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ZPRACOVÁNÍ, GELACE A CHARAKTERIZACE ATELOKOLAGENU

ATELOCOLLAGEN PROCESSING, GELATION AND CHARACTERIZATION

POJEDNÁNÍ KE STÁTNÍ DOKTORSKÉ ZKOUŠCE
DOCTORAL THESIS TOPIC

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BRNO 2018

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INTRODUCTION

Healthiness is one of the most important things for most of the persons. Current modern drugs can solve many of health problems, but when larger defects occurs, body is not able to regenerate and transplant is necessary. Autologous (from patient). Allogenic (from donor) transplants have longest history. Autologous transplants are often used in the practice, but there is always additional stress to donor site of the body, which limits their usage. On the other hand allogenic transplants can help to save the lives since transplantation of whole organs is possible, but the main drawback is induction of foreign body reaction which must be suppressed by high amount of immunosuppressive drugs.

To overcome limitations of immunosuppressives, people started to develop new concepts of modified transplants. These approaches are deeply focused on mimicking of native Extra Cellular Matrix (ECM), which should allow body to penetrate to the engineered transplant and form new tissues.

Decellularized ECM is made by processing of donor tissue (allogenic, xenogenic). This processing should remove cells which inducing foreign body reaction. Decellularized ECM products for smaller defects repair are available from 80^{nd} years of the past century with good results. But there is always problem with larger tissues decellularization, because penetration of decellularization agents is greatly limited by diffusion limits of the tissues. In the recent years people try to decellularize whole organs with great improvements, but just in academic field. Even if they succeeded in-vitro, they still did not succeed in transplantation into living organism.

Tissue engineered scaffolds are based on bottom up process. They are more difficult to bring them to the market since the knowledge of humans to design this kind of transplant is still limited. Rapid growth of this market is observed in the two past decades. The delay against the Decellularized ECM is not just because of complicated development, but also due lack of adequate clinical trials rules, which are mostly designed for the drug safety verification.

There are two main classes of Tissue Engineered scaffolds native ECM and synthetic one. Synthetic materials already succeeded as fixative during surgeries (suture, spike, etc.) thanks to their great mechanical strength and variable degradation. But usage of them for soft tissue repair is very limited because of imperfect biocompatibility and serious problems with their degradation products.

Native ECM Tissue Engineered scaffolds are often made from collagen, because it is the most abundant protein in the body responsible of many mechanical functions over all the body. Because of his nature, collagen exhibits great biocompatibility and low risk of immune response. In recent decade limited number of Tissue Engineered scaffolds products from collagen were introduced to the market, mainly in the skin

repair field. Products like Integra and Matriderm helped to improve life quality of heavily burned patients. Other Products like Apligraf and Epicel are made from collagen gel seeded by allogenic cells. They already shown outstanding performance in handling of leg ulcers saving mostly the diabetic legs. The main drawback of this modern products is the cost.

There are two main classes of collagens used in scaffolds - Insoluble and soluble . Insoluble collagen is relatively cheap to prepare, but it contains large collagen fibers in scale of few hundreds of microns limiting the processing. Soluble collagen is much better processable, but also more expensive. It allows to create homogenous fibrillary networks, allowing well integration with the cells in-vitro, in-vivo. Main drawback of soluble collagen gels are their poor mechanical properties.

Bringing medical products to the market consist not just of material development, there is need to make detailed in-vitro (cells), in-vivo (animal) tests prior the testing on first human. European (GMP) and US (FDA) rules becoming to be more and more complicated so the investment in this field is extremely risky [24, 25]. On the other hand successful production in Tissue Engineering field could be extremely beneficial.

1 INTRODUCTION TO COLLAGEN

1.1 Collagen in the Body

Collagen has several functions in the body. The most important is the mechanical function, but the others like morphogenesis and repair are also important [26]. Collagen is present in many tissues in the body - skin, ligament, bone, cartilage, muscles, blood vessels, cornea and others. There are 28 known types of collagen forming different macromolecular architectures **Fig. 1.1**. It is known that collagen interacts with more than 50 ligands and proteoglycans [1].

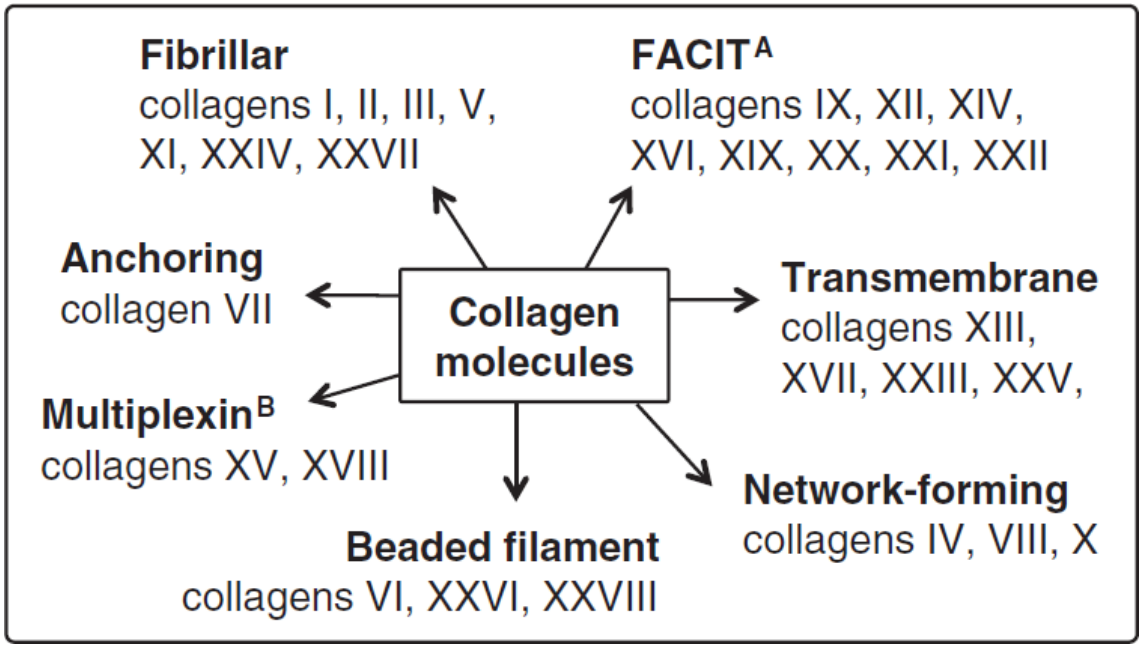

Fig. 1.1: Supramolecular assembly architectures of collagens I - XXVIII [1]

1.2 Insoluble Collagen

Insoluble collagen is made from chemically and physically processed tissue (bovine skin, etc.). Fats, some proteins, proteoglycans, cells etc. are removed during processing. Insoluble collagen contains highly cross-linked collagen fibril bundles mostly in micrometric scale. Possibly not just collagen can leave in resulting material but other ECM(Extra Cellular matrix) molecules (e.g. elastin for skin derived insoluble collagen) can be included. Diameter of fibril bundles is mostly dependent on source tissue. Length can be modified by physical processing (i.e. mixing/homogenizing). This type of collagen is not able to completely dissolve in acids or bases, but just swells into viscous suspension. Depending on processing, wide range of fibril sizes

	Type	Molecular formula	Polymerized form	Tissue distribution
Fibril-Forming (fibrillar)	I	$[\alpha1(I)]_2\alpha2(I)$	fibril	bone, skin, tendons, ligaments, cornea (represent 90% of total collagen of the human body)
	II	$[\alpha1(II)]_3$	fibril	cartilage, intervertebrate disc, notochord, vitreous humor in the eye
	III	$[\alpha1(III)]_3$	fibril	skin, blood vessels
	V	$[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha2(V)\alpha3(V)$	fibril (assemble with type I)	*idem* as type I
	XI	$\alpha1(XI)\alpha2(XI)\alpha3(XI)$	fibril (assemble with type II)	*idem* as type II
Fibril-associated	IX	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	lateral association with type II fibril	cartilage
	XII	$[\alpha1(XII)]_3$	lateral association with type I fibril	tendons, ligaments
Network-forming	IV	$[\alpha1(IV)]_2\alpha2(IV)$	Sheet-like network	basal lamina
	VII	$[\alpha1(VII)]_3$	anchoring fibrils	beneath stratified squamous epithelia

Fig. 1.2: Collagen types, forms and distribution in the body[2]

can be produced from sub-micrometer to few hundreds micrometers **Fig. ??**, **Fig. 1.3**.

1.3 Soluble Collagen

Soluble collagen is manufactured by various processes from various tissues(see isolation in 1.3.2 section). Unlike insoluble collagens (Chapter-1.2), soluble collagens are able to form solutions in acids,bases, or some organic solvents. Purity of soluble collagen is relatively high but perfect just for some scientific applications. Soluble collagen is able to form fibrous networks in-vivo and also in-vitro at neutral conditions [4] and mimics the native structure of various body tissues. Fibril formation and good processing capability of soluble collagen brings several advantages for TE. Limited mechanical properties are the main drawback of soluble collagen.

1.3.1 Soluble Collagen Types

1.3.1.1 Acid Soluble Collagen

Acid soluble collagen is obtained from various tissues by prolonged acid exposures. Bovine skin, achiles tendom, rat tails [27, 28], cartilage[29], fish scales [30, 31],

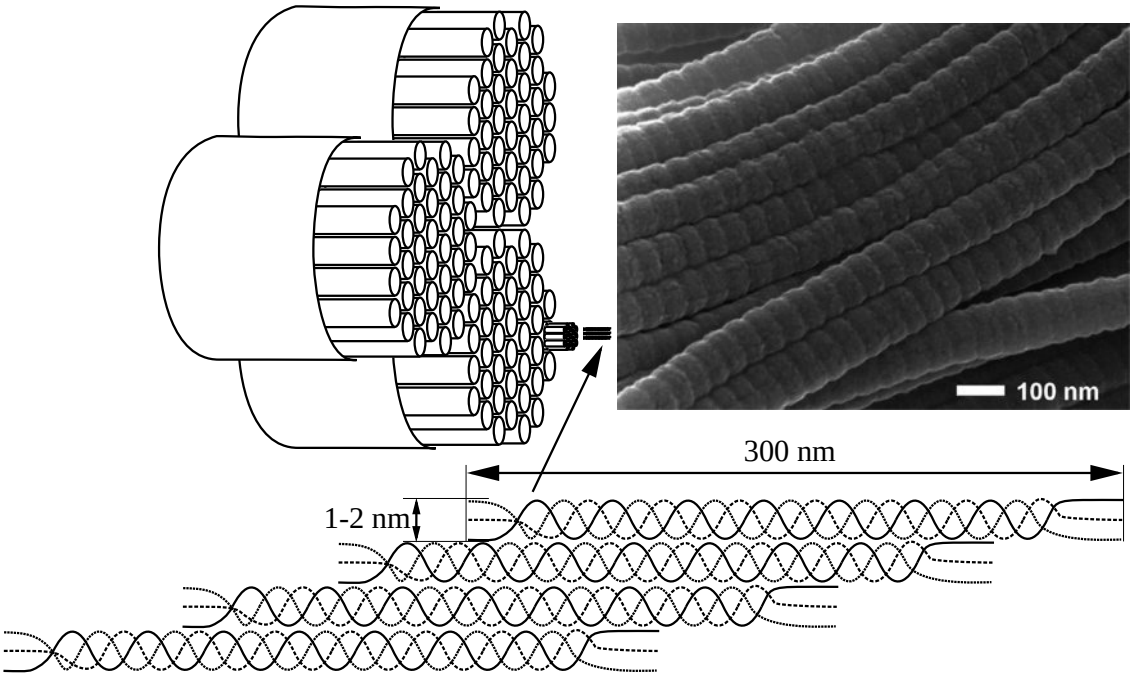

Fig. 1.3: Schematic view on collagen bundles. Upper right image showing collagen bundles visualized by Helium Ion Microscopy(Reprinted from [3])

fish skin [31, 32], fish bone [31] etc can be used to produce acid soluble collagen. By acid processing completely soluble material could be obtained but with limited yields. Resulting collagen molecules contain complete telopeptides [4]. Acid soluble collagen has higher risk to immune response, but his self-assembly and stability is very similar to atelocolagen [4]. This type of collagen is soluble in weak acids at pH less than 4 and in bases, urea or some organic solvents. Thermal stability of soluble collagen is much lower than insoluble collagen. Denaturation temperature for acid soluble collagen is dependent on animal origin. It could be near to 20-25 °C [31] for some cold sea fish collagens and 30-35 °C for most of mammalian collagens [33, 34].

1.3.1.2 Atelocollagen

Atelocollagen is also called Pepsin Soluble Collagen. It is obtained by processing tissue in acid in presence of pepsin. Various tissues are possible the use to atelocollagen extraction: skin[29, 35], aorta[29], muscle [29], human adipose tissue [33] and fish scales [30]. Telopeptides are partly cleaved by pepsin[4], **Fig. 1.4** so the molecule is slightly modified in comparison with acid soluble collagen. Resulting molecule showing slightly different electrophoresis results but thermal and self-assembly properties leaving almost unchanged **Fig. 1.4**. Atelocollagen is more commonly used thanks to shorter processing times, higher yields and lower immune response risk[4]. Usable solvents and thermal stability is similar to acid soluble collagen (Chapter 1.3.1.1).

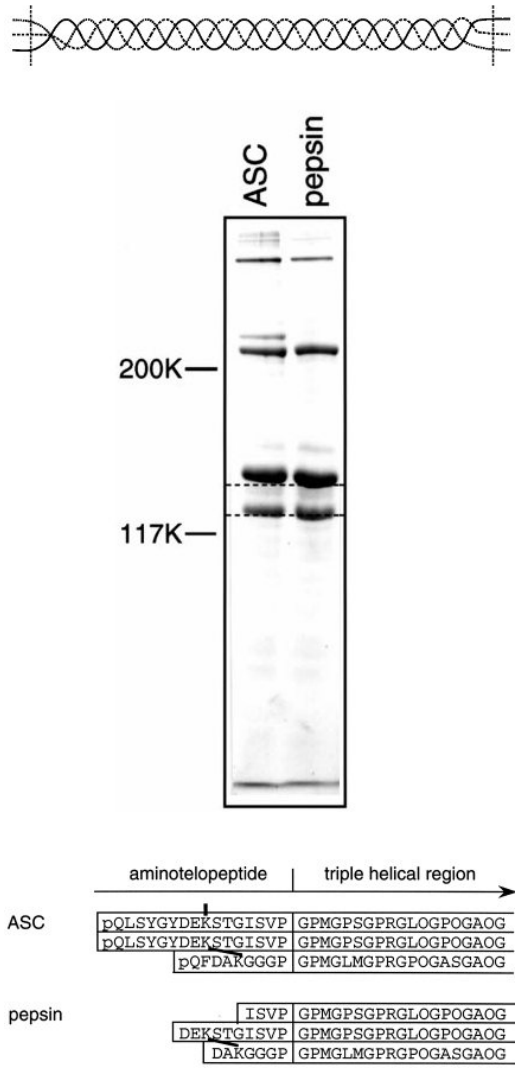

Fig. 1.4: Top - Schematic view of collagen molecule, Center - SDS-PAGE of Purified type I ASC (ASC), pepsin-treated ASC (PSC), Bottom - schematic view on ASC and PSC amino acid sequence cleavage sites. Reprinted from [4]

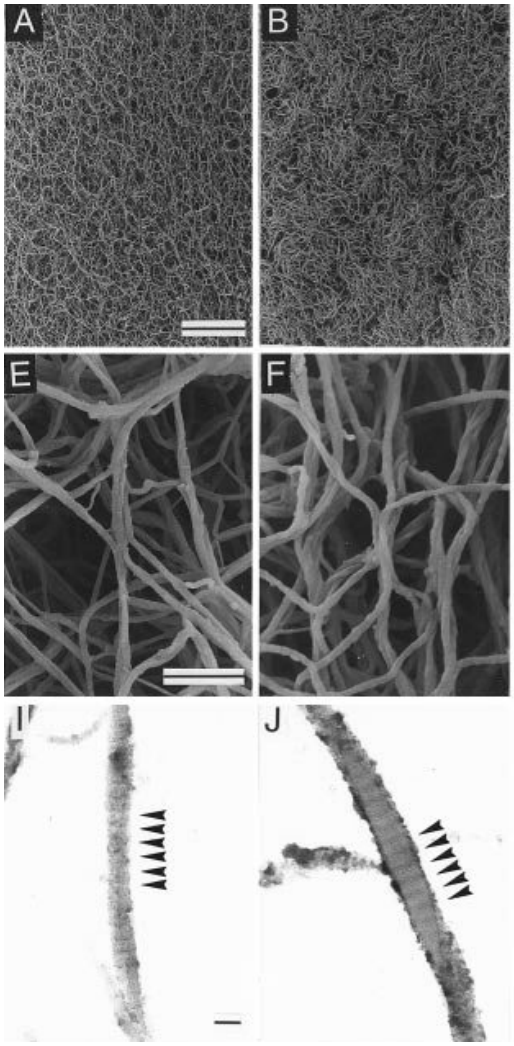

Fig. 1.5: Purified type I ASC (A, E, I), pepsin-treated ASC (B, F, J) gels . A, B (bar 20 μm), E, F (bar 1 μm) critical point dried gels visualized using SEM, I, J (bar 100 nm) fixed and negatively stained single collagen fibril visualized using TEM, reprinted from [4]

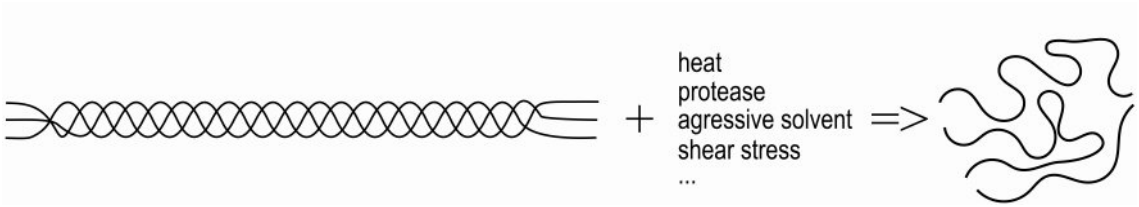

Fig. 1.6: Schematic conversion of collagen(left) to gelatine(right)

1.3.1.3 Gelatin

Gelatin is obtained by denatured collagen (see schematic view in **Fig. 1.6**). Denaturation can be done e.q. by heat, proteases, aggressive solvents, mechanical disruption. Gelatin is soluble at any pH and is not able to self-assemble into fibrous networks. Gelatin is able to form gel but at lower temperatures than physiological. This concludes necessary to chemically or physically crossling gelatin prior the use in-vitro or in-vivo, otherwise it melts and degrade rapidly. It can also happen that collagen can be completely or partially converted to gelatin during some processing steps. Electrophoresis 1.3.4.1, Circular Dichroism 1.3.4.2, Chromatographic techniques 1.3.4.7, Rheology 1.3.4.3, Differential Scanning Calorimetry 1.3.4.9, Assays and other techniques could be utilized to recognize collagen denaturation into gelatin.

1.3.2 Isolation

1.3.2.1 Quality control

First it is important to setup some methods which are able to recognize quality of freshly isolated material and compare it with commercial/clinical quality products. Collagen isolated for usage in Tissue Engineered Medical Products collagen I product should be manufactured according to recommendation guide [5]. This document suggesting to use many characterization methods(Concentration assays, Amino acid analysis, SDS-PAGE, Elastin assay, Peptide mapping, Elisa, Western blot, Cytotoxicity, Heavy metal content, Microbial profile, Carbohydrate analysis, Trypsin reaction analysis, DSC, TEM, other than target collagen type analysis, Endotoxin analysis, Lipid analysis, ..) to be absolutely sure of quality of collagen. Some of those methods are listed in characterization (1.3.4) section.
This list of those methods seems to be too wide for many researchers and unfortunately most articles use just a few methods [27, 28, 29, 30, 32, 4, 33, 34, 35] to describe atelocollagen quality. The quality of yielded material is then not clear, variant, with insoluble content. Material and biological response of those material will be then of course also variant. This keeps many of extraction processes closed

Characterization Method	Applicable to
Chemical	
Appearance	Soluble or Insoluble
Concentration	Soluble or Insoluble
Purity	Soluble or Insoluble
Amino acid analysis	Soluble or Insoluble
Peptide mapping	Soluble or Insoluble
Impurities profile, includes Heavy Metal Analysis	Soluble or Insoluble
Carbohydrate analysis	Soluble or Insoluble
Trypsin resistance	Soluble or Insoluble, Mainly Insoluble
Collagenase resistance	Soluble or Insoluble, Mainly Insoluble
pH of implantable	Soluble or Insoluble
Additives (cross-linkers, lubricants, drugs, sterilents)	Soluble or Insoluble
Physical	
Shrink Temperature (DSC)	Insoluble
Viscosity	Mainly soluble
TEM	Insoluble
SDS-PAGE	Soluble or Insoluble
Moisture Content (5 to 20 %), dependent on storage environment	Insoluble
Electron Micrograph (native banded 640 Å structure for fibrils)	Insoluble
Biochemical	
Endotoxin level	Soluble or Insoluble
Bioburden	Soluble or Insoluble
% Type I collagen/Total Protein	Soluble or Insoluble
% Other Types Collagen and List of which Types present	Soluble or Insoluble
Total DNA (ppm or %)	Soluble or Insoluble
Total Lipid	Soluble or Insoluble
% native collagen (by trypsin resistance, circular Dichroism)	Soluble or Insoluble

Abbreviation in Table:
DSC = Differential Scanning Calorimetry
TEM = Transmission Electron Microscopy
SDS-PAGE = Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Fig. 1.7: Characterization methods for Collagen type I, reprinted from [5]

in single lab, limiting scientific results and limiting potential of use in medical products.

For early stages of atelocollagen isolation seems to be very important to maintain well quality of filtration. Rheometer with microscopy should be robust tools to detect large particles before and after filtration and detect non molecular content in the freshly isolated material. Only well filtrated material is compatible with other characterization methods. Insufficient filtration leads to unexpected errors for many characterization methods. Well filtrated material should be subjected to SDS-PAGE or CD spectroscopy to detect collagen/gelatin content.

Methods listed above [5] should be used in later stages of isolation to ensure the quality from many points of view.

1.3.2.2 Pre-treatment

This work will use insoluble collagen as starting material for extraction. So no pre-treatment will be used.
If researchers starting they normally use NaOH,ethanol, NaCL to remove lipids and some other proteins [35, 33, 31, 36], but this is not topic of this work.

1.3.2.3 Extraction

Extraction is usually done by 0.5 M AA [27, 28, 35, 36, 4] for more than three days yielding Acid Soluble Collagen. Second common option is 0.5 M AA with pepsin extraction [33, 4, 29, 35, 30] for 2-3 days yielding pepsin soluble collagen(atelocollagen). Before extraction at least lipids must be removed from starting material. Material should be also well homogenized to sizes like few hundred micrometres or less. Otherwise used chemistry will be limited by diffusion.
Disassembling collagen bundles in AA pepsin buffer are visualized by microscopy **Fig. ??**. White arrows indicate untouched insoluble collagen bundles, black arrows indicating disassembling collagen bundles. The smallest visible fibres are still not single collagen molecules but still smaller bundles. Single collagen molecules(2nm thick, 300 nm diameter) are probably not clearly visible using light microscopy since they are much smaller than visible light wavelengths(300 - 700 nm).
Stirring is important variable influencing extraction dynamics. Because of possible shear stress denaturation of collagen triple helix stirring speed is somehow limited, precise number is unknown and should be experimentally set.

1.3.2.4 Filtration

Various filters could be used for collagen filtration after extraction (clarification). The final filtration must be equivalent to at least 0.45 µm filter otherwise the resulting solution contain aggregates of few collagen molecules and properties will be changed. **Fig. ??** shows example of various filtration to sample viscosity. Commercial collagen with 0.4 µm depth filter have lowest viscosities, common speed centrifuge (15 000 g) have significant higher viscosity and glass filter G3 (pore size 40-100 µm) with filter paper grade 390 shown highest viscosities.
If filters are used for clarification of collagen, resistance to acid should be carefully checked. If higher volume of collagen needs to be filtrated, filters with descending pore sizes should be used prior to use final 0.4 µmembrane. Direct filtration by membrane will result in their block after few ml. The depth filters could be used directly.
Researchers often use centrifuge because of their availability in most of the labs.

General use centrifuges (reaching 15 000 - 20 000 g) are used in many articles [35, 31, 33], but probably this kind of clarification does not lead to perfect results Figure **??**. High speed centrifuge (40 000 g and higher) should be used [29, 36] to improve filtration efficiency. In case of imperfect clarification by high speed centrifuge filters or precipitation in 0.3 M sodium chloride [36] with subsequent centrifugation could be used to improve final purity.

1.3.2.5 Purification

Final purification step consist of selective removal of smaller molecules than collagen (gelatine, elastin peptides, etc.). Sodium chloride is normally used for selective precipitation of collagen. Researchers uses various concentrations of sodium chloride 2.5 M [35], 0.9 M [33], 0.6 M [36] to precipitate collagen. As our best knowledge 0.6 - 0.7 M sodium chloride should be enough to fully precipitate collagen, higher sodium chloride concentration used could lead to co-precipitation of smaller molecules than collagen and resulting impurities. Precipitated collagen is normally removed by low speed centrifuge (3 000 - 5000 g), the supernatant is removed and the precipitate redissolved (mostly in 0.5 M AA). Subsequent dialysis(15 - 30 kDa) for 3 days with change of water each day is normally used to remove sodium chloride. Many researchers use lyophilisation to preserve collagen for extended time periods. For that purpose samples should be frozen at least at -45 °C to insure sample is completely frozen [37]. Adequate pressure (0.1 - 0.3 of vapour pressure of -45 °C ice) should be used to ensure safe (possible shear stress denaturation) and fast lyophilisation [38].

1.3.3 Fibrilogenesis

Collagen in the body is formed from tropocollagen (collagen precursor) molecules bundled together into fibrils. Detailed view of production and deposition of collagen fibrils by cells is described by Hulmes [39].On the smallest level of organization tropocollagen molecules forming parallel fibrils which are shifted each to other a bit less than one quarter. A bit less means that there is a gap 67 nm each fifth tropocollagen molecule as shown on **Fig. 1.8 top-right**. The gap in the collagen bundle results in the bands in the transversal direction of the collagen fibril, well observable on TEM (1.14), AFM (1.8) and recently on Helium Ion Electron microscopy(1.3). It is believed that the 67 nm gap is related to maximum interaction between charged chains hydrophobic acid of adjacent triple helices [11]. This phenomenon is commonly called “Quarter staggered stacking model”, because of the approx. quarter shift of the molecules.
The fibrils ranging from 10 to 300 nm could be interconnected with other fibrils

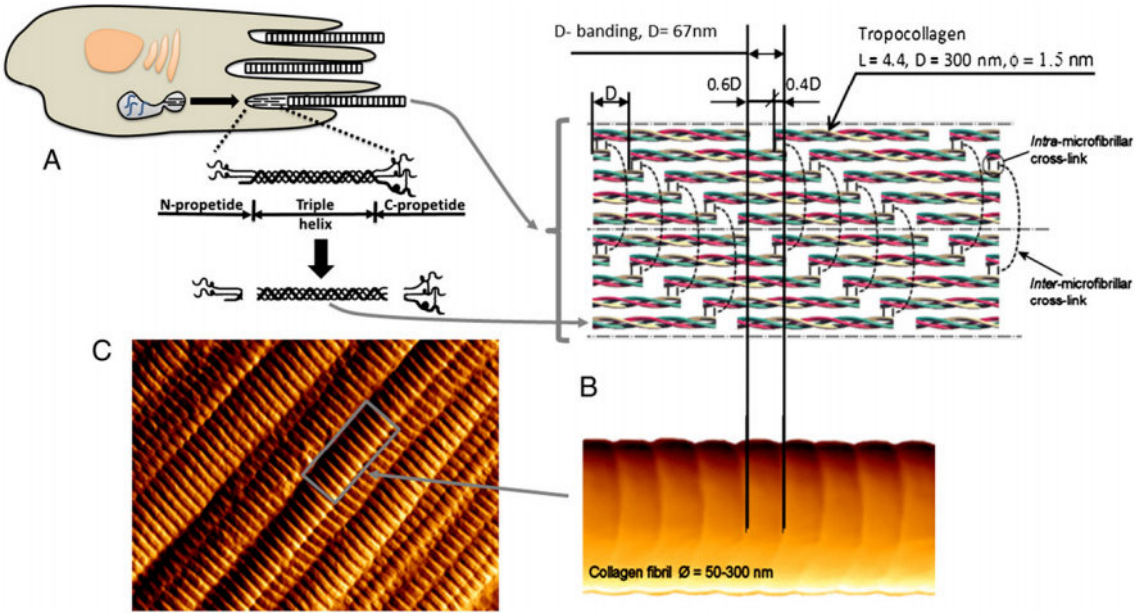

Fig. 1.8: Micro and ultra-structure of collagen. (A) Secretion of "procollagen" from osteoblast into extracellular matrix. "Procollagen" is then converted into "tropocollagen" by specific proteases that cleave the N- and C-propeptides; "tropocollagen" has the ability to spontaneously assemble into fibrils. (B) Collagen triple helix (right-handed coil of three left-handed polypeptide helices). Immature, intra-microfibrillar cross-links bond two collagen molecules head-to-tail; mature, inter-microfibrillar cross-links however bond two immature cross-links from adjacent microfibrils. "Quarter staggered stacking model" of a collagen fibril where five "tropocollagen" molecules are staggered side-by-side with an offset of D=67 nm with gap of 0.6D and overlap of 0.4D regions appear. (C) AFM image of aligned collagen fibrils in-situ of a tendon, displaying the very characteristic D-banding fibrillar annular periodicity.(Reprinted from [6])

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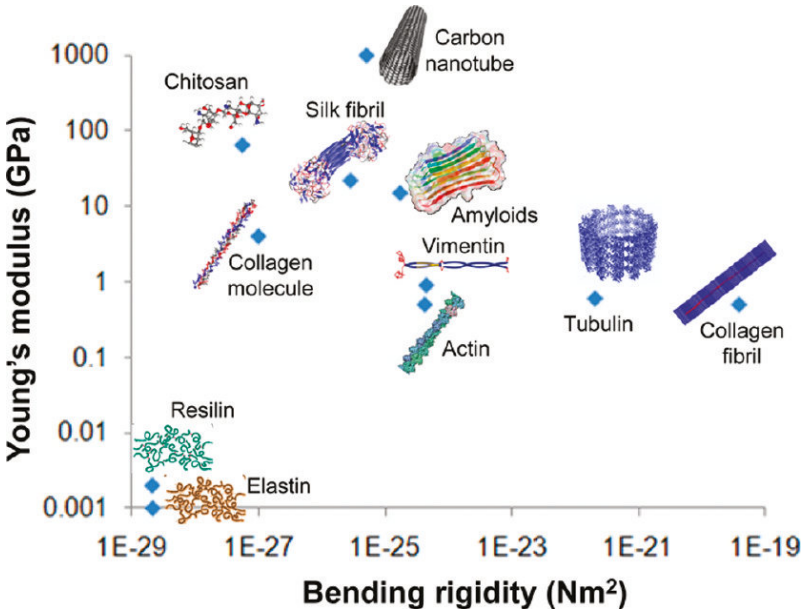

Fig. 1.9: Mechanical properties of various materials at nanoscale. Thanks to well packing collagen fibril present much higher bending rigidity than single collagen molecule with small drop of young moduli [7].

forming larger fibers of size 0.5 µm to 3 µm [2].
During experiments on single collagen molecule [8] researchers found, that there are four regimes of longitudinal molecule deformation: Decoiling up to 17% of deformation, stretching of covalent bonds up to 33%, molecular fracture due to breaking peptide bonds up to 50%, flowed by rapid decay of the force [8], **Fig. 1.10**.
Since collagen fibrils are well packed together, they have much higher bending rigidity and keeping the young moduli almost unchanged [7] **see Fig. 1.9**.
It is possible to induce fibrillogenesis also in-vitro [9, 15] at conditions near to physiological usually pH near to physiological, 37 °C, ionic strength. There is usually two phases of collagen gelation in-vitro lag (nucleation) phase and growth(exponential) phase[40, 41]. But fibrilogenesis also occur at different conditions Gobeaux et al. show fibril formation at acidic, low ionic strength in highly concentrated collagen solutions [11] **Fig. 1.13**, **Fig. 1.20**. Christiansen et al. [10] demonstrated fibril formation at pH range 5.5-8.5 and temperatures 25-37 °C and manufactured extruded fibers whose shown better mechanical properties for fibers formed at lower pH 1.3.4.4. This effect seems to be connected with prolonged lag phase and creation of larger nucleation sites and consequent larger fibrils [41].

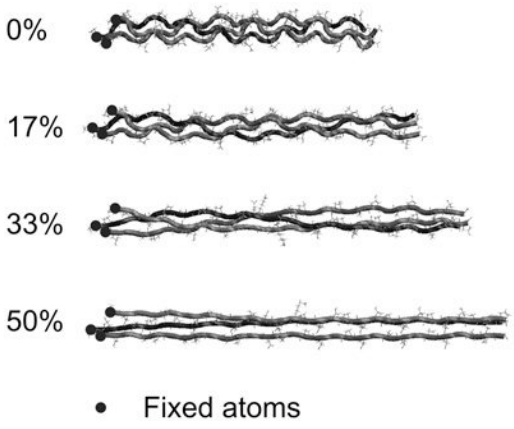

Fig. 1.10: Longitual deformation of single collagen molecule [8]

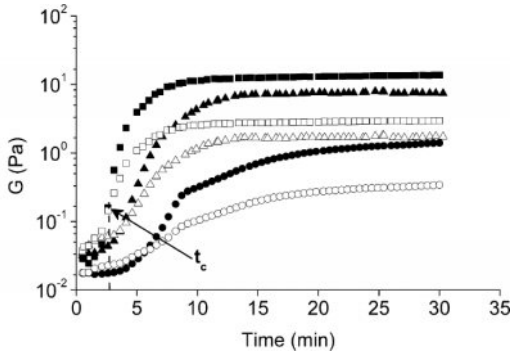

Fig. 1.11: Collagen gel formation at 37 °C evaluated by oscilatory rheology at 1Hz. Solid symbols indicate storage modulus G', and open symbols indicate loss modulus G". Collagen concentrations are 0.5 (circles), 1.0 (triangles), and 1.5 mg/mL (squares) [9].

1.3.4 Characterization

1.3.4.1 Electrophoresis

Electrophoresis is often useful during development of soluble collagen isolation protocols [33, 30, 31] or analysis of denaturation/changes in collagen solutions [4]. There are two main types of electrophoresis denaturalize and native:

- Denaturalize method originally introduced by Laemmli [42] is frequently used because of possibility of usage of molecular weight standards, but utilizes denaturalize conditions, so only disrupted (linearized) collagen fragments could be observed instead of complete collagen molecule. Then the real quantification of collagen is questionable.
- Native electrophoresis is also possible [4] (see **Fig. 1.4**). Molecular standard is not usable because of different electric and gel permeation properties of different molecules under native conditions (speed of native proteins in electric field is not just proportional to their size). Molecules are separated in native state so their analysis is much more quantitative, but their identification is more difficult. It also possible to cut the gels for later analysis as preparative electrophoresis.

1.3.4.2 Circular Dichroism

Circular dichroism(CD) is able to detect some properties of secondary structure of biomolecules thanks to their chirality. CD is often used for analysis of collagen thanks to its triple-helix secondary structure [4, 33, 12]. Because gelatin loses

triple-helix structure CD is able to sensitively detect difference between collagen and gelatin. It's known that CD signal at 221.5 nm is linearly proportional to collagen content **Fig. ??**. Gelatin signal at the same wavelength is always very close to zero (**Fig. ??**). Today's common CD spectrophotometers measures also UV signal (precision can be limited because of some calculations), many accessories are also available (peltier temperature control, fluorescence detector, titration equipment, stopped flow equipment, etc.). As for many other (spectroscopic) methods measured sample must be pure solution, otherwise researcher is risking of significant errors. Kelly et al. wrote recommendation review how to use CD properly [43].

1.3.4.3 Rheology

Flow rheology measurements are often used for determination of denaturation temperature and filtration quality (**??**) of isolated collagen [30, 31]. Also detailed analysis of the collagen solution possible [36]. Other researchers use oscillatory rheology measurements to observe gel formation and mechanical properties of collagen gels [9] (Fig. 1.11).

1.3.4.4 Stress-strain mechanical testing

Stress strain mechanical testing is useful tool for characterizing fibers made by extrusion of collagen into bath [10] Fig. **??**, lyophilized collagen sponges [44] or composites with other biopolymer [45]. Collagen gels are usually tested on rheometer.

1.3.4.5 Scanning Electron Microscopy

Scanning Electron Microscopy(SEM) is powerful imaging tool often used by material engineers. Resolution of this modality could be less than 10 nm. Samples for SEM must be dry, so biological samples must be dried prior to observing in microscope:

- **Freeze drying** is the simplest way to dry biological samples. Samples are normally frozen in liquid nitrogen and then dried in freeze dryer. Method suffers of several artefacts which are connected with crystallization of water during freezing [46, 12], so only very thin samples are recommended for this method. Some collagen products are directly produced by freeze drying method (collagen sponges), so they are ready to use in SEM.
- **Critical Point Drying(CPD)** is the most common way to dry biological samples. Thanks to negligible surface tensions of supercritical CO_2 liquid, drying of biological samples is possible without dramatic loose of their shape (still shrinkage up to 25% is possible). Glutaraldehyde is basically used for (pre)fixation, but another fixatives(like osmium tetroxide) have to be used to

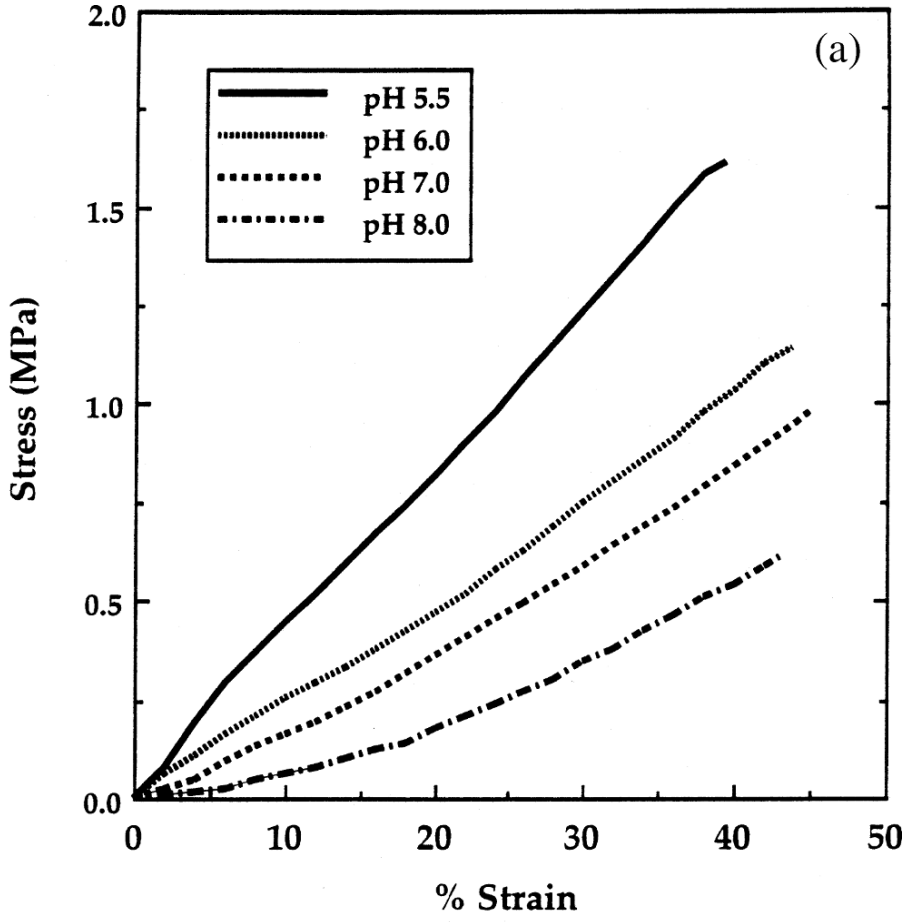

Fig. 1.12: Extruded collagen fibers shown different mechanical properties when formed at different pH [10].

obtain better (post)fixation [4, 9, 11] see **Fig. 1.5**. **Fig 1.13** show typical micrographs of if in-vitro formed gels.

- **Cryo SEM** is rare method for observing biological samples due its expensiveness.

1.3.4.6 Transmission Electron Microscopy

Transmission electron microscopy allows to visualize single collagen fibril or bundles and observe quarter staggered structure (**Fig. 1.5** bottom, **Fig. 1.14**, **Fig. 1.15**) [12, 4]. This is unexceptionally useful to confirm collagen ability to self-assemble into collagen fibrils under various chemical, physical and processing conditions. Thus scientists use TEM for observing collagen fibrils in gels [4], during cell culture [47], to verify recombinant collagen self-assembly ability [48].

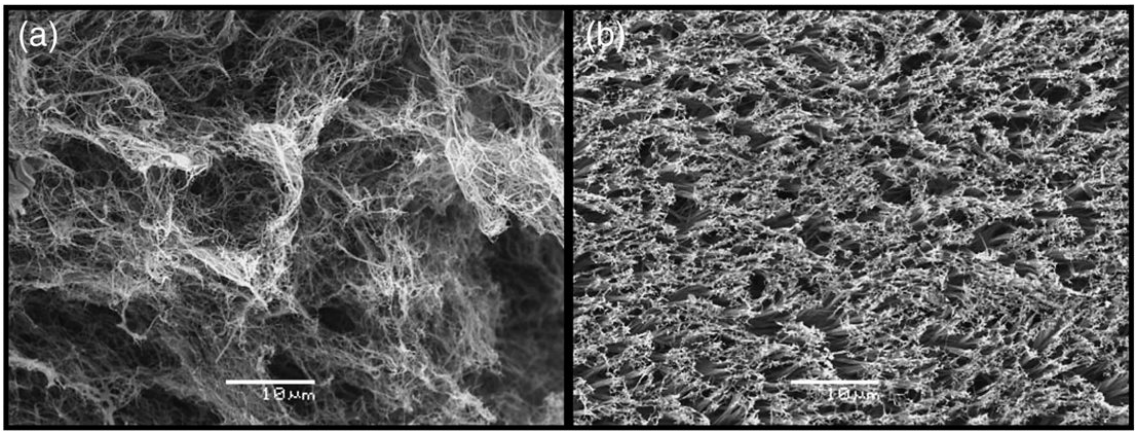

Fig. 1.13: Scanning electron micrographs of fibrillar gels: (a) 30 mg/ml, (b) 80 mg/ml.(Reprinted from [11])

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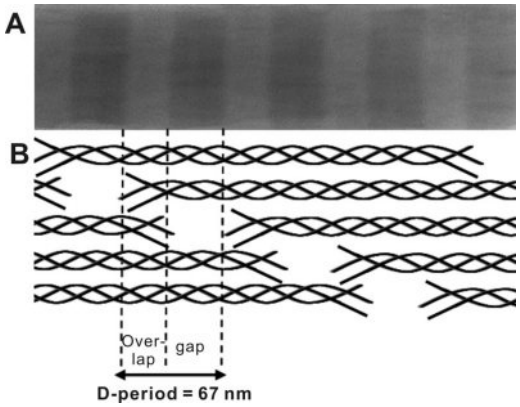

Fig. 1.14: Negativly stained single collagen fibril observed in TEM (reprinted from [12])

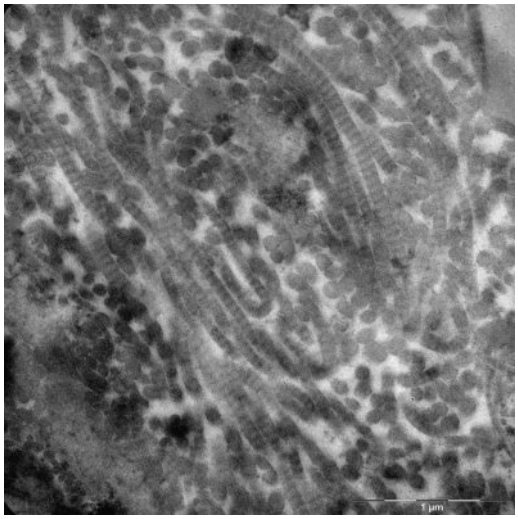

Fig. 1.15: Freeze dried adipose tissue collagen fibers observed by negatively stained TEM

1.3.4.7 Chromatography

Gel Permeation Chromatography(GPC) was previously used for separation of collagen in native state [29, 27, 28], in denatured state [30], because of avoid of column-protein interactions at acidic pH. Some special charged columns could be used to make

High Pressure Liquid Chromatography(HPLC) and Field Flow Fractionation are alternatives for separation.

Usable detectors are UV, refractive index, single angle light scattering, multi angle light scattering.

1.3.4.8 Atomic Force Microscopy

Atomic force microscopy is common tool to observe collagen at almost angstrom resolution with ability to see quarter staggered structure[12, 46, 6] fig 1.8. Collagen samples are normally observed in dry state, but wet state is also possible, but difficult with limited resolution and many possible artefacts. AFM can be also used for mechanical testing of single collagen fibril [49, 12].

1.3.4.9 Differential Scanning Calorimetry

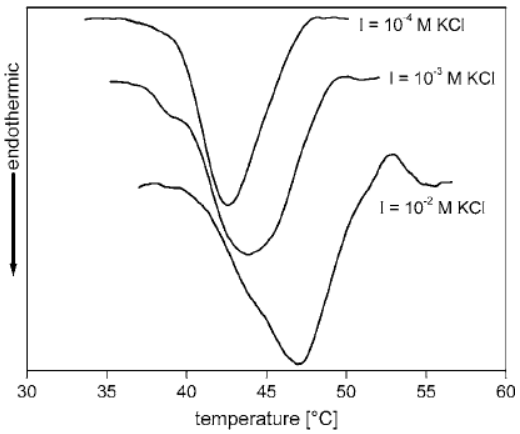

Fig. 1.16: DSC measuremnt shown increased stability of collagen solution in presence of KCL [13].

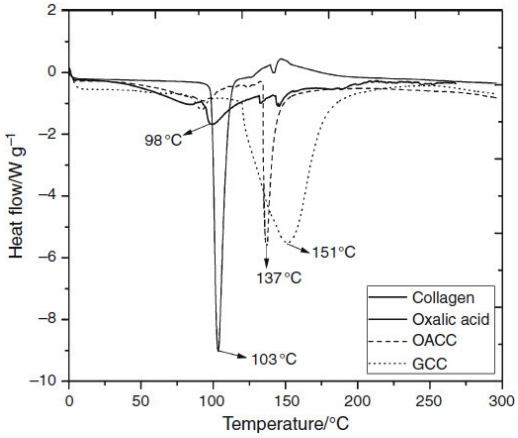

Fig. 1.17: Increased thermal stability of collagen membranes in presence of crosslinkers. Oxalic acid cross-linked collagen (OACC), and glutaraldehyde (GCC) [14].

Differential Scanning Calorimetry(DSC) is useful tool to evaluate collagen stability in the solution and also in solid state. DSC could be applied to evaluate quality of collagen when isolated from tissues[30], observe denaturation temperature change in presence of molecules in the solution [13] (**Fig. 1.16**), or observe effect of crosslinkers to thermal stability of collagen products [14].

1.3.4.10 Confocal Reflectance Microscopy

In recent years Confocal Reflectance Microscopy is used to visualize collagen gels in-vitro[9, 15]. Advantage of this method is simplicity of usage,no need of complicated sample preparation and possibility to measure in wet state. So for example fibrilogenesis could be observed continuously **Fig. 1.18**. Limiting factor is resolution of Confocal Laser Microscope. All imaging systems are limited by Raileigh Resolution Limit, so for visible light laser the resolution varies from 300-500 nm. In consequence collagen fibers of sizes 50-500 nm will be imagined nonlinearly. 500 nm

fibers with high contrast, 300 nm fibers with much lower contrast and 50 nm fibers are invisible.

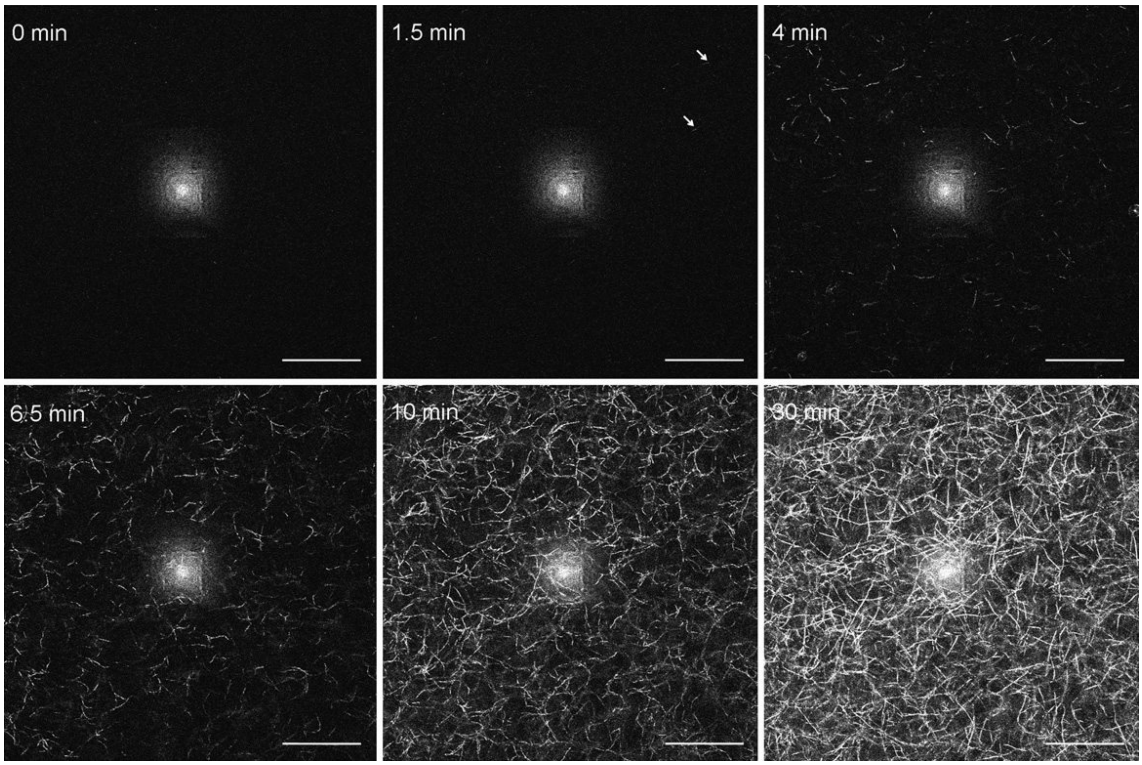

Fig. 1.18: Collagen 1 mg/ml during gelation at 37 °C., observed by confocal reflectance microscopy(Reprinted from [15])

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1.3.4.11 SAXS, WAXS

SAXS (Small Angle X-ray Scattering) could be used to quantify total amount of quarter staggered structure within the sample [11]. This method measures the scattering pattern within whole sample, which brings big advantage against microscopy methods SEM(1.3.4.5), TEM(1.3.4.6) and Confocal Reflectance Microscopy (1.3.4.10). Calculation of fiber orientation is described by Burger et al. [50].

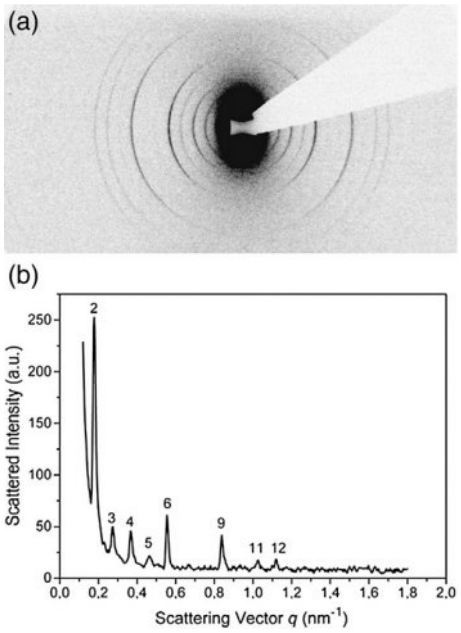

Fig. 1.19: (a) SAXS pattern of a 145-mg/ml fibrillar gel and (b) the corresponding linear plot of the scattered intensity I as a function of the scattering vector modulus q along the axial (equatorial) direction. The peaks labeled 2â€“ 12 correspond to the reflections arising from the staggered stacking of the collagen molecules in the fibrils (67-nm period). (reprinted from [11])

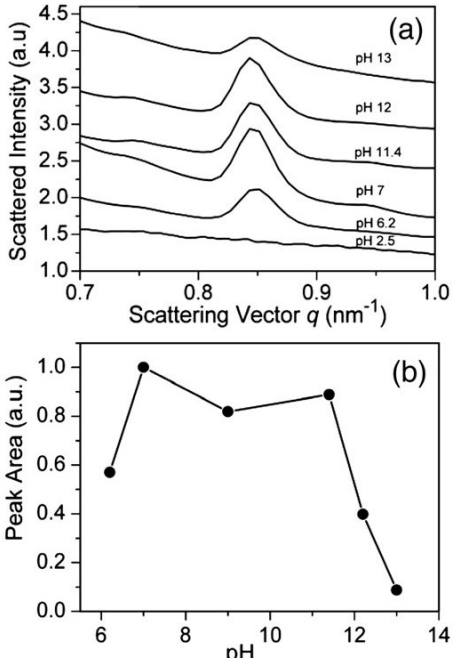

Fig. 1.20: SAXS detected quarter staggered structure(67 nm periodicity) of collagen samples at different pH (reprinted from [11])

2 TISSUE ENGINEERING OVERVIEW

2.1 Types of Transplants

This section describes some main types of transplants currently used in surgery and some types of transplants currently in the development

2.1.1 Tissue Transplants

2.1.1.1 Characteristic

Tissue transplants are autologous (from patient), allogenic (from donor) or xenogenic (animals) those are currently widely used in various surgery segments.

- **Autologous** transplants are removed from one part of body and used on another part of body of same patient. This technique is widely used in burn surgery, because currently there is no alternative to maintain full thickness burns. But usage is limited when burn coverage is higher than 40% of patients body and risk of scarring on the donor sites. In some countries (also in the Czech republic) are autologous transplants also used in Orthopedy surgery(various tendons) with good clinical results, but this techniques causes stress on the donor part of body.
- **Allogenic** transplants are harvested mainly from cadaveric donors. Bone, ligament, skin, cartilage, corneas and amniotic membranes are clinically used in tissue banks. Internal organs like heart, liver, kidney or whole part of bodies are transplanted immediately after harvesting from donor body (see solid organ section 2.1.2). After transplantation of allogenic tissue is normally necessary to use immunosuppression. In some cases just for limited time in the others permanent immunosuppression is necessary causing several complications [51, 52]. Some of these transplants are immunoprivileged(cornea, amniotic membranes) so the immunosurpession is not necessary [53]. Main complications of this transplants are associated with foreign body reaction. Despite this they are widely used because other there is no other possibility. It is also necessary screening of donors to prevent diseases transmission.
- **Xenogenic** transplants are made from animal bodies. They are often chemically modified to modulate immune response[54].

2.1.1.2 Benefits

- For long time best option for many types of operations
- Possibly autologous

- Long term results thanks to complete transplant replacement by recipient
- Price

2.1.1.3 Limitations

- Risk of rejection - need to immunosuppression
- Limited donor counts
- Risk of diseases transmission
- Transplant to transplant variation

2.1.2 Solid Organ Transplants

2.1.2.1 Characteristic

First attempts in the field of solid organ transplantations started at beginning of 19th century. While in the 1920s researchers found, that early transplant failures are associated with acute rejection by immune system [55]. This starts large research in the development of immunosuppressives, they block acute foreign body response [51, 52]. Immunosuppressives could modulate acute immune response but their long term side effects results to shorter life expectancy of recipients.

2.1.2.2 Benefits

- Currently only whole organ transplantation option
- Saves the lives

2.1.2.3 Limitations

- Long or short term need to immunosuppression which tends many side effects
- Limited donor numbers, waiting lists
- Risk of diseases transmission
- Development of various transplant pathologies
- Transplant to transplant variation

2.1.3 Decellularized ECM

2.1.3.1 Characteristic

At the second half of 20th century researches started to remove cells from organ using wide range of reagents, targeting to keep ECM (Extra Cellular matrix) and remove immunologically active cells, to prevent foreign body reaction. Today the process is called as decellularization process. Some of decellularized animal skins[54, 56],

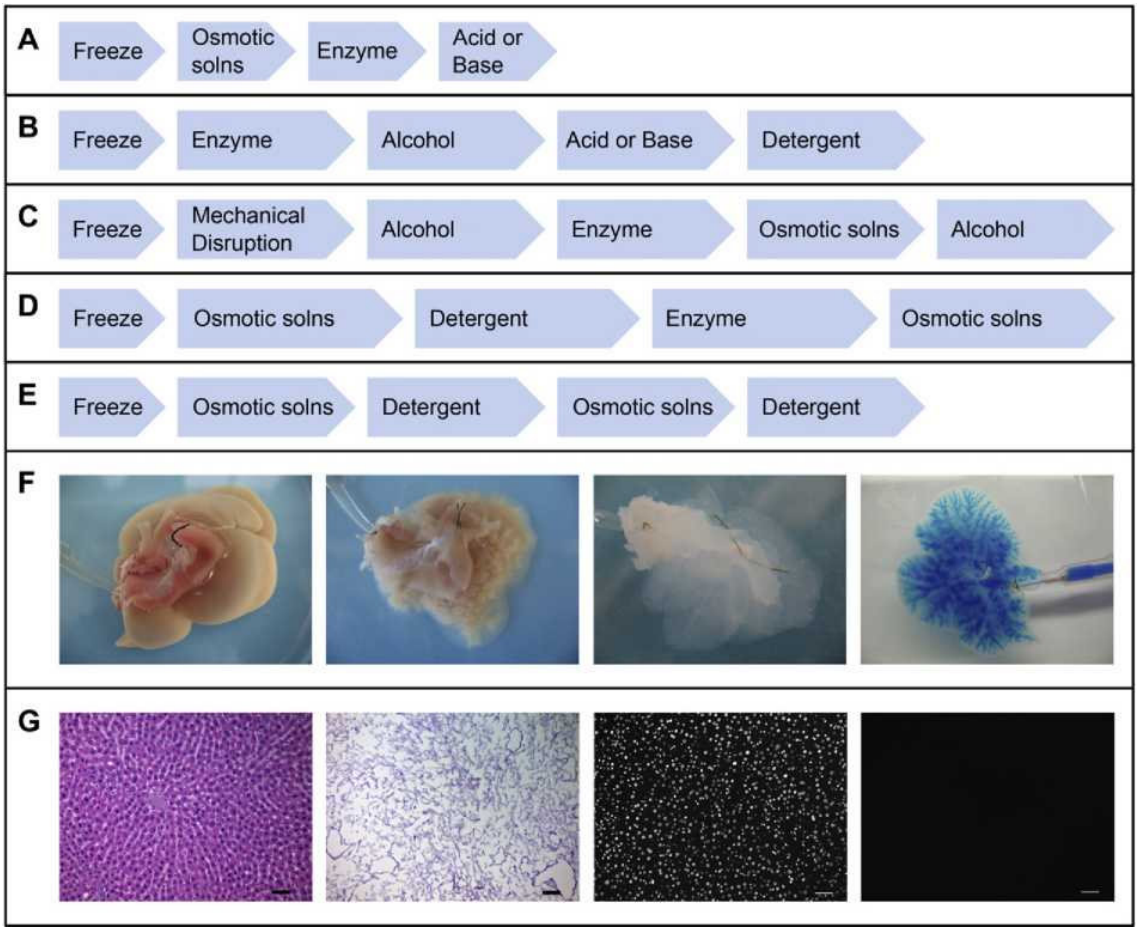

Fig. 2.1: Example decellularization protocols for (A) thin laminates such as pericardium, (B) thicker laminates such as dermis, (C) fatty, amorphous tissues such as adipose, (D) composite tissues or whole simple organs such as trachea, and (E) whole vital organs such as liver. Arrow lengths represent relative exposure times for each processing step. Rinse steps for agent removal and sterilization methods are not shown to simplify comparison. (F) Representative images of the gross appearance of intact rat liver subjected to decellularization: (left to right) before, during, and after decellularization; decellularized liver perfused with blue dye. (G) Representative photomicrographs showing no nuclear staining after whole organ decellularization: (left to right) native rat liver H&E; decellularized liver-ECM H&E; native rat liver DAPI; liver-ECM DAPI. Scale bars are 50 mm. Reprinted from [16]

heart valves[57, 58, 59], bladder[60] and pericardium are available in clinical practise. Almost all parts of human body is today decellularized in the laboratories e.q. adipose[61], heart[19], kidney[62], pancreas, lung, artery, bone, cartilage, tendom. Researchers found that decellularization protocols differs for different sizes and morphology of tissues[16], but this protocols always changes mechanical and chemical composition of native ECM [63]. It was found that chemical composition of decellularized tissues could induce spontaneous differentiation of stem cells [62, 19], which recovers partial function of recellularized organs. Only low levels of physiological performance were achieved [19].

2.1.3.2 Benefits

- On shelf availability (made under controlled manufacturing process)
- Low or no need to immunosuppression
- Ability to rapid recellularization in-vitro, in-vivo
- Possibly Allogenic or Xenogenic

2.1.3.3 Limitations

- Limited decellularization uniformity
- Reagent residuals, ECM molecules damage
- Transplant to transplant variation
- Various transplant pathologies
- Risk of diseases transmission

2.1.4 Tissue Engineered Scaffolds

2.1.4.1 Characteristic

Main purpose of this class of transplants is to mimic ECM and allow to restore some body function. Scaffolds should not induce foreign body reaction or calcifications. Tissue engineered scaffolds are made from synthetic or natural materials (2.1.4.4) using various fabrication methods(2.1.4.5).

2.1.4.2 Benefits

- Engineered reproducibility
- Engineered mechanical stability
- Engineered healing process
- Lower risk of diseases transmission
- Often ability to rapid recellularization in-vitro, in-vivo

2.1.4.3 Limitations

- Complicated manufacturing - often higher price
- Often insufficient stiffness for soft tissue applications
- Healing process and scaffold-tissue interaction is not well yet understand
- Lower biocompatibility than native natural materials
- Absence of vascular network

2.1.4.4 Materials

2.1.4.4.1 Natural Natural materials have longest history in tissue engineering. Especially collagen is well studied and used in today's clinical products [64, 65, 66] because collagen is most abundant protein in the body.
Biocompatibility of natural materials is generally better than synthetic. Cells are able to recognize naturally derived material so are able to easily adhere and interact with them [67] for example use MMPs for migration and remodelling.
All types of tissues contain different composition of biologic molecules, so researchers are trying various combinations of collagen-biomolecules to produce tissue-specific scaffolds.
There are currently two main ways to obtain ECM molecules or biomaterial:

- **Tissue/plant extracted** various methods are used for isolation of specific biomolecules from source tissues (using acid, bases, enzymes, etc.). Materials with different properties are obtained by different methods [6]. Look into 1.3.2 section to see how collagen is extracted.
- **Recombinant** Recombinant technology transfers biomolecule-producing sequences of genes(collagen, elastin, glycosaminglycans, fibronectin, etc.) from source organism(Human cells) into host organism (bacteria - E.coli, Hamster cells..). Recombinant production on bacteria cells allows production high amounts of well-defined target biomolecules [68, 69]. Some molecules can be produced in bacteria easily but more complex molecules (like collagen [69]) is difficult to produce in bacteria hosts because of need of some posttranslational modifications [69]. For example recombinant technology can produce different molecular weights with different properties [69, 70] and there are often problems with cytotoxicity of products.

2.1.4.4.2 Synthetic Synthetic polymers are also used as biomaterials. Their benefits are mechanical properties and they are well defined, but biocompatibility of this materials is in general limited. Synthetic materials acts very often inherent for cells, but degradation products could trigger complications [71]. Some of synthetic polymers degrade to acidic product which induce local change of pH and could induce later inflammation.

2.1.4.5 Fabrication methods

Tissue Engineered Scaffolds can be made from solutions or suspension. Selected fabrication technique must be used to obtain desired macroscopic shape and microscopic morphology.

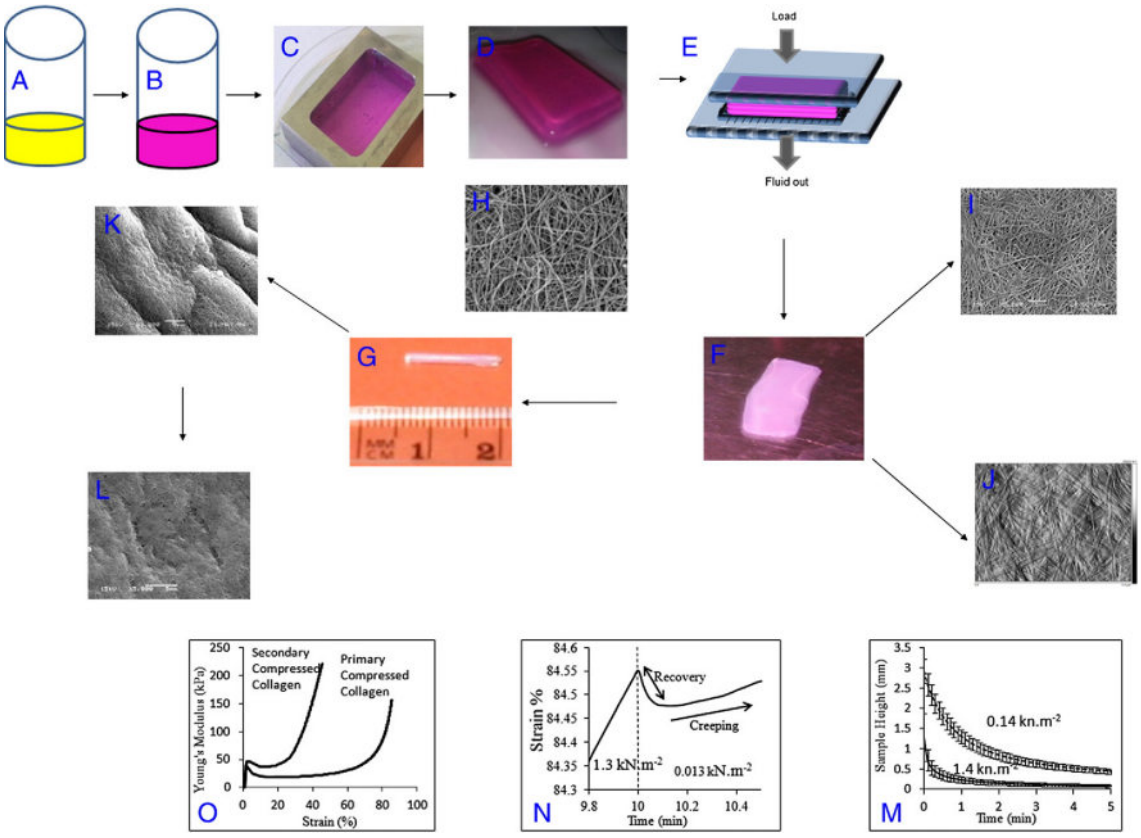

Fig. 2.2: Fabrication, microstructure and properties of plastic compressed collagen. (A) acid soluble collagen solution mixed with 10xDMEM, (B) collagen solution after neutralisation with few drops of 5 M NaOH solution, (C) neutralised collagen solution in polytetrafluroethylene mould, (D) post set hyper-hydrated collagen gel after being removed from the mould, (E) diagrammatic representation of plastic compression technique. (F) Plastically compressed collagen sheet that produced by exposing the post set hyper-hydrated collagen gel to a combination of unconfined compression and capillary action by absorbent tissues to eliminate 98% of casting water. This compressed collagen sheet is then rolled along its short axis using razor blade to form three dimensional highly dense biomimetic collagen construct (G). Post set hyper-hydrated collagen gel consists of randomly oriented fibrils with several nano-metres in diameter. Small interconnected voids are present between the fibrils, thereby forming a three-dimensional porous network structure (H). The density of collagen fibrils increased by plastic compression by approximately 7dž″10 folds and the pore size was smaller and more regular than those in hyper-hydrated gel (I), and the plastic compressed collagen maintains the characteristic D-banding as seen from atomic force microscopy (AFM) image using contact mode in air (J). The same increase in density was also observed from the longitudinal section of three dimensional plastic compressed collagen rolled construct (K). Applying another set of plastic "secondary" compression to three dimensional rolled construct produces a further increase in collagen density (L). This fluid loss is associated with a reduction in sample thickness that directly related to the level of load (M). These changes are permanent and there is no recovery seen after load removal (N). Secondary compression produces a stronger and stiffer construct than primary compression (O). Reprinted from [6]

2.1.4.5.1 Gel can be made from solutions of polymer or polymer blends by various methods. Time, temperature, pH, UV light and other stimuli could be used to induce gel formation. Gels are often too weak to be able to act as scaffold so various chemical reagents could be used to crosslink them [72, 73, 74]. Influence of croslinking reagents to cells must be then carefully in-vivo, in-vitro evaluated since biocompatibility can be altered [75]. Also physical methods are possible to use to reconstitute gels for example plastic compressed collagen[6]. Plastic compression induces better mechanical stability but denser biopolymer network can limit nutrition and oxygen transfer through the gel which concludes in limited cell integration.
In some cases (collagen, alginate, etc.) is also possible to mix polymer solution with cell suspension and induce gel formation later. This results in gel formation with homogeneously dispersed cells. Prolonged culture of those constructs could result in increase of mechanical properties due cell ECM deposition[76, 77]. But thickness of gels is limited by oxygen and nutrition transfer limits (see vascularization 2.2.1.4 for details).

2.1.4.5.2 Freeze-drying (FD) is common fabrication method used for production (**Fig. 2.3**) of commercial Tissue Engineered Scaffolds. Good preservation of biomolecules during drying and long term durability is the main advantages of FD. FD fabricated materials are sponge-like (**Fig. 2.6**), highly porous with highly interconnected pores, so they are sufficient for rapid cell colonization. Various techniques can be used to control pore size[78, 79] and in-vivo stability[72, 73, 74].

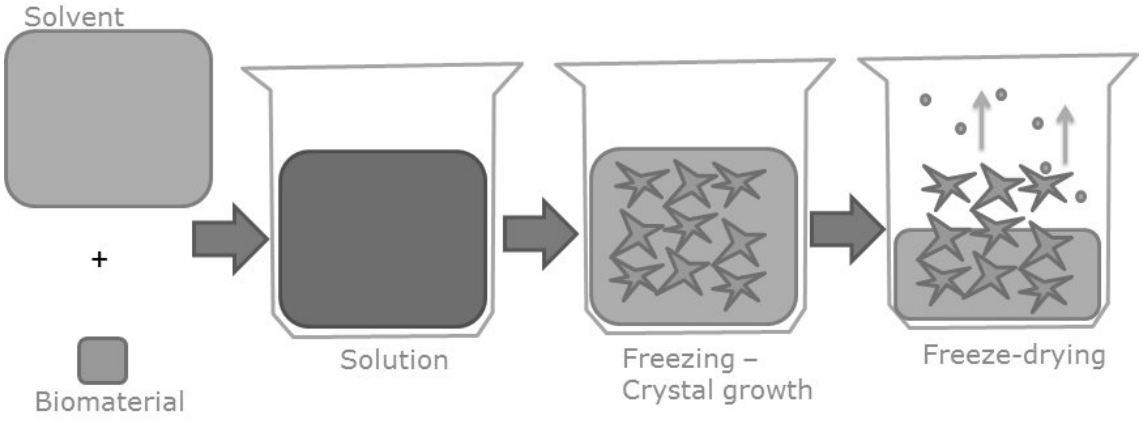

Fig. 2.3: Schematics of producing spongious scaffolds using freeze-drying

2.1.4.5.3 Electrospinning Since electrospinning was developed at start of 20 century PUT REFEERENCE, Tissue Engineers start to use this technique at the end of 20 century because of morphology similarity of electrospun networks and

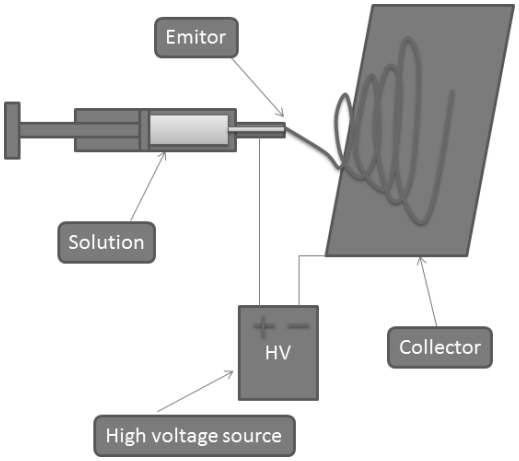

Fig. 2.4: Schematics of producing nanofibrous scaffolds using electrospinning

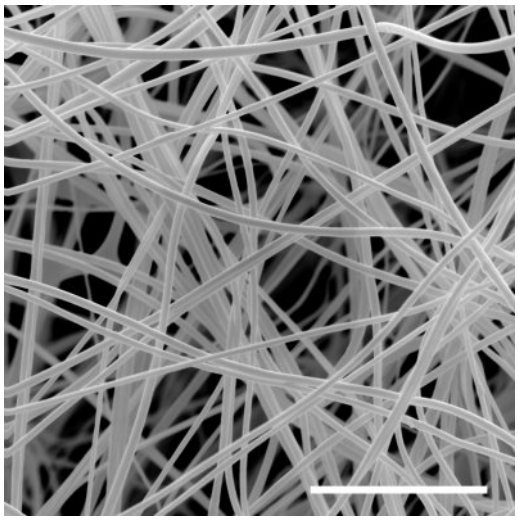

Fig. 2.5: Electrospun gelatin, bar 5 µm

native tissue. Electrospinning uses high voltage to accelerate solution extruded from needle tip (**Fig. 2.4**). This results in formation of Taylor cone and continuous (nano) fiber production. Materials produced by electrospinning forms dry, non-woven fiber networks with fiber diameters from 50 nm to 5 um. Electrospun collagen..

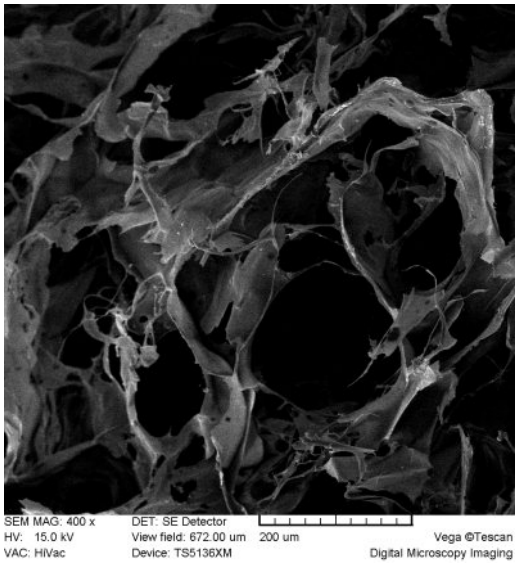

Fig. 2.6: SEM micrograph of collagen scaffold produced by FD, bar 200 µm

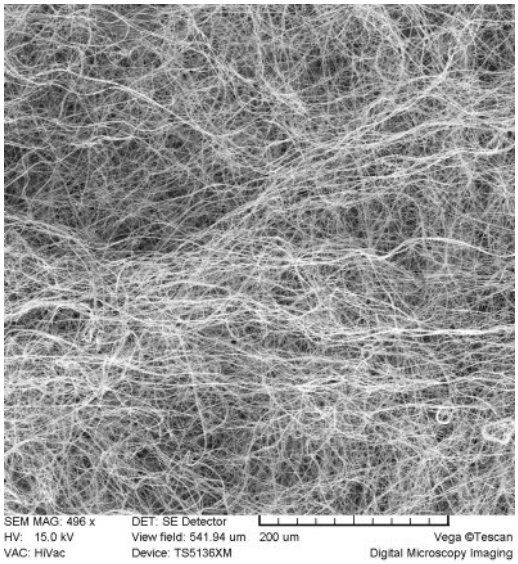

Fig. 2.7: SEM micrograph of gelatin scaffold produced by ES, bar 200 µm

2.1.4.5.4 Printing Engineers utilizes x-y-z driven stage controlled by stepper motors to print viscous solutions or melts from syringes or extruders into computer designed three dimensional scaffolds [80]. It is possible to print just material [81, 17]

or also material droplets with encapsulated cells [82, 83, 81, 17]. Resolution of produced materials is a few hundreds of micrometers (typically 200 µm) which is approximately similar to scaffolds produced by freeze drying. Some printed materials was already evaluated in-vivo [17]

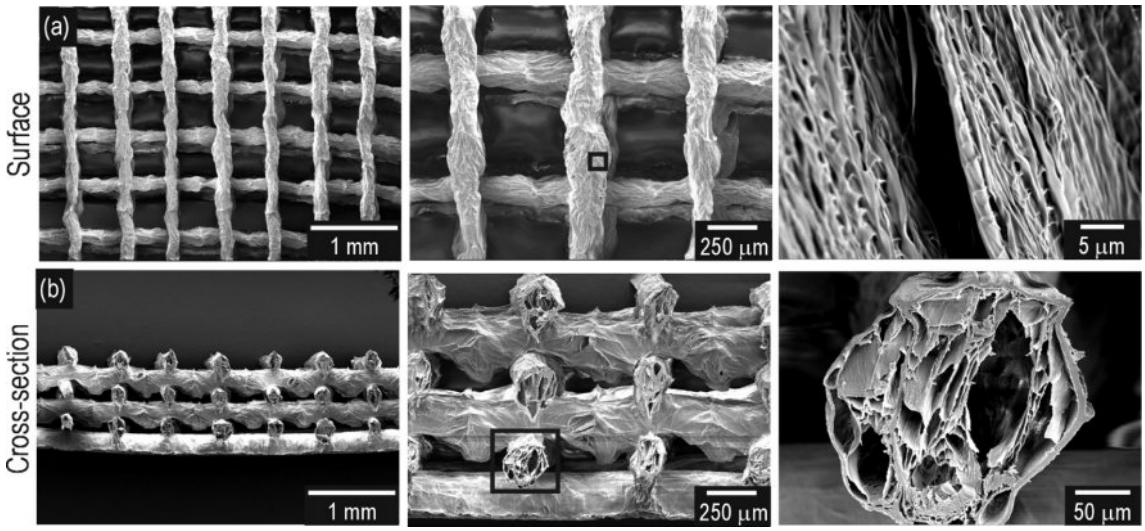

Fig. 2.8: 3D printed collagen scaffold. Reprinted from [17]

2.1.4.5.5 Cell-derived scaffolds Cells are able to secrete ECM components during in-vitro cell culture. This is basically observed by increased medium viscosity during prolonged culture. Researchers started to develop of cell-derived scaffolds, with [76] of without [18, 84] addition of collagen component. By this way scaffolds are produced without aggressive or toxic reagents. This results in production of scaffolds contain complex range of ECM components, which also shown ability of self-assembly in-vitro. Mechanical properties of Cell-derived scaffolds could be similar to synthetic scaffolds, but typically cells interacting better with natural scaffold [18].

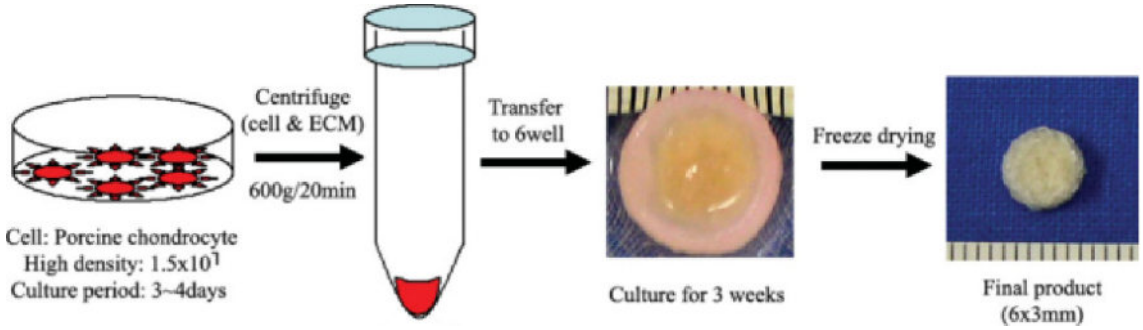

Fig. 2.9: Symplified example for derivation of ECM from cell culture. Reprinted from [18]

2.2 Response of Living Organism to Transplants

2.2.1 General Physiological Requirements of Transplants

2.2.1.1 Morphology

2.2.1.1.1 Macroscopic According to target tissue(skin, bone, cartilage, ligament, heart, liver, etc.), there are always macroscopic requirements for those tissues. For example by decellularized heart perfectly fits all macroscopic requirements (see **Fig. 2.10**).

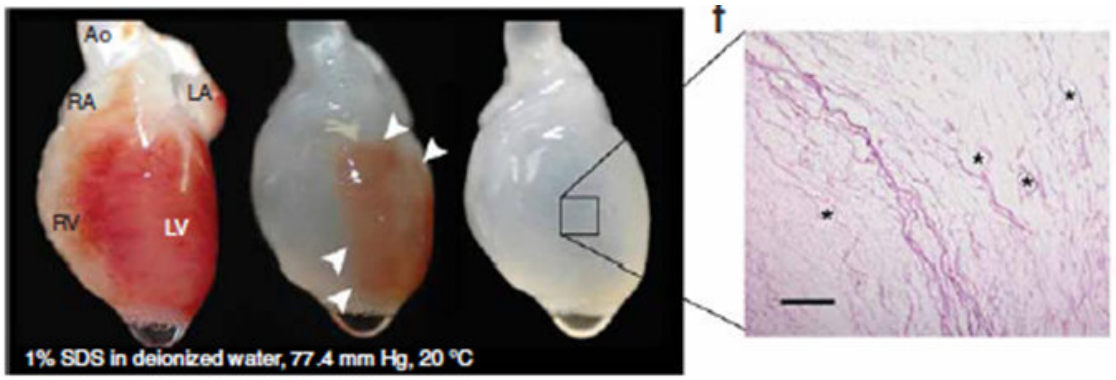

Fig. 2.10: Demonstration of morphologic - decellularized rat heart. Reprinted from [19]

2.2.1.1.2 Microscopic Borders between macroscopic and microscopic morphology are not clear. For gels, decelularized tissues and electrospun nanofibers are basic elements - fibres smaller than cells, so little changes in fibre diameters and pore sizes could be important in terms of cell penetration [85, 86] because of limits in cell plasticity **Fig. 2.11** [20]. But chemistry (biomaterial composition, crosslinking density, cytotoxicity,..) is often very important, because of high cell-scaffold interaction area **Fig. 2.12** [21]. Large pores of freeze-dried scaffolds allows rapid colonization of scaffolds but this concept suffers in terms of sub-cell sizes control. Researchers found different in-vivo behaviour for engineered skin fabricated using electrospinning and freeze-drying[87].

2.2.1.2 Biocompatibility

Important part of biocompatibility is the chemical composition of the material. Material should allow good attachment of cells, should be enough mechanically stable and their degradation products should be nontoxic.

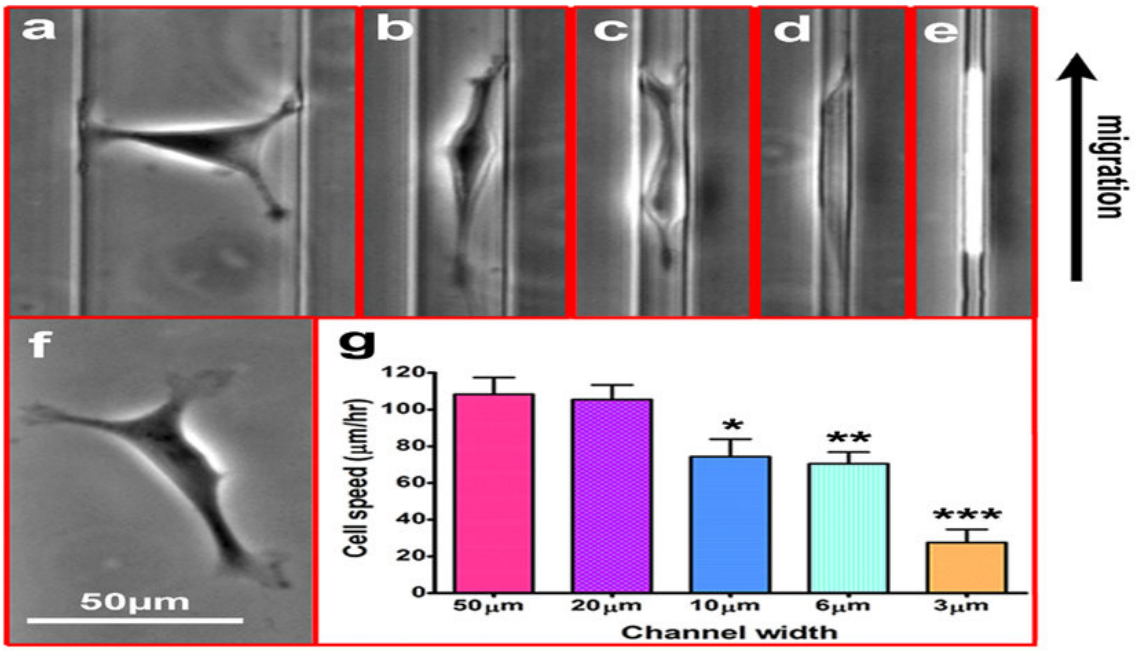

Fig. 2.11: The influence of channel width on migratory cell morphology and migration speed. Reprinted from [20]

Even if the choice of biomaterial is important, biocompatibility of whole transplant(scaffold) cannot be simplified to biocompatibility of base material. Biocompatibility is also closely linked with microscopic morphology, resulting scaffold surface area, cell-scaffold interaction area and local micromechanics [88].

Schematic view on the image **Fig. 2.13** demonstrates that in case of **microporous scaffold(a)**, only cells directly attached to scaffold are strongly influenced by the scaffold. Cells growing into center of scaffold will be less influenced by the scaffold. In consequence the phenotype, viability, apoptosis rate and other cell health parameters can differ for cells with direct or indirect contact with scaffold. E.q. if scaffold contains growth factors, directly attached cells will get the biggest growth factor dosage. In other case toxic products released from improper material will increase apoptosis rate of directly attached cells.

Using **microfibre scaffold (b)**, much more cells will be directly influenced by scaffold. Sensitivity to scaffold will be higher. Good material biocompatibility or growth factors can influence strongly the healing process. In the other case nutrition, waste, oxygen, carbon dioxide permeability will be lowered. Inadequate material biocompatibility will probably strongly decrease the viability.

Nanofibre material(c) - native ECM, biopolymer fibrous gels (collagen, fibrin, elastin), electrospun scaffold. This type of scaffold influences cellular response much stronger than the others, because of large surface area. Mechanism of cellular movement is also different. It was shown that cells are not able to grow in-vitro into many of types synthetic electrospun polymer materials [85], but they are able to grow into

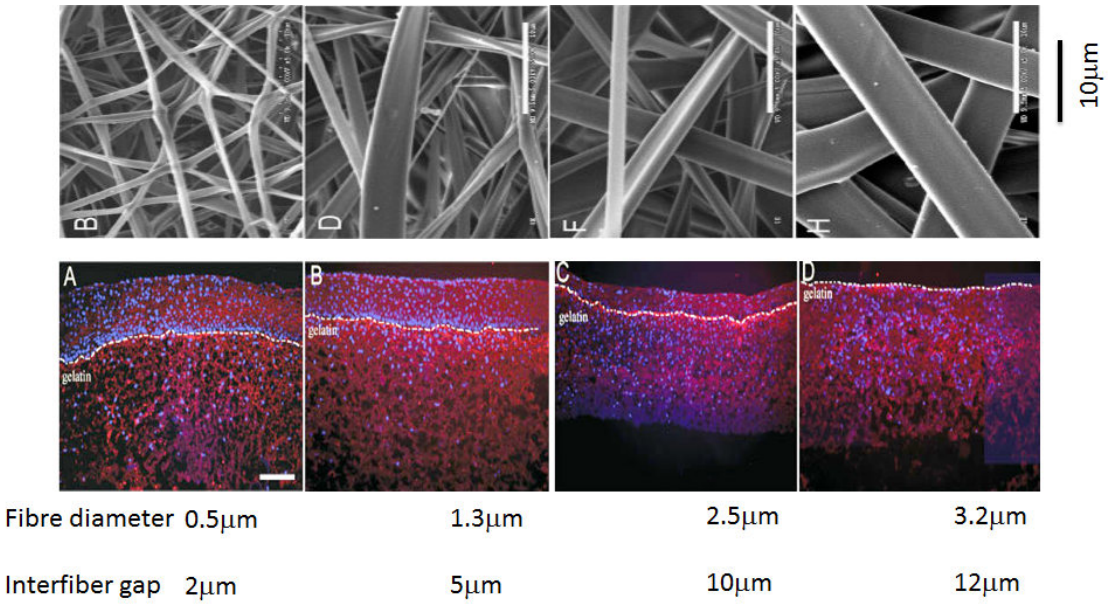

Fig. 2.12: Top: Gelatin electrospun fibres with different fibre diameters and pore sizes. Middle: In-vitro growth of human karatinocytes and fibroblast on electrospun materials. Bottom: Measured fibre diameters and pore sizes. Reprinted from [21]

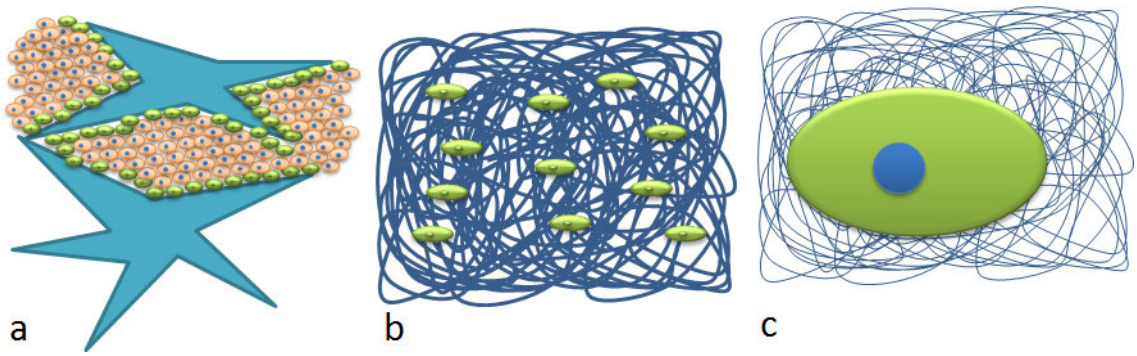

Fig. 2.13: Schematic interaction of cells and different transplants. Microporous scaffold(a), Microfibre scaffold(b), Nanofibre scaffold(c). Morphology of transplant could influence cell-scaffold surface area and biocompatibility

nanofibres made from collagen with similar morphology [21]. This is probably possible because cells are able to manipulate with single natural fibers (with adequate mechanics) and dissolve them by metalloproteinases [22].

2.2.1.2.1 Immune response Immune response is the main allogenic and xenogeneic material limitation. This complication results in development of decellularized ECM and Tissue Engineered Scaffold. Researchers found that DNA content is strongly associated with imune response [16], but is expected that many other molecules/mechanisms could trigger imune response. Today the only efficient method to detect immune response is preclinical testing on higher mammalian animal models. Cost and ethical problems connected with animal models motivates

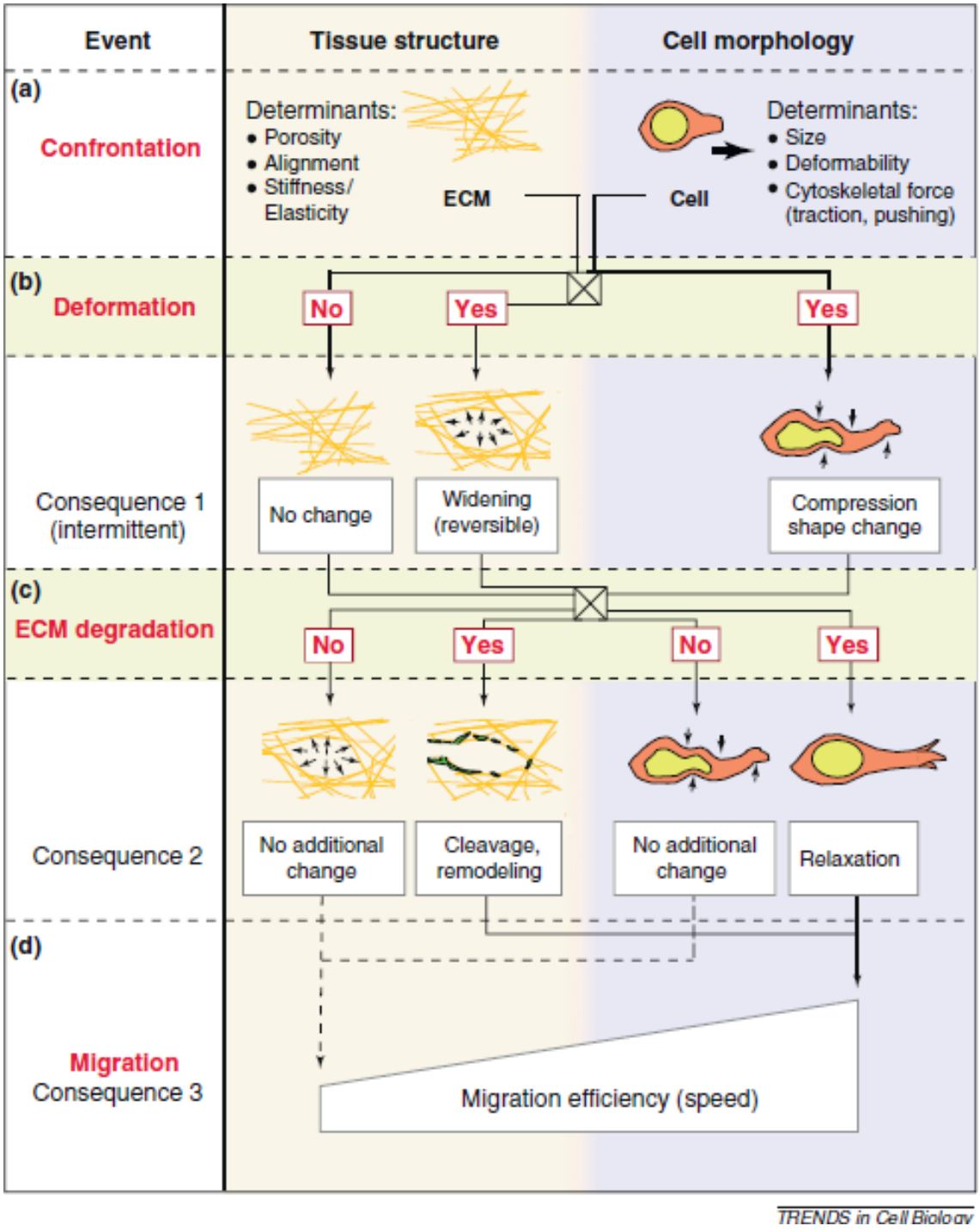

Fig. 2.14: ***Decision-making in cell invasion through 3D tissue(left).*** *Stepwise physicochemical events and decisions (the diagonally crossed square) during dynamic cell confrontation with 3D ECM depicting the perspectives of both the tissue structure and cell morphology, leading to either a proteolytic or non-proteolytic migration mode. The decision model and resulting migration efficiency assume a matching scenario between pore diameter smaller than the cell diameter, but large enough to allow protease-independent migration supported by combined ECM deformation, or by cell shape change alone. The rate-limiting spatial conditions leading to complete migration arrest in non-proteolytically moving cells remain to be determined. Reprinted from [22].*

researchers to develop other predictors which should be able to detect immune response in-vitro. Some of researchers believed that macrophage phenotype could be good predictor [89, 90], but high quality evaluation of this method is still missing and should be also evaluated on another types of transplants than decellularizedECM.

2.2.1.3 Mechanical properties

It is natural that each transplant must be able to match mechanical properties of target tissue. Not just mechanical strength is important, but stiffness or elasticity is for many applications also important e.q. skin [91] and vascular applications [92]. Mechanical stability in-vivo could be problematic especially for natural polymers, because their degradation could be modulated by immune response. On the other hand, degradation of synthetic scaffolds is normally well defined and not influenced by immune system. But their degradation products could directly induce later complications like inflammation (particles, low molecular weight polymers, lactic acid, glycolic acid, etc.) [71] and loss of their function.

2.2.1.4 Vascularization - Nutrient delivery and waste removal

Physiologicall maximum distance between capillaries is 200 um[93], this is because of diffusion limits which was also confirmed in-vitro models **Fig. 2.15** [23]. Decellularization protocols can preserve vascular system of donor tissues. But for other Tissue Engineered Scaffolds vascularization concepts are in early development. Cell based strategies of vascularization consist of in-vitro prevascularization, in-vivo prevascularization, growth factors, adhesion peptides and co-culture **Fig. 2.16** [23].

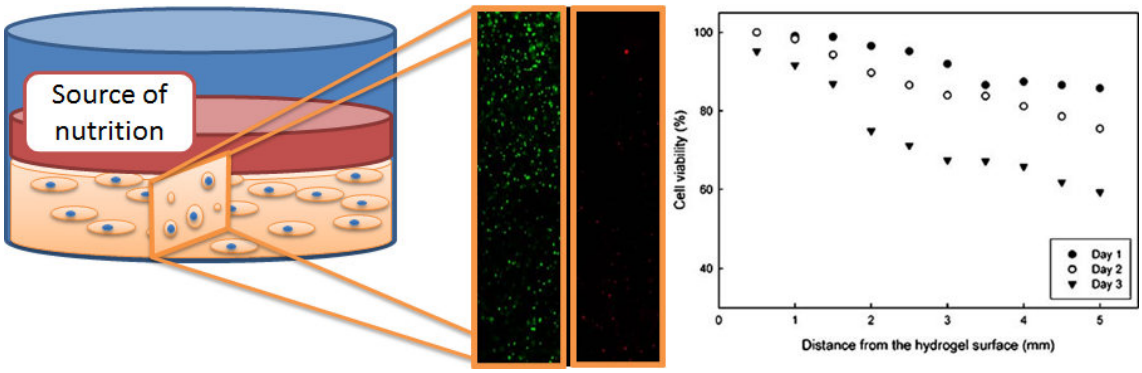

Fig. 2.15: Agarose gel diffusion model. Reprinted from [23]

.

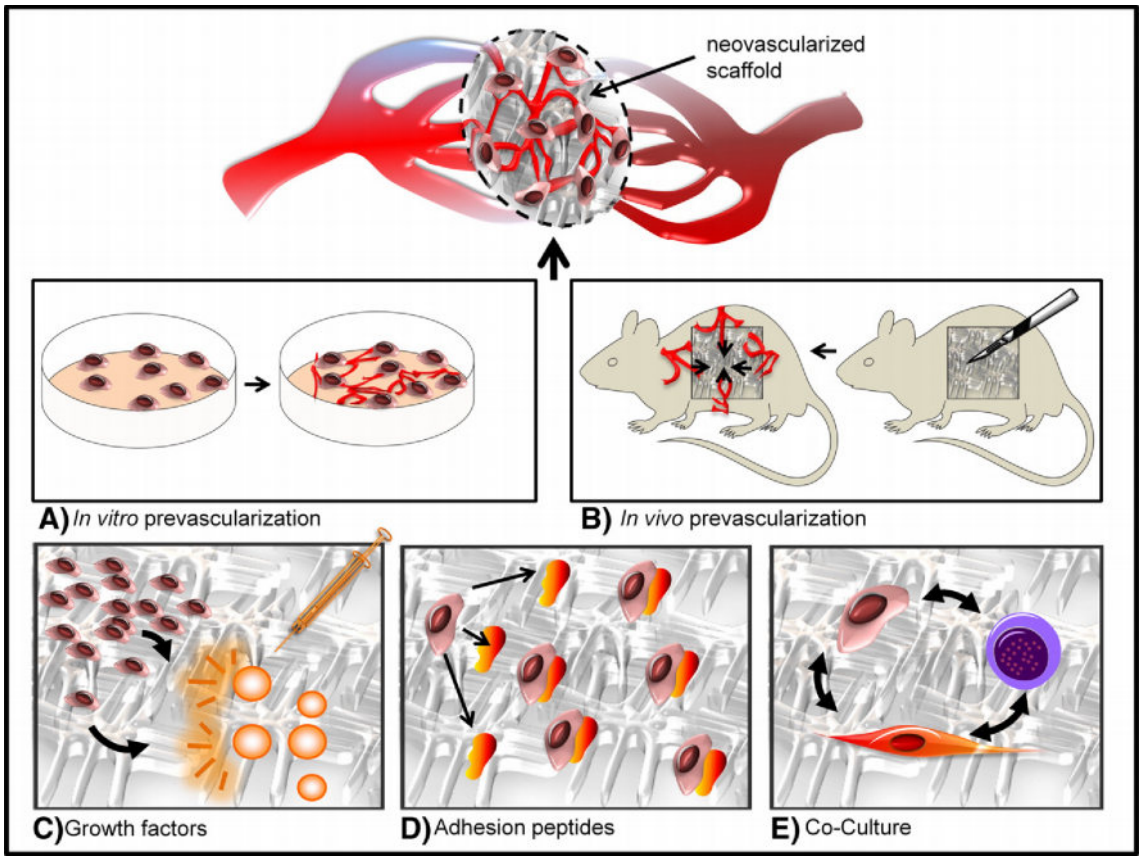

Fig. 2.16: Five different cell-based strategies to induce vascularization by stimulation of angiogenic processes. The strategies include the application of in vivo and in vitro prevascularization (A, B) as well as stimulation of vascularization by addition of growth factors (C), immobilization of adhesion peptides (D) and co-culture of different cell types (E). Reprinted from [23]

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2.2.1.5 Reproducibility

It's always expected to be able to reproduce products well. Reproducibility of Decellularized products will be always lower because of natural variability between individuals. For same reason there are problems in producing of naturally derived materials from tissues, but cell derived or recombinant produced materials are possibly better purified. Synthetic materials normally offer great reproducibility.

2.2.2 In-vitro Tissue Engineering

Culturing of mammalian cells outside of their native environment must be performed in clean environment, because only immune system cells are able to resist bacteria and viruses, other types of cells are unable to resist to another microorganisms which generally growing much faster than mammalian cells. There are about 300 distinct types of cells in the human body. Adherent(f.e. fibroblasts, chondrocytes, myblasts,

osteoblasts, etc.) and non-adherent(f.e. erytrocytes, lyphocytes, etc.) types of cells are cultured by very different protocols.

2.2.2.1 Cell culture of adherent cells

Adherent cells are able to adhere to many types of materials, by other mechanisms of adhesion. Adhesion is critical for adherent cells, if they don't adhere in such a period, they will die by apoptosis. Their phenotype development is strongly influenced by this material. Not only chemical composition of this material is important, but also surface morphology.
It's necessary to feed cells during their expansion or maintenance. Many of types of culture media were developed for different types of cells. Those can strongly influence their rate of expansion and their phenotype. Basically nutrition, proteins or growth factors should be included in cell culture media.

2.2.2.1.1 Cell culture on flasks is standard method of cell expansion of adherent cells. Cells obtained from biopsies or cell banks (defrozen) should be seeded into culture flasks at appropriate cell density. Majority of cells should adhere to the flask within few hours, non adherent cells or non-viable cells will be removed with media change about 1 or 2 days after cell seeding. Confluence(percent of surface of flask covered by cells) should be controlled by microscopy at least during each media change. When confluence reach desired level, cells should be released from flask by enzymes(trypsin, collagenase, triple etc.) and moved to another flasks (more flasks or different size) - passaging. Cell growth is only optimal at sub confluent cell concentrations, prolonged period of cell culture at higher confluences could conclude significant drop in cell metabolism or some pathologies. Cells are normally centrifuged when they are released from flask, supernatant is withdrawn(to remove enzyme) and pellet mixed with fresh media (enzymatic reaction is completely stopped). Concentration of cells and also their viability should be measured. Then they could be accurately spitted into new flasks. Cell growth, media change, confluence measurement, passaging and new seeding should continue until desired number of cells is reached. Limited number of passages is normally possible for primary cells (from biopsies), many passages concludes in phenotype change, low metabolism rate and other pathologies.

2.2.2.1.2 Cell culture on Tissue Engineered Scaffolds Different microscopic and macroscopic morphologies of each type of scaffold and their chemistry causes in different cell spreading inside the scaffold, different mechanism of cell penetration into scaffold, liquid(nutrition, waste, oxygen, carbon dioxide) permeability

characteristics.
Seeding and culturing techniques of different scaffolds will be different.

- **Decellularized 2.1.3** membranes are often too dense, so penetration of cells will be slow, but adhesion is normally excellent. Today best method for seeding of decellularized organs is using of natural vascular system[19], culture of this cells could results to partial recovery of organ function in-vitro.
- Some **gels 2.1.4.5.1** allow to be mixed with cells before polymerization, so cells could be well spread in the scaffold. Gels are normally weak, but expression of proteins from seeded cells could make gels much stronger[6], but shrinkage of gel-cell construct often occurs. Nutrition permeability limits usable thickness of the constructs (see Vascularization section2.2.1.4).
- **Freeze-dryed 2.1.4.5.2** collagen scaffolds could be easily seeded because of their large pores. There are some troubles with seeding homogeneity because of various pore sizes and proportion of scaffold. Seeded cells adheres on the walls of scaffold and populates pores.
- **Electrospun materials 2.1.4.5.3** shown very different cell behaviour for synthetic and natural materials. Cells are able to easily adhere and spread on electrospun materials from synthetic materials. But penetration into this scaffolds is only possible when there is sufficient pore size occurs, this can be handled by producing of thicker fibres or altering pore sizes[85]. Proteolysis and adequate mechanical properties of single natural fibers allows to penetrate cells trhough small pores [86]. Development in mechanical properties[94] during cell culture could be similar to gels.

3 AIMS OF THESIS

Collagen is most abundant protein in the body, which makes him important material for medicine and Tissue Engineering. Collagen forms highly crosslinked fibrils in-vivo, which are hardly processable for the the engineers. Thus researchers developed methods to dissolve them. This work will focus on the pepsin dissolved collagen called atelocollagen. This material is fully soluble, better processable and characterizable by many material engineering methods. Dissertation work will also be focused on atelocollagen gels which consist of semicrystalline bundles of atelocollagen molecules.

State of the art based on:

- Characterization
  As mentioned in Quality control 1.3.2.1 and Characterization 1.3.4 sections. Rheology, electrophoresis, CD spectroscopy are essential for atelocollagen extraction. SEM, TEM and rheology are essential for characterization of created gels. There are also many of other tools reviewed in Characterization section 1.3.4 currently used in the collagen research.
- Atelocollagen extraction and purification.
  Large part of this work is focused on soluble collagen extraction and isolation. Since our lab is still using insoluble collagen, this step will help us to start to work with soluble collagen. Full understanding of quality 1.3.2.1 of this material is essential to future development of the products in our lab. Any impurities can greatly alter collagen properties. Not just from material point of view, but also biological point of view.
  Soluble collagen is much more difficult to prepare, but allows much better processability than in comparison with insoluble form. There are currently many labs over the world working with their own soluble collagen, but the quality of their collagens is at least discussable because of lack of adequate characterizations 1.3.2.1. Reaching the maximum quality of the collagen at start of the development of the new product is very important, because subsequent optimizations of product, in-vitro and in-vivo tests are very expensive. Any change of source material can conclude in large drawbacks. There are currently limited number of sources of GMP, FDA quality collagen (Koken, Integra, etc.) but cost of this materials is extremely high. In case of using material from other manufacturers the only acceptable price is offered just for products at scientific quality. But there is always risk that those companies will later not cooperate in transition to medical quality production or their price could be limiting for introduction of new product to the market. So there is serious need to create own collagen source which will open the way for

possible medical application.

- Fibrilogenesis processes - gelation.
  Collagen forms 3D gel networks at neutral pH, physiological ionic strength and temperatures in-vivo and also in-vitro. The process of forming gels is called fibrilogenesis 1.3.3. Fibrilogenesis formed in-vitro doesn't match the mechanical properties of native ones formed in-vitro because of precise alignment and additional crosslinks. There is great need to overcome this limitation, there is just little improvement in the past years. People believe that the lower mechanical properties are connected with lower quality of crystallization due quality of material and processing conditions.
  We will try develop new solvent-gelation system which should improve properties of collagen gels by modification of their self-assembly behaviour.

4 METHODS

4.1 Electrophoresis

Laemli method was used to prepare electrophoresis gels (6.5%/4%). Samples at 3 mg/ml in 0.1 M AA were mixed 1:1 with sample buffer (100 mM TRIS-HCl buffer pH 6.8 - 4 % SDS, 20 % glycerol, 0.02 % bromphenol blue) and boiled for 2 min at 100 °C. Gels were run using Protean II aparatur (Biorad) at 180V.
Chemicals: acrylamide and bisacrylamide(36:1), Tris-HCL buffer (pH 8.8 resolving gel, pH 6.8 stacking gel), SDS, amonium persulphate, glycerol, bromphenol blue, glycine, TEMED.

4.2 CD Spectroscopy

Collagen should be dissolved at least overnight in 0.5 M Acetic acid at final concentration 0.3 mg/ml and placed in 1 mm path length quartz cell and scanned from 190-250 nm at 20 °C or less (depending of expected denaturation temperature of used collagen) [33]. For measurement on shorter wavelengths or longer path lengths 0.1 M Acetic Acid could be used to dissolve collagen, but two day dissolution period is recommended to completely dissolve collagen. Centrifuge 10 000 g for 30 min at 4 °C is recommended to remove insoluble material.

4.3 Rheology

Continuous flow experiments were done using method described by Gobeaux [36]. Collagen sample dissolved at least two days in 0.1 M AA at 3 mg/ml was placed on cone-plate geometry and measured at shear rates 0.001 s^{-1} - 400 s^{-1} at 20 °C without any pre-shearing using AR-G2 instrument (TA instruments).

4.4 Scanning Electron Microscopy

Collagen gels were prepared for SEM according to protocol previously used by Yang et al. [9], without Hyaluronic acid fixation. Collagen gels were fixed in 3% (v/v) glutaraldehyde containing 3%(w/v) paraformaldehyde and 2.5% (v/v) dimethylsulfoxide in 0.1M sodium cacodylate buffer (pH 7.4) for at least 12 h at room temperature, washed in 0.1 M sodium cacodylate buffer(3x30 min), dehydrated in graded ethanol solutions(50 - 100%, each 30 min) and critical-point dried. The dried gels

were coated by 20nm platinum/palladium and observed in a Carl Zeiss (Zeiss Supra 60) scanning electron microscope at 5 kV.

5 CONCLUSION

Rheology, electrophoresis, CD spectroscopy and SEM methods were used for collagen development. Very pure atelocollagen was extracted, with a yields of about 20 % with the purity of 90-95 %. Improved method is currently being in development. The most difficult step of atelocollagen extraction was filtration.
Transparent and flexible gels were produced by new process based on pressurized CO_2. Detailed results from those experiments cannot be currently published within this document.

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6 AUTHOR PUBLICATIONS AND OTHER OUTPUTS

- Bioimplantology 2014 lecture Izolace a charakterizace atelokolagenu,
- Bioimplantology 2015 lecture NovĂ″ zpĹŻsob zpracovĂˇnĂ atellokolagenu

LIST OF SYMBOLS, PHYSICAL CONSTANTS AND ABBREVIATIONS

Allogenic human origin, transplant is recieved other person donor

Autologous human origin, transplant is harvested from one part of body and recieved to other part of same person

CD Circuar dichroism - spectrophotometer which is able to detect secondary structure of chiral molecules

Confluence percent of surface of flask covered by cells

Decellularized Without cellular content

decellularizedECM Cell free tissue ECM

ECM Extra Cellular matrix

ES Electrospinning - method for producing nanofibres

FD Freeze-drying - class of strong enzymes secreted by cells

Gel Chemically or physically crosslinked polymer network

SEM Scanning Electron Microscopy

MMP Metalloproteinases - class of strong enzymes secreted by cells

TE Tissue Engineering

TEM Transmission Electron Microscopy

Tissue Engineered Scaffold Artificial ECM made from natural or synthetic molecules

UV Ultraviolet, range of wavelengths

Xenogenic Animal origin, transplant is harvested from animal and implanted to person