Sigma. Acetone, 1, 4-dioxane, n-hexane, cyclohexane, ethanol, isooctane, calcium chloride, mouse-NGF were purchased from Merck. Poly (vinyl alcohol) (PVA) with a molecular weight of 25,000 was purchased from Polyscience. RPMI 1640, Horse Serum, Fetal Bovine Serum, L-glutamine and antibiotic (penicillin, streptomycin), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

(XTT) were obtained from Biological Industries and glutaraldehyde used was from Alfa-Easer.

2.2. Preparation of nanofibrous scaffold

Macroporous nanofibrous gelatin scaffolds were prepared using a previously developed protocol (Liu and Ma 2009). Paraffin spheres were prepared as pore forming agent with the method suggested by Ma and Choi (2001). Briefly, spheres with different sizes (250-425 μm and 425-600 μm) were added to a custom made teflon mold (with diameter of 1.7 cm and depth of 1.5 cm) and the top surface was leveled. The mold was then heated at 37 °C to obtain interconnected spheres. Gelatin was dissolved in 50/50 (v/v) ethanol-water solution at 45 ºC and 0.35 ml of this solution was cast onto the paraffin sphere assemblies. The gelatin solution in the paraffin assembly was phase separated at -80 ºC for 5h. Next, the scaffolds were immersed in cold ethanol (-18 °C) and transferred into 1, 4-dioxane for 24h for solvent exchange. After freeze-drying, gelatin/paraffin composites were soaked in hexane at 37 °C for 3 days then, cyclohexane was used to exchange hexane in the scaffold. As a final step, the scaffolds were freeze-dried for 4 days.

2.3. Preparation of alginate microspheres

Alginate microspheres were prepared by external ionic gelation and reticulation of alginate with calcium ions in water-in-oil emulsion system. For this a method proposed by Ciofani et al. (2008) was modified. First, iso-octane was mixed with Tween 80 and Span 80, then alginate solution was added to this mixture and homogenized at 15,000 rpm (IKA, Ultra-turrax). During homogenization, 2 ml of 100% $CaCl_2$ (w/v) was added drop **wise** to crosslink alginate. After additional homogenization for 30 sec, the system was allowed to settle and **the supernatant** was removed. Next, the **microspheres** were stabilized with **an** acetone and water mixture (1:10 (v/v)) at 10,000 rpm for 5 min. To prepare protein loaded alginate microspheres, 100 μl of 10 μg/ml of NGF was mixed with alginate solution and this mixture

was added to the isooctane, Tween 80 and Span 80 mixture. **The sizes of microspheres were measured with a phase contrast microscope by dispersing the microspheres in water.** To control release rate of NGF, alginate microspheres were first coated with Poly(L-lysine) (PLL) and then with the alginate. In this step, harvested microspheres were suspended in 100 μg/ml of PLL solution for 30 min. After removing **the** PLL solution **by** centrifugation, **the** alginate solution was added **to** microspheres, shaken for 30 min, then samples were washed with deionized water and 20% $CaCl_2$ was added to crosslink alginate. Finally, $CaCl_2$ solution was removed by centrifugation, and microspheres were washed, and these steps were repeated **in** triplicate.

2.4. Chemical crosslinking of scaffold and the attachment of alginate microspheres to the gelatin scaffold

1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) with N-hydroxysuccinimide (NHS) were used as cross linkers of gelatin scaffolds with NHS to EDC mole ratio of 0.2. In the reaction the mole ratio of EDC to free COOH groups in gelatin was taken into consideration and this ratio was fixed as 2:1 (as described in Supporting Information). The reaction was carried out in 2-(N-morpholino) ethanesulfonicacid hydrate (MES) buffer (pH 5.3, 0.05 M) at +4 °C for 24h. Acetone/water with a ratio of 90/10 (v/v) was used instead of pure water to protect the nanofibrous structure (Liu and Ma 2009).

In the presence of EDC, amide linkages between lysine residue of gelatin and the carboxylate moieties of alginate polysaccharide is formed on polymer backbone (Hermanson 1996) as shown in Scheme 1. Therefore in this study EDC was used, not only for crosslinking the scaffold but also for attaching the alginate spheres to the gelatin matrix. For this purpose, 18 mg of 1% and 1.8 mg of 0.1% of alginate **microspheres** were suspended into 100 μL of crosslinking solution and 50 μL of this solution was added onto two sides of the disc shaped scaffold with a diameter of 16 mm and height of 2 mm. After waiting for 10 min for attaching

microspheres, the scaffolds were kept in crosslinking solution for 24h at +4 °C, finally washed with deionized water 3 times and freeze-dried for 3 days.

The amount of alginate **microspheres** attached **to the** gelatin scaffold was determined **using the** phenol sulfuric acid assay (Gu et al. 2004). For this aim, scaffolds integrated with microspheres were taken into 1 ml water, and 1 ml of 5% (w/v) phenol and 5 ml of concentrated sulfuric acid were added into the tube. The mixture was vortexed and kept at room temperature for 15 min. The sample was then transferred to a 96-well plate, and absorbance was measured at 488 nm. **In this experiment** negative **and positive controls were the** same size of plain scaffold and **alginate microspheres equal in number to the free** particles **in solution that was added** to the scaffold**, respectively**. Microspheres attached to the scaffolds were calculated using:

$$\% \text{ Attachment} = (A_1/A_0) \times 100$$

where A_0 is absorbance value of positive control and A_1 is absorbance value of microsphere attached gelatin scaffold after subtracting absorbance of plain scaffold (negative control).

2.5. Characterization of nanofibrous scaffold

2.5.1. Surface morphology examination

The morphology of the nanofibrous scaffolds and alginate microspheres were examined by scanning electron microscopy (SEM) on a Philips XL-30SFG model. The samples were coated with gold using a Magnetron Sputter Coating Instrument.

2.5.2. Mechanical test

A unidirectional compression test was performed on hydrated scaffold samples using a mechanical tester (Schimadzu AG-I 5 kN). All samples were circular discs 16 mm in diameter and 2 mm in thickness. Before **the** mechanical test, scaffolds were kept in PBS (pH=7.4) for 24h. At least, five specimens were tested for each sample. Values for the

compressive modulus of elasticity were recorded at a strain of 5% and the average values with standard deviations were reported.

2.6. Determination of NGF loading and release from alginate microspheres

Alginate microspheres were transferred to **a** siliconized tube and 1 ml of 3% (w/v) of Na-citrate solution was added. The tube was rotated at 300 rpm in an orbital shaker. Ca^{2+} ions in the microspheres were exchanged with Na^{+} ions destroying the structure of the particles. Next, the tube was centrifuged at 10,000 rpm for 5 minutes and **the** supernatant was kept at -20 °C. Encapsulation efficiency was calculated as **follows**:

$$\text{Encapsulation Efficiency (EE \%)} = \frac{\text{Loaded amount of NGF}}{\text{Initial amount of NGF}} \times 100$$

For the release study, alginate microspheres and microsphere attached scaffolds were put into **a** 1 ml starvation medium (RPMI 1640 and 1% horse serum) and shaked at 180 rpm at 37 ˚C. At the end of different time intervals, the tubes were centrifuged at 10,000 rpm for 5 min and **the** supernatant was analyzed to determine the concentration of released NGF. 1 ml fresh starvation medium was added on**to** the **microspheres** to continue release studies. The amount of NGF loaded into **microspheres** or released from **microspheres** was determined with mouse NGF ELISA kit (Boster Immunoleader).

2.7. *In vitro* cell culture

Rat adrenal pheochromocytoma (PC12) cell line was kindly donated by the Department of Medical Biochemistry **at Dokuz Eylul University**. Cells were cultured in 0.1 mg/ml poly-L-lysine coated 75 cm^2 flask in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, 1% L-glutamine, 1% Penicillin/Streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin) and incubated at 37 °C in 5% CO_2 and 95% air.

2.7.1. Proliferation of PC12 cells on macroporous nanofibrous gelatin scaffold integrated with empty alginate microspheres

The proliferation **behaviour** of PC12 cells **was investigated with** 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) **assay.** XTT **is** a yellow tetrazolium salt cleaved by the mitochondrial dehydrogenase in metabolically active cells to form an orange formazan dye. The formazan dye was measured in an optimal spectrophotometric range of 450 to 500 nm. All assays were performed in 96-well tissue culture plates coated with 0.1 mg/ml poly-L-ornithine. Cells were seeded onto pre-wetted scaffolds. **As a positive control, a scaffold which was not integrated with microspheres was used.** At the end of **the** determined time, 50 μL of XTT was added into to the wells and they were kept at 37 °C for 4 hours. Finally, **the** scaffolds were removed from wells and photometric measurement was carried out at 450 nm. All experiments were repeated a minimum of three times with assays performed in triplicate.

2.7.2. Differentiation of PC12 cell on macroporous nanofibrous gelatin scaffold integrated with the NGF loaded alginate microspheres

For NGF induced differentiation studies, complete culture medium was replaced with starvation medium which consisted of RPMI 1640 containing 1% horse serum. 30,000 cells were seeded onto the empty scaffold and the scaffold integrated with alginate microspheres (both scaffolds were 200 mm^3 in volume) after culturing in starvation medium for 24h. As a positive control, 50 ng/ml of NGF was added to the culture medium of scaffold incorporated with empty alginate microspheres. **The** medium was changed every 2 days and at the end of the 4^{th} and 10^{th} days **the** scaffolds were removed from the wells and washed in PBS, fixed with 2.5% glutaraldehyde for 20 min, dehydrated in a series of graded concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 100%) each for 5 min and vacuum-dried. Dehydrated constructs were cut, coated with gold and examined in SEM.

2.8. Statistical Analysis

The significant differences between the groups were evaluated by ANOVA analysis by Tukey's method with 95% confidence interval. The results were presented as mean ± standard deviations which were calculated from at least three independent experiments.

3. Results and Discussion

3.1. Preparation and characterization of interconnected macroporous nanofibrous scaffolds

Thermally induced phase separation (TIPS) technique **consists** of polymer dissolution, phase separation, gelation, solvent extraction, freezing and freeze-drying. Under a certain temperature, homogeneous polymer solutions separate into polymer-rich and polymer-lean phases. After removal of the solvent, a 3D porous structure can be obtained from the solidified polymer-rich phase (Kim and Lee 2007). In this study, gelatin was dissolved in **an** ethanol/water solvent mixture, where ethanol **is a** nonsolvent of gelatin and adjusts the interaction between gelatin and solvent molecules resulting in the formation of NF structures, as demonstrated by Liu and Ma (2009). Based on this finding, we used **a** fixed 50/50 (v/v) ethanol/water mixture ratio in all experiments to prepare 5% (w/v), 7.5% (w/v) and 10% (w/v) gelatin concentrations. When gelatin concentration was 5%, **a** bead-like scaffold structure was observed as shown in Figure 1A indicating that phase separation occurred in the metastable region (Figure 1B). Nam and Park (1999) have shown that at the low polymer concentration end of the metastable region, powder-like polymer solid is obtained as a result of droplets of polymer-rich phase dispersed in the polymer-lean phase. By increasing gelatin concentration from 5% to 7.5% and 10%, **characteristic nanofibrous** (NF) scaffold structures **(well-interconnected nanofibers with small uniform pores)** were observed as a result of phase separation that occurred in the spinodal region (Figure 1C-D). The average diameter and length of the **fibers in the** scaffolds were determined as 501 nm and 1267 nm

for **the** 7.5% gelatin concentration and 528 nm and 4390 nm for **the** 10% gelatin concentration. The fiber sizes of both NF scaffolds mimic the size of natural collagen fiber bundles that vary in diameter from 50 to 500 nm. **To** facilitate fluid flow for nutrient and metabolite waste transport and to support migration and differentiation of cells, **macropores were created using paraffin** spheres with different sizes (250-425 μm and 425-600 μm). **The** assembly was exposed to heat treatment for 200 min and 400 min, resulting in high interconnectivity between **the pores** (Figure 2A-B). **Gelatin is a water soluble polymer; hence, its mechanical stability was improved by crosslinking using a combination of carbodiimide with NHS. The degree of crosslinking was optimized to protect the nanofibrous structure in the scaffold (see Supporting Information).**

We considered the **elastic modulus of the scaffolds** as the **major design criterion in optimizing preparation conditions, since colonization, migration,** differentiation **of neural cells (Saha et al. 2008,** Leipzig and Shoichet 2009) and **altered neurite formation and trajectory (Balgude et al. 2001, Jiang et al. 2008) were found to be influenced by matrix stiffness. Figure 3 shows that all of the** scaffolds displayed a typical compressive stress-strain curve; a characteristic profile of the hydrated scaffolds with open-cell foams. In the stress-strain diagram, linear elastic, collapse plateaus and densification regimes were observed with a transition from the linear elastic to collapse plateau region at approximately 5% strain. Compressive modulus values were calculated from the slope of the linear elastic region and **are** listed in Table 1. As expected, the stiffness of the scaffolds increased by increasing gelatin concentration from 7.5% to 10%. On the other hand, preheating time had an influence only on the modulus of the scaffold prepared from 10% gelatin concentration. **It was seen that the scaffold** prepared from 7.5% gelatin solution with 250-425 μm sized paraffin spheres **which were exposed to** 200 min **of heat treatment,** had a modulus of 1.2±0.4 kPa, similar to that of brain tissue (0.5-1 kPa) (Pettikiriarachchi et al. 2010). **Thus,**

these conditions were chosen as optimum conditions to prepare suitable scaffolds for brain tissue engineering applications and used in further studies.

3.2. Preparation and characterization of NGF loaded alginate microspheres

NGF plays a critical role in the regeneration of neural cells. However appropriate techniques are needed to incorporate it into scaffolds due to its short half-life **(Tria et al. 1994)**. Target cells respond to NGF when the concentration is above a certain threshold level **(Barde 1989)**. Thus, an optimal delivery system is required to achieve controlled release of NGF. Although direct adsorption of free NGF on the scaffold **might** be the easiest way for its delivery, desired biological effects cannot be easily achieved due to loss of activity during adsorption and **the** short duration of release. **In addition, direct immobilization may alter the structure, degradation and mechanical properties of the scaffolds.** To overcome these disadvantages, we have encapsulated NGF in alginate microspheres. Alginate has been chosen since encapsulation can be carried out at a mild room temperature without using organic solvents and dissolution, and biodegradation of the system takes place under physiological conditions. We produced NGF loaded alginate microspheres by water-in-oil emulsification technique using a mixture of hydrophilic and lipophilic surfactants, Tween 80 and Span 80. Ciofani et al. (2008) prepared alginate **microspheres** with a similar method using only Tween 85 as a surfactant and reported 70% yield (ratio of mass of microspheres obtained to the mass of polymer used in the emulsion) while our yield was 82.5%. Although hydrophilic lipophilic balances (HLB) for the mixture of Span 80 and Tween 80 (HLB: 10.7) and pure Tween 85 (HLB:11) were similar, **the** higher yield we obtained with **a** blend of surfactants can be attributed to better dispersion and solubilization into continuous phase (Porras et al. 2004).

The **microspheres that were** synthesized with this technique had **a** smooth structure with a spherical shape (Figure 4A-D) and they were attached to the scaffolds with a high efficiency

(94%). As seen in Figure 4E, the general appearance of the NF scaffolds was white and opaque and attachment of alginate microspheres did not cause a morphological or structural change in the scaffolds (Figure 4F). **Uniform distribution of alginate microspheres was achieved due to seeding of particles from both sides of the scaffold (Figure S3).** Average diameter of the unloaded microspheres increased from 1.72 ±0.11 μm to 5.2 ±0.51 μm with **the increased** alginate concentration (Figure 5). NGF loading into 0.1% alginate **microspheres** did not change the **microsphere** size, while **1% alginate** particles reduced in size as loading level increased. This can be attributed to the presence of a higher number of free carboxylate groups and higher negative charge in 1% alginate **microspheres** causing a stronger interaction with positively charged groups in NGF, hence, yielding more compact particles. High charge density and large size of 1% alginate **microspheres** allowed 100% encapsulation of 1 μg NGF while encapsulation efficiency for 0.1% alginate **microspheres** was 85%. High encapsulation efficiency is a desired property for cost-effective utilization of growth factors.

3.2.1. NGF release from free and attached alginate microspheres

To determine release mechanism, we first measured the release of NGF from free microspheres directly added into the release medium and the results are shown in Figure 6A. In all cases burst release was observed in the first 5 hours due to diffusion of water into alginate gels and release of loosely attached NGF on the surface. At later times, the release was controlled by diffusion of NGF through the alginate matrix and dissolution of **microspheres** caused by bulk erosion and decrosslinking. As expected, slower **NGF** release **was observed** from the **1% alginate particles** due to **their** larger sizes compared to **the size of the** 0.1% alginate **microspheres**. In addition, **higher** negative charge on **the** 1% alginate **particles makes the removal of Ca^{+2} ions, hence, decrosslinking in the structure, difficult due to strong** electrostatic interaction between the alginate and positively-charged NGF

(Wee and Gombotz 1998). Consequently, fast release of NGF **was** prevented. Alginate can make a strong ionic interaction with polycations which can be used to stabilize the integrity of gel and to delay protein release (Smidsrod 1973). **We utilized** this **property of alginate and** selected poly-L-lysine (PLL), as the polycation, to coat the alginate microspheres. **As seen from Figure 6A, coated particles showed** a more sustained release profile as a result of modified surface properties and additional barrier against NGF **diffusion, in addition, coating on smaller microspheres (prepared with 0.1% alginate) was found more effective in delaying NGF release.** It has previously been reported that binding of PLL to the alginate is followed by the diffusion of PLL into the alginate network forming a complex membrane on the surface of the microspheres (Thu et al. 1996). This semi-permeable membrane decreases both the burst effect and the release rate (Lemoine et al. 1998). The alginate-PLL interactions are governed by the stoichiometric ratio of the charged groups, suggesting that **the** number of NH_3^+ groups in PLL is not sufficient to interact with most of the COO^- groups in **the** 1% alginate; hence, release from PLL coated 1% alginate **microspheres** is not significantly delayed.

Figure 6B shows that immobilization of the **microspheres** reduced the release of NGF, **likely** due to diffusional resistance of the scaffold and reattachment of the released NGF to the surface of the scaffold (Wei et al. 2006). The release patterns of free and attached **microspheres** were found **to be** different. **Total amount** of NGF **released** from both free **microspheres** at the **end of 100 hours was similar** (Figure 6A). **On the other hand**, NGF released from 1% immobilized **microspheres** was 1.4 times lower **compared to the amount released** from the 0.1% attached particles due to the presence of more negatively charged groups in 1% alginate causing redeposition of released NGF **on** the surface **of the particles.** The release of NGF from the scaffolds integrated with **PLL coated** 0.1% alginate microspheres was also measured (Figure 6B). PLL coating reduced the release rate of NGF

from 1040 pg/h to 680 pg/h **within** 10 days. The results in Figure 6B suggest that the release profile of NGF can be adjusted by **the** alginate concentration or PLL coating to achieve the desired **release** rate.

3.3. PC12 cell proliferation and differentiation

3.3.1. PC12 cell proliferation on macroporous NF gelatin scaffolds integrated with empty alginate microspheres

Proliferation of seeded cells in the scaffold is required to enable new tissue development. **In our preliminary study, we compared cell** proliferation **on petri dishes** and the gelatin scaffolds. **In these experiments, the same number of cells was seeded into the gelatin scaffold and petri dishes of the same size. The results in Figure S4 have shown that until the 9th day, the rate of cell growth in 2D (petri dish) and 3D (scaffold) cell culture media was similar. However, at the end of 12 days, the cell population on the scaffold was significantly higher than those on petri dish which clearly indicated the advantage of the 3D environment on cell proliferation.**

As seen from Figure 7, at the end of 10 days, the number of cells in the empty scaffold and the scaffold integrated with 0.1% **unloaded** alginate microspheres were similar ($p>0.05$). **The lower absorbance value, in 0.1% alginate group at the 6th day compared to empty scaffold, was due to the presence of impurities in the alginate which were removed during the exchange of cell culture medium, thus, the cell population significantly increased from 6th to 10th day. We** observed that 1% unloaded alginate particles in the scaffold inhibited the proliferation of cells. **The** inhibition is due to **a** high alginate concentration **which decreased** the pH of the cell culture medium from 7.2 to 6.7 **at** the **end of 24h** and **this stopped** proliferation process as **also reported by** Mackenzie et al. (1961). **A similar** inhibition effect was also reported for fibroblast and Schwann cells where the proliferation rates were **lower** when these cells were cultured in a high concentration of

alginate solution compared to the proliferation rates observed in the low concentration alginate solution (Bohari et al. 2011; Cao et al. 2012).

3.3.2. PC12 cell differentiation on macroporous nanofibrous gelatin scaffold integrated with NGF loaded alginate microspheres

We demonstrated the differentiation of PC12 cells using SEM images taken at the end of 4 and 10 days after seeding the cells. **Figures 8A and 8B show that the shapes of** PC12 cells attached on the empty **scaffolds, incubated with and without free NGF, are similar, which indicated that 150** ng/ml of **free** NGF added **within 4 days was** not **enough to** initiate **the** differentiation of t**he cells. Similarly, at the end of 4 days no differentiation was** observed **on** the scaffold integrated with **1% alginate microspheres (Figure 8C), on the other hand, NGF released from** 0.1% **alginate microspheres induced well-organized neurite structure formation (Figures 8D-8F). Figures 8D, 8E and 8F correspond to the SEM pictures of the same scaffold integrated with NGF loaded 0.1% alginate microspheres. The 3 elements of the construct, scaffold, cells and alginate particles, along with the PC12 cell neurites are shown with arrows (Figure 8D and Figure S5). Due to intense neurite extension, alginate particles cannot be seen in Figures 8E and 8F taken at higher magnification. Although the amounts of free NGF added to the scaffold and NGF released from 0.1% attached microspheres at the end of 4 days were almost equal (~ 150 ng/ml), the absence of neurite extension in the case of direct adsorption of free NGF to the scaffold indicated a loss of NGF activity. All of the scaffolds** supported differentiation of PC12 cells at the end of 10 days (Figure 9), however, the most extensive neurite outgrowth and tissue-like network formation was visualized in the **scaffold integrated with 0.1% microspheres (Figure 9A and 9B).** Local pH in the microenvironment of cells controls not only cell proliferation but also cellular activity (Mackenzie et al. 1961; Taylor 1962). Delayed **differentiation of cells in the scaffold in the presence of 1% alginate**

microspheres is likely caused by the decrease in pH, which also had an adverse effect on the proliferation of the cells. Another reason for delayed neurite outgrowth **is** lower NGF level in the microenvironment of the cells since NGF release from **1% alginate microspheres** is slower (Figure 6B). Neurite structure on 3D network (Figure 9A-C) was much more complex and intense than that observed in 2D cell culture medium (Figure 9D). This result **showed** that nanofibrous 3D scaffolds provided a microenvironment mimicking the extracellular matrix, a higher surface area for growth and migration and protected cells from environmental disturbances. In addition, encapsulating NGF into the alginate **microspheres** helped **in** preserving biological activity and maintained, controlled and sustained release of NGF. Consequently, survival and differentiation of the cells in the scaffolds were improved.

4. Conclusions

In this study, a new biomimetic gelatin based nanofibrous scaffold with nerve growth factor (NGF) loaded microspheres **was** prepared for the first time for brain tissue engineering application**s**. Thermally induced phase separation combined with a porogen leaching method allowed **control of** porosity, pore size, interpore connectivity, mechanical properties as well as pore morphologies of the scaffolds to promote neural regeneration. The scaffold with a modulus value similar to that of brain tissue **was** obtained and used for cell proliferation **studies**. NGF **was** encapsulated into alginate **microspheres** with a high loading efficiency without utilizing harsh conditions. Crosslinking of the scaffolds and attachment of the alginate microspheres to the scaffold **were** accomplished easily in a single step with a very high post seeding efficiency of the **microspheres**. The release rate of NGF from the microspheres **was** controlled by the alginate concentration and poly(L-lysine) coating. Alginate concentration **was** found **to be** critical in controlling the release rate of NGF, **as well as** proliferation and differentiation of PC12 cells. The scaffolds prepared in this study can

also be combined with other neurotrophins such as brain derived neurotrophic factor (BDNF), Neurotrophin-3(NT-3) and Neurotrophin-4 (NT-4). High encapsulation efficiencies of these factors into alginate **microspheres** are expected since they have similar molecular weights (13.2 - 15.9 kDa) and isoelectrical point**s** (in the range of 9-10) to those of NGF. **With interconnected macropore structure for axonal growth and nutrient transport, similar topologic and mechanical properties of brain tissue and capability of controlled release of nerve growth factor, gelatin based biomimetic scaffolds have great potential as implantable matrixes for the regeneration of destroyed tissues derived from traumatic brain injuries. In** future work, the *in vivo* performance of **these** scaffolds **will need to** be investigated.

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Conflict of interest

The authors have declared that there is no conflict of interest.

Table 1. Compressive modulus (kPa) of scaffolds prepared with 7.5% and 10% gelatin concentrations.

Gelatin concentration	**Diameter of paraffin spheres**	**Preheating time**	
		200 min	400 min
7.5 %	250-425 μm	1.2±0.4	2.5±1.7
	425-600 μm	3.3±0.9	3.8±1.2
10 %	250-425 μm	4.8±0.3	6.2±0.4
	425-600 μm	9.0±2.5	5.7±0.3

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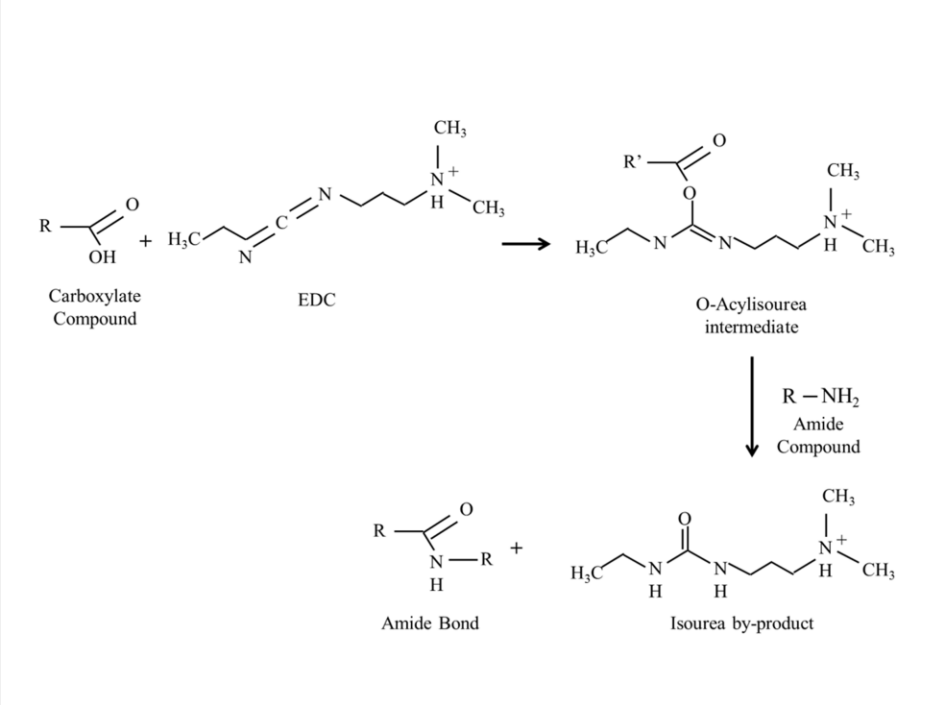

Scheme 1. Crosslinking of alginate and gelatin in the presence of EDC (Hermanson 1996).

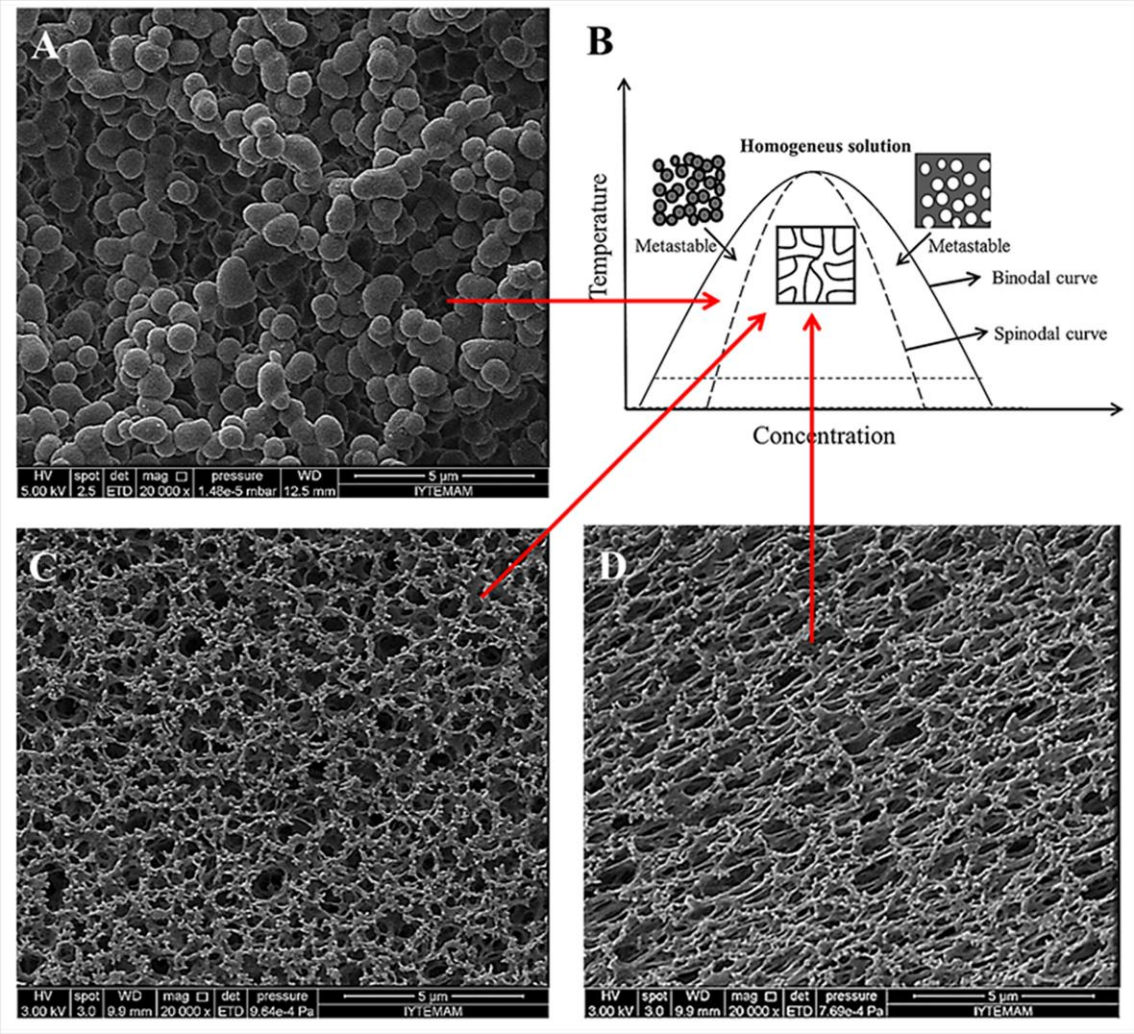

Figure 1. Scaffolds prepared from 5% (A), 7.5% (C) and 10% (D) gelatin solution X5000. (B) Schematic phase diagram of polymer solution.

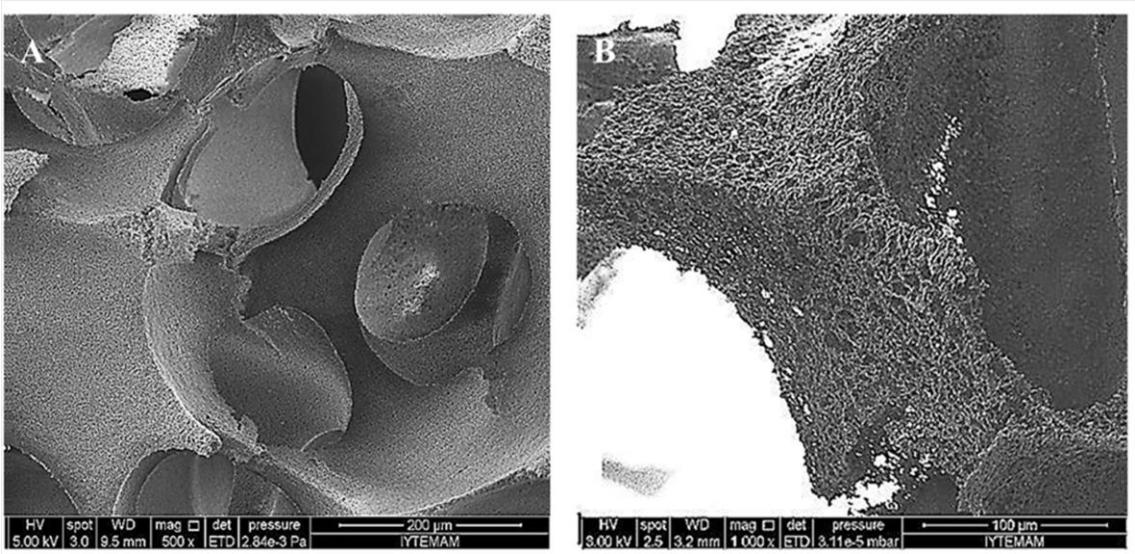

Figure 2. Nanofibrous scaffolds prepared with 10% gelatin concentration, 200 min preheating time and 425-600 µm sized paraffin spheres.

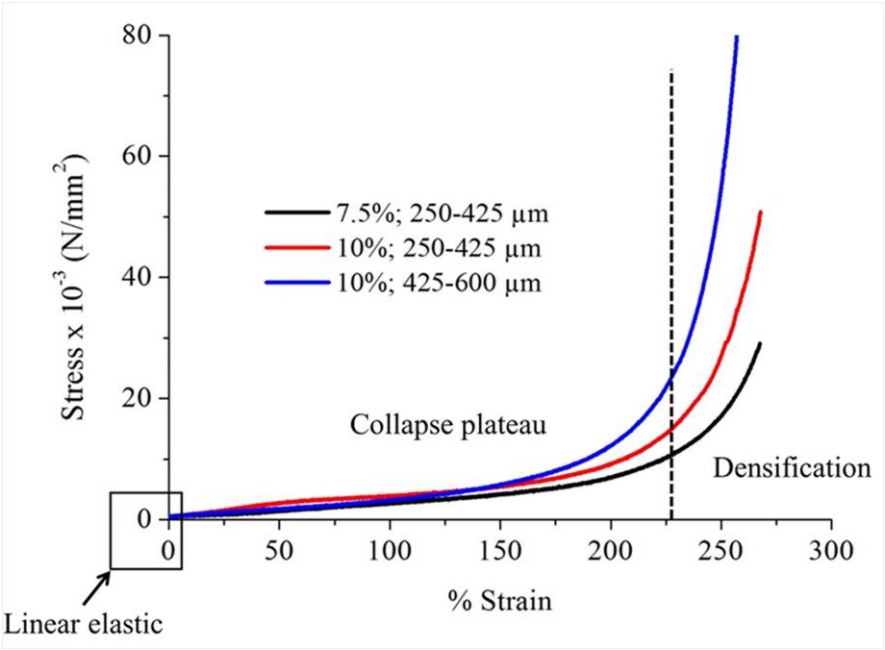

Figure 3. Stress-strain curves from the mechanical test of nanofibrous scaffolds.

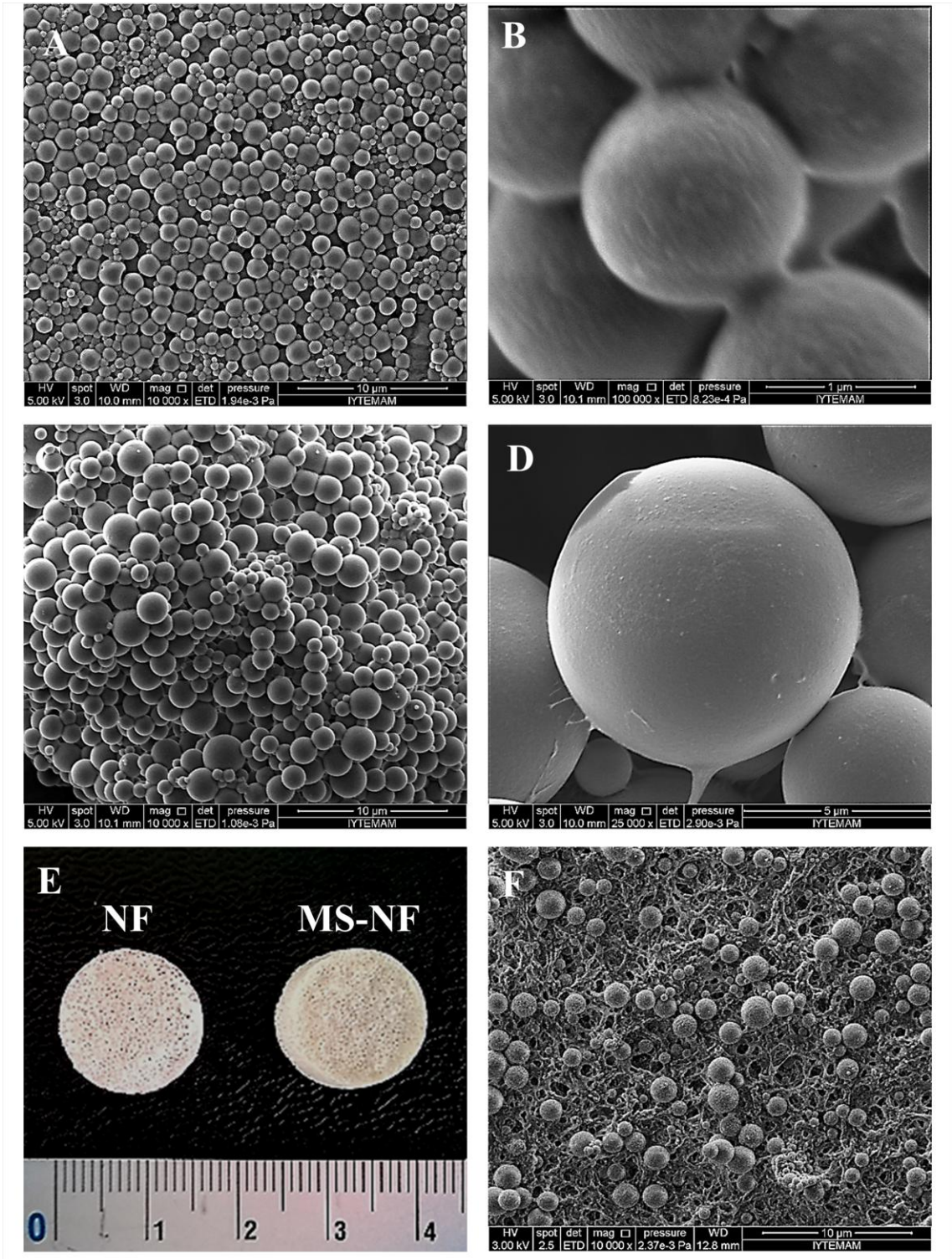

Figure 4. **SEM images of** (A-B) 0.1**% (w/v) (C-D) 1**% (w/v) alginate **microspheres**, (E) General appearance of nanofibrous scaffolds **before (NF)** and **after (MS-NF) microsphere attachment**, (F) SEM image of microsphere attached nanofibrous scaffolds **(MS-NF)**.

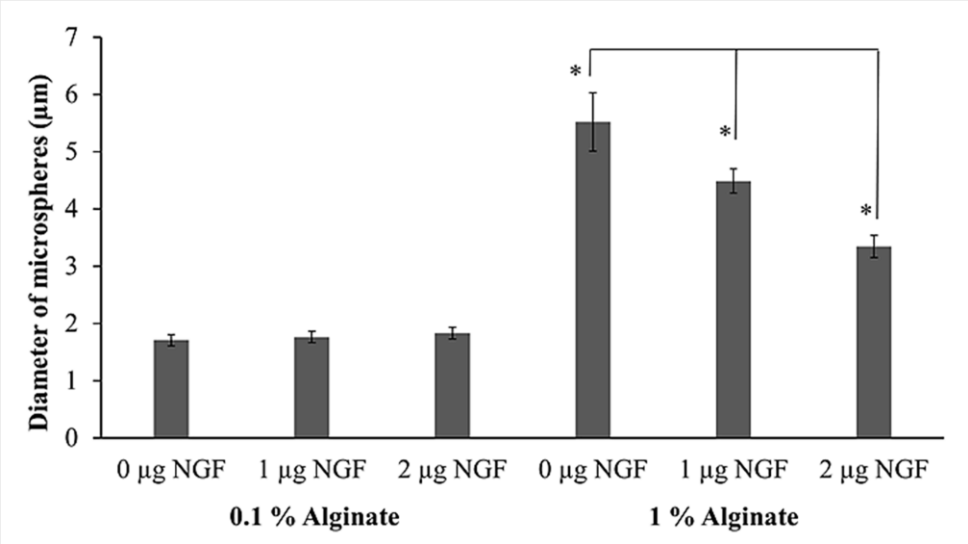

Figure 5. Effect of alginate concentration and NGF loading on the size of microspheres $^{*}p<0.05$.

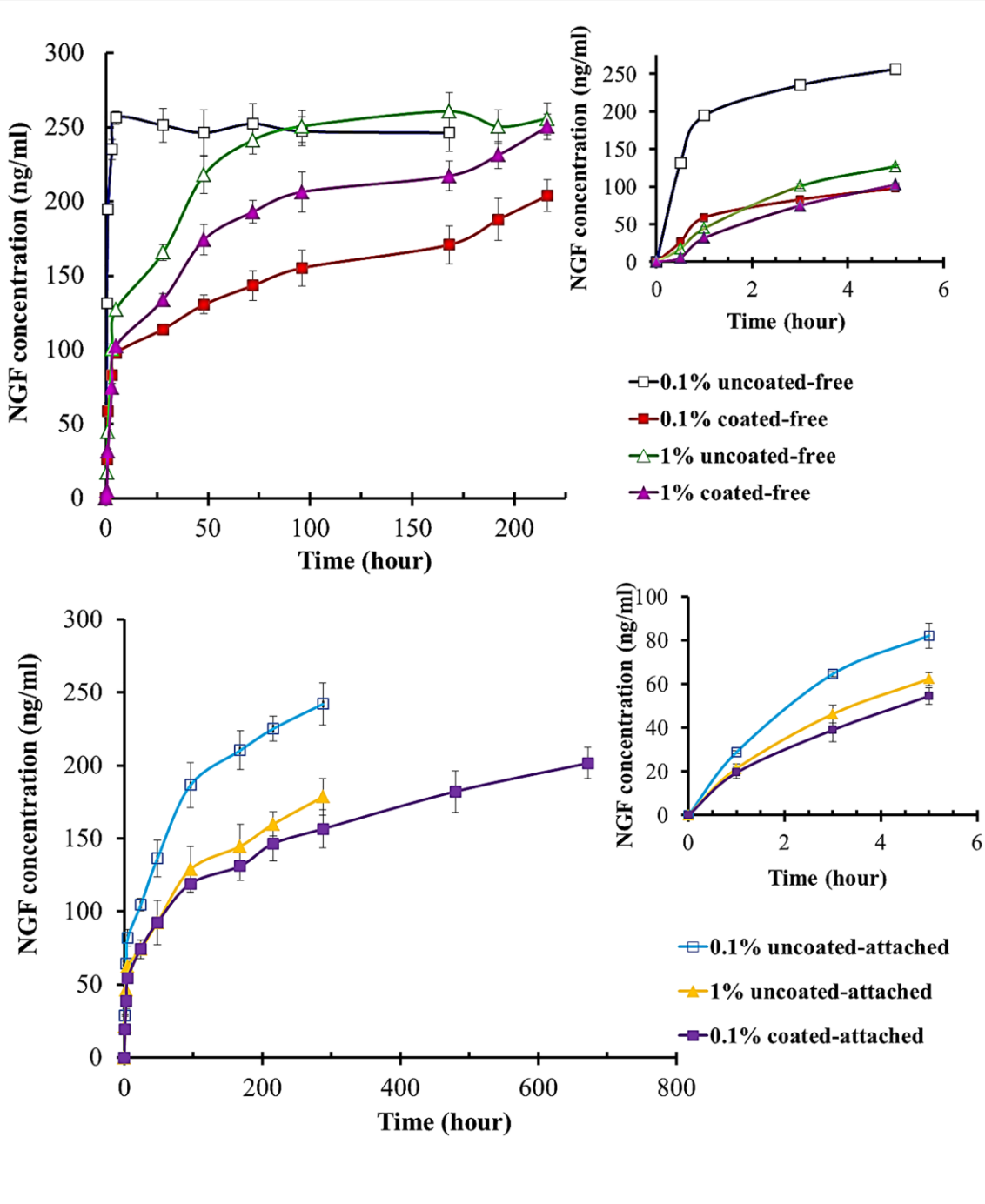

Figure 6. Release of NGF from (A) uncoated and coated **free** alginate microspheres and (B) **the scaffolds** integrated **with alginate microspheres**.

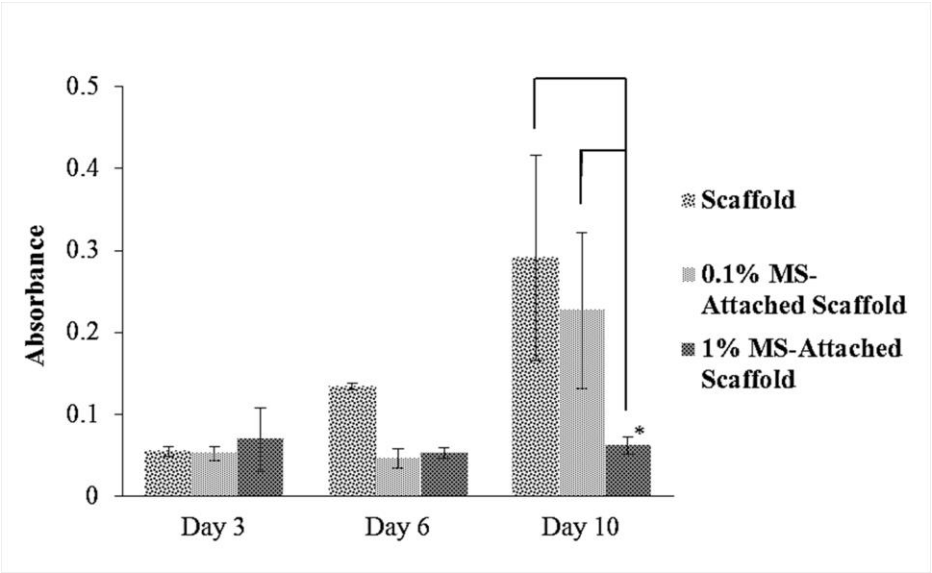

Figure 7. Absorbance value of PC12 cells cultured on **empty (Scaffold) and** alginate microspheres **attached (MS-Attached Scaffold) scaffolds**. $p<0.05$.

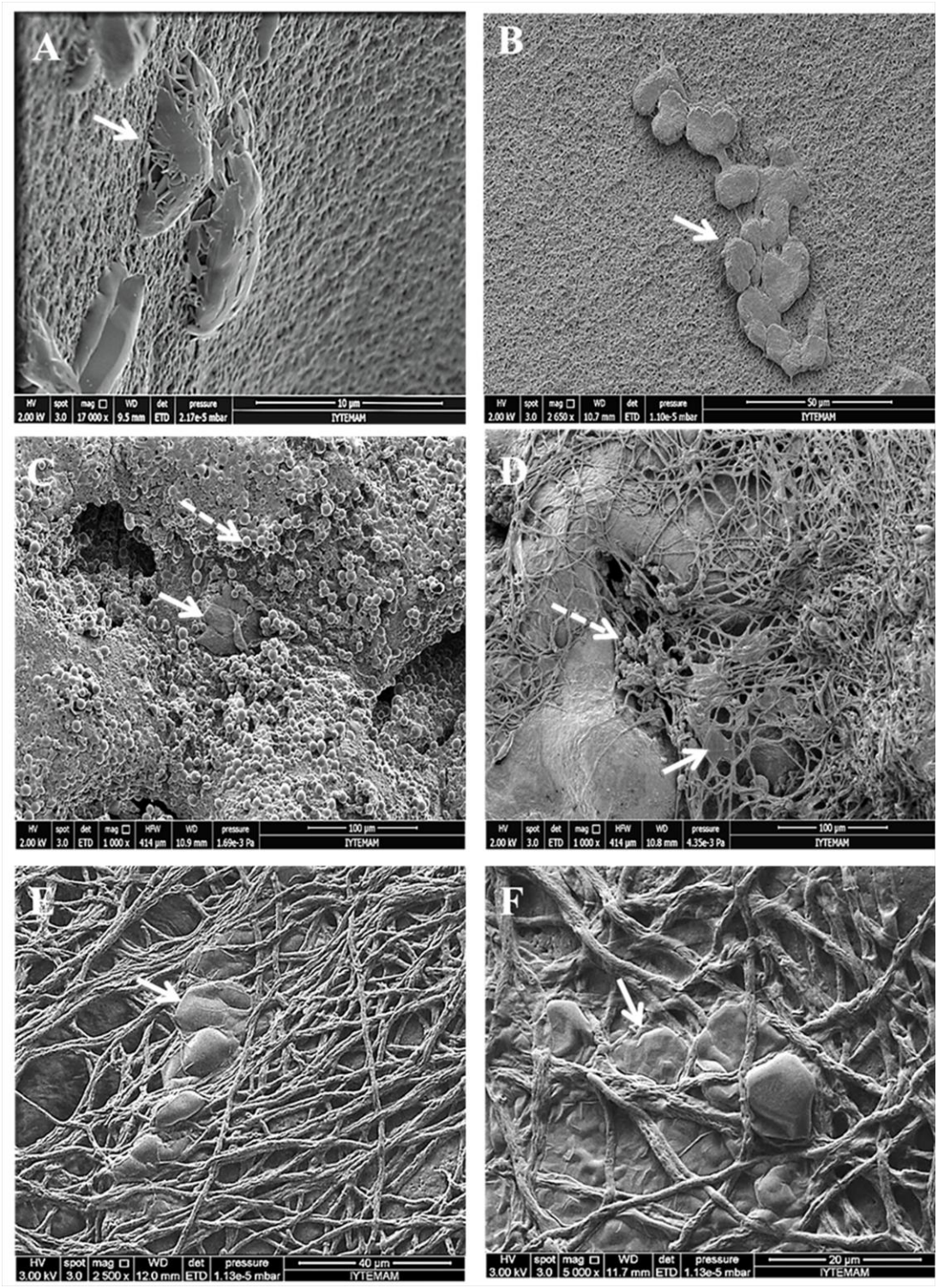

Figure 8. 4th day SEM images of PC12 cells seeded on (A) empty scaffold (B)the scaffold treated with 50 ng/ml/**2 days** of **free** NGF (C) the scaffold **attached** with NGF loaded **1%** alginate microspheres **and (D-F) 0.1%** alginate microspheres. (**Solid and dashed arrows indicate PC12 cells and** alginate microspheres attached on the scaffold).

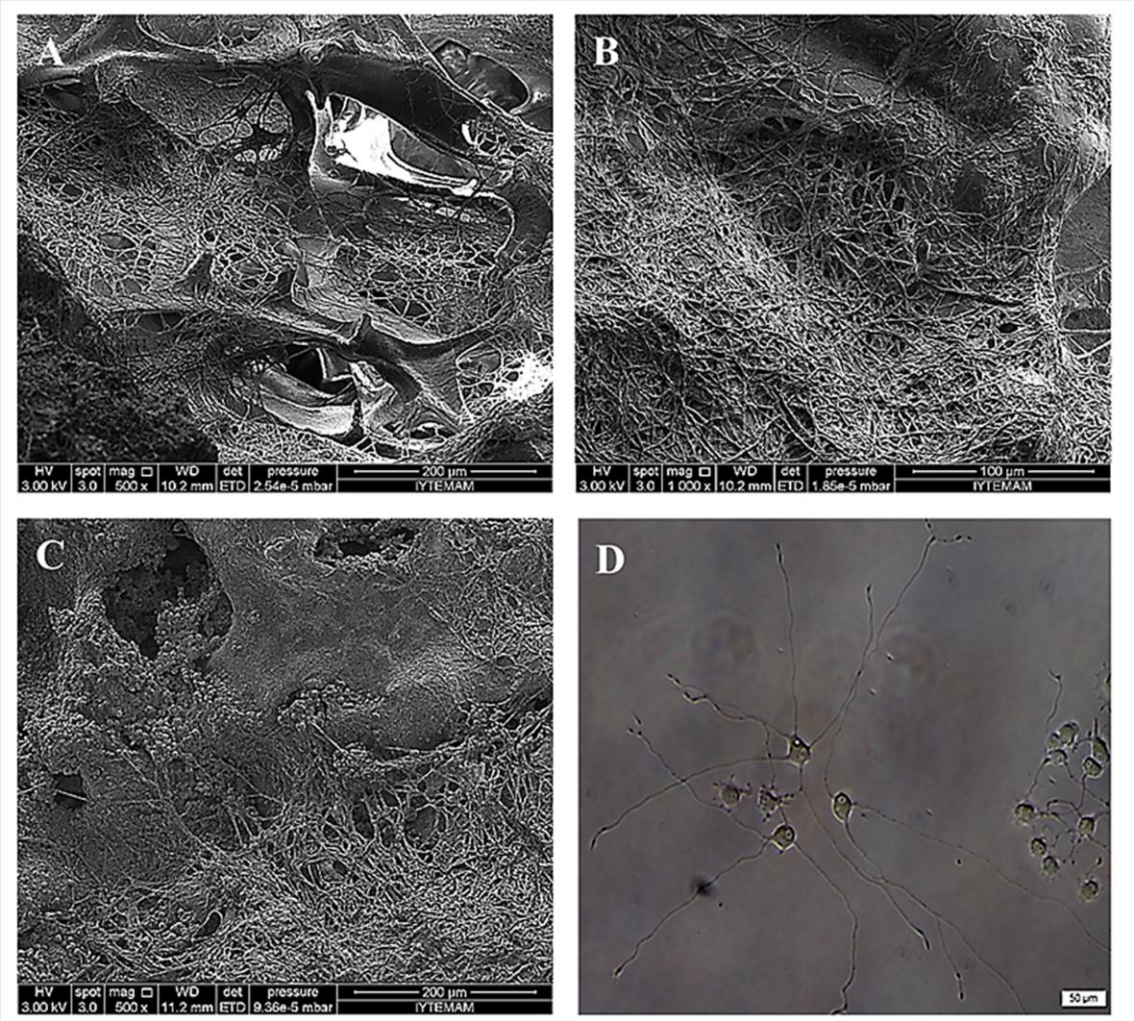

Figure 9. 10th day SEM images of PC12 cells seeded on the scaffold **attached** with **NGF loaded** (A-B) 0.1% (C) 1% alginate microspheres (D) 10th day **phase contrast microscope** images of PC12 cells in 2D cell culture flask in **starvation medium fed with** 50 ng/ml/2 days of NGF.