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Preparation of Suitable Nanofibre by Blending of Natural and Synthetic Polymer for Tissue Engineering Applications

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ABSTRACT

Tissue engineering field is an interdisciplinary field that has endeavoured to utilize a variety of processing methods with synthetic and natural polymers for various applications like scaffold preparation, drug delivery, wound healing and nanoparticle preparation. Blending of Polymers is one of the most effective method for providing new, desirable biocomposites for tissue engineering applications. Polymer exhibits a variety of physical and chemical properties from the constituent polymer. In this project preparation of chitosan and polyethylene oxide nanofibrewas performed by the process of electrospinning method. Chitosan is a natural polymer obtained from shell of shellfish and it has novel properties like biocompatibility, biodegradability, and antibacterial. Polyethylene oxide is a synthetic polymer which is also reported to be non toxic and highly degradable polymer. Blending ratio of these two polymers was varied to obtain uniform nanofibres. The blended polymer had been characterized by SEM, UTM and FTIR studies and their toxicity was studied by MTT assay and staining method.

Keywords: Nanofibres, Natural and Synthetic, Polymer, Tissue Engineering.

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INTRODUCTION

Polymeric materials are widely applied in the biomedical field especially in tissue engineering field. Although it is much easier to use synthetic polymers, natural polymers are also required due to their biocompatibility and biodegradability. Another method of preparation of polymeric materials for biomedical applications is to blend synthetic polymers with natural ones. Increasing applications and interest in new materials based on blends of two or more polymers has been observed during the last three decades. Blending of such synthetic and natural polymers can form a new class of materials with improved physical, chemical properties and biocompatibility compared with those of single components[1-3]. These kind of blended materials are called as bioartificial or biosynthetic polymeric materials. Natural polymers are usually biocompatible, whereas synthetic polymers can contain a residue of initiators and other compounds/impurities that do not allow cell to grow[4]. Synthetic polymers have good mechanical properties and thermal stability, much better than several naturally occurring polymers. There is also a limitation in the performance of several natural polymers in comparison to synthetic polymers. Synthetic polymers can be processed into a wide range of shapes, whereas for natural polymers several shapes are not easily obtained. To overcome these issues newly developed polymeric materials based on the blends of natural polymers and man-madeones should be biocompatible while, at the same time, possess good thermal and mechanical properties for use in biomedical applications[5].

Chitosan

Structure and properties of Chitosan

Chitosan, a (1–4)-linked 2-amino-2-deoxy-D-glucopyranose a natural polysaccharides which is derived from chitin sources a by product of shell fishes. Compared with other polysaccharides, chitosan has several important advantages, including biocompatibility, biodegradability, no toxicity, good film-forming characteristic chitosan and excellent chemical-resistant properties[6-10]. Chitosan is primarily produced from chitin by exhaustive alkaline deacetylation: this involves boiling chitin in concentrated alkali for several hours (40–45% sodium hydroxide, 120°C, 1–3 h) 170. Since this *N*-deacetylation is almost never complete, chitosan is considered as a partially *N*-deacetylated derivative of chitin. However, its mechanical properties and other physical/chemical properties are not good enough to meet this wide range of applications. In order to improve the inherent and poor solubility of chitosan, synthetic polymers like polyvinyl alcohol (PVA) or polyethylene oxide(PEO) which is amphiphilic and has unique mechanical properties can be

used to form the polymer. This gives improved characteristic chitosan of the nanofibre formed[10-14].

Solubility of chitosan

The most commonly used solvent is 1% acetic acid(as a reference) at about pH 4.0. CHITOSAN is also soluble in 1%hydrochloric acid and dilute nitric acid but insoluble in sulfuric and phosphoric acids[14-16]. But concentrated acetic acid solutions at high temperature can cause depolymerization of chitosan. Chitosan is not soluble in any organic solvents such as dimethyl formamide and dimethyl sulfoxide. There are several critical factors that contribute to chitosan solubility.They may include factors such as temperature and time of deacetylation, alkali concentration, prior treatments applied to chitin isolation, ratio of chitin to alkali solution, particle size, etc.

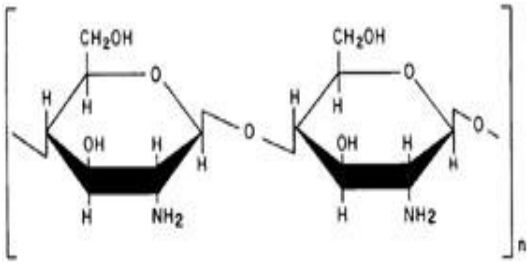

Figure 1: Structure of Chitosan

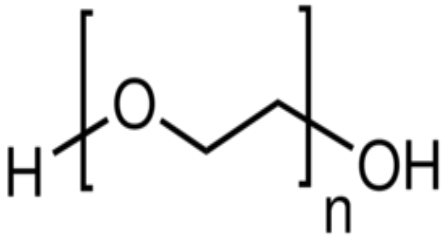

Figure 2: Structure of Polyethylene oxide

MATERIALS AND METHOD

Materials

Chitosan was obtained from Sigma Aldrich, India with deacetylation of 88% and medium molecular weight. Polyethylene oxide was also obtained from Sigma Aldrich, India with a molecular weight of 1,00,000 g/mol. The deionized water and 80% acetic acid was used as solvent without further purification.

Electrospinning

Chitosan solution was prepared by dissolving 2% and 3% chitosan in 80% acetic acid and polyethylene oxide of 7% was dissolved in distilled water. The apparatus consists of

- A capillary, through which the liquid to be electro spun is forced
- A high voltage source with positive or negative polarity, which injects charge into the liquid; and a grounded collector
- A syringe pump, gravitational forces, or pressurized gas are typically used to force the liquid through a small-diameter capillary forming a pendant drop at the tip.

Figure 3: Electrospinning setup

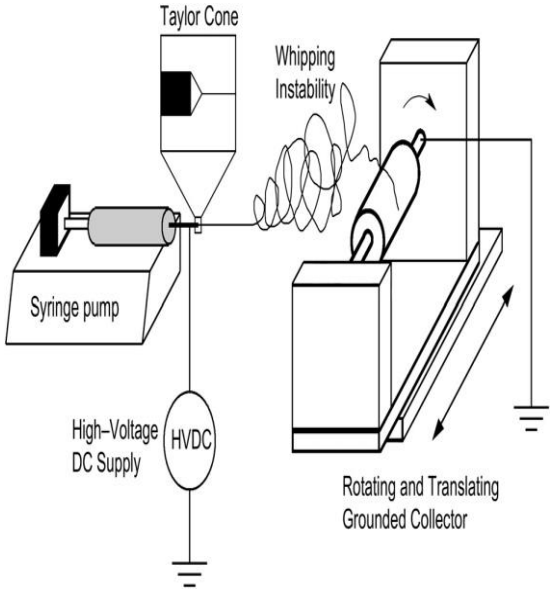

Figure 4: Schematic representation of Electrospinning Setup

Characterization

Characterization of the newly blend polymer was done to study the bonding characteristic chitosan, surface morphology and its tensile and compressive strength. These characteristics chitosan were interpreted by the help of Fourier transform infrared spectroscopy (FTIR), Scanning electron microscopy (SEM), and Universal tensile machine (UTM). For FTIR study an IR radiation was passed through a sample (it is coated with 0.3g of potassium Bromide). Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. An infrared spectrum represents a finger print of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. Therefore, infrared spectroscopy can result in a positive

identification (qualitative analysis) of every different kind of material[1,3]. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present.In this project, PerkinElmer (USA) FTIR spectrometer RX100 in the range of 400 – 4000cm^{-1}was used to know about the molecules present and to confirm the bonding nature of these fibres. UTM stands for Universal Tensile Machine which measures the ultimate tensile strength of the given sample. In this study, Tiniusolsen H10KS Universal tensile machine was used to calculate the tensile stress of the fibres. The specimen was prepared suitable for gripping into the jaws of the testing machine type that will be used. The specimen used is approximately uniform over a gauge length. It involves taking a small sample with a fixed cross-section area, and then pulling it with a tensometer (device used to evaluate the Young's modulus), gradually increasing force until the sample breaks. The major parameters that describe the stress-strain curve obtained during the tension test were the ultimate tensile strength, Yield strength, elastic modulus, percentage elongation, and reduction in area. SEM stands for scanning electron microscope operates at a high vacuum. The Scanning Electron Microscope, SEM uses electrons instead of light to form an image. The signals that arise from the electron-sample interactions reveal information about the sample including external morphology, chemical composition, crystalline structure and orientation of materials making up the sample. In this study S-3400N fully automated variable pressure SEM from Hitachi Company (Japan) was used to study the surface morphology for the confirmation of regular nanofibress and to find out the diameter of the nanofibres. All non-metals need to be made conductive by covering the sample with a thin layer of conductive material. Here the sample was prepared by gold coating of the fibers to change the conductivity of the nanofibers. The sample was placed in a small chamber that is at vacuum. Argon gas and the electric field cause an electron to be removed from Argon, making the atoms positively charged. The Argon ion gets attracted to a negatively charged gold foil. The Argon ions knock gold atoms from the surface of the gold foil. These gold atoms settle on the sample surface producing a thin gold coating. Data were collected over a selected area of the surface of the sample and was analyzed.

RESULTS AND DISCUSSION

Scanning Electron Microscopic analysis

Figure 5a and 5b shows the fibre formation chitosan at 2 different concentrations 2% and 3%. At 2%, the fibres formed consists of minute beads but 3% chitosan showed uniform nanofibres with less number of beads in it. So, the optimum chitosan concentration to form uniform fibres was

chosen to be 3%. Figure 5c shows the fiber formation of 7% Polyethylene oxide which is free of beads and uniform in nature. Figure 5d shows the nanofibre formed by blending chitosan and PEO in the ratio of 60:40. This shows uniform nanofibres with an average diameter of 135nm.

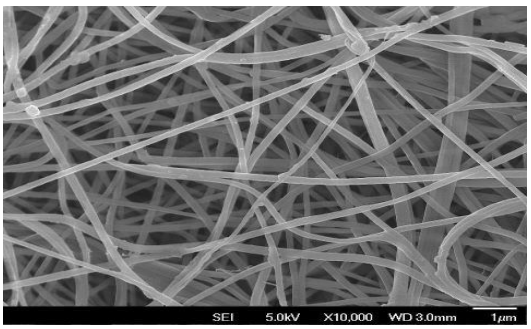

Figure 5a SEM image of 2% chitosan

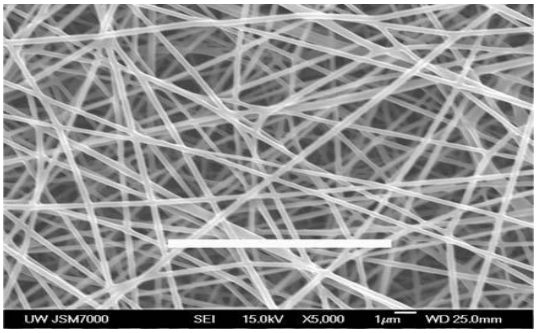

Figure 5b: SEM image of 3% chitosan

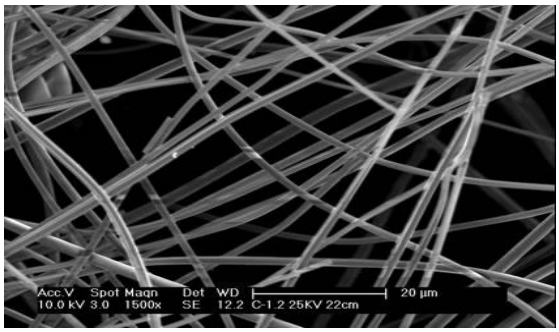

Figure 5c: SEM image of 7% PEO

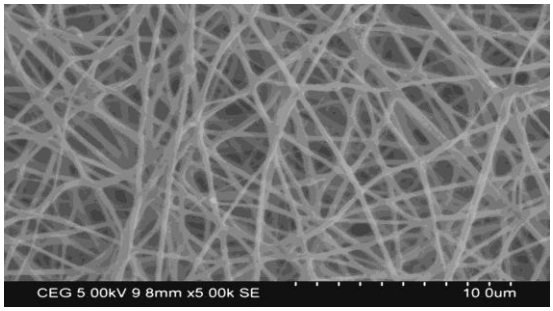

Figure 5d: SEM image of chitosan and PEO blend

Universal Tensile Machine Analysis

Tensile strength of the fibres was calculated from by UTM. Figure 5a shows the tensile strength of the chitosan fibre formed, which is 1.365Mpa. Figure 5b shows the tensile strength of PEO fibres formed and it is 2.716Mpa. Then Figure 5c shows the tensile strength of the blended polymer and it is 2.445Mpa. So with this results, it is interpreted as the fibres formed by blending shows the average strength obtained from both the polymers and tensile strength of the fibres can be varied by varying the composition of the blend.

Table 1: Tensile strength of Chitosan nanofibre

Specimen	Diamet. mm	Yield MPa	Yield Force N	Elong. at Yield %	Tensile MPa	Max Force N	Elong. at max %	Elong. %	Stress at break MPa	Force at Break N
Chitosan	0.2500	1.365	0.0670	115.3	1.365	0.0670	115.3	116.0	1.365	0.0670

Table 2: Tensile strength of PEO nanofibres.

Specimen	Diamet. mm	Yield MPa	Yield Force N	Elong. at Yield %	Tensile MPa	Max Force N	Elong. at max %	Elong. %	Stress at break MPa	Force at Break N
PEO	0.25000	1.901	0.0933	-	2.716	0.1333	1.300	1.800	1.698	0.0833

Table 3: Tensile strength of the blended nanofibres.

Specimen	Diamet. mm	Yield MPa	Yield Force N	Elong. at Yield %	Tensile MPa	Max Force N	Elong. at max %	Elong. %	Stress at break MPa	Force at Break N
Blended Fibre	0.2500	0.1369	0.0067	1.300	2.445	0.1200	4.400	4.400	2.445	0.1200

FTIR Analysis

Figure 6 shows the FTIR analysis of the blended polymer chitosan and PEO, where the OH stretching vibrations were seen at 3396cm^{-1}, C-O-C asymmetric stretching vibration at 1056cm^{-1}, symmetric and asymmetric C-H stretching at 2920cm^{-1}, vibrations of CH_2 groups at 1424cm^{-1}, and asymmetric stretching vibration of the C-O group at 842cm^{-1} conforms that bonding has taken place between chitosan and PEO. This further confirms the blending of chitosan and PEO and also about the chemical modifications taking place.

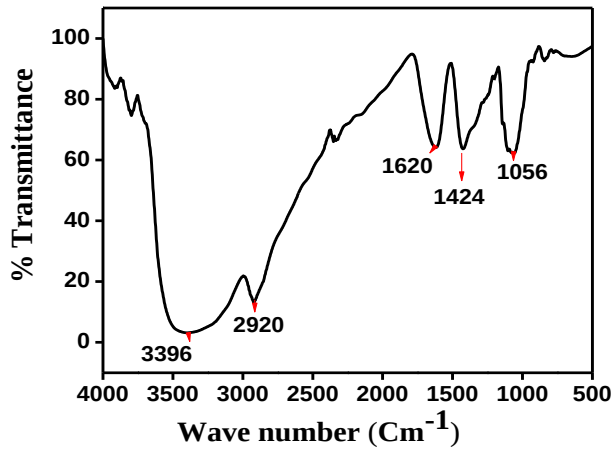

Figure 6: FTIR analysis of chitosan and PEO

***IN VITRO* STUDIES**

MTT Assay

The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple color. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH to purple formazan in living cells. In this results, cell viability when compared to control the percentage of visbility was found to be 90% in chitosan but viability was found to be 80% in PEO. Blending of chitosan and PEO showed 100% viability which is a higher level of cell viability compared with their seperate cell viability assays. This

shows that the cells showed improved viability when the polymers were blended than its individual form of fibres.

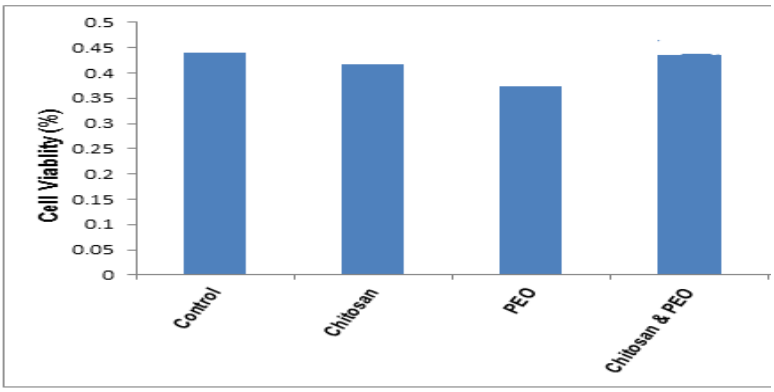

Figure 7: MTT assay of the fibres

Morphology of cells

The morphology of the cells were studied in the presnce of nanofibres when they are treated with MTT. This changes in cell morphology was studied usind the optical microscpe. The images obtained conformed that the morphology of cells remained the same when treated with chitosan fibres and PEO fibres seperately. The cells were found intact and there were no harm to the cells. When they were treated with blended fibre of chitosan and PEO together they cells were found clumpsy, which means the firm attchment of cells with the fibres.

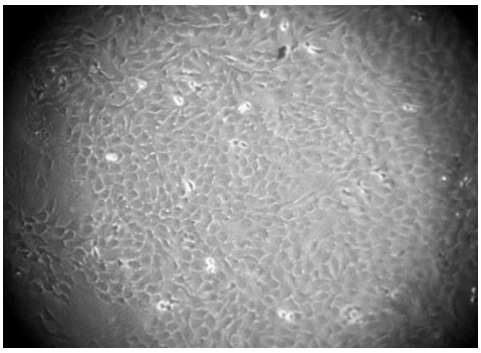

Figure 8a: Morphology of cells when treated with chitosan

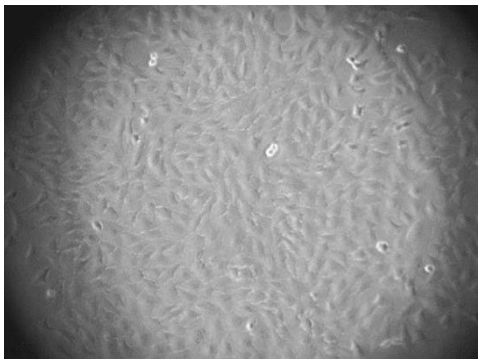

Figure 8b: Morphology of cells when treated with PEO

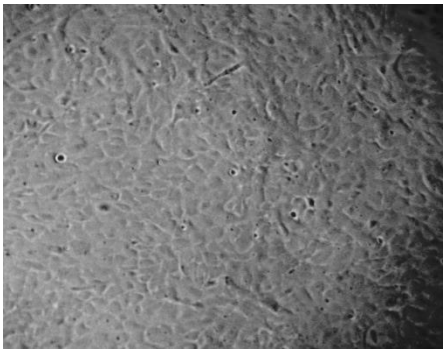

Figure 8c: Morphology of cells when treated with chitosan and PEO blend.

Acridine orange/Ethidium bromide staining

Acridine orange can be used in conjunction with ethidium bromide to differentiate between viable, apoptotic and necrotic cells. Ethidium bromide intercalates and stains DNA, providing a fluorescent red-orange stain. This EB/AO combined stain causes live cells to fluoresce green whilst apoptotic cells retain the distinctive red orange fluorescence. Figure 9a shows the control, which doesn't contain fibres. All the samples were compared with this control to study the viability of cells. Figure 9b shows the staining of cells when treated with chitosan fibres. There is only least number of orange spots which indicates the dead cells. So, there is more number of live cells coloured in green indicating that they are non toxic. Figure 9c shows the stained cells treated with PEO nanofibres, which show more number of orange coloured cells which are considered as dead cells. Figure 9d indicates the stained cells when treated with blended nanofibres of chitosan and PEO. It is found that there are no orange coloured cells and they remain same as the control, which indicates that they are completely non – toxic to the cells.

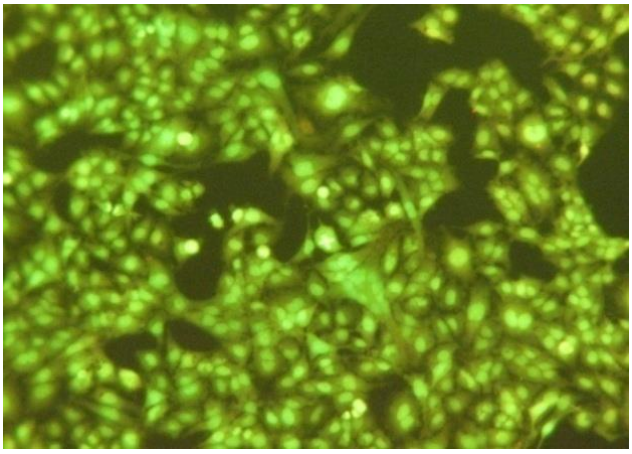

Figure 9a: AcOr/EtBr stained cells with control

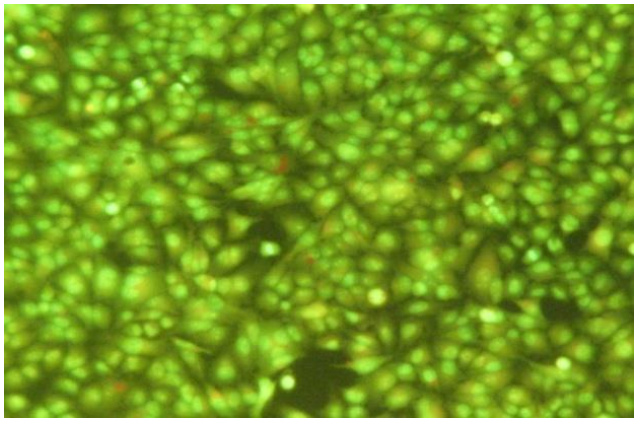

Figure 9b: AcOr/EtBr stained cells with chitosan

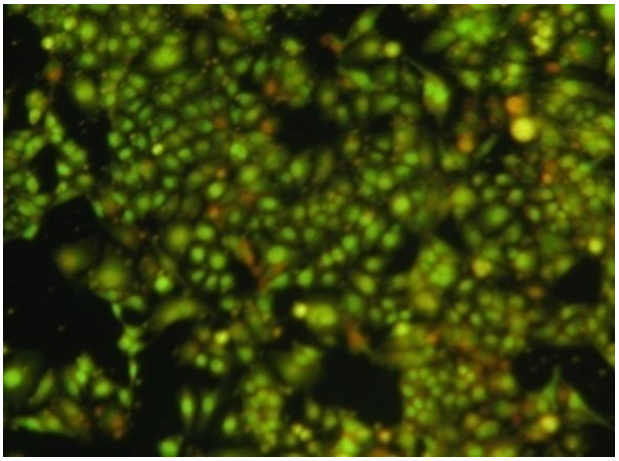

Figure 9c : AcOr/EtBr stained cells with PEO

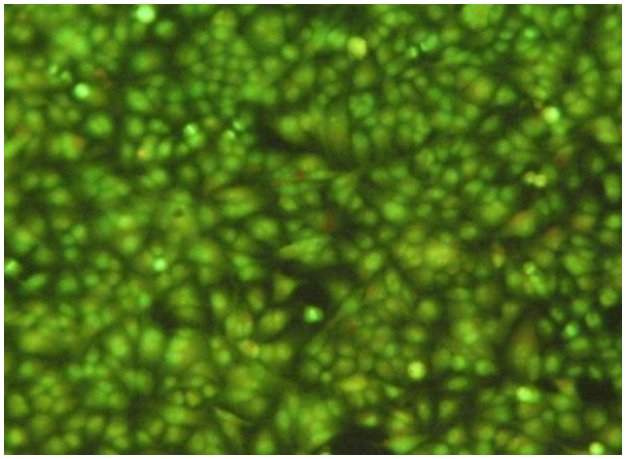

Figure 9d: AcOr/EtBr stained cells with chitosan and PEO blend.

CONCLUSION

Polymeric nanofibres of chitosan and PEO were formed individually, which is then blended together to form improved nanofibres. Structural analysis of fibres were done by SEM, which showed uniform nanofibres free of bead formation in the ratio of 70:30.Chemical and bonding characteristics chitosan of fibres were studied using FTIR analysis, which showed the different

stretching vibrations at 3396cm^{-1}, 1424cm^{-1},1056cm^{-1} conforming the bonding of chitosan and PEO fibres. Tensile strength of the fibres was studied using UTM analysis, in which the blended fibre showed average tensile strength of 2.445Mpa.These characterization studies confirmed the formation of fibres. Then *in vitro* studies were done by performing MTT assay and acridine orange/ ethidium bromide staining assays. In MTT assay, the individual fibres of chitosan and PEO showed viability percentage of 90% and 80%. Blended fibres of chitosan and PEO showed viability percentage of 100% which confirms that these fibres were not cytotoxic to the cells. Morphology of the cells were studied using optical microscope which also didn't show any changes in the cell structure, further confirming the non – toxicity of fibres. In the staining assay, discrimination between live and dead cells were made which didn't show any dead cells and it coloured green throughout in the blended fibres of chitosan and PEO. All this together confirmed that the fibres formed by blending chitosan and PEO can be used for tissue engineering applications and further studies are needed to confirm it's suitable application area in tissue engineering.

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