Review

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Two-photon polymerization nanolithography technology for fabrication of stimulus-responsive micro/nano-structures for biomedical applications

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Abstract: Micro/nano-fabrication technology via two-photon polymerization (TPP) nanolithography is a powerful and useful manufacturing tool that is capable of generating two dimensional (2D) to three dimensional (3D) arbitrary micro/nano-structures of various materials with a high spatial resolution. This technology has received tremendous interest in cell and tissue engineering and medical microdevices because of its remarkable fabrication capability for sophisticated structures from macro- to nano-scale, which are difficult to be achieved by traditional methods with limited microarchitecture controllability. To fabricate precisely designed 3D micro/nano-structures for biomedical applications via TPP nanolithography, the use of photoinitiators (PIs) and photoresists needs to be considered comprehensively and systematically. In this review, widely used commercially available PIs are first discussed, followed by elucidating synthesis strategies of water-soluble initiators for biomedical applications. In addition to the conventional photoresists, the distinctive properties of customized stimulus-responsive photoresists are discussed. Finally, current limitations and challenges in the material and fabrication aspects and an outlook for future prospects of TPP for biomedical applications based on different biocompatible photosensitive composites are discussed comprehensively. In all, this review provides a basic understanding of TPP technology and important roles of PIs and photoresists for fabricating high-precision stimulus-responsive micro/nano-structures for a wide range of biomedical applications.

Keywords: micro/nano-fabrication, two-photon polymerization, stimulus-responsive structure, biomedical applications

1 Introduction

Well-defined three-dimensional (3D) structures with micro- or nano-features are of great interest for diverse bioapplications including cell engineering [1], tissue engineering [2,3], biorobots [4], microfluidic systems [5] and drug delivery [6,7]. In order to fabricate desirable hierarchical 3D architectures, various kinds of manufacturing techniques, such as selective laser sintering (SLS) [8], stereolithography (STL) [8,9] and electrospinning [10,11], have been developed. Although many two-dimensional (2D) nanopatterns and simple 3D microstructures can be manufactured by the aforesaid technologies, precise control of the submicron to nanometer-sized features is still difficult to be achieved. Direct laser writing (DLW) via two-photon polymerization (TPP), as a novel emerging prototyping technique, has attracted enormous research attention in the past few decades because of its high spatial resolution and ultraprecision in photopolymerization not only on the microscopic scale but also on the nanoscale [12]. Unlike conventional single-photon polymerization induced by an ultraviolet (UV) laser, a photoinitiator (PI) molecule in a polymerizable resist consisting of monomers or oligomers absorbs two photons simultaneously to initiate polymerization in a highly localized region around the center of the focused...
beam via nonlinear absorption. A solid volume pixel, known as a voxel in micro/nano-fabrication, is then created [13]. Based on this voxel-by-voxel approach, sophisticated design features can be realized in specific areas of structures by a tightly focused laser beam without the need to use photomasks. The scanning path of the laser beam is commonly moved along a 2D scanning path, while layer-by-layer scanning along the vertical axis enables the printing process of 3D architectures. The polymerization rate is proportional to the square of the equipment laser intensity. Therefore, sub-100 nm resolution can be achieved using a high numerical aperture (NA) objective lens with the advent of femtosecond and picosecond laser pulse [14]. Other polymerization mechanisms triggered by nonlinear absorption of photons such as three-photon absorption [15] are also possible.

To exploit the potential applications of TPP, various optically transparent photosensitive materials have been engineered, leading to the fabrication of 3D microstructures with remarkable functions due to the presence of unique micro/nano-structure features which are difficult to be produced by conventional methods. Most of the typical photopolymerizable materials for TPP nanolithography resemble those utilized for traditional UV lithography. The difference of absorption in photons distinguishes the spatial resolution of structures. The most popular materials developed for single-photon STL decades ago are acrylate-based and epoxy-based resins which are photosensitized by an excimer laser at 308 nm wavelength or Hg-lamp at 365 nm [16–18] and can also be applied in the TPP-DLW nanolithography system. A large diversity of micro-objects, including micro/nano-electromechanical systems (MEMS/NEMS) [19–21], micro/nano-photonic [22], biomimetic interfaces and architectures [23,24], have been fabricated by TPP based on these materials.

In particular, when biocompatible and non-cytotoxic photoresists (hydrogel, photo-crosslinkable proteins, commercial IP-series photoresists and other hybrid materials) and water-soluble PIs are used, TPP is capable of generating 3D matrices featured by outstanding stability and excellent biocompatibility with natural tissue environments [25–28]. In addition, TPP uses a light source lying in a near-infrared (NIR) region, which makes it possible to conduct the photopolymerization in photoresist loaded with living cells [29]. The native microenvironments where cells reside, namely, extracellular matrix (ECM), possess complex networks from the macroscale to nanoscale which can regulate cell behavior and tissue differentiation [30–32]. Although some scaffolds with micro/nano-features can be manufactured by some approaches, such as solvent casting and particle leaching [33], electrospinning [34] and emulsion freeze-drying [35], micro/nano-features generated by the aforementioned technologies cannot be controlled in terms of size and shape for mimicking the natural microenvironments. Therefore, how structural features affect the interaction between cells and scaffolds have been hindered by limitations of the conventional methods. Instead, well-organized scaffolds fabricated by TPP can mimic the natural microenvironment of human tissue with respect to multi-scale structures. They serve as a versatile platform to promote the regulation of cell behavior, including cell attachment, proliferation, differentiation and cell-to-cell interaction [36]. Development of novel drug transportation and release systems has also been enabled with the coming of precise structural design by computer-aided CAD software and the minimization of microdevices by TPP [37]. Desirable temporal and spatial distribution of drugs in vitro and in vivo can be achieved by the design of a special drug-loaded scaffold. Moreover, microneedle enhancing drug delivery is of advantage in solving many issued problems associated with intravenous drug administration, including pain to the patients resulting from the traditional metal needles, trauma in the injection site and long-term sustainable release of the medicine [38].

This paper aims to provide an up-to-date and comprehensive account of the TPP research field, with emphasis on conventional and stimulus-responsive photosensitive materials and their biomedical applications. As PIs and photoresists play crucial roles in fabricating stimulus-responsive micro/nano-structures via TPP technology such as spatial resolution of the structures and their manufacturing time, currently widely used PIs and different photoresists are summarized systematically in terms of their properties and two-photon absorption capability. Meanwhile, a selection of distinctive previous studies that can potentially project the future trends of TPP in the biomedical field is discussed with featured versatile applications of 3D nano/micro-constructs based on the aforementioned materials. Finally, current limitations and challenges in the material and fabrication aspects as well as the outlook on future prospects of TPP for biomedical applications are described.

2 TPP PIs for biomedical applications

Unlike the single photon absorption process, to trigger the TPA process, one atom or molecule must simultaneously absorb two photons to arrive at an excited state
from the ground stage to induce the transition, which can be realized by an ultrafast laser beam with a high intensity of the pulse peak. After immediate excitation, the TPA process occurs within a small focal volume in the photopolymerizable materials to ensure a high resolution. Then chemical polymerization between the initiators and monomers will occur [39,40]. Based on this process, photosensitive monomers with active moieties can react with reactive species (e.g., free radicals or cations) generated by photosensitive molecules to form 3D micro/nano-structures. A typical photoresist system contains (1) one or more PIs or photosensitizer(s) with absorption near the two-photon excitation wavelength; (2) transparent photocurable polymeric monomers with functional groups in the NIR wavelength. In most research, some other materials (cross-linkers, nanoparticles, etc.) are added into the aforementioned photoresists to achieve special properties. During this modification procedure, organic or inorganic solvents are usually used to dissolve and dilute the above individual components for liquid-based photoresists with controlled viscosity.

The overall photopolymerization procedures can be divided into the following three steps: initiation, propagation and termination [41]. For the initiation step, it is critically significant to choose highly active PIs so that full potential of the TPP technique can be realized. PIs can affect the mechanism of chemical polymerization, as well as the polymerization reaction rate and the final properties of the formed polymer including its hardness and viscosity [42]. Molecules with a large TPA cross section ($\delta_{\text{TPA}}$) and high-initiating efficiency are in tremendous demand [43]. $\delta_{\text{TPA}}$ is widely used in the evaluation of TPA performance of molecules, and it is measured in Goeppert–Mayer (GM) units ($1\text{GM} = 10^{-15} \text{cm}^4 \text{ per photon molecule}$). Currently, a variety of molecules with enhanced $\delta_{\text{TPA}}$ have been designed and synthesized. The structure–property relationship between the molecule structure and $\delta_{\text{TPA}}$ was summarized by Perry et al. [44]. The intermolecular charge transfer efficiency (ITCE) has been demonstrated to be a key parameter in evaluating the $\delta_{\text{TPA}}$ performance of one molecule. Several research studies have been reported to increase the $\delta_{\text{TPA}}$, by extending the length of the π-conjugated chains, introducing strong electronic acceptor and donor moieties and increasing the molecular coplanarity [45–47].

Thereby, PIs with a high TPA cross section and high initiation efficiency undoubtedly play an important role in the TPP micro/nano-fabrication process. The size of TPA-triggered polymerized voxels is a significant feature for fabricating micro/nano-structures because it determines the capacity of achieving a sub-diffraction-limited structural resolution. Moreover, the chemical polymerization can be carried out at a low excitation laser power (less than 180 mW) and short exposure time, which further brings about a fast scanning speed and desired printing quality. The selection of an appropriate PI is essential for achieving a desirable initiation rate and structural properties. With the low photon energy of NIR light used in TPP, some polymerization processes can be manipulated in the presence of cells or whole organisms [48,49]. When being applied in biomedical fields, PIs are required to be nontoxic to the surrounding cells or tissues. In most biocompatible photoresist systems such as hydrogel-based natural or artificial materials, they also need to be soluble and thermally stable in the monomer aqueous microenvironments [42]. Currently, a lot of PIs, such as rose bengal, methylene blue, lithium phenyl-2,4, 6-trimethylibenzoylphosphinate (LAP) and Irgacure 369, are available commercially and commonly used for fabricating micro/nano-structures for biomedical applications [26,36,50–52]. In Table 1, PIs for the TPP fabrication are summarized along with their water solubility, $\delta_{\text{TPA}}$, and biomedical applications as reported in some studies.

To improve the water solubility of PIs in aqueous solutions, non-ionic surfactants have been added to conventional hydrophobic PIs. Jhaveri et al. [54] undertook the microfabrication of hydrogels via TPP in aqueous solution by adding a surfactant Pluronic F127 into a commercially available initiator Irgacure 651. Although this method is effective, a high surfactant concentration is needed to achieve initiation efficiency. Excessive surfactants may further reduce the biocompatibility of the final developed structures or even bring about cytotoxicity, which is unacceptable to the subsequent biomedical applications. Some other hydrophilic PIs and xanthene dyes, such as Irgacure 2959 [55], rose bengal and methylene blue [56], have gained a lot of research attention for TPP. Irgacure 2959, one of the first commercially available PIs, has been widely used in the fabrication of 3D hydrogel scaffolds because of its biocompatibility and low cytotoxicity, but it is only processable for TPP at a wavelength of 515 nm, which may cause the denaturation of proteins [55,57]. Moreover, its low water solubility below 2% leads to unsatisfactory polymerization efficiency. Rose bengal, methylene blue and Eosin-Y have very low $\delta_{\text{TPA}}$ values (only 10GM at 800 nm), which require long exposure time (300–400 μs) and high laser intensity, resulting in a time-intensive process.
In order to synthesize efficient water-soluble PIs, the most effective method is to introduce water-borne functional groups such as quarternary ammonium salts or different sodium carboxylic salts into the well-investigated chromophore core structures which have high TPA activity. Among the synthesized PIs, a large covalent structure like alkyl chains of different lengths and benzylidene are usually needed to avoid shifting the electronic structure of the TPA chromophore, as shown in Figure 1. For example, when quarternary ammonium cations are introduced, the synthesized water-soluble WSPI initiator \((\,4-\text{(N,N-bis(6-(N,N,N-trimethyl-ammonium)hexyl)amino)-styril)-2,5-dimethoxybenzene tetraiodide})\) shows a high \(\delta_{\text{TPA}}\) value of 120 GM. Torgersen et al. [48] first fabricated 3D woodpile scaffolds with high water content using poly(ethylene glycol)diacylate (PEGDA) and WSPI in the presence of living organisms, which laid the foundation for in vivo microfabrication via TPP for tissue engineering applications. WSPI was also used in a thiol–vinyl copolymer system to fabricate 3D protein hydrogels [58]. Sodium carboxylic groups were also incorporated at the terminal alkyl chains to generate a series of PIs for direct encapsulation of living cells by TPP under aqueous conditions, such as P2CK [53] G2CK [59], BSEA [60] and E2CK [61].

Recently, a newly emerging effective method to increase the solubility of hydrophobic PIs in aqueous solution was completed by a host–guest chemical interaction. Some macrocyclic host molecules, such as cyclodextrins (CDs) and cucurbit[n]urils (CB[n]s), can encapsulate hydrophobic PIs as guests to form host–guest inclusion complexes [62–64]. Due to their supramolecular structures, CDs and CB[n]s both had one hydrophobic cavity and a hydrophilic outer surface contributed by the hydroxyl and carbonyl functional groups, respectively. The driving force for the generation of the host–guest structure between the macrocyclic host molecules and guest molecules mainly resulted from the following two factors: (1) the hydrophobic effect to release the high-energy water stuck in the host cavities; (2) electrostatic attraction between the electron-rich surface of the host molecules and the electron-deficient parts of the guest molecules [65–68]. Therefore, many researchers have synthesized different host–guest complexes with different types of CDs and CB[n]s, one example with good aqueous solubility and low cytotoxicity in the CB[n]s family. Duan’s group [69] has done a lot of work and studied their \(\delta_{\text{TPA}}\) properties. For example, when hydrophobic 2,7-bis(2-(4-pentaneoxy-phenyl)-vinyl) anthraquinone was assembled with
2-hydroxypropyl-β-CDs, this water soluble TPP initiators had a $\delta_{\text{TPA}}$ value of around 200GM at a 780 nm wavelength. At the same time, the TPP threshold energy in water was 8.6 mW when using PEGDA with this PI [70]. 3,6-Bis[2-(1-methyl-pyridinium) vinyl]-9-pentyl-carbazole diiodide (BMVPC) exhibited a high $\delta_{\text{TPA}}$ value attributed to its A–π–D–π–A structure. However, it has limited biomedical application via the TPP technology due to its low solubility and initiating effect in spite of its good biocompatibility and hypotoxicity [71]. By incorporating BMVPC with CB[7]s, Zheng et al. [72] created a host–guest complex BMVPC-CB[7]s with a very high $\delta_{\text{TPA}}$ value up to 2,999GM at a wavelength of 800 nm. When being applied in PEGDA photoresist, a high spatial resolution of 180 nm was achieved by using a relatively low laser threshold of 4.5 mW.

### 3 TPP-compatible photoresists

Most of the materials for TPP are similar to those used in the conventional UV lithography method, in which the materials are usually in the form of viscous liquid, amorphous solid or gel. Negative- and positive-tone photoresists are the two typical photosensitive materials. For negative-tone photoresists, two-photon exposure leads to a cured 3D structure on a substrate directly via crosslinking of polymer chains, leaving the unpolymerized photoresists to be washed out by a developing solvent. In contrast, for the positive-tone photoresists, they are first solidified by an UV laser or heat. Then the NIR laser beam exposure results in the breakup of the photoresist polymer chains through photoacid degradation, generating shorter units which can be dissolved and washed away in the development process [73]. Negative-type photoresists, including the commercially available epoxy-based photoresists (e.g., SU-8; MicroChem) [74], acrylate-based photoresists (e.g., IP-Series resists; Nanoscribe GmbH) [75] and the hybrid sol–gel ORMOCER (Microresist Technologies) [76] with low shrinkage and superior chemical stability, are the most widely used. Positive-tone photoresists are efficient in fabricating hollow structures with only a small fraction removed from the whole material in the original workpiece [77]. Moreover, such hollow constructs can serve as good master templates/molds for indirect manufacturing. Although it is convenient to carry out 3D scaffold fabrication using the aforementioned photoresists, they are proprietary materials and cannot be easily modified or incorporated with other components for customized functionalities such as tunable modulus or single- or multi-stimulus-responsive behavior. This has limited their use for bioapplications via TPP, especially fabrication of dynamic cell or tissue engineering scaffolds, microactuators in medical...
Table 2: Types of microstructures fabricated from various IP-series photoresists and their application

<table>
<thead>
<tr>
<th>Photoresist systems</th>
<th>Bioapplication</th>
<th>Structures</th>
<th>Performance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP-L 780</td>
<td>Drug screening</td>
<td>3D cell cage assembly</td>
<td>Enable multilayer stacking of different types of cells</td>
<td>[91]</td>
</tr>
<tr>
<td></td>
<td>Cell engineering</td>
<td>3D architectures with different Young's modulus and stiffness gradients</td>
<td></td>
<td>[92]</td>
</tr>
<tr>
<td>IP-Dip</td>
<td>Cell engineering</td>
<td>3D woodpile scaffold</td>
<td>Reveal the mechanisms of cell proliferation and colonization</td>
<td>[93]</td>
</tr>
<tr>
<td>IP-S</td>
<td>Regenerative therapy</td>
<td>3D porous scaffolds</td>
<td>Support induced pluripotent stem cell (iPSC) adhesion, proliferation and differentiation</td>
<td>[94]</td>
</tr>
<tr>
<td></td>
<td>Drug delivery</td>
<td>Microneedle arrays</td>
<td>Achieve more effective multicomponent cutaneous vaccination using undercut microneedle arrays</td>
<td>[37]</td>
</tr>
<tr>
<td>IP-G 780 with Ni/Cr layers</td>
<td>Microswimmers</td>
<td>Helical structures</td>
<td>Possess high sensitivities to both the magnetic propulsion and the fluorescence</td>
<td>[95]</td>
</tr>
<tr>
<td>IP-Visio</td>
<td>Microfluidics</td>
<td>3D microfluidic channel</td>
<td>Tailor gas dynamic virtual nozzles function to achieve jet diameters below 100 nm for single-particle imaging</td>
<td>[96]</td>
</tr>
</tbody>
</table>
Table 3: Types of stimulus-responsive photoresists and their microstructures fabricated by TPP for biomedical applications

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Photoresists and PIs</th>
<th>Bioapplication</th>
<th>Microstructures</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>BSA and rose bengal</td>
<td>Actuators</td>
<td>Dual-pillar cross-shaped microstructures and microtrap</td>
<td>[81]</td>
</tr>
<tr>
<td></td>
<td>BSA and rose bengal</td>
<td>Microfluidics, cell micropatterning</td>
<td>Geometrical shape-shifting microstructures with sandwich-based design</td>
<td>[97]</td>
</tr>
<tr>
<td></td>
<td>BSA and rose bengal, PEGDAs, CEA and Irgacure 819</td>
<td>Microdevices, tissue engineering</td>
<td>3D microsieves with changing pore size</td>
<td>[98]</td>
</tr>
<tr>
<td></td>
<td>LC monomers, LC crosslinker, azo dyes and Irgacure 369</td>
<td>Biosensing</td>
<td>Micropyramid and microdome with different swelling action</td>
<td>[99]</td>
</tr>
<tr>
<td></td>
<td>LCE, AuNRs and Irgacure 369</td>
<td>Biorobots</td>
<td>A cuboid body and conical legs</td>
<td>[100]</td>
</tr>
<tr>
<td>Light</td>
<td>PNiPAAm, PEGDA, Fe3O4 nanoparticles and Benzil</td>
<td>Microactuators or robotic devices</td>
<td>Woodpile structures, microclamp structures with gripping behavior.</td>
<td>[101]</td>
</tr>
<tr>
<td>Electromagnetic</td>
<td>SU-8, Fe3O4 nanoparticles</td>
<td>Microactuators</td>
<td>Microstructures with deformable two microcantilevers and micropedestal</td>
<td>[78]</td>
</tr>
<tr>
<td>field</td>
<td>SU-8, Ni/Ti bilayer</td>
<td>Drug delivery, single-cell manipulation</td>
<td>3D cube and helical microswimmers</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td>Acrylamide, PEGDA, Fe3O4 NPs and benzyl</td>
<td>3D cell transportation and drug delivery</td>
<td>3D porous microneiches</td>
<td>[103]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sensors and actuators</td>
<td>Largely bendable micronail</td>
<td>[104]</td>
</tr>
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</table>

3.2 Customized smart materials for TPP

3.2.1 pH-responsive photoresists

Proprietary IP photoresists from Nanoscribe GmbH are developed for TPP DLW via non-linear absorption of 780 nm laser beams for direct laser writing (DLW) or micro-3D printing processes, such as atomic layer deposition, chemical vapor deposition or galvanization, can be used to modify 3D structures for additional functions.

Recent research has reported that these photoresists can undergo dynamic swelling changes that influence their volume and elasticity in response to pH fluctuations. This property is particularly useful in the fabrication of microfluidic devices and microchannels with pH-responsive behavior. By utilizing these photoresists, researchers can create microstructures that change their shape or open/close in response to pH changes, making them ideal for applications in biosensing, drug delivery, and tissue engineering.

In addition, the use of pH-responsive photoresists in TPP technology can lead to the development of stimuli-responsive systems that can be used for self-assembly, self-healing, or adaptive microfluidic systems. These systems have potential applications in the areas of biomedical devices, micro- and nanotechnology, and nanomedicine.

3.2.2 Light-responsive photoresists

Light-responsive photoresists are designed to respond to light stimuli, such as visible light, UV light, or infrared light. These photoresists undergo changes in viscosity, solubility, or shape upon exposure to light, which can be used to control the formation of micro- and nanoscale structures.

Some light-responsive photoresists are based on photochromic compounds, which can undergo a color change upon exposure to light. These photoresists can be used to create microfluidic devices, microactuators, and other light-responsive microstructures.

However, the development of light-responsive photoresists is still in its early stages, and more research is needed to understand their properties and applications. Further studies are required to optimize the performance of light-responsive photoresists, including their sensitivity, reversibility, and stability under light exposure.
response to the slight altering of the surrounding pH value because they have functional moieties such as carboxylic (–COOH) and amino (–NH₂) groups that can respond to a pH change [81,97]. pH-responsive hydrogels are the mostly studied photoresponsive monomers. The swelling behavior of hydrogels with carboxylic or amino pendant groups depends on the difference of the pKᵦ values of carboxylic groups or the pKᵦ values of amino groups relative to environmental pH. A lower or a higher pH value of the surrounding medium than the dissociation constants of the pendant groups could induce the increase of charges in the pendant groups, leading to the electrostatic repulsion between the polymer chains [105].

A representative example of a pH-sensitive hydrogel used in TPP is bovine serum albumin (BSA)-based hydrogel whose globular structure resembles to that of human serum albumin. It is a highly water-soluble monomeric protein containing 583 amino acid residues. Therefore, it is the most widely used protein for the preparation of 3D structures via TPP for cell behavior studies and tissue engineering. At the isoelectric point of pH, BSA proteins are hydrophobic without a net charge, leading to an increase of the aggregation of protein molecules. Less water is absorbed by the polymer network, which results in shrinkage of the protein gel [106]. On the other hand, at the altered pH point, amino acids in the protein chains become ionized due to protonation of their amino functional groups or deprotonation of carboxylic groups. The increasing number of ions in the protein can further facilitate the repulsion of the molecules and the interaction with water via ion–dipole interaction, thereby causing the protein structures to swell [97,98].

BSA proteins have been used to fabricate microstructures with anisotropic and programmable shape deformation ability by means of TPP. Using TPP, a dynamic and reversible BSA-based Venus fly trap was created. When the pH value was changed from 5 to 11, four tips of the structure would bend inward to generate a closed trap [81]. Additionally, by patterning microstructures with low and highly cross-linked changing parts, dynamic circle-to-polygon and polygon-to-circle shape-shifting can be realized by changing the pH [97]. This will potentially enable controllable connectivity and porosity in 3D microfluidics for biosensors.

Other hydrogels can also be used to achieve pH-responsive action by the chemical functionalization of carboxylic-based materials. Among these biocompatible hydrogels, polyethylene glycol-based hydrogels have been the most attractive for bioapplications for many years [107,108]. A number of advantages, including low-protein absorption, in vivo safety with negligible inflammatory profile and easy functional modification, have made it become “gold standard” material for cell and tissue engineering. Scarpa et al. [99] first used biomolecule, 2-carboxyethyl acrylate (CEA), to confer high-molecular-weight PEGDA with pH sensitivity. Different microstructures showed different deformation actions under varying pH values. Micropyramids exhibited a large degree of shape change between high and low pH values, while microdomes maintained their shape with only a dimensional change.

pH-responsive biomaterials composed of BSA and other hydrogels have also been investigated. Kaehr and Shear [109] incorporated BSA proteins into poly(methyl methacrylate) (PMMA) for the fabrication of microscopic 3D structures via TPP. The protein microstructures were demonstrated to maintain ligand-binding and catalytic functionality. Therefore, after the TPP fabrication process, electrostatic and hydrophobic interactions that control protein conformation remained intact in the microchamber. By well-defining the spatial gradients of protein in the PMMA hydrogel matrix, the microchamber had distinct capacity for expansion and contraction in response to changes in a chemical environment. Then, for example, this responsive microstructure could trap, incubate and release motile cells such as *Escherichia coli* passively. At the pH 7, the microchamber could capture motile cells and provide a suitable environment for the growth. When the pH value was changed from 7 to 12.2, the hydration level of the protein-based microchamber walls was changed greatly, inducing a temporary compression of the internal microchamber. Trapped cells were then released into the outside aqueous environment.

Although BSA-based pH-responsive photoresists are promising for biomedical applications, there remain some limitations that need to be considered. As the single component BSA lacks mechanical stability, it may be overcome by combining BSA with other hydrogels to form hybrid materials. In future, more types of hybrid photoresists composed of proteins and hydrogels can be considered to be explored for TPP due to their great demand for various biomedical applications.

### 3.2.2 Light-responsive photoresists

Light, one of the remotely controllable stimuli, has attracted the most research interest for smart materials. In general, light-induced actuation of polymers has two main types: photoisomerization of chromic moieties in a
chain of monomers and the photothermal effect of nanoparticles in heat-responsive polymer matrices [110–112]. The cis–trans isomerization photoresponse of azobenzene function groups is commonly used in light-responsive photoresists. Most LCEs contain azobenzene photochromic molecules characterized by –N≡N– linkage, which are able to induce the phase transition isothermally [113]. The azobenzene moiety can undergo reversible isomerization between its two geometric isomers under light of appropriate wavelength. The trans form of azobenzene has a rod-like shape that can stabilize the liquid crystalline phase, while the cis form can disrupt this stable phase order. Therefore, macroscopic deformation of LCEs could be induced by the change of phase order of the azobenzene moieties in LCE chains [114]. Moreover, LCEs are known as smart materials because of the anisotropic properties of the liquid crystals and the elastic behavior of their polymeric networks similar to that of traditional rubber [115,116]. These properties depend on their glass transition temperature ($T_g$), which is usually below room temperature. When the surrounding environment temperature is below $T_g$, the mesogens (liquid crystalline phases) in the monomer chains have an anisotropic and elongated conformation. Once the temperature is higher than $T_g$, the mesogens can become randomly aligned, leading to random coil conformation of chains [117].

A large number of applications have been undertaken for micro/nano-robotics based on the capability of LCEs to transform light into mechanical force. A typical one is the light-fueled microscopic walkers made of an azobenzene-containing LCE body (their own muscle) and four IP-Dip conical rigid legs, as proposed by Zeng and co-workers [100]. Actuated by a 532 nm laser beam at 50 Hz, this tiny LCE walker with a dimension of $60 \times 30 \times 10 \mu m^3$ could contract along one side, which further triggered the locomotion of the four legs. This LCE body was also demonstrated to have different types of behaviors including random or directional walking, rotation or jumping. Using a similar method, a micro-gripper also made of azobenzene-containing LCE inspired by the hominid-type hand was able to capture different microscopic objects under a light stimulus [118]. Although azobenzene functional groups have been demonstrated to have good biocompatibility, the light wavelength that can trigger deformation of azobenzene-containing LCEs falls within the visible light range (400–700 nm) [119,120]. Visible light has poor penetration ability in the tissue, which may result in limited application of deformable scaffolds prepared by azobenzene-containing LCEs in cell and tissue engineering.

Apart from the photochromic functional groups in the polymer monomers, some nanoparticles, such as gold nanorods (AuNRs) [121] and Fe$_3$O$_4$ nanoparticles, were also introduced as the heating element in the photoresists system for achieving light-response and thus conversion of light into heat energy. The light-responsive mechanism is based on the dissipation of energy from light into heat through these dopants. For example, for our research group, Chen et al. [101] introduced AuNRs into an LCE matrix for the fabrication of several 3D microstructures. At a wavelength of 810 nm, the photon energy could be effectively converted into heat energy. The two arms of the microclamp could move toward the middle location under the stimulus of NIR light, which proved promising to work as microarms for clamping or gripping small items. The distance between the two arms is about 15 μm, which would be used to manipulate a single cell for biomedical application. Poly (N-isopropylacrylamide) (poly(N-isopropylacrylamide) (PNIPAAm)) has been widely used for research studies of thermo-responsive polymers. Their chains have a reversible lowest critical solution temperature (LCST) phase transition from a swollen, hydrated state to a shrunken and hydrophobic one [79]. An increase in temperature above LCST leads to the expelling of absorbed water in PNIPAAm chains, resulting in the phase separation and volume shrinkage [122]. Zheng et al. [78] introduced Fe$_3$O$_4$ nanoparticles into the photoresist system of N-isopropylacrylamide (NIPAM) and PEGDA and fabricated a double-armed NIR-light strip of only ~26 μm. Black Fe$_3$O$_4$ nanoparticles were found to be highly effective photothermal dopants triggered by NIR light [123]. The photothermal effect was remarkable with a very fast response time of about 0.033 s under NIR light with a power of 29.2 mW.

The photothermal effect induced by nanoparticles has attracted lot of research interest in the biomedical fields, especially in photothermal therapy for cancer and tumor diseases. However, such a photothermal effect cannot be ignored, because the extra heat due to the incorporated nanoparticles in the light-responsive photoresist could cause a temperature rise, leading to its unsuitability for the constant temperature environment of cell culture and tissue regeneration. Therefore, it would be crucial to confine the nanoparticle concentration in the light-responsive photoresists within a suitable range.

### 3.2.3 Electromagnetic field-responsive photoresists

In remote manipulation of the locomotion of micro/nano-devices, it may be difficult to conduct light actuation in
some opaque scaffolds or liquid environments. Electromagnetic actuation is considered as another promising method compared with light actuation due to its large actuation force in different media with low or high light transparency. Most approaches for electromagnetic actuation are involved in the incorporation of magnetite nanoparticles such as Fe₃O₄ into the pre-polymerized photoresist [124] or modification of the micro/nano-structures with a thin Ni layer via surface metallization modification techniques, such as e-beam evaporation [125]. For example, Suter et al. [102] prepared a magnetic polymer composite consisting of SU-8 photoresist and Fe₃O₄ nanoparticles for subsequent TPP fabrication. A helical microswimmer and cube were fabricated using the composite with 2 vol% Fe₃O₄ nanoparticle concentration. Stimulated by a uniform rotating magnetic field, superparamagnetic helical microstructures could complete cork-screw swimming behavior for about 12 μm (forward plus drift motion) during 4 s in water. Moreover, the composite with up to 10 vol% Fe₃O₄ nanoparticles was found not to influence the cell viability of NHDFs. These 3D magnetic-field-driven microstructures could serve as microrobots for target drug delivery platforms in the complex human body liquid environment. However, due to agglomeration of the Fe₃O₄ nanoparticles induced by the van der Waals interactions, it became very difficult to achieve a high nanoparticle concentration and homogenous dispersion in the composite photoresist. Thus, surface functionality and coating were generally required to overcome such limitations.

Although the helical structures have shown remarkable advantages in magnetic actuation, the capability of cell and drug loading within the structures remains limited because of their low surface area. Porous scaffolds have been demonstrated to improve the transport efficiency. Kim et al. [103] reported a 3D porous microniche using the photoresist of SU-8 by means of TPP. Then a 150 nm Ni layer and a 20 nm Ti layer were deposited on a polymer scaffold for generating a magnetic and biocompatible microrobot. Hexahedral microrobots and cylindrical microrobots were both designed to study their magnetic moments. They found that a cylindrical structural design was favorable for minimizing the resistive force against manipulation under the actuation magnetic field of 800 mT m⁻¹. Moreover, after a 96 h culture of human embryonic kidney (HEK) 293 cells, the porous microrobots with customized pore sizes indicated good cell behavior, including attachment, migration and proliferation. In another research study, Li et al. [126] fabricated burr-like porous spherical microrobots with diameters ranging from 70 to 90 μm using a method similar to that of Kim et al. [103].

Generally, most photoresists can be incorporated with magnetite nanoparticles for achieving magnetic field actuation. Due to the bi-stimulus-responsive function of the Fe₃O₄ nanoparticles triggered by light and magnetic field simultaneously, it is believed that more advanced microactuators based on light-magnetic field responses will be further developed for biomedical applications. Multiple-stimulus-responsive photoresists deserve to be developed largely due to their outstanding functions in different situations. Recently, several studies have been reported to endow the photoresist with combined pH- and temperature-responsive behavior by employing acrylic acid and PNIPAAm as monomers. Based on these well-known stimulus-responsive monomers, Zarzar et al. [127] fabricated micropillar arrays which displayed independent responsiveness to the respective stimulus. A small temperature change and pH value could lead to large bending angles of the micropillars. Recently, Jin and co-workers [128] also reported reconfigurable compound micromachines which possessed a rapid and reversible 3D-to-3D shape deformation in response to changes in temperature and pH values.

4 TPP for medical applications

4.1 Cell engineering

Living cells in the body are typically sensitive to their surrounding microenvironment with physicochemical features including surface roughness ranging from nano- to micrometers and stiffness. In the field of cell engineering, the realization of culture conditions is of critical significance for the investigation of cell behavior such as cell growth, proliferation, alignment and colonization. Many studies have suggested that specific cell behavior can be in vitro resembled and investigated by mimicking the corresponding in vivo culture conditions using different strategies [129,130]. TPP technology has allowed researchers to control the surface topography at the multiscale for investigation of the cell–substrate interaction.

Cell alignment plays a vital role in cell behavior, which has shown remarkable influence in tissue functionality [131], strongly influenced by the 3D topography rather than a single cell level. Grigoropoulos et al. [132]
prepared a series of well-designed microstructures, including aligned high aspect ratio fibers between two glass plates, patterned ridges and grooved surfaces [133] using non-toxic ORMOCER®. The alignment of fibroblast cell lines (NIH-3T3) and epithelial cell lines (MDCK) could be controlled by changing the distance of the adjacent fibers. They found that MDCK cells should have a stronger adherence force to the fiber than the NIH-3T3 cells. They also compared the cell alignment on the orthogonally ridge-patterned surfaces with small differences of height and found that NIH-3T3 cell elongation was enhanced by increasing the ridge height. The threshold obstacle height for obtaining cell alignment was found to be about 1 µm. Similarly, Engelhardt et al. [26] reported the fabrication of a gelatin methacrylamide polymeric substrate containing a series of ridges of 1 µm width, 2 µm height and a separation of 4 µm for cell studies. The ridge-patterned substrates also exerted influence on cell alignment. To evaluate the cell alignment direction, chondrocytes were seeded on the substrates. Compared with culture petri dishes, chondrocytes were strongly aligned along the direction of the gelatin lines.

Investigation of cell migration is very important in pathological and physiological processes, such as the formation of cancer metastasis, blood vessel formation and inflammatory response [134]. Particularly in the field of tumor invasiveness, the degree of cancer malignancy was evaluated by the ability of tumor cells to invade the surrounding cells or tissues [135]. Initially, the mechanisms and regulations of cell migration were not fully understood because most studies were performed on 2D substrate models. However, the real growth of cells in the human body is a 3D multiscale microenvironment. To address this problem, Tayalia and coworkers [136] prepared a 3D microstructure with precisely controlled parameters using TPP for investigating controlled cell adhesion and migration behavior. In this work, interconnected woodpile structures with different lateral pore sizes were fabricated by a mixture photoset composed of tris(2-hydroxy ethyl)isocyanurate triacrylate (SP499) and ethoxylated (6) trimethylol-propane triacrylate (SR499). Aided by commercial 3D imaging software, HT1080 fibrosarcoma cells showed a higher migration speed in this 3D woodpile scaffold than that in the 2D substrate. Furthermore, different pore sizes in the 3D matrix structures also changed the cell migration speed.

In addition, 3D structures prepared by TPP can be used to mechanically stimulate cell behavior, while local traction forces exerted by cells can deform these elastic structures. It is worth mentioning that the scaffolds fabricated by TPP can be used to measure traction forces produced by single cells. The measurement of contraction forces is of paramount importance for the investigation of their influence on the mechanical properties and functions of stretchable tissues. Particularly, the contraction forces of cardiomyocyte and neurite have been measured by utilizing the deformability of TPP structures [137,138]. Similar to the topographic complexity in in vivo 3D ECM, a 3D cobweb-like structure based on 15 µm height pillars connected by beams of varying diameter was fabricated using OrmoComp® photoresist to measure cardiomyocyte force by Klein et al. [137]. During the fabrication, it was proven that the 3D structures could be tuned over a wide range of dimensions and the beam stiffness could be easily and accurately manipulated. Taking advantages of atomic force microscopy and time-lapse microscopy, the contraction forces by single cardiomyocytes were analyzed quantitatively for the first time. This work offers the possibility to explore the mechanical control of different types of cell growth by TPP technology. Concerning neural tissue engineering, Marino et al. [138] prepared sub-micrometric patterned substrates based on aligned ridges by biomimicking the axonal outgrowth and guidance environment. Using scanning probe techniques, an elongated PC12 neurite was estimated to exert a force of about 3 nN for bending of the ridge.

Micro/nano-stimulus-responsive actuators have been found to be useful for capturing microparticle and cell manipulation in the biomedical field. The use of stimulus-responsive photoresists for TPP has facilitated development of different micro/nano-actuators. Recently, Li et al. [139] reported a pH-triggered soft microgripper (<100 µm) with four fingers using a poly(acrylic acid)-based hydrogel for capturing microparticles and cells. As discussed above, carboxyl (–COOH) in the side chain played an important role in the deformation triggered by a pH value change. When the pH value was changed to be lower than 9, the straight fingers would bend to grip a target. However, the sequent cell culture indicated that the catching procedure did little harm to the cells.

Therapeutic cell delivery to target disease situations is a potential treatment for some diseases such as neurodegenerative diseases. However, it remains challenging to complete the targeted transportation of cells and subsequent in situ differentiation in current clinic applications. Micro/nano-robots loaded with therapeutic cells display enormous advantages in performing therapeutic tasks [140]. Dong et al. [141] prepared 3D helical magnetoelectric microswimmers via TPP to deliver
neuron-like cells. These helical structures were prepared using gelatin-methacryloyl as monomers and P2CK as the PI by TPP. More importantly, gelatin-methacryloyl could be degraded by the enzymes generated by the cells. After incubation in water containing magnetoelectric nanoparticles composed of a CoFe₂O₄ (CFO) core and a BiFeO₃ (BFO) shell, magnetoelectric microswimmers were produced. When SH-SY5Y cells were cultured on the microswimmers using an alternating magnetic field, the whole structures gradually collapsed as time passed by. Moreover, obvious neuronal differentiation was observed by testing relevant protein formation after 7 days. Although this work demonstrated the possibility of the potential value of micro/nano-robots in neurodegenerative diseases, a large number of therapeutic cells were required for such a therapy method. As the helical structures had only a low surface area, more effort would be needed to enhance the micro/nano-structures with more efficient cell loading ability.

### 4.2 Tissue engineering

Tissue engineering is recognized as an interdisciplinary field of research that applies material and biological science to create artificial organ and tissue substrates [142]. 3D scaffolds function as the desirable skeleton for the development of drug-loaded cells and further promotion of tissue formation. Therefore, it is essential for the scaffolds to mimic the natural ECM structures of the target tissues. The peculiar features offered by the TPP technique have enabled the preparation of versatile 3D structures with precisely defined interconnected pores and topographical stimulation in vitro on the microscale and nanoscale for specific tissue formation. Currently, various types of tissue engineering research studies, such as retina, heart, bone and cartilage tissue, have been carried out based on the scaffolds prepared via TPP. Nevertheless, highly engineered scaffolds with specific features and functions are in great demand for biomedical applications.

Degenerative retinal diseases are important health matters that are urgently needed to be solved by new and reliable therapeutic methods because of the limitation of current strategies and the shortage of substrates from donors. Because photoreceptor cells are tightly packed and aligned with the path of light entering the eye, the scaffolds used in retinal tissue repair require sophisticated multilayered structures [143]. Tucker et al. [94] prepared an innovative 3D hexagonal scaffold with interconnected horizontal pores for oxygen and nutrient element delivery, and with closely packed vertical pores of varying size for facilitating the growth of human induced pluripotent stem cell (iPSC)-derived retinal progenitor cells (RPCs) loaded in commercially available IP-S photoresists. By tuning the slicing distance and hatching distance, the printing time and design-to-structure fidelity were optimized for fabricating larger scaffolds. The expression of neural progenitor-specific protein TUJ1 aligned in the vertical pores demonstrated the successful transplantation and correct cell orientation of RPCs into the scaffold. Recently, they used poly (caprolactone) (PCL) modified by acrylate groups to prepare similar degradable photoreceptor cell delivery scaffolds [144]. In this work, for improving the design-to-structure fidelity, different formulations of PCL functioned by various numbers of acrylate groups per prepolymer molecule were explored except the aforementioned printing parameters. After staining by the nuclear marker DAPI, it was demonstrated that RPCs successfully grew and proliferated in the pores, which further indicated that they could be transported to the target site of the eye with little influence of the shear forces in surgery because of the protection of the interconnected scaffold. Most importantly, when the cell-free scaffolds were implanted into the sub-retinal space of a pig model, no inflammation, infection and tumor formation were detected after 1 month. Therefore, therapy based on a porous 3D scaffold via TPP would provide a robust, promising and repeatable tool for photoreceptor cell replacement for those suffering from late-stage retinal degeneration.

TPP has also shown great advantages in the creation of microstructured scaffolds made of biodegradable polymers for bone defect repair. Among diverse biodegradable polymers, synthetic polylactic acid (PLA)-based copolymers are the most widely used for bone tissue engineering because of their tunable mechanical properties [145]. Timashev et al. [146] synthesized star-shape methacrylate functionalized poly(ε-caprolactone) for fabricating 3D porous scaffold structures composed of two layers of hollow cylinder arrays in a hexagonal arrangement. Young’s modulus and microhardness were found to be 4.11 and 0.36 GPa, respectively, which are comparable to that of human bone. This scaffold was demonstrated to provide human mesenchymal stem cells with a suitable environment in vitro for cell growth and further differentiation toward the osteogenic lineage without osteogenic stimulation. In another work, Hauptmann and coworkers [147] fully explored how the monomer ratio of poly(ε-caprolactone) and poly-ε-caprolactone...
influenced the elastic modulus and provided a material platform for bone and cartilage reconstruction in combination with the TPP lithography technique. With a ratio of 2:8, the Schwarz primitive scaffold showed very good performance as an artificial tumor environment.

In order to reduce the immune rejection and chronic inflammation after the scaffold implantation, drugs were generally needed to be incorporated in the scaffolds and released on demand for reducing some side effects. Dexamethasone is a widely used pharmaceutical agent which not only can solve the aforementioned problems but also stimulate the differentiation of osteogenic cells in bone tissue engineering [148, 149]. Paun et al. [150] reported electrically responsive microreservoirs with tunable dexamethasone release kinetics. Vertical microtubes arrays were first fabricated using IP-L780 photoresist via TPP, then loaded with a polypyrrole/dexamethasone film by an immersion process. Polypyrrole is a kind of conductive polymer and its intrinsic conductivity can stimulate the growth of bone tissue. Under the stimulation of electrical field, the drug can be released from the redox polymer film because of the electrical switching of the polymer redox states [151]. Using a voltage cycle between −1 V and +1 V, dexamethasone could be released at the desired time. Meanwhile, the osteogenic efficiency of osteoblast-like MG-63 using electrically responsive microreservoirs was improved by 2.2 times as compared to that using unstimulated samples.

4.3 Biomedical devices

With the aforementioned advantages, TPP offers highly efficient reproduction of small-scale 3D medical devices with micro- or nano-features, including microneedles for drug delivery, microfluidic systems and biosensors. Some microneedles require complex shape and small tip angle for low microneedle penetration forces. Gittard et al. [152] fabricated round-tip microneedle arrays made of a photoreactive acrylate-based polymer by using TPP and polydimethylsiloxane (PDMS) micromolding. First, a master structure for a solid microneedle array with a height of 500 µm and a base diameter of 150 µm was fabricated via TPP. Subsequently, a negative mold was fabricated using PDMS based on the master structure. Finally, a number of microneedle arrays could be cast using the negative mold. The microneedle arrays could maintain their elastic deformation over a 10 N axial load and successfully created pores in human stratum corneum and epidermis. Recently, Balmert et al. [37] first prepared dissolving undercut microneedle arrays as cutaneous vaccine delivery platforms with tip-loaded antigen plus adjuvant vaccine components using a similar method. In this work, a square pyramid head (250 µm × 250 µm base area) was designed to increase skin insertion for delivering inside biological cargos. Importantly, cutaneous vaccination with antigen-loaded microneedle arrays could simultaneously deliver adjuvant and antigen to the same skin microenvironment, which further triggered more potent antigen-specific immune responses than conventional immunization by intramuscular injection. These studies indicate that TPP enabled fast reproduction of microneedles, which would facilitate the use of microneedles for future clinical application.

Different microfluidic components have been created for lab-on-a-chip applications. Kumi et al. [153] reported the fabrication of large-area microfluidic master structures via high-speed TPP. They used FLUOR-SU8 to fabricate master relief structures with different cross-sections and features with a high aspect ratio for the PDMS molds. This technology has been useful to prepare microfluidic structures that are challenging to fabricate using conventional photolithographic methods, including microfluidic channels with non-rectangular cross-sections. Micropumps fabricated by stimulus-responsive hydrogel materials have attracted a growing interest in bioanalytical areas. Xiong et al. [154] fabricated a solvent-driven micropump made of solvent-responsive polyacrylamide hydrogel. The micropump system was composed of a hydrogel valve, a microfluidic channel and a hydrogel film wall. When the solvent was changed from water to solvent, the 25 µm-thick hydrogel film wall would bend to trigger the closed and open state of the hydrogel valve. A fluid change of 9.2 × 10−2 pL in the microchannel was completed in only 0.17 s. Thus, the micropump would have a great potential in achieving ultra-microdosage drug release in vivo.

It is also possible to produce other stimulus-responsive microscale actuators via TPP. Zheng et al. [78] introduced Fe3O4 nanoparticles by a surface-modifier to the mixture of poly(N-isopropylacrylamide) (PNIPAAm) and PEGDA for fabricating a double-armed NIR-light hydrogel actuator with only ~26 µm thick. Two arms in this microdevice could close and open to achieve a very fast response time of about 0.033 s in response to NIR light with a power of 29.2 mW. The biocompatibility and small size rendered its potential application in the biomedical MEMS field. It is worth noting that Fe3O4
nanoparticles can also respond to a magnetic field, which is expected to be actuated under multiple external stimuli. In a previous work, this group reported a 3D hydrogel micronail with a size of only ∼10 μm which consisted of a rod and a cap out of surface-modified Fe₃O₄ nanoparticles and magnetic gelphotoresists [104]. The rod part could be reversely deformed to a wide angle of 52.4° under a magnetic field. Taking the biodegradability into consideration, Wang et al. [155] fabricated enzymatically biodegradable magnetic-field-driven soft helical microswimmers based on a protein photoresist by means of TPP. Gelatin methacryloyl (GelMA), which is highly cytocompatible and bioactive, is often used as a material for cell culture. For instance, GelMA and photoinitiator P2CK were first polymerized into helical microstructures, followed by a decoration with magnetic (Fe₃O₄) nanoparticles in water. Under a rotating magnetic field, the soft microswimmers had some advantages with respect to the forward velocity and drift velocity as compared to other rigid helical microswimmers. The helical structures also showed better human skin fibroblast cell attachment performance. Moreover, the GelMA microswimmers could be degraded by cell-secreted proteases after a 1-week culture of HaCaT cells, providing opportunities for the next generation of target site therapy using biodegradable small robots.

5 Summary, current challenges and future perspectives

This paper presents a comprehensive review of the properties of widely used PIs and photoresists which play crucial roles for designing and developing stimulus-responsive micro/nano-structures for biomedical applications via the TPP nanolithography technology. Due to significance of water-soluble PIs in biomedical fields, the properties of the traditional commercial and newly emerging PIs are also described. Moreover, the design and synthesis principles of the PIs are summarized with respect to TPA cross section and water solubility. Most importantly, stimulus-responsive materials can offer the 3D micro/nano-structures “life” with changeable properties in a controllable approach, which cannot be achieved by the static type of micro/nano-structures. With the availability of different PIs, various types of stimulus-responsive photoresists facilitating the generation of enormous 3D micro/nano-structures via TPP for various biomedical applications, such as cell engineering, tissue engineering and medical devices, are also described. As reflected from a number of distinctive recent TPP-related works, the TPP technology has been demonstrated to possess many advantages in producing well-defined 3D structures over conventional miniaturization fabrication technology, including high spatial resolution, easy handling, repeatability and ample material choice.

Despite the aforesaid advantages, TPP as a powerful and versatile 3D micro/nano-fabrication tool still exhibits certain drawbacks and challenges, which need to be addressed in future work. For the most unique feature of the TPP known as the submicron-size voxel, a long printing time is generally needed for producing millimeter-scale structures, which may hinder its application to mass manufacturing. Moreover, the voxel-to-voxel printing method makes the final sample have inevitable connection gaps, which are especially obvious in some unstable photoresists in the development process. Furthermore, the biomaterial choice for the preparation of 3D scaffolds used as soft tissues such as muscle and retina remains limited. Although hydrogels are widely used candidate materials in cell engineering and tissue engineering, 3D hydrogel scaffolds prepared by TPP exhibit a low spatial resolution because of their high water content. Precise manipulation of the topography and pore dimensions at the nanoscale is still difficult to achieve. Particularly, the absence of suitable PIs for TPP nanolithography with a high efficiency in aqueous solution remains a great barrier for the fabrication 3D hydrogel micro/nano-structures for biomedical applications.

Recently, some of these challenges have been mitigated. For instance, the special resolution of TPP can be improved by modification of the scanning speed and laser power. Therefore, highly efficient aqueous-based PIs or monomers with photoactive moieties were developed to optimize the two parameters [49]. A host–guest chemical reaction was proposed to prepare water soluble PIs with high efficiency for polymerization in an aqueous medium [66,67,69,72,156]. To address the time-consuming issue, the recent TPP printing equipment has been enhanced with faster scanning technologies [157]. Another more efficient and effective strategy for the TPP is the application of an array for preparing periodic constructs and multi-microfabrication [158,159], so as to enable a series of small microlenses to be produced in one pass. New biocompatible and biodegradable photopolymerizable materials are being enriched so that users can create novel micro/nano-structures with the industry-standard CAD software. With this vision, it is evident that TPP potentially would solve many current challenges in conventional
technologies in regard to spatial resolution. In order to realize its full range of capabilities, it is expected that the photopolimerizable material pool and water-soluble TPPs can be further developed.

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