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In Vitro Characterization of Scaffolds for Tissue Engineering - A Review

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Abstract— *Scaffolds are artificial structures that replace the natural extracellular matrix (ECM). Scaffolds are capable of supporting three dimensional tissue formations. The natural ECM performs functions, like regulating intercellular communication along with providing support to the cells. A variety of fabricating methods are adopted for preparing scaffolds from a wide range of available biomaterials. The scaffolds thus produced can function as a successful scaffold only if they satisfy the necessary parameters. The suitability of any scaffold for tissue engineering application is tested by using a battery of tests that is a combination of mechanical and biological tests. This paper reviews the test methods adopted by researchers to test the suitability of scaffolds for tissue engineering applications.*
Keywords— Biodegradability, Cytotoxicity, Porosity, Proliferation, Scaffolds.

I. INTRODUCTION

A biomaterial is any substance (other than drugs) or combination of substances synthetic or natural in origin, which can be used for any period of time, as a whole or a part of a system which treats, augments, or replaces any tissue, organ or function of the body. Biomaterials may be natural or synthetic. Polymers, biopolymers, metals, ceramics and composites have been researched for their suitability as a biomaterial. Attempts are made to replace petrochemical products by renewable, bio sourced materials like starch, collagen, gelatin, alginate, cellulose and chitin [1,2]. The biomaterials are used to design scaffolds by the scaffold fabrication techniques like textile technologies, particulate leaching techniques, phase separation and rapid prototyping techniques [3]. Each fabricating technique produces a scaffold of a different characteristic nature. There are a few basic requirements that a scaffold must satisfy. The scaffold provides a framework and initial support for the cells to attach, proliferate, differentiate and form an extracellular matrix (ECM) [4]. The design aspect along with the choice of the material for the artificial scaffold is very crucial to cell differentiation, adhesion, proliferation, and the transport of the growth factors or other bio molecular signals [5].

An ideal scaffold should have the following characteristics: (i) an extensive network of interconnecting pores so that cells can migrate, multiply and attach deep within the scaffolds; (ii) channels through which oxygen and nutrients are provided to cells deep inside the scaffold, and the waste products can be easily carried away; (iii) biocompatibility with a high affinity for cells to attach and proliferate; (iv) right shape, however complex as desired by the surgeon; and (v) appropriate mechanical strength and biodegradation profile. Tissue engineering would greatly benefit from such scaffolds [6].

The aim of this paper is to review the test methods like cytotoxicity, MTT assay, DAPI assay, FTIR adopted to judge the suitability of a scaffold for tissue engineering applications.

II. CYTOTOXICITY

The cytotoxicity of the biomaterial selected for tissue engineering application is the first step towards producing a suitable scaffold for tissue engineering.

2.1 Test for cytotoxicity

International Standard ISO 10993-5:2009(E), Biological Evaluation of Medical Devices, Part 5: Tests for In vitro cytotoxicity are designed to determine the biological response of mammalian cells in vitro using appropriate biological parameters. An extract of the scaffold sample is tested for cytotoxicity. It measures cell viability via metabolic activity. Yellow water-soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide) is metabolically reduced in viable cells to a blue-violet insoluble formazan. The number of viable cells correlates to the colour intensity determined by photometric measurements after dissolving the formazan in alcohol.

Cells are seeded into 96-well plates and maintained in culture for 24 h. They are then exposed to the test compound over a range of concentrations. After 24 h exposure, the formazan formation is determined for each treatment concentration and compared to that determined in control cultures. For each treatment the percentage inhibition of growth is calculated.

A decrease in number of living cells results in a decrease in the metabolic activity in the sample. This decrease

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directly correlates to the amount of blue-violet formazan formed, as monitored by the optical density at 570 nm. To calculate the reduction of viability compared to the blank, the following equation (1) is used.

$$Viab\% = \frac{OD\ 570\ e}{OD\ 570\ b} \text{ x } 100 \qquad (1)$$

where

OD570e is the mean value of the measured optical density of the 100 % extracts of the test sample;

OD570b is the mean value of the measured optical density of the blanks.

The lower the Viab.% value, the higher the cytotoxic potential of the test item is. If viability is reduced to < 70 % of the blank, it has a cytotoxic potential. The 50 % extract of the test sample should have at least the same or a higher viability than the 100 % extract; otherwise the test should be repeated.

III. PORE SIZE

3.1 Importance of Pore Size

The next important property to be tested for a scaffold is its pore size. The porosity of the scaffold allows the transport of oxygen and nutrients to be provided to cells deep inside the scaffold, and the waste products can be easily carried away. Simply producing a highly porous scaffold and seeding it with the appropriate types of cells in most cases does not reproduce the desired feature of a normal tissue [6].

The subcellular structures (1–10 mm) to control the cellular environment, cell scale structures (10–100 mm) to control cell–cell inter-relationships, and supracellular scale structures (100–1000 mm) to build the essential functional units of the tissue are the pore sizes that are essential for various functions as described by Bhatia and Chen, 1999 [7].

3.2 Measurement of Pore Size:

Pore size is measured in different ways. K Katoh et al measured the porosity by using the formula Porosity(%) =(ρ keratin– ρ sponge)/ ρ keratin x 100, where ρ keratin was the density of a dense S-sulfo keratin plate prepared by compression-molding the S-sulfo keratin–urea mixture, dissolving out urea and lyophilizing. The density of keratin plate was measured using a 50 ml pycnometer with ethanol, where the density of ethanol was 0.7893 g/cm3 at 20oC [8].

Nayak and Gupta determined the apparent porosity of the scaffold by applying the Archimedes principle and its calculation was done by using dry, soaked and suspended weights of the scaffolds using the equation (2):

$$\%AP = \frac{W2-W1}{W2-W3} \text{ x } 100 \qquad (2)$$

where

W1 = dry weight of the scaffold,

W2 = soaked weight of the scaffold, and W3 = suspended weight of the scaffold [9].

Pore size is also observed under a scanning electron microscope (SEM).

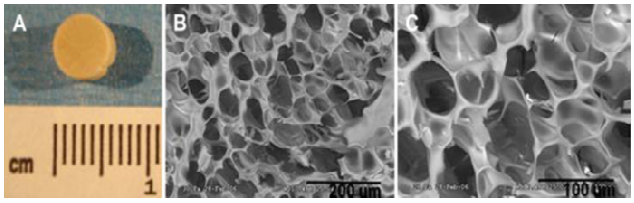

Fig. 1: Images of kerateine disks prior to subcutaneous implantation. A digital photo shows a macroscopic view of a 6 mm4 mm disk created by lyophilization of a kerateine solution (A). Scanning Electron Microscopy (SEM) of lyophilized kerateine disks at (B) 100and (C) 300. This homogenous porous network was formed by spontaneous re-crosslinking of the proteins upon exposure to air [10].

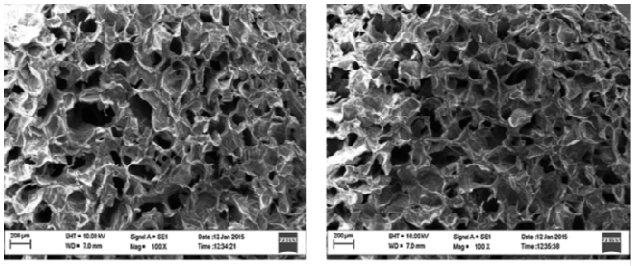

Fig. 2: SEM image of the keratin/agar scaffold illustrates that the pores were available in the range of 50–300 m in size. The magnification of the SEM was adjusted at 100×.(A) illustrates the transverse section, while (B) demonstrates cross-sectional area of the scaffold [9].

The pore size distribution of PLLA and PLLA/NaC1 composite membranes was determined by a mercury intrusion porosimeter (model Poresizer 9220, Micromeritics, Norcross, GA) [11].

IV. CELL ADHESION AND PROLIFERATION

4.1 MTT assay

International Standard ISO 10993-5:2009(E), Biological Evaluation of Medical Devices, Part 5, also describes a test by direct contact which allows both qualitative and quantitative assessment of cytotoxicity. The sterilised scaffold sample is used for MTT assay and the investigation for adhesion and proliferation of cells is made by the observation of the optical density (OD) under MTT assay [12].

Observations are done on the 1st, 3rd, 5th and 7th day of cell culture and attached cell percentage is calculated by using the following equation (3):

$$Attached\ Cell\ \% = \frac{ODt2}{ODt1} \text{ x } 100 \qquad (3)$$

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where

ODt1= optical density at time t1,

ODt2=optical density at time t2.

The OD observed on the 1st day was considered as the value of ODt1, while the value of ODt2was opted from the OD value taken on the 3rd, 5th and 7th day of culture [9].

3.2 DAPI assay

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA. It is used extensively in fluorescence microscopy. As DAPI can pass through an intact cell membrane, it can be used to stain both live and fixed cells. It passes through the membrane less efficiently in live cells and therefore the effectiveness of the stain is lower.

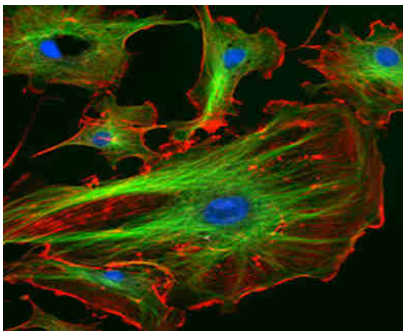

Fig.3: Endothelial cells stained with DAPI (blue), phalloidin (red) and through immunofluorescence via an antibody bound to fluorescein isothiocyanate (FITC) (green) image from- https://en.wikipedia.org/wiki/DAPI.

V. BIODEGRADABILITY

5.1 Weight loss%

The scaffolds are allowed to swell in physiological conditions (37^{o} C in serum-free medium) for 28 days. Before test, the initial weight of each vacuum dried films (Wo) was recorded. Every four days, three replicate specimens were withdrawn from the medium and blotted with a filter paper to remove the excess surface water. Then the films were vacuum-dried at room temperature for 24 h, weighed again (W dry), and subjected to analysis using the equation (4) given below [13].

$$\text{Weight Loss } \% = \frac{\text{Wo} - \text{W dry}}{\text{Wo}} \text{x } 100 \qquad (4)$$

5.2 Fourier transform infrared spectroscopy (FTIR)

PLLA/keratin scaffolds were immersed in 50 ml phosphate buffer saline (PBS, pH 7.4) at 37^{o} C for various periods up to 4 weeks. The degradation medium was changed daily for the first week, once at day 10 and day 14, and then weekly for the rest of the remaining period. Scaffolds were taken out at 1, 3, 7, 14, and 28 days. The samples were examined by FTIR and XPS (Perkin-Elmer, PHI 1600ESCA), respectively [14].

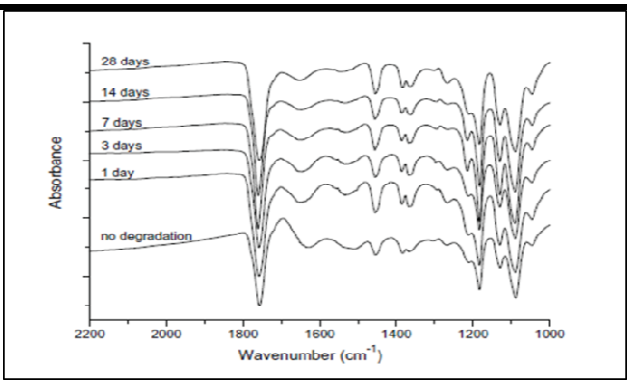

Fig.3: FTIR spectra of PLLA/keratin scaffolds as a function of degradation periods [14].

VI. CONCLUSION

The suitability of a scaffold for tissue engineering is decided by its non-cytotoxic nature, porosity, cell proliferation, cell adhesion and biodegradability property. These properties are tested using the cytotoxicity test, SEM, porosimeter, MTT assays, DAPI assay and FTIR. The biomaterial used for the preparation of scaffold for the tissue engineering application must fulfil these requirements.

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