Virtual and Physical Prototyping

Design, physical prototyping and initial characterisation of 'lockyballs'

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This paper reports the fabrication of interlockable microscale scaffolds using two photon polymerization (2PP) and proposes a "lockyball" approach for tissue self-assembly for biofabrication

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Directed tissue self-assembly or bottom-up modular approach in tissue biofabrication is an attractive and potentially superior alternative to a classic top-down solid scaffold-based approach in tissue engineering. For example, rapidly emerging organ printing technology using self-assembling tissue spheroids as building blocks is enabling computer-aided robotic bioprinting of three-dimensional (3D) tissue constructs. However, achieving proper material properties while maintaining desirable geometry and shape of 3D bioprinted tissue engineered constructs using directed tissue self-assembly, is still a challenge. Proponents of directed tissue self-assembly see the solution of this problem in developing methods of accelerated tissue maturation and/or using sacrificial temporal supporting of removable hydrogels. In the meantime, there is a growing consensus that a third strategy based on the integration of a directed tissue self-assembly approach with a conventional solid scaffold-based approach could be a potential optimal solution. We hypothesise that tissue spheroids with 'velcro®-like' interlockable solid microscaffolds or simply 'lockyballs' could enable the rapid *in vivo* biofabrication of 3D tissue constructs at desirable material properties and high initial cell density. Recently, biocompatible and biodegradable photo-sensitive biomaterials could be fabricated at nanoscale resolution using two-photon polymerisation (2PP), a development rendering this technique with high potential to fabricate 'velcro®-like' interlockable microscaffolds. Here we report design studies, physical prototyping using 2PP and initial functional characterisation of interlockable solid microscaffolds or so-called 'lockyballs'. 2PP was used as a novel enabling platform technology for rapid bottom-up modular tissue biofabrication of interlockable constructs. The principle of lockable tissue spheroids fabricated using the described lockyballs as solid microscaffolds is characterised by attractive new functionalities such as lockability and tunable material properties of the engineered constructs. It is reasonable to predict that these

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building blocks create the basis for a development of a clinical *in vivo* rapid biofabrication approach and form part of recent promising emerging bioprinting technologies.

Keywords: organ printing; lockyballs; tissue spheroids; two-photon polymerisation

1. Introduction

Organ printing is a rapidly emerging computer-aided robotic layer-by-layer additive biofabrication of three-dimensional (3D) functional human tissue and organ constructs using self-assembling tissue spheroids (Mironov *et al.* 2003, Mironov *et al.* 2008, Mironov *et al.* 2009). The tissue spheroids could be used as building blocks in organ printing technology due to their intrinsic capacity to fuse (Mironov *et al.* 2009). Tissue fusion driven by surface tension forces is an ubiquitous process during embryonic development (Pérez-Pomares and Foty 2006) and at the same time it constitutes a fundamental biological and biophysical principle of organ printing technology (Jakab *et al.* 2004, Mironov *et al.* 2009). However, it has been demonstrated that the kinetics of tissue spheroid fusion depends on their material properties (Rago *et al.* 2009). Tissue spheroids with increased stiffness fuse much slower (Hajdu *et al.* 2010). Thus, the development of alternative methods of tissue bioassembly using different approaches is highly desirable.

Recently, two-photon polymerisation (2PP) has been introduced as a novel technology for the biofabrication of scaffold for tissue engineering (Kasko and Wong 2010, Schade *et al.* 2010, Tayalia *et al.* 2011). Several biocompatible materials have been successfully employed for scaffold fabrication using 2PP (Infuehr *et al.* 2007, Stampfl *et al.* 2008, Correa *et al.* 2009, Ovsianikov *et al.* 2011a). It has been shown that 2PP enables the fabrication of delicate microscaffolds from gelatin-based enzymatically degradable material at a high level resolution (Ovsianikov *et al.* 2011b). The concept of so-called digital materials or material capable of self-assembly using locking mechanisms (Hiller and Lipson 2009, 2010) and directly assembled cell-laden hydrogel modules (Du *et al.* 2008, Nichol and Khademhosseini 2009, Fernandez and Khademhosseini 2010) have been recently introduced as new perspective bottom-up modular approaches in tissue biofabrication and tissue engineering of functional 3D tissues. Moreover, it has been suggested in a recent influential review that the '...directed tissue assembly process possesses immense potential for advancing the field of tissue engineering and there will be increased integration of directed assembly-based approach with conventional scaffold-based approach to create more complex functional tissue with physiological architecture for clinical applications' (Kachouie *et al.* 2010).

We hypothesise that tissue spheroids with 'velcro®-like' interlockable microscaffolds ('lockyballs') fabricated using 2PP from biodegradable, and in certain case non-biodegrad-

able biomaterials, materials could enable the rapid biofabrication of 3D tissue constructs with desirable material properties. Thus, the new approach will allow the limitation of relatively slow tissue fusion kinetics in the case of tissue spheroids with advanced material properties to be overcome. Furthermore, it will enable the rapid assembly of 3D tissue constructs with mechanical properties that can be tailored over a wide range to meet specific demands. In this paper we present the results of the initial geometrical design studies and the experimental functional testing of the lockability of large lockyballs. Shortly, lockyballs could potentially solve two outstanding problems of emerging directed tissue self-assembly approaches in tissue engineering: i) assembly of rigid tissue spheroids with slow kinetics of tissue fusion, ii) provision of desirable and tunable material properties for 3D tissue engineered constructs facilitated by the biofabrication from lockable tissue modules. In addition the bottom-up nature of employed variants of solid scaffolding will not compromise the high cell density typical for tissue spheroids. Their unique 'processability' or capacity to be 'dispensable', 'sprayable', 'placable', 'injectable' and even 'printable' as well as their ability for directed tissue self-assembly or capacities for 'bio-assembly' and to be 'fusable' can be maintained with the proposed approach.

2. Materials and methods

2.1 *Computer-aided designing of lockyballs*

Computer-aided software, 3D Studio Max 2009 (Autodesk®), was used to design three different variants of lockyballs. Virtual locking of two lockyballs has been extensively used for designing the virtual screening and the optimisation of the best lockability.

2.2 *Fabrication of lockyballs using selective laser sintering*

Lockyballs with diameters of 6 cm were fabricated at the Center for Information Technology Renato Archer (CTI) from Polyamide Nylon-6 using a selective laser sintering (SLS) rapid prototyping machine HIQ (High Quality). Fifteen lockyballs of three different designs were fabricated.

2.3 *Estimation of the lockability of the lockyballs*

The lockability of fabricated lockyballs was tested experimentally using simple manual opposition-based locking of two lockyballs and pressure-mediated locking of multiple lockyballs placed in a confined space.

2.4 Fabrication of 2D and 3D constructs from lockyballs

The lockyballs have been used to manually fabricate scaffold of different desirable geometry. Lockyballs were photographed in two positions before and after bending. The bending angles were measured on correspondent images.

2.5 Estimation of the bending angle of two locked lockyballs

The bending angle of two locked lockyballs with different degrees of locking has been measured on a photograph before and after bending. At least 10 bending experiments were performed for every type (hook-to-hook or hook-to-loop) and degree (number of locking points) of locking.

2.6 Measurement of the material properties of scaffolds fabricated from locked lockyballs

The material properties of rod-like scaffolds constructed from lockyballs were estimated using the three points flexure test. Rod-like linear structures fabricated from six interlocked lockyballs were used for the flexure test and six monolithically printed lockyballs were used as reference.

2.7 Fabrication of microlockyballs using two-photon polymerisation

The lockyball structures were produced by 2PP of Zr-based hybrid photopolymer. The detailed description of the material synthesis can be found elsewhere (Ovsianikov *et al.* 2008a). For the presented experiments 0.2 wt.% of the photoinitiator (4,4'-bis(diethylamino) benzophenone; Sigma-Aldrich) was added to the material. For 2PP a Ti:sapphire laser (Femtotrain EC-800-100FS, HighQ) delivering 100 fs pulses at a repetition rate of 73 MHz at around 810 nm was used. The rest of the experimental setup is similar to the one reported previously (Ovsianikov *et al.* 2008b). The laser beam was focused into the material by a conventional 20 x microscope objective (NA = 0.8; Carl Zeiss). The structures were produced in a layer-by-layer fashion, with the computer-aided design (CAD) model (STL format) sliced into 1 μm thick layers. Each layer was produced by patterning in a linear scanning fashion at a distance of 0.5 μm between the neighbouring scans. An average laser power of 400 mW at the scanning speed of 5 mm/s was used to induce 2PP. The unpolymerised material was removed by a 50/50 blend of 1-propanol and isopropanol. Due to a slight material shrinkage a reduction of the lockyball's diameters by about 4%, compared to the CAD model dimensions, was observed (Ovsianikov *et al.* 2009). For the scanning electron microscope (SEM) imaging (Quanta FEI) the samples were sputtered with a 15 nm thick Pd/Au coating.

2.8 Scanning electron microscopy

After sputtering with a gold/palladium coating, microlockyballs fabricated by 2PP were observed at different magnifications by means of SEM (FEI Quanta). Light and laser scanning microscopes (LSM 600, Zeiss) were used for preliminary analysis.

2.9 Statistical analysis

A standard software (NIH ANOVA) was used for the statistical analysis of the measurement of bending angles and the three point bending test. At least 10 measurements were used for every experiment.

3. Results

3.1 Lockability testing

It has been shown that only one of three proposed original lockyballs designs (Figure 1) shows a desirable level of lockability functionality under applied pressure. An increasing number of hooks interferes with the capacity for locking. The spatial separation of hooks and loops (elevated pentagons) provided the best interlocking results. Two basic mechanisms of locking include hook-to-hook locking and hook-to-loop locking (Figure 2). 'Hook-to-hook' locking provides the maximal possible bending angle, whereas the 'hook-to-loop' locking mechanism dramatically reduces the bending angle or the rotation mobility of locked lockyballs (Figure 3). Furthermore, it could be shown that locking-induced immobilisation of two lockyballs is a direct function of the types and numbers of individual locking sites (Figure 4).

3.2 Fabrication of 2D and 3D constructs using lockyballs

Lockyballs with a flexible moderate level of locking enable the fabrication of linear and 2D constructs such as rods, rings or toruses, 2D sheet-like structures and 3D constructs such as tubes, cubes and spirals (Figure 5).

3.3 Material properties of rod-like constructs fabricated from lockyballs

Material properties of the rod-like constructs fabricated from locked lockyballs have been estimated using the three point flexure test. The data was compared with the material properties of fabricated continuous constructs having similar geometry and size. The material properties of the constructs fabricated by 'hook-to-loop' locked lockyballs are comparable with the material properties of the continuous 'monolithic' constructs. The three point bending test has been performed to estimate the deviation from the
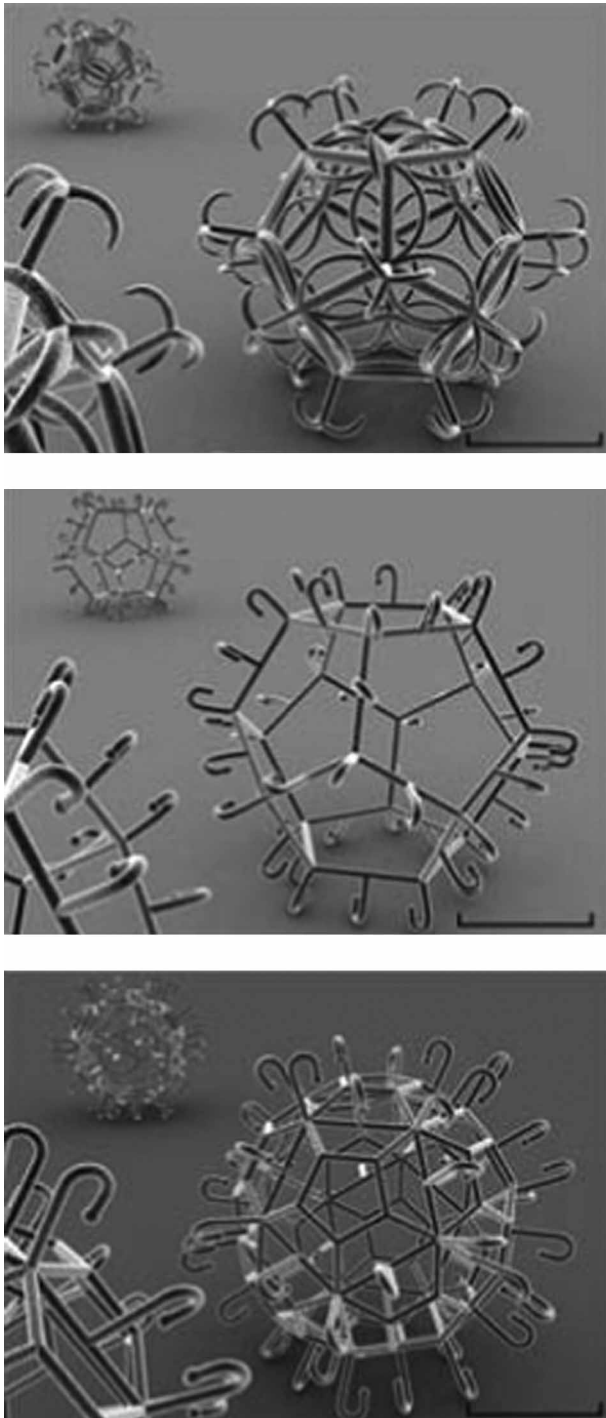
Figure 1. Computer-aided design of three types of lockable microscaffold (lockyballs). Scale bar = 3 cm.

interlocked constructs' linearity when stressed by bending. Manually interlocked linear constructs and monolithic printed linear constructs have been taken as reference. Each tested linear constructs consisted of five lockyballs. Bending has been realised by sequentially loading with 449 g, 898 g and 1347 g. The manually interlocked and monolithically printed linear constructs' ends were fixed. The fixed constructs were sequentially loaded and bended with one (449 g), two (449 g x 2) and three (449 g x 3) standard weight-blocks. The deflection or bending displacement of linear constructs under load has been measured with respect to a reference in the middle of the bended constructs (Figure 6).

3.4 *Light microscopy, confocal microscopy and scanning electron microscopy of microlockyballs*

The diameter of cells is much smaller than pores in lockyballs (Figure 7). Data gained by light, confocal and scanning electron microscope measurements of the specimens confirmed that the level of porosity of microlockyballs fabricated by the 2PP method is permissible for the cell seeding and thus for the biofabrication of living tissue spheroids inside lockyballs from cell suspension.

3.5 *Virtual cell seeding experiment*

In order to estimate how many cells could be placed into lockyballs we assumed that realistic cell diameters could be 10, 15 and 20 μm. Furthermore, we assumed that cells do not change their volume during the formation of cell aggregates or tissue spheroids. Even if the internal diameter of the lockyball will be as small as 150 μm, the number of cells inside the formed tissue spheroids will be correspondently 3375, 1000 and 422 after maximal cell packing. This relatively simple virtual experiment indicates that cells of different diameters can seed into the lockyball to form tissue spheroids without any problems.

4. Discussion

The main goal of this study was to develop the foundation of a novel technology or an enabling technological platform which allows the combination of the advantages of two competing approaches in tissue engineering: the conventional solid scaffold-based approach as well as the rapidly emerging novel bottom-up directed tissue self-assembly approach (Kachouie *et al.* 2010). Classic biomimetic 'velcro®' technology has been used as an inspiration and as a starting point for the development of a lockyball-based, bottom-up modular tissue engineering (technological) platform. More specific, the focus of this study was to design, fabricate, characterise and test novel solid lockable scaffolds for bottom-up modular tissue engineering.

We have tested different designs of mechanical locking mechanisms. It has been shown that locking under pressure is probably the most attractive form of locking. We tested different types of geometrical configurations that can enable an interlocking mechanism. Traditional classic
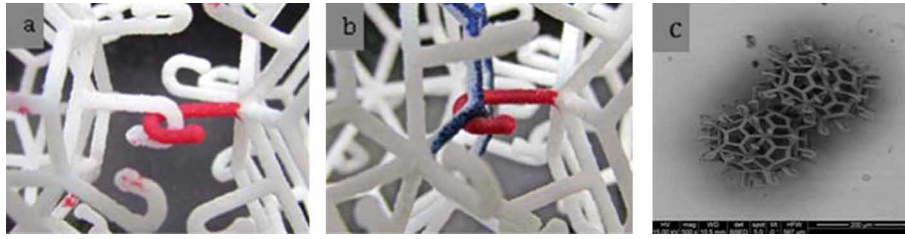
Figure 2. Two types of locking mechanism: (a) hook-to-hook and (b) hook-to-loop locking. (c) Scanning electron micrograph of two interlocked lockyballs.
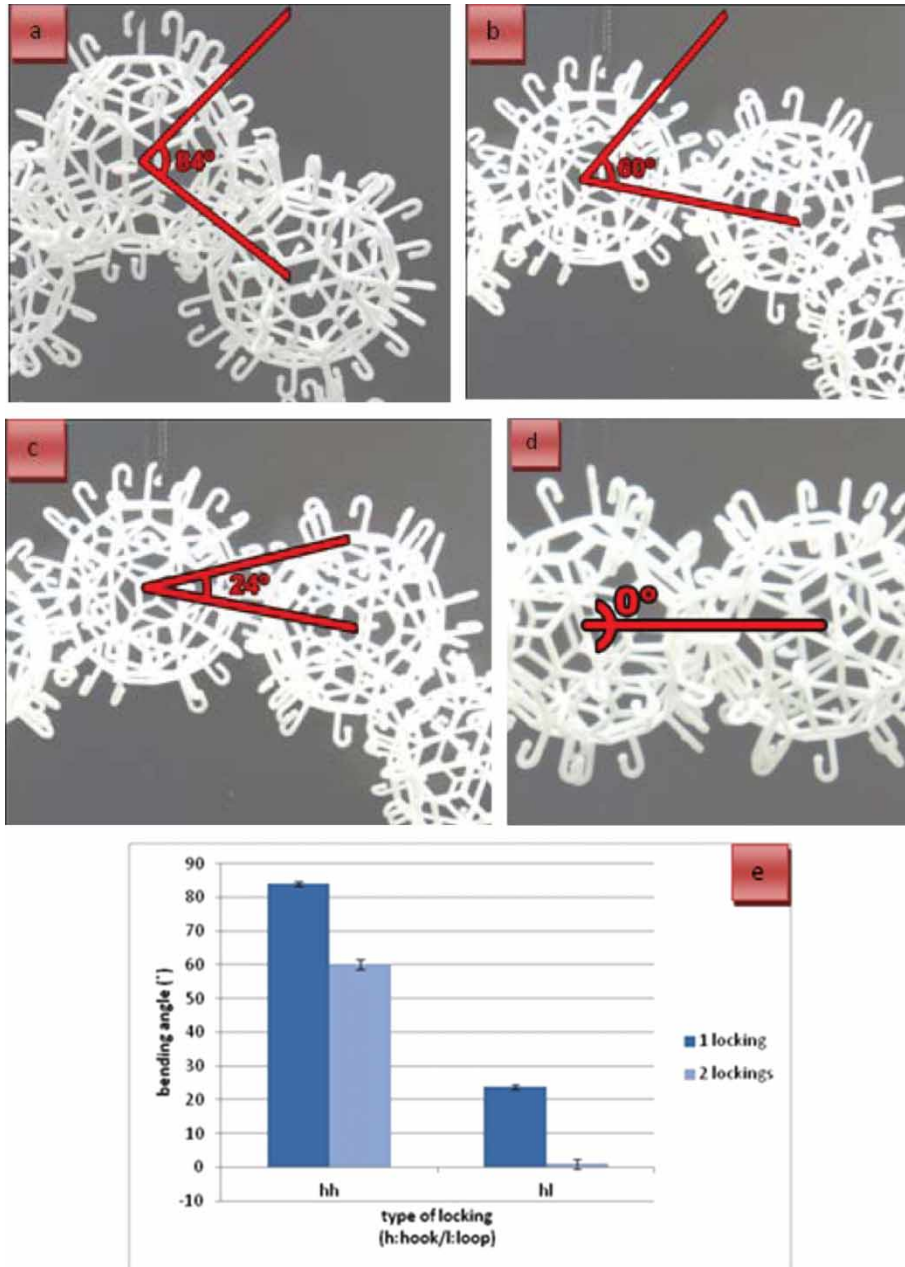

Figure 3. Estimation of bending angle of locked lockyballs (diameter = 6 cm): (a-b) bending angle for two lockyballs with (a) one hook-to-hook locking (84°) and (b) two hook-to-hook lockings (60°); (c-d) bending angle for two lockyballs with (c) one hook-to-loop locking (24°), (d) two hook-to-loop lockings (0°) and (e) bending angle as a function of locking type with standard deviation.
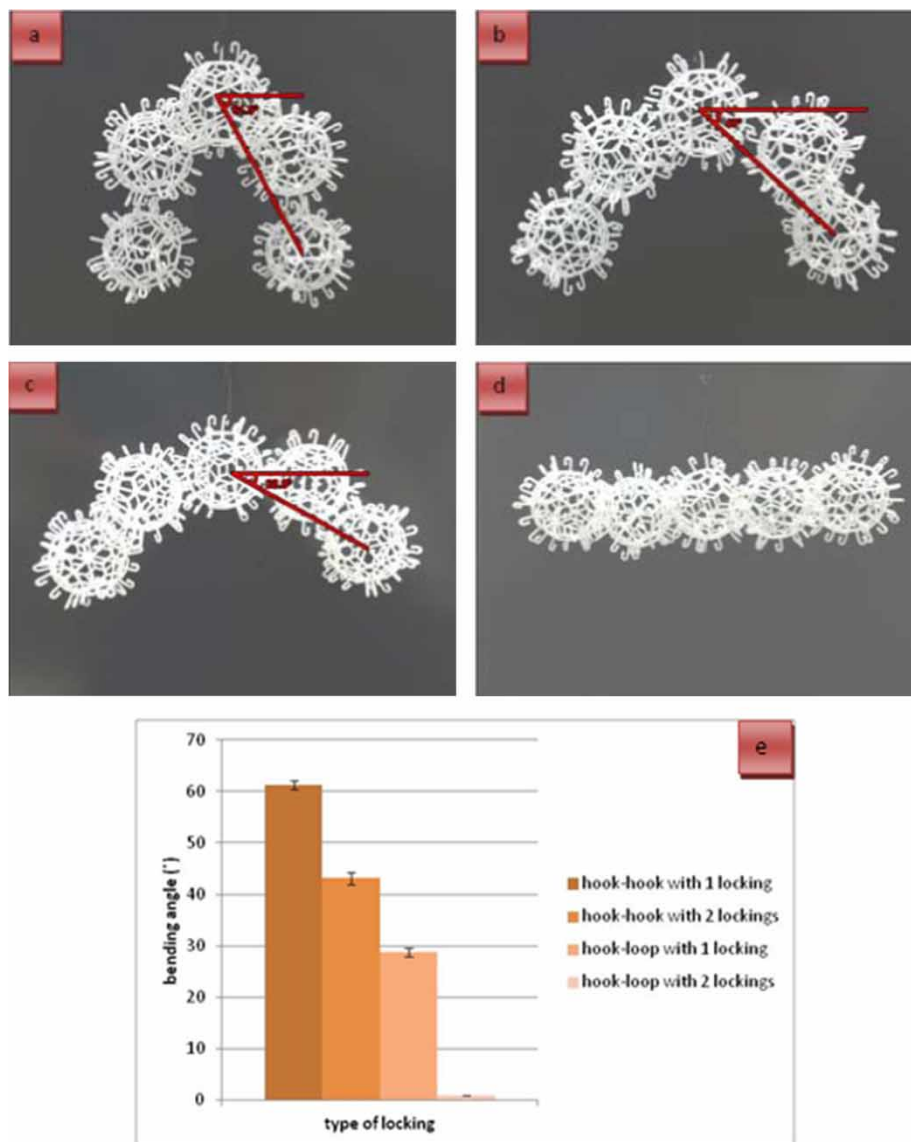
Figure 4. Bending angle as a function of number of locking sites: (a-d) representative experimental data [a) 61.5°, b) 43°, c) 28.8°] and (e) bending angle as a function of number of locking sites with standard deviation (lockyballs with 6 cm diameter).

velcro® devices consist of two types of strips: one containing only hooks and another only loops. The initial logical idea was to fabricate two types of lockyballs: one type with only hooks and another with loops. However, in this case the effective locking will require proper redistribution or precise placing of hooks-balls and loops-balls. The sphere-like design was selected based on the assumptions that a precise, expensive and potentially slow placement device would be needed to handle the opposition hook populated surface and the loop populated surface of the miniscaffolds of different geometries. We decided to design sphere-like lockyballs with both types of locking structures (hooks and loops) on the same ball. A high density of hooks prevents an effective locking of hooks to loops. Accordingly, zones

or areas on the ball containing hooks and loops were spatially separated. The elevated pentagon was selected as the most effective analogy to velcro® loops.

Our data show that there are two basic locking mechanisms: i) hook-to-hook (H-H) locking and ii) hook-to-loop (H-L) locking. The H-H locking provides a higher freedom of bending of two locked lockyballs with maximal bending angle. The H-L locking mechanism provides much stronger fixation demonstrating lower bending angle. Finally, it has been shown that the bending angle is a direct function of the types of locking and the number of locking sites. Higher bending angles provide an opportunity to create circular constructs such as toruses, tubes or spirals. Locking not only requires proper opposition but also pressure and likely
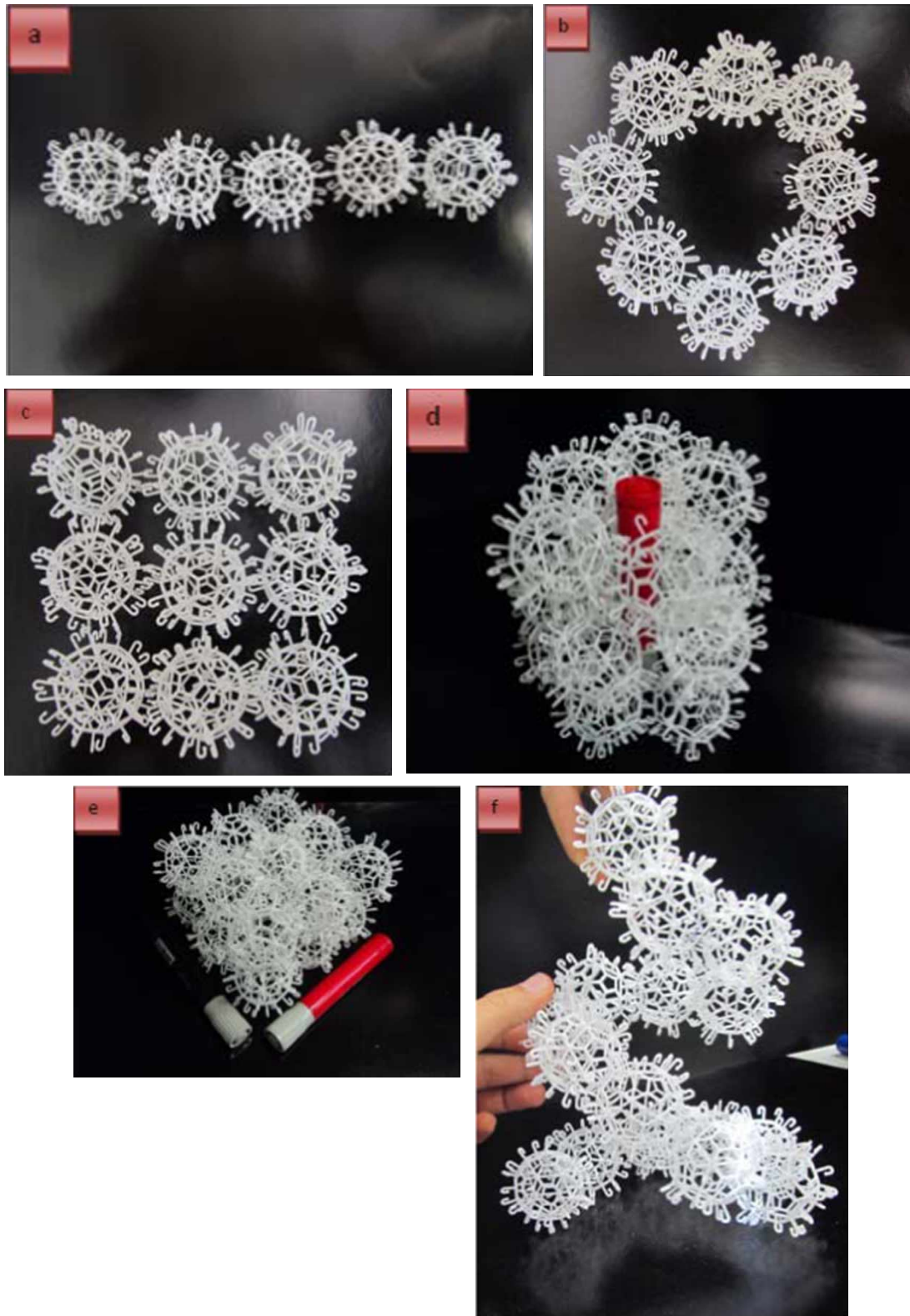
Figure 5. Fabricated 2D (a-c) and 3D (d-f) constructs: a) rod; b) ring; c) sheet; d) tube; e) cube; f) spiral (lockyballs with 6 cm diameter).
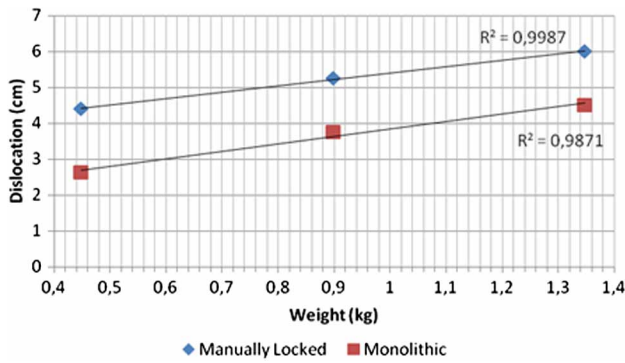
Figure 6. Comparative material properties of assembled and monolithic rod-like constructs under three point flexure test: dislocation caused by weight.

associated rotation. Locking under pressure has been confirmed for multiple lockyballs placed into a confined space.

Lockyballs enable the fabrication of diverse 3D constructs of desirable geometrical configuration like rods, toruses, sheets, tubes, spirals and cubes. Theoretically, lockyballs can be randomly placed into cartilage or bone defects of irregular form. They can fill these defects using random sphere packing mechanisms. By applying external manual pressure, they can be integrated into single constructs. Finally, fibrin hydrogel can be used to fill the remaining gap. Mechanical testing experiments confirmed that the material properties of such 'assembled'-by-locking constructs are comparable with the material properties of monolithic (one block) constructs of similar geometrical configuration. Linear chain-like constructs fabricated from

lockyballs with H-H locking mechanisms can be used to assemble tubular spiral-like constructs. The spiral-like approach can be effectively used for the rapid biofabrication of branched segments of vascular trees.

For initial testing and the selection of the optimal design, selective laser sintered macrolockyballs with diameters of 6 cm have been used as a geometrically identical prototype of the minilockyballs. It has been shown that the first designs were functionally not optimal. Thus, only the third design of microlockyballs was selected for 2PP with microlockyballs of 150 μm in diameter. 2PP has been successfully employed for the biofabrication of biocompatible scaffolds for tissue engineering (Kasko and Wong 2010, Schade *et al.* 2010, Tayalia *et al.* 2011). It has been shown that living cells can attach, survive, migrate, proliferate and differentiate on these microscaffolds (Ovsianikov *et al.* 2011b). Moreover, due to the high precision level of porosity, the porous microscaffolds fabricated by 2PP have been suggested as ideal objects for examining the effects of pore size on cell migration (Tayalia *et al.* 2011) and for the evaluation of physical forces attached cells create on the delicate scaffold structures. The development of new photo-sensitive biomaterials for two-photon scaffold fabrication is a booming area of research (Infuehr *et al.* 2007, Correa *et al.* 2009, Ovsianikov *et al.* 2011a, Ovsianikov *et al.* 2011b). Recently, biocompatible gelatin (Ovsianikov *et al.* 2011b) and chitosan (Correa *et al.* 2009) based/derived photopolymers have been successfully fabricated by means of 2PP. It has been demonstrated that the material properties
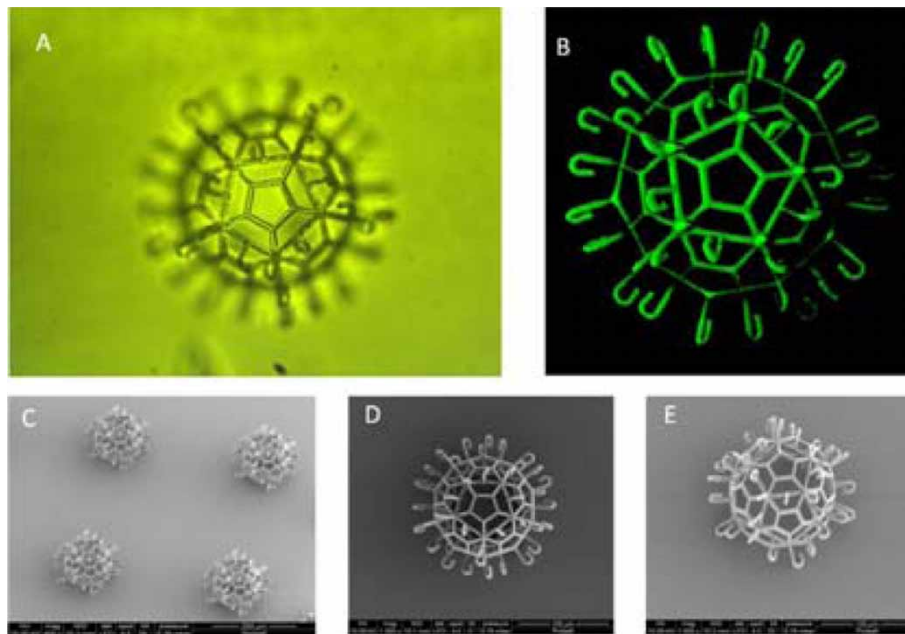

Figure 7. Light microscopy (a), confocal microscopy (b) and scanning electron micrographs (c,d,e) of lockyballs microscaffold fabricated by means of two-photon polymerization technology.
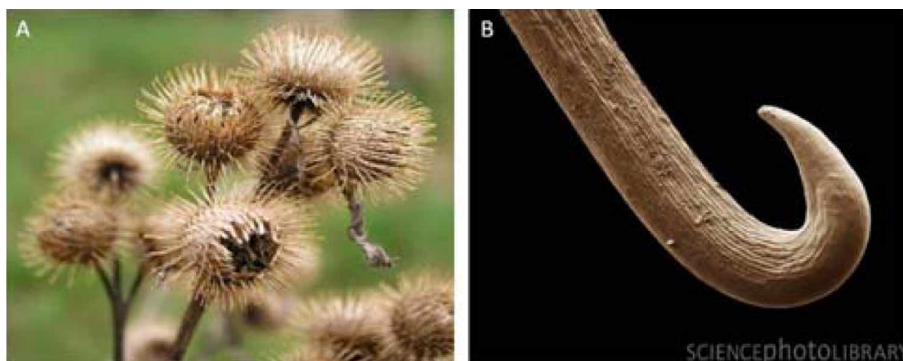
Figure 8. Biomimetic approach: a) burdock locking hook structure (http://www.indiamart.com/phytohomeoremedies/products.html); b) scanning electron microscopic photograph of burdock hook (http://www.sciencephoto.com/image/79690/530wm/C0015288-Hook_of_Burdock_Bract-SPL.jpg).

of these scaffolds can be tailored over a wide range to match the specifications of potential applications (Infuehr *et al.* 2007; Stampfl *et al.* 2008). Thus, one of the main advantages of two-photon polymerised lockyballs as a solid microscaffold is a combination of a bottom-up modular tissue engineering approach together with the capacity to fit desirable material properties of 3D tissue constructs by particular interlocking of lockyballs.

The presented data demonstrates that the selected and the implemented geometrical design of the interlocking mechanism is sufficiently effective at least under applications of pressure. The further potential improvement and the optimisation of the interlocking mechanisms' design are possible and desirable. As in the case of 'velcro®', it is important to be inspired by already existing natural solutions of locking. Learning as much as possible from Mother Nature in future biomimetic approaches is the key issue to design the next generation of lockyballs (Figure 8).

Some potential geometric designs of hooks for interlocking microscaffolds are summarised in Figure 9.

Another potential great advantage of lockable microscaffold or micro-lockyballs is related to their very small size and their high level of porosity. We anticipate the logical combination of lockyball technology with the recently developed technology of scalable robotic biofabrication of tissue spheroids using moulded non-adhesive hydrogel (Figure 10). In this case, lockyballs will be placed in recessions with similar diameters fabricated in non-adhesive hydrogels such as agarose and later cell suspension will be robotically dispensed (Napolitano *et al.* 2007; Nagy-Mehesz *et al.* 2011). The lockyball microscaffold will be encapsulated or embedded into resulting self-assembled tissue spheroids (Figure 11).

Sequential incubation of these tissue spheroids (with encapsulated microlockyballs) with specific differentiation factor cocktails will allow the generation of chondrospheres
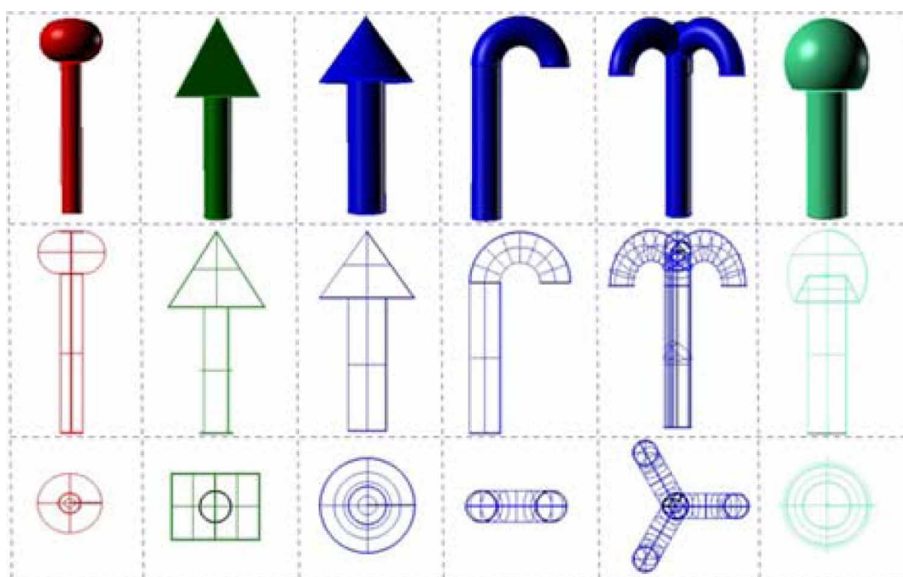

Figure 9. Scheme depicting different types of biomimetically inspired arrows (hooks) for lockyball locking.
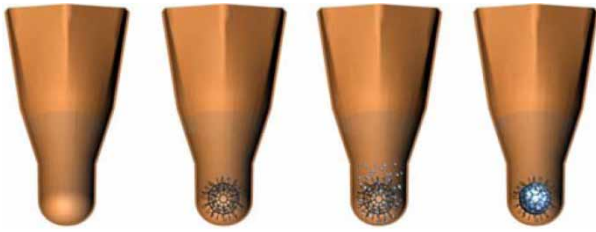
Figure 10. Schemes demonstrating biofabrication of lockable tissue spheroids in moulded non-adhesive hydrogel.

or osteospheres examples with strong material properties. However, in this case, the propensity for low speed tissue fusion due to the rigidity and the stiffness of these types of tissue spheroids will not undermine or interfere with their new lockable microscaffold mediated capacity to be rapidly assembled into 3D tissue constructs. Lockable osteospheres or chondrospheres can be potentially ideal filler biomaterials for cartilage and bone regeneration and *in vivo* tissue engineering. It is important to mention that scalable robotic biofabrication of tissue spheroids as well as the biofabrication of osteospheres and chondrospheres is already a well-established and proven technology (Schubert *et al.* 2009, Nagy-Mehesz *et al.* 2011, Osteosphere, LLC 2011). At least five companies are already involved in the commercialisation of the different variants of tissue biofabrication technologies. Moreover, chondrospheres are already in clinical trial in Europe as tissue engineered therapy for cartilage diseases (see for details codon, Germany website: http://www.codon.de/). Lockable tissue spheroids could be the potential next
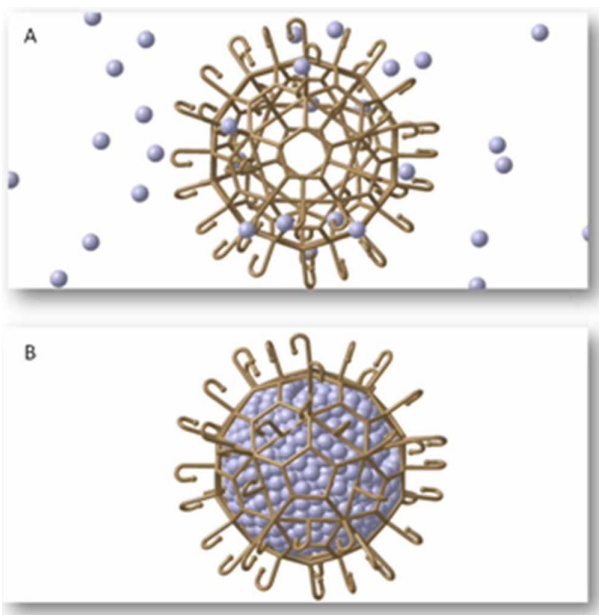

Figure 11. Schemes demonstrating virtual experiment with cell seeding and tissue spheroid formation inside lockyball a) cells seeding in lockyball; b) formation of tissue spheroid inside lockyball (or lockable tissue spheroids).

generation for more sophisticated and most essentially living filler biomaterials for skeletal tissue engineering.

There are several main directions in the development and the optimisation of the proposed minilockyball scaffolds technological platform. Novel photo-sensitive biomaterials with tunable material properties can be incorporated with carbon nanotubes. The functionalisation of lockyball microscaffolds for the enhancement and the acceleration of cell's attachment, their spreading and their seeding as well as the provision of new functionalities, such as electroconductive coating or coating with inducible magnetic properties, are other potential areas of research. Encapsulation and cryopreservation of composite lockable tissue spheroids are also promising areas of further investigations. Finally, the effective vascularisation of lockable tissue spheroids will allow the increasing of their size.

The lockable tissue spheroids or the pre-cellularised lockyballs will represent an excellent example of the postulated integration of the classic solid scaffold-based approach in tissue engineering with a rapidly emerging bottom-up modular tissue engineering approach based on the directed tissue self-assembly (Kachouie *et al.* 2010). 2PP, enabling the fabrication of microlockyballs, is a very promising and powerful technological platform for the bottom-up composite 3D tissue engineering. Moreover, the integrative approach based on the usage of pre-cellularised lockyballs or lockable tissue spheroids renders a broader selectable range of mechanical properties and will enable a cost-effective rapid *in vivo* biofabrication of 3D composite tissue constructs. The lockable tissue spheroids fabricated using lockyballs as solid microscaffolds could be utilised as more sophisticated building blocks due to several new functionalities such as their lockability, their tunable material properties that can carry out the development of an *in vivo* rapid biofabrication approach and subsequently, a bioprinting technology for clinical regenerative medicine (See related scheme on Figure 12).

It is important to mention, however, that effective interlocking is a function of the selection of a proper geometry and the distribution of 'hooks' and 'loops' as well as their optimal spatial redistribution. For example, the idea of so-called 'elevated pentagons' with a 'loop' function was crucial for implementing the design of the lockyballs and for achieving the effective interlocking. The selection of the proper material properties of the employed biomaterials and the diameters of the hooks and loops could also be potentially very important.

We would like to specially outline that the fabrication of lockyballs with inducible magnetic properties can eventually lead to the development of a novel type of magnetic digital bioprinter with magnetic lockyballs as a digital material (Hiller and Lipson 2009, 2010). The development of a magnetic digital bioprinter will be one of the most
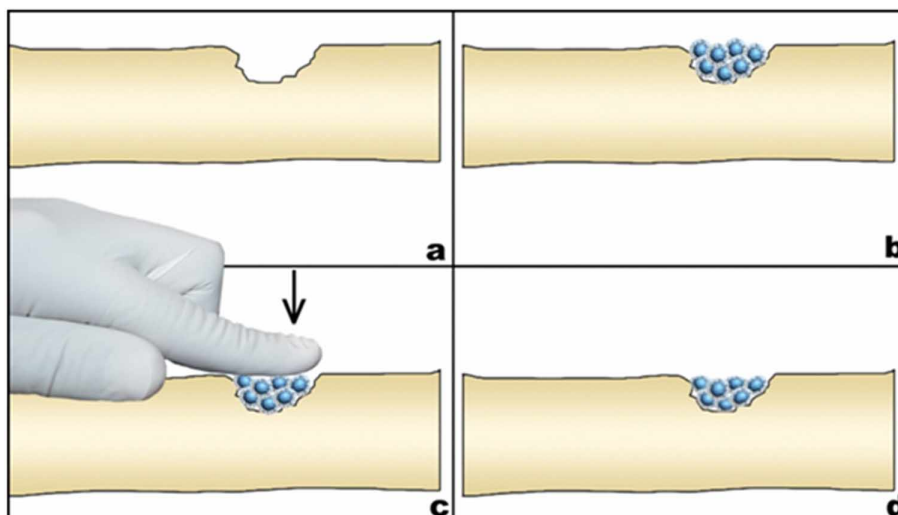
Figure 12. Schemes demonstrating *in vivo* rapid 3D tissue biofabrication using lockable tissue spheroids.

exciting applications of the lockyballs technological platform in tissue engineering.

Our data demonstrated a principal feasibility of two-photon polymerisation-based biofabrication of lockyballs using commercially available organic-nonorganic photosensitive polymers such as SZ2080 Ormosil (organically modified silica) and Ormocer (organically modified ceramics). Previously published data on mechanical testing of constructs fabricated from these photosensitive polymers have shown sufficient levels of their material properties to ensure their function as a scaffold and enabling the mechanism of lockyballs interlocking. For example, nanoindentation testing of Ormocer® US-S4 and for Ormocor demonstrated Young's modulus values of ∼90 MPa and ∼2100 MPa, respectively (Doraiswamy *et al.* 2010). Considerable viscoelastic/plastic creep as well as viscoelastic recovery behaviour were observed during test-

ing. The high hardness and modulus values observed in Ormocer® US-S4 material may be attributed to the strong interlinkages between ceramic and polymer components in the material. Moreover, material properties of scaffold fabricated by two-photon polymerisation technology could be tuned to a desirable level by modification of chemical composition of Ormosil and Ormocer. It has been also shown by several independent groups that these polymers have a high level of biocompatibility (Figure 13). All tested scaffolds engineered from acrylate based AKRE37 and hybrid organic–inorganic SZ2080 (Ormosil) as well as Ormocore b59 and biodegradable PEG-DA-258 photopolymers were biocompatible and thus can be characterised as being suitable for artificial tissue engineering (Doraiswamy *et al.* 2010, Raimondi *et al.* 2012).

Finally, it has been shown on fabricated scaffold of similar geometry that even pores of 20 micrometres
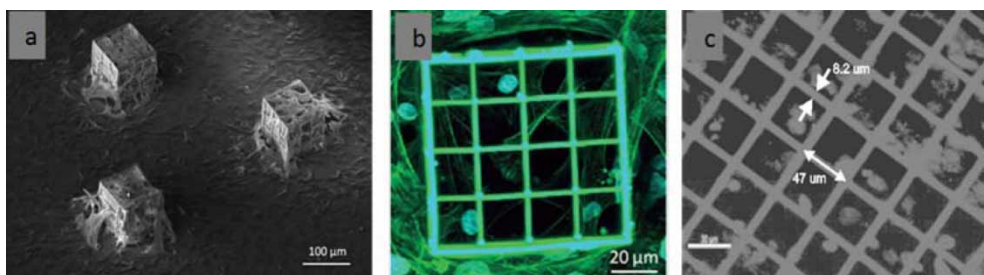

Figure 13. a) Scanning Electron Micrograph (SEM) of MG63 osteosarcoma cells cultured for six days on synthetic structural niches engineered by 2PP in the SZ2080 photoresist. Cells have invaded the external walls and internal volume of the niches with extensive adhesion according to Raimondi *et al.* (2012; b) Fluorescence images acquired by confocal microscopy at six culture days on mesenchymal stem cells (MSC) populated synthetic niches engineered by 2PP in the SZ2080 photoresist. The cell nuclei stain blue and the cytoskeletal actin stains green. Cells adhere extensively to the niche internal lattice and show a roundish morphology: 20 mm lattice, according to Raimondi *et al.* (2012; c) Fluorescence microscope images of adult rabbit myogenic stem cells *in vitro* (highlighted 4′,6-diamidino-2-phenylindole (DAPI) stained nucleuses having size of 10 μm and invisible cell body) expanded in the 2D artificial scaffolds fabricated out of SZ2080, according to Malinauskas *et al.* (2010).
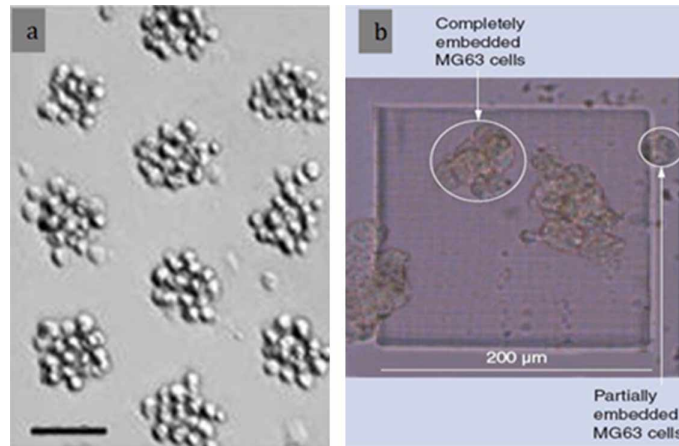
Figure 14. Dielectrophoresis cell patterning and living cells encagement by two-photon polymerisation. a) Clustering of bovine chondrocyte into cell aggregates by dielectrophoresis (according to Albrecht *et al.* 2006), scale bar = 100 micrometres; b) Encaging of living cell cluster into photo-sensitive hydrogel by two-photon polymerisation (according to Ovsianikov *et al.* 2012), scale bar = 200 micrometres.

diameter are sufficient for cell invasion into scaffold (Raimondi *et al.* 2012). In our case, the diameter of pores in lockyballs was 30 micrometres and it could be also increased. Thus, already reported material properties of scaffolds fabricated from organically modified photo-sensitive polymers using two-photon polymerisation and their confirmed biocompatibility strongly indicates that a designed and fabricated lockyballs miniscaffold could be successfully used in clinical tissue engineering (paper in preparation).

Cellularisation or placing of tissue spheroids inside lockyballs is not a trivial issue. We envision three principal approaches to encaging tissue spheroids inside lockyballs.

The first approach involves placing already fabricated tissue spheroids inside photo-sensitive hydrogel and then forming lockyballs around them using two-photon polymerisation. Moreover, tissue spheroids could be formed
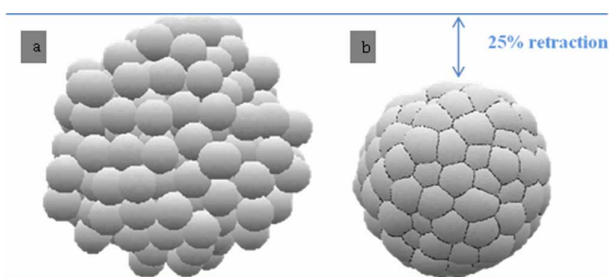

Figure 15. Computer simulation of cell aggregates compaction into cohesive tissue spheroids using open source Surface Evolver software (see. http://www.susqu.edu/brakke/evolver/evolver.html). a) Cell aggregate before compaction; b) Cell aggregate after compaction and evolution into compact cohesive tissue spheroid with 25% reduction of diameter.

directly in photo-sensitive hydrogel using dielectrophoresis technology with sequential encaging of cell clusters into lockyballs fabricated by two-photon polymerisation. The development of sophisticated cell patterning technique using dielectrophoresis technology and special masks by Prof. Sangeeta Bhatia's group at Massachusetts Institute of Technology (Albrecht *et al.* 2006) (Figure 14a) and our preliminary data on encapsulation of living cell clusters into microscaffolds (see Figure 14b from our recent review about two-photon polymerisation technology, Ovsianikov *et al.* 2012) strongly indicate that it is a technologically feasible approach.

The second approach is much more simple and based on using a non-adhesive hydrogel mould which has been used for biofabrication of tissue spheroid of standard size (http://www.microtissues.com/). However, even in this approach the critical issue is a careful adaptation of diameter of lockyballs with diameter of microrecession in non-adhesive agarose hydrogel. One potential problem with this approach is that lockyball hooks could be embedded into tissue spheroids. However, our theoretical modelling and computer simulation using Surface Evolver software indicates that during compaction of cell aggregates into tissue spheroid, diameter of tissue spheroids will be 25% smaller (Figure 15). Thus, lockyball hooks after tissue spheroid compaction will be exposed (Figure 16).

The third approach is based on using magnetic levitation and lockyballs functionalised with magnetic nanoparticles. Again it is not a trivial approach. Firstly, we must fabricate functionalised lockyballs labelled with magnetic nanoparticles. Secondly, it is essential in this approach that there is optimal adaptation of lockyballs pore size to diameter of human chondrocytes. We estimated that the average diameter of human chondrocyte is around 12 micrometres
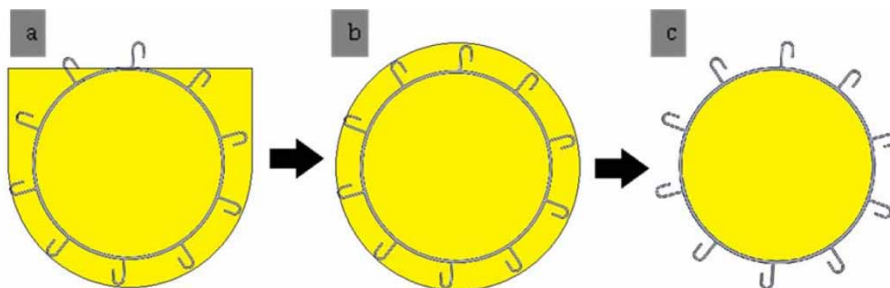
Figure 16. Scheme of the evolution of cellular pellet from cell sedimented cells in moulded microrecession of non-adhesive hydrogel. a) Cellular pellet (yellow) formed from sedimented cells in moulded recession of non-adhesive hydrogel containing lockyball; b) Rounding of cellular pellet into cell aggregate with embedded lockyball; c) Compaction of cell aggregate into compact tissue spheroid encaged into lockyballs.
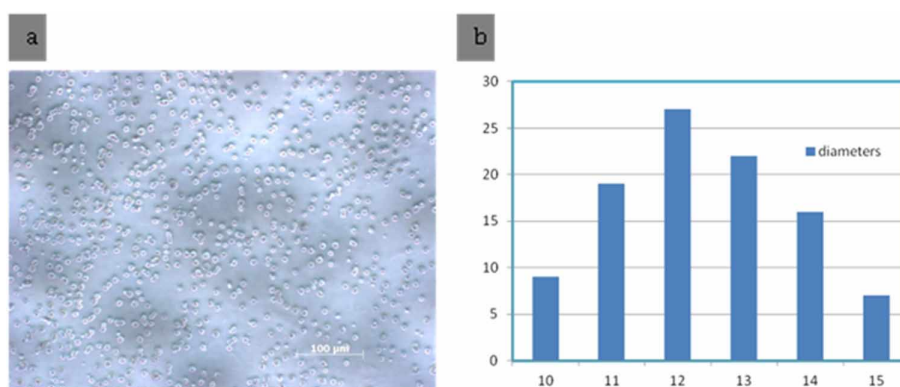

Figure 17. Morphometry of human chondrocytes. a) Human chondrocytes on agarose hydrogel (image for morphometric analysis was kindly provided by Monica and Silvio Duailibi from Federal University of São Paulo - UNIFESP); scale bar = 100 micrometres; b) Distribution of diameters of human chondrocytes.
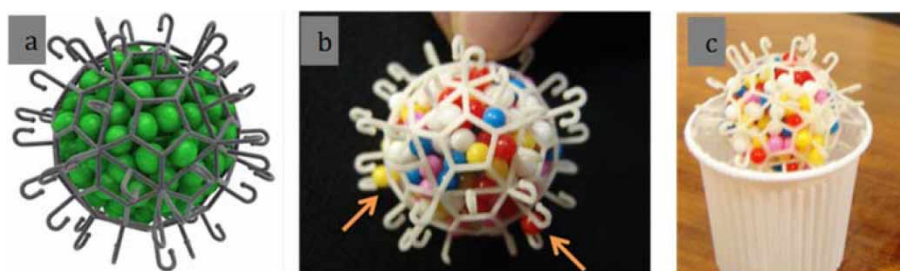

Figure 18. Explanation of lockyball 'cellularisation' experiment using physical models of human cells. a) Scheme explaining important precondition for successful cellularisation of lockyballs - diameter of balls (physical geometrical model of living cells) must be equal or close to diameter of lockyball pore; b) 'Cellularised' lockyballs have been removed from the plastic cap (which served as a physical model of microrecession in non-adhesive hydrogel) without losing cells if diameter of coloured balls ('physical geometrical model of living cells') is equal or close to diameter of lockyball pores. In control, with lockyballs with larger lockyball pore diameter, all cells were lost. Only two balls ('cells') were left attached outside of lockyball; c) Plastic cap (which served as a physical model of microrecession in non-adhesive hydrogel) loaded with coloured balls (physical geometrical model of living cells) with cellularised lockyball which was used in virtual experiment.

(Figure 17). The scheme demonstrating importance of adapting size of lockyballs pores to estimated diameter of human chondrocytes and results of physical cell harvesting experiment using plastic beads as physical analogy of living human chondrocytes is presented in Figure 18. Finally, scheme of magnetic levitation-based harvesting of cell aggregates from dense cell suspension in recession moulded in non-adhesive agarose hydrogel using functionalised lockyballs labelled with magnetic nanoparticles is presented in Figure 18.

It remains to be seen whether it will be possible or challenging to fabricate microlockyballs using not only, still unique, two-photon polymerisation machines but also more popular selective laser sintering machines (Wiria *et al.* 2010). Increasing the size of lockyballs is not desirable due to the possible negative effect of hypoxia and apoptosis in the centre of large size tissue spheroids. Also we must not forget that 2PP has nanolevel resolution and offers polymerisation not only on the surface but also inside a 3D photo-polymer solution.

5. Conclusion

The design and the fabrication of the lockable microscaffold ('lockyballs') described in this study open up new opportunities and represent a novel enabling technological platform for bottom-up modular tissue engineering. The presented technological platform will facilitate the development of new integrated solid microscaffold-based and directed tissue self-assembly in 3D tissue engineering. The lockable tissue spheroids approach will combine the advantage of the classic solid-scaffold-based approach in tissue engineering with the use of self-assembling tissue spheroids based on bottom-up modular tissue engineering. More specifically, lockable tissue spheroids will allow the slow tissue fusion of rigid tissue spheroids such as chondrospheres or osteospheres to be overcome and will enable the rapid biofabrication of 3D tissue modules. It is reasonable to predict that the unique design characteristic of lockable tissue spheroids will allow them to be used as more sophisticated self-assembling building blocks for *in vivo* manual or robotic tissue biofabrication.

Acknowledgements

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