From scaffold to tissue: design, realisation and characterization of 3D architectures for regenerative medicine

Giovanni Vozzi, Carmelo De Maria*, Annalisa Tirella#, Arti Ahluwalia#

Research Center “E. Piaggio”, University of Pisa
Dipartimento di Ingegneria dell’Informazione, University of Pisa
*FABLlab PISA
#CNR-IFC, NanoBioscopy Lab, Pisa
Outline

• Scaffold definition
• Scaffold requirements
• History of scaffold fabrication
• New approaches in scaffold design: Bioprinting, Nano-in-Micro
• Scaffold characterisation
What is a scaffold?
A 3D structure which supports 3D tissue growth
What are the features of an ideal scaffold?

- Biocompatible, cell adhesive, bioerodable and *bioactive*
- Mechanical properties *similar* to those of natural tissue
- Optimal meso, micro- pores
- Well-defined, or *quantifiable* topology at meso- micro- and nanoscales
Stimuli- the tripartite axis

Biochemical stimuli in scaffolds

- Synthetic biomaterials with ligands (next talk!)
- Natural biomaterials
- Decellularized Tissue
# Biochemical stimuli in biomaterials

<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Reproducibility</th>
<th>Processability</th>
<th>Biochemical features</th>
<th>Mechanical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic</td>
<td>✓</td>
<td>✓</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Natural</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Decellularized</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
Extracellular matrix features

- High degree of porosity
- Appropriate pore size
- High surface to volume ratio
- High degree of pore interconnectivity
- Biochemical factors & ECM features able to guide cell function

We need a bottom–up approach
Mechano-structural stimuli
Methods for generating MS stimuli in scaffolds

- **Designer Scaffold**
  - Subtractive
  - Additive

- **Random Scaffold**
  - Organ processing
  - Biomaterial processing
Designer or Random?

Structure

Function

Retina

Liver

Bone

serial components parallel components
Designer Scaffold
Additive = rapid prototyping
CAD FILE

STL FILE

GCODE

File

3D object

Triangle and vertex

Toolpath

Description

FreeCAD

FreeCAD (MeshLab)

Slic3r

Printrun to 3DP

Software

Designer Scaffold

Additive

Subtractive
3D Printing/Digital Fabrication & RP
Designer Scaffold

Three main groups:
- laser systems
- nozzle based systems
- direct writing systems

Materials?
Speed?
Price?
Fidelity?
Three main groups:
- laser systems
- nozzle based systems
- direct writing systems

Materials?  
Speed?  
Price?  
Fidelity?
Designer Scaffold

Three main groups:
- laser systems
- nozzle based systems
- direct writing systems

Materials?
Speed?
Price?
Fidelity?
Stereolithography

Laser for polymerisation of liquid monomer or resin

Materials?
Speed?
Price?
Fidelity?
Fused Deposition Modeling

Materials? Speed? Price? Fidelity?

Hutmacher & coworkers

Figure 1: Platform technology for patient specific scaffolds TE.
Pressure Assisted Microsyringe (PAM)

Materials? Speed? Price? Fidelity?

Piston Assisted Microsyringe (PAM2)

Plunger driven

Materials?
Speed?
Price?
Fidelity?
The PAM2 system
Robotic 3 axis micropositioner.

- Position controlled brushless motors (resolution of 10 µm ± 1 µm)
- Working space 100x100x80 mm
- Working velocity 1-15 mm s⁻¹
- Design of z-stage to locate several modules

Materials?
Speed?
Price?
Fidelity?

Tirella, De Maria, Vozzi, Ahluwalia Rapid Prot. J (2012);
Smart-tunable modular scaffolds...

Resolution, fidelity, viscosity

PEG-DA 5% w/v
alginate 4% w/v
PCL 10% w/v
PCL 20% w/v with 0.75% w/w CNTs

Development of a modular microfabrication system to engineer complex tissues
Inkjet technology is a *contact free dot matrix printing* procedure. Ink is issued from a small aperture directly onto a specific position on a substrate.
Penelope Ink-Jet printer

Materials?
Speed?
Price?
Fidelity?
Agarose, water and DMSO

• **Printing water** on a substrate of agarose dissolved in DMSO induces a higher (local) concentration of water that allows the agarose to form a gel with a **well defined topology**.

• Starting from **digital images**, arbitrary gel structures can be printed.

• **Layer-by-Layer** approach
Membrane Lamination

Laser as a cutter

Materials?
Speed?
Price?
Fidelity?
Indirect Rapid Prototyping (iRP)

- Molds realised with RP devices (CAD/CAM)
- Casting of the desired (bio-) material
- Extraction of the final object

Advantages? Limitations?

Materials? Speed? Price? Fidelity?

DW Hutmacher et al., Trends in Biotechnology, 22(7):354 – 362, 2004
<table>
<thead>
<tr>
<th>Technique</th>
<th>Material used</th>
<th>RTM ratio (cm³/min)</th>
<th>Resolution (μm)</th>
<th>Cells used</th>
<th>Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane Lamination</td>
<td>Bioerodable polymers (PLA, PLGA, etc), bio-ceramics</td>
<td>Low (&lt;1)</td>
<td>1000</td>
<td>Osteoblasts</td>
<td>Structures not really porous, low resolution</td>
</tr>
<tr>
<td>Laser Sintering</td>
<td>Calcium Phosphates, polymers (PLA, PLGA, etc)</td>
<td>Medium to high</td>
<td>&lt; 400</td>
<td>Osteoblasts</td>
<td>Presence of polymeric grains and of excess solvent</td>
</tr>
<tr>
<td>Photo-polymerisation</td>
<td>Photo-polymeric resins</td>
<td>0.5 (medium)</td>
<td>250</td>
<td>Osteoblasts</td>
<td>Use of photo sensitive polymers and initiators which may be toxic</td>
</tr>
<tr>
<td>Fused Deposition Modelling</td>
<td>Bioerodable polymers (PLA, PLGA, etc)</td>
<td>7 (very high)</td>
<td>200</td>
<td>Various types</td>
<td>Limited to non thermo labile materials. Layered structure very evident</td>
</tr>
<tr>
<td>3D™ Printing</td>
<td>Bioerodable polymers, (PLA, PLGA, etc) and hydroxyapatite</td>
<td>Medium (about 1)</td>
<td>300</td>
<td>Various types, mainly skeletal</td>
<td>Presence of polymeric grains and of excess solvent</td>
</tr>
<tr>
<td>iRP</td>
<td>Bioerodable polymers (PLA, PLGA, etc), collagen</td>
<td>0.1 (low)</td>
<td>300</td>
<td>Various types</td>
<td>Complex to realise, build materials limited, low fidelity.</td>
</tr>
<tr>
<td>PAM²</td>
<td>Bioerodable polymers (PLA, PLGA, etc) and gels (alginate, gelatin)</td>
<td>1 (medium)</td>
<td>5-100</td>
<td>Neurons, endothelial cells, fibroblasts, hepatocytes, muscle</td>
<td>Highly water soluble materials cannot be used. Extrusion head very small.</td>
</tr>
<tr>
<td>InkJet</td>
<td>Water, solvents, nanoparticle suspensions</td>
<td>Very low (&lt;0.01)</td>
<td>10</td>
<td>Various</td>
<td>Only low viscosity liquids.</td>
</tr>
</tbody>
</table>
Summary

• Resolution vs manufacturing time trade off

• Softness (and wetness) vs resolution and fidelity trade off

Organ Processing

Whole Organ Perfusion
- Detergents
- Intact microvasculature
- Slow and costly

Tissue Decellularization
- Detergents
- Rapid, less wasteful
Biomaterial Processing

- Freeze drying
- Phase separation
- Gas foaming
- Salt leaching

Price?
Materials?
Speed?
Repeatability?
Biomaterial Processing

Freeze drying
Phase separation
Gas foaming
Salt leaching
Biomaterial Processing

Freeze drying
Phase separation
Gas foaming
Salt leaching

- Polymer
- \( \text{CO}_2, 55 \text{ atm, RT} \)
- Polymer
- \( \text{CO}_2 \text{ dissolves in the polymer} \)
- Polymer
- \( \text{CO}_2 \text{ forms gas bubbles} \)

Price?
Materials?
Speed?
Repeatability?
Biomaterial Processing

- Freeze drying
- Phase separation
- Gas foaming
- Salt leaching

Price?
Materials?
Speed?
Repeatability?
Electrospinning

- Random Scaffold
- Organs processing
- Biomaterial processing

Syringe pump
Syringe metallic needle
High-voltage power supply
Grounded collector
Solution
High Voltage power supply
Electrospinning jet
Collector

(a) Image: Material sample - 0.5 mm
(b) Image: Material sample - 100 μm

Price?
Materials?
Speed?
Repeatability?
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<th>Cells used</th>
<th>Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze drying</td>
<td>Proteins, carbohydrates, polyesters, hydroxyapatite</td>
<td>High</td>
<td>Variety</td>
<td>Wide distribution of pore size</td>
</tr>
<tr>
<td>Phase Inversion</td>
<td>Polyesters, PVA, polyurethanes, biogels (gelatin)</td>
<td>High</td>
<td>Variety</td>
<td>Low interconnectivity, difficult to control pore size</td>
</tr>
<tr>
<td>Salt leaching</td>
<td>Polyesters, polyurethanes, hydroxyapatite</td>
<td>High</td>
<td>Variety</td>
<td>Salt residues, limited connectivity</td>
</tr>
<tr>
<td>Gas foaming</td>
<td>Polyesters, PVA, polyurethanes, biogels (gelatin)</td>
<td>High</td>
<td>Variety</td>
<td>Quite expensive</td>
</tr>
<tr>
<td>Whole organ decell</td>
<td>Organs</td>
<td>High</td>
<td>Heart, liver, lung, etc</td>
<td>Whose organ? Detergents are aggressive</td>
</tr>
<tr>
<td>Tissue decell</td>
<td>Pieces of tissue</td>
<td>High</td>
<td>Many</td>
<td></td>
</tr>
<tr>
<td>Electrospinning</td>
<td>Bioerodable polymers (PLA, PLGA, etc), proteins and gels (collagen, alginate, gelatin)</td>
<td>Very low (&lt;1)</td>
<td>Variety</td>
<td>Gives rise to pseudo 3D &quot;squashed&quot; scaffolds</td>
</tr>
</tbody>
</table>
Cell Printing

• Cell Printing (Boland-inkjet)
• Organ Printing (Mironov-Forgacs)
• Living Inks, bioinks, bioprinter, bioplotter

Olivetti NanoBioJet
Cell dispensers and Bioprinters

Fig. 3. Bioprinters: a) 3D dispensing Laboratory Bioprinter – ‘LBP’ (designed by Neatco, Toronto, Canada in cooperation with MUSC Bioprinting Research Center, Charleston, SC); b) 3D robotic printer – ‘Fabber’ (designed by Cornell University, USA); c) 3D robotic industrial bioprinter — ‘BioAssembly Tool’ (designed by Sciperio/nScript, Orlando, USA).
NanoBioJet for Living Inks

- **2D**...
- *Small volumes* in high spatial resolution patterns
- *Biolink* (i.e. protein based solutions)
- Particle based inks
- *LivingInk* (i.e cell suspensions)

Tirella et. al, *Substrate stiffness influences high resolution printing of living cells with an ink-jet system*. J Biosci Bioeng. 2011
Nozzle system for Living inks

• *pseudo-3D or 3D*

• *Micro-resolution* of viscous biomaterials

• *Complex pattern* and 3D architecture

• Liquid and viscous inks (including Biolnk, particle based inks and LivingInks)
Inkjet vs. direct writing for living scaffolds

**NanoBioJet (inket system)**
- Impact energy on drop
- Importance of substrate stiffness

**PAM2 (nozzle based direct writing)**
- Forces exerted on cell membrane during extrusion
- Volumetric flow rate is more important than impact force

*Tirella A and Ahluwalia A. Biotechnol Prog (2012)*
Organ Printing using cell suspensions as a material


fusion is a ubiquitous process during embryonic development and can be recapitulated in vitro [45]. It has been shown that the kinetics of tissue fusion of two rounded embryonic heart cushion tissue explants placed in an hanging drop fits perfectly to fusion kinetics described for two droplets of fluids [46]. Moreover, based physical laws and Malcolm Steinberg’s “differential adhesion hypothesis” [28–30]. From another point, motile living cells, cytoskeleton and number, and redistribution and activation of cell adhesion receptors are also essential for the tissue fusion process [46,47]. The accumulation of ECM and associated restriction of cell
Live scaffold fabrication

- Direct Fabrication
- Composite materials
- Live Engineered Scaffold
- Cells
- Biomaterial Processing
Nano-in-micro (NIM) Live Scaffold Fabrication

Recreate an *in vitro* microsystem able to interact and monitor living constructs in a non-invasive manner

Assembling:

- **Living micro-spheres** with controlled mechanical and properties and biomimetic composition;
- Having:
  - Cells
  - Tissue matrix
  - Release of known moieties (e.g. ROS, exogenous molecules)
  - **Scavenger properties**
  - **Sensitive detectors**

Spherical Hydrogel Generator

Sensitive/Functional domains can be easily fabricated controlling sphere dimension, shape and composition

Size controlled hydrogel micro-spheres as function of system working parameters and solution properties:

- Solution viscosity (e.g. alginate w/v ratio, NPs concentration, cell concentration)
- Nozzle diameter
- Volumetric flow rate
- External air flow

Shape is fixed via rapid physical gelation, e.g. for alginate microspheres form a gel in a beaker containing a 0.1 M CaCl₂ solution in water.
NIM Live Scaffold

200 μm spheres immersed in buffering solutions
A. pH reversibility detection

B. Calibration curve (spectrofluorimeter vs confocal acquisition)

Alginate hydrogel μsphere
hepatocytes
Digested liver matrix
Ratiometric pH-NSs

ddECM/pH-NS / HepG2 μsphere
Confocal acquisition
DAPI / pH-sensitive / pH-reference

Figure. Reversibility test of pH measurements in alginate micro-spheres including pH sensitive nanoparticles. The starting pH is buffered respectively at 5.8 (A), 7.0 (B) and 8.2 (C).
Future of Live Scaffold Fabrication

Concept: European Bioprinting Network
Scaffold Characterisation

**Without cells**

- **Topological** (porosity, interconnectivity, & related scaffold features)
- **Physico-chemical** (swelling, degradation, ligand release, presentation, ligand localisation)
- **Mechanical**: compressive, tensile, viscoelastic

**With cells**

- In-vitro
- Quasi-vivo
- In-vivo

Wet vs. Dry
Scaffold Characterisation

Topological

**Dry methods**

\[\mu\text{CT scan of a 200-\text{\textmu}m (A) and 500-\text{\textmu}m (B) pore scaffolds. SEM micrographs depicting the scaffold architecture of the 200-\text{\textmu}m (C) and 500-\text{\textmu}m (D) pore scaffolds. In (E) is shown a representative higher magnification image of the scaffold walls as they appear on both types of scaffolds.} \]
Scaffold Characterisation (wet)

Swelling

Mechanical

Mechanical characterisation of soft wet materials

Tirella, Mattei, Ahluwalia, Strain rate viscoelastic analysis of soft and highly hydrated biomaterials, JBMRA, 2013
The problem of characterising living scaffolds

They are alive

They are 3D

Small features

High resolution, non destructive, fast
# 3D characterization

<table>
<thead>
<tr>
<th>Technique</th>
<th>Principle</th>
<th>Depth</th>
<th>Lateral (micron)</th>
<th>Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrasound (20 MHz)</td>
<td>Acoustic impedance</td>
<td>20 cm</td>
<td>250</td>
<td>no</td>
</tr>
<tr>
<td>Microscope</td>
<td>Phase/Transmitance</td>
<td>100 µm</td>
<td>5-10</td>
<td>no</td>
</tr>
<tr>
<td>Fluorescent microscope</td>
<td>Fluorescent label</td>
<td>50 µm</td>
<td>5</td>
<td>yes</td>
</tr>
<tr>
<td>Confocal</td>
<td>Laser scanning, confocal planes</td>
<td>100-200 µm</td>
<td>1</td>
<td>yes</td>
</tr>
<tr>
<td>OCT</td>
<td>Interferometry (optical impedance)</td>
<td>Several mm</td>
<td>100</td>
<td>no</td>
</tr>
</tbody>
</table>
Measures difference in path length between reference and sample beam. Highly focused white light source. The back-scattered light travels to the detector where the unique phase delay for each wavelength is detected. Depth information is acquired using a Fast Fourier Transformation.

Fercher et al.  