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Review Article

Microporous annealed particle hydrogels in cell culture, tissue regeneration, and emerging application in cancer immunotherapy

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Abstract: Microporous annealed particle (MAP) hydrogels consist of densely crosslinked and annealed hydrogel particles. Compared to common hydrogels, the inherent porosity within and among these hydrogel particles offers interconnected channels for substance exchange in addition to sufficient growth space for cells, thereby forming a three-dimensional culture system that highly mimics the in vivo microenvironment. Such characteristics enable MAP hydrogels to adapt to various requirements of biomedical applications, along with their excellent injectability and mechanical properties. This review initially provides a comprehensive summary of the fabrication methods and material types of MAP hydrogels, alongside an assessment of their mechanical properties and porosity. In vitro studies are evaluated based on the impact of MAP hydrogels on cellular behaviors, focusing on cell proliferation, differentiation, migration, activity, and phenotype. In vivo research highlights the promising applications of MAP hydrogels in tissue regeneration, as well as their innovative use in cancer immunotherapy. Current challenges and future research directions are outlined, underscoring the potential of MAP hydrogels to significantly improve clinical outcomes in cancer treatment and regenerative medicine.

Keywords: Hydrogel, microporous annealed particles, cell culture, tissue regeneration, cancer immunotherapy

Introduction

Porosity naturally exists within the human body, with pore sizes ranging from nanometer scale (e.g. vascular system [1]) to micrometer scale (e.g. bones [2], skin [3], lymphatic system [4] and gastrointestinal mucosa [5]). Porous structures are known for their low density, large surface area, and interconnected channels that allow for substance transportation. These features play a vital role in maintaining and enabling the normal functions of the tissues and organs. Over the past few decades, extensive research has been conducted to develop porous materials with varying pore sizes, shapes, and densities. Porous materials are widely used in medicine, including cancer immunotherapy, artificial joints, soft tissue repair, dental repair, facial plastic surgery and drug delivery systems. In biomedical applications, the main benefits provided by porous materials are the accessible void space and interaction with surrounding cells/tissues. Microporous structures can be delicately and flexibly designed to mimic and provide the required microenvironment for cells, which creates new possibilities for restoring superior performance and functionality in biological systems [6].

Hydrogels are 3D network structures with high water content. Because of their inherent porosity and excellent hydrophilicity, they are similar to the extracellular matrix and thus provide various application potentials in the biomedical field [7]. Hydrogel microporous particles (HMPs) are formed when hydrogels are confined within the shape of microparticles, and they are endowed with injectability. Therefore, they are promising for biomedical applications such as cell and drug therapies, tissue scaffolds, biosensors, and medical imaging [8]. When the concentration of hydrogel particles builds up

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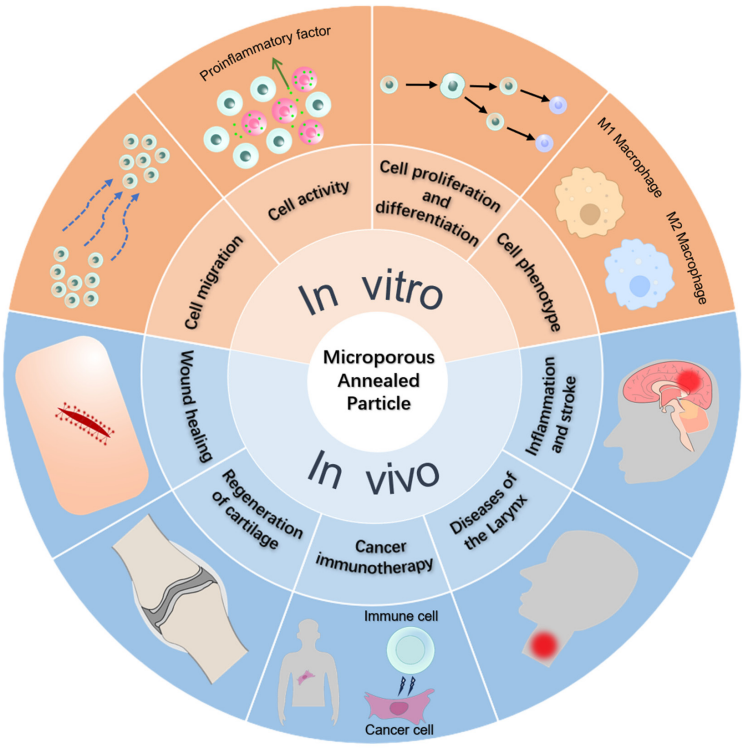


Figure 1. Schemes of both in vitro and in vivo application scenarios of MAP hydrogels. MAP, Microporous annealed particle.

and interaction forces occur, HMPs become granular hydrogels, existing in a clogged state. Cross-linking and annealing among these hydrogel particles further stabilize and fabricate microporous annealed particle (MAP) hydrogels with powerful mechanical properties [9]. Griffin and coworkers were the first to propose the concept of MAP in 2015 [10]. With an interconnected network of micropores, MAP hydrogels could be more effective than dilute HMP solutions in certain implementations.

Tissue regeneration is one application scenario where MAP hydrogels stands out among other biomaterials (e.g. synthetic and natural polymers, bioceramics and extracellular matrices [11]), effectively aiding in the healing process. The focus is on harnessing the body's regenerative potential to control cellular function and direct endogenous cells (including immune cells and precursor or stem cells) to move, proliferate, or differentiate to promote tissue healing, integration, and regeneration [12]. This cellular potential positively impacts the inward growth of cells and regeneration of tissues, inspiring numerous research works on the properties and utilizations of MAP hydrogels. Due to similar reasons, researchers are intrigued by the potential application of MAP hydrogels in the continuous recruitment of diverse immune cells for postoperative cancer treatment [13]. Despite being in its nascent stage and requiring further documentation, research in this field holds promising prospects.

This review aims at summarizing the recent advancements in MAP hydrogels, highlighting how they act on cells to influence tissue regeneration and cancer treatment. The fabrication and properties of MAP hydrogels are firstly introduced, and then the effects of MAP hydrogels on cells are elaborated from four perspectives: cell proliferation and differentiation, migration, activity, and phenotype. Following the progress of these in vitro studies, the in vivo research are illustrated on how the cell responses in MAP hydrogels in turn influence the applications in tissue regeneration (wound healing, inflammation and stroke, laryngeal diseases, and cartilage regeneration) and cancer immunotherapy. Both in vitro and in vivo application scenarios are depicted in **Figure 1**. Finally, the current challenges and future directions are discussed.

Fabrication of MAP hydrogels

As the precursor of MAP, HMP can be synthesized from natural (e.g. alginate, gelatin, hyaluronic acid, chitosan, silk, agarose) or synthetic (e.g. polyethylene glycol (PEG), polyvinyl alcohol) materials [9]. HMP synthesis methods include two broad categories: 1) "bottom-up": polymerization from monomers (or prepolymers, precursors), such as batch emulsions [14], microfluidic emulsions [14], electrohydrodynamic spraying [15]; 2) "top-down": breakdown from large hydrogels into tiny particle, such as mechanical fragmentation [16], lithography [17].

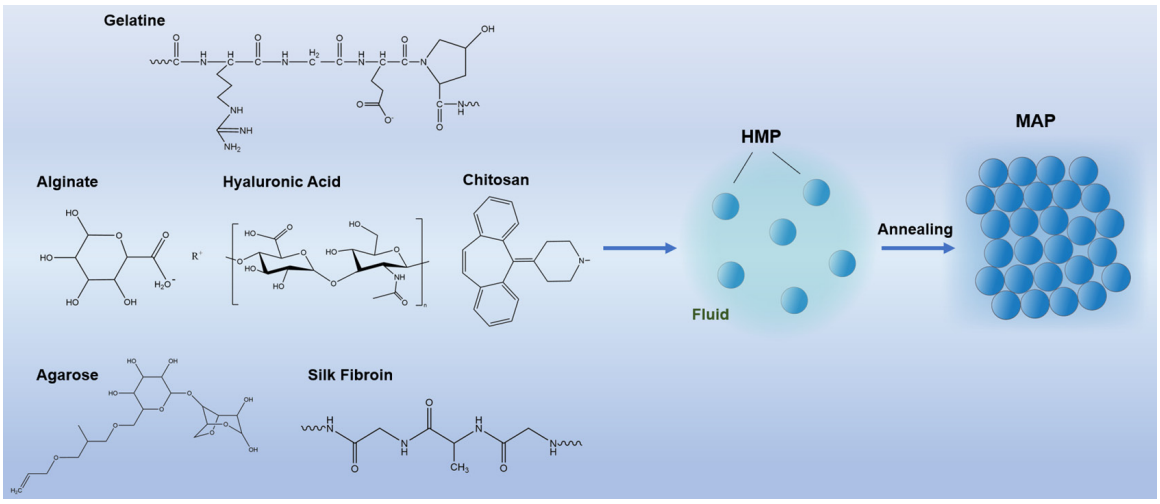


Figure 2. Schematic illustration of the materials and fabrication steps for MAP hydrogels.

In much of the literature, the critical transition from HMP to MAP is termed as "annealing" to distinguish it from the cross-linking method applied in forming HMP [18, 19]. Originally, annealing is a process commonly used in metallurgy, where the metal is heated to a specific temperature and held there for a considerable period of time for structural relaxation and defect control. Over time, the concept of annealing is expanded to the field of polymer materials to bring kinetically trapped structures towards thermodynamic equilibrium, and the performance of these macromolecules during thermal processing could be similar to metals under certain circumstances [20, 21]. In the biomedical field, it is well known that high temperatures are not suitable for living organisms, especially for in vivo applications. Consequently, annealing at a body temperature of 37°C, also known as self-annealing, has become the most common thermal treatment method to maintain the stability of biomedical materials and ensure their stability and safety in vivo [22-24]. Therefore, the "annealing" step in forming stable MAP hydrogels from HMPs, to be exact, incorporates interparticle cross-linking and self-annealing (**Figure 2**).

Interparticle cross-linking affects material properties in MAP. Conventional chemical cross-linking forms irreversible bonds via cross-linking agents [25], whose multiple reaction pathways include enzymatic (FXIIIa) chemistry [26, 27], carboxylic acid/amine chemistry [28], light-mediated radical reactions [28], light-mediated thiol-alkene reactions [29-33], norbornene-tetrazine cycloadditions [34-37], azide/alkyne click chemistry [38-41], and tetrazine-norbornene click reaction [9, 18] (summarized in **Table 1**). Through these covalent bonds, chemical cross-linking can form stable and reliable scaffolds for cell adhesion, creating a mild environment for cell survival. However, the rapid gelation produced by covalent cross-linking can make it difficult to inject, failing to meet the rising needs for minimally invasive applications. As a result, research is increasingly focusing on dynamic and reversible cross-linking that provides MAP hydrogels with the ability to shear-thin and self-repair, and makes it injectable in liquid form. Examples include hydrogen bonding [42], guest-host interactions [43], electrostatics [44], biorecognition motifs [45], hydrophobicity [46], metal-ligand coordination [47] and dynamic covalent chemistry [48] (summarized in **Table 2**).

Properties of MAP hydrogels

Mechanical properties

The mechanical properties of MAP hydrogel can help to support cell proliferation and spreading as well as good physiological stress. They can be affected by HMP concentration, hardness, interparticle cross-linking chemistry among HMPs, and particle packing density [9]. Such tunable mechanical properties have been demonstrated through rheological analysis and mechanical compression testing [18, 52, 53].

Table 1. Chemical cross-linking pathways for interparticle cross-linking in MAP hydrogels

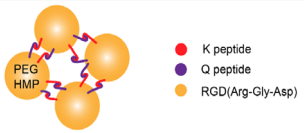
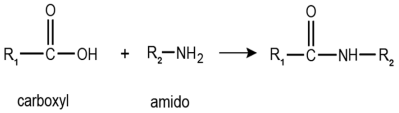
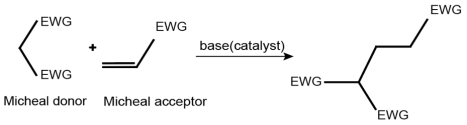
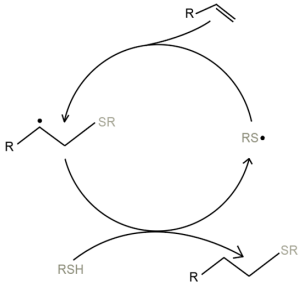
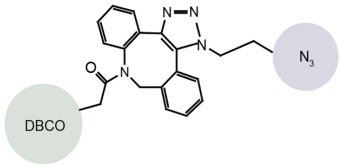
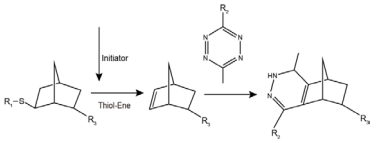
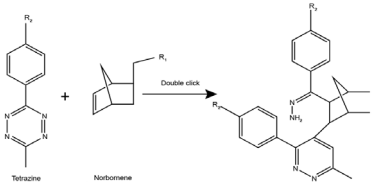
Interaction	Materials	Functional group	Applications	Ref.
Enzymatic (FXIIIa) chemistry	Bioactive 4-arm polyethylene glycol (PEG)		Accelerated wound healing. This crosslinking method enables the scaffold to be formed through injection and rapidly self-assemble after injection.	[10]
Carboxylic/amine chemistry	Hyaluronic acid		Cell migration and diffusion. This crosslinking method can significantly enhance the mechanical strength of the material, forming a stable three-dimensional network structure that provides sufficient physical support in biological environments.	[28]
Light-mediated radical reactions	4-arm PEG	 *EWG: electron withdrawing group	Enhanced photo-annealing. Compared to other crosslinking methods, it offers a faster photoinitiated crosslinking speed, achieving higher crosslinking efficiency within a given time.	[19]
Light-mediated thiol-ene reactions	PEG-norbornene macromers and PEG-dithiol		The proliferation and multiplication of stem cells. This crosslinking method significantly influences cellular responses to different ligands.	[49]
Azide/alkyne click chemistry	PEG end functionalized with azide and alkyne moieties		Adjust microgel size and control cell morphology. This method significantly improved cell encapsulation efficiency while maintaining a cell survival rate of up to 95%.	[50]
Norbornene-tetrazine cycloadditions	PEG		Drug delivery and cell encapsulation applications. The rapid crosslinking kinetics enabled efficient crosslinking without the need for additional initiators or catalysts.	[35]
Tetrazine-norbornene click reaction	Hyaluronic acid		Reduced levels of inflammation and astrocyte activity. The mechanical properties of the scaffold can be precisely controlled by adjusting the ratio of the crosslinking agent.	[18]

Table 2. Reversible cross-linking pathways for interparticle cross-linking in MAP hydrogels

Interaction	Materials	Functional group	Applications	Ref.
Hydrogen bond	Methacrylic acid, acrylamide and potassium persulfate	Functional groups on polymer chains	Influence of chain flexibility and hydrogen bonding on the mechanical properties of gels. The introduction of double and multi-chain hydrogen bonds significantly enhances the stability of hydrogels in water and complex environments.	[42]
Guest-host interactions	PEG	Guest molecule Adamine and host molecule β-cyclodextrin	To evaluate the effect of guest-host interaction on the mechanical properties of PEG microgels. MAP hydrogels based on guest-host crosslinking exhibit excellent shear-thinning and self-healing properties. These features allow the material to withstand shear forces during injection and restore structural integrity after injection, making it suitable for tissue repair and cell delivery.	[43]
Electrostatics	Copolymers of neighboring cationic-aromatic sequences	Aromatic	Exhibits strong adhesion to negatively charged surfaces in saline environments such as seawater. This hydrogel exhibits excellent dynamic crosslinking properties, enabling it to absorb energy and quickly self-recover. This characteristic is suitable for scenarios requiring repeated use or dynamic loading, such as tissue engineering and soft robotics.	[44]
Biorecognition motifs	Triblock protein polymers	Repeated heptapeptide sequences	Protein copolymers can self-assemble into reversible hydrogels. Hydrogels based on protein block copolymers exhibit a sensitive response to changes in temperature and pH. By adjusting the amino acid sequence of the coiled-coil blocks, the self-assembly and disassembly behavior of the hydrogels can be precisely controlled.	[51]
Hydrophobicity	Cellulose nanocrystal and N-[3-(dimethylamino)propyl] methacrylamide	C18 alkyl	Can be easily repaired by tetrahydrofuran when damaged, maintaining good tension and elongation behavior. Through dual physical crosslinking, combining hydrophobic interactions and hydrogen bonding, the mechanical properties of the hydrogel are significantly enhanced.	[46]
Metal-ligand coordination	Polyacrylic acid	Fe^{3+} and polyampholyte	By varying the concentration of metal ions (e.g., Fe^{3+}), the mechanical properties of hydrogels can be significantly improved. By altering the types of metal ions (such as Fe^{3+}, Zn^{2+}, Ca^{2+}) and their concentrations, the network structure and mechanical properties of the hydrogel can be precisely tuned. This tunability allows the hydrogel to meet various application requirements.	[47]
Dynamic covalent chemistry	N-Dimethylacrylamide	Schiff base bond	The introduction of dynamic covalent bonds enables the hydrogel to respond to environmental changes. Under low pH conditions, the material's structure can dissociate and recover, offering potential applications in drug delivery and environmental monitoring.	[48]

Various cross-linking methods lead to diverse mechanical properties of the MAP hydrogels prepared. Among them, dynamic acetylhydrazone bonds by photo-cross-linking were found to play an important role in enhancing the mechanical properties of the hydrogel while maintaining the injectability of the particles [54]. The pH also affects the cross-linking of the microgels [55]. Widener and coworkers [43] detailed two unique types of MAP-PEG hydrogels using two different secondary cross-linking methods based on reversible guest-host interactions, where guest molecules can be wrapped or embedded in host molecules. One type is the dual microgel MAP-PEG hydrogel (Inter-MAP-PEG), which utilizes two microgels functionalized with the guest molecule Adamine and host molecule β-cyclodextrin, respectively. The other type is the single microgel MAP-PEG hydrogel (Intra-MAP-PEG), which uses the same microgel functionalized as both guest and host molecules. Significant differences in mechanical properties were observed, as Intra-MAP-PEG hydrogels are softer and exhibit a significantly reduced yield stress required to initiate material flow. These findings highlight the potential of guest-host interactions in regulating hydrogel properties. Zoratto [53] studied a new gelatin methacryloyl (GelMA) microgel scaffold formed from physically cross-linked GelMA microscale beads (microgels) through chemical annealing and semi-processing via ultraviolet irradiation. Compression testing and rheological analysis of the scaffolds showed that their biomechanical properties are like those prepared using non-chemically cross-linked microbeads. Optimization of the semi-photo cross-linking time is crucial for maintaining the thermal stability and annealing ability of the microgels.

Due to its excellent hygroscopicity and water retention capacity, MAP hydrogel is in a gel state with yield stress after annealing. Different cross-linking methods produce different yield stresses. Since MAP hydrogel is chemically inert to a certain extent, when it is introduced into the body by injection or oral administration, it needs to be cross-linked in a specific way. MAP hydrogel cross-linked by non-covalent bonds (reversible bonds) still behaves as a liquid under high shear stress. The stiffness of the MAP scaffold has an important influence on the diffusion, proliferation and gene transfer of cells in the hydrogel. Increasing the cross-linking ratio can enhance the stiffness of the scaffold, but excessive cross-linking will reduce the porosity of the scaffold [23, 56].

Porosity

The porous structure of MAP scaffolds is crucial for cell study and is directly related to the diameter of HMP. As mentioned above, the higher the degree of cross-linking, the lower the porosity of the scaffold. The MAP scaffold cross-linked and annealed by FXIIIa enzymatic reaction showed low cross-linking, which was conducive to cell proliferation and diffusion [18]. By adjusting the pore size of the microparticles, the recruitment and phenotypic transformation of immune cells can be effectively affected [57]. For example, Liu et al. [24] used PEG as a raw material to make three HMPs with different diameters. After annealing, they formed MAPs, which showed different effects on cells. This direct correlation emphasizes the importance of HMP diameter to MAP performance and cell interaction. Therefore, by regulating the physical structure of MAP, we can more finely control its role at the cellular level, providing useful inspiration for future biomedical research. Such impact will be furthered described in the following sections.

In vitro applications

As microporous scaffolds, MAP hydrogels exhibit great potential as cell culture platforms, offering a number of significant advantages over ordinary Petri dishes. Through the precise regulation of micro-nanostructures, MAP scaffolds are able to provide a more realistic and three-dimensional environment for cell growth, guiding the orderly orientation and localization of cells while simulating the complex hydrodynamic environment to which cells are subjected in vivo. High biocompatibility, strong permeability, and highly customizable properties make MAP scaffolds ideal for biomedical research and tissue engineering, facilitating a deeper understanding of cell behavior and tissue regeneration.

MAP hydrogels act on cells through their diverse properties, such as the size, shape, density, material and composition of hydrogels. These factors have significant effects on cell proliferation, differentiation, migration, activity and phe-

notype [58], which would indirectly affect the formation of tissues.

Cell proliferation and differentiation

Cell proliferation and differentiation are vital functions of living cells. Cell proliferation involves the creation of new cells through cell division, while cell differentiation refers to the gradual development of cells into specific cell with distinct functions and morphologies [59]. These processes are crucial for evaluating the health of living organisms. In this regard, mesenchymal stem cells exhibit superior pluripotency, possessing the self-renewal and multidirectional differentiation capabilities of stem cells. As a result, they are commonly used in cell proliferation and differentiation experiments, but the success of these experiments depends on various factors.

Regarding the scaffolds where stem cells grow, factors including stiffness, degradability, cell adhesion, and pore structure would significantly impact the survival and function of stem cells [50, 56, 60-62]. In a study by Koh et al., stem cells showed better retention and proliferation in MAP scaffolds made from PEG [56]. The researchers used in vitro experiments and an in vivo model of C57BL/6 mice, an immunocompetent mouse strain, to test the ability of stem cells to proliferate, survive, and migrate. They found that the scaffolds provided an effective microenvironment for the stem cells, which eventually enhanced their retention and function in vivo. Another in vitro cell culture experiment has revealed that the micropore structure of photo-annealed microgels plays a crucial role in cell spreading and aggregation [60]. It is found that human mesenchymal stem cells exhibit different responses depending on the stiffness and degradability gradients of the microgel [63]. Cell spreading, proliferation, and extracellular matrix protein secretion were significantly enhanced in MAP hydrogels that were made from PEG and degraded by matrix metalloproteinase with faster degrading efficiency. Moreover, MAP hydrogels functionalized with cyclized RRETAWA peptide significantly increased the secretion of bone morphogenetic protein-2 on a per-cell basis. However, this effect was heavily dependent on the degradability of the microgels. On the other hand, RGDS (Arg-Gly-Asp-Serine) functionalization resulted in higher overall vascular endothelial growth factor secretion in degradable scaffolds due to the high cell number, where the MAP hydrogel scaffold provided a suitable three-dimensional microenvironment that promoted cell attachment [49].

Newly synthesized proteins in hydrogels can contribute to cell spreading, YAP/TAZ (Yes-associated protein/transcriptional coactivator with PDZ-binding motif) nuclear translocation and osteogenic differentiation [64]. Different types of extracellular matrix proteins, distinguished by their varying densities, exert distinct impacts on osteogenic differentiation of stem cells [65]. When mesenchymal stem cells are cultivated within hydrogels that incorporate natural extracellular matrix components, their proliferative capacity, value-added properties, and osteogenic differentiation potential are significantly enhanced [66]. When bound to MAP hydrogels composed of PEG through a bioorthogonal site-selective protein-binding strategy, the recombinant proteins (such as green fluorescent protein and human bone morphogenetic protein-2) have been found to maintain their bioactivity and exhibit efficacy in stimulating the proliferation and osteogenic differentiation of human mesenchymal stem cells within the hydrogel matrix [22]. Wilson and colleagues [67] created MAP scaffolds using hyaluronic acid. The behavior of neural progenitor cells can be influenced by chemically modifying the hydrogel particles within MAP scaffolds (**Figure 3A**). This can affect their migration, spreading and differentiation. Different peptide modifications, such as IKVAV (Isoleucine-Lysine-Valine-Alanine-Valine), YIGSR (Isoleucine-Lysine-Valine-Alanine-Valine) and RGD (Arginine-Glycine-Aspartic Acid), can lead to neural stem cells forming neurospheres or spreading out and further differentiating into nerve cells.

Cell migration

Cell migration is a complex and purposeful process that involves the movement of cells through a tissue. It typically encompasses several crucial steps, including sophisticated sensing of the surrounding environment, cell adhesion, and coordinated cell movement. The MAP scaffold has been noted to enhance cell migration in vivo [68]. As for in vitro assessment, 3D MAP scaffolds demonstrated their effectiveness in promoting the migration and mass growth of human dermal fibroblasts that are

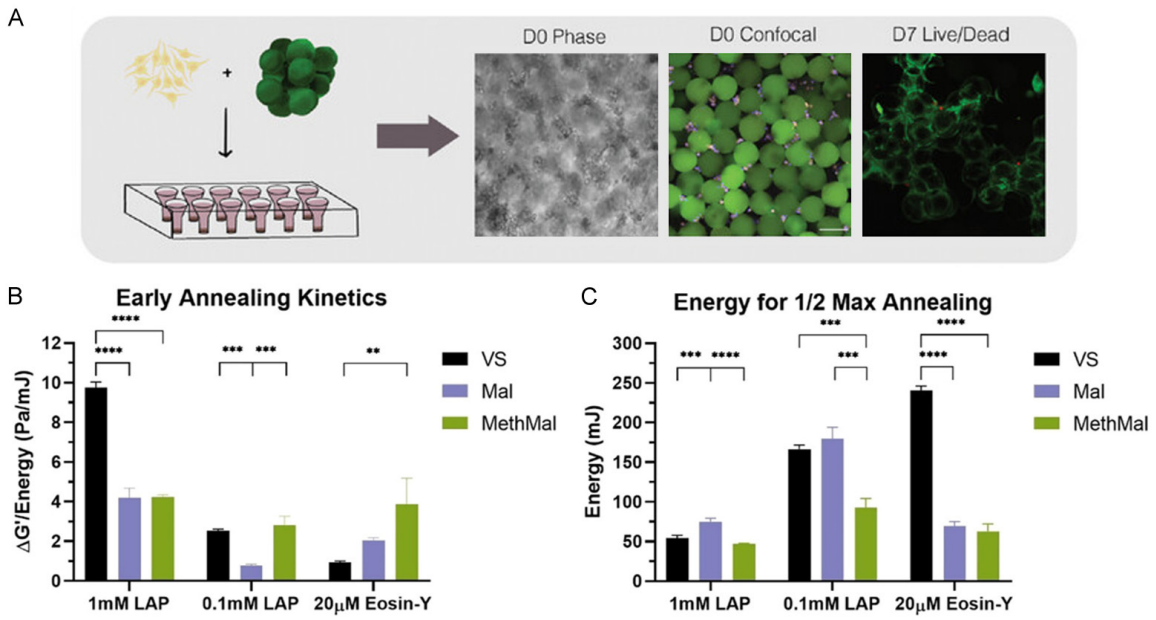


Figure 3. Representative characterization results of MAP hydrogels. (A) Neural progenitor cells can be influenced by chemically modifying the hydrogel particles within MAP scaffolds. (Reproduced with permission from Ref. [67]. Copyright 2022, Wiley). MAP scaffolds of MethMal showed improved photopolymerization capabilities with rheological analysis: (B) early annealing kinetics and (C) light energy required for half-maximum storage moduli (Reproduced with permission from Ref. [19]. Copyright 2021, American Chemical Society).

the primary cellular constituents of loose connective tissues and frequently chosen to be applied in such kind of experiments. It has been found that enlarging the aspect ratio of the microgel could result in a significant increase in porosity and pore sizes of the scaffolds, leading to further enhancement in the migration and growth of cells, which was achieved with the help of 3D printing technology [69].

Researchers have developed a hydrogel made from annealable natural protein microbeads, also known as GelMA microbeads [63]. These microbeads can be physically crosslinked and UV-chemically annealed to provide stability and 3D structures. The GelMA microbeads were tested for biological performance using fibroblasts and were found to be excellent for 3D cell culture, cell viability, adhesion, and proliferation [70]. It's worth noting that Pfaff et al. [19] have developed a specific heterogeneous macromolecule called MethMal for use on microgels fabricated by microfluidic devices and photo-annealing techniques, which can be used to enhance the photo-annealing ability of MAP scaffolds. They found that the activity of human dermal fibroblasts was not affected after cell activity assays. MethMal demonstrated improved photo-annealing ability and can be easily integrated into any formulation of MAP hydrogels, provided that the microgels were cross-linked using the Michael-type addition mechanism (**Figure 3B** and **3C**). Additionally, the incorporation of other substances such as epidermal growth factor (EGF) into MAP hydrogels can further promote cell migration, which can be beneficial for specific applications, such as in wound healing and tissue engineering, to help accelerate the repair process [71]. By optimizing the concentration and release rate of EGF, the therapeutic efficacy of the hydrogel can be improved to better support tissue repair and regeneration. This improvement not only enhances the biocompatibility of the hydrogel, but also expands its potential for clinical therapeutic applications.

Cell activity

Cellular activity refers to the level of activity displayed by a cell throughout its life cycle including the cell's metabolic activity, growth rate, division capacity, motility, and response to external stimuli. Active cells exhibit higher biological activity and are able to perform their

functions effectively, participating in tissue development, physiological processes, and immune responses.

Injection of MAP hydrogel provides mechanical support and reduces atrophy of brain tissue, which reduces the stress response of astrocytes and thus the proportion of active astrocytes [72]. Under normal physiological conditions, astrocytes maintain homeostasis in the central nervous system. When astrocytes are exposed to pathological stimuli (e.g., infection, injury, or neurodegenerative disease), astrocyte activity increases significantly, a state known as astrocyte activation or reactive gliosis, which activates and produces a variety of pro-inflammatory factors [73]. MAP hydrogels made of hyaluronic acid were injected into the stroke core, helping to promote axonal regeneration, provide mechanical support, and modulate the microenvironment [72], all of which attenuated the stress response of astrocytes, thereby decreasing astrocyte activity. The application of hydrogels in the context of inflammation and stroke will be further elaborated in Section 5.2 below.

Cell phenotype

Cell phenotype refers to the observable characteristics of a cell in terms of its shape, structure, and function, which reflect the appearance and biological properties of the cell. Taking macrophages as an example, their cellular phenotypes mirror the biological functions they manifest in maintaining homeostasis and immune defense within the body [74]. The activation of macrophages in the given space and time is called macrophage polarization, which is not permanent since macrophages possess sufficient flexibility [75]. The particle size of the MAP hydrogel exerts an influence on macrophage polarization, enabling the modulation of macrophage cellular phenotype by adjusting its particle dimensions. Consequently, this alteration can impact the macrophage's restriction ability. Activated macrophages fall into two categories: M1-like macrophages and M2-like macrophages. The dynamic equilibrium between them is critical for immune response, inflammatory regulation and tissue repair [76, 77].

Liu et al. have developed a microporous annealed particulate scaffold (D-MAPS) that has been demonstrated to promote a balanced M1/M2 macrophage phenotype at an early stage through various characterization tools, including spectral flow cytometry and multicolor flow cytometry [78]. Spatial confinement is found to influence the behavior of macrophages in MAP hydrogels. They used 8-arm PEG Vinylsulfone with K-peptide, Q-peptide, and Arginylglycylaspartic acid to create microgel particle of three different diameters (40 μm, 70 μm, and 130 μm), which were then annealed to form MAP hydrogels [24]. The morphology, motility, and nuclear shapes of macrophages within the hydrogels were evaluated (**Figure 4**). Macrophages exhibited smaller cell volumes to fit into the over-constrained pores in the 40 μm MAP hydrogels, displaying an increase in the transmembrane glycoprotein CD11c expression and Arg1+CD206+ macrophage subpopulation. In the 70 μm MAP hydrogels, both cells and nuclei of macrophages showed more spherical morphologies, which was linked to higher levels of iNOS (inducible nitric oxide synthase), CD206 (M2 macrophage markers), CD86 (co-stimulatory molecule), and MCHII (major histocompatibility complex class II) and represented a balance of M1/M2 labeling in the macrophage population. As for the 130 μm MAP hydrogels with even lower spatial restrictions, larger volume and surface area allowed more space for cells to stretch and exchange among 3D pores. Additionally, in vitro cell culture experiments indicated that small-sized microgels promoted the formation of M1 macrophages, whereas large-sized microgels tended to induce the M2 phenotype [60]. As a result, the 130 μm MAP scaffold effectively facilitates wound healing, reduces inflammation, and accelerates the formation of mature collagen [57].

In vivo applications

While in vitro cell experiments have been established as an effective means to pre-evaluate and verify the ability of MAP hydrogels to regulate cell behavior, the ultimate vision of studies is to safely and efficiently apply carefully designed and optimized MAP hydrogels to the human body. This section focuses on core application scenarios of MAP hydrogels in vivo for tissue regeneration (i.e. wound healing, inflammation and stroke, laryngeal diseases and cartilage regeneration) and cancer immunotherapy.

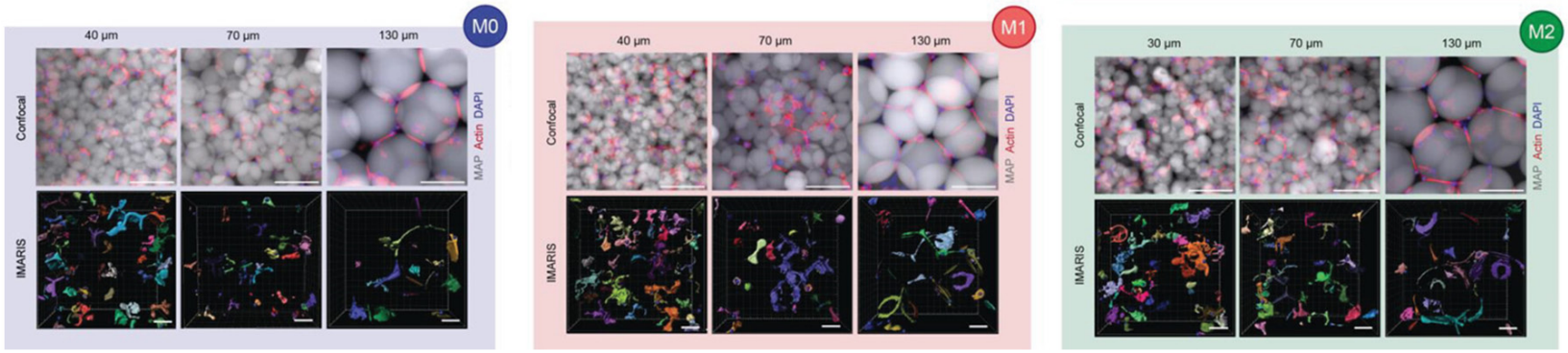


Figure 4. The morphology, motility, and nuclear shapes of macrophages within the MAP hydrogel scaffolds were evaluated with M0, M1, and M2 activation (Reproduced with permission from Ref. [24]. Copyright 2023, Wiley).

Wound healing

Wound healing is a complex process that is essential for maintaining the skin's barrier function [79-82]. MAP hydrogels have great potential in accelerating wound healing due to their injectable nature [83-85]. The pores of the MAP scaffold also provide space for cell migration and adhesion, thus promoting tissue regeneration [86].

The size and shape of the hydrogel particles in the MAP can impact wound healing. For example, the 130 μm MAP scaffolds mentioned in Section 4.4 are more effective in promoting wound healing and reducing inflammation compared to smaller-sized ones [57]. MAP scaffolds are able to modulate immune cell recruitment and phenotype in a pore size-dependent manner, and in particular 130 μm MAP scaffolds induce the regeneration of mature collagen, which influences their pro-healing effect. Another hydrogel with granular texture and rod-shaped particle has been found to promote cell invasion and tissue repair more effectively [86]. Compared to conventional spherical particles, rod-shaped MAP hydrogels are able to modify the pore shape and interconnectivity of granular hydrogels. In vitro experiments have revealed that, within these rod-shaped hydrogels, the neovascularization of endothelial cells extending from embedded cell spheres exhibited greater length and density.

Variations in molecular structures of MAP hydrogels also affect the regeneration process. Griffin and coworkers [87] created a microporous annealed particle (d-MAP) hydrogel that can be rapidly degraded in vivo with the chirality of the cross-linked peptides transitioning from L- to D-amino acids. Through immunoreaction analysis, they found that this MAP can activate adaptive immune responses and significantly promote regenerative healing of wounds, which is critical for skin regeneration. However, this hydrogel has only been tested in mouse models with further research required to determine its efficacy and safety for human clinical application.

MAP has the ability to encapsulate drugs, and such combined mechanism has the potential to significantly expedite the wound healing process. EGF, as an excellent wound healing agent that stimulates skin cell growth, proliferation, and differentiation [88], is one of the commonly utilized substances that can be loaded into MAP. In a study by Pruett et al., it was discovered that loading 5% heparin-containing EGF into MAP hydrogels made of PEG significantly promoted cell migration and thus improved wound closure [71].

Inflammation and stroke

Stroke, also known as cerebrovascular accident, occurs when the blood supply to the brain is interrupted or severely reduced. After a stroke, the influx of astrocytes and microglia causes intense inflammation and scarring in the brain, which impairs its ability to self-repair [89, 90]. Inflammation is a crucial response that the immune system provides to ensure survival during infections and tissue damage. However, prolonged inflammation can have serious detrimental side effects on health [91]. MAP hydrogel is able to modulate the activity of astrocytes and microglia and to promote the infiltration of reparative astrocytes into the lesion, which offers great promise for the treatment of stroke disease and clinical development [52].

Injecting MAP hydrogel directly into the stroke core reduced astrocyte activity, significantly decreased the inflammatory response, and promoted the repair environment [68, 72, 85, 92]. Evaluation of the hydrogel's pore structure and distribution in the brain through fluorescence tracing and microscopic imaging showed that the MAP hydrogel of hyaluronic acid encourages the penetration of reparative astrocytes and axons into the core of the stroke area, which is vital for neural regeneration and repair [52]. In addition, the molecular weight of hyaluronic acid has a significant impact on its cross-linking and angiogenic potential in biomaterial scaffolds [93]. This MAP hydrogel shows potential in treating stroke by facilitating reparative astrocyte and axonal penetration into the core of the stroke area, increasing the proportion of more reparative astrocytes and microglial cells, reducing brain atrophy and preserving axon bundles NF200, and simultaneously promoting neurological repair [52, 94].

Laryngeal diseases

Vocal folds insufficiency is a common disorder that affects the larynx, and can cause dyspho-

nia and dysphagia impairing an individual's quality of life. Pruett and coworkers focused on applying MAP hydrogels to treat such diseases. They developed a PEG-based MAP hydrogel using a water-oil emulsification method, and matched the stiffness modulus of the natural vocal fold muscle [95]. In the rabbit model, the results showed that the MAP hydrogel had superior tissue integration, stiffness matching, and durability, as well as biomechanical retention of function. This makes it a promising therapeutic approach for vocal fold dysphonia, especially when compared to other injectable implants. But it was tested for only six months without long-term effects evaluated. Four years later, Pruett et al. prepared a new MAP hydrogel made of PEG-maleimide and PEG-thiol crosslinker that was found to be more effective than the current clinical standard of hyaluronic acid through MRI imaging and acoustic analysis [96]. Over the 14-month experiments using a rabbit model of vocal fold paralysis, most of the MAP scaffolds were replaced by new tissues that matched the mixture of fibrotic and nonfibrotic collagen in the contralateral vocal folds. The rabbits treated with MAP gel retained tissue enhancement, airway volume, and functional improvement despite varying degrees of hydrogel degradation.

Cartilage regeneration

Cartilage is a translucent and elastic tissue. Due to the limited regenerative capacity of damaged cartilage and the potential morbidity associated with implanted or transplanted bone and cartilage, cartilage regeneration emerges as an attractive alternative [97].

Zhu and collaborators [98] studied the MAP hydrogel made from PEG in treating osteochondral defects in knee joints. The defects were introduced into the rats' knee joints, with one group treated with the MAP hydrogel while others injected with saline as a control. The MAP hydrogel was cured to form a scaffold by photo-annealing. The results showed that the rats injected with MAP hydrogel achieved stable incorporation and regeneration of chondrocytes in articular cartilage defects. During the 12-week observation period, it was found that the MAP-treated group also showed tissue endogenization and increased glycosaminoglycan production in the area of the cartilage defects compared to the control group, without significant inflammatory response (**Figure 5**).

Similarly, Schaeffer et al. [99] used PEG as a raw material along with hyaluronic acid and gelatin to create a photo-annealed granular hydrogel (GH) by in vivo photo-annealing. The in vitro studies revealed that GH caused chondrocyte volume expansion and morphology restoration and significantly improved their chondrogenic phenotype as compared to non-granular hydrogel (nGH). The subcutaneous cultures and in vivo studies in a rat model of full-length cartilage defect for 12 weeks showed that GH-loaded chondrocytes promoted hyaline cartilage matrix deposition and connectivity, leading to hyaline cartilage-like regeneration.

Cancer immunotherapy

Cancer, as a major disease threatening human health, has always been a significant challenge in medical research. Among various treatment methods, cancer immunotherapy is gradually emerging as a research hotspot. It works by stimulating the body's own immune system to recognize and attack cancer cells, thereby controlling and eradicating tumors. Research on the application of hydrogels in cancer immunotherapy has gained increasing interest. While hydrogels of 10-100 nanometers in diameter being suitable for drug delivery technologies [100], larger pores are required for therapeutic immune cells to directly destroy cancer cells. The unique porous nature of MAP hydrogels allows them to establish an immune microenvironment, providing favorable conditions for cell growth (as described in the previous Chapter 4), thus making them feasible as a platform for immunotherapy.

Kuang et al. [13] presents a microfluidic-assisted MAP scaffold for continuous recruitment of immune cells for cancer postoperative therapy (**Figure 6**). The scaffold is made from GelMA, using physical and photo-cross-linking of GelMA droplets prepared by microfluidic electrospraying. Encapsulating liquid nitrogen-inactivated tumor cells and immune stimulants, the MAP scaffold recruits various immune cells, including T cells, macrophages, dendritic cells, B cells, and natural killer cells, forming biomimetic tertiary lymphoid structures (TLSs) in vivo. When combined with immune checkpoint inhibitors, it triggers a strong immune response to

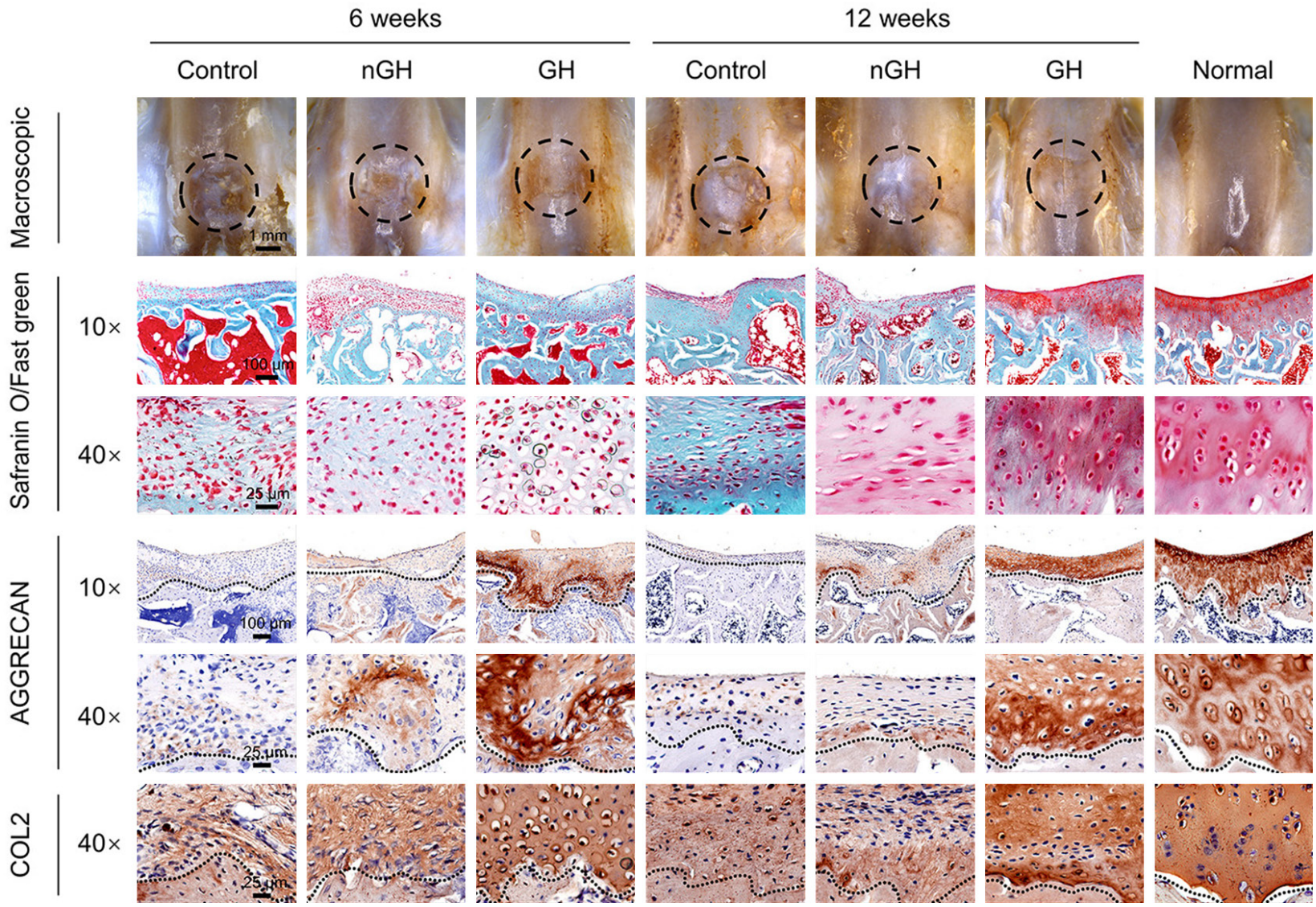


Figure 5. Representative macroscopic, safranin O/fast green staining and immunohistochemical staining images for the rat knee cartilage defect site for 6 and 12 weeks after surgery. The MAP-treated group achieved faster regeneration (Reprinted with permission from Ref. [98]. Copyright 2022, American Chemical Society).

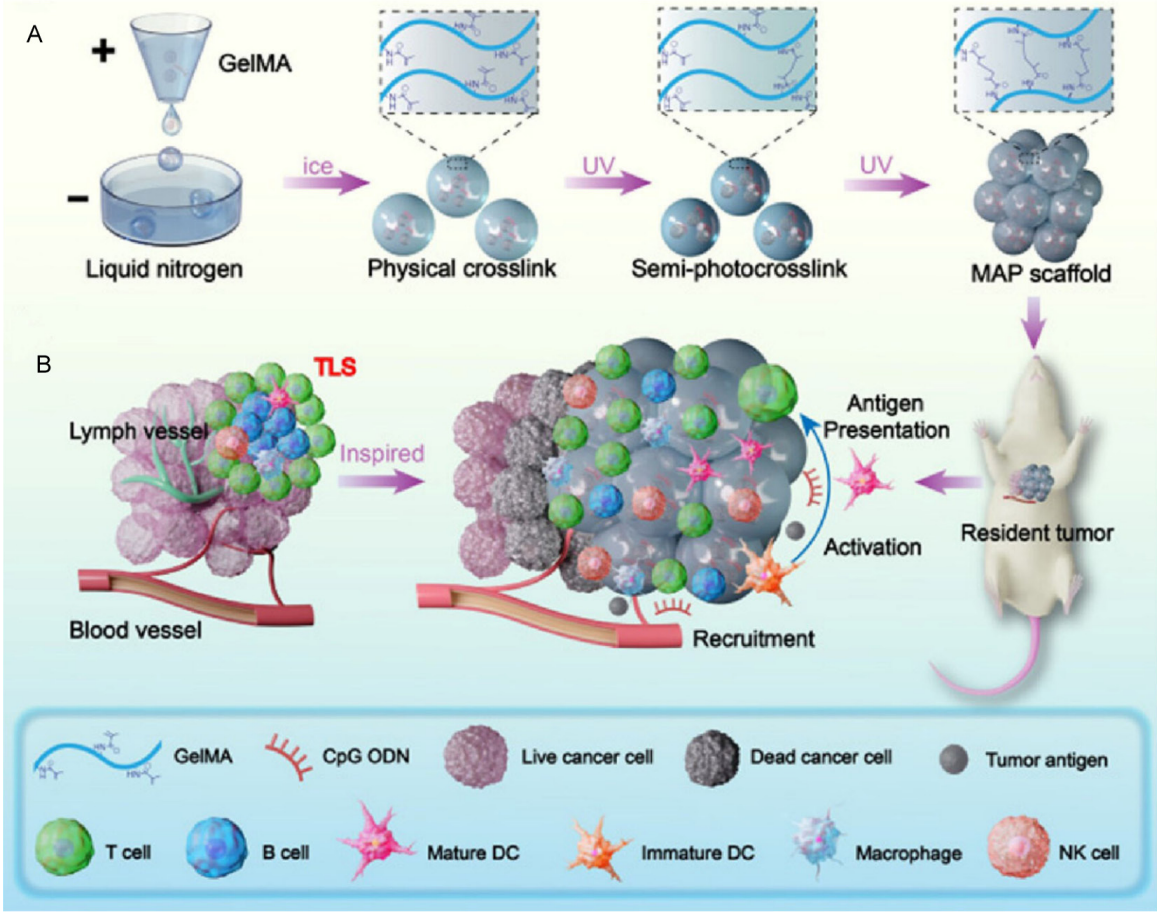


Figure 6. Schemes of a gelatin methacryloyl (GelMA) MAP scaffold for cancer immunotherapy. A. The preparation process of the MAP scaffold. B. The MAP scaffold could recruit a large number of immune cells in the tumor postoperative region (Reprinted with permission from Ref. [13]. Copyright 2024, American Chemical Society).

prevent tumor recurrence and metastasis. These features make the MAP scaffold-based TLSs valuable for effective cancer postoperative treatment. This method uses the MAP scaffold to recruit immune cells and offers a new way to treat cancer.

The current research on the application of MAP hydrogels in cancer immunotherapy has just started, yet its potential extends far beyond what has been explored. In fact, MAP not only possesses large void spaces between its hydrogel particles, which are conducive to constructing an immune microenvironment, but also features nanostructures within these hydrogel particles that may be suitable for drug delivery. This unique combination of MAP microstructures holds promise for achieving synergistic immunotherapy, potentially enhancing therapeutic efficacy further. Although such type of research is still in early stages, with limited documentation and clinical data to prove feasibility, it offers a glimmer of new hope for postoperative cancer treatment.

Challenges and future directions

Overall, MAP exhibits a well-organized microporous structure that creates an optimal environment for cell growth, facilitating cell attachment, movement, and proliferation. Cell attachment supports tissue regeneration, while the micropores play a significant role in recruiting immune cells for cancer treatment. Common biomaterials that lack a microporous structure may encounter difficulties in effectively attracting immune cells, often necessitating additional drugs or immune boosters to enhance the immune response. In contrast, MAP is more complex to design and manufacture, requiring

specialized processes to ensure that its microporous structure is both uniform and stable.

These innovative studies on MAP hydrogels are primarily limited to animal experiments, posing many challenges for their practical clinical application. One of the primary challenges is the ambiguous nature of human immune specificity and the extent of human adaptation. Specifically, the focus is on how to develop a broader variety of materials with diverse functions to address the needs of different scenarios while ensuring biocompatibility. Another question is how to achieve precise control of the hydrogel microenvironment during manufacturing and application to ensure consistency and reproducibility in experimental and clinical applications. Due to these uncertainties, the popularization and application of MAP hydrogels in the medical field has been somewhat constrained.

To overcome these challenges, future research could focus on exploring novel materials and carriers, the application of biomimetic strategies, and innovative cross-linking methods.

(1) By mimicking the structure and function of natural tissues (bone, cartilage, nerves, etc.), dynamic biomimetic materials can be developed that enable MAP hydrogels to respond to microenvironmental changes in living organisms and mimic the complex and dynamic environment in vivo with similar mechanical properties and bioactivity. (2) The development of MAP hydrogels with various functions, such as temperature responsiveness, moisture responsiveness, and electrical responsiveness, is expected to suit different application needs and to solve a series of challenges currently faced by improving material properties. (3) Immune rejection and clinical application strategies arising from the interaction of MAP hydrogels with organisms may require surface modification of the materials to improve the biocompatibility of hydrogels and to reduce the immune response. (4) The degradation mechanism of MAP hydrogel in vivo needs further studies. By optimizing the degradation rate of hydrogels, it can better match the speed of tissue regeneration, ensuring that the hydrogel provides sufficient support while not impeding the formation of new tissue to avoid secondary surgeries.

Despite the numerous challenges still encountered in the application of MAP hydrogels, this field will continue to thrive with innovation and refinement. The future of MAP hydrogels will see more application scenarios and opportunities, including further advancements in MAP-based cancer immunotherapy and promotions in clinical translation.

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Disclosure of conflict of interest

None.

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