**Review**

**A Review of the 3D Designing of Scaffolds for Tissue Engineering with a Focus on Keratin Protein**

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**Abstract:** In tissue engineering scaffolds take the place of the natural extra cellular matrix (ECM). The natural ECM is the extracellular part of animal tissue that usually provides structural support to the animal cells in addition to performing various other important functions. 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. In addition to the material and design of the scaffolds, it is necessary to replicate the normal physiological situation if the scaffold has to function as an implant. The cells have to be located in the porous scaffold to form a three dimensional assembly. The article discusses the important factors to be considered while designing a scaffold for tissue engineering and regenerative medicine.

**Keywords:** biomaterial; scaffold; protein; keratin; tissue engineering

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**1. Introduction**

Scientists have dreamed of a ‘body shop’, where a patient goes with a prescription for a lost limb or a failing organ and the limb/organ is grown in the shop using details from the patient like DNA or bone marrow for cell multiplication. This regenerated limb/organ is then grafted onto the patient. The limb/organ shall grow naturally as a part of the patient’s body [1]. Tissue engineering has the potential to make this dream come true. This approach has gained importance over a few decades due to the drawbacks in traditional tissue or organ transplant such as insufficient number of donors, traumatic procedures and rejections [2]. The basis for this technology is that cells from the patient’s own body are isolated and allowed to proliferate, either in vivo or ex vivo, so as to form extra-cellular matrix, and ultimately new tissue [3].

Tissue engineering is a multidisciplinary field that applies the principles of engineering and life sciences [4]. Materials and fabrication technologies are critically important for tissue engineering in designing temporary, artificial extracellular matrices (scaffolds), which support three-dimensional tissue formation [1]. The cell is the basic structural, functional and biological unit of all known living organisms. Cells are the smallest unit of life that is classified as a living thing and are often called the ‘building blocks of life’. The cells of plants, animals, fungi, algae are all eukaryotic cells i.e. they contain a nucleus. The nucleus is the information centre for a cell. Tissue is an ensemble of similar cells from the same origin that together carry out a specific function. It is a cellular organizational level intermediate between cells and a complete organism. Organs are formed by the functional grouping together of multiple tissues. Tissue engineering is the use of combination of cells, engineering and materials with suitable biochemical and physio-chemical factors to improve or replace biological functions. The term regenerative medicine is often used synonymously with tissue engineering. Tissues are fabricated in laboratories by a combination of ECM, cells and biologically active molecules.
The ECM can serve many functions, such as providing support, segregating tissues from one another, and regulating intercellular communication. The extracellular matrix regulates a cell’s dynamic behaviour. In addition, it sequesters a wide range of cellular growth factors and acts as a local depot for them. Formation of the extracellular matrix is essential for processes like growth, wound healing and fibrosis. Fibrosis is the formation of excess fibrous connective tissue in an organ or tissue in a reparative or reactive process. Fibrosis can be used to describe the pathological state of excess deposition of fibrous tissue, as well as the process of connective tissue deposition in healing. Scaffolds are artificial structures that are capable of supporting 3D tissue formation. The cells are implanted or seeded in scaffolds and are critical both ex vivo as well as in vivo. The terms ex- vivo and in vitro are not synonymous. In vivo studies are those that are conducted with living organisms in their normal intact state. Ex vivo studies are conducted on functional organs that have been removed from the intact organism. In vitro studies are conducted using components of an organism that have been isolated from their usual biological surroundings. They are commonly called as test tube experiments. In- vitro means ‘in glass’ in Latin. To restore function or regenerate tissue, a scaffold is necessary that will act as a temporary matrix for cell proliferation and extracellular matrix deposition, with subsequent ingrowth until the tissues are totally restored or regenerated. Scaffolds have been used for tissue engineering such as bone, cartilage, ligament, skin, vascular tissues, neural tissues, and skeletal muscle and as vehicle for the controlled delivery of drugs, proteins, and DNA [5].

One of the first considerations when designing a scaffold for tissue engineering is the choice of material. The three main material types which have been successfully investigated to be applied in developing scaffolds include Synthetic polymers, Natural polymers, and Ceramics. Keratin has been widely researched as a biomaterial in tissue engineering applications. Hair keratins are a family of structural proteins found abundantly in hair and fingernails, and are often referred to as “hard” keratins (as opposed to soft keratins that are found in epithelial tissues) [6]. Keratin in the form of films has been found to be biocompatible. Electro spun and wet spun keratin fibres in the form of non-woven nanostructures have been researched for their application in tissue engineering [7].

2. Requirements of scaffolds:

There are three approaches in tissue engineering: 1) the use of isolated cells or cell substitutes to replace those cells that supply the needed function; 2) the delivery of tissue inducing substances, such as growth and differentiation factors, to targeted locations; 3) growing cells in three dimensional scaffolds [1].

Scaffolds are defined as three-dimension porous solid biomaterials designed to perform some or all of the following functions: (i) promote cell-biomaterial interactions, cell adhesion, and ECM deposition, (ii) permit sufficient transport of gases, nutrients, and regulatory factors to allow cell survival, proliferation, and differentiation, (iii) biodegrade at a controllable rate that approximates the rate of tissue regeneration under the culture conditions of interest, and (iv) provoke a minimal degree of inflammation or toxicity in vivo [8].

There are a few basic requirements that have been widely accepted for designing polymer scaffolds. An ideal scaffold should possess the following characteristics to bring about the desired biological response (1) the scaffold should possess inter-connecting pores of appropriate scale to favor tissue integration and vascularization, (2) be made from material with controlled biodegradability or bio-resorbability, (3) appropriate surface chemistry to favour cellular attachment, differentiation and proliferation, (4) possess adequate mechanical properties to match the intended site of implantation and handling, (5) should not induce any adverse response and, (6) be easily fabricated into a variety of shapes and sizes (Liu et al., 2007; Sachlos et al., 2003) [9].
The importance of porosity for scaffold has been stressed by many researchers. A three-dimensional structure supports cell growth similar to the natural phenomenon. The scaffold has interconnected macro-pores that host the cells and newly formed tissue, while the pore walls should be micro-porous to transport nutrients and waste products [10]. Sandwich-type scaffolds consisting of radially-aligned nanofibers at the bottom, nanofiber membranes with square arrayed micro wells and nanostructured cues at the top, and micro skin tissues in between as micro skin grafts for use in skin regeneration and the void area in the scaffolds was well suitable for exudate drainage in wound [11]. The high porosity achieved in the collagen scaffold, combined with the increased mechanical properties and improved permeability, seen as a result of the addition of the osteoinductive HA phase, make this scaffold an ideal template for the promotion of cell ingrowth and in vivo vascularisation [12].

The biocompatibility and the surface interaction of scaffolds with cells is another important factor that needs careful consideration. The experimental results indicated that Schwann Cells (SCs) could grow onto chitosan materials with two different shapes: spherical and long olivary. It was also found that the cells on the chitosan fibers migrated faster than those on the chitosan membranes. There was a good biological compatibility between chitosan and SCs. [13]. The ideal tissue engineering scaffold should positively interact with cells, including enhanced cell adhesion, growth, migration, and differentiated function. To achieve these positive cell-scaffold interactions, surface or bulk modifications of the polymers are often employed [1]. Immunostaining for sarcomeric myosin revealed surface chemistry-dependent differences in myogenic differentiation following the pattern OH > CH3 > NH2 = COOH [14].

The wettability of a scaffold influences the adhesion of cells. Atomic force microscopy (AFM) was used to directly measure the adhesion forces between three test proteins and low density polyethylene (LDPE) surfaces treated by glow discharge plasma to yield various levels of water wettability and it was found that LDPE shows a stark transition between protein adherent and protein non-adherent materials in the range of water contact angles 60–65°, consistent with known changes in protein adsorption and activity [15].

3. Biomaterials for scaffolds:

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’.

One of the primary reasons that biomaterials are used is to physically replace hard or soft tissues that have become damaged or destroyed through some pathological process. They are used in Orthopedics, Cardiovascular applications, Ophthalmics, Dental applications and wound healing [16].

Proteins have been widely researched as a biomaterial for tissue engineering. Keratin is a protein found in skin, hair, nails, horns and hooves. Keratins in its various forms have been fabricated by using a variety of techniques from freeze drying to electro spinning. This review lays its focus on keratin as a biomaterial for the fabrication of an appropriate scaffold for tissue engineering applications.

Materials that have been used as biomaterials include, 1) Polymers: Nylon, Polytetrafluoroethylene, Polyurethane, Silicone rubber, Polycaprolactone, 2) Biopolymers: Alginate, Chitin/Chitosan, Collagen, 3) Metals: Ti, Co-Cr alloy, Stainless steel, Pt, Au, 4) Ceramics: Aluminium oxide, Calcium phosphate, Hydroxyapatite, 5) Composites: Fibre reinforced bone cements.
The synthetic polymers approved by FDA are Poly-e-caprolactone (PCL), Poly lactic acid (PLA, PDLA, PLLA), poly lactic-co-glycolic acid (PLGA), Poly ethylene glycol (PEG) and poly glycolic acid (PGA). The synthetic polymers do not possess the surface chemistry that is familiar to the cells but they can be produced in a wide range of mechanical properties and degradation rates. They are a reliable source of raw materials. The natural polymers on the other hand like Collagen, Alginate and Chitosan lack in the mechanical properties. Collagen alone does not possess enough mechanical strength. Along with hydroxyapatite, collagen is one of the two major components of bone. It makes up 89% of the organic matrix and 32% of the volumetric composition of bone [17]. Hydroxyapatite (HA) is widely used for bone tissue engineering. Addition of HA to collagen scaffolds was investigated for mechanical strength, porosity and in vivo healing potential [12]. Silicon-substituted hydroxyapatite offers the prospect of rapid and ordered bone repair [3].

4. Various forms of scaffolds:

Scaffolds are used in its various forms like, films, membranes, their composites, fibre composites and nanofibers. Chitin and Chitosan has been prepared in its various forms as scaffolds.

| Form         | Species | Applicable clinical case                              | Reference                  |
|--------------|---------|-------------------------------------------------------|----------------------------|
| Cotton       | Dog     | Abscess, Bite Wound, Contused Wound, Fracture         | Okamoto et al. 1992, 1993  |
|              | Cat     | Abscess, Bite Wound, Contused Wound, Fracture         | Okamoto et al. 1992, 1993  |
|              | Human   | Decubitus ulcer                                       | Ueyama et al. 1994         |
| Sponge       | Cow     | Decubitus ulcer                                       | Wada et al. 1990           |
|              |         | Abscess, Arthritis, Contused Wound, Surgical Dead Space (Umbilical Hernia) | Minami et al. 1992, Okamoto et al. 1993 |
|              | Dog     | Abscess, Bite Wound, Contused Wound, Surgical Dead Space (Tumor Resection, Inguinal Hernia, Fracture), Lacerated Wound, Alveolitis | Okamoto et al. 1992, 1993  |
|              | Cat     | Abscess, Bite Wound, Surgical Dead Space (Tumor Resection, Inguinal Hernia, Fracture) | Okamoto et al. 1992, 1993  |
|              | Rabbit  | Abscess                                              | Fukumoto et al. 1994       |
|              | Monkey  | Bite Wound                                           | Fukumoto et al. 1994       |
|              | Human   | Maxillossinusectomy (Chitin sponge coated gauze)     | Nagashima et al. 1991, Maeda et al. 1992 |
| Composite    | Cow     | Fetlock deformity, Capsuloplasty, Herniorrhaphy, Tendoplasty, Redressment of entropion | Okamoto et al. 1992, Minami et al. 1992 |
| with NWF     | Ring tailed Lemur | Skin defect of tail                                | Fukumoto et al. 1994       |
A biomaterial has to be analysed for its advantages and disadvantages considering the requirements of a scaffold. F. Croisier and C. Jérôme have presented the advantages and disadvantages of Chitosan biomaterials for various applications [19].

| Biomaterial type | Specifications | Main advantages | Main Disadvantages | Main biomedical applications |
|------------------|----------------|-----------------|--------------------|-----------------------------|
| Hydrogels (3D)   | Physically associated (reversible) | Soft flexible non-toxic | Not stable (uncontrolled dissolution may occur) Low mechanical resistance Pore size difficult to control | Tissue replacement/engineering drug growth factor delivery |
|                  | Chemically cross-linked (irreversible) | Soft flexible stable controlled pore size | May be toxic Cross-linking may affect chitosan intrinsic properties | |
| Sponges (3D)     | Free-standing | High porosity Soft | May shrivel Low porosity | Tissue engineering (filling material) Wound dressing skin substitutes |
| Films (2D)       | Thin (LB) | Material coating | Laborious for the construction of multilayers | Coatings for a variety of scaffolds wound dressings skin substitutes |
|                  | Thin (LBL) | Material | Many steps | |
5. Fabrication techniques:

A number of techniques are used for the fabrication of scaffolds. Textile technologies, Particulate leaching, Phase separation and Computer assisted design and manufacture (CAD/CAM) are the popular techniques [1].

Electrospinning and freeze drying are the popular methods for the preparation of scaffolds for tissue engineering. Electrospinning provides the porosity that is one of the most important properties for the success of any scaffold. A multi-layered composite (MC) was prepared by Cédryck Vaquette and Justin Cooper-White using the electrospinning technique along with the thermally induced phase separation technique which highlighted the importance of electro spun fibres for increasing the tensile strength [20].

![Diagram of scaffold fabrication strategies](image-url)

Fig.1. Scaffold fabrication strategies utilized (a) Morphology of the MC scaffold. (b-d) Scaffold cross-section showing that the TIPS treatment “glued” the layers together without filling the pores of the electrospun membranes. The black arrows show the connectivity at the interface between two adjacent electrospun membranes. (e) The surface of the MC scaffold, indicating that the pores were not filled with PLCA; the black arrows show some PLCA around or in between the PCL fibres. (f,i) The morphology of the MC scaffold produced using a round patterned collector and showing the locally increased pore size. (g,h) The microstructure of the 5% PCL-TIPS scaffold. (Reproduced from [20])

Table 2. Main advantages, disadvantages and applications of the Chitosan biomaterials presented. (Reproduced from [19]).
Freeze extraction and freeze gelation techniques are time and energy-saving, with less residual solvent, which also resolves the problem of formation of surface skin [21].
Clinical applications for tissue engineering has been limited because of their brittleness, difficulty of shaping for implantation and new bone formed in a porous HA network cannot sustain the mechanical loading needed for remodelling [16,22].
Combination of an outer concentric layer of circumferentially oriented stiff PLA fibres and an inner concentric layer of randomly oriented and elastic PCL fibres resulting in a desirable strong and pliable scaffold mimicking morphological and mechanically a blood vessel was prepared by the multi layering electrospinning technique [23].

Incorporation of chitosan fibres into porous chitosan scaffolds improved the scaffold strength and stiffness in proportion to the fiber/scaffold mass ratio [24].

Fig.2. SEM micrographs of the bilayered tubular construct: (a) bilayered tube (entire view); (b) bilayered tube wall; (c)–(d) details of the interface (mixing zone) between inner and outer layers; (e)–(f) details of the outer layer (PLA); and (g)–(h) details of the inner layer (PCL). Reproduced from [23]

Fig.3. (A) Unigraphics ® Image of trileaflet heart valve mold. The outer cylinder rendered transparent to allow visualization of interior features. (B) Ventricular view of porous chitosan trileaflet heart valve scaffold showing the leaflet (L) and the cylinder (C). (C) Aortic view of trileaflet heart valve scaffold showing the sinus (S). reproduced from [24].
6. Keratin Biomaterial:

Proteins such as such as fibrin, collagen, zein, silk fibroin, keratin, casein and albumin are used as scaffolds. Proteins are one of the important candidates for tissue engineering materials based on their superior biocompatibility, biodegradation, bioreorbability, and so on limited only by their inferior mechanical properties [2]. Keratin scaffolds have been fabricated in the form of films, sponges and fibres. Keratin biomaterials possess many distinct advantages over conventional biomolecules, including a unique chemistry afforded by their high sulfur content, remarkable biocompatibility, propensity for self-assembly, and intrinsic cellular recognition [7].

| Protein           | Source                       | Form                  | Advantages                                                                 |
|-------------------|------------------------------|-----------------------|----------------------------------------------------------------------------|
| Fibrin            | Derived from blood           | Used as coatings and films | Promotes cell attachment and proliferation                                  |
| Collagen and Gelatin | Found in connective tissues, bones, skin etc | Membranes | Excellent biocompatibility, negligible immunogenicity, and high bio-absorbability |
| Zein              | Extracted from corn and maize | Coating               | Biocompatible with the endothelial cells of human umbilical veins, human liver cells and mice fibroblast cells |
| Silk fibroin      | Produced by spiders and insects | Films                 | Easy processing, impressive mechanical strength, environmental stability, biocompatibility |
| Keratin           | Found in skin, hair, nails, horns and hooves | Films and 3D porous constructs | Good biocompatibility and been used in tissue engineering, owing to its cell adhesion sequences, such as arginine-glycine-aspartic acid (RGD) and leucine-aspartic acid-valine (LDV) |
| Casein            | Extracted from milk          | Microspheres          | Inexpensive, readily available, non-toxic and highly stable                 |
| Albumin           | Found in egg white, blood serum and milk | Microspheres | Water-soluble                                                              |

Table 3 Adapted from [2]

Scaffold fabricated from keratin and agar showed good biocompatibility against the myofibroblast cell line (C2C12) and have promising characteristics for tissue engineering applications. Good degradability and biocompatibility properties, also favor its competent candidature to construct a porous scaffold for biomaterial application [25]. Chitosan can considerably modify the properties of keratin film when the biological or mechanical properties are considered [26].

Keratin film obtained by the compression molding of the S-sulfo keratin powder, extracted from wool supported attachment and proliferation of fibroblast cells indicating the biocompatibility of
keratin [27]. A compression-molding/particulate-leaching (CM/PL) method developed to fabricate the sponge scaffolds from S-sulfo keratin had high water tolerability and the pore size and porosity could be controlled [28]. Calcium phosphate composite keratin sponges made by two rapid fabrication methods mimic the matrix of gamma carboxyglutamicacid protein, which is responsible for osteoblast calcification and addition of the hydroxyapatite particle suspension suggested that both hybrid sponges, CaP-precipitated and trapped sponges, alter the differentiation pattern of preosteoblasts, MC3T3-E1 [29].

A new type of oxygen generating scaffold composed of human keratin, silk, gelatin and calcium peroxide (CPO) was fabricated by drying at room temperature for 24 hours, which enhanced the repair in dog urethral defect models, resulting in patent urethra [30].

Wool fibre which is similar to human hair was the starting material for experiments, both confirmed and expanded earlier findings that keratins demonstrate excellent compatibility in biological systems [6].

Fig. 4. Schematic of a wool fibre. Reproduced from [6]

12 Cell lines Caco-2, HaCaT, SIRC, CEPI, CEPI serum reduced, HENC, HCK, HUFIB, HCE-T, PHK, Calu-3, RPMI 2650 of human origin were investigated for their growth behaviour on coating of keratin extracted from human hair and compared with standard polystyrene substrate. It was found that the Keratin CLEAR coating best supports the attachment and proliferation of most cell types, and seems to offer advantages over the traditional polystyrene approach i.e. a low cost, standardized alternative to already known and frequently used coating materials, e.g., extracellular matrix like collagen or basement membrane components like fibronectin [31].
7. Conclusion:

The suitability of any biomaterial for any application in tissue engineering has to be studied thoroughly from the point of view of all the requirements of the scaffolds i.e. toxicity, biodegradability, porosity, difficulties in fabrication, tensile strength and the ease of its preparation into 3D constructs.

The process begins with the choice of a biomaterial that must be non-cytotoxic and biodegradable. The biomaterial must be biocompatible and allow cell adhesion. The design of scaffold must allow sufficient porosity in the scaffold for nutrient and waste transport. One must consider the difficulties in the fabrication of scaffolds using the chosen biomaterial. The scaffold should possess sufficient tensile strength for any practical use. Lastly, it is necessary the scaffold be, able to be produced in any shape so that it can be advantageous for its application as implants.

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