

Review

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

Aarti Baliga * and Shashikant Borkar

Textile Manufactures Department, Veermata Jijabai Technological Institute (VJTI), Matunga, Mumbai 400019, India; spborkar@vjti.org.in

* Correspondence: anbaliga@vjti.org.in; Tel.: +91-022-2419-8262

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

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 $\text{OH} > \text{CH}_3 > \text{NH}_2 = \text{COOH}$ [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	Maxilloinsectomy (Chitin sponge coated gauze) Skin and soft tissue defect	Nagashima et al. 1991 Maeda et al. 1992
Composite with NWF	Cow	Fetlock deformity, Capsuloplasty, Herniorrhaphy, Tendoplasty, Redressment of entropion	Okamoto et al. 1992 Minami et al. 1992
	Ring tailed Lemur	Skin defect of tail	Fukumoto et al. 1994

	Dog	Perineocele, Skin Defect, Prosthesis of Subcutaneous Tissue	Minami et al. 1994
	Cat	Skin Defect	Saito 1995
Powder	Dog	Contused Wound	Okamoto et al. 1993
	Cat	Bite Wound	Okamoto et al. 1993
	Human	Surgical wound, Slow healing surgical incision, Perineal wound, Excision of a keratosis, Ulcer, Trauma, Amputation	Balassa et al. 1978
Film	Human	Dermatomed wound, Fresh burn Artificial Skin	Kishimoto et al. 1985 Maeda et al. 1986, Yasuse et al. 1992, Oura et al. 1992, Yamamoto et al. 1990, Oshima et al. 1986

Table. 1 Applications of Chitin Biomaterials. (Reproduced from [18]).

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	

		coating Multilayer constrution		
Porous Membranes (2D)	Nanofibers	High porosity Mimic skin extracellular matrix	ESP of pure chitosan difficult	Coatings for a variety of scaffolds wound dressings skin substitutes

Table.2 Main advantages, disadvantages and applications of the Chitosan biomaterials presented. (Reproduced from [19]).

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].

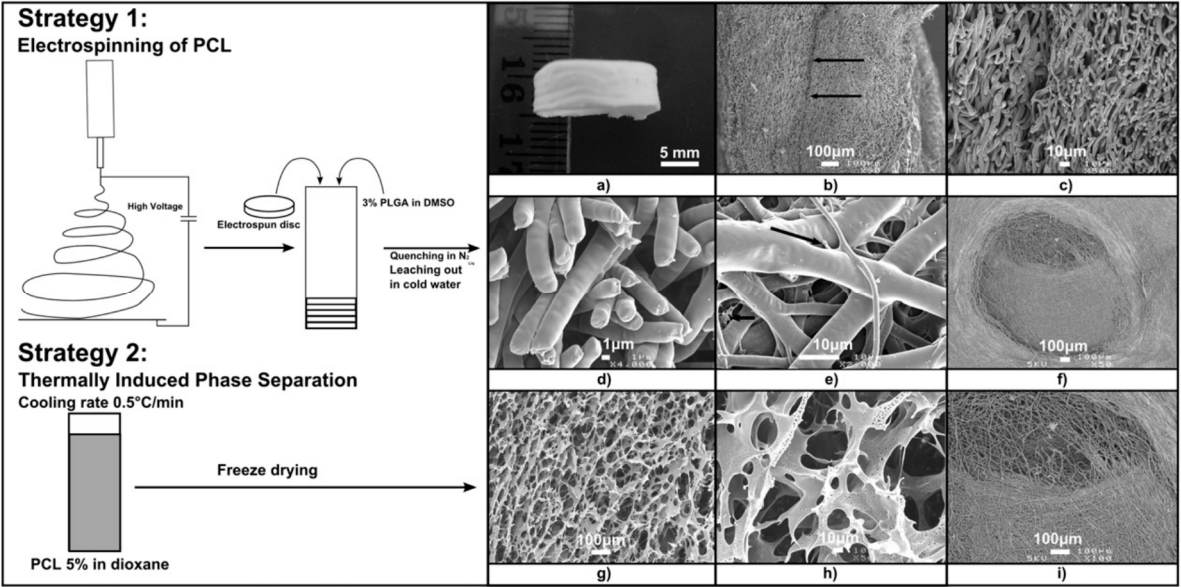


Fig.1. Scaffold fabrication strategies utilized (a) Morphology of the MC scaffold. (b-d) Scaffold cross-section showing that the TIPS treatment “glued” the layers together without filling the pores of the electrospun membranes. The black arrows show the connectivity at the interface between two adjacent electrospun membranes. (e) The surface of the MC scaffold, indicating that the pores were not filled with PLGA; the black arrows show some PLGA 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])

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].

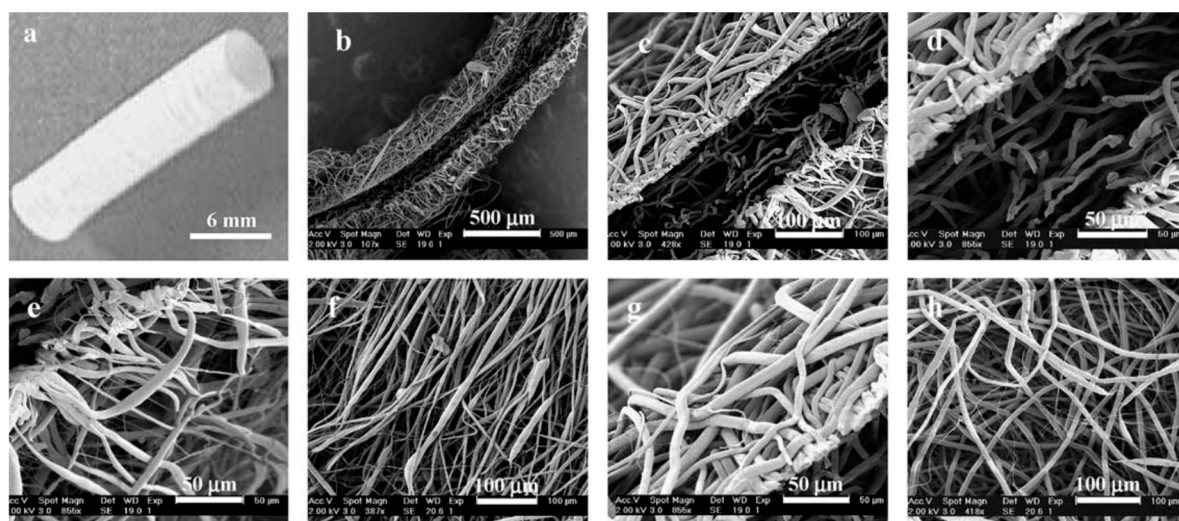


Fig.2. SEM micrographs of the bilayered tubular construct: (a) bilayered tube (entire view); (b) bilayered tube wall; (c)–(d) details of the interface (mixing zone) between inner and outer layers; (e)–(f) details of the outer layer (PLA); and (g)–(h) details of the inner layer (PCL). Reproduced from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

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

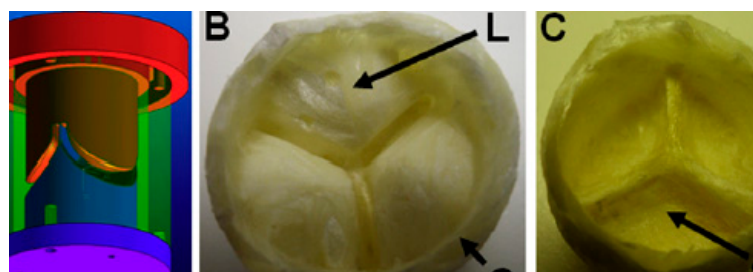


Fig.3. (A) Unigraphics® Image of trileaflet heart valve mold. The outer cylinder rendered transparent to allow visualization of interior features. (B) Ventricular view of porous chitosan trileaflet heart valve scaffold showing the leaflet (L) and the cylinder (C). (C) Aortic view of trileaflet heart 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, bioresorbability, 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 flms	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 carboxyglutamic acid 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].

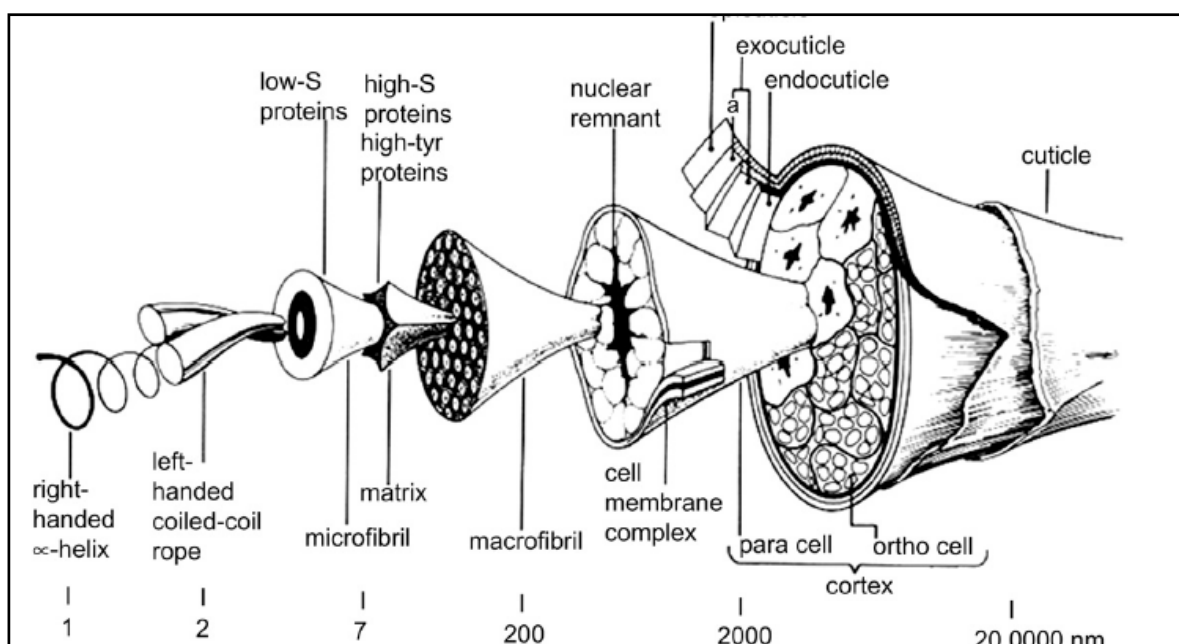


Fig. 4. Schematic of a wool fibre. Reproduced from [6]

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.

References

1. Peter X. Ma, Scaffolds for tissue fabrication, Materials Today, May 2004, ISSN: 1369 7021 © Elsevier Ltd 2004.
2. Hua-Jie Wang 1, Ling Di 1, Qiu-Shi Ren 1 and Jin-Ye Wang, Applications and Degradation of Proteins Used as Tissue Engineering Materials, Materials 2009, 2, 613-635; doi:10.3390/ma2020613.
3. William Bonfield, Designing porous scaffolds for tissue engineering, Phil. Trans. R. Soc. A 2006 364, doi: 10.1098/rsta.2005.1692, published 15January 2006.
4. Langer, R., and Vacanti, J. P., Science (1993) 260, 920.
5. Brahatheeswaran Dhandayuthapani, Yasuhiko Yoshida, ToruMaekawa, and D. Sakthi Kumar, Polymeric Scaffolds in Tissue Engineering Application: A Review, Hindawi Publishing Corporation, International Journal of Polymer Science, Volume 2011, Article ID 290602, 19 pages, doi:10.1155/2011/290602.
6. Paulina Hill, Helen Brantley, Mark Van Dyke, Some properties of keratin biomaterials: Kerateines, Biomaterials 31 (2010) 585–593.
7. Jillian G. Rouse and Mark E. Van Dyke, A Review of Keratin-Based Biomaterials for Biomedical Applications, Materials 2010, 3, 999-1014; doi:10.3390/ma3020999.
8. R. Langer and D. A. Tirrell, "Designing materials for biology and medicine," Nature, vol. 428, no. 6982, pp. 487–492, 2004.
9. Ghassem Amoabediny, Nasim Salehi-Nik, and Bentolhoda Heli, The Role of Biodegradable Engineered Scaffold in Tissue Engineering, Chapter 7, Biomaterials Science and Engineering, Edited by Rosario Pignatello, Published by InTech Janeza Trdine 9, 51000 Rijeka, Croatia.
10. L. Vikingsson, B.Claessens, J.A.Go´mez-Tejedor, G. GallegoFerrer, J.L.Go´mez Ribelles, Relationship between micro-porosity, water permeability and mechanical behaviour in scaffolds for cartilage engineering, Journal of the mechanical behavior of biomedical materials, 48 (2015), 60-69.
11. Bing Ma, Jingwei Xie , Jiang Jiang, Jun Wu, Sandwich-type fiber scaffolds with square arrayed microwells and nanostructured cues as microskin grafts for skin regeneration, Biomaterials 35 (2014) 630-641.
12. J.P. Gleeson, N.A. Plunkett, and F.J. O'Brien, Addition of Hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue engineering, European Cells and Materials, Vol 20, 2010, pages 218-230.
13. Ying Yuan, Peiyun Zhang, Yuming Yang, Xiaodong Wang, Xiaosong Gu, The interaction of Schwann cells with chitosan membranes and fibers in vitro, Biomaterials 25 (2004) 4273–4278.
14. Michael A. Lana,, Charles A. Gersbach, Kristin E. Michael, Benjamin G. Keselowsky, Andre´ s J. Garcí'a, Myoblast proliferation and differentiation on fibronectin-coated self-assembled monolayers presenting different surface chemistries, Biomaterials 26 (2005) 4523–4531.

15. Li-Chong Xu, Christopher A. Siedlecki, Effects of surface wettability and contact time on protein adhesion to biomaterial surfaces, *Biomaterials* 28 (2007) 3273–3283.
16. Overview of Biomaterials and Their Use in Medical Devices, Chapter 1, published by ASM International, Materials Park, Ohio, USA.
17. Fergal J. O'Brien, Biomaterials and scaffolds for tissue engineering, *materialstoday*, March 2011, Vol 14, number 3.
18. Yoshihiro Shigemasa, Saburo Minami, Applications of Chitin and Chitosan for Biomaterials, *Biotechnology and Genetic Engineering Reviews*, Vol 13, Dec 1995.
19. Florence Croisier, Christine Jérôme, *European Polymer Journal* 49 (2013) 780–792.
20. Cédryck Vaquette, Justin Cooper-White, A simple method for fabricating 3-D multilayered composite scaffolds, *Acta Biomaterialia* 9 (2013) 4599–4608.
21. Ming-Hua Ho, Pei-Yun Kuo, Hsyue-Jen Hsieh, Tzu-Yang Hsien, Lein-Tuan Hou, Juin-Yih Lai, Da-Ming Wang, Preparation of porous scaffolds by using freeze-extraction and freeze-gelation methods, *Biomaterials* 25 (2004) 129–138.
22. Min Wang, Developing bioactive composite materials for tissue replacement, *Biomaterials* 24 (2003) 2133–2151.
23. C.M. Vaz, S. van Tuijl, C.V.C. Bouten, F.P.T. Baaijens, Design of scaffolds for blood vessel tissue engineering using a multi-layering electrospinning technique, *Acta Biomaterialia* 1 (2005) 575–582.
24. Mohammad Z. Albanna, Therese H. Bou-Akl, Henry L. Walters III, Howard W.T. Matthew, Improving the mechanical properties of chitosan-based heart valve scaffolds using chitosan fibers, *Journal of mechanical behaviour of biomedical materials*, 5 (2012) 171–180.
25. Kush Kumar Nayak, Pratima Gupta, In vitro biocompatibility study of keratin/agar scaffold for tissue engineering, *International Journal of Biological Macromolecules* 81 (2015) 1–10.
26. Toshizumi Tanabe, Naoya Okitsu, Akira Tachibana, Kiyoshi Yamauchi, Preparation and characterization of keratin–chitosan composite film, *Biomaterials* 23 (2002) 817–825.
27. Kazunori Katoh, Mikio Shibayama, Toshizumi Tanabe, Kiyoshi Yamauchi, Preparation and physicochemical properties of compression-molded keratin films, *Biomaterials* 25 (2004) 2265–2272.
28. Kazunori Katoh, Toshizumi Tanabe, Kiyoshi Yamauchi, Novel approach to fabricate keratin sponge scaffolds with controlled pore size and porosity, *Biomaterials* 25 (2004) 4255–4262.
29. Akira Tachibana, Sumika Kaneko, Toshizumi Tanabe, Kiyoshi Yamauchi, Rapid fabrication of keratin–hydroxyapatite hybrid sponges toward osteoblast cultivation and differentiation, *Biomaterials* 26 (2005) 297–302.
30. XiangGuo Lv, Zhe Li, ShiYan Chen, MinKai Xie, JianWen Huang, XuFeng Peng, RanXing Yang, HuaPing Wang, YueMin Xu, Chao Feng, Structural and functional evaluation of oxygenating keratin/silk fibroin scaffold and initial assessment of their potential for urethral tissue engineering, *Biomaterials* 84 (2016) 99e110.
31. Stephan Reichl, Films based on human hair keratin as substrates for cell culture and tissue engineering, *Biomaterials* 30 (2009) 6854–6866.

