Expert Reviews

Engineering 3D cell-culture matrices: multiphoton processing technologies for biological and tissue engineering applications

Expert Rev. Med. Devices 9(6), 613–633 (2012)

Aleksandr Ovsianikov*[1], Vladimir Mironov[2], Jürgen Stampfl[1] and Robert Liska[1]

[1]*Technical University of Vienna, Favoritenstrasse 9–11, 1040 Vienna, Austria*
[2]*Renato Archer Center for Information Technology, Division of 3D Technologies, Campinas, Brazil*
**Author for correspondence: a.ovsianikov@gmail.com*

Cells respond to topographical, mechanical and biochemical characteristics of the surrounding environment. Capability to reconstruct these factors individually, and also acting in accord, would facilitate systematic investigations of a multitude of related biological and tissue engineering questions. The subject of the present review is a group of technologies allowing realization of customized cell-culture matrices. These methods utilize photochemistry induced by multiphoton absorption and are carried out using essentially identical equipment. Fabrication of 2D microstructured substrates, complex 3D scaffolds, containing actively induced topographies, and immobilization of biomolecules in a spatially defined manner was demonstrated with these techniques. The reviewed reports indicate that multiphoton processing is a promising technology platform for the development of standard biomimetic microenvironments for 3D cell culture.

Keywords: biodegradable • femtosecond laser • functionalization • hydrogel • microfabrication • multiphoton • photoinitiator • photopolymerization • polymer • rapid prototyping • scaffold • tissue engineering

Conventional cell-culture systems used in biology do not accurately reproduce the structure, function or physiology of living tissue [1]. As a result, there are substantial discrepancies in behavior and responses of cells compared with *in vivo* environments. For example, Bokhari *et al.* have shown that HepG2 hepatocytes grown on 3D polystyrene scaffolds are less susceptible to certain toxicological challenges [2]. Their work suggested that testing drugs on liver cells should be performed in a 3D cell culture, since observed responses are more similar to that of natural tissue. Stegemann *et al.* have shown that the response of vascular smooth muscle cells (VSMCs) to the biochemical and mechanical signals differs depending on the extracellular environment [3]. The evaluation of interplay between different biomimetic factors is particularly challenging. For example, MJ Bissel's group has shown that both appropriate matrix elasticity and presence of laminin are required in order for mammary epithelial cells to maintain their differentiated phenotype [4]. Similarly, Peyton *et al.* revealed a synergetic effect of matrix elasticity and RhoA activation on the VSMC phenotype [5]. Numerous other examples can be found in related reviews on this topic [6–8].

Until recently, cell-culture matrices were mainly considered from a standpoint of support and guidance of cell proliferation and tissue development. The early designs of tissue engineering constructs focused on bulk properties, while disregarding the individual cell environment. A number of approaches for the realization of porous matrices, in the form of hydrogels or scaffolds, have been developed in recent years; with a different degree of success they all aim at approximating the complexity of the natural cell environment. However, the majority of current methods for realization of 3D cell-culture matrices allow the study of only particular biomimetic aspects of a natural extracellular matrix (ECM) without the possibility to evaluate the interplay of different effects. The main reason for this is that, owing to the technical limitations of these

 10.1586/ERD.12.48 ISSN 1743-4440

methods, quite limited variability of produced matrix parameters is possible. Consequently, only cellular processes characteristic to each particular matrix (fabrication method) can be studied. For example, particulate leaching and gas foaming are capable of producing sponge-like structures from many relevant materials, but provide virtually no control over the pore distribution [9,10]. Electrospinning yields a fibrous mesh with nanoscale architecture similar to the prevalent ECM components such as collagen and fibrin. However, one often faces a choice between producing a thick mechanically stable matrix and porosities relevant for cell migration [11]. Rapid prototyping-based techniques gained popularity since they are able to produce ordered structures in accordance with the provided computer-aided design model [12]. By the same virtue, they are capable of producing a set of exactly identical scaffolds or introducing intentional variations of the design. The spatial resolution of classical rapid prototyping techniques is inferior to the previously mentioned methods. Natural ECM is characterized by elements at multiple length scales, from micrometer-sized pores to nanofibers. As different individual methods fail to approach the complexity of the natural cell environment, a tendency to use a combination of methods is observed [13,14]. Combining different fabrication methods comes at a price of increasingly complex processing procedures, which set new limitations and might compromise the reproducibility of such approaches.

A large portion of the *in vivo* versus *in vitro* divergence is caused by the lack of biomimetic signals. Current findings show that the role of the ECM extends beyond a simple structural support to regulation of cell and tissue function [15]. The ECM is constantly remodeled and through its intimate interaction with cells it regulates cell differentiation, proliferation and cell death. In this regard, local properties of an artificial matrix on a single-cell level play a crucial role. It has been demonstrated that cells respond to topographical, mechanical and biochemical cues [16–18]. In order to control and direct cell behavior, it is important to understand the mechanisms of these responses. Owing to limitations set by most experimental systems, it is still hard to recapitulate on the general aspects of such interactions for different cell types. Most of the groundbreaking research in this area has been performed on flat 2D substrates. The transition to studies with *in vitro* native-like 3D systems relies on the availability of methods capable of reproducing key biomimetic aspects and varying different properties of artificial cell-culture matrices on demand. Some important advances in this direction have been recently demonstrated with engineered 3D cell-culture matrices [19,20].

In order to recapitulate on the basic aspects of cell matrix interactions in 3D, a standardized method for the fabrication of a cell-culture matrix is needed. Most importantly, such a method has to provide independent control over the key properties of the produced matrix: geometry, surface topography, and biochemical and mechanical material properties. The main requirements for the standardization of 3D cell-culture systems are: capability to mimic the environment of *in vivo* tissues; reproducibility and flexibility similar to that of 2D culture systems, allowing one to control experimental variables, study different cell types and ask a wide range of biological questions; and the possibility to integrate with current analysis tools (e.g., optical microscopy) [21]. Apparently, a single artificial matrix that is suitable for every cell and tissue type does not exist. However, the authors believe that a single technological platform, versatile and accurate enough for the fabrication of a wide variety of well-defined matrices, can be established to study responses of different cells. This study reviews a recent progress of a set of technologies grouped by the term 'multiphoton processing'. They make use of highly localized photochemical reactions, induced by a multiphoton absorption (MPA) of laser radiation. Reviewed methods facilitate complexity and allow design variability of produced structures. In addition to realization of well-defined 2D and 3D artificial cell-culture matrices, multiphoton processing can be used for spatially defined immobilization of biomolecules. Along with an expanding portfolio of processable materials, these techniques demonstrate the great potential towards development of standard laboratory systems for 3D cell culture.

Multiphoton processing

Multiphoton processing has its origins in multiphoton microscopy, which relies on MPA to locally excite fluorescence [22]. MPA was predicted in 1929 by Maria Göppert-Mayer [23]. Three decades prior to the experimental observation of this process, her work described a theoretical model gathering the principles of multiphoton interaction between light and matter. For MPA, two or more photons combine to bridge an energy gap larger than the energies of each photon individually (Figure 1A). It has to be differentiated from the sequential absorption process, where a real intermediate state (energy level) is involved. Hence, materials used with MPA are essentially transparent at the wavelength of the utilized laser. An important consequence of this is the possibility to induce MPA deep within the sample, which creates a fundamental advantage for all MPA-based methods from microscopic to material processing. For the description of the MPA process, a notion of virtual state (dashed line in Figure 1A) is often used. Such a short-lived ($\sim 10^{-15}$ s) virtual state results from absorption of the first photon ($h\upsilon_{IR1}$). The excited S_1 state can be reached if a second photon ($h\upsilon_{IR2}$) is absorbed within this short lifetime. In multiphoton microscopy, relaxation of the state S_1 induces fluorescence utilized for high-resolution imaging. Mutiphoton processing relies on initiation of reactive species, such as ions or radicals produced from the photoinitiators (PIs) after the inter system crossing from S_1 to the triplet state, for example.

The probability of MPA scales as the square of the incident light intensity for two-photon absorption (TPA), third power for three-photon and so on. Therefore, in contrast to conventional light attenuation via one-photon absorption, MPA is often referred to as nonlinear absorption. In practice the TPA is most commonly utilized, since it is much more effective than higher order processes. By adjusting the applied laser power, the conditions can be met when the light–material interaction region is limited to the volume of largest laser intensity – the focus of the beam

– while the rest of the laser radiation passes the material without inducing any photochemistry. Figures 1B & C show a comparative example of fluorescence excitation by one-photon absorption and TPA processes. In contrast to a one-photon excitation, TPA probability is rapidly reduced away from the focal plane of the beam. As a consequence, fluorescent volume is strongly reduced, whereby higher spatial resolution is achievable. For effective initiation of the process, light sources providing high intensities are advantageous. Conventionally, femtosecond lasers are used, since they can generate high peak intensities at moderate average laser power.

This review discusses microfarbication methods relying on the MPA of laser radiation in photosensitive materials. By translating the laser beam within the sample, the material is modified along the trace (Figure 1D). Photochemical reactions induced by MPA can result in cross-linking/polymerization of the material, immobilization of molecules by photografting or in photocleavage of the material. Therefore, three main approaches for engineering of cellular microenvironments can be differentiated: build up of 3D matrices by means of two-photon cross-linking or two-photon polymerization (2PP); spatially resolved functionalization of cell-culture matrices with bioactive molecules; and modification of

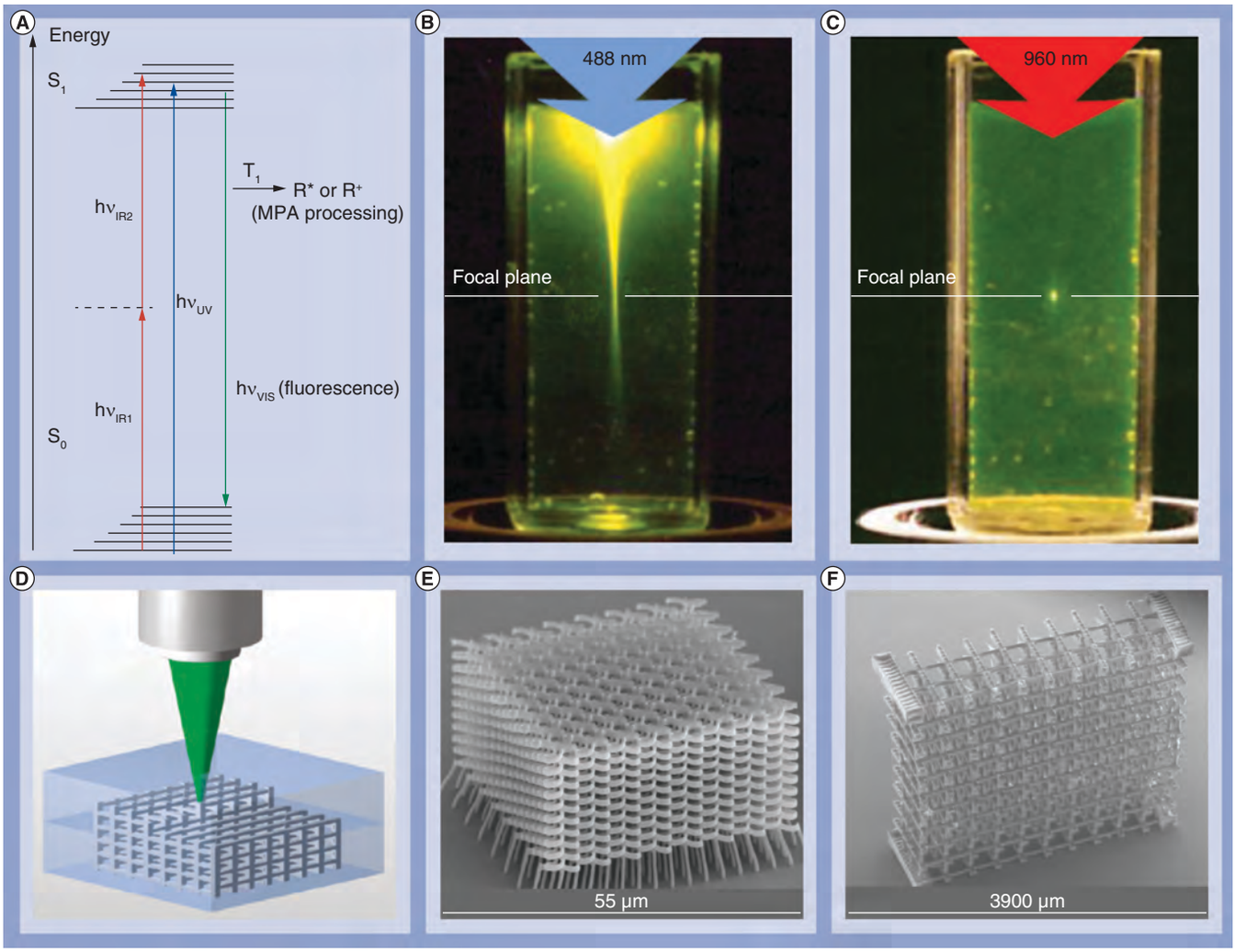


Figure 1. Two-photon absorption. (A) Jablonski diagram of the process resulting in fluorescence or production of reactive species, usually via first excited triplet state (for simplicity of representation, the possibility of nonradiative decay is not considered here). **(B)** Example of one- (488 nm) and **(C)** two-photon (960 nm) excitation of fluorescein. **(D)** How the structures/patterns are produced by scanning the focussed laser beam within the photosensitive material. Scanning electron microscope images of **(E)** a 3D helix-based photonic crystal structure and **(F)** a 3D scaffold. The scale bars in **(E & F)** differ more than 70-times.
Please note that denoted spectral ranges are a mere example and should not be considered a necessary prerequisite of the process.
$h\nu_{IR1}$: First photon in near-infrared spectral range; $h\nu_{IR2}$: Second photon in near-infrared spectral range; $h\nu_{UV}$: Photon in the UV spectral range; $h\nu_{VIS}$: Photon in a visible spectral range; MPA: Multiphoton absorption; R^*: Radical; R^+: Ion; S_0: Ground state; S_1: Excited state; T_1: Triplet state.
(B & C) Adapted with permission from [22].
(D) Reprinted with permission from [92]. © American Chemical Society (2011).
(E & F) A Ovsianikov. Reproduced with permission of The Laser Zentrum Hannover.

local matrix properties via photodegradation. All three approaches can be pursued by using essentially the same experimental setup. The main advantages of multiphoton processing for biological and tissue engineering applications are:

- Capability of true 3D patterning within the sample;
- High spatial resolution, often below the diffraction limit set by focussing optics;
- Mild reaction conditions, allowing processing of bioactive materials.

Owing to high spatial resolution, multiphoton processing technologies allow the realization of structures with characteristic features at multiple length scales (Figures 1E & F). Usually, such two-photon-induced processes of UV-sensitive materials (λ_{UV}) are activated by irradiation with a near-infrared laser light at approximately the double wavelength ($\lambda_{IR} \approx 2 \times \lambda_{UV}$).

Photo-induced cross-linking

Photo-induced cross-linking of peptides in aqueous solutions was performed at nearly the same time as the more famous 2PP was being developed. From a mechanistic point of view, the two-photon active compound forms radicals that are efficiently quenched by hydrogen donation from the aromatic OH group of tyrosine units [24,25]. The formed Ar-$O^{\cdot}$ radical is resonance-stabilized by the aromatic ring (Figure 2A). The main reaction is based on the recombination of two tyrosine radicals and, therefore, cross-linking of the protein. The big disadvantage of this mechanism compared with 2PP is that two radicals have to be formed by TPA to make one cross-link.

Two-photon polymerization

In 2PP, even a single initial radical is able to form thousands of cross-links until chain termination occurs by recombination or disproportion. The 2PP is sometimes also referred to as two-photon-absorbed photopolymerization [26], two-photon-induced polymerization [27,28], two-photon lithography [29], two-photon laser scanning lithography [30,31], multiphoton-excited microfabrication [32], 3D multiphoton lithography [33], 3D laser lithography [34] or direct laser writing [35,36]. Special PIs (please see the section on PIs) form radicals or ions to start radical chain growth polymerization of unsaturated monomers or cationic polymerization of epoxy monomers or vinyl ethers (Figure 2B).

For both above discussed methods, the resulting photochemical reaction leads to a polymerization/cross-linking of the material within a highly localized volume (down to <1 μm^3). By moving the beam focus within the material, 3D patterns are produced by direct laser writing (Figure 1D). After the 2PP processing is complete, the material, which was not cross-linked, is removed by an appropriate solvent to reveal the produced structure. Owing to the intensity-threshold behavior of the 2PP process, not only true 3D structuring, but also realization of structures with subdiffractional spatial resolution is possible. The unrivalled advantage of 2PP for tissue engineering is the possibility to produce 3D constructs with feature sizes adjustable in the range from <100 nm to a few tens of microns. Figure 1E shows a 3D helix-based photonic crystal structure and Figure 1F shows a scaffold produced by the 2PP technique. The scale bars in both images differ by more than 70-times.

Multiphoton grafting

Multiphoton grafting has been introduced very recently and it uses the classical principle of aromatic azide chemistry [37]. Photolysis of the azide causes the dissociation of the N–N bond from the excited singlet state, followed by the generation of nitrogen and highly reactive electron-deficient nitrene species. The short-lived nitrene intermediate is able to insert into any C–H bond (Figure 2C). In contrast to 2PP utilizing chain-growth polymerization, here a true single molecule grafting is performed. It was shown that aromatic azides with appropriate chromophores can be grafted in 3D on a poly(ethylene glycol) (PEG)-based matrix [37].

Two-photon-induced photocleavage

Finally, two-photon-induced photocleavage [38] was recently introduced as a tool for precise 3D modification of hydrogel properties [39,40]. In both cases, classical photocleavable groups such as 7-hydroxy coumarin or aromatic ortho-nitro esters were used. Photocleavage of the excited singlet state in such coumarins has been proven to proceed in the presence of water as a solvent via a photo-SN1 mechanism (Figure 2D, II). The ortho-nitro ester is a classical protecting group for carboxylic acids, phosphates, alcohols, phenols and amines. In any case, the nitro group is reduced to a nitroso group under simultaneous cleavage of the functional group in ortho position (Figure 2D, I). Aromatic ortho-nito esters were employed in a diacrylate monomer to loosen the network after two-photon excitation [39]. In addition, a photolabile RGDS peptide monomer has been reported that it can be cleaved under two-photon conditions. The coumarin dye has been used to mask amino [40] and thiol groups [41]. These thiol groups were subsequently used for selective protein immobilization [42].

Materials for two-photon polymerization

Initially, 2PP was used with commercially available photopolymers developed for UV lithography. Owing to its high spatial resolution and good optical properties of processable materials, the main studied applications of 2PP were in the field of nanophotonics. With the increasing importance of biomedical applications, the portfolio of 2PP compatible materials extended in the direction of biocompatible and biodegradable materials. The following sections describe the recent developments of the specialized PIs for 2PP, and review the materials utilized for biomedical applications of this technology.

Photoinitiators

At the initial stage of 2PP development, research groups used typical commercially available radical one-photon PIs such as Irgacure® 369 (Ciba Specialty Chemicals, now part of BASF) (Figure 3A) because of the lack of optimized two-photon PIs [26,43,44]. These PIs have their origin in classical photopolymerization and

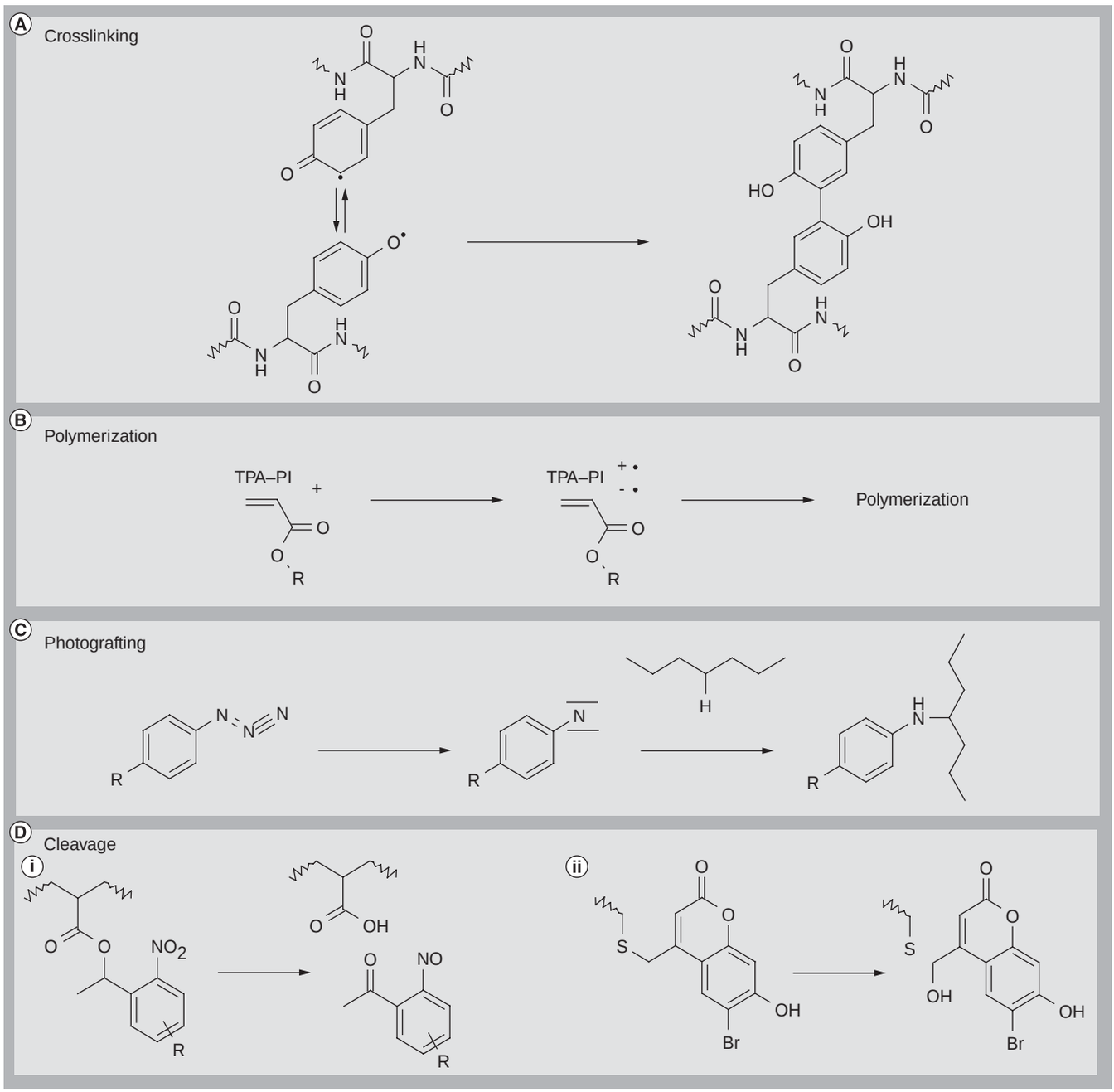


Figure 2. Multiphoton processing. (A) Cross-linking: the TPA-induced PI forms radicals, which are able to abstract hydrogen from tyrosin units. Subsequently, formed radicals are resonance stabilized and react under recombination reactions. **(B)** Polymerization: the PI interacts with the radical by charge transfer and subsequently forms radicals on the monomer that initiate the polymerization **(C)** Grafting: aromatic azides undergo multiphoton absorption. From the singlet excited state nitrogen and a nitrene are formed; the nitrene then undergoes C–H insertion reactions. **(D)** Cleavage: classical photoremovable protecting groups are used to break down the network structure **(i)** or liberate functional groups **(ii)**.
PI: Photoinitiator; TPA: Two-photon absorption.

are widely used in various fields of application, such as coatings and printing inks for metal, wood, cardboard or paper. Their high photoinitiation activity is based on either bimolecular type II (e.g., hydrogen abstraction benzophenone/amine systems, Figure 3B) or more efficient monomolecular type I mechanisms (e.g., α-cleavage of Irgacure 369), forming highly reactive radicals for the initiation process. However, relatively high PI concentrations and laser intensities are required for structuring with 2PP [45–47]. The limited efficiency of these PIs under TPA conditions can be ascribed to the rather low TPA cross-section (σ^{TPA}), normally less than 40 GM, especially for a typical wavelength of 800 nm.

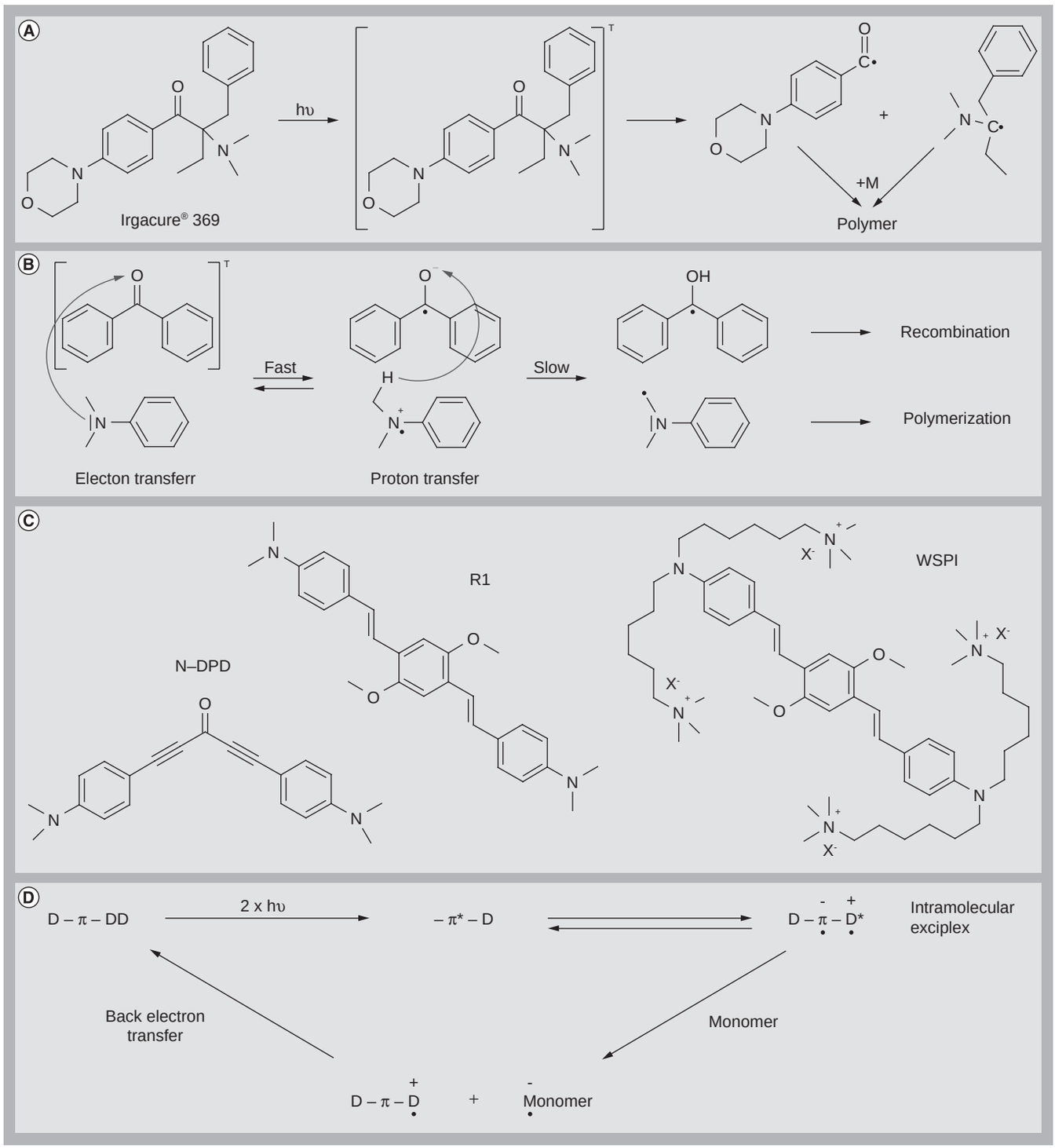


Figure 3. One- and two-photon initiators. (A) Type I photoinitiators undergo α-cleavage reaction from the excited triplet state under the formation of two radicals that initiate the polymerization. **(B)** Bimolecular Type II photoinitiators are based on chromophores, such as benzophenone and tertiary amines. Fast electron transfer to the excited ketone from the lone pair of the amine occurs first. Rate-determining proton transfer competes with the back electron transfer. **(C)** Typical chromophores with high two-photon absorption cross-section. Planar-conjugated π-systems with strong donor/acceptor groups are essential. **(D)** Principle of intra- and inter-molecular charge transfer interaction between an excited photoinitiator and monomer, and subsequent radical formation to initiate the radical polymerization.
N-DPD: N,N-dimethyl-p-phenylenediamine dihydrochloride; M: Monomer; WSPI: 1,4-bis[4′-(*N,N*-bis[6′[bis[trimethylammoniumiodide-6-hexyl]-aminohexyl]amino)styryl]-2,5-dimethoxybenzene.

Until recently, a full understanding of the relationship of the molecular structure and the TPA properties has remained a big challenge [44,48–51]. In order to form a push–pull system, an extended [52] and coplanar [49] π-conjugated core (π), with strong donor and/or acceptor functionalities is of importance to give high σ^{TPA}. For the characterization of the PIs, the σ^{TPA} is determined and 2PP structuring tests are carried out. The σ^{TPA} is the equivalent value to the extinction coefficient in Lambert–Beers law, and can be determined by nonlinear transmission [53], upconverted fluorescence emission [54], transient absorption [55], four-wave mixing [56] and z-scan analysis [57]. 2PP-structuring tests provide information about the practical performance of the PI, different parameters are measured such as the polymerization/fabrication threshold [58], the ideal processing window or the dynamic power range [59], estimated voxel size [45] and pseudo rate of polymerization [60].

Studies on the initiation mechanism of these typical PI concepts revealed that a two-photon-induced intermolecular electron transfer from the TPA chromophore to the functional monomer [61,62], or a senzitation of classical PIs [63–65] is the initiating step (Figure 3D). Based on the knowledge of two-photon active compounds from confocal laser scanning fluorescence microscopy, different scientific groups also used these chromophores for 2PP. One of the frequently cited molecules is R1, shown in Figure 3C. More compounds can be found in the detailed reviews of Lin *et al.* [66], Rumi *et al.* [67] and Lee *et al.* [68]. Research frequently focused on getting enormous numbers for the TPA cross-section but this value only gives a hint as to the probability to reach the excited state. One has to keep in mind that these molecules usually have a high quantum yield of fluorescence, which means that they either undergo an ultrafast deactivation of the excited singlet state or *cis–trans* isomerization reactions due to the presence of double bonds. The most up to date concept combines the chemistry of classical ketone-based PIs with the requirements for TPA active compounds (e.g., N,N-dimethyl-p-phenylenediamine dihydrochloride), which have negligible fluorescence due to the presence of the ketone group and no *cis–trans* isomerization reactions due to the exchange of the double bond by a triple bond [69,70]. Despite these recent developments, for 2PP classical PIs are often used due to the lack of commercially available two-photon PIs.

For the application of 2PP in the field of tissue engineering and hydrogels, water soluble initiators are required. For appropriate solubility, one-photon PIs [71] have been substituent with quaternary ammonium cations, salts from sulfonic or phosphonic acids, or long PEG-based chains [65,72]. The advantage of the latter nonionic PIs is that these exhibit a good solubility in organic solvents and there is no sensitivity to the pH value in water [73]. In case of TPA, there are only very few articles describing two-photon active compounds in water borne systems. Most of them were synthesized for the use as chromophores for the two-photon microscopy of biological samples such as living cells [74–77]. One example of such a compound is 1,4-bis[4′-(*N*,*N*-bis[6′[bis[trimethylammoniumiodide-6-hexyl]-aminohexyl] amino)styryl]-2,5-dimethoxybenzene (WSPI) (Figure 3C). It has been shown that these molecules have a substantial drop of the σ^{TPA} in water, probably due to hydrogen bonding or changes in the chromophore geometry and/or multichromophore aggregation. Using water-soluble TPA dyes, such as rose bengal, eosin, Rhodamine B, and erythrosine as initiators, simple 3D objects were fabricated from acrylamide [78]. In order to optimize the low efficiency of this system, other groups used *N*-methyldiethanolamine and Omnicat 820 as co-initiators [79,80].

Truly TPA-optimized systems are still quite rare. Very recently, Wan *et al.* described a water-soluble derivative of a ketone-based two-PI with trietanolamine as co-initiator, a water-soluble acrylate (SR610, Sartomer) as monomer, and 20 wt% water as solvent [81]. A higher water content of approximately 40% and a classical organosoluble PI (Irgacure 651) have been described by Jhaveri *et al.* [82]. By using water soluble WSPI, the author's group succeessfully fabricated 3D scaffolds from (PEG diacrylates) PEGda with up to 50% water content [83]. A complete list of other initiators applied in 2PP for applications in biology and tissue engineering can be found in Table 1.

Photopolymers

The first report on multiphoton processing of biopolymers was published in 2000 [84]. Rose Bengal was used as a water-soluble PI suitable for TPA at 800 nm. Simple 2D structures from bovine serum albumin (BSA) were produced and evaluated for their stability and capability to entrap enzymes. The results suggested that cross-linking of the tyrosine units, and not simple denaturation and precipitation, is the enabling mechanism of BSA processing. In 2005, the same group reported successful cross-linking of cytoplasmic proteins within live starfish oocyte cells [32]. Again, Rose Bengal was used to produce patterns of cross-linked proteins, which were later analyzed by two-photon fluorescence microscopy. At low initial concentrations, structures produced from cross-linked proteins exhibit inferior mechanical properties and stability. However, a few fold increase of the protein concentration facilitates realization of mechanically stable complex 3D structures [85,86].

The first porous 3D scaffolds were produced by 2PP of synthetic commercially available photoresins [87]. By comparing the proliferation rates of cells grown on flat material surfaces of resulting polymers and under control conditions, it was demonstrated that structures produced from Ormocomp® (micro resist technology GmbH) and SU8 (MicroChem) are biocompatible and are not cytotoxic. The use of other nondegradable commercially available acrylate- and methacrylate-based photoresins such as PEGda, SR368 and SR499 triacrylate blends (Sartomer), Accura® SI10 (3D Systems Corporation), ORMOCER®s (The Fraunhofer ISC), and other sol-gel hybrids for fabrication of 3D scaffolds followed (Table 1). These studies demonstrated the great potential of 2PP for fabrication of well-defined matrices with features at the micro- and nano-scale for studies of cell behavior in a porous 3D environment.

However, typically the tissue engineering scaffolds are expected to provide only temporary support. An important advance in the direction of 2PP processable biopolymers came with the development of hydrolytically degradable polycaprolactone-based

Table 1. Materials used for two-photon cross-linking and two-photon polymerization.

Type	Hydrogel yes/no	Polymer	Photoinitiator	Ref.
Nondegradable	No	ORMOCER®/Ormocomp®	Irgacure® 369	[36,87,102]
	No	SR368+SR499	Lucirin® TPO-L	[103,104]
	Yes	PEGda	Irgacure® 369	[97, 105, 111,132]
	Yes	PEGda	WSPI†	[83]
	No	Accura® SI10	Not reported	[31]
	No	Ti- and Zr-based sol-gels	Irgacure® 369	[113,133]
	No	Chitosan-doped	Lucirin® TPO-L	[134]
	No	UDMA	Irgacure® 369	[90]
Biodegradable	No	PCL based	Michlers ketone	[88]
	Yes	gelMOD	Irgacure® 2959†	[92,95]
	No	OLMA	Irgacure® 369	[90]
	No	PLA based	Michlers ketone	[91]
	Yes	BSA and fibrinogen	RoseBengal†	[84]
	Yes	Biotinylated BSA	Flavin mononucleotide†	[135]
	Yes	Collagen	Benzophenone dimer†	[136]

†Water-soluble and biocompatible photoinitiators.
BSA: Bovine serum albumin; gelMOD: Methacrylated gelatine; OLMA: Methacrylated oligolactones; PEGda: Poly(ethylene glycol) diacrylate; PLA: Poly(lactic acid); SR368: Tris (2-hydroxyethyl) isocyanurate triacrylate; SR499: Ethoxylated (6) trimethylolpropane triacrylate; UDMA: Urethandimethacrylate; WSPI: 1,4-bis[4′-(*N*,*N*-bis[6′[bis[trimethylammoniumiodide-6-hexyl]-aminohexyl]amino)styryl]-2,5-dimethoxybenzene.

material containing photopolymerizable methacrylate groups [88]. Previous studies on this triblock co-polymer have shown that not only is it biocompatible and biodegradable, but it also degrades on a similar time scale as tissue formation [89]. The 2PP fabricated structures were of good quality and had 4-µm resolution. The cell culture tests showed absence of cytotoxicity and support of cell adhesion and proliferation [88]. Recently, 2PP processing of hydrolytically degradable poly(lactic acid)-, oligolactones- and urethane-based photopolymers [90,91] was demonstrated.

A further important advance came with utilization of enzymatically degradable methacrylamide-modified gelatin (gelMOD) [92]. The gelatin is derived from collagen, which is one the main structural adhesive and load-bearing proteins of the native ECM. Thus, from a chemical point of view the 2PP scaffolds produced from gelMOD closely mimic the natural cellular microenvironment. Furthermore, the mechanism of ECM remodeling *in vivo* relies on the capability of cells to degrade the matrix via enzymes. Scaffolds produced from enzymatically degradable materials are likely to provide optimized conditions for the generation of bioartificial tissues. The gelMOD was cross-linked employing a femtosecond laser emitting at 515 nm and the water soluble PI Irgacure 2959, known to be highly biocompatible [93,94]. Produced 3D scaffolds were shown to support adhesion, proliferation and differentiation of both mesenchymal and adipose-derived stem cells [92,95]. By adjusting the physical versus chemical cross-linking of gelMOD, and by altering the synthesis protocol, it is theoretically possible to control the degradation rate and the stiffness of resulting materials. These parameters are known to be important for control over cell phenotype and proliferation [96].

The material biocompatibility is critically important for successful application of 2PP in tissue engineering. Residuals of monomer and PI, not consumed during the 2PP fabrication, are potentially cytotoxic and represent legitimate concern. A recent report on systematic studies of cytotoxicity of commercially available photopolymer, using standard ISO10993–5 material extract protocol, demonstrated that PIs make a significant contribution to the cytotoxic effects [97]. The relative level of cytotoxicity is different for various PIs, but higher PI concentrations tend to increase the toxic effects. On the example of commercially available PEGda material and three different initiators, it was demonstrated that postprocessing/extraction protocols allowed the reduction or complete elimination of such cytotoxic effects. These results emphasize the importance of optimizing material composition, not only with regard to 2PP processability, but also to biocompatibility of resulting polymeric structures. They also suggest that by employing appropriate extraction procedure and solvents, postprocessing can be used to reduce toxic residuals from 2PP-structured polymers. In general, more effective PIs allow minimization of the potential toxic effects by reducing the amount of the necessary PI content. Of course, the comparative cytotoxicity of different PIs has to be taken into account as well.

Applications

Cell biology

2PP is capable of manufacturing complex 3D structures, but it can also be used to produce simple 2D patterns. Kaehr *et al.* employed 2PP to cross-link BSA directly in the presence of cells [98]. For this, the cell-culture medium was exchanged with a photo-cross-linkable solution for the duration of multiphoton processing, which had no observable negative effect on cells. On the example of neuroblastoma-glioma (NG108–15) cells, it was shown that BSA topographies can be used to affect neurite elaboration and pathfinding. Furthermore, it was observed that the capacity of produced structures to bind biotin can be tuned by adjusting the composition of cross-linking solution, adding another parameter that is useful for controlling cell behavior. This particular

study demonstrated potential of multiphoton processing for realization of 2D models for investigation of neuronal signaling and plasticity. However, reconstructive therapies could also profit from 3D systems, allowing neuronal development and repair in a controlled fashion [99].

Migration is one of the fundamental cellular functions. It plays a key role in normal physiological processes, including tissue development and repair [100]. Jeon *et al.* investigated cell migration using 2PP-produced ORMOCER grids (cross patterns) and gratings (parallel lines) [101]. Structures with different parameters were produced on the same substrate, allowing to directly compare the influence of the microstructures on cells cultured under exactly the same conditions. On the example of NIH3T3 fibroblast cells, it was shown how different width-to-length ratios of the grid influences cell morphology and motility (Figure 4A). Compared with flat surfaces, migration of cells was clearly directed by more elongated grids, with the effect being slightly different for various grid heights.

A similar, 2PP produced, grid structure was utilized by Kiyan *et al.* to study differentiation of VSMCs [102]. By comparing the behavior of cells cultured on flat surfaces and on ORMOCER grids, a new molecular mechanism controlling phenotypic modulation of VSMCs *in vitro* and *in vivo* was identified (Figure 4B). These findings demonstrate a possibility of controlling the differentiation of VSMCs, and regulation of their phenotype by microstructured prosthetic biomaterials.

3D scaffolds made of a few layers of gratings, stacked on top of each other, were used by Tayalia *et al.* for investigation of HT1080 cell migration [103]. By varying the grating period (110, 52, 25 and 12 µm), scaffolds with different (square) vertical pore sizes were produced (Figure 4C, i). Migration of cells transfected with fluorescen protein was tracked using confocal microscopy, a typical migration path is shown in Figure 4C, ii. The results demonstrated an increase in the mean migration speed of the cells residing on a 3D scaffold compared with those on flat substrates. Measured migration speed was smaller for scaffolds with smaller pores. It was suggested that a decrease in mean speed was due to obstruction from the matrix (represented by scaffolds cross-bars in this case). Unfortunately, no control experiment was performed on a grid composed out of just two layers of gratings. Taking into account that the produced pore sizes are quite large, the cells can only migrate along the beams of the grid, with the change in direction possible at the intersection of two beams. This way, the scaffolds with smaller pores represent a system where a cell is provided with more possibilities to change the direction of its migration. Using the same type of scaffolds, placed in the middle (corner) of an L-shaped two-chamber system, migration of bone marrow-derived dendritic cells, stimulated by chemotactic factors diffusing from a neighboring compartment, was investigated (Figure 4D) [104]. It was shown that using a lymph node chemokine, directed migration of bone marrow-derived dendritic cells can be induced and that the 3D architecture of the scaffolds has a strong influence on chemotactic cell motility. These reports demonstrate the capability of 2PP for microfabrication of standardized systems for studies of cell migration in 3D.

Linear beams supported by a set of pillars were produced by 2PP of ORMOCER by Klein *et al.* [36]. These structures essentially represent 2D gratings lifted above the glass substrate. However, since every section of such beam is suspended between two pillars, observation of beam deflection induced by cellular contractile forces is possible. The mechanical properties of the beams, characterized by an atomic force microscope, allowed the author's group to calculate the force exerted by a single chicken cardiomyocyte. A later report by the same group described a sequential fabrication of structures composed from two photopolymers with distinct protein binding properties [105]. A set of pillars with linear beams on top were produced by 2PP of protein-repelling PEGda-based photopolymer. In a second step, the structure was covered with Ormocomp, in order to produce a set of protein-binding cube-like structures in the middle of each beam (Figure 4E, i). Finally, Ormocomp was selectively coated with fibronectin, thus producing cell-binding sites throughout the PEGda-based frame. As it was anticipated, chicken fibroblast cells exhibited clear preference for attachment to fibronectin-coated parts of 2PP produced structures (Figure 4E, ii). The results are reported for cells cultured on top of produced structures for only 2 h. It is not clear if the adherence preference is sustained beyond this extremely short cell-culture duration.

In summary, a great advantage of 2PP for cell biology is the possibility to gradually 'build up' the system for cell culture, starting from simple 2D patterns up to an arbitrarily complex 3D structure. This way, a complete transition from a classical 2D system to a more complex 3D-like architecture can be studied. Compared with other approaches, 2PP not only allows great variability in structural parameters, but also to produce series of exactly identical 2D or 3D structures. A number of structures directly adaptable to current biological observation methods were produced by 2PP. It was demonstrated with these systems that it is possible to study cell migration, differentiation, as well as adhesion and mechanobiological aspects of cell interaction with materials.

Scaffolds for tissue engineering

Scaffold or temporal biodegradable support is a key element of a classic top-down tissue-engineering approach [106]. Scaffolds give shape to future tissue-engineered constructs, provide initial material properties and structural support for cell attachment and seeding [15,107,108]. In order to perform systematic studies on cell–scaffold and cell–cell interactions in a 3D environment, scaffolds have to be created in a reproducible manner according to a defined design. In this context, rapid prototyping or additive manufacturing techniques have gained increasing popularity [12,108]. However, owing to their low resolution, most rapid prototyping methods alone do not satisfy the requirements associated with mimicking the multiscale physical architecture of the natural cell environment. Few recent reports have addressed this drawback by combining different techniques to produce large scaffolds containing features at the micro- and nano-scale [13,14]. Combining different fabrication methods comes at a price of increasingly complex processing procedures, which set new limitations and compromise the reproducibility of such approaches.

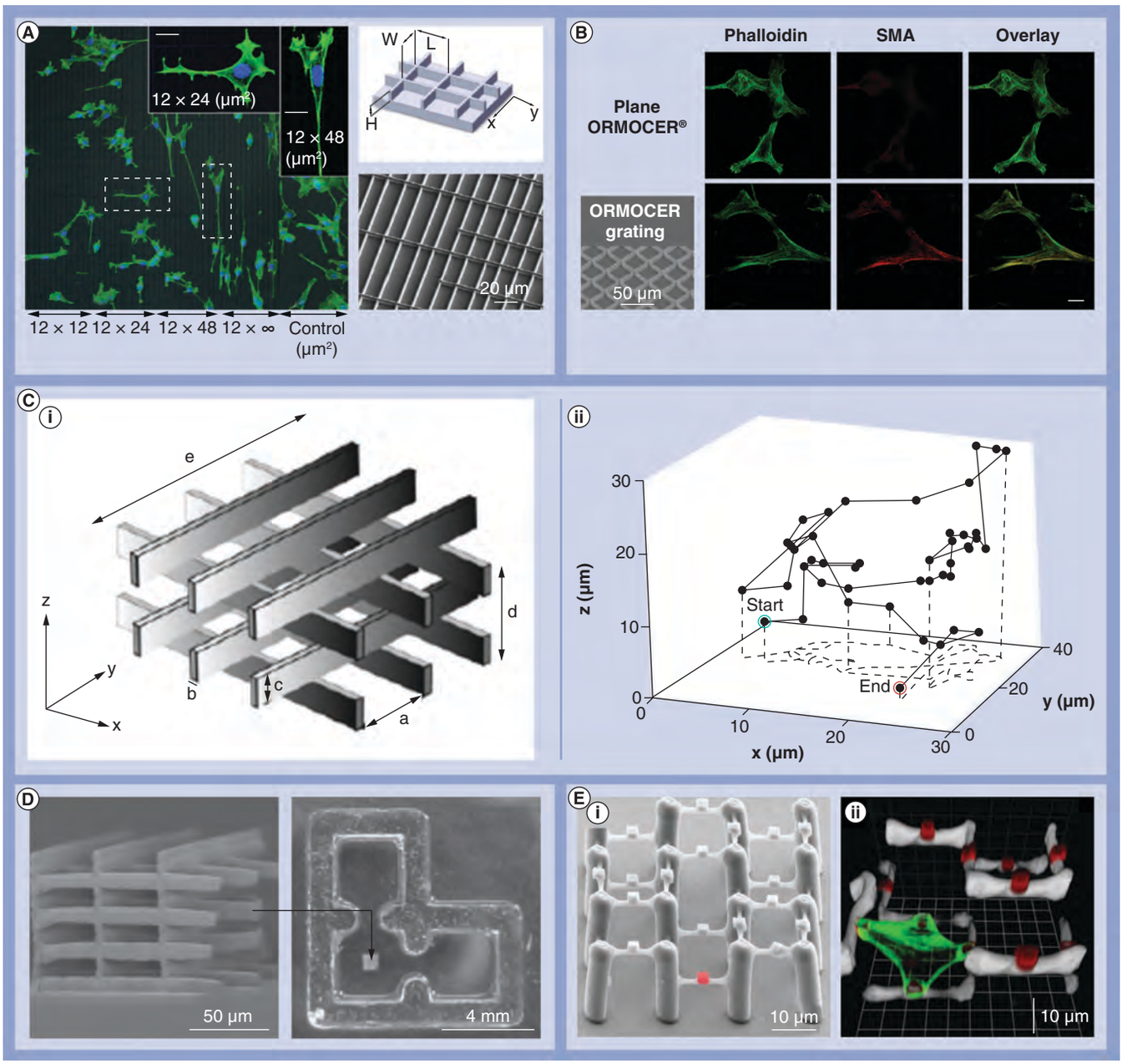


Figure 4. Two-photon polymerization structures for studies of cell behavior. (A) Cell migration on a grid with different length/width aspect ratio [101]. **(B)** Smooth-muscle actin expression in muscle cells cultured on flat and structured substrates [102]. **(C)** A model of a 3D scaffold and a typical migration path of a cell within such a structure [103]. **(D)** L-chamber system for chemotactic studies within 3D scaffolds [104]. **(E)** A two-component scaffold, with the cubes made of cell-adherent Ormocomp® and a confocal image showing cells selectively adhering to cubes at different heights [105].
W: Weight; H: Height; L: Length; SMA: Smooth muscle actin.
Adapted with permission from [102–105,101].

The advances in the fabrication of 3D scaffolds for tissue engineering and regenerative medicine constructs using the 2PP technique started from the series of works at the Nanotechnology Department of the Laser Zentrum Hannover (Germany) led by Chichkov. Initially, 2PP was applied for the generation of 3D scaffold-like structures, from the nonbiodegradable Ormocomp [109,110]. On the example of different cell types, it was demonstrated that utilized materials support cell adhesion and formation of intercellular gap junctions, which is critical for functional tissue growth and cell communications. Finally, the possibilities of seeding 3D structures fabricated by means of 2PP with cells were analyzed. These results demonstrated for the first time the great potential of 2PP technology for the manufacturing of biocompatible scaffolds with controlled

topology and properties, enabling systematic studies of cell behavior and tissue formation in 3D.

Most natural tissues contain more than one cell type. Arrangement of different cells within the tissue or organ defines its functionality. Specially designed scaffolds with open vertical pores were produced by 2PP of PEG-based material. The scaffold design facilitated homogeneous high-density cell seeding throughout the scaffold by means of laser-induced forward transfer [111]. With the laser-induced forward transfer method, it is possible to introduce cells at almost arbitrary density precisely into the desired location of a scaffold. Most importantly, two different cell types – endothelial cells and VSMCs, were distributed in a predefined way. In addition to the advantage of 2PP for designer scaffolds manufacturing, this work also reported for the first time a combination of 2PP and laser-induced forward transfer for biofabrication of 3D construct containing different cell types [111].

Hsieh *et al.* reported 2PP fabrication of relatively large (>15 mm^3) 3D scaffolds from commercially available nonbiodegradable photopolymer (Figure 5A) [31]. Produced scaffolds were coated with collagen Type I in order to improve cell adhesion. Primary rat hepatocytes have been used as test cells. In order to prevent cells from just falling though the 250 × 250-µm pores during seeding, five out of six facets of this cubic scaffold were sealed with a nylon membrane. The results showed that cells cultured within a 3D scaffold maintained higher liver-specific functions over a period of 6 days, compared with a monolayer culture [31].

The first enzymatically degradable biomimetic 3D scaffolds produced by means of 2PP were reported recently (Figure 5B) [92,95]. The gelMOD utilized in this study was shown to preserve its enzymatic degradation capability after 2PP processing. The gelatin was derived from collagen, which is one of the main structural adhesive and load-bearing proteins of the native ECM. Thus, from a chemical point of view, produced scaffolds closely mimic the natural cellular microenvironment. It was demonstrated that 2PP is capable of producing relatively large scaffolds (3 × 3 mm) with actively induced microtopographies in a single fabrication step. Produced gelMOD scaffolds supported adhesion, proliferation and differentiation of stem cells. Porcine mesenchymal stem cells were successfully differentiated into osteogenic lineage, as shown by the calcium phosphate deposition onto the scaffold [92]. Human adipose-derived stem cells were differentiated into adipogenic lineage, as indicated by accumulation of intracellular lipid [95].

Melissinaki *et al.* have used 2PP to produce various 3D structures from hydrolytically degradable methacrylated poly(lactic acid)-based material [91]. A thorough investigation showed that the utilized material supports proliferation of PC12 and NG108–15 neuronal cell lines. Cells seeded onto suspended polymeric beams were shown to organize and extend their neurites along such guide wires. Sea-shell-shaped structures produced by 2PP facilitated adhesion of neuronal cells. The neurite projections extended over more than one sea-shell structure, indicating that a 3D interconnected network could be produced in a similar fashion (Figure 5C). In addition, small size (~100 µm) scaffolds were produced to verify proliferation of PC12 cells in a 3D environment. It was suggested that such microscaffolds can be used as a cell-delivering building block, which can later self assemble into large tissues [112].

Hybrid organic–inorganic nondegradable materials were used by Psycharakis *et al.* for 2PP manufacturing of permanent 3D structures for cell culture [113]. Proliferation of mouse NIH3T3 fibroblasts on flat surfaces showed preference for materials with higher inorganic content. The cells could also attach and proliferate within small 3D scaffolds fabricated from the selected material formulations.

Depending on the 2PP microfabrication system, the reactivity of the material, and the size of the particular scaffold, its fabrication can take from a few minutes to a few hours. Combining 2PP with some serial technology could greatly decrease the time and the cost of scaffold production. Koroleva *et al.* have used 2PP to fabricate millimeter-sized master scaffolds, which were later replicated with elastic stamps [114]. The design is based on the 2PP scaffolds developed by Ovsianikov *et al.* for cell seeding by laser printing [111]. The advantage of this design is that all of the pores are vertically oriented within different layers of the scaffold. This way, using essentially a 2D soft lithographic technique, it was possible to replicate up to four-layer thick 3D scaffolds containing overhangs and small features. The cell-culture results demonstrated that human fibroblasts could attach and proliferate to high densities throughout the 50-µm pores of the replicated scaffolds. In addition to the possibility of producing a series of identical scaffolds in a cost-effective manner, such replication approach allows realization of scaffolds from materials not directly processable with 2PP [115].

Hydrogels are widely used as 3D matrices for cell growth owing to similarity of their mechanical and diffusivity properties to the natural cell environment. Furthermore, encapsulation of living cells within the hydrogel produces constructs with high initial cell loading and intimate cell-matrix contact, similar to that of the natural ECM. A number of synthetic and natural hydrogels has been developed and used as 3D scaffold for studies of cell behavior and tissue formation [116]. In a recent study Torgersen *et al.* have shown that 2PP is capable of producing 3D scaffolds from hydrogels (Figure 5D) [83]. A highly sensitive two-photon PI was synthesized and verified for biocompatibility using nematode *Caenorhabditis elegans* as a multicellular test organism. The utilization of this water-soluble photoninitiator allowed fast 2PP processing of PEGda-based hydrogels with up to 80% water content. Furthermore, 2PP scaffolds were successfully produced around and in direct contact with living *C. elegans*. Our recent study showed that encapsulation of live cells within hydrogels is also possible by 2PP (Figure 5E) [Ovsianikov A *et al.*, Unpublished Data]. These studies demonstrate that in addition to conventional photopolymers, 2PP can also make use of hydrogels, which present a highly relevant and well-established biomimetic material platform. Utilization of hydrogels for 2PP will facilitate development of systems for live-cell encapsulation within 3D scaffolds in a spatially defined manner.

An important issue for live cell encapsulation and for matrix modification (please see section on modification of 3D matrices) is a risk of cell damage by femtosecond laser radiation. While

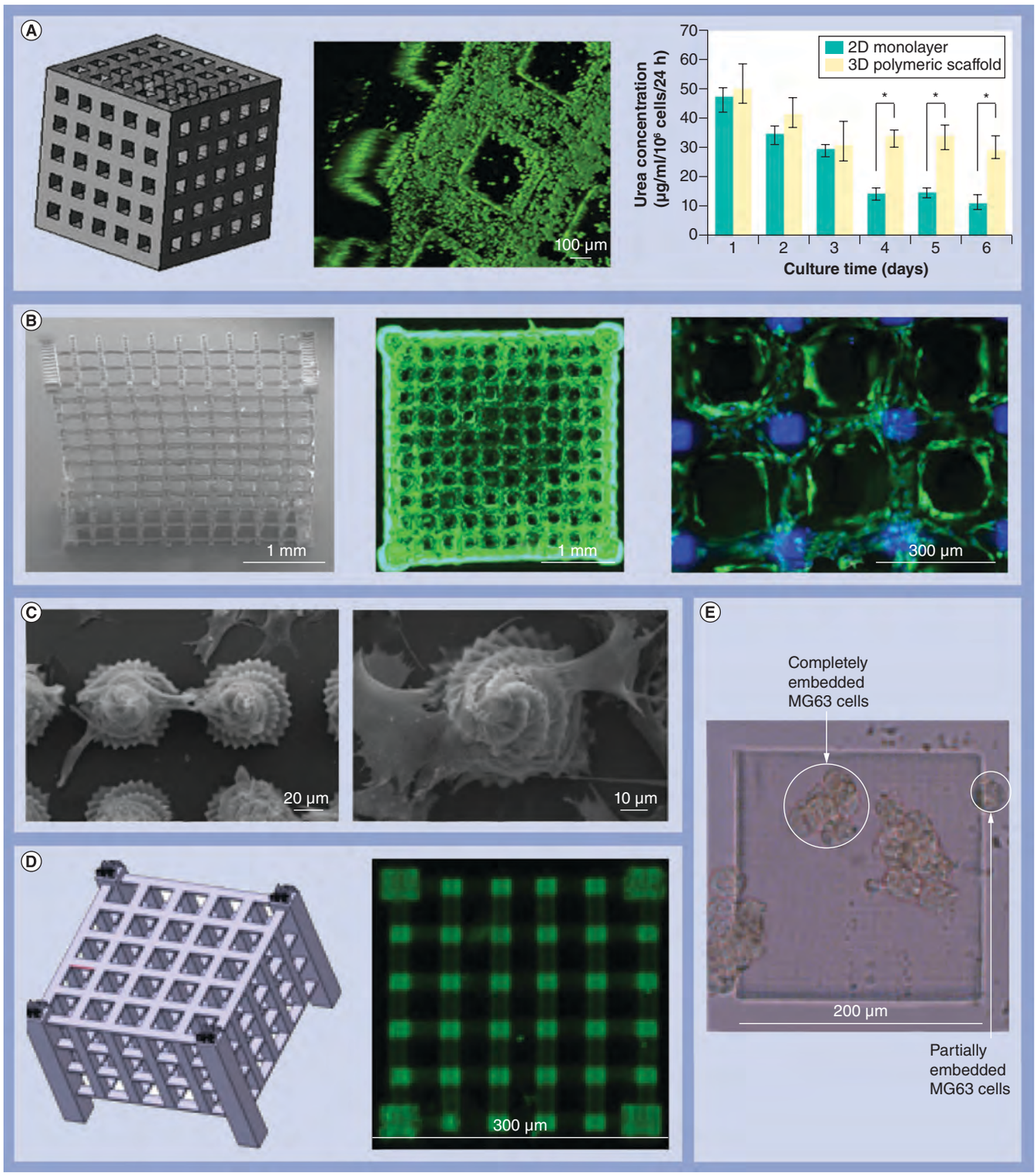


Figure 5. 3D scaffolds for tissue engineering applications. (A) Rat hepatocytes seeded onto a nondegradable 3D scaffold exhibit higher urea synthesis, compared to 2D monolayer culture; *$p < 0.05$ [31]. **(B)** 3D scaffold from enzymatically degradable gelatin-based photopolymer, seeded with adipose-derived stem cells [92,95]. **(C)** Two-photon polymerization produced sea-shell structures seeded with neuronal cells [91]. **(D)** 3D scaffolds produced from a poly(ethylene glycol)-based hydrogel with a 50% water content [83]. **(E)** MG63 cells completely and partially embedded in a two-photon polymerization-produced hydrogel cube. **(A–D)** Adapted with permission from [31,91,92,95].
(E) Courtesy of A Ovsianikov.

multiphoton microscopy is conventionally utilized for imaging living cells, femtosecond laser pulses have also been used for precise dissection of fibers within the cytoskeleton [117–119]. A report on photodisruption of cell mitochondria by femtosecond pulses focused with a high numerical aperture lens, indicated that pulse energies below 3 nJ are not expected to compromise cell viability [120]. The investigations of ablation threshold values for a similar system showed that pulse energies below 1.5 nJ can induce photobleaching but are not sufficient for ablation [117]. A recent study on *in situ* hydrogel degradation confirmed that two-photon-induced photochemistry can be realized at pulse energies of 1.25 nJ, in other words, at cytocompatible irradiation conditions [121].

In summary, the 2PP technique was shown to be capable of producing a variety 3D scaffold designs from different degradable and nondegradable materials. The high nano- and micro-level resolution of 2PP technology, the growing arsenal of photosensitive materials, especially biocompatible photosensitive hydrogels, as well as recent advances in increasing speed of 2PP process and volume of polymerized constructs makes 2PP a very attractive alternative to more traditional methods of tissue-engineered scaffold fabrication. Thus, it is not surprising that scaffold biofabrication is becoming a rapidly growing and increasingly popular biomedical application of 2PP technology. Despite the substantial progress in the field, there is still a lot of potential for 2PP-fabricated scaffolds. In addition to the adequate level of porosity and high resolution 3D features, the ideal scaffolds should be produced from material with desirable properties. It should be biocompatible and biodegradable with properly controllable degradation kinetics. Scaffolds' surfaces should promote cell attachment, spreading, proliferation and migration. In most cases it is desirable for scaffolds to function as a signaling and instructive molecule delivery device, or be able to present and elute differentiation and growth factors and other signaling molecules. Such combination of properties should ultimately facilitate cell and tissue differentiation and, in some cases, be able to induce angiogenesis.

Modification of 3D matrices

In addition to the build-up of 3D structures and scaffolds for cell biological investigations, MPA can be used for configuring already preformed 3D matrices, such as hydrogels. The general procedure for such processing consists of several steps (Figure 6A): the hydrogel is soaked in a photoreactive solution; the multiphoton patterning is performed; and finally the sample is rinsed in order to remove the unreacted reagents. The procedure can vary depending on the method, or be repeated in order to produce another pattern within the sample hydrogel sample.

The group of JL West at Rice University (TX, USA) used 2PP to produce 3D patterns within UV-polymerized PEGda-based hydrogels [122]. In their work, immobilization of functional acrylate moieties was achieved by means of PI-mediated co-polymerization with acrylate groups of the hydrogel matrix. The sample was soaked within a solution of a PI and a lower molecular weight PEGda monomer, in order to distribute them throughout the preformed hydrogel network. Biomechanical 3D patterns are obtained by 2PP of this photoreactive solution, as a result of locally increased cross-linking density. Biochemical patterning was demonstrated by using a fluorescently labeled monoacrylate derivative processed in a similar fashion – the fluorescent molecules are locally immobilized within the network, without substantially changing the density of the hydrogel. Furthermore, by immobilization of PEGylated cell-adhesive peptides (RGDS), migration of HT-1080 fibrosarcoma cells was guided in accordance to produced 3D patterns [122].

The same functionalization approach was later extended to hydrogels, which were made biodegradable by the incorporation of a collagenase-sensitive peptide sequence into the PEG network [30]. For verification of cell-guiding capability, human dermal fibroblast clusters were first produced by encapsulating cells within fibrin gel. Produced clusters were than embedded via photopolymerization within biodegradable PEG-based hydrogels. Finally, the cell-interactive 3D patterns were produced by localized 2PP of cell-adhesive ligand (PEGylated RGDS peptides) within the resulting hydrogel. Within such 2PP functionalized hydrogels, the cells migrated out from the clusters along peptide-patterned regions (Figure 6C) [30]. By repeating the micropatterning procedure, two distinct fibronectin derived peptides were immobilized within the same hydrogel sample [123]. The same group later demonstrated that by 2PP-patterning of integrin ligands and signaling factors within otherwise biologically passive hydrogels, tubulogenesis could be accelerated, along with the upregulation of angiogenic genes in endothelial cells [124]. The key requirement for the presented approach is the availability of free acrylate groups (not consumed during the initial UV polymerization) throughout the hydrogel matrix.

Seidlits *et al.* utilized a similar method for localized cross-linking of BSA proteins in the presence of a photosensitizer within hyaluronic acid-based hydrogels [125]. In contrast to the aforementioned biomechanical matrix modification, here the 2PP-produced BSA and the existing hyaluronic acid networks interpenetrate without covalent bonding between the two. The use of biotinylated BSA for 2PP patterning facilitated later modification of the functionalized sites with cell-adhesive peptides. Glial and neuronal cell guidance was demonstrated on the surface and within the volume of hyaluronic acid hydrogel surfaces with such 2PP-produced adhesion patterns. These reports show the top-down capability of the 2PP technique for biomechanical and biochemical modification of 3D cell-culture matrices, including patterning of cell interactive peptides in a user-defined fashion.

Another option for spatially resolved photochemical modification of 3D matrices is the two-photon uncaging, developed in a series of comprehensive works by the group of M Shoichet at the University of Toronto (ON, Canada) [40–42]. In this approach the matrix already contains biomolecules, and the two-photon excitation is used for deprotection of their functional groups via photocleavage of a photoliable groups. For this, an agarose hydrogel was functionalized with coumarin-caged amines [40]. The natural polymer agarose was chosen

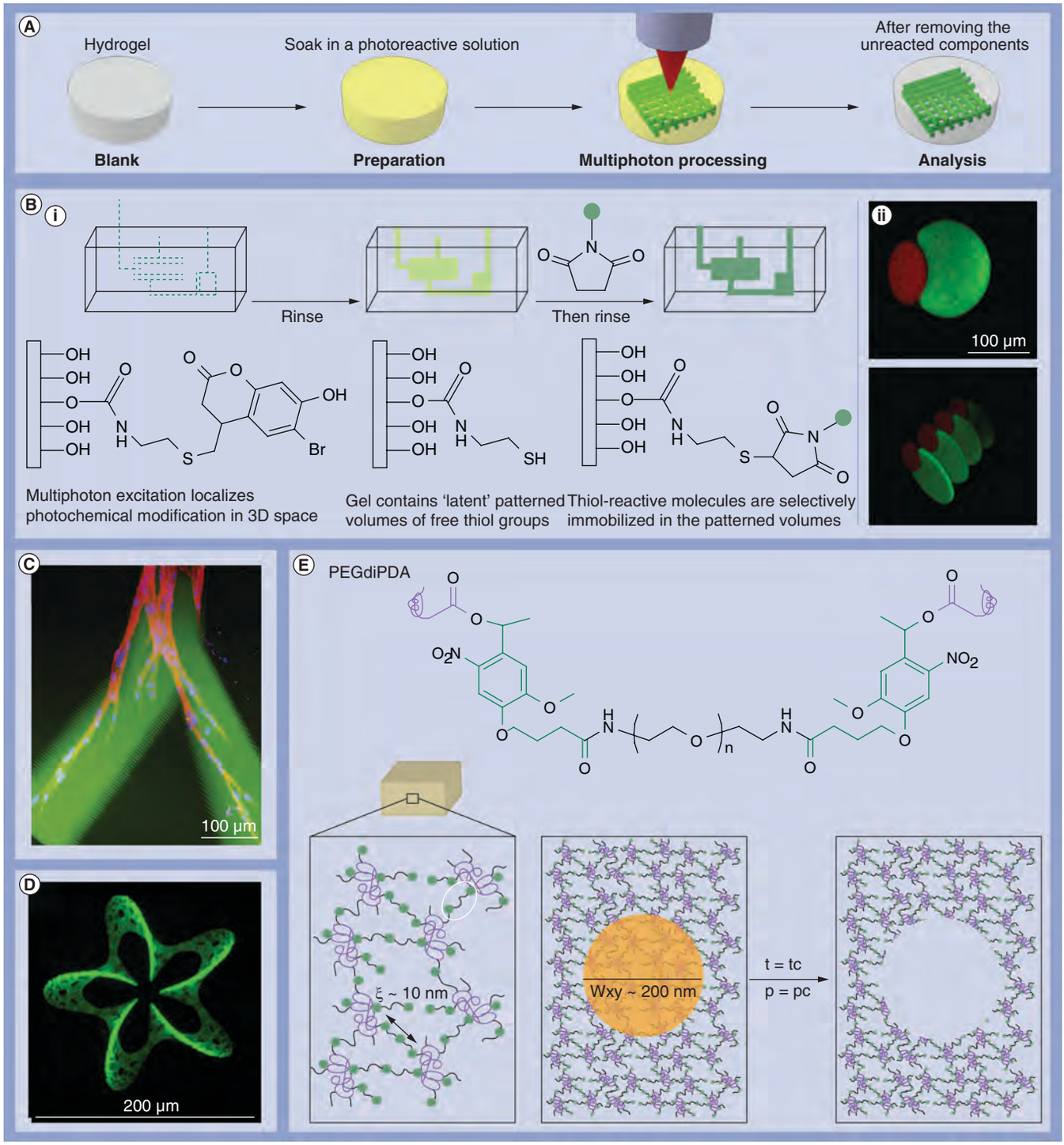


Figure 6. Matrix modification by means of multiphoton processing. (A) General sample processing procedure for methods, resulting in the immobilization of molecules [37]. **(B)** Procedure for two-photon-induced uncaging and chemical modification of a hydrogel matrix with a fluorescent molecule and 3D patterns produced by sequential uncaging and functionalization with two different fluorescently labeled peptides (**[i]**: [41]; **[ii]**: [42]). **(C)** Cells migrating into the RGDS region produced by two-photon polymerization within a poly(ethylene glycol)-based hydrogel [30]. **(D)** 3D patterns produced by multiphoton-induced photographing [37]. **(E)** Two-photon-induced photodegradation [121].
(A) Adapted with permission from [37].
(B) Adapted with permission from [41]. © American Chemical Society (2008).
(E) Adapted with permission from [121]. © The Royal Society of Chemistry (2010).
p: Extent of degradation; pc: Extent of complete erosion; t: Time; tc: Critical exposure time; Wxy: Lateral size of the laser focal point.

since it is transparent, which allows diffusion of biomolecules, and is itself nonadhesive to cells. 3D patterns were produced by scanning the selected volume region of the hydrogel with tightly focused femtosecond laser pulses (Figure 6B). Successful deprotection via TPA was confirmed by reduced fluorescence of the hydrogel throughout the modified region, resulting from eliminated coumarin fluorescence. The functional groups of the produced patterns are primary amines, which can either directly serve as a site for cellular interaction or be further modified with cell-specific molecules [41]. In a very recent report this approach has been further elaborated, taking advantage of the orthogonal chemistry of peptide binding sites in order to pattern two different growth factors, amino-terminal sonic hedgehog (SHH) and ciliary neurotrophic factor [42]. Again, the TPA was used for deprotection of thiol groups within similar agarose hydrogels. In a multistep process the hydrogel was uncaged in the presence of different thiol-reactive proteins, in order to produce distinct volumes of barnase- and then streptavidin-containing patterns. Finally, the sample is soaked in a mixture of fusion proteins, barstar–SHH and biotin–ciliary neurotrophic factor, which specifically bind to barnase and streptavidin, respectively. A final washing step is carried out to remove unbound proteins, yielding two immobilized bioactive factors in spatially defined volumes within the agarose hydrogel (Figure 6B, ii). The bioactivity of immobilized proteins was confirmed with retinal precursor cells of mice. It was demonstrated that the developed coupling method is nontoxic. Furthermore, migration of neural precursor cells into the hydrogel was shown to be stimulated by the gradient patterns of SHH proteins.

Our group has recently reported multiphoton-induced photografting of a reactive aromatic azide compound onto a highly permeable PEG-based matrix [37]. The utilized chemistry involves a single-molecule insertion mechanism, which represents a compromise between the two aforementioned techniques, since it is potentially more efficient, but less precise than uncaging; and more precise, but less efficient than chain-growth mechanisms of 2PP. Nevertheless, a highly selective molecule immobilization with the lateral resolution down to 4 µm in 3D was achieved by use of conventional optics with moderate numerical aperture (Figure 6D). Along with high scanning speeds of over 550 mm/s, the results emphasise the competiveness of the multiphoton grafting approach for modification of hydrogels. In the second step the photografted patterns were successfully functionalized via click chemistry. The developed two-step method is very universal since it is applicable to a wide variety of matrices containing C–H or N–H bonds (Figure 2C). Furthermore, owing to the distinct features of click reactions, the application range of 'functionalization' can be largely extended by utilizing various alkyne derivatives. Among the main drawbacks of the reported approach is the potential toxicity of the utilized aromatic azide compound. If the multiphoton grafting is to be performed in the presence of living cells, a different compound has to be selected.

At the group of KS Anseth at the University of Colorado (CO, USA), monomers, capable of polymerizing in the presence of cells to produce photolytically degradable hydrogels, whose physical or chemical properties are tunable temporally and spatially with light, were developed [126]. PEG-based hydrogels were rendered photodegradable by the utilization of a nitrobenzyl ether-derived moiety, susceptible to two-photon photolysis. The photoliable moiety is incorporated into the network backbone. This way the TPA causes cleavage of hydrogels, resulting in a decreased cross-linking density of the local network (Figure 6E). The developed method was used to erode channels for guiding fibrosarcoma cell migration [39], and local cavities inducing detachment of mesenchymal stem cells [121]. Recently, this group reported development of cytocompatible click-based hydrogels, supporting wavelength-specific photocleavage and photoconjugation reactions [127]. These novel hydrogels can serve as 3D cell-culture matrix with dynamically tunable biomechanical and biochemical properties. Inducing these reactions via MPA provides spatiotemporal control. A further report described photoreversible patterning of biomolecules within similar hydrogels [128]. Depending on the applied wavelength, a specially designed peptide could be immobilized or removed to provide the hydrogel with distinct cell adhesive characteristics. This work demonstrated the potential of two-photon-induced reactions for real-time spatially resolved manipulation of the cell microenvironment.

In summary, a number of methods for spatially resolved modification of hydrogel matrices by means of multiphoton-induced photochemistry were developed. They allow biomechanical modification of hydrogels by increasing (2PP) or reducing (photodegradation) the cross-linking density in a controlled fashion. In addition, biochemical modification of hydrogels by utilization of a variety of materials, including biomolecules was demonstrated. Most of the above described methods were verified to be cytocompatible. Therefore, modification of hydrogels containing living cells, that is, dynamic manipulation of the cellular microenvironment, is possible.

Expert commentary

The development of multiphoton processing technologies showed considerable advances within just a little more than a decade. Their recent applications in cell biology and tissue engineering indicate the emergence of a new technology platform in response to an urgent need for customized cell microenvironments mimicking the complexity of the natural ECM. The following aspects of the multiphoton processing speak in favor of using this approach for development of standardized 3D cell-culture systems:

- The excellent control over the experimental variables, due to flexibility and reproducibility of structure or pattern design;
- Tunability of both biochemical and biomechanical properties. Along with the high spatial resolution and manifold properties of available materials, this should allow selective mimicking of various parameters of the natural ECM;
- Owing to optical transparency of utilized materials (a prerequisite for multiphoton processing), most produced matrices are automatically compatible with optical analysis tools that are already well established in biology.

The reviewed reports demonstrate that the multiphoton processing methods can be used to study a wide variety of relevant biological questions. Fabrication of complex 3D constructs in accordance to a computer-aided design model offers a true engineering dimension to designing of cell-culture matrices. Their parameters can be adapted to study specific biological and tissue processes. The possibility of precisely adjusting the biomechanical and biochemical properties of produced matrix might finally allow separation of the influence of these two parameters on cell behavior in 3D. The further development of the reviewed techniques will facilitate realization of elegant biological *in vitro* experiments, helping to elucidate biomimetic aspects of cell interaction with the surrounding environment. In perspective, multiphoton processing technologies are sufficiently versatile to produce standard biomimetic 3D matrices for different tissue types. Finally, the authors expect them to contribute to a paradigm transition from accepting the cell-culture matrix as a standard based on its availability, to constructing and selection of a standard matrix based on its performances in particular cell culture. These considerations further emphasize the potential impact of the multiphoton processing methods for realization of rationally engineered biomimetic cell-culture matrices.

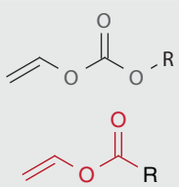
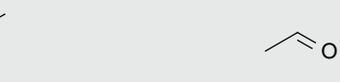
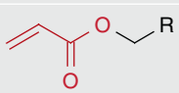
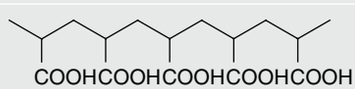
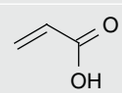
Five-year view

The progress in this multidisciplinary field was possible owing to close cooperation between physicists, chemists, mechanical engineers and biologists. The further pace of development will depend in contributions from these respective fields. Advancement of the turnkey femtosecond lasers has considerably simplified maintenance and operation of multiphoton processing systems. A rapidly increasing number of groups are now using adapted two-photon microscopes, home-built, or even commercially obtained 2PP systems. Despite the fact that essentially the same experimental setup can be used for all the reviewed multiphoton processing methods, 2PP is clearly the most widespread; admittedly, because multiphoton grafting, uncaging and erosion rely on specialized chemistry, while most materials currently used for 2PP are readily available. However, there is still a lot of room for development in the field of two-photon initiation. Since the principles and rules for two-photon excitation are now better understood, we expect notable progress in this direction within the next 5 years. The combination of specialized chromophores for TPA with classical strategies for photoinitiation seems to be a reasonable path. Particularly, the lack of the water soluble two-photon PIs has to be addressed.

An important issue for widespread adoption of the reviewed multiphoton techniques is the speed of processing. The majority of current experimental systems provide the maximum scanning speed of approximately 10 mm/s. Depending on the required resolution and the photosensitivity of utilized material, fabrication of a millimeter-scale scaffold/pattern might take from a few hours to longer than a day. For systematic biological studies, series of such scaffolds are necessary and for tissue engineering much larger structures are desirable. Necessary upscaling relies on utilization of highly efficient photochemistry but also on improvements on the hardware side. Our group at the Vienna University of Technology (Vienna, Austria) has recently developed an experimental system facilitating a drastic increase of multiphoton processing speed to at least 550 mm/s [37].

In the field of biocompatible materials, the two approaches of using natural and synthetic sources will persist. While natural polymers have the advantage of specific functions, the drawback of possible immunogenic reactions remains. In entirely synthetic polymers, possible degradation products and remaining unreacted groups in polymerizable systems have to be considered. From this point of view vinylesters and vinylcarbonates [129] could substitute currently used acrylate chemistry, which is associated with problems of irritancy and cytotoxicity [130,131]. Vinylcarbonates, in particular, have no acidic degradation products that allow a preferred surface erosion mechanism (Table 2). Since there is always at least 10–20% nonreacted group present in the polymer after the 2PP, attention should be paid not only to the cytotoxicity of the group itself, but also to possible degradation products. Acrylates form rather cytotoxic acrylic acid, while the new monomers form acetaldehyde that can be easily oxidized to acetic acid by acetaldehyde dehydrogenase. In

Table 2. Acrylates and alternative monomers for two-photon polymerization.

Monomer	Polymer backbone and degradation product	Cleavage product of nonreacted polymerizable groups
Vinylesters and vinylcarbonates			
Acrylates			

Monomers have similar reactivity to acrylates in thiol-ene polymerization. Cytotoxicity is two to three orders of magnitude lower than acrylates. The degradation product polyvinylalcohol is nontoxic and approved by the US FDA. Nonreacted groups form less irritant acetaldehyde under degradation that can be easily oxidized to harmless acetic acid.

addition, the authors' recent results show that the reactivity of thiol-ene chemistry can compete with that of acrylates [Mautner A, Qin X, Macfelda GK *et al*. Efficient curing of vinyl carbonates by thiol-ene polymerization (2012), Submitted].

Finally, the authors expect that availability of water-soluble PIs will facilitate advances in the area of water-based photopolymers for realization of hydrogel constructs. Hydrogels are already a well accepted and highly relevant biomimetic material platform. The possibility to use 2PP for microstructuring of hydrogels adds a new degree of freedom to this area of research. Equally attractive is the use of 2PP with the near-infrared light for encapsulation of cells within a hydrogel already during the fabrication process.

Still being in an early development stage, multiphoton processing technologies have a lot to offer to the fields of biology and tissue engineering. The most likely applications within the next 5 years are investigations of the role of the ECM in stem cell differentiation, angiogenesis, studies of cell and tissue mechanobiology and mechanisms gathering migration of different cells, including tumor cell invasion and metastases development. In a long-term perspective, the authors anticipate that multiphoton-processing technologies will contribute to development of tissue models, allowing the *in vitro* reproduction of some crucial aspects of natural cell behavior. Availability of such reliable artificial tissues will help to reduce the number of animal studies and the cost of research.

Financial & competing interests disclosure

R Liska kindly thanks the ESF for financial support in the P2M network. V Mironov would like to acknowledge FAPESP visiting professor fellowship. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- Commercially available systems for two-photon polymerization (2PP) processing are quite expensive and are not very easy to adapt to individual needs.
- The portfolio of biocompatible and biodegradable 2PP photopolymers is still very scarce and has to be further extended in order to address different biological issues.
- Mainly commercially available photoinitiators, designed for one-photon polymerization, are used for 2PP. Highly effective two-photon initiators will contribute to increasing the processing speed, while minimizing the possibility of cytotoxic effects.
- Processability of photopolymers with high water content is mainly limited by the lack of water-soluble photoinitiators suitable for 2PP.
- Current 2PP systems are fairly precise, but are relatively slow. Most reported scaffolds are miniature and, as such, are hard to handle in cell culture. In addition, statistically relevant experiments require a large series of identical structures. In order to keep up with these requirements, processing speed has to be increased manifold.

References

1 Cukierman E. Taking cell-matrix adhesions to the third dimension. *Science* 294(5547), 1708–1712 (2001).

2 Bokhari M, Carnachan RJ, Cameron NR, Przyborski SA. Culture of HepG2 liver cells on three dimensional polystyrene scaffolds enhances cell structure and function during toxicological challenge. *J. Anat.* 211(4), 567–576 (2007).

3 Stegemann JP, Nerem RM. Altered response of vascular smooth muscle cells to exogenous biochemical stimulation in two- and three-dimensional culture. *Exp. Cell Res.* 283(2), 146–155 (2003).

4 Alcaraz J, Xu R, Mori H *et al.* Laminin and biomimetic extracellular elasticity enhance functional differentiation in mammary epithelia. *EMBO* 27(21), 2829–2838 (2008).

5 Peyton SR, Kim PD, Ghajar CM, Seliktar D, Putnam AJ. The effects of matrix stiffness and RhoA on the phenotypic plasticity of smooth muscle cells in a 3-D biosynthetic hydrogel system. *Biomaterials* 29(17), 2597–2607 (2008).

6 Cukierman E, Pankov R, Yamada KM. Cell interactions with three-dimensional matrices. *Curr. Opin. Cell Biol.* 14(5), 633–640 (2002).

7 Abbot A. Cell culture: biology's new dimension. *Nature* 424(6951), 870–872 (2003).

8 Schmeichel KL. Modeling tissue-specific signaling and organ function in three dimensions. *J. Cell Sci.* 116(12), 2377–2388 (2003).

9 Mooney DJ, Baldwin DF, Suh NP, Vacanti JP, Langer R. Novel approach to fabricate porous sponges of poly(D,L-lactic-*co*-glycolic acid) without the use of organic solvents. *Biomaterials* 17(14), 1417–1422 (1996).

10 Mikos AG, Thorsen AJ, Czerwonka LA *et al*. Preparation and characterization of poly(L-lactic acid) foams. *Polymer* 35, 1068 (1994).

11 Kumbar SG, James R, Nukavarapu SP, Laurencin CT. Electrospun nanofiber scaffolds: engineering soft tissues. *Biomed. Mater.* 3(3), 034002 (2008).

12 Hutmacher DW, Sittinger M, Risbud MV. Scaffold-based tissue engineering: rationale for computer-aided design and solid free-form fabrication systems. *Trends Biotechnol.* 22(7), 354–362 (2004).

13 Liu X, Ma PX. Phase separation, pore structure, and properties of nanofibrous gelatin scaffolds. *Biomaterials* 30(25), 4094–4103 (2009).

14 Moroni L, Schotel R, Hamann D, de Wijn JR, van Blitterswijk CA. 3D Fiber-deposited electrospun integrated scaffolds enhance cartilage tissue formation. *Adv. Funct. Mater.* 18(1), 53–60 (2008).

15 Tsang KY, Cheung MCH, Chan D, Cheah KSE. The developmental roles of the extracellular matrix: beyond structure to regulation. *Cell Tissue Res.* 339, 93–110 (2009).

16 Guilak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS. Control of stem cell fate by physical interactions with the extracellular matrix. *Cell Stem Cell* 5(1), 17–26 (2009).

17 Wheeldon I, Farhadi A, Bick AG, Jabbari E, Khademhosseini A. Nanoscale tissue engineering: spatial control over cell–materials interactions. *Nanotechnology* 22(21), 212001 (2011).

18 Dvir T, Timko BP, Kohane DS, Langer R. Nanotechnological strategies for engineering complex tissues. *Nat. Nanotechnol.* 6(1), 13–22 (2010).

19 Lutolf MP. Integration column: artificial ECM: expanding the cell biology toolbox in 3D. *Integr. Biol.* 1(3), 235 (2009).

20 Tibbitt MW, Anseth KS. Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol. Bioeng.* 103(4), 655–663 (2009).

21 McGuigan AP, Bruzewicz DA, Glavan A, Butte M, Whitesides GM. Cell encapsulation in sub-mm sized gel modules using replica molding. *PLoS ONE* 3(5), e2258 (2008).

22 Zipfel WR, Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in the biosciences. *Nat. Biotechnol.* 21(11), 1369–1377 (2003).

23 Göppert-Mayer M. Elementary processes with two quantum transitions. *Ann. Phys. (Berlin)* 18(7–8), 466–479 (2009).

24 Malencik DA, Anderson SR. Dityrosine as a product of oxidative stress and fluorescent probe. *Amino Acids* 25(3–4), 233–247 (2003).

25 Spikes JD, Shen H-R, Kopečková P, Kopeček J. Photodynamic crosslinking of proteins. III. Kinetics of the FMN- and rose bengal-sensitized photooxidation and intermolecular crosslinking of model tyrosine-containing *N*-(2-hydroxypropyl)methacrylamide copolymers. *Photochem. Photobiol.* 70(2), 130–137 (1999).

26 Maruo S, Nakamura O, Kawata S. Three-dimensional microfabrication with two-photon-absorbed photopolymerization. *Opt. Lett.* 22(2), 132 (1997).

27 Lu W-E, Dong X-Z, Chen W-Q, Zhao Z-S, Duan X-M. Novel photoinitiator with a radical quenching moiety for confining radical diffusion in two-photon induced photopolymerization. *J. Mater. Chem.* 21(15), 5650 (2011).

28 Yokoyama S, Nakahama T, Miki H, Mashiko S. Two-photon-induced polymerization in a laser gain medium for optical microstructure. *Appl. Phys Lett.* 82(19), 3221 (2003).

29 Juodkazis S, Mizeikis V, Seet KK, Miwa M, Misawa H. Two-photon lithography of nanorods in SU-8 photoresist. *Nanotechnology* 16(6), 846–849 (2005).

30 Lee S-H, Moon JJ, West JL. Three-dimensional micropatterning of bioactive hydrogels via two-photon laser scanning photolithography for guided 3D cell migration. *Biomaterials* 29(20), 2962–2968 (2008).

31 Hsieh TM, Benjamin Ng CW, Narayanan K, Wan ACA, Ying JY. Three-dimensional microstructured tissue scaffolds fabricated by two-photon laser scanning photolithography. *Biomaterials* 31(30), 7648–7652 (2010).

32 Basu S, Rodionov V, Terasaki M, Campagnola PJ. Multiphoton-excited microfabrication in live cells via rose bengal cross-linking of cytoplasmic proteins. *Opt. Lett.* 30(2), 159 (2005).

33 Haske W, Chen VW, Hales JM *et al.* 65 nm feature sizes using visible wavelength 3-D multiphoton lithography. *Opt. Express* 15(6), 3426 (2007).

34 Mizeikis V, Seet KK, Juodkazis S, Misawa H. Three-dimensional woodpile photonic crystal templates for the infrared spectral range. *Opt. Lett.* 29(17), 2061–2063 (2004).

35 Ventura MJ, Bullen C, Gu M. Direct laser writing of three-dimensional photonic crystal lattices within a PbS quantum-dot-doped polymer material. *Opt. Express* 15(4), 1817–1822 (2007).

36 Klein F, Striebel T, Fischer J *et al.* Elastic fully three-dimensional microstructure scaffolds for cell force measurements. *Adv. Mater.* 22(8), 868–871 (2010).

37 Ovsianikov A, Zhiquan L, Torgersen J, Stampfl J, Liska R. Selective functionalization of 3D matrices via multi-photon grafting and subsequent click chemistry. *Adv. Funct. Mater.* doi:10.1002/adfm.201200419 (2012) (Epub ahead of print).

38 Judkewitz B, Roth A, Häusser M. Dendritic enlightenment: using patterned two-photon uncaging to reveal the secrets of the brain's smallest dendrites. *Neuron* 50(2), 180–183 (2006).

39 Kloxin AM, Kasko AM, Salinas CN, Anseth KS. Photodegradable hydrogels for dynamic tuning of physical and chemical properties. *Science* 324(5923), 59–63 (2009).

40 Wylie RG, Shoichet MS. Two-photon micropatterning of amines within an agarose hydrogel. *J. Mat. Chem.* 18(23), 2716–2721 (2008).

41 Wosnick JH, Shoichet MS. Three-dimensional chemical patterning of transparent hydrogels. *Chem. Mat.* 20(1), 55–60 (2008).

42 Wylie RG, Ahsan S, Aizawa Y, Maxwell KL, Morshead CM, Shoichet MS. Spatially controlled simultaneous patterning of multiple growth factors in three-dimensional hydrogels. *Nat. Mat.* 10(10), 799–806 (2011).

43 Sun H-B, Kawakami T, Xu Y *et al.* Real three-dimensional microstructures fabricated by photopolymerization of resins through two-photon absorption. *Opt. Lett.* 25(15), 1110–1112 (2000).

44 Nguyen LH, Straub M, Gu M. Acrylate-based photopolymer for two-photon microfabrication and photonic applications. *Adv. Funct. Mater.* 15(2), 209–216 (2005).

45 Wu S, Serbin J, Gu M. Two-photon polymerisation for three-dimensional micro-fabrication. *J. Photochem. Photobiol. A Chem.* 181(1), 1–11 (2006).

46 Heller C, Pucher N, Seidl B *et al.* One- and two-photon activity of cross-conjugated photoinitiators with bathochromic shift. *J. Polymer Science A Polymer Chem.* 45(15), 3280–3291 (2007).

47 Baldacchini T, LaFratta CN, Farrer RA *et al*. Acrylic-based resin with favorable properties for three-dimensional two-photon polymerization. *J. Appl. Phys.* 95(11), 6072–6076 (2004).

48 Albota M. Design of organic molecules with large two-photon absorption cross sections. *Science* 281(5383), 1653–1656 (1998).

49 Reinhardt BA, Brott LL, Clarson SJ *et al.* Highly active two-photon dyes: design, synthesis, and characterization toward application. *Chem. Mater.* 10(7), 1863–1874 (1998).

50 Rumi M, Ehrlich JE, Heikal AA *et al.* Structure–property relationships for two-photon absorbing chromophores: bis-donor diphenylpolyene and bis(styryl) benzene derivatives. *J. Am. Chem. Soc.* 122(39), 9500–9510 (2000).

51 Xing J-F, Chen W-Q, Gu J *et al.* Design of high efficiency for two-photon polymerization initiator: combination of radical stabilization and large two-photon cross-section achieved by *N*-benzyl 3,6-bis(phenylethynyl)carbazole derivatives. *J. Mater. Chem.* 17(14), 1433–1438 (2007).

52 Terenziani F, Painelli A, Katan C, Charlot M, Blanchard-Desce M. Charge instability in quadrupolar chromophores: symmetry breaking and solvatochromism. *J. Am. Chem. Soc.* 128(49), 15742–15755 (2006).

53 Clay GO, Schaffer CB, Kleinfeld D. Large two-photon absorptivity of hemoglobin in the infrared range of 780–880 nm. *J. Chem. Phys.* 126(2), 025102 (2007).

54 Xu C, Webb WW. Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm. *JOSA B* 13(3), 481–491 (1996).

55 Bhawalkar JD, He GS, Prasad PN. Nonlinear multiphoton processes in organic and polymeric materials. *Rep. Prog. Phys.* 59(9), 1041–1070 (1996).

56 Yang Q, Seo J, Creekmore S *et al.* Z-scan and four-wave mixing characterization of semiconductor cadmium chalcogenide nanomaterials. *J. Phys. Conf. Ser.* 38, 144–147 (2006).

57 Sheik-Bahae M, Said AA, Wei T-H, Hagan DJ, Van Stryland EW. Sensitive measurement of optical nonlinearities using a single beam. *IEEE J. Quantum Electron* 26(4), 760–769 (1990).

58 Xue J, Zhao Y, Wu J, Wu F. Novel benzylidene cyclopentanone dyes for two-photon photopolymerization. *J. Photochem. Photobiol. A Chem.* 195(2–3), 261–266 (2008).

59 Cicha K, Li Z, Stadlmann K *et al.* Evaluation of 3D structures fabricated with two-photon-photopolymerization by using FTIR spectroscopy. *J. Appl. Phys.* 110, 064911 (2011).

60 Zhang X, Yu X, Sun Y *et al.* Synthesis, structure and nonlinear optical properties of two new one and two-branch two-photon polymerization initiators. *Chem. Phys.* 328(1–3), 103–110 (2006).

61 Lu Y, Hasegawa F, Kawazu Y, Totani K, Yamashita T, Toshiyuki W. Investigation of mechanism of photo induced polymerization excited by two-photon absorption. *FIBER* 60(6), 165–172 (2004).

62 Lu Y, Hasegawa F, Goto T *et al.* Highly sensitive measurement in two-photon absorption cross section and investigation of the mechanism of two-photon-induced polymerization. *J. Lumin.* 110(1–2), 1–10 (2004).

63 Jin M, Malval J-P, Versace D-L *et al.* Two-photon absorption and polymerization ability of intramolecular energy transfer based photoinitiating systems. *Chem. Commun.* (48), 6540–6542 (2008).

64 Lu Y, Hasegawa F, Ohkuma S *et al.* Highly efficient two-photon initiated polymerization in solvent by using a novel two-photon chromophore and co-initiators. *J. Mater. Chem.* 14(9), 1391–1395 (2004).

65 Wu J, Zhao Y, Li X, Shi M, Wu F, Fang X. Multibranched benzylidene cyclopentanone dyes with large two-photon absorption cross-sections. *N. J. Chem.* 30(7), 1098–1103 (2006).

66 Lin T-C, Chung S-J, Kim K-S *et al.* Organics and polymers with high two-photon activities and their applications. In: *Polymers for Photonics Applications II.* Lee K-S (Ed.). Springer, Berlin, Heidelberg, Germany, 157–193 (2003).

67 Rumi M, Barlow S, Wang J, Perry JW, Marder SR. Two-photon absorbing materials and two-photon-induced chemistry [internet]. In: *Photoresponsive Polymers I.* Marder SR, Lee K-S (Eds). Springer, Berlin, Heidelberg, Germany, 1–95 (2008).

68 Lee K-S, Kim RH, Yang D-Y, Park SH. Advances in 3D nano/microfabrication using two-photon initiated polymerization. *Prog. Poly. Sci.* 33(6), 631–681 (2008).

69 Li Z, Siklos M, Pucher N *et al.* Synthesis and structure-activity relationship of several aromatic ketone-based two-photon initiators. *J. Polym. Sci. A-Polym. Chem.* 49(17), 3688–3699 (2011).

70 Pucher N, Rosspeintner A, Satzinger V *et al.* Structure−activity relationship in D-π-A- π-D-based photoinitiators for the two-photon-induced photopolymerization process. *Macromolecules* 42(17), 6519–6528 (2009).

71 Green WA, Timms AW. Water-soluble photoinitiators. In: *Radiation Curing in Polymer Science and Technology.* Fouassier J-P, Rabek JF (Eds). Springer, NY, USA, 375–434 (1993).

72 Ullrich G, Ganster B, Salz U, Moszner N, Liska R. Photoinitiators with functional groups. IX. Hydrophilic bisacylphosphine oxides for acidic aqueous formulations. *J. Poly. Sci. A-Poly. Chem.* 44(5), 1686–1700 (2006).

73 Lougnot DJ, Turck C, Fouassier JP. Water-soluble polymerization initiators based on the thioxanthone structure: a spectroscopic and laser photolysis study. *Macromolecules* 22(1), 108–116 (1989).

74 Fang Z, Zhang X, Hing Lai Y, Liu B. Bridged triphenylamine based molecules with large two-photon absorption cross sections in organic and aqueous media. *Chem. Commun.* (8), 920–922 (2009).

75 Hong JW, Woo HY, Liu B, Bazan GC. Solvatochromism of distyrylbenzene pairs bound together by [2.2]paracyclophane: evidence for a polarizable 'through-space' delocalized state. *J. Am. Chem. Soc.* 127(20), 7435–7443 (2005).

76 Woo HY, Liu B, Kohler B, Korystov D, Mikhailovsky A, Bazan GC. Solvent effects on the two-photon absorption of distyrylbenzene chromophores. *J. Am. Chem. Soc.* 127(42), 14721–14729 (2005).

77 Tian Y, Chen C-Y, Cheng Y-J, Young AC, Tucker NM, Jen AK-Y. Hydrophobic chromophores in aqueous micellar solution showing large two-photon absorption cross sections. *Adv. Funct. Mater.* 17(10), 1691–1697 (2007).

78 Campagnola PJ, Delguidice DM, Epling GA *et al.* 3-Dimensional submicron polymerization of acrylamide by multiphoton excitation of xanthene dyes. *Macromolecules* 33(5), 1511–1513 (2000).

79 Farsari M, Filippidis G, Sambani K, Drakakis TS, Fotakis C. Two-photon polymerization of an Eosin Y-sensitized acrylate composite. *J. Photochem. Photobiol. A Chem.* 181(1), 132–135 (2006).

80 Li S, Li L, Wu F, Wang E. A water-soluble two-photon photopolymerization initiation system: Methylated-β-cyclodextrin complex of xanthene dye/aryliodonium salt. *J. Photochem. Photobiol. A Chem.* 203(2–3), 211–215 (2009).

81 Wan X, Zhao Y, Xue J, Wu F, Fang X. Water-soluble benzylidene cyclopentanone dye for two-photon photopolymerization. *J. Photochem. Photobiol. A Chem.* 202(1), 74–79 (2009).

82 Jhaveri SJ, McMullen JD, Sijbesma R, Tan L-S, Zipfel W, Ober CK. Direct three-dimensional microfabrication of hydrogels via two-photon lithography in aqueous solution. *Chem. Mater.* 21(10), 2003–2006 (2009).

83 Torgersen J, Ovsianikov A, Mironov V *et al.* Photo-sensitive hydrogels for three-dimensional laser micro-fabrication in the presence of whole organisms. *J. Biomed. Opt.* (2012) (In Press).

84 Pitts JD, Campagnola PJ, Epling GA, Goodman SL. Submicron multiphoton free-form fabrication of proteins and polymers: studies of reaction efficiencies and applications in sustained release. *Macromolecules* 33(5), 1514–1523 (2000).

85 Khripin CY, Brinker CJ, Kaehr B. Mechanically tunable multiphoton fabricated protein hydrogels investigated using atomic force microscopy. *Soft Matter* 6(12), 2842–2848 (2010).

86 Nielson R, Kaehr B, Shear JB. Microreplication and design of biological architectures using dynamic-mask multiphoton lithography. *Small* 5(1), 120–125 (2009).

87 Ovsianikov A, Schlie S, Ngezahayo A, Haverich A, Chichkov BN. Two-photon polymerization technique for microfabrication of CAD-designed 3D scaffolds from commercially available photosensitive materials. *J. Tissue Eng. Regen. Med.* 1(6), 443–449 (2007).

88 Claeyssens F, Hasan EA, Gaidukeviciute A *et al.* Three-dimensional biodegradable structures fabricated by two-photon polymerization. *Langmuir* 25(5), 3219–3223 (2009).

89 Mizutani M, Matsuda T. Photocurable liquid biodegradable copolymers: *in vitro* hydrolytic degradation behaviors of photocured films of coumarin-endcapped poly(ε-caprolactone-*co*-trimethylene carbonate). *Biomacromolecules* 3(2), 249–255 (2002).

90 Weiß T, Schade R, Laube T *et al.* Two-photon polymerization of biocompatible photopolymers for microstructured 3D biointerfaces. *Adv. Eng. Mater.* 13(9), B264–B273 (2011).

91 Melissinaki V, Gill AA, Ortega I *et al.* Direct laser writing of 3D scaffolds for neural tissue engineering applications. *Biofabrication* 3(4), 045005 (2011).

92 Ovsianikov A, Deiwick A, Van Vlierberghe S *et al.* Laser fabrication of three-dimensional CAD scaffolds from photosensitive gelatin for applications in tissue engineering. *Biomacromolecules* 12(4), 851–858 (2011).

93 Fedorovich NE, Oudshoorn MH, van Geemen D, Hennink WE, Alblas J, Dhert WJA. The effect of photopolymerization on stem cells embedded in hydrogels. *Biomaterials* 30(3), 344–353 (2009).

94 Bryant SJ, Bender RJ, Durand KL, Anseth KS. Encapsulating chondrocytes in degrading PEG hydrogels with high modulus: engineering gel structural changes to facilitate cartilaginous tissue production. *Biotechnol. Bioeng.* 86(7), 747–755 (2004).

95 Ovsianikov A, Deiwick A, Van Vlierberghe S *et al.* Laser fabrication of 3D gelatin scaffolds for the generation of bioartificial tissues. *Materials* 4(1), 288–299 (2011).

96 Boontheekul T, Hill EE, Kong H-J, Mooney DJ. Regulating myoblast phenotype through controlled gel stiffness and degradation. *Tissue Eng.* 13(7), 1431–1442 (2007).

97 Ovsianikov A, Malinauskas M, Schlie S *et al.* Three-dimensional laser micro- and nano-structuring of acrylated poly(ethylene glycol) materials and evaluation of their cytoxicity for tissue engineering applications. *Acta Biomaterialia* 7(3), 967–974 (2011).

98 Kaehr B. Guiding neuronal development with *in situ* microfabrication. *Proc. Natl Acad. Sci. USA* 101(46), 16104–16108 (2004).

99 Murray-Dunning C, McArthur SL, Sun T, McKean R, Ryan AJ, Haycock JW. Three-dimensional alignment of schwann cells using hydrolysable microfiber scaffolds: strategies for peripheral nerve repair [internet]. In: *3D Cell Culture.* Haycock JW (Ed.). Humana Press, Totowa, NJ, USA, 155–166 (2011).

100 Friedl P, Brücker E-B. The biology of cell locomotion within three-dimensional extracellular matrix. *Cell Mol. Life Sci.* 57(1), 41–64 (2000)

101 Jeon H, Hidai H, Hwang DJ, Healy KE, Grigoropoulos CP. The effect of micronscale anisotropic cross patterns on fibroblast migration. *Biomaterials* 31(15), 4286–4295 (2010).

102 Kiyan Y, Limbourg A, Kiyan R *et al.* Urokinase receptor associates with myocardin to control vascular smooth muscle cells phenotype in vascular disease. *Arterioscler. Thromb. Vasc. Biol.* 32(1), 110–122 (2011).

103 Tayalia P, Mendonca CR, Baldacchini T, Mooney DJ, Mazur E. 3D Cell-migration studies using two-photon engineered polymer scaffolds. *Adv. Mater.* 20, 4494–4498 (2008).

104 Tayalia P, Mazur E, Mooney DJ. Controlled architectural and chemotactic studies of 3D cell migration. *Biomaterials* 32(10), 2634–2641 (2011).

105 Klein F, Richter B, Striebel T *et al.* Two-component polymer scaffolds for controlled three-dimensional cell culture. *Adv. Mater.* 23(11), 1341–1345 (2011).

106 Langer R, Vacanti J. Tissue engineering. *Science* 260(5110), 920–926 (1993).

107 Meng D, Erol M, Boccaccini AR. Processing technologies for 3D nanostructured tissue engineering scaffolds. *Adv. Eng. Mater.* 12(9), B467–B487 (2010).

108 Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues – state of the art and future perspectives. *J. Biomater. Sci. Polym. Ed.* 12(1), 107–124 (2001).

109 Ovsianikov A, Schlie S, Ngezahayo A, Haverich A, Chichkov BN. Two-photon polymerization technique for microfabrication of CAD-designed 3D scaffolds from commercially available photosensitive materials. *J. Tissue Eng. Regen. Med.* 1(6), 443–449 (2007).

110 Schlie S, Ngezahayo A, Ovsianikov A *et al.* Three-dimensional cell growth on structures fabricated from ORMOCER® by two-photon polymerization technique. *J. Biomater. Appl.* 22(3), 275–287 (2007).

111 Ovsianikov A, Gruene M, Pflaum M *et al.* Laser printing of cells into 3D scaffolds. *Biofabrication* 2(1), 014104 (2010).

112 Bible E, Chau DYS, Alexander MR, Price J, Shakesheff KM, Modo M. The support of neural stem cells transplanted into stroke-induced brain cavities by PLGA particles. *Biomaterials* 30(16), 2985–2994 (2009).

113 Psycharakis S, Tosca A, Melissinaki V, Giakoumaki A, Ranella A. Tailor-made three-dimensional hybrid scaffolds for cell cultures. *Biomed. Mater.* 6(4), 045008 (2011).

114 Koroleva A, Schlie S, Fadeeva E *et al.* Microreplication of laser-fabricated surface and three-dimensional structures. *J. Opt.* 12(12), 124009 (2010).

115 Koroleva A, Gittard S, Schlie S, Deiwick A, Jockenhoevel S, Chichkov B. Fabrication of fibrin scaffolds with controlled microscale architecture by a two-photon polymerization–micromolding technique. *Biofabrication* 4(1), 015001 (2012).

116 Zhu J, Marchant RE. Design properties of hydrogel tissue-engineering scaffolds. *Expert Rev. Med. Devices* 8(5), 607–626 (2011).

117 Heisterkamp A, Maxwell IZ, Mazur E *et al.* Pulse energy dependence of subcellular dissection by femtosecond laser pulses. *Opt. Express* 13(10), 3690 (2005).

118 Kumar S, Maxwell IZ, Heisterkamp A *et al.* Viscoelastic retraction of single living stress fibers and its impact on cell shape, cytoskeletal organization, and extracellular matrix mechanics. *Biophys. J.* 90(10), 3762–3773 (2006).

119 Maxwell I, Chung S, Mazur E. Nanoprocessing of subcellular targets using femtosecond laser pulses. *Med. Laser Appl.* 20(3), 193–200 (2005).

120 Watanabe W, Arakawa N, Matsunaga S *et al.* Femtosecond laser disruption of subcellular organelles in a living cell. *Opt. Express.* 12(18), 4203 (2004).

121 Tibbitt MW, Kloxin AM, Dyamenahalli KU, Anseth KS. Controlled two-photon photodegradation of PEG hydrogels to study and manipulate subcellular interactions on soft materials. *Soft Matter* 6, 5100 (2010).

122 Hahn MS, Miller JS, West JL. Three-dimensional biochemical and biomechanical patterning of hydrogels for guiding cell behavior. *Adv. Mater.* 18(20), 2679–2684 (2006).

123 Hoffmann JC, West JL. Three-dimensional photolithographic patterning of multiple bioactive ligands in poly(ethylene glycol) hydrogels. *Soft Matter* 6(20), 5056 (2010).

124 Leslie-Barbick JE, Shen C, Chen C, West JL. Micron-scale spatially patterned, covalently immobilized vascular endothelial growth factor on hydrogels accelerates endothelial tubulogenesis and increases cellular angiogenic responses. *Tissue Eng. A* 17(1–2), 221–229 (2011).

125 Seidlits SK, Schmidt CE, Shear JB. High-resolution patterning of hydrogels in three dimensions using direct-write photofabrication for cell guidance. *Adv. Funct. Mater.* 19, 3543–3551 (2009).

126 Kloxin AM, Tibbitt MW, Anseth KS. Synthesis of photodegradable hydrogels as dynamically tunable cell culture platforms. *Nat. Protoc.* 5(12), 1867–1887 (2010).

127 DeForest CA, Anseth KS. Cytocompatible click-based hydrogels with dynamically tunable properties through orthogonal photoconjugation and photocleavage reactions. *Nat. Chem.* 3(12), 925–931 (2011).

128 DeForest CA, Anseth KS. Photoreversible patterning of biomolecules within click-based hydrogels. *Angew. Chem. Int. Ed. Engl.* 51(8), 1816–1819 (2012).

129 Husár B, Liska R. Vinyl carbonates, vinyl carbamates, and related monomers: synthesis, polymerization, and application. *Chem. Soc. Rev.* 41(6), 2395–2405 (2012).

130 Heller C, Schwentenwein M, Russmueller G, Varga F, Stampfl J, Liska R. Vinyl esters: low cytotoxicity monomers for the fabrication of biocompatible 3D scaffolds by lithography based additive manufacturing. *J. Poly. Sci. A Poly. Chem.* 47(24), 6941–6954 (2009).

131 Heller C, Schwentenwein M, Russmüller G *et al.* Vinylcarbonates and vinylcarbamates: Biocompatible monomers for radical photopolymerization. *J. Poly. Sci. A Poly. Chem.* 49(3), 650–661 (2011).

132 Ovsianikov A, Ostendorf A, Chichkov B. Three-dimensional photofabrication with femtosecond lasers for applications in photonics and biomedicine. *Appl. Surface Sci.* 253(15), 6599–6602 (2007).

133 Danilevicius P, Rekstyte S, Balciunas E. Micro-structured polymer scaffolds fabricated by direct laser writing for tissue engineering. *J. Biomed. Opt.* 17, 081405 (2012).

134 Correa DS, Tayalia P, Cosendey G *et al.* Two-photon polymerization for fabricating structures containing the biopolymer chitosan. *J. Nanosci. Nanotechnol.* 9(10), 5845–5849 (2009).

135 Turunen S, Käpylä E, Terzaki K *et al.* Pico- and femtosecond laser-induced crosslinking of protein microstructures: evaluation of processability and bioactivity. *Biofabrication* 3(4), 045002 (2011).

136 Basu S, Cunningham LP, Pins GD *et al.* Multiphoton excited fabrication of collagen matrixes cross-linked by a modified benzophenone dimer: bioactivity and enzymatic degradation. *Biomacromolecules* 6(3), 1465–1474 (2005).